THE CHEMISTRY OF EXTRACTIVES FROM ACACIA SPECIES.

A THESIS PRESENTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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by
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STATEMENT

All the experimental work reported in this thesis has been carried out by myself, and this thesis does not contain any material which has been previously submitted for a degree in any University either by myself or by any other person.

P. I. Mortimer.

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CONTENTS.

I N T R O D U C T I O N

THE GENUS ACACIA AND ITS PHYTOCHEMISTRY.

This thesis is concerned with the chemistry of some constituents of various species of Acacia, a genus of the family Leguminosae. This is a large genus, of about 500 (1) or 800 (2) species, of which about two-thirds are native to Australia, where they form a considerable part of the flora. Within Australia, the Acacias are widely distributed, about 90 species occurring in South Australia (1), about 80 in Victoria (2), and "several hundred" in New South Wales (3). The genus is represented in Asia, Africa and the Americas, but no species are native to Europe or New Zealand (1).

Of the Asian and African species, several have long-established uses, e.g. A. senegal Willd. and a few other species as sources of gum acacia, A. arabica Willd. and A. catechu Linn as sources of tanning material, and A. farnesiana (L.) Willd. in perfumery. Of the Australian species, A. melanoxylon R.Br. is used in furniture manufacture, and several species have been used in the tanning industry. The Australian species A. mollissima Willd. is now extensively cultivated in South

Africa where about 120,000 tons of tanning material is obtained per year from its bark (4).

The genus Acacia has not been systematically investigated by chemical methods, as has been done with the eucalypts (5) and the conifers (6). The sporadic chemical work may be grouped with respect to the types of products investigated.

(a) Odoriferous constituents of the flowers (7). A. farnesiana and A. cavenia Hook et Arn. are used commercially in perfumery. The odoriferous constituents represent less than 0.1% of the weight of the fresh flowers. The following terpene alcohols occur as esters - linalool, terpineol, nerolidol, farnesol; other constituents have been identified (8).

(b). The bases 2-phenylethylamine, N-methyl-phenyl-ethylamine and tryptamine have been obtained from the flowers of various Australian species growing in New Zealand (9).

(c) Mucilagenous gums are exuded by many species of Acacia. When marketed, they are known as gum acacia or gum arabic irrespective of specific origin. Such gums are composed of residues of D-galactose, L-rhamnose, L-arabinose and D-glucuronic acid (10). Recent work on gums of known specific origin (A.mollissima (11), A.pycnantha Benth. (12), A. cyanophylla Lindl. (13), A. karroo Hayne (14)) has shown that these sugar

residues do not occur in constant proportions, but the composition of the gums varies from species to species. Variation within a species does not seem to have been examined.

(d) The seedfats of eight species have been examined; the seeds contain 3-8% oil, of iodine number 85-103 (15,16); an oil content of 10%, iodine number 137 has been reported for A. cyclops - (17). The fat from the seed pod of A. giraffae Willd. (1-2% yield) has been closely examined; it contained the usual saturated, mono-unsaturated and di-unsaturated fatty acids, and 1.1% of 9,12-hexadecadienoic acid (18), the only reported occurrence of this acid.

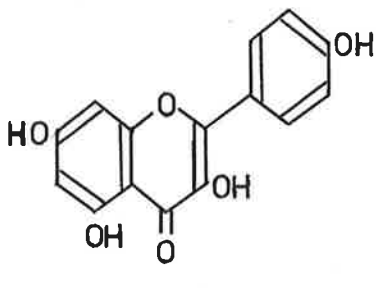
(e) Sterols have been reported to occur in the seed oils (16,17). A "steroid" m.p.160-161°, acetate m.p. 167-169° has been isolated from the heartwood of A. mollissima in 0.06% yield (19); its structure is not known.

(f) Carotenoids have been isolated from A. dealbata Link pollen (20) and β-carotene from A. acuminata Benth heartwood (21) in which it occurs to the extent of about 0.02%, together with phytofluene. The funicles of A. cyclops are reported to contain about 0.2% β-carotene (17).

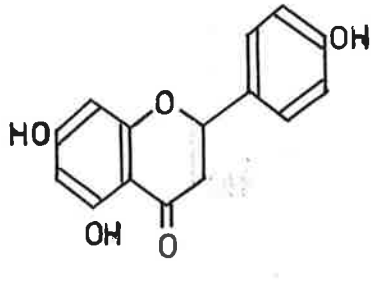
(g) Cyanogenetic glucosides have been isolated from the leaves of some Acacia species. A.Glaucescens L. and A. Cheelii Blakely have yielded sambunigrin (the glucoside of L-(+)-mandelonitrile) (22), and A. lasiopetala Oliv. and A. stolonifera Burch. have yielded acacipetalin, which was considered to be the glucoside of dimethylketene

cyanhydrin (23). Many other Acacias have been found to be cyanogenetic, but isolation of the cyanogenetic constituent has not been carried out.

(h) Ketonic flavonoids. Petrie (24) was the first to examine the bright yellow flowers; he extracted the flowers with hot water, precipitated the colouring matters with lead acetate, and then hydrolysed the recovered pigments with sulphuric acid. The flavonoid material was extracted from the deep red solution with ether, and subjected to several manipulations, including extraction with aqueous alkali, precipitation with acid and crystallisation from aqueous alcohol. From four New South Wales species he obtained kaempferol (I) as follows: A. discolor - 0.08%, A. linifolia Willd. 0.07%, A. decurrens - 0.006%, and A. longifolia - 0.06%. He detected rhamnose in the hydrolysate. Using this technique, other workers have obtained naringenin (II) from Argentine A. longifolia flowers (25), and myricetin (III) from A. leucophloeia Willd. flowers (26). In the latter case, the sugar obtained on hydrolysis was considered to be glucose.



(I)



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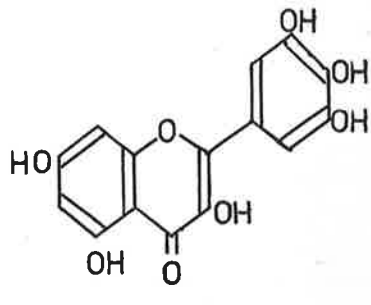
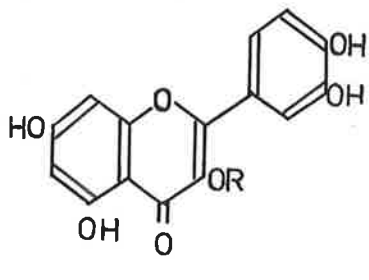


(III)

Paris has examined the flowers of some Acacias grown in southern France (27). A. longifolia var. floribunda F.v.M = A. floribunda Sieber (to be distinguished from the horticultural Acacia floribunda which is A. retinodes Schlecht) yielded naringenin - 5 - glucoside (26). A. cyanophylla Lindl. yielded quercetin - 3 - glucoside (IV, R = glucose). From A. linifolia flowers he was not able to isolate any components, but the leaves yielded a flavonol glycoside; the flavonol was similar to, but not identical with kaempferol.

The pollen of A. dealbata Link. (A. decurrens var. dealbata) has been examined by Spada et al. They have obtained a naringenin diglucoside (29), and evidence of glucosides of flavonols which were suggested to be robinetin (V) and morin (VI) (30).



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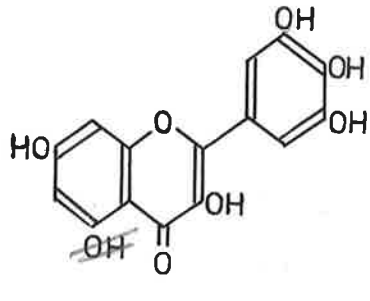

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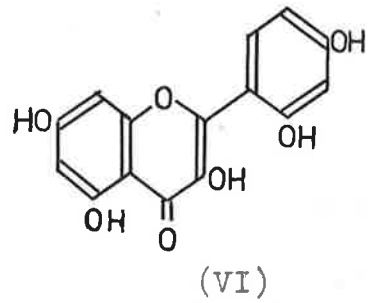
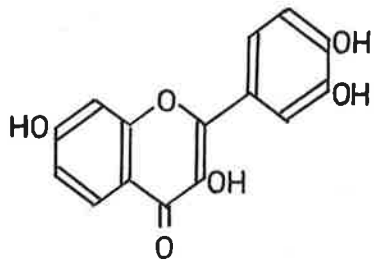


(VI)

It might be expected that a common pattern of hydroxylation would be found among the flower pigments of Acacia species. The evidence so far available, suggests that no such common pattern occurs, but the evidence is so unsatisfactory that it would seem unwise to draw any conclusions. Much further work is required.

Paris (27) has also examined the leaves of some species for flavonoids. A. retinodes yielded rutin (IV, R = rhamno glucose). The flavonols from A. linifolia, A. verticillata Willd. and A. dealbata were not identified, and that from A. farnesiana appeared to be kaempferol (I).

From A. catechu extract, Perkin (31), obtained a very small quantity (0.012%) of quercetin. Other flavonols occur in this extract; from paper chromatographic evidence they are thought to be fisetin (VII) and quercetagetin (VIII) (32). King and Bottomley (33) isolated 7,8,3',4'-tetrahydroxy-flavonol in small yield (0.01%) from A. melanoxylon heartwood.

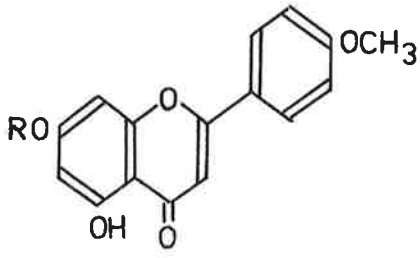


(VII)

(VIII)

The extract of A. mollissima bark is reported to contain fisetin (VII) and robinetin (V) (4).

The compounds acacetin (IXA) and its glycosides (IX B and IXC) are not derived from Acacias despite



IXA R = H

IXB R = rhamnoglucose

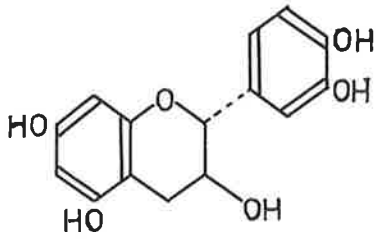
IXC R = xylorhamnoglucose

the name; they are obtained from Robinia pseudacacia leaves, and some other plants (34).

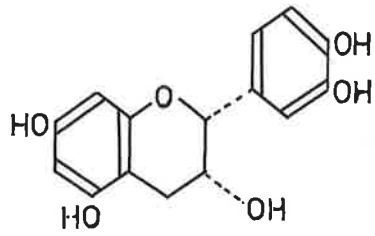
(i) Non-ketonic flavanoids. This class of compound includes the flavan-3-ols (catechins) and the flavan-3,4-diols, the latter being leucoanthocyanidins.

For many years, it has been considered that natural tanning materials of the "condensed tannin" or "phlobaphene" class consist of molecules formed of C_6-C_3-C_6 units at the flavan-3-ol oxidation level (35,36,37,38,32). It has been recently shown that about 25% of these units in A. mollissima extract may be at the flavan-3,4-diol level of oxidation (39). However, no compounds with two or more C_6-C_3-C_6 units have yet been isolated from Acacia sp.

The flavan-3-ols catechin (X) and epicatechin (XI) have been obtained from A. catechu. The first record appears



(X)

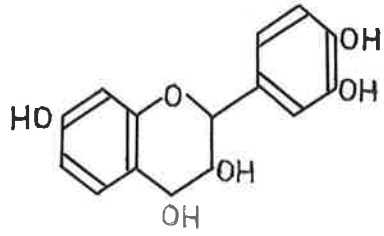


(XI)

to have been in 1839 (40); later isolations are considered in the discussion on methods of isolation. (+)-Catechin, (±)-catechin, (-)-epicatechin and (±)-epicatechin have been obtained. (+)-Catechin and (-)-epicatechin are reported to have been isolated from A. confusa Merr. bark (41) and to occur in A. mollissima extracts (4). Roux (42) has isolated (+)-catechin, (+)-gallocatechin and a 7,3',4',5'-tetrahydroxyflavan-3-ol from A. mollissima extract.

The flavan-3, 4-diol melacacidin (XII) was obtained from A. melanoxylon heartwood in an impure form by King & Bottomley (33), and the flavan-3, 4-diol mollisacacidin (XIII) from A. mollissima heartwood in a

(XII)

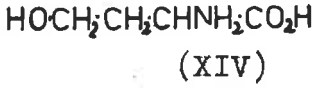


(XIII)

crystalline form by Keppler (19). Besides mollisacacidin, A. mollissima is reported to contain a leucocyanidin, a leucopelargonidin and a leucodelphinidin, but these have not been isolated(4).

(j) Other polyphenols. Ethyl gallate has been obtained from leaves of A. adonsonii - and A. seyal - (43). Maclurin (2,4,6,3',4' - pentahydroxybenzophenone) has been obtained from the sapwood of A. catechu, A. catechuoides - and A. sundra - in 0.04% yield (44). White (4) lists syringic acid, gallic acid and resorcinol as having been found to occur in A. mollissima.

(k) Amino acids: Apart from the normal amino-acids, L (+) - homoserine (XIV), (45), 4-hydroxypipecolic acid (XV) and 5-hydroxypipecolic acid (XVI) (46) have been reported by Virtanen as present in the leaves of A. pentadena -,



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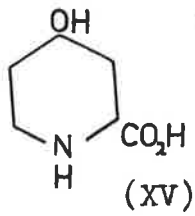

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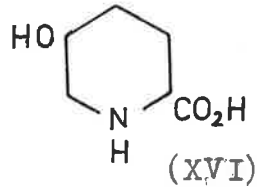
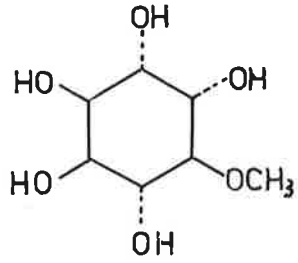


(XVI)

the latter pair also occurring in A. retinoides - leaves.

(l) Carbohydrates: Fructose has been detected in A. mollissima wood by paper chromatography and isolation of glucosazone (47). (+)-Pinitol (XVII) has been isolated from



(XVII)

the leaves of A. longissima Wendl., A. stolonifera and A. lasiopetala (23), and from the wood of A. mollissima (47). It is of widespread occurrence among the leguminosae (48).

(m). Calcium oxalate has been reported to occur in appreciable amounts in Acacias e.g. 18% in the bark of A. cambagei - (49).

There are several reports of "saponins" in Acacia pods, seeds, and bark, e.g. (50), but no specific substance has been isolated.

THE ISOLATION OF POLYPHENOLIC FLAVANOIDS.

The isolation of polyphenolic substances from plant material is attended by the difficulty that these compounds, particularly the non-ketonic flavanoids, do not crystallise readily in the presence of other polyphenols, and, except in comparatively rare instances, they are accompanied by other polyphenols, often of high molecular weight. The flavanoids may not be distilled without decomposition. Final purification is thus of necessity by crystallisation, and the techniques available for preliminary purification are limited.

The range of methods utilized for the isolation of the relatively few non-ketonic flavanoids is illustrated by the following summary.

Catechin (X), and epicatechin (XI) have been isolated from the hot-water extract of Acacia catechu twigs by several workers. Perkin and Yoshitake (51) extracted the dry extract with ethyl acetate, and crystallised the ethyl acetate - soluble portion from water to obtain a catechin m.p. 204-205° (probably partly racemic catechin). From "cube gambier", using the same method but with purification of the ethyl acetate-soluble portion by addition of lead acetate to an aqueous solution to remove colouring material, these workers isolated a catechin m.p. 175-177° ((-) - catechin?) and a small amount of a catechin

m.p. 235-237° (apparently (±)- or (-)-epicatechin). (The use of ethyl acetate as a solvent had been suggested by Löwe in 1874 and the use of lead acetate had been introduced by Berzelius in 1837). Clauser (52) extracted powdered cube gambier with ether for 15-18 hours; the extracted material crystallised on trituration under water, and was dissolved in four volumes of hot water (quercetin remained undissolved), and allowed to crystallise. Re-crystallisation gave a catechin m.p. 210° decomp. when anhydrous.

Freudenberg (53) triturated the dried Acacia catechu extract with one half part of acetone and added 5 parts of ether, repeating this selective-solution process on the residue. The ether-soluble portion so obtained was dissolved in water, treated with a little lead acetate, and concentrated in vacuo to obtain a crystalline catechin, penta-acetyl derivative m.p. 156°, $[\alpha]_D$ -20.5° (c, 10 in $C_2H_2Cl_4$). Freudenberg later (54) used Clauser's method of extraction; by attention to the fractional crystallisation, (±)-catechin containing some (-)-catechin, (-)-epicatechin, and (±)-epicatechin were obtained. Lead acetate was used for the removal of impurities. This fractional crystallisation was described in more detail later (55). Freudenberg also used A. catechu heartwood as a source of (-)-epicatechin (56), the milled heartwood was extracted with ether for 250 hours, the extract was dissolv-

ed in water (quercetin remained undissolved) and allowed to crystallise, yielding (-)-epicatechin. The mother liquor was extracted with ether for 2 days, and the extracted material was fractionally crystallised from water yielding more (-)-epicatechin and some (±)-catechin. During the ether extraction of the wood, (-)-epicatechin may be deposited (57). (The isolation of (-)-epicatechin from A. catechu heartwood has been repeated by Seshadri (58): the cold acetone extract was crystallised from ethyl acetate). From cube gambier, by kneading with water to remove water-soluble material, and extracting with ether for 240 hours, a fraction was obtained which gave on fractional crystallisation, (+)-catechin, (±)-catechin and (-)-epicatechin (56). (+)-Catechin was isolated from Chinese rhubarb by extracting the moist plant material with ether, dissolving the product in water, filtering, purifying the solution by extraction with chloroform, and lead acetate treatment, followed by concentration and crystallisation (54). From mahogany wood (+)-catechin was obtained by extracting moist shavings with ether, dissolving the product in water, purifying with lead acetate, followed by concentration and crystallisation (54). (+)-Catechin was obtained from Paullinia cupana seeds in a similar manner (54). From a cold alcoholic extract of kola nuts, by solution in water, extraction with chloroform to remove caffeine, then with ethyl acetate, and crystallisation of this extract from wate

(+)-catechin and (-) - epicatechin were obtained (59).

Nierenstein (60) obtained (±)-and (+) - catechin from Krameria argentea root bark by extracting with ether after a chloroform extraction, and fractional crystallisation of the ether soluble material from water. From a variety of plant material (61), he obtained catechin and epicatechin by either of two methods; (a) wood material was extracted in a Soxhlet with ethyl acetate, the extractives were mixed with sand, ether extracted, and these extractives crystallised from water, (b) leaf material was extracted in a Soxhlet with chloroform, then with ether, and the ether extract crystallised from water.

Schmidt and Hüll (62) isolated (+) - catechin from dried unripe shells of Castanea vesca by hot alcohol extraction, removal of solvent from the extract, solution in warm water, and continuous extraction with ether (a preliminary benzene extraction was made). Some product crystallised from the ether during the extraction. Purification was by solution in dry acetone followed by precipitation with benzene. The catechin mostly remained in the benzene-acetone solution; it was crystallised from water. Hergert & Kurth (63) obtained (+)-catechin and (-)-epicatechin from Abies concolor, bark by ether extraction (24 hours) after a preliminary benzene extraction; the ether extract was recrystallised from water. From some bark fractions, (+) - catechin could be obtained by extraction with hot water, followed by concentration to 1% solids; crystallisation

was assisted by addition of a small amount of sodium bisulphite.

The only other flavan-3-ols isolated from natural products have been (-)-epiafzelechin ((-)2H,3H cis, 5,7,4'-trihydroxyflavan-3-ol), (+)-gallocatechin and (-)-epigallocatechin (epimeric 5,7,3',4',5' - pentahydroxyflavan-3-ols). The first was obtained by King, Clark-Lewis & Forbes (64) from a sample of Afzelia sp. heart-wood by ether extraction of the wood after a petroleum ether extraction, and crystallisation of the ether extractions from water. (-) - Epigallocatechin was obtained by Tsujimara (65) from green tea by extraction with water, precipitation with lead acetate, removal of lead as sulphate, extraction from water with ethyl acetate, and again from water with ether, at which stage a crystalline product was obtained. (+)-Gallocatechin has been obtained only by multiple partition methods, to be discussed later.

The first flavan-3, 4-diol to be isolated (in crude form) was melacacidin from A. melanoxylon (33). The heart-wood, after extraction with light petroleum, was extracted for 3-4 days with ether, when crude melacacidin deposited from the ether solution. Seshadri has reported the isolation of leucodelphinidins (66) and a leucocyanidin (67) by fractional precipitation from a solution in ethyl acetate by light petroleum. A cold acetone extract of defatted plant material was used, and the ethyl acetate-

solution was dried over magnesium sulphate. Other flavan-3, 4-diols have been isolated by the use of multiple partition methods, discussed below.

The compounds cyanomaclurin and peltogynol contain a flavan nucleus. Cyanomaclurin was obtained from Artocarpus integrifolia by hot water extraction, treatment with lead acetate (which removed morin), removal of excess lead with hydrogen sulphide, concentration and extraction with ethyl acetate; from this extract, cyanomaclurin crystallised on concentration (68). Peltogynol was isolated from Peltogyne porphyrocardia by hot water extraction, addition of sodium chloride, extraction with three portions of ethyl acetate, drying the extract and precipitation with light petroleum (69). Both these compounds have been isolated in recent years by multiple partition procedures.

The isolation of ketonic flavonoids, is in general, much easier than that of non-ketonic flavanoids, because they crystallise more readily. Two techniques are noteworthy; that of Erdtman and Lindstedt, and that of Hasegawa. Erdtman developed his method for use on Pinus spp. heartwoods (70); an example of its use is the investigation of Pinus contorta var. latifolia by Lindstedt (71). The heartwood was extracted with ether (which extracted little polyphenolic material) and then with acetone. The acetone extract was concentrated to a syrup, then diluted with ether. The deposit which

was obtained was considered to be "membrane substances", a term introduced by Erdtman (72) because ether extraction of the intact wood failed to extract ether-soluble materials subsequently obtained by acetone extraction. The ether solution obtained from the acetone-extract was shaken successively with saturated aqueous sodium bicarbonate, with a sodium carbonate solution, with dilute sodium hydroxide (0.2 to 0.4%) and with stronger sodium hydroxide (4.5%). Each aqueous extract was acidified with sulphuric acid and extracted with ether. The carbonate solution yielded pinocembrin (5,7-dihydroxyflavanone) and pinobanksin (5,7-dihydroxyflavanonol). The dilute hydroxide solution yielded pinocembrin and pinosylvin (3,5-dihydroxystilbene). The strong hydroxide solution yielded pinosylvin monomethyl ether. Hasegawa and co-workers have based their technique on solubilities; an example is their examination of Prunus verecunda heartwood (73). The crude methanol extract was divided into ether-soluble and ethyl acetate-soluble fractions by extraction of the dry solid. The ether-soluble portion was subdivided into benzene-soluble, water-soluble and remainder. The benzene-soluble portion yielded pinocembrin and isosakuranetin (5,7-dihydroxy-4-methoxyflavanone); the water soluble portion yielded taxifolin (5,7,3',4'-tetrahydroxyflavanonol); the remaining ether-soluble portion yielded genkwanin (5,4'-dihydroxy-

7-methoxyflavone), genistein (5,7,4'-trihydroxyisoflavone), and prunetin (5,4'-dihydroxy-7-methoxyisoflavone). The ethyl acetate-soluble portion yielded verecundin (5,7-dihydroxyflavanone 5-glucoside) and isosakurenin (5,7-dihydroxy-4'-methoxyflavanone 7-glucoside). This example is interesting in that compounds with more than one free (not H-bonded) hydroxyl were not benzene-soluble, only the polyhydroxy flavanonol was soluble in both ether and water, and the glucosides were not water-soluble.

The procedures of both Erdtman and Hasegawa are dependent finally upon crystallisation for the separation of mixtures.

The methods of isolation of non-ketonic flavanoids summarised above were developed for use with plant materials yielding relatively simple fractions on extraction. The approach to each isolation was largely empirical. In the main, the materials investigated were traditional tanning agents, rich in polyphenolic material, yet success was achieved only in a few cases. Until the introduction of paper chromatography into this field about 1948, the only real knowledge of the polyphenol composition of extracts was that derived from actual isolations. Extension of knowledge of the occurrence of known polyphenols, and discovery of new polyphenols, were very slow.

The development of paper chromatography brought about a complete change in approach to the problem of the

isolation of polyphenols. The types of polyphenols in a mixture, their relative abundance, and occasionally their identity became ascertainable. It became possible for the chemist to develop a systematic approach to isolations, and to follow readily the progress of isolations.

Besides its analytical use, paper chromatography, has been used for isolation of polyphenols in amounts sufficient for characterisation and identification. Roux (74) used preparative paper chromatography for the separation of (+) - catechin and (+) - gallo-catechin (from Casuarina equisetifolia); these compounds could not be separated by crystallisation from water. The amount of polyphenol which may be easily handled on paper sheets is however usually too small; the use of columns of cellulose powder permits somewhat larger amounts to be handled. Examples are the isolations of mollisacacidin (19), peltogynol and peltogynol B (75), (-) - epicatechin (76) and dihydromorin and dihydrokaempferol (77).

A similar partition procedure is the use of columns of silica gel. These were pioneered by Bradfield (78) and proved suitable for the isolation of (-) - epicatechin, (-) - epicatechin gallate, (-) - epigallo-catechin and (-) - epigallocatechin gallate from green tea. Hathway (79) has recently used this system for the isolation of (+) - catechin and (+) - gallocatechin from oak bark extract, and Mayer (80) for the isolation of (+) - gallo-

catechin.

A further increase in scale is achieved using liquid/liquid partition distribution. After Corse (81) used this procedure for the separation of chlorogenic acids, Forsyth (82) applied it to flavanoids. He separated (-) - epicatechin from a leucoanthocyanidin using ethyl acetate and water in a Bush and Densen (83) procedure; (-) - epicatechin was more readily extracted by ethyl acetate than the leucoanthocyanidin. Recently counter-current distribution has been used for the isolation of a 7,3',4' - trihydroxyflavan-3, 4-diol, from quebracho (ether/water, k about0.15), and cyanomaclurin from Artocarpus integrifolia (ether/water, k not available) (84).

Hörhammer and Wagner (85) have attempted to correlate the partition coefficients of some flavonols and flavonol glycosides in a series of solvent systems, with the Rf the flavonoids exhibit on paper chromatograms, with some success.

DISCUSSION.

ISOLATION OF HEARTWOOD CONSTITUENTS.

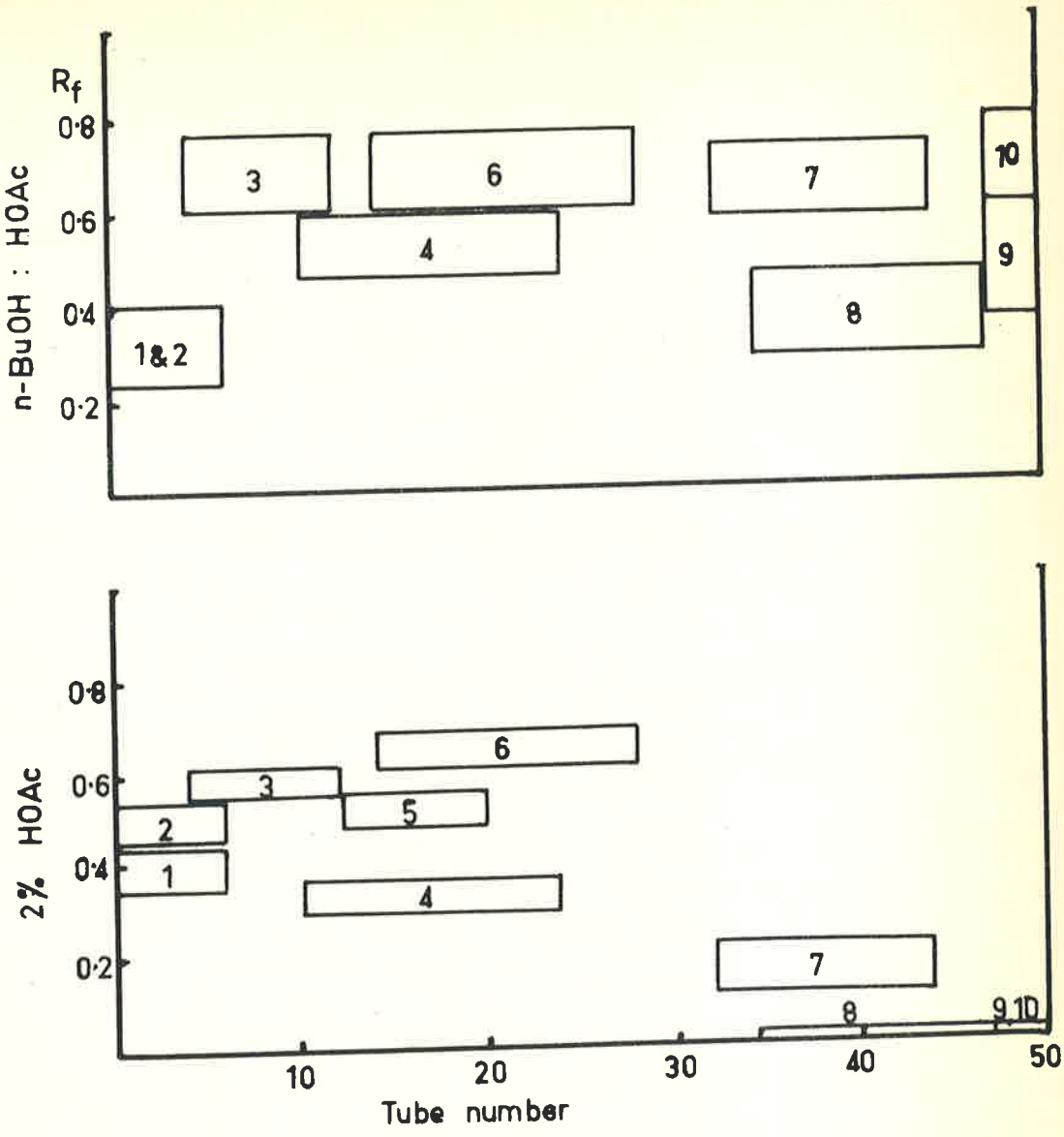
The work on heartwoods was directed towards the isolation of the leucoanthocyanidins. In two cases ether extraction of the wood gave crude melacacidin, but the fractions were very impure, and represented a very small proportion of the melacacidin actually present in the wood. After considerable experimentation, the best line of attack seemed to be firstly, to obtain a fraction containing the low molecular weight polyphenolic material, and then to obtain from this a mixed leucoanthocyanidin fraction which could be further subdivided.

Extraction of the polyphenols from the woods was usually fairly complete with refluxing acetone, although the time required was longer than was desirable when dealin with compounds susceptible to damage by heat. Refluxing ether proved very inefficient, and an experiment with cold acetone extraction gave incomplete extraction. This cold acetone extraction gave a mixture of polyphenols in which the leucoanthocyanidin fraction was similar to that obtained by prolonged hot extraction with acetone. That hot acetone did not extract the polyphenols completely was shown in Extraction B. In this extraction, hot acetone extracted from A. harpophylla heart-

wood 5% of its weight as polyphenols; after hot acetone ceased to extract much more material, hot ethanol extracted a further 0.6% of polyphenols, mainly leucoanthocyanidins.

The acetone and ethanol extracts were concentrated and taken up in water. This gave much brown amorphous insoluble material which did not prove readily susceptible to purification procedures. The polyphenols sought were found in the aqueous solution, from which they were readily recovered by extraction by ethyl acetate. The distribution constants of the leucoanthocyanidins of the melacaidin series are such that extraction with ethyl acetate required the use of continuous extraction. This meant that the polyphenols were kept at 80°C in either water or ethyl acetate for about 8 hours. Owing to the presence of much polymeric material in the water-soluble fraction it was difficult to determine whether this treatment produced any decomposition or condensation of the leucoanthocyanidin. The products were similar to those obtained in other extractions where continuous ether extraction was used.

The nature of the mixture present in the crude polyphenols of A. harpophylla is shown in Figure 1. The polyphenol mixture of A. excelsa was found to be similar (Extraction H); that of A. melanoxylon was not examined in such detail but one-dimensional chromatograms were generally similar to the chromatograms of corresponding



Major components:- 1 melacacidin 2 isomelacacidin
6 O-ethyl isomelacacidin 7 flavanonol?
8 flavonol 9 chalcone
Minor components:- 4 flavan-3-ol? 3,5,10 unidentified

Figure 1 : Polyphenols of Acacia harpophylla heartwood extract separated by counter-current distribution and paper chromatography.

fractions from the first two species. To obtain the separation shown in Figure 1. the polyphenols were subjected to counter current distribution and fractions examined by paper chromatography. Apart from traces of polymeric material (not shown in Figurel.), the components fall into two main groups; that group not readily extracted from water by organic solvents which includes melacacidin and an isomer, isomelacacidin, and that group readily extracted from water by organic solvents, which includes a third leucoanthocyanidin subsequently found to be O-ethyl isomelacacidin, and ketonic flavonoids. The differences in the various distribution constants is such that it was possible to obtain an almost complete separation of these two groups using separatory funnels; this enabled considerable quantities of polyphenols to be handled.

Further work was assisted by two circumstances. First the amount of non-leucoanthocyanidin material in the less-readily extracted fraction was very small (in the case of A. harpophylla and A. excelsa) and secondly, it was found that isomelacacidin and O-ethyl isomelacacidin were readily interconvertible. Thus when this fraction was boiled in ethanol containing a trace of weak acid, and the O-ethyl isomelacacidin removed by a distribution procedure, the remaining polyphenol was largely melacacidin. The isolation of crude melacacidin by King and Bottomley

by ether extraction of A. melanoxylon suggested that ether extraction from an aqueous solution of this crude melacacidin fraction might give a deposit of melacacidin in the ether. This was found to be so in some instances, and the melacacidin proved to be crystalline and sufficiently pure to recrystallise from ethanol. The conditions required to obtain crystalline melacacidin in this ether extraction seem to be that other low-molecular weight polyphenols should be almost completely absent, and that the concentration of melacacidin in the aqueous solution being extracted should be high.

Crystalline melacacidin was obtained fortuitously during handling of the mixed leucoanthocyanidin fraction. It was found that heating an alcoholic syrup of the mixed leucoanthocyanidin resulted in the formation of crystalline melacacidin. The explanation for this seems to lie in the conversion of the isomelacacidin present into 0-ethyl isomelacacidin, which would appear to inhibit the crystallisation of melacacidin less than isomelacacidin does.

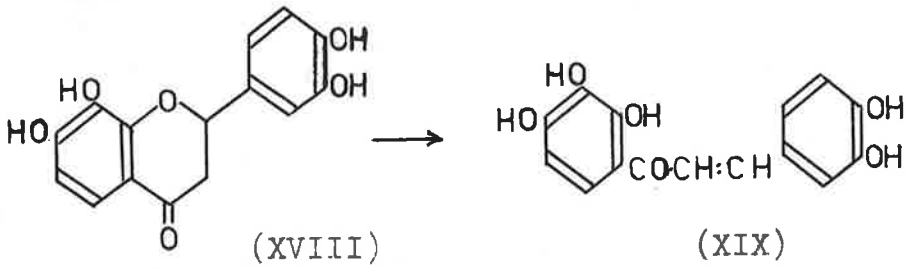
The ready interconversion of isomelacacidin and 0-ethyl isomelacacidin permitted the isolation of the latter. When heated in water, slightly acid, the 0-ethyl isomelacacidin present in the more readily extracted fraction of the polyphenols was converted to isomelacacidin which could be isolated by a distribution process and reconverted to 0-ethyl isomelacacidin, which could be obtained crystalline.

While no experimental work has been done on the question, it is almost certain that O-ethyl isomelacacidin does not exist as such in the plant. The evidence suggests that isomelacacidin does occur in the plant. Experimental work on the interconversion of the leucoanthocyanidins showed that melacacidin is comparatively stable in neutral or weak acid solution, and yet extraction of A. harpophylla heartwood with cold acetone (Extraction E) gave an extract containing isomelacacidin in quantity comparable with that in hot acetone extracts. Roux (86) has reported that A. melanoxylon contains two leucoanthocyanidins.

Crystalline melacacidin could not be obtained from the sample of A. melanoxylon heartwood examined, but was obtained from the heartwoods of A. harpophylla (first sample, 0.6 and 1.0%; second sample, 0.2%) and A. excelsa (0.4%). The most direct and reliable procedure for the isolation of melacacidin is that used in Extraction I (from A. excelsa). O-Ethyl isomelacacidin was prepared in yields of about 0.2% from the heartwoods of all three species, but in the preparation of this compound from extracts of A. harpophylla and A. excelsa not all the isomelacacidin was utilised. As isomelacacidin was not isolated crystalline, the efficiency of the procedure by which it was isolated as its O-ethyl derivative cannot be assessed accurately. In the example given for the preparation of O-ethyl isomelacacidin, the yield of crystall-

ine product was about 15% of the theoretical assuming that the material used was pure isomelacacidin.

Although no attempt was made to isolate the non-leucoanthocyanidin polyphenolic components, three were readily obtained. 7,8,3',4'-Tetrahydroxyflavonol was obtained on concentrating the acetone extract of A. harpophylla heartwood. This had been obtained from A. melanoxylon by King and Bottomley (33). During continuous extraction of A. harpophylla heartwood ketonic polyphenols with light petroleum okanin (3,4,2',3',4'-pentahydroxychalcone) crystallised from the aqueous solution. This compound had not previously been isolated from Acacia sp. but had been obtained by King and King (87) from Cylicodiscus gabunensis, and occurs as the 4'-glucoside (marein) in Coreopsis spp. (88). The circumstances of its isolation in the present case suggests that the corresponding flavanone (XVIII) was present in the extract, and was being converted into okanin (XIX).



(XVIII) (XIX)

The third non-leucoanthocyanidin isolated (compound A) was not identified. Its colour reactions suggested that it was a flavanonol; as the other polyphenols isolated had the melacacidin hydroxylation pattern, it is possibly

a 7,8,3',4' - tetrahydroxyflavanonol. It was obtained from A. excelsa heartwood and was present also in A. harpophylla heartwood.

The heartwood of A. intertexta yielded a new leucoanthocyanidin, teracacidin. This compound was obtained in crude form by counter-current distribution of the polyphenolic fraction, and was characterised as its trimethyl ether. The extracts from A. intertexta contained three leucoanthocyanidins, apparently inter-related in the same way as the leucoanthocyanidins of the melacacidin series. However, an attempt to obtain O-ethyl isoteracacidin failed; the solution on reaction with p - toluenesulphinic acid, yielded isoteracacidin p - tolyl sulphone.

The work with A. intertexta was made difficult by the presence of comparatively large quantities of brown, apparently high molecular weight, polyphenols. Several methods of processing extracts were tried in attempts to reduce their amount, but without success. Among the methods tried was that used by Lindstedt & Erdtman (e.g. 71), namely dilution of the acetone extract with ether. It was found that with an undried acetone extract, the leucoanthocyanidins were distributed between the ether layer and the other phases so that several ether extractions were necessary to recover them. This procedure gave a comparatively high yield of total polyphenols and of crude teracacidin.

The yield of purified teracacidin was 0.5% from the first sample, and about 0.05% from the second sample.

A brief examination of the polyphenols of A. mollissima was also carried out.

Pinitol was present in all the heartwoods examined. It was obtained as a deposit during some acetone extractions of A. melanoxylon heartwood (about 0.9%) and A. mollissima heartwood (1.5%). In both cases, no deposit was obtained in other extractions of the same sample. Pinitol was isolated from A. intertexta heartwood (0.3%) and A. harpophylla heartwood (0.05%). It was detected in A. excelsa heartwood by paper chromatography. The isolation of pinitol from A. mollissima was reported by Stephen (47) and by Keppler (19).

The first sample of A. excelsa heartwood yielded 4-hydroxypipecolic acid in 0.2% yield. This stimulated examination of the wood samples for amino-acids; the results are given in Table 1.

The sapwoods examined contained little, if any, polyphenols, and no detailed investigation was made. A small amount of mannitol was isolated from A. melanoxylon sapwood.

Table 1.

Imino-acids of Acacia heart and sap-woods.

Species	Wood	4-Hydroxy-pipecolic	5-Hydroxy-pipecolic	Pipecolic	Proline
harpophylla	heart	nil	nil	trace	trace
"	sap	X	X	0.15	X
excelsa	heart (1)	2.0	nil	0.2	nil
"	heart (2)	0.2	nil	0.001	trace
"	sap (2)	0.05	nil	0.01	trace
melanoxylon	heart	nil	nil	trace	nil
"	sap	nil	X	trace	trace
mollissima	heart	0.01	nil	trace	nil
"	sap	0.03	nil	trace	trace
intertexta	heart	nil	nil	nil	nil

Trace = detected by paperchromatography.

X = present ca. 0.01-01%, not isolated.

Figures give % isolated.

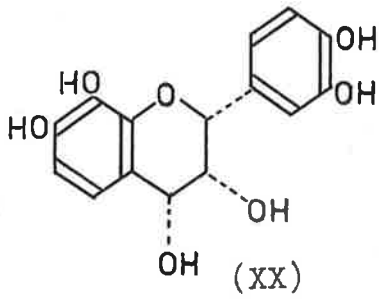
THE CHEMISTRY OF LEUCOANTHOCYANIDINS: O-ETHYL-ISOMELACACIDIN.

Although they did not obtain pure melacacidin, King and Bottomley (33) established that it was a 7, 8, 3',4'-flavan-3,4-diol. It was shown to be a cis-glycol as the tetramethyl ether formed a cyclic carbonate from which the tetramethyl ether diol was obtained on hydrolysis. King and Clark-Lewis (89) hydrogenated 7,8,3',4'-tetramethoxyflavonol to a 7,8,3',4'-tetramethoxyflavandiol and obtained the racemate corresponding to melacacidin. This indicates that the substituents at C_2 and C_3 are cis in melacacidin. One of the aims of the present work on melacacidin was the clarification of this point. It was expected that it would be possible to remove the 4-hydroxyl group from melacacidin tetramethyl ether by selective hydrogenation, to give a flavan-3-ol. If the substituents at C_2 and C_3 are cis, the stereochemistry of this flavan-3-ol would be similar to that of epicatechin, and the 3-toluenesulphonate on heating with hydrazine would undergo a trans-elimination reaction to give the flaven, as eith epicatechin (90) and epiafzelechin (64). If the substituents at C_2 and C_3 are trans, the toluenesulphonate of the flavan-3-ol would not react so readily and would undergo fragmentation, as with catechin (90,91). The use of this reaction to establish the relative configuration at

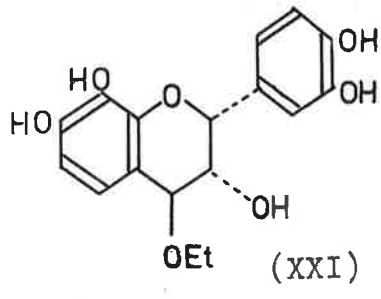
C_2 and C_3 in flavan-3-ols, is discussed by King, Clark-Lewis and Forbes (64).

The isolation of crystalline melacacidin has meant that the hydrogenation to remove the benzylic alcohol may be carried out on the phenolic compound, which may be of advantage.

The experimental relationship between melacacidin and the two other leucoanthocyanidins also present in the extracts of A. harpophylla, A. excelsa and A. melanoxylon is shown in Figure 2. O-Ethyl isomelacacidin was isolated in crystalline form. It is considered to be 4-O-ethyl isomelacacidin (XXI), melacacidin being represented as (XX).



(XX)



(XXI)

The evidence for this structure for O-ethyl isomelacacidin is as follows: analytical figures for the anhydrous material agree with the formula $C_{17}H_{18}O_7$, and show that it contains an O-ethyl group (one O-alkyl and one C-methyl). Methylation with diazomethane followed by reaction with

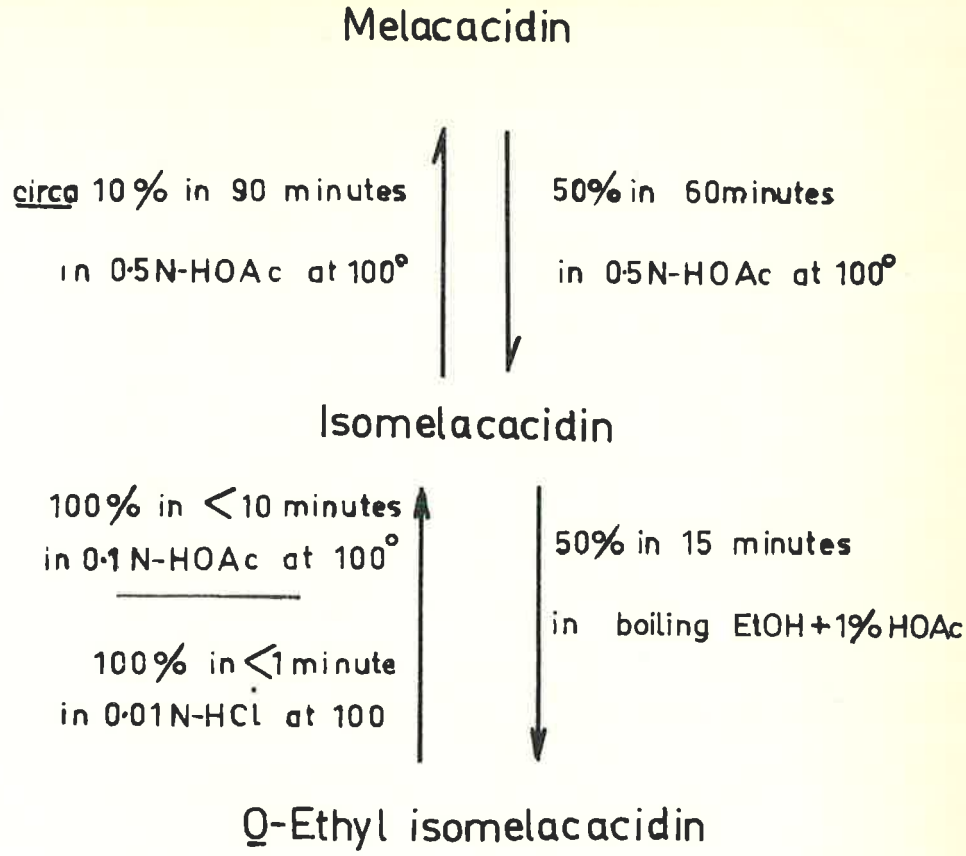


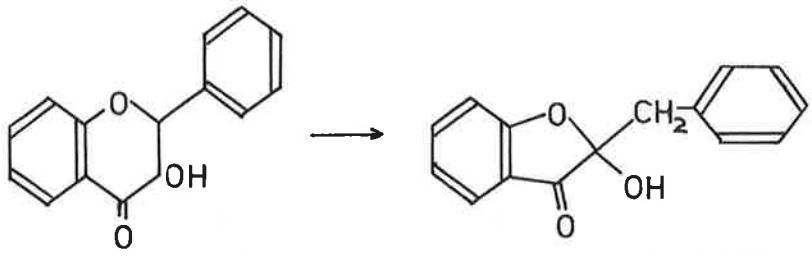
Figure 2 : Interconversion of melacacidin, isomelacacidin and O-ethyl isomelacacidin

p-toluenesulphonyl chloride in pyridine gave a stable crystalline tetramethyl ether toluenesulphonate. This shows firstly, that the molecule contains four phenolic-hydroxyl groups and, secondly, that it contains one non-phenolic hydroxyl group, and, thirdly, that this group is not a benzylic alcohol as these, especially with a p-methoxy substituent, give unstable toluenesulphonates[92]. That the compound contains a flavan nucleus is shown by its conversion by hot mineral acid to an anthocyanidin. Also, reaction with toluenesulphinic acid gave the same product, a sulphone, as was obtained from melacacidin. As the non-phenolic hydroxyl group must be located at C_3, the ethoxyl group must be on the only remaining carbon atom not containing an oxygen-substituent, that is at C_4.

That the ethoxyl group is an integral part of the molecule is shown by several facts. O-Ethyl isomelacacidin was, before its structure was realised, recrystallised from methanol-water mixtures but the O-ethyl group remained. The O-methyl compound may be readily distinguished from O-ethyl isomelacacidin by paper chromatography in the solvent systems n-butanol:acetic acid:water and 2% aqueous acetic acid, where compounds differing in alcohol of crystallisations lose their separate identities. O-Ethyl isomelacacidin may be readily separated from isomelacacidin by partition distribution between water and ether or water and ethyl acetate, which would not be the case if it were a simple alcoholate. It was

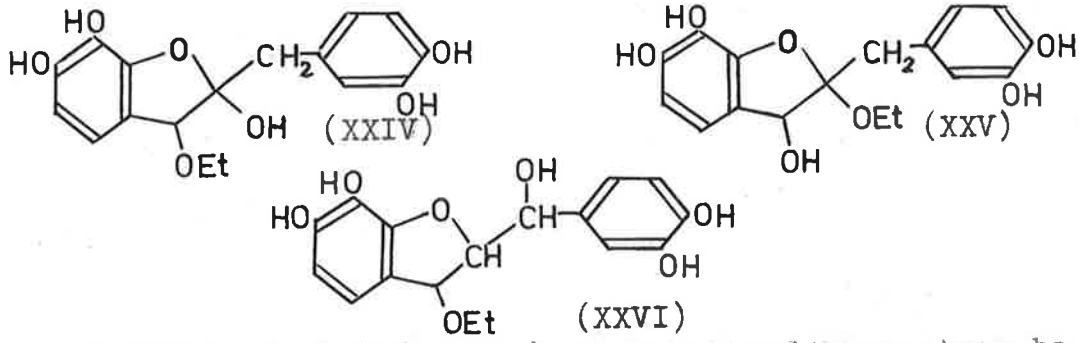
shown by Bate-Smith and Westall (93) that the Rf values of polyphenols such as flavonols, anthocyanidins and flavan-3-ols in n-butanol:acetic acid:water are dependent on the number of hydroxyl groups present in the molecule. O-Ethyl isomelacacidin has an Rf value similar to those of (+) - catechin, (-) - epicatechin and mollisacacidin and much higher than that of melacacidin. Bate-Smith and Westall have used the R_m value, i.e. $\log_{10}(\frac{1}{Rf}-1)$ to correlate structure and chromatographic behaviour. The R_m values for melacacidin, isomelacacidin and O-ethyl isomelacacidin in butanol;acetic acid:water are 0.41, 0.25 and -0.21. The differences between that of O-ethyl isomelacacidin and the others (0.62, 0.46) are similar in size to the Δ R_m values found by Bate-Smith and Westall for flavonols and leucoanthocyanidins and by Bradfield and Bate-Smith for flavan-3-ols (94).

In connection with the formulation of O-ethyl isomelacacidin as (XXI), there are two further points which must be considered. The first is the possibility of a rearrangement preceded by an opening of the pyran ring during conversion of isomelacacidin to the O-ethyl compound. Such a ring opening occurs readily with flavanones (XXII) to give chalcones and, with flavanonols, 2-benzyl-2-hydroxycoumaranones (XXIII) (95). The ring opening is favoured by alkaline conditions. It is



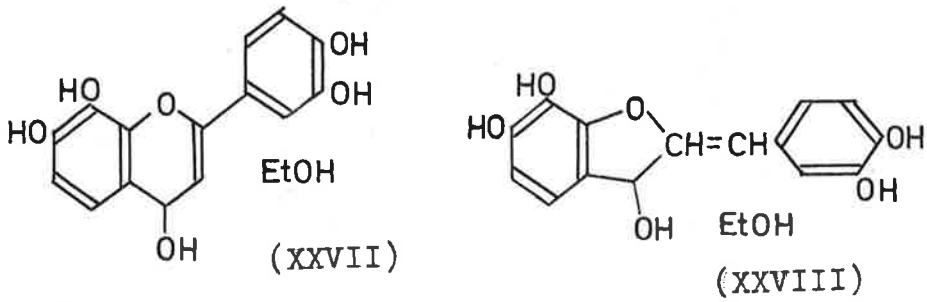
(XXII) (XXIII)

undoubtedly due to the presence of the carbonyl group and is either initiated by or assisted by removal of a proton from the α-carbon atom. Such a ring opening has not been observed with flavan-3-ols; in these the ring is cleaved only on reduction e.g. by sodium in ethanol. Possible formulae such as (XXIV), (XXV), (XXVI) would not give a stable tetramethyl ether toluenesulphonate.



(XXIV) (XXV) (XXVI)

Unsaturated alcoholates such as (XXVII) and (XXVIII) may be excluded as the compound has the ethoxy group covalently



(XXVII) (XXVIII)

bound in the molecule. The benzocoumaranonol and isoflavan formulae may also be excluded as O-ethyl isomelacacidin

gives an anthocyanidin on heating in acid.

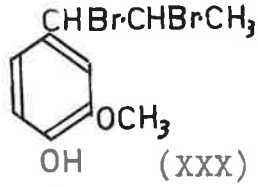
A second point to be considered is the report by Roux and Bill (96) that some leucoanthocyanidins on heating with anhydrous hydrogen chloride in propanol give two anthocyanidins, one of which appears to have an alkoxy group. O-Ethyl isomelacacidin, on heating with aqueous acid, gave the same anthocyanidin as derived from melacacidin. This confirms that the ethoxyl group is at C_4 where it would be removed. Examination of the effect of aqueous acid using paper chromatography, shows that the ethoxyl group is removed very rapidly by mineral acid, giving isomelacacidin. It thus seems that the report of Roux and Bill does not have any bearing on the problem of the structure of O-ethyl isomelacacidin.

The formulation of O-ethyl isomelacacidin as (XXI) is supported by data obtained on its formation from isomelacacidin, and on its solvolysis in aqueous acid to isomelacacidin as this behaviour resembles that of other benzyl alcohol derivatives. Most of the evidence available concerns benzyl halides. These hydrolyse by mechanisms which vary from near S_N2 to near S_N1 depending on solvent and on substituents (97,98). Substituents which are capable of releasing electrons to stabilise an intermediate carbonium ion increase the rate of reaction (examples are given by Kornblum (99) and figures are given by Burkhardt *et al* (100)). A *p* - methoxy substit-

uent results in an abnormally large increase in the reaction rate (101,102), compared with its effect on other types of reactions correlated by the Hammett equation. It appears that the intermediate carbonium ion is stabilised particularly well by a resonance from (XXIX)

(XXIX)

It is to be expected that solvolysis of p.- hydroxylbenzyl halides would be extremely rapid. That substituted p - hydroxybenzyl halides undergo solvolysis very readily has been known for some time; the compounds (XXX) and (XXXI) were found in 1902 to undergo replacement



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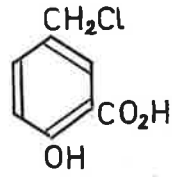
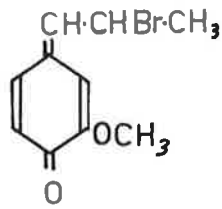


(XXXI)

of the ∝-halogen by hydroxyl in aqueous acetone or by alkoxyl in methanol or ethanol in the cold (103,104). In alkaline solution, (XXXI) forms a "quinone-methide" formulated as (XXXII) (105)



(XXXII)

The reactions of compounds of such structures have been investigated by chemists working on lignin; short reviews have been given by Adler (106,107). It has been

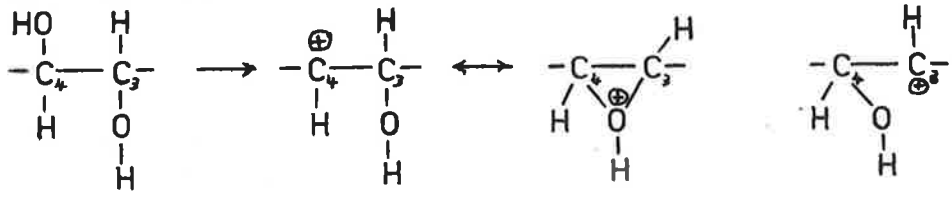
known for some time that lignin, in acidic alcohol, undergoes an increase in number of alkoxyl groups. This has been studied for the last 20 years, and variously ascribed to acetal formation from ketones, conversion of hemiacetals into acetals, and replacement of p - hydroxybenzylic alcohol hydroxyls by alkoxyls. The last is now accepted and the reaction has been shown to proceed under very mild conditions e.g. 0.5% methanolic hydrogen chloride at room temperature for 48 hours gave apparently complete reaction (106).

Such a p - hydroxybenzyl alcohol grouping is present in melacacidin, and presumably also in the accompanying leucoanthocyanidin, isomelacacidin, (An o - ether group is also present). It is therefore to be expected that melacacidin and isomelacacidin would give 4-alkoxy compounds with alcoholic acids. The experimental work shows that, in boiling ethanol containing acetic acid, only isomelacacidin does so to any extent, melacacidin remaining unchanged. It seems that this difference in rate must be the effect of a stereochemical difference.

The sulphones obtained from p - toluenesulphinic acid and melacacidin (treated with strong acid) and O-ethyl isomelacacidin (treated with weak acid) were shown to be identical. This confirms the paper chromatographic evidence of the identity of the intermediate leucoanthocyanidin, and shows that melacacidin and O-ethyl

isomelacacidin have identical stereochemistry except at the position or positions affected by acid. Of the three centres of asymmetry, the one at C_4 is most likely to be affected, the hydroxyl or alkoxyl group, being removed after the addition of a proton. The experimental evidence shows that the rates of conversion of both compounds into isomelacacidin are increased by increased acid concentration.

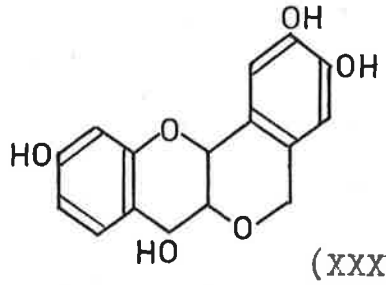
The stereochemical difference between melacacidin and isomelacacidin must therefore be sought at C_4. This is confirmed to some extent by the catalytic hydrogenation work, both melacacidin and O-ethyl isomelacacidin giving the same product, as judged by paper chromatography, this product being apparently the flavan-3-ol obtained by hydrogenolysis of the groups at C_4. Melacacidin has been shown to be a <u>cis</u> - glycol (33); isomelacacidin must therefore be the <u>trans</u>-glycol of opposite configuration at C_4. The greater reactivity of isomelacacidin may be explained by the participation of the 3-hydroxyl group in the reaction intermediate (XXXIII↔XXXIV), (written here as the carbonium ion). An intermediate of partial structure similar to (XXXIV) has been postulated to occur



(XXXIII) (XXXIV) (XXXV)

in the reaction of trans-1-bromo-2-methoxycyclohexane with silver acetate in dry acetic acid which gives a product with the same configuration at C_1 as the bromo - compound (108). The intermediate (XXXIII ⟷ XXXIV) is of a unique type in that the two carbon atoms of the epoxide structure differ greatly in power to sustain a positive charge, and the form (XXXV) would contribute very little to the stability of the intermediate, the main contribution being probably from forms in which the positive charge is dispersed over C_4, the p - hydroxyl and the O-ether oxygen atoms. The p - methoxystilbeneepoxides are of interest in this connection; it appears that in some conditions opening of the epoxide ring proceeds via an intermediate containing the partial structure (XXXIII) and cis - glycol derivative is formed (109). However, the product of nucleophilic attack on the intermediate (XXXIII ⟷ XXXIV) from isomelacacidin would probably take place from the less hindered side, resulting in a trans-compound. As O-ethyl isomelacacidin is formed by the attack of an ethanol molecule, it is formulated as the trans-compound (XXI).

A similar epimerisation at C_4 occurs with peltogynol and peltogynol B (75) which have the structure (XXXVI). When peltogynol was heated with aqueous oxalic acid, a

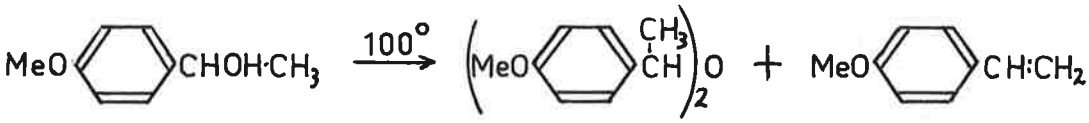


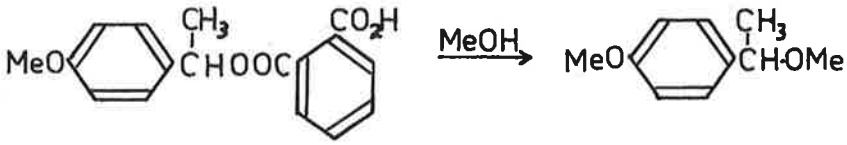
(XXXVI)

small proportion of peltogynol B was isolated. It is not clear if the mixture had reached equilibrium or if peltogynol B may be converted back into peltogynol.

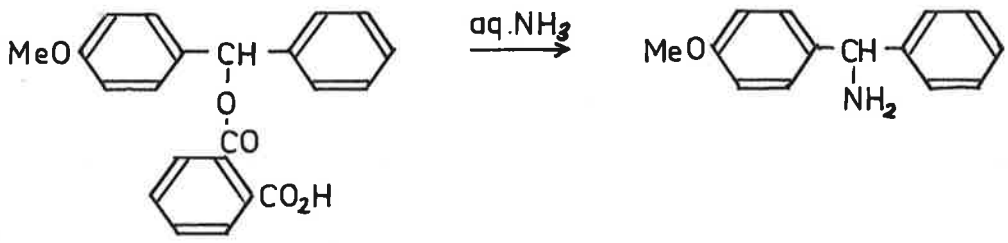
A synthetic 8,3',4' - trimethoxy-flavan-4-ol exists apparently as a mixture of epimers giving a single acetyl derivative (110).

Compounds containing p - (and o-) methoxybenzyl alcohol groups have been much studied by Kenyon (111). The esters of such compounds are hydrolysed by fission between the alkyl carbon and the ester oxygen. If sufficient groups are present to contribute towards the stability of an intermediate carbonium ion, other evidence of carbonium ion behaviour may be obtained, e.g. formation of the ether from the carbinol, or from an ester in an alcohol (112), formation of an amine from



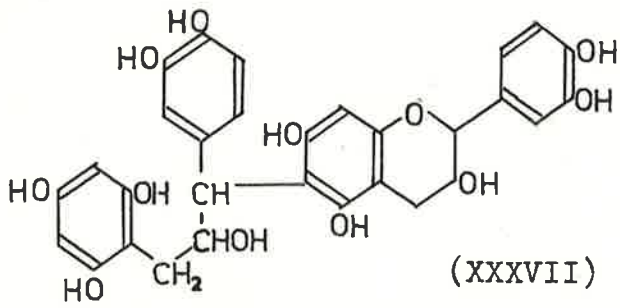


an ester and ammonia (113) and, in the case of



2,4,6-trimethoxydiphenyl-methanol alkylation of ethyl aceto-acetate (114). Kenyon has shown that such compounds react with p - toluenesulphinic acid forming sulphones; the reaction is reversible and is demonstrable when conditions are such that the sulphone is deposited from the mixture.

The tendency for p - hydroxy - and p - methoxybenzyl alcohols to react with nuleophilic reagents is of great interest in connection with the occurrence of "condensed tannins". For many years, these have been considered to be derived from flavan-3-ols (35,36,37). A summary is given by Freudenberg and Alonso (115) who describe the isolation of a "catechin dimer" as its acetyl derivative from the action of acid on (+)-catechin. The



(XXXVII)

structure assigned to this compound is derived from Freudenberg's idea that in 7,4' - dihydroxyflavans, the benzylic ether is readily cleaved, giving an intermediate reactive at C2 which then attacks a second molecule at C6 or C8. In this paper, Freudenberg suggested that flavan-4-ols would also react (as the electrophilic reagent) at C4. This type of condensation has been

studied recently by B. R. Brown (116) with simpler benzyl alcohols and phenols. The formation of "phlobaphene" type compounds from the action of acid on melacacidin was observed by King and Bottomley (33) who pointed out its significance.

It is to be expected that a carbonium intermediate such as that from isomelacacidin would not only condense to form carbon-carbon linkages, but would also form ethers. It is unlikely that carbon-carbon bonds would be cleaved by acid, with the formation of monomeric anthocyanidins, but, as with O-ethyl isomelacacidin, ethers would be cleaved. Condensed tannins, such as those of A. mollissima (39) often yield appreciable amounts of monomeric anthocyanidin on treatment with acid and the C_{15} units responsible must be present with either hydroxyl or alkoxy (aryloxy) groups at C_4.

Besides the O-ethyl derivative, O-methylisomelacacidin was isolated crystalline. Both ethers showed mutarotation in acetone-water and dioxan-water on the addition of acid. The mutarotation in dioxan-water was complex; the optical rotations first increased (became more negative) then decreased. It was therefore not possible to fix upon a definite figure for the molecular rotation of the product in this solvent. In acetone-water, the products at equilibrium had a molecular rotation of -232° (from O-methyl isomelacacidin) and -218° (from O-ethyl isomelacacidin). The molecular

rotations of melacacidin, O-methyl isomelacacidin and O-ethyl isomelacacidin are given in Table 2:

Table 2.

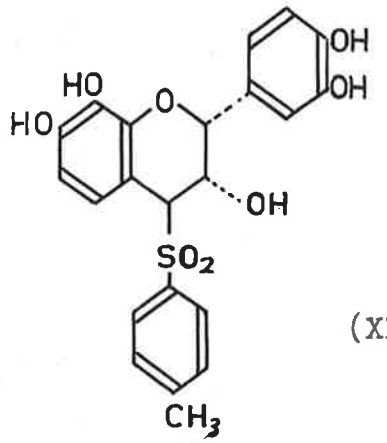
Molecular rotations of leucoanthocyanidins.

	Ethanol	Acetone-water	Dioxan-water
Melacacidin	-229°	-260	-229
O-Methyl isomelacacidin	-196 (methanol)	-236	-179
O-Ethyl isomelacacidin	-102	-124	-123

As has been mentioned above, a sulphone was obtained from both O-ethyl isomelacacidin and melacacidin, using p - toluenesulphinic acid in dilute, weakly acid solution. It gave the following crystalline derivatives; penta-acetate, tetramethyl ether and tetramethyl ether acetate. The phenolic sulphone therefore has four phenolic hydroxyl groups and one alcoholic hydroxyl. On heating with acid, the sulphone gave an anthocyanidin; the flavan structure is therefore present. The position of the phenolic hydroxyls must be at 7,8,3',4' and one of the alcoholic hydroxyl groups of melacacidin has been replaced by the sulphone group.

The preparation of the sulphone was carried out under conditions in which alkylation of the sulphinic acid by a p - hydroxybenzyl alcohol would be expected, and as there is no reason to expect reaction at C3, it seems

reasonable to assume that in the sulphone, the alcoholic hydroxyl group is at C_3 and the sulphone at C_4. (The alkylation of sulphinic acids to form sulphones is reviewed in Houben-Weyl (117)). The sulphone is therefore a p - tolyl 7,8,3',4' - tetrahydroxy flavan-3-ol-4 - sulphone. The sulphone group probably enters the reactive intermediate (XXXIII ↔ XXXIV) trans - to the C_3 - hydroxyl group as with solvolyses leading to isomelacacidin and O-ethyl isomelacacidin. The configuration of the sulphone is therefore probably (XXXVIII)



(XXXVIII)

The sulphone was notable for its crystallinity and the ease with which it could be decolourised with carbon, in contrast to melacacidin and O-ethyl isomelacacidin.

CATALYTIC HYDROGENATION OF LEUCOANTHOCYANIDINS.

It was found that melacacidin and O-ethyl iso-melacacidin could be reduced using palladium in acetic acid at elevated temperatures and pressures. Both tended to give a mixture of products; in each case, the major product appeared, from paper chromatography, to be the same flavan-3-ol. This was not obtained crystalline.

The most successful experiment was carried out on a non-crystalline mixture of leucoanthocyanidins; the compound sought was separated by counter-current distribution, and although neither the polyphenols or its methyl ether could be obtained crystalline, a small yield (0.63g. from 10.45g. leucoanthocyanidins) of a crystalline tetramethoxyflavan-3-ol toluenesulphonate was obtained. As the polyphenol mixture used in this hydrogenation was not crystalline, the possibility exists that the flavan-3-ol was originally present as such in the mixture. Hydrogenation of melacacidin and conversion of the crude product to the methyl ether toluenesulphonate gave an impure sample of this toluenesulphonate.

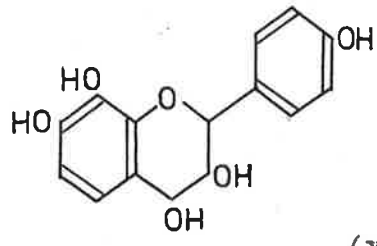
An attempt was made to increase the specificity of the hydrogenation by addition of a small quantity of mineral acid. This has been used to assist the palladium-catalysed reduction of tetracycline to

6-deoxytetracycline (120a) which consists of removal of a hydroxyl group from a p - hydroxybenzyl structure. In the present instance, reduction of melacacidin was accelerated, and the formation of by-products substantially eliminated, provided the amount of added acid (hydrochloric) was not too great.

Catalytic hydrogenation has often been used for the preparation of flavan-4-ols. Examples are: 7,8,3',4' - tetramethoxyflavonol to ( ) - melacacidin tetramethyl ether, with nickel (64); dihydrorobinetin to a 7,3',4',5' - tetrahydroxyflavan-3, 4-diol, with platinum (118); flavanonol to a flavan - 3,4-diol (119), and flavanone to flavan-4-ol (120) with palladium. The last compound suffered cleavage of the pyran ring on further hydrogenation. An interesting series of examples is provided by Freudenberg and Weinges (84). 5,7,3',4' - Tetrabenzyloxyflavanonol acetate (from taxifolin) was debenzylated with palladium on barium sulphate in dimethylformamide in 72% yield, and the corresponding tetrabenzyloxyflavan-3, 4 - diol was also smoothly debenzylated under these conditions to a 5,7,3',4' - tetrahydroxyflavan-3, 4 - diol, but with palladium without support in dioxan, hydrogenation gave ( ) - catechin. Hydrogenation of 5,7,3',4' - tetrabenzyloxyflavan-3, 4 - diol diacetate gave, under both conditions, ( ) - catechin 3 - acetate.

TERACACIDIN.

This leucoanthocyanidin was isolated from *A. intertexta* heartwood by countercurrent distribution of the polyphenols after they had been heated in acidic ethanol. The phenolic leucoanthocyanidin was not obtained crystalline. It gave a nonphenolic trimethyl ether $C_{18}H_{20}O_6$ which was shown by the standard degradation to contain methoxyl groups at positions 7, 8 and 4'. The distribution and chromatographic behaviour of the polyphenol, in particular its Rf in butanol: acetic acid:water and the Rf of its anthocyanidin in Forestal solvent, suggest that the polyphenol contains three phenolic hydroxyls (i.e. no methoxyl groups). As teracacidin gave an anthocyanidin when heated with acid, it is a 7,8,4'-trihydroxyflavan-3,4-diol (XXXIX).



(XXXIX)

This particular hydroxylation pattern does not appear to have been reported in a natural product (cf.121).

Three leucoanthocyanidins were present in the extracts from A. intertexta; these appeared to be inter-related in the same way that melacacidin, isomelacacidin and O-ethyl isomelacacidin are inter-related. Thus isoteracacidin was converted into the O-ethyl derivative, permitting isolation of teracacidin. An attempt to obtain crystalline O-ethyl isoteracacidin failed, either because of the presence of other polyphenols or because the mixture still contained acid which resulted in hydrolysis. However the solution gave a crystalline sulphone, characterised as the tetra-acetate.

As is to be expected from the occurrence of the unusual hydroxylation pattern in the A ring in both series, the teracacidin - series leucoanthocyanidins were qualitatively closely analogous to the melacacidin-series, and it seemed that the stereochemistry of teracacidin might exactly duplicate that of melacacidin. The optical rotation figures available (Table 3) are compatible with this assumption.

Table 3.

Molecular rotations.

	Melacacidin series	Teracacidin series
Methyl ether	-309°	-214
Iso-sulphone	-118	-110
Acetylated iso-sulphone	-88	-86

4 - HYDROXYPIPECOLIC ACID.

This compound was isolated from A.excelsa heartwood and subsequently from A. mollissima heartwood and sapwood. It was then found to occur in the leaves of A. Oswaldii F.v.M., from which it was readily isolated. The amino-acids were nitrosated and the N - nitroso imino-acids were extracted with ether as described by Witkop and Foltz (122). Crystallisation of the recovered imino-acids from aqueous ethanol gave 4-hydroxypipecolic acid.

This acid had been reported to occur in Acacia leaves by Virtanen and Kari (46) but had not been adequately characterised, a melting point (270° decomp. after turning brown at 250°), the ninhydrin colour (yellow changing to blue) and its Rf values in various solvents, being reported. As the imino-acid from A. excelsa had m.p. 294° decomp., and gave with ninhydrin, followed by heating at 100-110° a brown-grey colour, it was at first thought to be a new amino-acid. The occurrence in Armeria maritima of the same compound as Virtanen's was reported by Fowden (123). The amino-acid from Armeria maritima was chromatographically indistinguishable from the acid from A. excelsa when examined by Dr. Fowden and by me. The ninhydrin and isatin colours were identical; the colour from 4 - hydroxy-

pipecolic acid and ninhydrin was found to vary with the temperature at which the paper was heated, being a yellow-brown after heating at about 80°, and developing a purple shade at higher temperatures.

Determination of gross structure.

Virtanen and Kari (46) showed that the amino-acid was a hydroxy-pipecolic acid by detecting pipecolic acid on a chromatogram of the products from reaction of the amino-acid with hydriodic acid and red phosphorus. As they were able to detect aspartic acid on a chromatogram of the products of the oxidation of the amino-acid with permanganate in dilute sulphuric acid, whereas 5 - hydroxypipecolic acid gave glutamic acid under the same conditions, they concluded that the amino-acid was 4 - hydroxypipecolic acid. Fowden (123) also obtained pipecolic acid by reduction with hydriodic acid and red phosphorus, but detected among the products of oxidation with acid permanganate, γ-aminobutyric acid. Fowden synthesised both 3 - and 4 - hydroxypipecolic acids, and only the former yielded any γ-aminobutyric acid on oxidation, as was to be expected. Fowden therefore considered that the amino-acid was 3 - hydroxypipecolic acid.

That the amino-acid was 4 - hydroxypipecolic acid was readily shown in this work by comparison with samples of synthetic 3 - and 4 - hydroxypipecolic acids, and by epimerisation in barium hydroxide

solution at elevated temperatures (about 150°). The synthetic 3 - hydroxypipecolic acid did not correspond to either epimer, but the synthetic 4 - hydroxypipecolic acid was not separated from the cis-epimer on chromatography (1 solvent), and gave the same colours with ninhydrin and isatin. When epimerised, the synthetic 4 - hydroxypipecolic acid gave a mixture of epimers, similar to that obtained by epimerising the natural amino-acid. On the other hand attempted epimerisation of 3 - hydroxypipecolic acid destroyed the acid and gave a mixture of products. Wieland (124) showed that acyclic β-hydroxy - α - amino-acids, when heated with alkali under conditions similar to those used for epimerisation, gave a mixture of products resulting from a retro-aldol reaction, transamination and dehydration. That the natural amino-acid epimerised without formation of other products was therefore further evidence that it was not 3 - hydroxypipecolic acid. 3 - Hydroxypipecolic acid also gave a different colour with ninhydrin.

The amino-acid from A. excelsa was shown to be different from 5 - hydroxypipecolic acid by paper chromatography. With phenol as a solvent, 5 - hydroxypipecolic acid is separated from both epimers (Virtanen and Kari showed that it was separated from the natural epimer). Also, as Virtanen and Kari noted, the

ninhydrin colours differ.

As Bragg and Hough (125) showed that proline reacted with periodate, consuming one mole in about three hours, the reaction of pipecolic acid and the amino-acid from *Acacia* with periodate was investigated, but the reaction proved to be too slow to be useful. Hydroxy-piperidines which have been found to react with periodate are the conhydrine isomers, (ethyl-2-piperidyl-carbinols) (126), and piperidin-3-ol, the latter reaction being carried out at 100° for the preparation of Δ^1-pyrroline (127).

Conclusive evidence that the amino-acid is 4 - hydroxy-pipecolic acid was obtained in the present work by decarboxylation of the amino-acid in acetophenone to piperidin-4-ol, identified by comparson with a synthetic sample by melting point, mixed melting point, I.R. spectrum, and the melting point and mixed melting point of the N - p - toluene-sulphonyl derivatives. This method of decarboxylation had been found by Chateleu (128) to be effective with acyclic unsubstituted and N - methyl amino-acids. The yield with the natural amino-acid, a hydroxylated, cyclic imino-acid, was much lower (37%), than that reported by Chateleu for his examples (80-95%). The mechanism of this decarboxylation reaction is not known.

The position of the carboxyl group was shown to be at C2 by the isolation of pipecolic acid on reduction

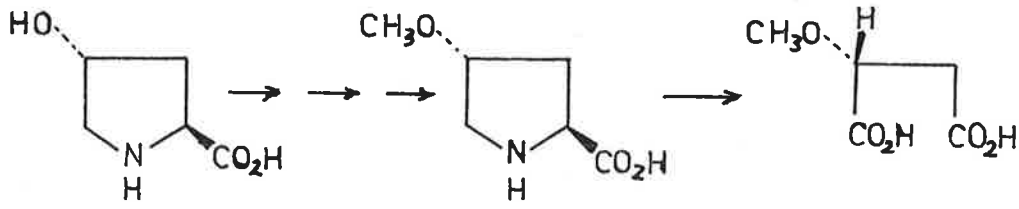
with hydriodic acid and red phosphorus, by the formation of a phenylhydantoin, and by the fact that the keto-amino-acid obtained by oxidation of the hydroxy-acid was stable, and therefore was not a β - keto-acid.

Relative configuration at C2 and C4.

Having determined that the amino-acid was a 4 - hydroxypipecolic acid, there remained the questions of the relative configurations of the carboyxl and hydroxyl groups, and the absolute configuration of the molecule. The amino-acid was optically active, and from comparison of its optical rotation in neutral and acid solution, it is thought to have the L - configuration at C_2; this point will be discussed later.

The problem of determining the relative configuration of 4 - hydroxypipecolic acid is similar to that with natural 4 - hydroxyproline and 5 - hydroxypipecolic acid, both of which have been shown to have the trans - configuration. The relative configuration of hydroxyproline was solved by Neuberger (129) in 1945 when he determined the absolute configuration at C_4. From biochemical work, and consideration of the change in optical rotation with change in pH of the solution, hydroxyproline was considered to have the L - configuration at C_2. With the absolute configurations at both centres known it was possible to deduce that the relative configuration was trans

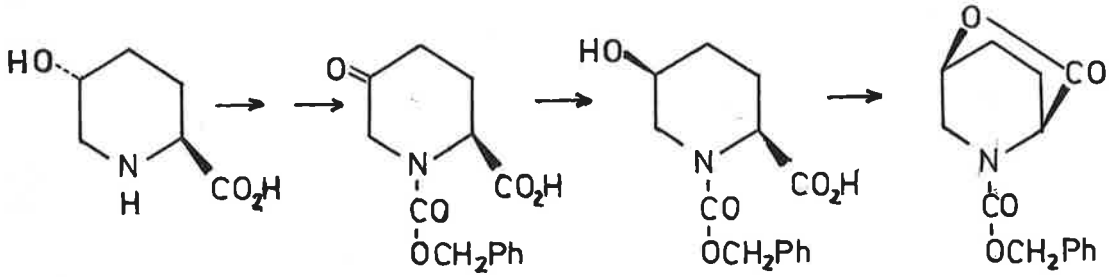
(cf.130) Neuberger converted hydroxyproline by a series of steps to its O-methyl ether, and oxidised this to methoxysuccinic acid which was isolated as the (+)-diamide. This degradation is of no use with 4 - hydroxypipecolic acid, as 3 - methoxyglutaric acid which would be the product, no longer contains a centre



of asymmetry. The piperidin-4-ol obtained by decarboxylation similarly provides no assistance, as it also contains no centre of asymmetry. (3 - Methoxyglutaric acid and its derivatives appear not to have been described; they could probably be obtained via 3 - chloroglutaric acid (131) and sodium methoxide).

That natural hydroxyproline was the trans-isomer was confirmed by Patchett and Witkop (132) who obtained the lactones of both N - carbobenzyloxyallohydroxy - L - proline and the D-isomer. The same procedure was used by Witkop and Foltz (122) to demonstrate that 5 - hydroxypipecolic acid from dates had the trans-configuration. The N - carbobenzyloxy-derivative was oxidised to the keto-acid, and reduced with sodium borohydride to the cis-epimer, which, with acetic anhydride, gave the lactone. The con-

figuration at C_2 was deduced from the change in



optical rotation with change in pH of the solution. The configuration at C_5 could also be deduced from the change of rotation on opening the lactone ring (133).

It seemed desirable to obtain the lactone of a derivative of cis-4-hydroxypipecolic acid in order to provide conclusive evidence on the relative configuration of the groups at C_2 and C_4 of natural 4-hydroxypipecolic acid. As stated above, synthetic 4 - hydroxypipecolic acid used in comparison of the hydroxypipecolic acids by paper chromatography which had been supplied by Professor Vanderhaeghe of Louvain, was found to correspond to the epimer of the natural acid. This compound was designated "allo" by Professor Vanderhaeghe, but no evidence on its stereochemistry has been published. There were two items of evidence which indicated that the natural compound was the trans-epimer; both derive from conformational analysis and require the assumption that in 4 - hydroxypipecolic acid, the carboxyl group is normally equatorial.

It was found that on chromatography using butanol:

acetic acid:water, the natural epimer had a higher Rf value than the allo-compound obtained by epimerisation in barium hydroxide solution. When a pair of epimer alcohols is chromatographed on paper, the epimer which is retained preferentially by the more polar phase is usually the epimer with the equatorial hydroxy group. This has been found to be the case with hydroxylated steroids (134, cf 135) and yohimbines (136). In the monohydroxycyclohexanecarboxylic acids, the epimer which would have the hydroxyl group axial if the carboxyl group were regarded as being equatorial, has the higher Rf value in ammoniacal butanol (137), although the differences in Rf values are very small. On this basis, the natural 4-hydroxypipecolic acid, having a larger Rf value than its epimer, would be considered to have its hydroxyl group axial; this implies a trans-configuration. However, in the solvent system which separated the epimeric 5 - hydroxypipecolic acids (collidine:lutidine 3:1) (138) the natural epimer had a higher Rf than the cis-compound, probably because of the nature of the solvent.

The second item of evidence that natural 4 - hydroxypipecolic acid has the trans-configuration is derived from the fact that reduction of 4 - oxopipecolic acid leads predominantly to the allo-compound. This reduction would be expected to give a

product with equatorial hydroxyl, whether considered as a relatively unhindered ketone to which Barton's "rule" (139) is to be applied or as a reaction which is "product development controlled" (140). This implies that the allo-acid is the cis-isomer. It has been found by Beyerman and Boekee (141) that reduction of 5-oxo-pipecolic acid with sodium borohydride gave, apparently specifically, trans-5-hydroxypipecolic acid, as expected. On the other hand, it was found by Witkop and Foltz (122) and confirmed by Beyerman and Boekee (141), that reduction of N - carbobenzyloxy-4-oxopipecolic acid gave the cis-isomer.

Efforts were therefore directed towards obtaining the allo-acid to attempt the preparation of the lactone from a derivative. The cis-epimer could be obtained from the trans-compound either by a specific inversion at C_4, or by attainment of an equilibrium at C_2 which would result in a mixture of epimers requiring separation. Such a mixture was conveniently obtained by heating N - benzoyl - 4 - hydroxypipecolic acid and hydrolysis of the product. However, it was found that separation using the copper salts, an efficient procedure when applied to a mixture of epimeric hydroxyprolines (142, 143) could not be achieved, as the copper salt crystallising from the mixture contained both epimers, apparently in equal proportions.

Preparation of the allo-acid, by oxidation of the natural acid to the keto-acid, followed by reduction, was investigated, and, after some exploratory work, the keto-acid was obtained in 36% yield; reduction with sodium borohydride gave *cis* - 4 - hydroxypipecolic acid in 78% yield.

The oxidation to the ketone was carried out on the free amino-acid as the determination of suitable reaction conditions, and the isolation of the product could then be assisted by paper chromatography. In other cases where the *cis* - hydroxy compound has been obtained *via* the ketone, namely with hydroxyproline (132) and 5 - hydroxypipecolic acid (122), it has been necessary to work on *N* - substituted acids because, as shown by Kuhn and Osswald (144), the group $-CO-CH_2-NH-$ as in 4 - oxoproline exists largely in the tautomeric form - $C(OH) = CH-NH-$ and confers reducing properties on the molecule. Witkop and Foltz (122) found that even *N* - benzyloxycarbonyl - 5 - oxopipecolic acid was unstable. In this work it has been found that 4 - oxo-pipecolic acid is quite stable in acid solution, but shows slight decomposition in neutral and alkaline solutions.

The oxidant chosen was chromium trioxide which has been used previously with amino-acids in at least two cases; Baker *et al* (145) used it in the preparation of pyrrolidine-2-acetic acid and Olomucki and

Marszak (146) for the oxidation of 4 - dimethylamino-butan-1-ol, 1 - dimethylaminoethanol and 4 - dimethylaminobut - 3 - yn - 1 - ol to the corresponding acids. These workers do not report any difficulty due to chromium ions; crystallisation of 4 - oxo-pipecolic acid appeared to be hindered by the presence of chromium, which formed a purple co-ordination compound. Removal of chromium ions was achieved by fractional elution from an ion-exchange column.

A large excess of oxidant was used in order that oxidation of 4 - hydroxypipecolic acid to the ketone should be complete although this precaution was probably unnesessary as the keto-acid was crystallised as the hydrochloride which would leave any 4 - hydroxypipecolic acid in the mother-liquor.

The only other product of the oxidation that was detected was β-alanine, isolated in 2.2% yield as N - phenylcarbamoyl- β - alanine.

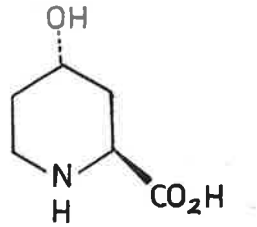
The reduction of 4 - oxopipecolic acid with sodium borohydride yielded both epimers, in the proportion cis : trans about 10-20 to 1 . The cis-acid was readily obtained pure by crystsllisation.

Attempts to obtain a lactone from N - benzoyl - cis - 4 - hydroxypipecolic acid were unsuccessful.

Absolute configuration.

The absolute configuration of (-) - pipecolic acid

is known to be L - , as (-) - baikiain, which gave (-) - pipecolic acid on hydrogenation, has been converted into a derivative of L - aspartic acid by oxidation (147). The absolute configuration of L - 5 - hydroxy-pipecolic acid has been deduced (122) from application of Lutz and Jirgenson's empirical rule, which states that the optical rotation of an α-amino-acid of the L - configuration becomes less negative on protonation of the carboxyl group. The applicability of this rule has been examined by Winitz, Birnbaum and Greenstein (148), and its application to 4 - hydroxypipecolic acid appears to be justifiable. As its optical rotation is made less negative on addition of acid, natural 4 - hydroxypipecolic acid therefore has the L - configuration at C_2, and is completely defined as (XL).



(XL)

The molecular rotations for the various pyrrolidine- and piperidine - α - carboxylic acids of the L - configuration in water and in acid solution are given in Table 4. In these figures,

Table 4.

Molecular rotations in water and in acid.

	$(M)_D$ water	$(M)_D$ 1 equiv. acid	$(M)_D$ 5N-HCl	Change water→ acid	
Proline	-99.9	-71.0		+28.9	(a)
Hydroxyproline	-99.6		-66.2	+33.4	(b)
cis-4-Hydroxy-proline	-78.0		-24.7	+53.3	(b)
Pipecolic	-32.5		-13.5	+19.0	(c)
4-Hydroxy-pipecolic	-18.9		+3.8	+22.7	(c)
cis-4-Hydroxy-pipecolic	-31.5		+12.9	+44.4	(c)
5-Hydroxy-pipecolic	-33.5	-19.8		+14.7	(d)
cis-5-Hydroxy-pipecolic	-45.0	-16.7		+28.3	(d) (e)

(a) At 20°C, calculated from (149); (b) at 25°C (148); (c) at room temperatures (20°-25°C), this work; (d) at 20°C, (122); (e) hydrobromic acid used.

there exists the regularity that the change in molecular rotation is greater with the cis - than with the trans - compounds. This confirms the assignment of the trans - configuration to natural 4 - hydroxy-pipecolic acid.

The hydroxypipecolic acids differ from hydroxy-proline in that, with the hydroxypipecolic acids, the cis - acids have the more negative rotation in water whereas, with hydroxyproline, the trans - form has the more negative optical rotation. This is illustrated in Figure 3, where the molecular rotations for aqueous solutions are given. (The

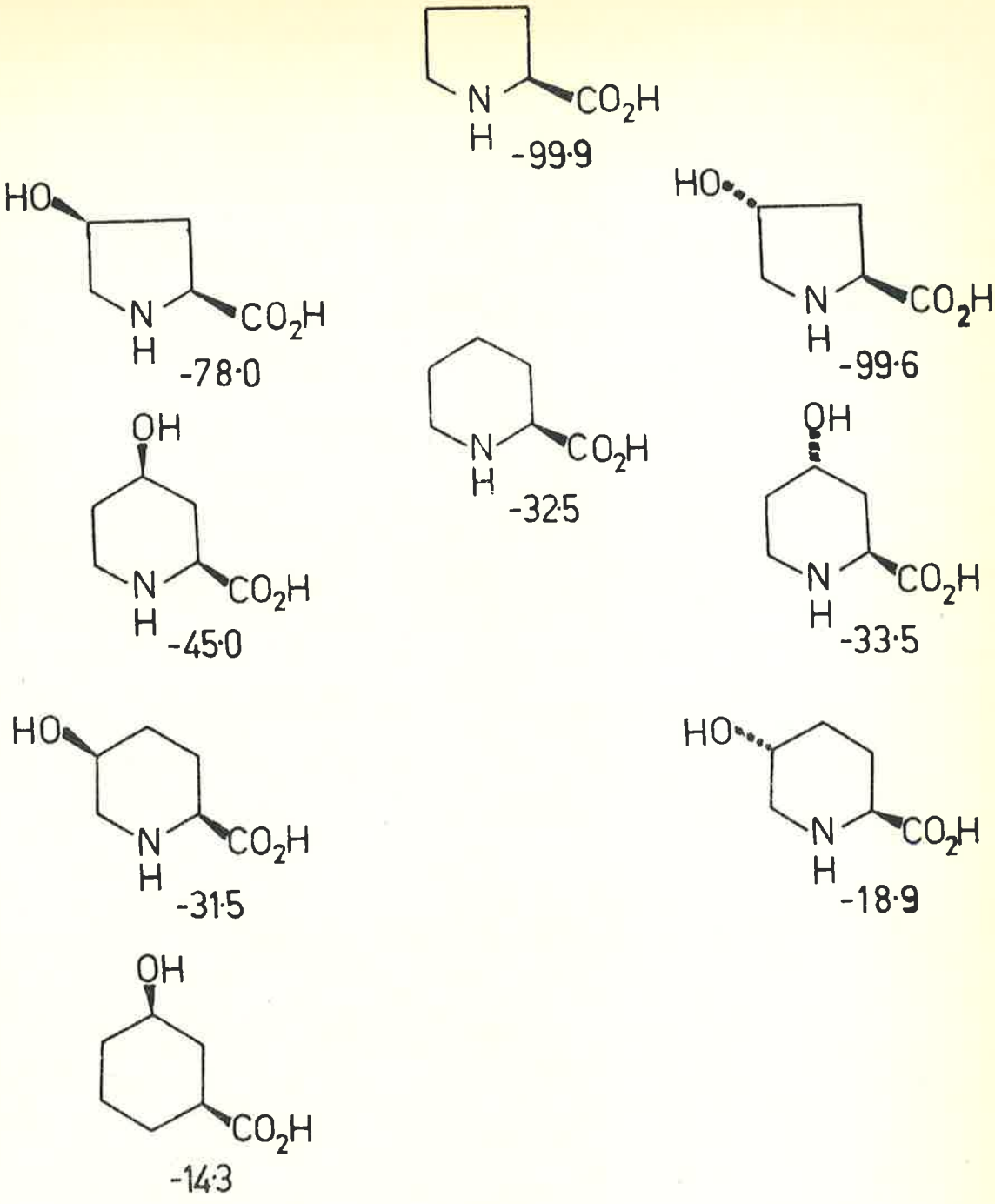


Figure 3 : Molecular rotations

molecular rotation of (-) - cis - 3 - hydroxycyclohexanecarboxylic acid is for methanol and ethanol (150).

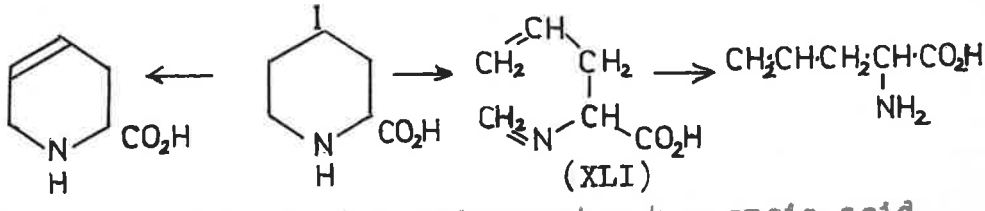
The reaction of 4 - hydroxypipecolic acid with hydriodic acid and red phosphorus.

As found by Virtanen and Kari (46), and by Fowden (123), the mixture resulting from this reaction contained pipecolic acid and two components having a higher Rf in butanol:acetic acid, considered in both cases to be iodine-containing amino-acids. Fischer (151) isolated (±) - proline from the reaction of hydroxyproline with hydriodic acid and red phosphorus, followed by treatment of the products with sodium amalgam to remove iodine - containing compounds which, according to Leuchs and Felser (142) amounted to about 14% calculated as mono-iodo-prolines. In this work, pipecolic acid was isolated without a dehalogenation step, but it proved to be racemic. The yield was small (8.5%). Neither Leuchs and Felser (142) nor Fischer (151) quote yields of proline from hydroxyproline.

The two products of high Rf did not give a positive test for ionic iodine (nitrous acid, then starch) but with silver nitrate they formed brown spots. When the products from the hydriodic acid and red phosphorus reduction were treated with excess silver carbonate, the two products of high Rf disappeared, and the

mixture contained baikiain and 2 - aminopent - 4 - enoic acid ("allyl - glycine") identified by comparison with authentic samples by paper chromatography in two solvent systems which gave different orders of Rf values, and by their colours with ninhydrin and with isatin. These colours were distinctive, particularly that given with ninhydrin by baikiain which varied from a yellow to a grey-green. As the colour given by the reaction product always matched that given by a reference spot of baikiain, it seems that the isomer, 1,2,5,6 - tetrahydropicolinic acid, was absent. The identity of 2 - amino - pent - 4 - enoic acid was confirmed by catalytic hydrogenation of the mixture, which yielded 2 - aminopentanoic acid ("norvaline"), identified by paper chromatography.

The formation of these products is explained as the removal of the elements of hydriodic acid from epimeric 4 - iodo - pipecolic acids formed in the hydriodic acid mixture, either by <u>trans</u> - elimination when the iodine is axial, to give a tetrahydropicolinic acid or by a ring cleavage when the iodine is equatorial, to give methyleneaminopentenoic acid (XLI),

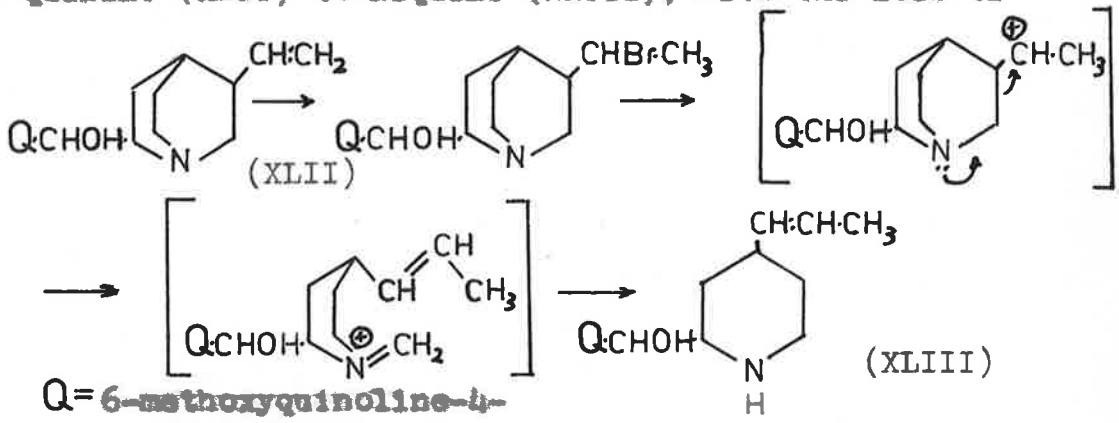


(XLI)

which decomposes to 2 - aminopent - 4 - enoic acid.

Apparently the carboxyl group influences the approach of the silver ion to the iodine atom in such a way that only the Δ^4- unsaturated pipecolic acid (baikiain) is formed.

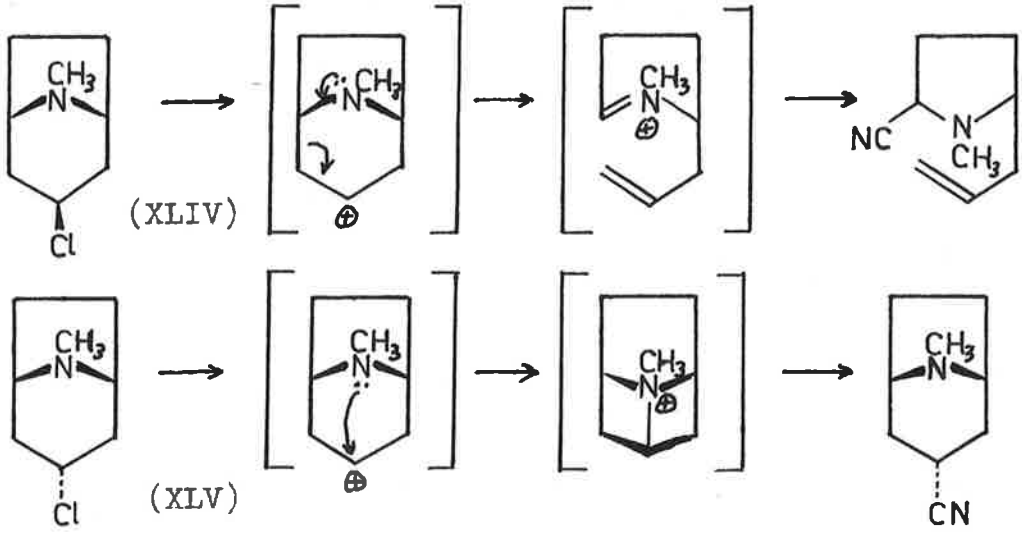
The above cleavage is of a type observed in many 1,4-halo-amines (152). An example is the conversion of quinine (XLII) to niquine (XLIII), with the loss of



(XLII) (XLIII)

Q= 6-methoxyquinoline-4-

one carbon atom. Archer (153) has shown that a similar reaction occurs with 3β-chlorotropane (XLIV), but not with 3α-chlorotropane (XLV), when treated with potassium cyanide in aqueous ethanol.



(XLIV) (XLV)

Investigation of the synthesis of 4 - hydroxypipecolic acid.

The first method of synthesis of 4 - hydroxypipecolic acid investigated was the catalytic hydrogenation of the corresponding pyridine carboxylic acid with platinum. As published methods of preparing 4 - hydroxypicolinic acid (4 - pyridone - 2 - carboxylic acid) (154) were unattractive, the benzyl ether was prepared by reaction of methyl 4 - chloropicolinate (155) with sodium benzyloxide. This benzyl ether was cleaved by hydrogenation under mild conditions, or when the hydrochloride was heated. In trials, it was found that hydrogenation of 4 - benzyloxypicolinic acid in strong acid yielded pipecolic acid, but in acetic acid or in water, some 4 - hydroxypipecolic acid (mainly cis -) was also formed; in ammoniacal solution, no hydrogenation occurred.

From hydrogenation of 4 - benzyloxypicolinic acid in water, a very small yield (1.4%) of crystalline (±) - cis - 4 - hydroxypipecolic acid hydrochloride was isolated; this method is obviously unsuitable as a preparation. Fowden (123) obtained about 3% of crude product in his synthesis of 4 - hydroxypipecolic acid by catalytic hydrogenation of 4 - hydroxypicolinic acid.

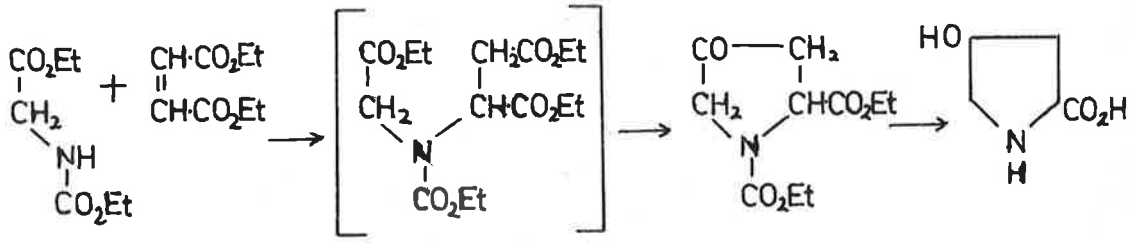
There are several reasons for the low yield in this hydrogenation. Firstly, the hydrogenation was not

complete; some 4 - hydroxypicolinic acid remained, and the solution darkened considerably in air, indicating the presence of partially hydrogenated products. Secondly, it is possible that some decarboxylation of 4 - hydroxypicolinic acid occurred. Thirdly, an appreciable amount of pipecolic acid (but probably less than 5%) was formed. This "hydrogenolysis" has been found to occur when 5 - hydroxypicolinic acid is hydrogenated in acidic, but not in ammoniacal solution (138). It occurs during catalytic hydrogenation of hydroxybenzoic acids (156); the proportion increased from 20% for the o - compound, through 68% for m - to 84% for the p - compound (137). An explanation of this hydrogenolysis is that allylic alcohols are present as intermediates during hydrogenation, and these would be susceptible to hydrogenolysis. This explanation is supported by the fact that methoxy - and acetoxybenzoic acids also suffer hydrogenolysis (156).

As stated above, the predominant isomer produced during the hydrogenation was the cis - isomer. This is to be expected, and could be used as evidence for the configuration of hydroxypipecolic acid. Hegarty (138) found that hydrogenation of 5 - hydroxypicolinic acid gave more cis - than trans - 5 - hydroxypipecolic acid when carried out in acid solution, but approximately equal amounts in ammoniacal solution.

It was stated by Balas and Crol (157) in 1929 that hydrogenation of 3 - and 4 - hydroxybenzoic acids with platinum gave mainly the cis - isomer of the hydroxycyclohexanecarboxylic acids, but later workers (e.g. 137) avoided a decision.

The second method of synthesis of 4 - hydroxypipecolic acid investigated followed that of hydroxyproline by Kuhn and Osswald (144). Inasmuch as the

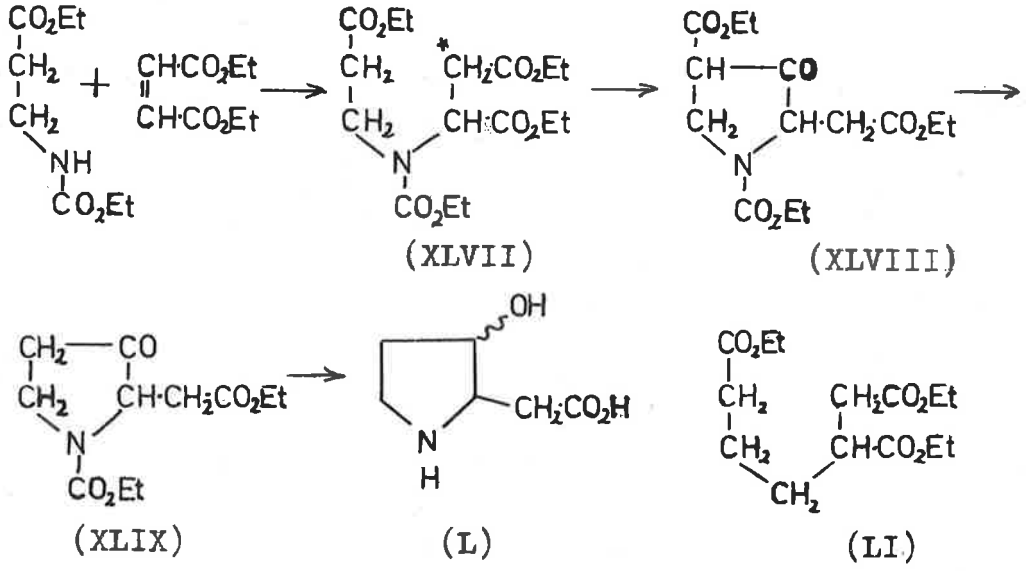


(XLVI)

initial step in this synthesis is probably addition of the amine to ethyl fumarate, to give (XLVI), the next step may be regarded as a Dieckmann cyclisation, first used to prepare a heterocyclic ring ketone by Ruzicka (158) and subsequently used as a general method by McElvain.

Reaction of the ethyl ester of N - ethoxycarbonyl - β - alanine with ethyl fumarate, followed by isolation of the β - keto-ester as its sodio derivative, and subsequent acid cleavage gave a ketone $C_{11}H_{17}O_5N$ which, by reduction and hydrolysis, gave an amino-acid $C_6H_{11}O_3N.H_2O$. This product was

chromatographically quite distinct from the 4 - hydroxypipecolic acids; as it did not give a purple colour with ninhydrin, and its N - phenylcarbamoyl derivative did not cyclise to a hydantoin, it was apparently not an α - amino-acid. It is therefore probable that the Dieckmann cyclisation gave a five-membered ring as (XLVlll), and the amino-acid obtained was one of the steroisomers of (L).



This result was unexpected as formation of the anion was expected to occur on the carbon atom marked * in (XLVll). It is possible that formation of the five-membered ring was favoured by the potential crowding in the six-membered ring. However, ethyl pentan - 1,2,5 - tricarboxylate (Ll) cyclises to a cyclohexanone (159).

GENERAL EXPERIMENTAL CONDITIONS.

Unless otherwise stated, melting points were determined in pyrex capillaries, and are not corrected.

Elementary analyses were by Dr. K. W. Zimmermann, C.S.I.R.O., Melbourne.

Optical rotations are calculated from the mean of six determinations, the scale being read at both left - and right-hand positions for each determination. Unless stated otherwise, a 1 dm. tube was used.

Paper chromatography:

Analytical paper chromatography was carried out on Whatman No. 1 paper using descending solvent flow. The paper was not equilibrated with the solvent. The chromatography tank was placed in a cupboard, but no other temperature regulation was used. The rate of solvent flow increased with temperature, but Rf values did not vary appreciably. In this report, the time the chromatogram was allowed to run and the distance of flow of the solvent front are given when important.

Much of the paper chromatography was done using Partridge's n - butanol:acetic acid:water 4:1:5 mixture (160). This solvent was found most convenient for use with polyphenols and amino-acids and gave useful information with carbohydrates and cyclitols. The abbreviation BAW will be used to describe

this solvent, which was made up immediately before use.

Rf values for amino-acids, carbohydrates and cyclitols are given with respect to the point of apparent maximum concentration, but the Rf values of polyphenols are given as the range from the rear of the spot to the front. This method of presentation has been chosen because it gives an indication of the length of a spot, which varied from compact (leucoanthocyanidins in BAW) to very long (7,8,3',4' - tetrahydroxyflavonol in BAW).

As a second solvent for the chromatography of flavanoids, 2% acetic acid (161) proved most satisfactory.

For the chromatography of anthocyanidins, the "Forestal" solvent (water:acetic acid:concentrated hydrochloric acid, 10:30:3) was used (162).

Detection of polyphenols on chromatograms.

The following reagents were used for detection and classification of polyphenols on chromatograms:

(a) Ferric chloride - 1% solution in ethanol.

(b) Ferric chloride - potassium ferricyanide reagent (163) was most useful as a spray or dip, detecting polyphenols of all types.

(c) Vanillin-HCl (about 1% vanillin in concentrated hydrochloric acid, the vanillin being brought into solution by dissolving

it in a small amount of ethanol), was used to detect polyphenols containing a phloroglucinol nucleus without ketonic substituent e.g. (+)-catechin, which give a red colour in the cold within five minutes. Leucoanthocyanidins gave a red colour visible after about 30 minutes in the cold. No vanillin - positive substances were detected in the Acacia heartwood polyphenols examined.

(d) Leucoanthocyanidins were initially detected by spraying with strong hydrochloric acid, (about 3N) followed by short heating (about 5 minutes at 100°). The texture of the paper was destroyed on heating and the paper charred if heating were prolonged. Roux's spray (3% p - toluenesulphonic acid in ethanol) (164) was as effective in locating leucoanthocyanidins, without such a deleterious effect on the paper. The latter spray was used for most of the work.

(e) Silver nitrate in acetone as a dip reagent, followed by an alkaline spray, was introduced by Trevelyan, Procter and Harrison (165) for the detection of polyols.

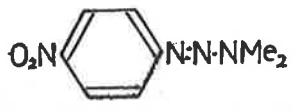
It was found that some polyphenols e.g. melacacidin, and (+) - catechin could be detected by a neutral silver nitrate spray, followed by exposure to light. It appeared that, for a positive result, the polyphenol required two or more hydroxyl

groups on a benzenoid ring. Leucoanthocyanidins gave a strong positive. The response of the polyphenol to neutral silver nitrate seemed to depend also on the presence or absence of the carbonyl group (C_4), flavans being apparently more readily oxidised than flavanones. The range and specificity of this effect were not explored.

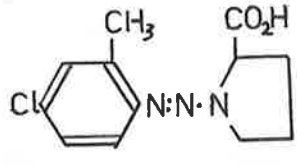
After examining the paper for substances reducing neutral silver nitrate, the paper was sprayed with alcoholic alkali (166). Some components e.g. reducing sugars, gave a positive spot without being heated, but other components e.g. pinitol required short heating.

(f) As a diazo reagent for phenols, diazotised sulphanilic acid (167) was used initially, but it was found more convenient to use bis-diazotised benzidine (168). This spray was not of much use in the polyphenol work.

As noted by Harris and Pollock (169) this reagent gave orange colours with imino-acids. In this connection, it is of interest that diazotised anilines give water-insoluble yellow compounds with secondary amines, that from diazotised p - nitroaniline and dimethylamine being (LII) (107). The structure of the



(LII)



(LIII)

compound from diazotised 5 - chloro - 2 - methyl-aniline and proline is reported to be (Llll) (171). The coloured products obtained from pipecolic and hydroxy-pipecolic acids rapidly diffused in the sprayed paper, forming large coloured areas. For this reason the reagent was not generally useful for detection of imino-acids.

Detection of amino-acids on chromatograms:

For amino-acids, the spray reagents used routinely were ninhydrin in ethanol or butanol and isatin in acetone. As the solvent usually used for chromatography was BAW, acetic acid was not added to these reagents. For spraying, butanol was more convenient than ethanol as a solvent for ninhydrin.

The colours given by 4 - hydroxypipecolic acids and other imino-acids with ninhydrin, isatin, and some other spray reagents are given in Table 22 (in section on 4 - hydroxypipecolic acid).

Origins of wood samples.

All wood samples except the Acacia mollissima sample were supplied by W.T. Jones, C.S.I.R.O., Brisbane. The A. mollissima sample was supplied by the South Australian Woods & Forests Department. Details are given in Table 5.

Table 5.

Details of origins of wood samples.

Sample Number	Species	Herbarium Number	Place of Origin	Date collected	Colour of Heartwood	Diameter of Heartwood
5583	harpophylla	54	Yarral Creek Goondiwindi	18.4.56	yellow-brown (a)	15cm
5919	"	500	Yuleba	21.11.57	"	10-15cm
5584	excelsa	55	Yarral Creek Goondiwindi	18.4.56	brown	20cm
5920	"	890	Yuleba	21.11.57	black	15cm
5905	Melanoxylon	481	Acacia plateau	21.7.57	brown	20cm
-	molliissima	-	--	-.12.57	pink	15-10cm
5891	intertexta	473	Whian Whian	12.7.57	light brown	Chips
5977	"	"	"	"	"	25-30cm

(a) Yellow fluoresence in UV light

Extraction apparatus and procedure.

The extractor used for solvent extraction of the wood material was a stainless-steel container, either 46 x 20cm or 45 x 15cm. totally enclosed within a stainless-steel shell, with a copper-coil water condenser at the top. The detachable bottom portion (17 x 22.7cm) served as boiler and as receiver for extract from the wood. A neoprene gasket was used between boiler and cover. The wood container had a

hole in the bottom and condensed solvent percolated directly through with minimum hold-up. As the container was completely surrounded by vapour of boiling solvent, the extraction was at the boiling point of the solvent. Heat was supplied by a water-bath for acetone or ether, or by a hot-plate for solvents of higher b.p.

The light pretroleum used had b.p. 60-80°. Light petroleum, acetone and ether were redistilled from a water-bath before use.

The wood samples were prepared for extraction by either of two methods. Initially, the wood was split into small pieces (smaller than a pencil) and ground in a "C & N" 8" laboratory mill. Most of the extractions were of wood which had been planed into shavings by a "buzzer". The amount of wood which could be packed into the extractor varied. Examples of loads for the larger container are 2.24kg. A. mollissima sapwood, 2.7kg. A. harpophylla heartwood, 1.8kg. A. melanoxylon heartwood, 2.0kg. A. intertexta heartwood. The smaller container held about half as much as the larger.

A solvent charge of 5-7 l. was required. Much of this was retained by the wood, and evaporated when the wood was left in thin layers overnight.

Counter-current distribution apparatus.

The apparatus used for counter-current distributions was a 50 - tube semi-automatic machine from J. H. Towers & Co. Ltd., Widnes, England (171A). In this machine, the bank of tubes is tipped manually for transfer. The volumes of moving phase and stationary phase were both 50 ml. The shaking and settling times used were 2½ minutes shake and 4 minutes settle.

Both phases were equilibrated before use.

Bush and Densen distribution.

In the work described in the section "isolation of Q-ethyl isomelacacidin; exploratory work" it was found that the distribution constants of the polyphenols between ethyl acetate and aqueous buffer pH 7.0 were such that it was possible to design a relatively simple Bush and Densen (83) distribution to obtain melacacidin and isomelacacidin, in the aqueous phase, and Q-ethyl isomelacacidin and ketonic flavonoids in the ethyl acetate phase.

With these solvents, the distribution coefficients were found to be as follows: melacacidin 0.56, isomelacacidin 0.39, Q-ethyl isomelacacidin 5.2. As most of the ketonic flavonoids had distribution coefficients similar to, or higher than, Q-ethyl isomelacacidin they would be carried into the ethyl acetate phase to the same extent as, or more than,

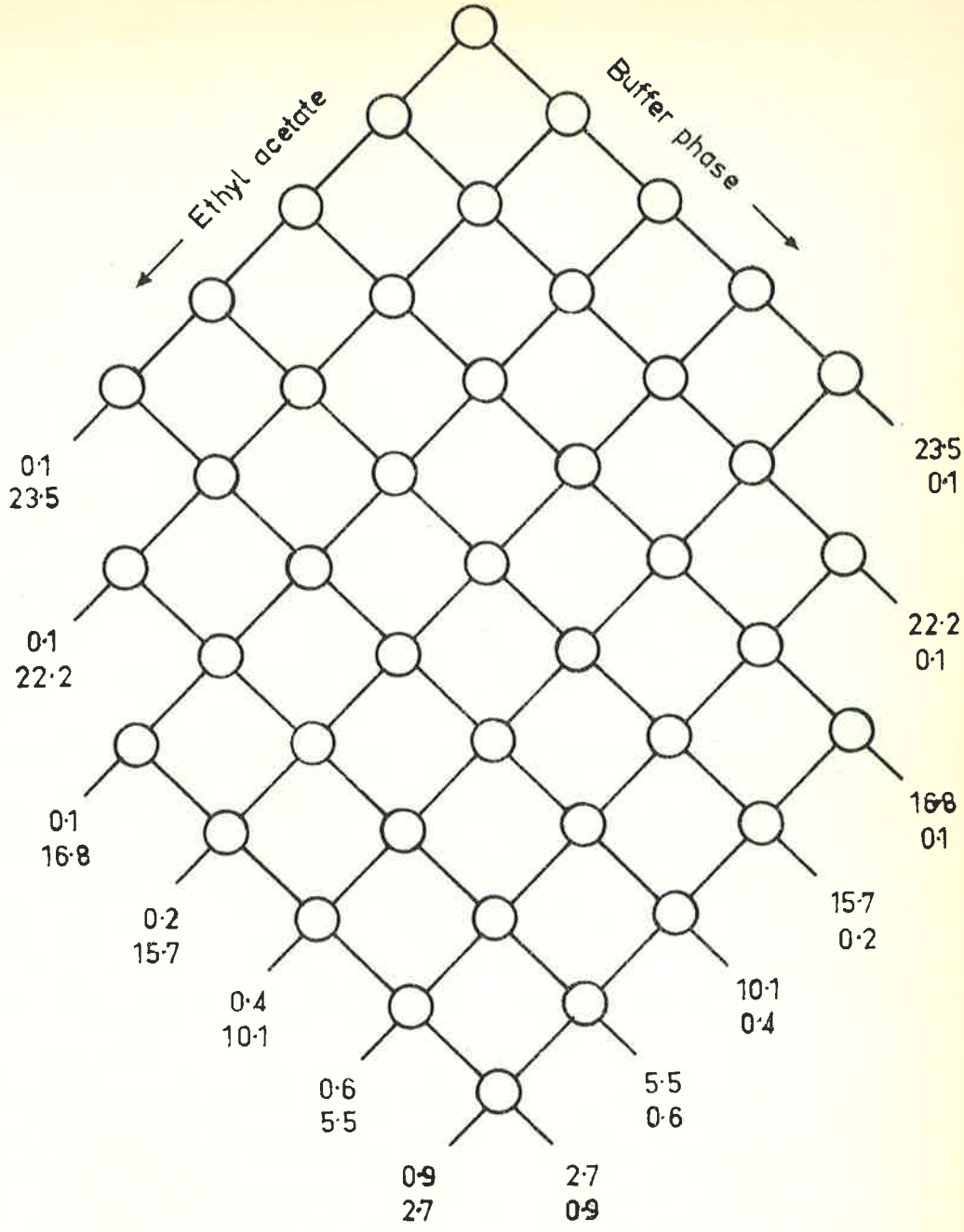
O-ethyl isomelacacidin. In order to obtain maximum separation of melacacidin (with isomelacacidin) from O-ethyl isomelacacidin, the phase volume ratio ethyl acetate: aqueous buffer 6:10 was used. The actual system used is shown diagramatically in Figure 4., with the calculated distributions of melacacidin and O-ethyl isomelacacidin.

This distribution was used repeatedly as an operation in processing heartwood extractives, and will be referred to subsequently as the "usual" Bush and Densen distribution.

Ion exchange resins:

Two cation exchange resins were used for isolation of amino-acids. Some work was done with a resin supplied by L. Light & Co. England. For most of the isolations and fractionations, Zeokarb 225 , a sulphonated polystyrene 8% cross-linked, standard size beads (particle size 14 - 52) supplied by Permutit Co. Ltd., Gunnersbury Avenue, London W4, was used.

In order to avoid blockage of columns by precipitation of acid - insoluble material in the strongly acid environment of the resin, extracts from wood were heated in a boiling water bath in dilute hydrochloric acid (0-5-1N) for 2 - 3 hours, cooled, filtered and evaporated to dryness under reduced pressure.



Phase volume ratio: $\frac{\text{Ethyl acetate}}{\text{Buffer}}$ $\frac{240}{400}$

Upper figure — calculated % of melacacidin in the fraction
Lower figure — calculated % of O-ethyl isomelacacidin in the fraction

Figure 4: Bush and Densen distribution for the separation of melacacidin and O-ethyl isomelacacidin.

The residue was dissolved in water and filtered from insoluble material. The acid treatment was repeated until no furtherinsoluble material was obtained on evaporation.

Table 6.

Extractions of wood samples.

Extraction	Species	Sample No.	Sap or Heartwood	Weight (g).	Page
A	harpophylla	5583	HW	2756	79
B	"	"	"	2407	91
C	"	"	"	2165	93
D	"	5919	"	8400	95
E	"	"	"	3970	98
F	"	"	SW & HW	10,350	101
G	excelsa	5584	HW	2094	104
H	"	5920	"	1905	107
I	"	"	"	2335	110
J	"	"	SW	1689	115
K	"	"	SW & HW	2470	117
L	melanoxylon	5905	HW	1293	119
M	"	"	"	1562	122
N	"	"	"	5934	125
O	"	"	SW	991	127
P	"	"	"	2781	129

Table 6 cont.

Extraction	Species	Sample No.	Sap or Heartwood	Weight (g).	Page
Q	mollissima	--	HW	1841	131
R	"	--	"	6788	135
S	"	--	"	6250	136
T	"	--	SW	7390	138
U	"	--	"	10,510	141
V	intertexta	5891	HW	579	141
W	"	5977	"	3132	147
X	"	"	"	2900	148
Y	"	"	"	3100	149
Z	"	"	"	2400	151

EXTRACTIONS OF WOOD SAMPLES.

A list of the extractions is given in Table 6.

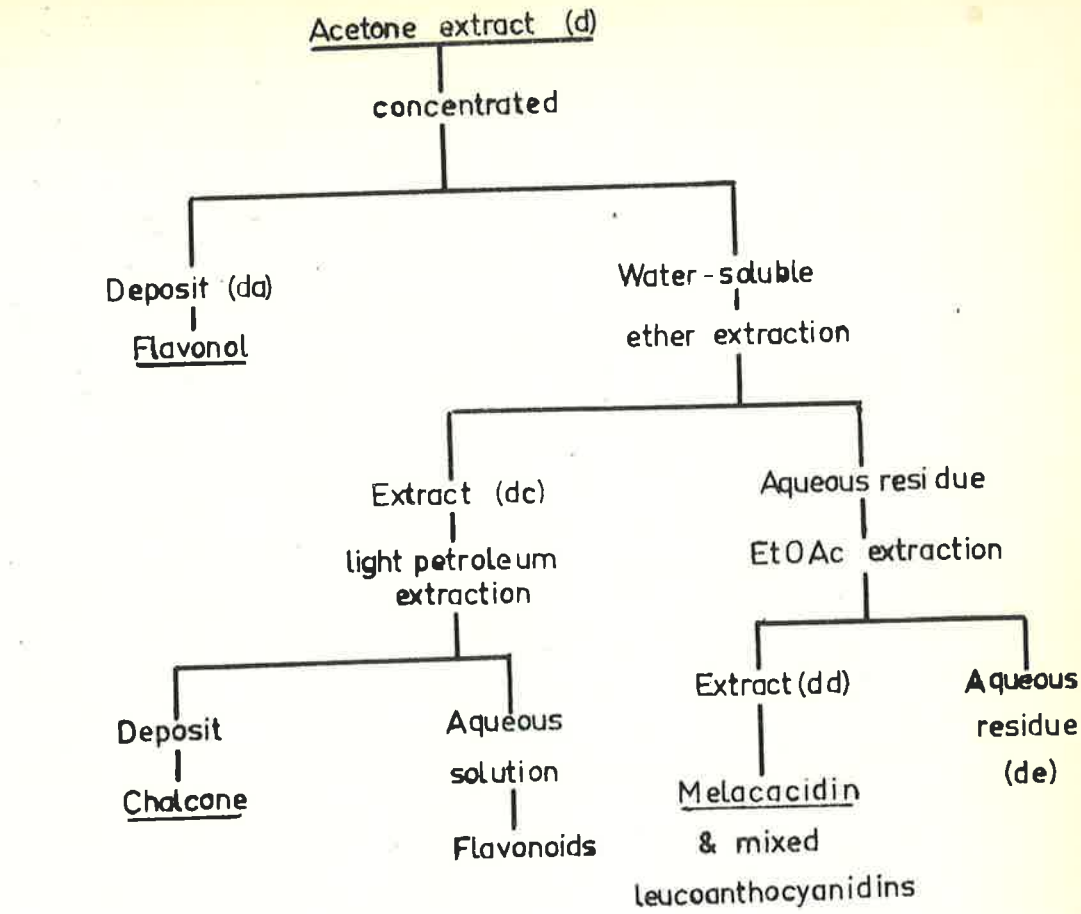
Extraction of A. harpophylla SN5583 heartwood.

Extraction A: (See figure 5). Milled heartwood (2,756g.) was extracted with light pretroleum for 10 hours. The cold extract contained a white deposit (4.8g.) and, on removal of solvent left an orange-coloured, soft wax (7.7g.). Ether extraction (31 hours) gave a brown deposit ((a), 8.4g.); the ether solution (about 2l.) contained 9.3g. of material (fraction (b)). Ether extraction for a further 36 hours resulted in no further deposit; the ether solution contained 9.0g. of material (fraction (c)). The wood was then extracted with acetone (7 hours, extract (d)), and with ethanol (7 hours, extract (e)).

Fraction (a), on paper chromatography (BAW, 12 hours, 24cm.) appeared to contain only two polyphenolic components:-

(a1) A compact spot Rf 0.25-0.33, reducing neutral silver nitrate, slowly giving a red colour with vanillin in hydrochloric acid (visible after 30 minutes), giving a khaki brown colour with ferric chloride, and a brown colour with diazotised sulphanilic acid. It was not visible under ordinary or UV light.

(a2) A streak Rf 0.25-0.44, yellow in visible light, fluorescing bright yellow in UV light, and re-



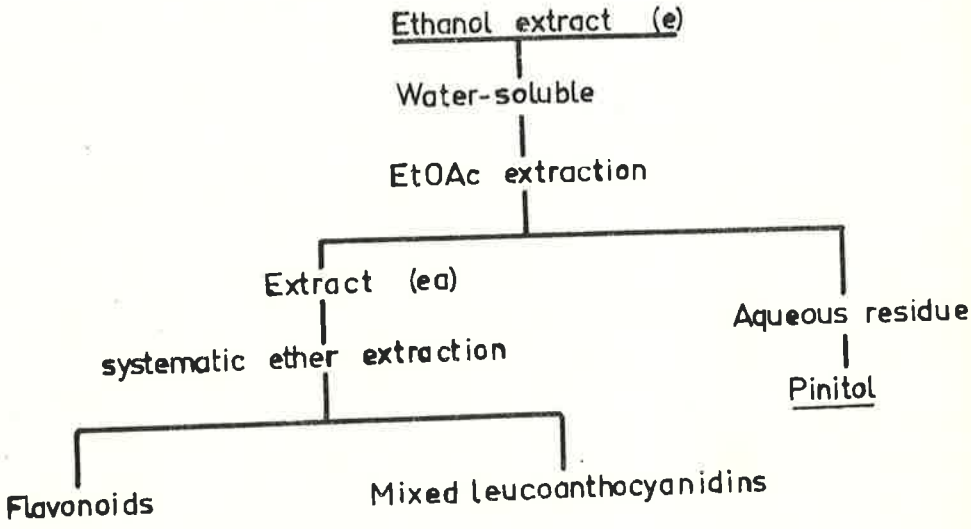


Figure 5: Extraction A (simplified)

ducing silver nitrate only when alkaline. The yellow colour was deepened by vanillin in hydrochloric acid, but other reagents gave negative results.

A portion of fraction (a) was boiled for a few minutes with 3N - hydrochloric acid, the deep red solution was extracted with isoamyl alcohol, and the extract was chromatographed as above. Substance (a1) was not detected, but (a2) was still present. The red colour was present as a faint spot Rf 0.35-0.40. Substance (a1) was therefore considered to be a leucoanthocyanidin.

Fraction (a) was extracted with hot water (3 lots of 20 ml each), then ethanol (50 ml). The brown aqueous extracts gave slight deposits on standing overnight (0.22g. in the first extract, very small in the others). After filtration, the solutions were charcoaled, but without removal of much colour. The solutions were then taken to dryness under reduced pressure giving 3.7,1.2,0.27g of material respectively. These fractions were combined, dissolved in ethanol (200 ml) and again charcoaled; the colour remained. The solution was taken to dryness under reduced pressure, leaving 3.7g. of brown powder. The colour was not removed by carbon from a solution of this material in ethanol:ether 1:1.

At this stage, paper chromatography (BAW, 28cm) showed two spots, Rf 0.31-0.41 and 0.69-0.77, which gave red colours when sprayed with concentrated hy-

drochloric acid and heated. These reduced neutral silver nitrate, and gave brown spots with diazotised benzidine. A portion of the material was methylated (2.74g. of polyphenol with methyl sulphate (5.5g.) and potassium carbonate (19.0g.) 45 minutes in boiling acetone; the excess of methyl sulphate was destroyed with ammonia); no crystalline product was isolated.

The water-insoluble portion of fraction (a) was recovered by diluting the ethanol extract with water (150 ml.) as a yellow-brown powder (1.2g.). Paper chromatography showed that it contained substance (a2).

Fractions (b) and (c) were found by paper chromatography (BAW) to be complex mixtures, containing components (a1) and (a2), a second component giving a red colour with acid (Rf 0.72-0.77) and other polyphenols Rf 0.4 to 0.8, not separately distinguished. These fractions were not further investigated.

The deep-brown acetone extract (d) was concentrated by distillation from a water-bath to about 220 ml. A deposit which formed was removed and washed with dilute acetone, then water (fraction (da), 5.9g.) The solution and washings were diluted to 2l. with water, and let stand. After several days, the solution was filtered (residue = fraction (db)) and concentrated to 400 ml. under reduced pressure. Continuous ether extraction for three 8 - hour periods yielded 21.1, 9.5,5.6g. of extractives (fraction (dc)). Ether was

then removed from the aqueous solution, and extraction continued with ethyl acetate. Successive 8 - hour periods yielded 4.3g. (fraction (dd)), 1.5, 0.2g. (fraction (de)). The residual aqueous solution contained 41g. of solids; it was discarded.

Fraction (da) appeared to be essentially a single substance, but it could not be recrystallised from methanol, ethanol, or acetone. The fractions obtained from methanol and acetone were yellow-brown powders, melting with decomposition in the range 290-210°, but the melting points were not reproducible. Paper chromatography showed the presence of substance (a2) only. By solution in hot ethanol (300 ml.), addition of water (100 ml.), treatment with carbon and concentration under reduced pressure to about 200 ml., there was obtained a yellow-brown powdery deposit (1.66g.). This was identified as 7,8,3',4' - tetrahydroxyflavonol (see below).

Fraction (db) was subjected to fractionation from methanol but no single compound was isolated. The hot methanol solutions gave tarry deposits on cooling, and gummy products on addition of water. Paper chromatography showed the flavonol to be present.

Fraction (dc) was obtained as a yellow powder. Paper chromatography showed that it was a complex mixture, components (a1) and the flavonol being present together with other polyphenolic components

Rf 0.3 to 0.7 (BAW). The fraction was dissolved in water (25ml) and the orange syrup was continuously extracted with light petroleum. In 24 hours, this removed 0.06g. of non-crystalline material. During the extraction, orange-red crystals formed in the aqueous solution. These were collected and washed with water (1.47g.). Recrystallisation by solution in ethanol (30 ml.) and addition of water (100ml.) gave loose aggregates of arange needles, m.p.238°. This was identified as okanin (3,4,2',3',4', - pentahydroxychalcone) (see below).

The aqueous solution after removal of the chalcone was continuously extracted with benzene for 96 hours. The extracted material (2.8g.) contained polyphenolic components of Rf. 0.6 to 0.95 (BAW). It was not further investigated.

The aqueous solution was then continuously extracted with ether; successive eight-hour extractions gave 15.8, 5.7g. of extractives. Continuous extraction with ethyl acetate gave, in eight-hour periods, 7.1 and 0.04g. extractives. No separation of components was apparent in this fractionation, and the fractions obtained were not utilised.

Fraction (dd) deposited a white powder during distillation of ethyl acetate. The remaining solvent was removed under reduced pressure, and the white powder isolated by trituration with cold ethanol, which

dissolved the brown amorphous material. After filtering and washing with ethanol, there remained a fine, almost white powder (9.2g.), which was identified as melacacidin (see below).

The ethanol-soluble portion was concentrated, and heated in small batches (about 5g. of solids and 3g. of ethanol) on a boiling water-bath so that the solvent evaporated slowly. A white deposit formed in 2-3 minutes. The mixture was cooled, diluted with cold ethanol, and filtered. In this way a further 8.4g. of melacacidin was obtained.

Paper chromatography showed that the brown mother liquors from the isolation of melacacidin contained three leucoanthocyanidins and traces of the flavonol. One of the leucoanthocyanidins was identifed as melacacidin by paper chromatography (BAW and 2% acetic acid). The other two leucoanthocyanidins were characterised by their Rf values in these solvents, as shown in Table 7.The nomenclature is derived from subsequent work (see discussion).

Table 7.

Rf values of A. harpophylla leucoanthocyanidins.

	BAW	2% acetic acid
Melacacidin	0.25-0.32	0.30-0.42
Isomelacacidin	0.32-0.40	0.45-0.52
O-Ethyl isomelacacidin	0.58-0.67	0.58-0.70

These mother liquors were used subsequently in

attempted isolation of O-ethyl isomelacacidin by column chromatography on cellulose powder, and in counter-current distributions.

Fraction (de) seemed to be mainly polymeric material; paper chromatography (BAW) showed that some melacacidin was present, but most of the polyphenolic material either remained at the origin or was present as a brown streak to Rf 0.2.

The ethanol extract from the heartwood, fraction (e), was concentrated under reduced pressure to a black syrup (71g.), taken up in water (350ml.), let stand 3 days, and filtered. The water-insoluble material (7.1g.) contained some flavonol, but was mainly polymeric material; no pure compounds were obtained from it. The aqueous solution was concentrated under reduced pressure to about 200 ml., and continuously extracted with ethyl acetate to yield, in successive eight-hour periods, 46, 1.2 and 0.4g. of extractives (fraction (ea)). The residual aqueous solution was evaporated on a water bath to a thick syrup (7.9g.) which was diluted with about 5 ml. water. After several weeks, crystals appeared (1.05g; colourless prisms). These were recrystallised from water-ethanol, m.p. 181-182°, $[\alpha]_D^{21}$ +63.6° (c, 3.7 in water). The melting point on admixture with pinitol from A. intertexta was 182-183°. Paper chromatography (BAW) showed only one component, Rf 0.14 (pinitol

from A. intertexta had Rf 0.14).

Fraction (ea) was found by paper chromatography to be a complex mixture containing the three leucoanthocyanidins, the flavonol, the chalcone and unidentified components. It was dissolved in water (150ml.) and continuously extracted with ether for two eight-hour periods, and then, to recover remaining polyphenols, with ethyl acetate for eight hours. The material extracted by ether in the first eight-hour period was redissolved in water, and continuously extracted with ether for eight hours. The material extracted from the first aqueous solution by ether during the second eight-hour period was then added, and the solution continuously extracted with ether for eight hours, followed by eight hours of ethyl acetate extraction to recover remaining polyphenols. This extraction series was continued systematically as shown in Figure 6. This is similar to systematic fractional crystallisation, with the operation of fractional crystallisation replaced by the operation of continuous liquid/liquid extraction for unit time. The system was designed to obtain separation of the ketonic flavonoids and other components readily extracted from water by ether from the less readily extracted leucoanthocyanidins.

The resulting fractions were examined by paper chromatography. The results are given in Table 8.

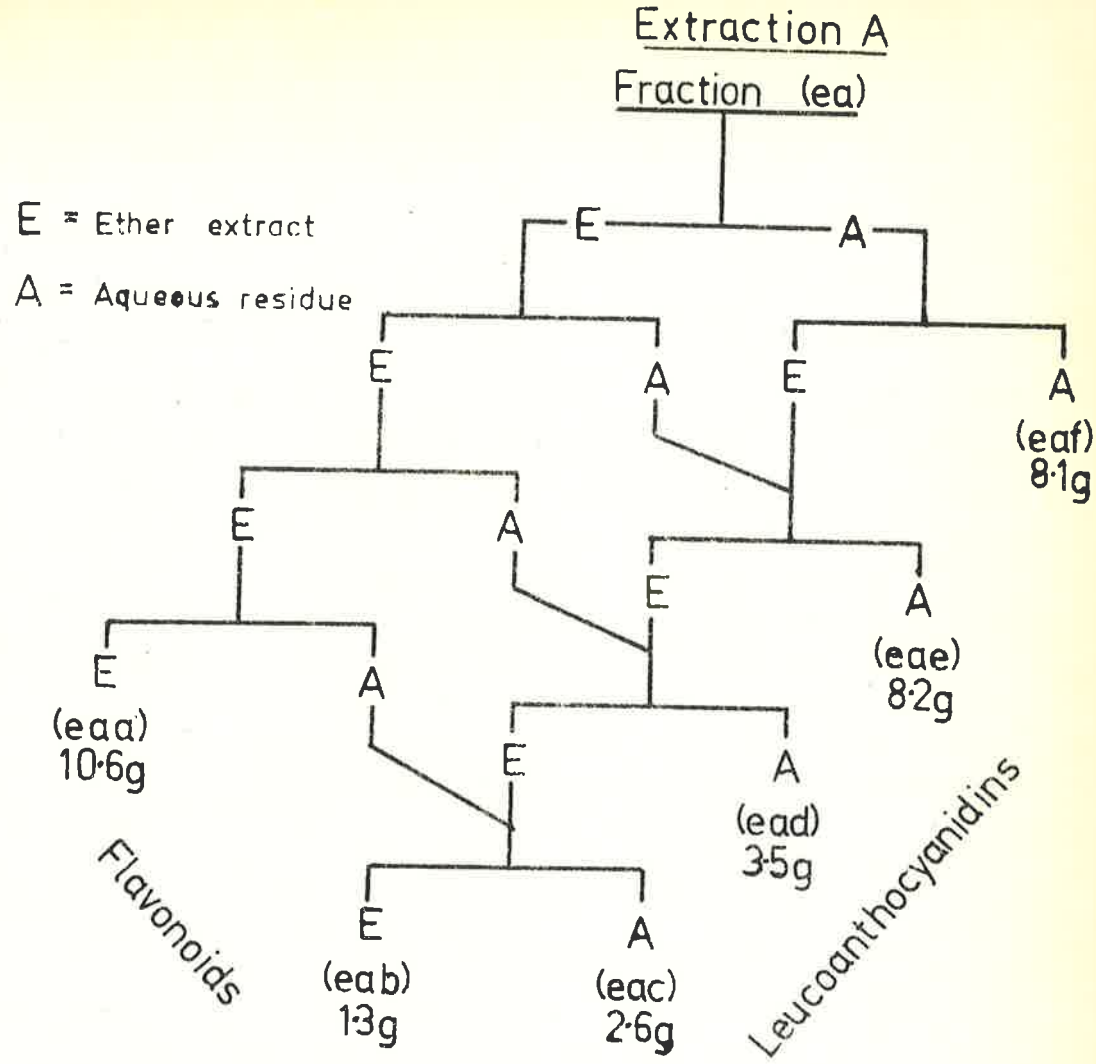


Figure 6 : Fractionation of polyphenols by systematic ether extraction.

Table 8.

Composition of fractions from systematic ether extraction.

Fraction		eaa	eab	eac	ead	eae	eaf
Component	Rf (BAW)						
Melacacidin	0.25-0.23	+	++	+++	+++	+++	+++
Isomelacacidin	0.32-0.39	+	+++	++	++	++	++
Flavonol	0.28-0.44	+	++	+	—	—	—
Chalcone	0.45-0.53	++	—	—	—	—	—
? A	0.53.0.74	+	—	—	—	—	—
? B	0.58-0.66	+	+	—	—	—	—
O-ethyl iso-melacacidin	0.69-0.75	++	+	+	—	—	—

Unknown A characterised by yellow fluorescence in UV light.

Unknown B characterised by positive $FeCl_3$ and diazotised benzidine reactions.

Fraction (eaa) was further examined by counter-current distribution between ether and M/15 phosphate buffer pH 6.5 over 50 tubes. Even numbered tubes were chromatographed in BAW (6 hours, 16.7 cm.) and in 2% acetic acid (2 hours, 25 cm.) The components detected with Roux's spray and with the ferric-ferricyanide reagent are given in Table 9, and are shown in Figure 1. Component 10 had a yellow fluorescence in UV light. Component 5 was present in very small amount.

Table 9.

Counter-current distribution of A. harpophylla

Extraction A fraction (EAA).

Component	Identity	Rf BAW	Rf 2% acetic acid	Peak	Extent
1	Melacacidin	0.24-0.41	0.35-0.43	1	1-6
2	Isomelacacidin		0.45-0.54	1	1-6
3	?	0.60-0.77	0.54-0.62	9	3-12
4	?	0.46-0.60	0.29-0.38	17	10-24
5	?	?	0.48-0.55	14	12-16
6	Q-Ethyl Isomel.	0-60-0.77	0.61-0.68	20	14-28
7	?	0.58-0.74	0.13-0.21	39	32-44
8	Flavonol	0.28-0.46	0.00	42	34-46
9	Chalcone	0.35-0.60	0.00	50	48-50
10	?	0.60-0.79	0.00	50	48-50

Fractions (eac), (ead), (eae) and (eaf) were used in the isolation of Q-ethyl isomelacacidin by counter-current distribution, described later.

Identification of 7,8,3',4' - tetrahydroxyflavonol: 3,7,8,3',4' - penta-acetoxyflavone: The crude flavonol from fraction (da) of Extraction A (0.5g.) was acetylated with acetic anhydride (1.6ml.) and pyridine (1.6ml.) (cf. 172). After 5 hours at room temperature the reaction mixture was diluted with water. The crystalline product was recrystallised from methanol (5ml.) as yellow crystals with a slight

green tint (0.33g.), m.p. 176° unchanged on further recrystallisation; lit.(173), m.p. 172-173°. (Found: C,58.6; H,4.0; acetyl, 43.4. Calc. for $C_{25}H_{20}O_{12}$: C,58.6; H,3.9; acetyl, 42.0).

3,7,8,3',4' - Pentamethoxyflavone. (a) From the natural flavonol: The crude flavonol (0.5g.) with methyl sulphate (2.0g.) and potassium carbonate (10g.) was heated in boiling acetone (50ml.) for 24 hours. The product was isolated as crystals from ethanol; on recrystallisation from ethanol, 3,7,8,3',4' - pentamethoxyflavone was obtained as yellow needles, m.p. 151°; lit. (33), m.p. 151°. (Found C,64.5; H,5.5; OCH_3, 40.9. Calc. for $C_{20}H_{20}O_7$: C,64.7; H,5.4; OCH_3, 41.7%).

(b) From synthetic 7,8,3',4' - tetramethoxyflavonol: The synthetic flavonol (0.5g.) with methyl sulphate (0.2g.) and potassium carbonate (10g.) was heated in boiling acetone for 24 hours. The product (85%) had m.p. 151°, alone and when mixed with that described in (a) above.

Identification of okanin:

The product from A. harpophylla had m.p. 238°. A mixed m.p. with an authentic sample of okanin m.p. 245° (87) from Dr. T. J. King was 240°. (Found: C62.2, H4.2, OCH_3, nil. Calc. for $C_{15}H_{12}O_6$: C,62.5, H,4.2; OCH_3, nil).

Both the material from A. harpophylla and the

authentic sample gave, on chromatography in BAW, a tailing spot with front Rf 0.22, and indefinite tail. This spot was yellow-orange in day-light, and brown with yellow edges in U.V. light. Chromatography of both samples together gave a single spot.

The product from A. harpophylla gave, in test-tube colour tests, a brown-green with ferric chloride in ethanol, a red-orange with sodium hydroxide, and an orange-red with magnesium and aqueous-ethanolic hydrochloric acid.

The penta-acetyl derivative, prepared as described by King and King (87) had m.p. 136°; lit., m.p. 141°.

Identification of melacacidin:

Melacacidin tetramethyl ether: Crude melacacidin from fraction (dd) of Extraction A (2.0g.) with methyl sulphate (4.0g.) and potassium carbonate (15g.) was heated in boiling acetone (50ml.) for 20 hours. The solution was cooled and filtered, the potassium salts were washed with hot acetone, and the washings were added to the main filtrate. Concentrated ammonia (2ml.) was added. Next day, acetone was distilled from the product, which was dissolved in ether with ethanol and the solution washed with water and aqueous alkali. The ethereal solution was dried over sodium sulphate, and left at 0°. The methyl ether formed clusters of colourless needles, m.p. 144-145°, $[\alpha]_D^{25}$ -83.5° (C, 1 in EtOH); lit. (33) m.p. 145-146°, $[\alpha]_D$

- 84.4.° Yield, 0.59g. (25%). For analysis a sample was recrystallised from ethanol-ether; the m.p. was unchanged. (Found: C,63.0; H,6.1; OCH_3, 34.3. Calc. for $C_{19}H_{22}O_7$: C,62.9; H,6.1; OCH_3, 34.2%). A mixed m.p. with an authentic sample of melacacidin tetromethyl ether (m.p. 135-138°) was 138-140°.

The mother liquors yielded two further crops of crystals; 0.61g. m.p. 110° and 0.16g. m.p. 110°.

Melacacidin tetramethyl ether diacetate: Acetylation of the tetramethyl ether (0.1g.) with acetic anhydride (0.5g.) and pyridine (0.5ml.) for 24 hours at room temperature gave melacacidin tetramethyl ether diacetate (75 mg.), m.p. 191-192°, $[\alpha]_D$ -39.5° (C, 0.182 in EtOH, 4 dm. tube); lit. (33), m.p. 193-194°, $[\alpha]_D$ -39.2° (in EtOH).

Extraction B: (See Figure 7). The milled heartwood (2,407g.) was extracted for 5 eight-hour periods with acetone, and the extracts concentrated by distillation at atmospheric pressure. The resulting syrups weighed 176,31,13.3,7.7,1.4g. respectively. They were combined (fraction (a)). The residual wood was extracted with ethanol for two 12 hour periods and the extracts were concentrated under reduced pressure. The resulting syrups weighed 43 and 6.0g. respectively. They were combined (fraction (b)). The alcohol -wet wood was extracted with water for 8 hours, which

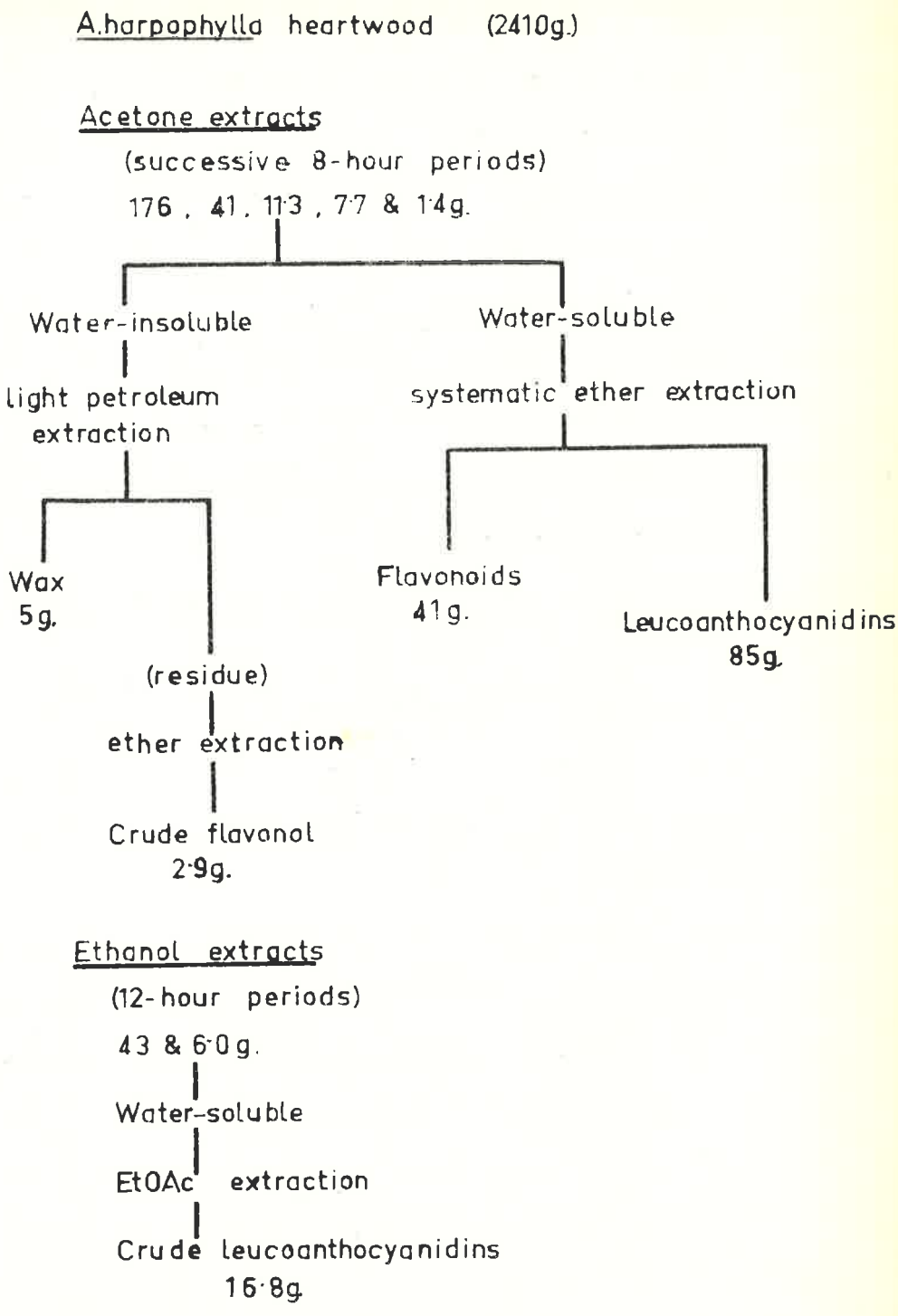


Figure 7 : Extraction B

yielded extractives (21g.) partly soluble in cold water (7.6g.). No ninhydrin-positive components were detected in a chromatogram (BAW) and this fraction was discarded.

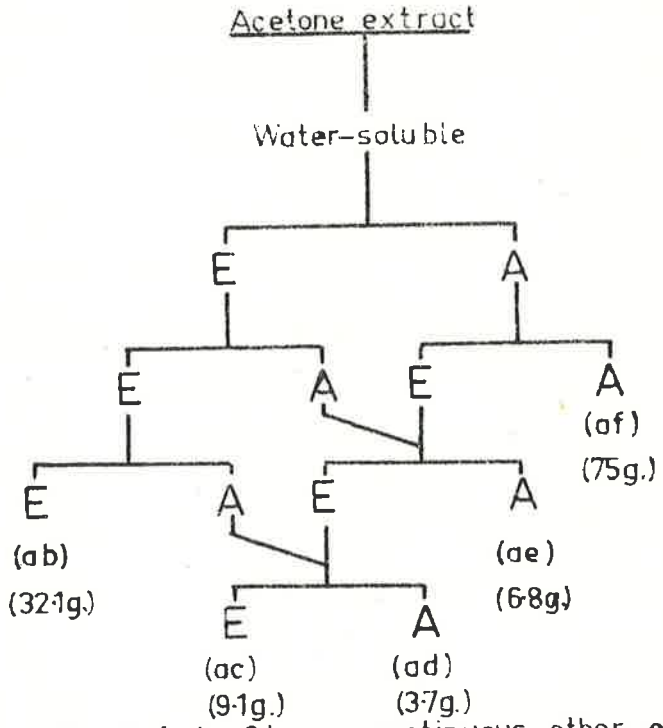
The acetone extracts were taken up in about 2l. of water, left overnight, filtered (residue 23.4g., fraction (aa)) concentrated under reduced pressure to about 400 ml., and then subjected to a systematic separation by continuous liquid/liquid extraction with ether as shown in Figure 8.

Fraction (ab) was dissolved in 400 ml. water and the orange solution was continuously extracted with light petroleum for 32 hours. Only a trace of material was extracted, but an orange crystalline deposit of chalcone formed (2.76g.)

As with the extractive distribution of the flavanoids of Extraction A, the fractions readily extracted by ether i.e. (ab) and (ac) contained a mixture of flavonoids whereas those fractions not readily extracted by ether, but recovered from the aqueous phase by ethyl acetate extractions, contained only leucoanthocyanidins. In this case, the chromatograms showed the presence of little isomelacacidin and O-ethyl isomelacacidin, most of the leucoanthocyanidin being melacacidin.

Fraction (aa), the water-insoluble portion of the acetone extract, was extracted in a soxhlet apparatus

Extraction B



E Extracted in 8 hours continuous ether extraction

A Remaining in the aqueous solution

Figure 8 : Fractionation of the polyphenols of Extraction B by systematic continuous ether extraction.

with light petroleum (about 5g. of wax was extracted in 100 hours) then with ether to yield crude flavonol (2.9g., 40 hours extraction).

The ethanol extracts, fraction (b), were taken up in about 2l. water, left overnight and filtered (residue discarded) the solution was concentrated under reduced pressure to about 200ml. and continuously extracted with ethyl acetate for two eight-hour periods yielding 15.5, 1.3g. of extractives respectively. These extractives, apart from a trace of flavonol, did not show any polyphenols other than leucoanthocyanidins. The residual aqueous phase contained 3.1g. of solids; paper chromatography showed the presence of pipecolic and a hydroxypipecolic acid both in amounts too small for isolation.

The combined crude leucoanthocyanidin fractions obtained in this extraction weighed 102g. (4.1%). This material was used in exploratory work on the isolation of O-ethyl isomelacacidin, described below.

Extraction C: (See Figure 9). This was carried out in almost the same system as Extraction B, A preliminary light petroleum extraction was made of the wood (2,165g.) Nine hours extraction gave a deposit of 1.4g. in the still, with 4.8g. of wax in solution. Acetone extraction for 23 hours and then for 30 hours gave extracts which were concentrated to syrups (196 and 17g.respectively) (Fraction (a)). Ethanol extraction

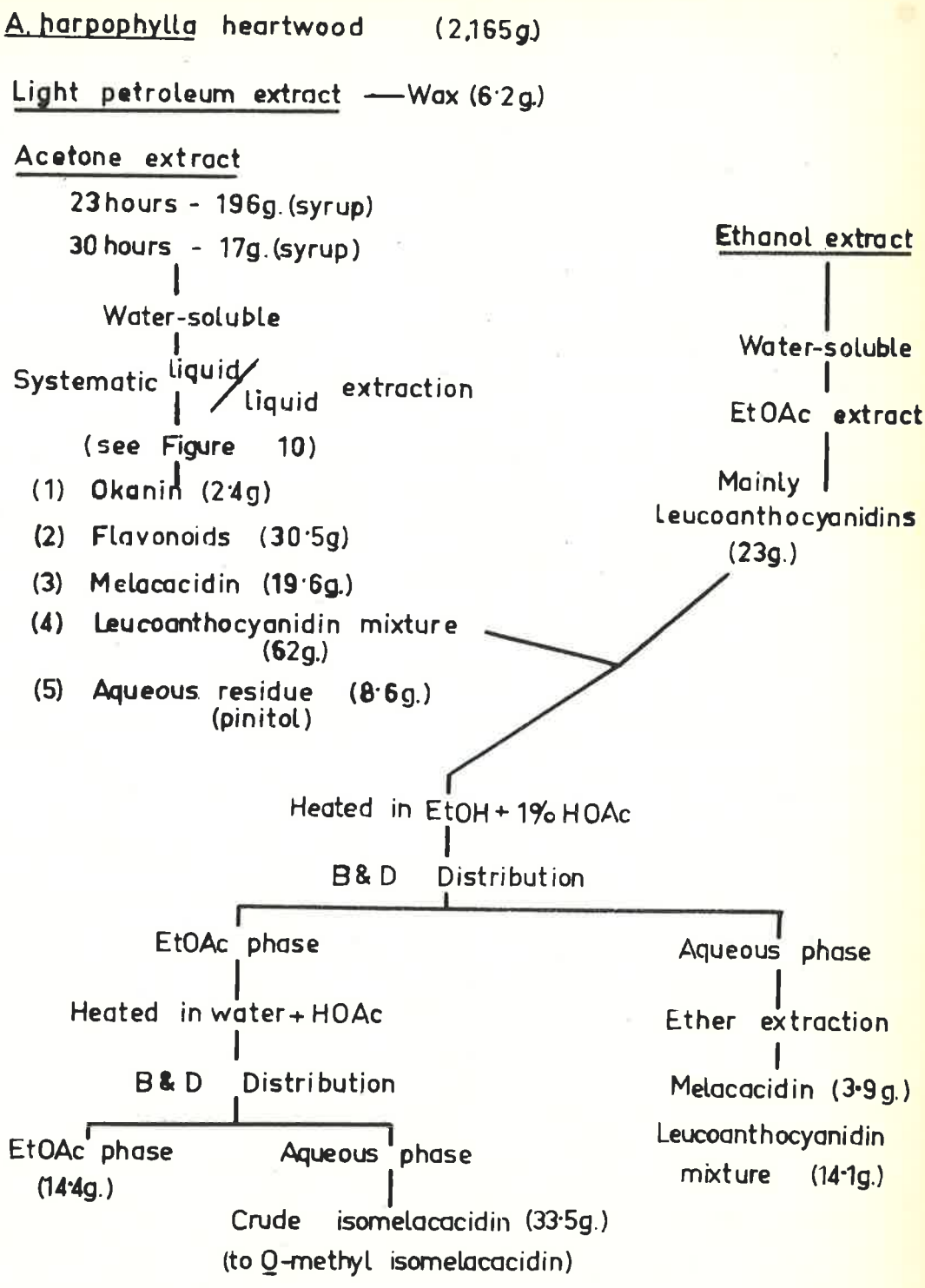


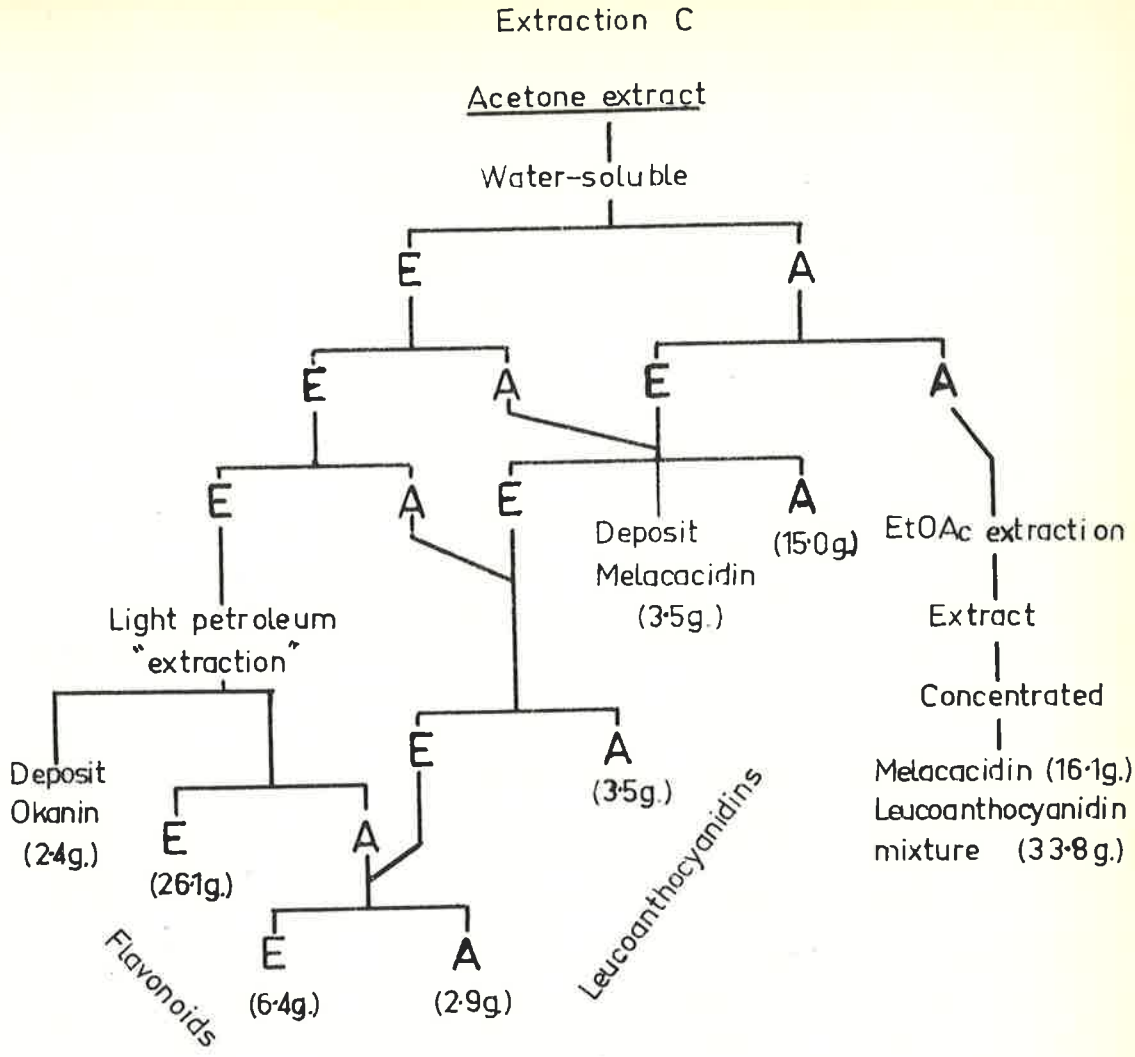
Figure 9: Extraction C

for 8 hours, processed as for the ethanol extract of Extraction B, yielded 23g. of mixed leucoanthocyanidin fraction. The water-soluble, non-ethyl acetate extracted portion of the ethanol extract showed a trace of pipecolic acid on paper chromatography.

The acetone extracts were dissolved in water, filtered, concentrated and subjected to systematic liquid/liquid extraction fractionation as shown in Figure 10. The water-soluble portion of the acetone extracts remaining after ethyl acetate extraction showed a strong pinitol spot on paper chromatograms.

As indicated in Figure 10., a deposit of melacacidin was obtained during one ether extraction. On removal of solvent from the ethyl acetate extract containing the major mixed leucoanthocyanidins fraction, deposition of white solid occurred as in Extraction A. This was melacacidin; the total amount obtained on repeated evaporation with ethanol was 16.1g.

The mixed leucoanthocyanidin fractions from both the acetone and ethanol extracts were combined (total, 85g.) and boiled with ethanol containing 1% acetic acid (750ml.) for 2 hours. The solvent was removed and the residue was dissolved in 400ml. of M/15 phosphate buffer, pH 7.0. It was submitted to the usual Bush and Densen distribution. The combined aqueous phases were concentrated under reduced pressure (40-47°C) to about 200ml., and continuously



E Extract from 8-hours continuous ether extraction.

A Material remaining in aqueous phase.

Figure 10 : Systematic liquid/liquid extraction of the acetone extract, Extraction C.

extracted with ether. After 4 hours, 2.7g. of non-crystalline extract was obtained. Extraction was continued, and seed crystals of melacacidin added after several hours; melacacidin then deposited continuously. In 24 hours, 3.9g. of crystalline melacacidin and 5.9g. of non-crystalline extract was obtained. Further extraction for 46 hours yielded 5.5g. of product containing a little crystalline melacacidin.

The combined ethyl acetate phases were taken to dryness and then heated in a boiling water bath with 200ml. 1% aqueous acetic acid for 2 hours. The Bush and Densen distribution was repeated, using half the usual phase volumes. The combined ethyl acetate phases from this distribution contained 14.4g. of polyphenols, including some leucoanthocyanidin. The combined aqueous phases were concentrated under reduced pressure to 500ml; extraction with ethyl acetate (24 hours) yielded crude isomelacacidin (33.5g.). This was used in the preparation of O-ethyl isomelacacidin.

Extraction of A. harpophylla SN 5919 heartwood.

Extraction D: (See Figure 11). Milled heartwood was extracted in two batches of 4.2kg. each. The solvents used were light petroleum (12 hours), acetone (36 hours), ethanol (12 hours), and water (12 hours). The light petroleum extract contained 49.7g. of orange wax. The ethanol and water extracts were combined.

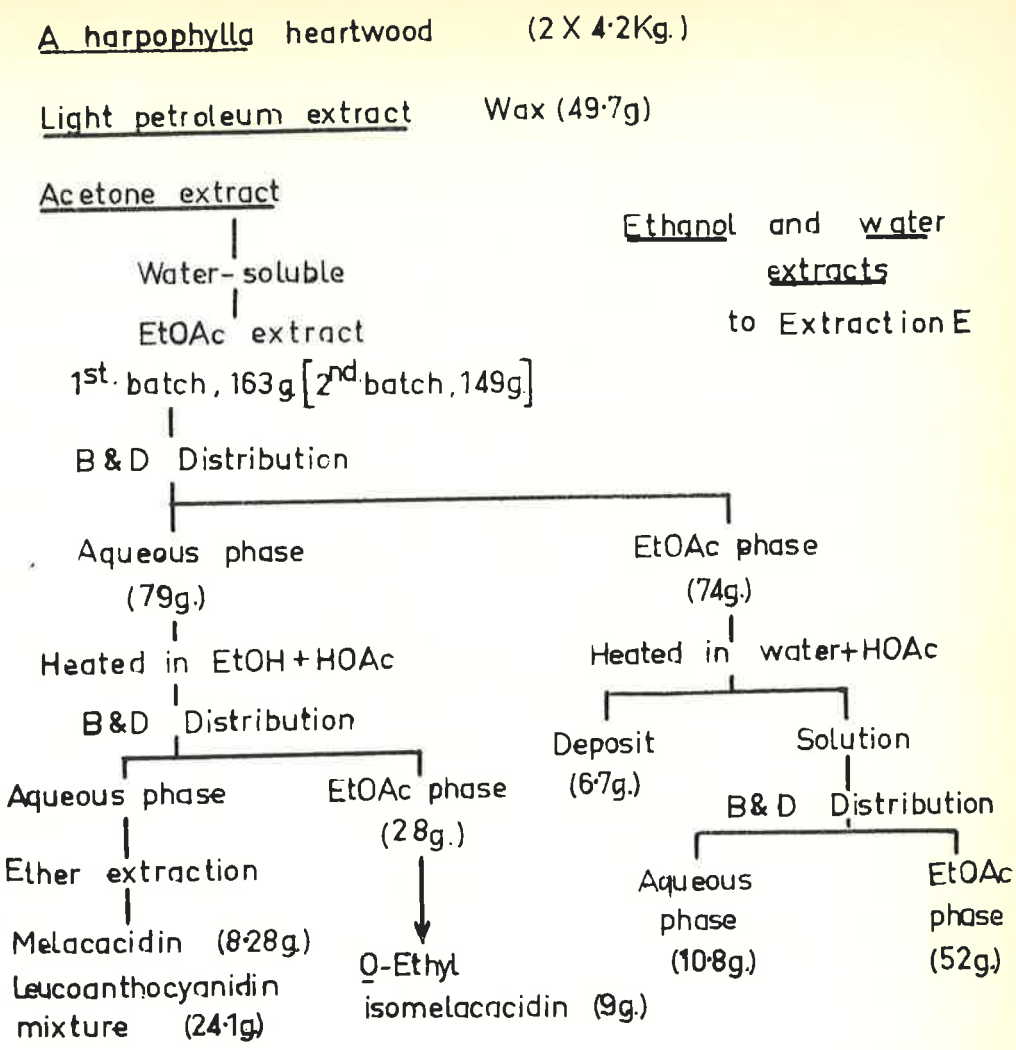


Figure 11 : Extraction D

A.harpophylla heartwood (3·97Kg.)

Cold acetone extract
Water-soluble, extracted by ether — 32g.

Hot acetone extract
Water-soluble, extracted by ether — 45g.

Ethanol extract (+ ethanol extract from Extraction D)
Water-soluble, extracted by EtOAc — 79g.
Amino-acid fraction — 0·50g.

Water extract (+water extract from Extraction D)
Amino-acid fraction — 1·96g.

Figure 12 : Extraction E

with the corresponding fractions of Extraction E.

The acetone extracts from each batch were processed separately. The extract from the first portion of wood was concentrated to 700ml., then diluted to 4l. with water and left overnight. Next day the filtered solution was concentrated under reduced pressure (40°C) to 400ml., and continuously extracted with ethyl acetate for a total of 20 hours. The extract was removed from the still flask at intervals of about 3 hours to reduce the amount of heat to which it was being subjected. Considerable solid deposit formed in the still flask during the latter stages. The total ethyl acetate extract was 163g..

This was dissolved in aqueous M/15 phosphate buffer pH 7.0 (800ml.) and subjected, in two portions, to the usual Bush and Densen distribution. These distributions yielded, from the ethyl acetate phases, a mixture (a) (74g.) and, from the buffer phases concentrated to about 500ml. under reduced pressure, by continuous extraction with ethyl acetate for 14 hours, a mixture (b) (79g.). Much deposit formed in the ethyl acetate during the extraction of fraction (b).

Fraction (a) was dissolved in water (400ml.) and acetic acid (8ml.) added. An oil began to deposit in five minutes, and crystallise. The mixture was heated in a boiling water bath for 15 minutes, then left at room temperature overnight. The deposit was

collected as a yellow-grey powder (6.7g.) found by paper chromatography (BAW and 2% acetic acid) to be a mixture of 7,8,3',4'-tetrahydroxyflavonol and "Compound A" of A. excelsa (see Extraction I). The solution was subjected to the usual Bush and Densen distribution. The initial aqueous phase, which contained acetic acid (2%) gave rise to emulsion but later aqueous phases, of M/15 phosphate buffer pH7.0, gave no trouble. The ethyl acetate phases contained 52g. of material. The buffer phases were concentrated to 130ml. under reduced pressure, and continuous extraction with ethyl acetate for 20 hours then gave 10.8g. of material.

Fraction (b) was dissolved in ethanol containing 1% acetic acid (800ml.) and the solution was boiled for 8 hours. After removal of solvent under reduced pressure, the residue was subjected to the usual Bush and Densen distribution. The ethyl acetate phases contained 28g. of material. This was dissolved in ethanol (56ml.) and the solution, cooled to 0°, was added to ice-cold water (224ml.). After 8 days at 0°, 9g. of O-ethyl isomelacacidin had crystallised. This was dissolved in boiling ethanol (about 35ml.), the solution treated with carbon and concentrated on a hot plate to a solution weight of 27g. This solution was cooled to 0° and diluted with 72ml. of

cold water. After 4 days at 0°, 6.5g. of O-ethyl isomelacacidin was collected.

The aqueous solution obtained from the distribution carried out on fraction (b) was concentrated to 300ml. under reduced pressure and continuously extracted with ether. The still contents were seeded during extraction with crystals of melacacidin. The results for successive periods of extraction were as follows: 4 hours, no crystalline material, 8.15g. in solution; 12 hours, 6.41g. of crystals, 5.67g. in solution; 12 hours, 2.76g. of crystals, 3.76g. in solution; 8 hours, 0.11g. of crystals, 1.87g. in solution; 12 hours, no crystals, 1.20g. in solution; 12 hours, no crystals, 1.77g. in solution; 32 hours, no crystals, 1.69g. in solution. (Total non-crystalline extract, 24.1g.). Recrystallisation of the crystalline melacacidin (8.28g.) from ethanol gave 6.1g. of pure melacacidin.

The acetone extract from the second batch of heartwood yielded 149g. of ethyl acetate-extract, not further processed.

Extraction E: (see Figure 12). Milled heartwood (3.97Kg.) was extracted with cold acetone. The wood was left overnight in acetone, which was drained off daily. The fifth extract was very small, and extraction was discontinued. After removal of solvent by distillation, the extracts were combined,

(fraction (a)). The wood was extracted with hot acetone for four 10-hour periods (fraction (b)), then with ethanol (12 hours) and water (12 hours).

Fraction (a) was taken up in water (500ml.), the mixture was filtered and the residue washed with water. The filtrate and washings (total, 900ml.) were continuously extracted with ether, yielding in the first two 8-hour periods, 24.9 and 8.5g. of material.

The hot acetone extracts (fraction (b)) were concentrated to a syrup which was taken up in water (500ml.) and the mixture was filtered. The residue was washed with water, and the filtrate and washings were continuously extracted with ether. The amounts of extracted material in successive periods of extraction were: 12 hours, 30g.; 12 hours, 8g.; 12 hours, 4.65g.; 8 hours, 1.8g.; 8 hours, 1.4g..

The extracts from fractions (a) and (b) were examined by paper chromatography (BAW, 12 hours, 22cm. and 2% acetic acid, 2.5 hours, 23cm.). All extracts contained melacacidin, isomelacacidin and O-ethyl isomelacacidin. Other polyphenols (including the flavonol and chalcone) were present in the first ether extracts.

The ethanol extract was added to that from Extraction D, and the solvent removed. The residue was mixed with water (3l.), and the mixture was fil-

tered next day. The filtrate was concentrated under reduced pressure and continuously extracted with ethyl acetate. This yielded in successive 8-hour periods, 72, 4.6, 2.5g. of material. Much black deposit formed in the ethyl acetate during this extraction. The residual aqueous solution was treated to remove acid-insoluble material, and passed through a column of cation exchange resin. The amino-acid fraction was eluted with 0.5 to 1 N-ammonia. This fraction was black and non-crystalline (0.50g.).

The aqueous extract was combined with that from Extraction D, and acid insoluble material removed. The amino-acid fraction (1.96g.) obtained *via* the cation exchange resin column was dark, but partly crystalline.

The amino-acid fractions were added together to a column of Zeokarb 225 (50g., 20 x 1.8cm.) which was then eluted with the following series of eluents (fractions of 70ml.); fractions 1-5, solution and water; 6-15, 750ml. of 0.2N-HCl; 16-18, 250ml. of 0.3N-HCl; 19-21, 250ml. of 0.4N-HCl; 22-24, 250ml. of 0.8N-HCl; 23-26, 250ml. of water; 27-32, 3N - ammonia. The eluate fractions were evaporated to dryness, the residue was dissolved in water (2-3ml.) and examined by paper chromatography (BAW, 12 hours). The following components were detected: pipecolic acid, 13.8cm. on chromatogram (identical with stan-

dard; characterised by its ninhydrin and isatin reactions) in fractions 14-24; proline, 10.2cm. (blue with isatin) in fractions 13-22; an α-amino-acid, 7.3cm., in fractions 5-14; an α-amino-acid, 10.2cm., in fractions 8-20; an α-amino-acid, 15.8cm., in fractions 13-20; and traces of component, 20.2cm., in fractions 19-22. Hydroxypipecolic acids appeared to be absent (4-hydroxypipecolic acid was run as a standard). A substance present in fractions 8-12 was detected as a blue-grey spot at 8cm.; subsequent spraying with Ehrlich's reagent gave a cerise colour. Fractions 9-11 were combined and treated with excess nitrous fumes. Paper chromatography showed only one component detectable with ninhydrin (orange-brown colour). With isatin this component gave a clear blue colour, changing to cerise on spraying with Ehrlich's reagent. This behaviour is characteristic of hydroxyproline (174). The amount present was very small.

Extraction of A. harpophylla SN5919 sapwood.

Extraction F: Sapwood containing some heartwood was extracted in lots of about 3Kg. with light petroleum (8 hours), acetone (24 hours), ethanol (10 hours) and water (8 hours). A total of 10.35Kg. was extracted. The combined light petroleum extracts weighed 31g. The water-insoluble portion of the acetone extracts was extracted with light petroleum to give 16g. of wax.

The ethanol and the aqueous extracts were freed of acid-insoluble material and the amino-acid fractions were isolated. The ethanol extracts yielded 19.7g., and the aqueous extracts, 9.5g. The former was crystalline but the latter contained much impurity.

The combined amino-acid fractions were dissolved in water (100ml.) and the solution, cooled in ice, was treated with sodium nitrite (14g.), and then, slowly, concentrated hydrochloric acid (20ml.). The solution was left at room temperature 1 day; nitrous acid was still present. The solution was filtered from a tarry deposit, and continuously extracted with ether for 48 hours. Ether was distilled from the extract, and the residue dissolved in water (100ml.), and concentrated hydrochloric acid (100ml.) added. Urea (2g.) was added gradually. The solution was brought to boiling slowly, then boiled 1½ hours. Evaporation on a boiling water bath left 22.9g. of syrup. The aqueous solution remaining from the ether extraction was found by paper chromatography to contain pipecolic acid and proline. It was treated with sodium nitrite (7g.) and concentrated hydrochloric acid (10ml.) and was continuously extracted with ether for 48 hours. The extract was boiled with an excess of hydrochloric acid and then evaporated to a syrup (2.75g.) containing pipecolic acid, proline, and traces of other imino-acids.

The combined products were passed through an ion-exchange column and the imino-acids (16.9g.) were eluted with 2N-ammonia. It appeared from chromatography (BAW 24 hours), that this material contained pipecolic acid, proline, 4-hydroxypipecolic acid and possibly 5-hydroxypipecolic acid. From water-ethanol, 1.8g. needles were obtained; these contained the three major components but not proline.

The total imino-acids, in 150ml. of water, were added to a Zeokarb 225 column (200g.), and eluted as follows (250ml. fractions): fractions 1-2, feed and water; 3-6, 1 l. of 0.1N-HCl; 7-8, 500ml. of 0.2N-HCl; 9-12, 1 l. of 0.4N-HCl; 13-16, 1 l. of 0.8N-HCl; 17-18, 500ml. of water; 19-23, 3N-ammonia. The eluates were evaporated to remove excess of hydrochloric acid and examined by paper chromatography (BAW, 12 hours). Fractions 12-14 contained a mixture of 4- and 5-hydroxypipecolic acids, and fractions 14-19 contained proline and pipecolic acid, which were also present in the ammonia wash. The hydroxypipecolic acid spot on the chromatogram contained a portion (7.6cm.) of the normal 4-hydroxypipecolic acid -ninhydrin colour, and a portion (8.3cm.) of the blue-purple with bright red fluorescence in UV light characteristic of 5-hydroxypipecolic acid. Chromatograms in phenol:water (24 hours) gave partial separation, the lower portion having the same Rf as

4-hydroxypipecolic acid and the upper portion having the same Rf as 5-hydroxypipecolic acid from dates.

Pipecolic acid hydrochloride m.p. 256-7° decomp. (1.5g.) crystallised after evaporating to a syrup the fractions which contained pipecolic acid.

Extraction of A. excelsa SN5584 Heartwood.

Extraction G: (See Figure 13). This extraction was carried out at the same time as Extraction A. The only compounds isolated were 4-hydroxypipecolic acid and pipecolic acid.

The milled heartwood (2094g.) was extracted with light petroleum (12 hours), yielding 1.8g. of yellow wax. Ether extraction (34 hours) gave no deposit, and 7.4g. of extractives. Attempts to obtain a crystalline product from this were fruitless. Acetone extraction (7 hours) yielded 34g. of extractives (fraction (a)), then ethanol extraction (8 hours) gave fraction (b).

Fraction (a) was taken up with water, the deep purple insoluble material was removed, and the solution was extracted with ethyl acetate for three 8-hour periods, to give 19.8, 0.3, 0.3g. of extractives respectively. (The aqueous residue, 3.7g., was discarded). The ethyl acetate extractives were dissolved in water and subjected to continuous ether extractions, giving in successive 8-hour periods, 8.4, 1.5, 0.8, 0.7, 0.3g. extractives. Attempts

A. excelsa SN5584 heartwood (2094g.)

Light petroleum extract (1·8g.)

Ether extract (7·4g)

Acetone extract (34g.)
(Water-soluble, extracted by EtOAc – 20·4g)

Ethanol extract (73g., syrup)

Water-soluble

EtOAc extraction

Extract (19·7g.)

Aqueous (20·7g.)

4-Hydroxypipecolic acid (4g.)
Pipecolic acid (0·35g.)

Figure 13 : Extraction G.

A. excelsa SN5920 heartwood (1905g.)

Light petroleum extract (1·4g.)

Acetone extract
Water-soluble, extracted by EtOAc — (100g.)

Ethanol extract
Water-soluble

EtOAc extraction

Aqueous

to Extraction I
for amino-acids

Extract (34g.)

B & D Distribution

Flavonoids (40g.)

Leucoanthocyanidin mixture (82g.)

Figure 14 : Extraction H

were made to obtain crystalline products from these but without success. Paper chromatograms showed that they contained melacacidin and isomelacacidin and other polyphenols.

Fraction (b) was concentrated to a syrup (73g.) which on dilution with water gave 12.8g. of brown, amorphous powdery deposit. The solution was extracted with ethyl acetate for three 8-hour periods giving successively 17, 2.1, 0.6g. of extractives. Paper chromatography showed that these were mixtures containing melacacidin and O-ethyl isomelacacidin. Attempts to crystallise them were fruitless. The residual aqueous solution was evaporated and the residue (20.7g.) was dissolved in water (10ml.) and ethanol (100ml.) was added. This gave a rapid deposition of crystals with much amorphous brown impurity (total, 9.85g.). Paper chromatograms (BAW) of the deposit were examined; pinitol was not detected, and, apart from polymeric material near the origin, polyphenols were absent. Two components, Rf 0.25 and 0.38, were present and gave a yellow area with diazotised benzidine. The crystals were recrystallised from aqueous ethanol four times, the second time from a slightly alkaline solution which failed to retain coloured impurities, and the third time from a slightly acidified (acetic) solution, after a carbon treatment which removed the colour.

There was obtained 4-hydroxypipecolic acid as colourless prisms, m.p. 294° decomp.

For the isolation of further quantities of 4-hydroxypipecolic acid, the material in the mother liquors was boiled for 24 hours in 6N-HCl which converted the polyphenolic material to an insoluble powder. The solution was filtered and evaporated. The residue was dissolved in water and added to a cation exchange resin column. The amino-acids were eluted with 0.5N-ammonia. Fractional crystallisation from water gave further crops of 4-hydroxypipecolic acid, m.p. 294° decomp.

The mother liquors (2g. of syrup) were chromatographed on Whatman 3MM paper (0.30ml. of a solution in 2 parts of water per 25cm.) using BAW (from 25ml. n-butanol for 25cm. sheet) for 12 hours. The amino-acids were located by dipping a centre strip in an acetone solution of ninhydrin and heating at 100°; 4-hydroxypipecolic acid was present 8-12cm. from the origin and pipecolic acid 14-18cm. The zones were extracted with refluxing 90% ethanol. 4-hydroxypipecolic acid was not obtained crystalline from the fraction containing it. The pipecolic acid fraction yielded 0.35g. (-) -pipecolic acid m.p. 273-275° decomp., $[\alpha]_D^{23}$ -25.2° (C, 2.2 in water).

The total yield of 4-hydroxypipecolic acid was about 4g.

Chromatograms showed the presence of a polyol, Rf 0.14 in BAW, presumably pinitol, in the aqueous fraction from which the amino-acid was obtained. After this fraction was boiled in 6N-hydrochloric acid, this polyol was no longer detectible, and another polyol, Rf 0.08 in BAW, was present.

Extraction of A. excelsa SN5920 heartwood.

Extraction H: (See figure 14). The milled heartwood (1905g.) was extracted with light petroleum (11 hours) yielding 1.41g. of orange wax, then with acetone (40 hours, fraction (a)), ethanol (15 hours, fraction (b)) and water (10 hours, fraction (c)).

Fractions (a) and (b) were concentrated to syrups (308g. and about 300ml. respectively) which were each diluted to 2l. with water. Next day, the solutions were filtered (residues discarded) and concentrated under reduced pressure to 350ml. and 150ml. respectively. Continuous ethyl acetate extraction of fraction (a) gave, in successive 8 hour periods, 94, 3.6, 2.7g. of extracted material, much of the last two being water-insoluble. The residual aqueous solution contained 61g. of material (fraction (aa)). Continuous ethyl acetate extraction of fraction (b) gave, in successive 8-hour periods, 32, 1.3, 1.1g. of extracted material. The residual aqueous solution contained 19.3g. of meterial (fraction (ba)).

The combined ethyl acetate extracts weighed 134g.

(7.0%). This was submitted to the usual Bush and Densen distribution; the individual fractions were examined separately. From the aqueous phase fractions, the polyphenols were recovered by continuous ethyl acetate extraction for 8 hours; the aqueous phases retained much colouring matter, apparently anthocyanidin, and much dark amorphous material separated during the extractions. The results of examination of the fractions by paper chromatography (2% acetic acid, 19.2cm.) are shown in Table 10; the fractions are numbered from the ethyl acetate end to the aqueous end. Other polyphenols not listed were present in fractions 1 to 4.

Table 10.

Results of Bush and Densen Distribution of A. excelsa polyphenols.

Fraction	1	2	3	4	5	6	7	8	9	10	11	12
Weight (g.)	28	12	8	6	45	3.5	5	8	14	8	13	12
Melacacidin	—	+	+	+	+	++	++	++	+++	+++	+++	+++
Isomelacacidin	—	—	—	+	+	+	+	+	++	++	++	++
Q-Ethyl isomel.	+++	+++	+	+	+	—	—	—	—	—	—	—
Flavonol	+++	++	+	+	+	—	—	—	—	—	—	—

The melacacidin-rich fractions were used for the isolation of Q-ethyl isomelacacidin by counter-current distribution.

The polyphenols of fractions 1 and 2 were examined by counter-current distribution between ether and M/15

phosphate buffer pH7.0. Samples of alternate tubes were examined by paper chromatography. The results are shown in Table 11. The components were located by the ferricferricyanide spray, and identified by inspection in UV light and by the effect of Roux's spray. Components 7 (flavonol) and 10 had a yellow fluorescence in UV light, 9 (chalcone) was brown in UV light and component 6 (flavanonol?) gave a yellow colour with Roux's spray, intensified on heating. Component 8 gave a red colour with Roux's spray. Components 3 and 4 gave brown colours with Roux's spray followed by heating. The melacacidin and isomelacacidin spots in the BAW chromatograms were obscured by a streak of polmeric tannin material.

Table 11.

Counter-current distribution of A. excelsa heartwood polyphenols.

Component	Identity	Rf BAW	Rf 2% acetic acid	Peak	Extent	Amount
1	Melacacidin	-	0.30-0.38	1	1-4	Medium
2	Isomelacacidin	-	0.41-0.48	1	1-4	Medium
3	?	0.67-0.76	0.55-0.66	6	4-10	Medium
4	?	0.54-0.64	0.27-0.37	16	8-22	Major
5	O-Ethyl isomel.	0.67-0.78	0.56-0.65	20	14-26	Major
6	?	0.66-0.81	0.11-0.17	36	32-42	Small
7	Flavonol	0.25-0.45	0.00	42	34-48	Small
8	?	0.82-0.91	0.58-0.65	42	36-46	Trace
9	Chalcone	0.45-0.59	0.00	48	44-48	Small
10	?	0.60-0.77	0.00	48	46-48	Trace

The aqueous residues of the acetone and ethanol extracts after liquid/liquid extraction with ethyl acetate (fractions (aa) and (ba))and also the aqueous extract from the wood (fraction (c)) were chromatographed and examined for amino acids using ninhydrin and isatin. Fractions (aa) and (c) showed no ninhydrin-positive components; fraction (ba) showed traces of pipecolic acid and 4-hydroxypipecolic acid. This fraction was added to the corresponding fraction from Extraction I for the isolation of the amino acids.

Extraction I: (See Figure 15). Milled heartwood (2,335g.) was extracted with acetone (24 hours), ethanol (8 hours), and water (8 hours).

The acetone extract was concentrated to a syrup (376g.) which was taken up in water (4l.), and the mixture, after being left overnight, was filtered. The filtrate was concentrated under reduced pressure to 600ml., and, was then continuously extracted with ethyl acetate, which gave in successive 8-hour periods, 116,8,4g. of extractives. Much black deposit formed during the extraction. The residual aqueous solution was evaporated; the black residue (75g.) was now mainly insoluble in cold water and was discarded.

The ethanol extract was processed similarly, and gave 14g. of ethyl acetate extract.

The combined ethyl acetate extracts were used for the preparation of melacacidin and O-ethyl isomelacac-

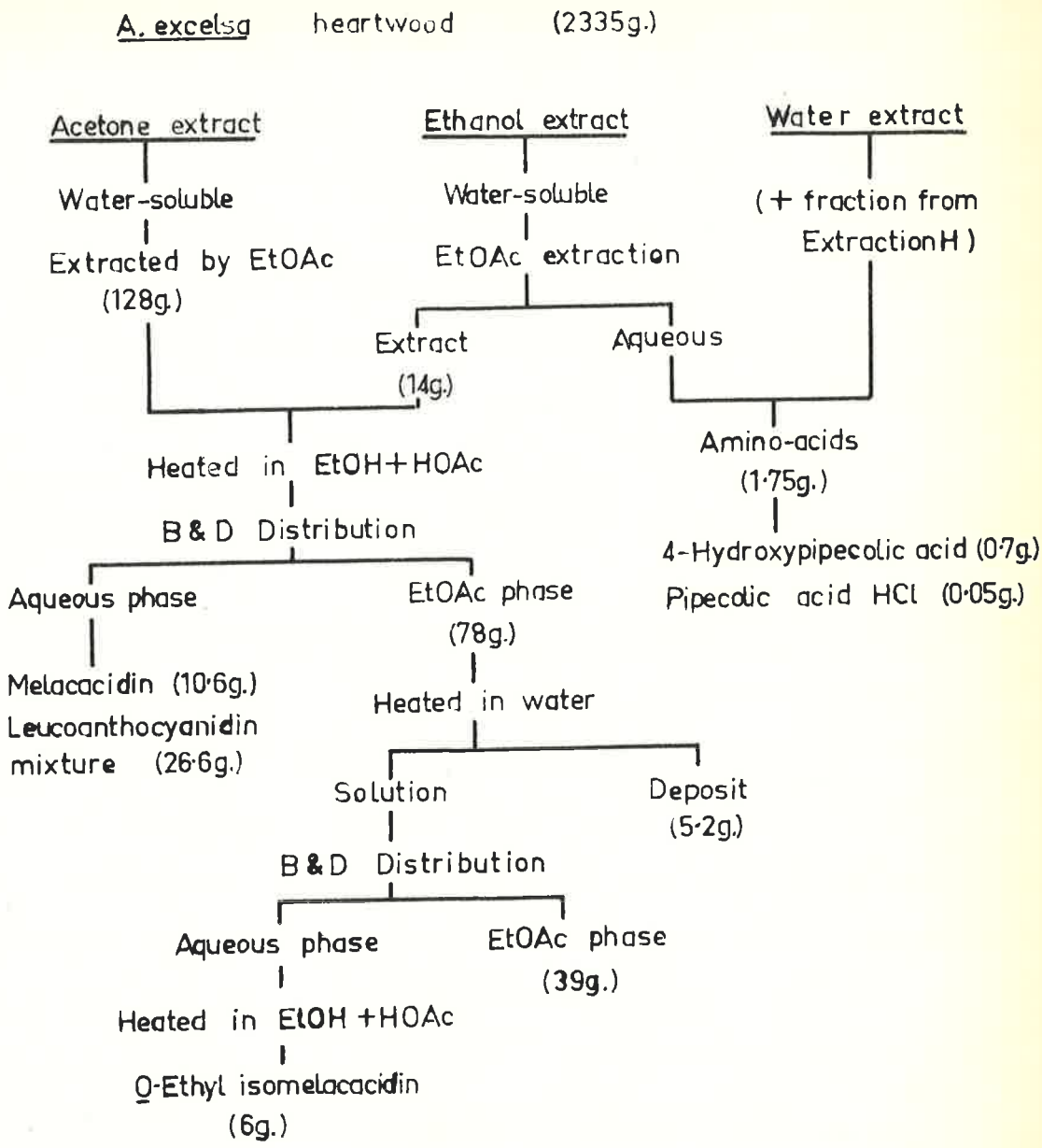


Figure 15 : Extraction I.

idin. The mixture was dissolved in ethanol (1l.) with acetic acid (10ml.) and the solution was boiled for 2 hours. The solution was then taken to dryness under reduced pressure, and the residue in water (400ml.) was subjected to the usual Bush and Densen distribution, but with water as the aqueous phase. The initial separations were made difficult by the formation of emulsions requiring about 30 minutes to settle.

The ethyl acetate fractions from the distribution were taken to dryness; the residues weighed, in order of occurrence of the fractions; 17.7, 24.3, 10.6, 8.7, 6.9, 5.3, 4.4g. (Total 77.7g., fraction (a)).

The aqueous fractions were combined and after concentration under reduced pressure (55°C) to 400ml., were continuously extracted with ether. Crystallisation of melacacidin occurred in the ether extract in the early extractions. The amounts of extract obtained in successive 8 hour periods of extraction were as follows: no crystals, 5.2g. in solution (this material, dried and triturated with cold ethanol, gave 2.4g. of crystalline melacacidin); 3.4g. of crystals, 4.4g. in solution; 2.9g. of crystals, 3.4g. in solution; 1.9g. of crystals, 5.0g. in solution. Subsequent extraction for 56 hours gave no more crystalline product; the extracts contained 11.0g. of material. The total yield of melacacidin was 10.6g.; non-crystalline material weighed 26.6g. Very little of the colour of the deep brown aqueous solution

was extracted by ether; the non-crystalline product was fawn-coloured.

Fraction (a) was heated in water (400ml.) for 2 hours in a boiling water bath. A crystalline deposit formed during the heating. The solution was left at room temperature; crystallisation appeared complete in 2-3 days, and after 14 days, the solution was filtered. The residue (5.2g.) was Compound A (see below). The solution was subjected to the Bush and Densen distribution with water as the aqueous phase. The combined ethyl acetate fractions contained 39.0g. of material. The combined aqueous phases were taken to dryness and the residue was dissolved in ethanol (300ml.). The solution, with acetic acid (3ml.) was boiled for 2½ hours. The solution was then taken to dryness, and the residue was dissolved in water (120ml.) containing sodium bicarbonate (3g.). This solution was continuously extracted with ether for 8 hours. On removal of solvent, the residue was obtained as very small crystals; trituration with a few ml. of cold ethanol, in which the residue was very soluble, gave crystalline O-ethyl isomelacacidin (3.6g.), and a solution containing 9.0g. of material, from which a second crop (about 4g.) of O-ethyl isomelacacidin was obtained on concentration. The ether extraction of the residual aqueous solution was continued; subsequent 8 hour periods of extraction gave 2.4, 0.8, 0.5, 0.6g. of material, found by paper

chromatography to contain melacacidin and O-ethyl isomelacacidin as well as O-ethyl isomelacacidin.

The aqueous portion from the ethanol extract of the wood was found by paper chromatography to contain pipecolic and 4-hydroxypipecolic acids, as did the aqueous extract from the wood. These two fractions were combined with that fraction from the previous extraction which contained amino-acids, and freed from acid-insoluble material. The amino-acids were adsorbed on a Zeokarb 225 column (200g.) and eluted with 0.1 N-ammonia. The amino-acids were eluted without separation. Paper chromatography (BAW, 15 hours) showed the presence of pipecolic acid and 4-hydroxypipecolic acid (identified by their distinctive ninhydrin colours, the red fluorescence of the ninhydrin product in UV light and the green colour given with isatin) and a small amount of proline (detected by isatin). The crude amino-acid fraction was crystalline (1.75g.).

The amino-acids were added to a column of Zeokarb 225 resin (50g.) and eluted with dilute hydrochloric acid, the eluate being collected in fractions of 70ml. The eluant series was; fractions 1-2, feed and water; 3-7, 1l. of 0.1N-HCl; 18-21, 250ml. of 0.2N-HCl; 22-25, 250ml. of 0.4N-HCl; 26-33, 500ml. of 0.8N-HCl; 34-37, water; 38-44, 3N-ammonia. The eluate fractions were evaporated to dryness and examined by

paper chromatography (BAW, 12 hours, 26cm.). 4-Hydroxypipecolic acid (Rf 0.18) was present in fractions 6-23, and pipecolic acid (Rf 0.35) in fractions 22-29. Another compound (Rf 0.11) giving with ninhydrin a purple colour not fluorescent in UV light, was present in fractions 6-23. The ammonia eluates contained traces of only pipecolic acid.

Fractions 6-23 were combined and nitrous fumes were passed in for about 1 hour. Next day the solution was boiled for 30 minutes, and the amino-acids were recovered by absorption on a Zeokarb 225 column (50g.) and elution with ammonia. From water-ethanol, 4-hydroxypipecolic acid, m.p. 286-287° decomp. (0.77g.) crystallised. Its identity was confirmed by paper chromatography in BAW and by the ninhydrin colour.

Fractions 25-28 were combined and evaporated to dryness. Extraction with ethanol and concentration of the extract yielded pipecolic acid hydrochloride (52mg.) identified by paper chromatography in BAW, and by the ninhydrin colour.

Characterisation of "Compound A". The crude material separated from boiling water as shining pale yellow flakes (about 50mg. per 100ml. of water). This product (300mg.) was recrystallised by solution in ethanol (5ml.) and addition of water (10-20ml.), a layer of light petroleum being used to prevent oxidation. After two such recrystallisations, the product had m.p. 284-285°, after sintering at 175-180°;

this behaviour was not changed by another recrystallisation. For analysis, a sample was dried over phosphorus pentoxide at room temperature in vacuo for 24 hours. (Found: C,57.6; H,4.4. $C_{15}H_{12}O_7 \cdot 0.5H_2O$ requires C,57.6; H4.2%). This material gave red colours, stable for several hours, when solutions in aqueous-ethanolic hydrochloric acid were treated with magnesium or with zinc, which indicates that it is a flavanonol (175, 176).

When chromatographed in BAW, it gave a streak, Rf. 0.57-0.79 (traces of 7,8,3',4'-tetrahydroxy-flavonol were also present) not visible in day-light when fresh but gradually becoming yellow (3-4 hours), and, when fresh, visible as a faint blue fluorescence in U.V. light. Hydrochloric acid or Roux's spray gave an immediate deep yellow colour. In 2% acetic acid, it gave a spot Rf 0.15 tailing to the origin; a spot due to flavonol was also present (Rf 0.0).

Extraction of A. excelsa SN5919 sapwood.

Extraction J: Milled sapwood (1,690g.) was extracted with light petroleum (13 hours), acetone (32 hours), ethanol (10 hours), and water (8 hours). The light petroleum extract contained 1.8g. of wax. The acetone extract was concentrated to a syrup (74g.); a brief investigation did not result in any crystalline products and polyphenols were not detectable.

Chromatograms of the water-soluble portion of the ethanol extract, and of the water extract, showed the presence in both of much pipecolic and 4-hydroxy-pipecolic acid (Rf 0.37 and 0.21 respectively in BAW). A component (Rf 0.15) giving a blue colour with ninhydrin was also present. These fractions were combined, freed from acid-insoluble material, and passed through a column of Light's cation exchange resin (200g.). The amino-acids were eluted with 2N-ammonia. The amino-acid fraction was evaporated to a dark syrup, partly crystalline (7.6g.), which was redissolved in water; carbon removed little colour, but passage of the mixture through a column of alumina (20g.) resulted in an almost colourless solution. The solution was again evaporated, and the residue was dissolved in a minimumof hot water; addition of ethanol gave 4-hydroxypipecolic acid. After several recrystallisations with the mother liquors processed systematically, a total amount of 0.56g. of 4-hydroxy-pipecolic acid, m.p. 288-290° decomp., was obtained.

The amino-acids in the mother liquors (1.8g.) were chromatographed on Whatman 3MM paper (0.10g. of mixture in 0.50ml. of solution per 25cm.; solvent BAW, about 25ml. of moving phase giving a 12 hour run on a paper 30cm. long). The amino-acids were located by spraying central strips with ninhydrin; 4-hy-

droxypipecolic acid, 5.3 to 11.0cm.; proline, 11.0 to 14.0cm.; pipecolic acid, 14.0 to 19.0cm. The amino-acids were recovered by extraction with 70% ethanol in a Soxhlet apparatus for 8 hours, yielding the following crude fractions: 4-hydroxypipecolic acid, 0.85g.; proline, 0.19g.; pipecolic acid, 0.29g. Recrystallisation of the 4-hydroxypipecolic acid fraction from aqueous ethanol yielded 0.47g., free from pipecolic acid. The pipecolic acid fraction was recrystallised from ethanol, yielding 0.16g., m.p. 257-260° decomp., $[\alpha]_D^{25}$ - 24.6° (C,2 in water). The proline fraction contained much 4-hydroxypipecolic acid and was discarded.

Extraction K: Mixed sapwood and outer heartwood, in the proportion about 70:30 (2,470g.) was extracted for the isolation of amino-acids. The wood was extracted with acetone (20 hours), ethanol (8 hours), and water (8 hours). The acetone extract was discarded. The ethanol and water extracts were found by paper chromatography (BAW, 12 hours, 25.8cm.) to contain pipecolic acid (Rf 0.39), 4-hydroxypipecolic acid (Rf 0.24) and some α-amino-acids. These extracts were combined, freed from acid insoluble material, and passed through an ion exchange column (Zeokarb 225, 200g.). The amino-acids were eluted with 0.1N-ammonin; no separation of amino-acids occurred during the elution. The amino-acid fraction crystallised when taken to dryness (4.14g.).

The amino-acids were dissolved in water (50ml.) and nitrous fumes were passed in at intervals over 1 day. The solution was acidified with hydrochloric acid, boiled and evaporated to a syrup. This was dissolved in water (50ml.) and added to an ion exchange column (Zeokarb 225, 50g.). The imino-acids were eluted with dilute hydrochloric acid, the eluate being collected in 70ml. fractions. The eluant series used was as follows: fraction 1-3, feed and water wash; 4-14, 750ml. of 0.2N-HCl; 15-17, 250ml. of 0.3N-HCl; 17-20, 250ml. of 0.4N-HCl; 21-24, 250ml. of 0.8N-HCl; 25-28, water; 29-34 ammonia. The fractions were evaporated to dryness and examined by paper chromatography. Pipecolic acid was present in fractions 10-21, with traces in fractions 7-9 and 22-24, proline was present in fractions 11-24 and 4-hydroxypipecolic acid was present in quantity in fractions 4-14, with small amounts in fractions 15-17 and traces in fractions 18-24. (When compared with results obtained when less amino-acid was applied to the column, these results show that the column was overloaded. With lesser loads, about 1g., pipecolic acid did not elute with hydrochloric acid less than 0.8N). The ammonia eluate was found to contain 0.33g. of coloured and non-crystalline material, in which much pipecolic and some 4-hydroxypipecolic acid was present; this was discarded.

Fractions 4-14 were collected and returned to the ion-exchange column. The amino-acids were eluted with dilute ammonia; crystallisation from aqueous ethanol gave 4-hydroxypipecolic acid, m.p. 294° decomp. (1.34g.).

Fractions 15-24 were combined, treated with carbon, and concentrated to a syrup which crystallised in part. This gave crude pipecolic acid hydrochloride, m.p. 236-238° (0.28g.), in which pipecolic acid was identified by chromatography in BAW and by its ninhydrin colour.

Extraction of A. melanoxylon SN5905 heartwood.

Extraction L: (See Figure 16). The milled heartwood (1293g.) was extracted with light petroleum (12 hours), yielding 2.0g. of yellow wax. Ether extraction (33 hours) gave a deposit (fraction (a)) and a solution containing 11.2g. of material (fraction (b)). The wood was extracted with acetone (25 hours, fraction (c)), again with acetone (20 hours, fraction (d)), with ethanol (12 hours, fraction (f)) and with water (10 hours, fraction (g)).

Fraction (a) was triturated with cold ethanol, thus dividing it into an insoluble portion (grey needles, 7.9g., fraction (aa)) and a soluble portion (20.2g., fraction (ab)). Chromatograms of fractions (aa), (ab) and (b) showed the presence of melacacidin; fractions (ab) and (b) also contained O-ethyl isomel-

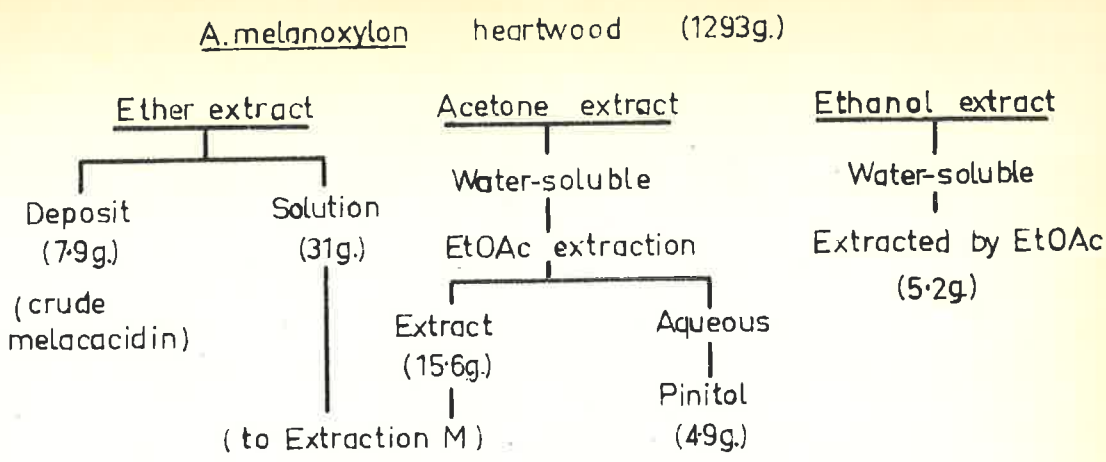


Figure 16 : Extraction L.

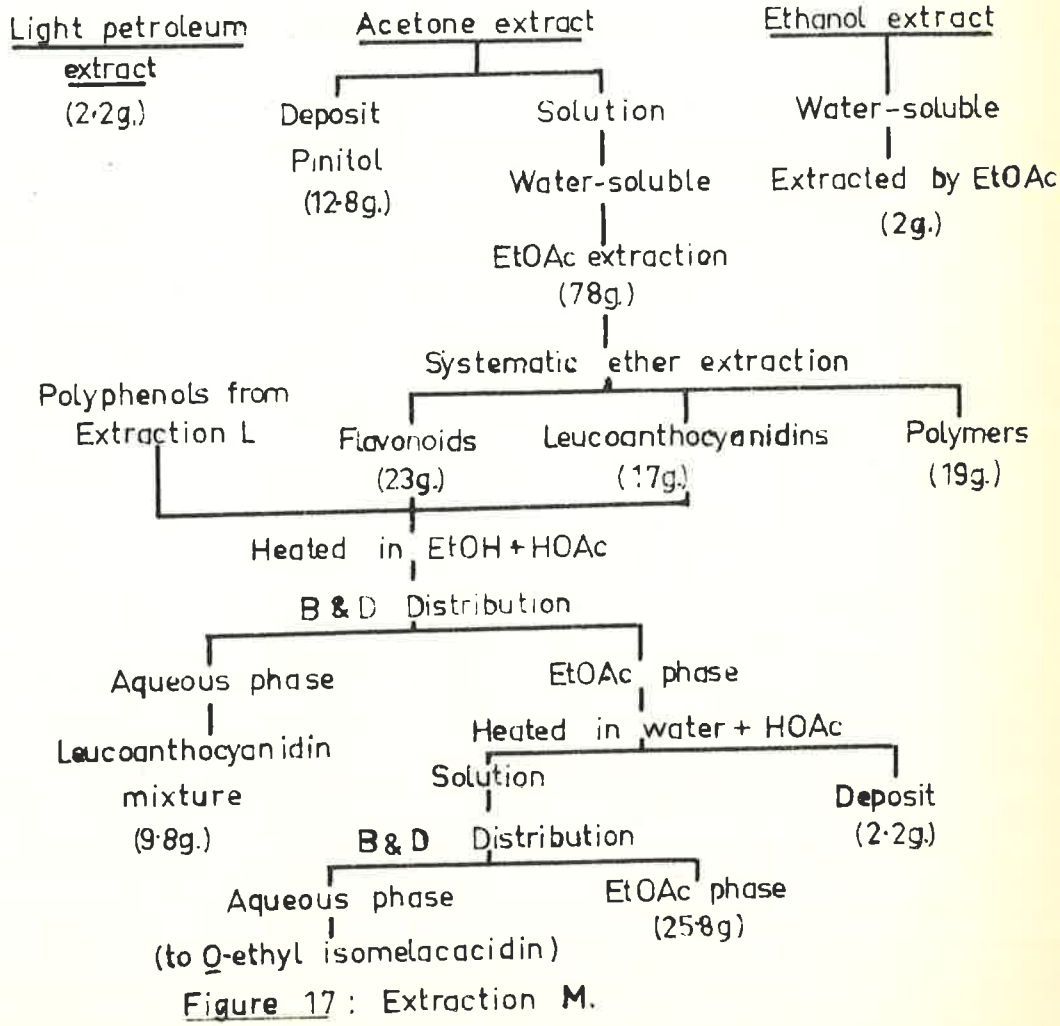


Figure 17 : Extraction M.

acacidin. Fraction (ab) contained much polymeric material, and both (ab) and (b) contained polyphenols.

Portion of fraction (aa) (5g.), with methyl sulphate (5g.) and potassium carbonate (35g.), was heated in boiling acetone for 4 hours. The acetone soluble products were treated with ammonia (10ml. of strong solution) to remove excess of methyl sulphate. The solvent was removed and the residue was dissolved in ether with the addition of sufficient ethanol to achieve solution; the solution was washed with dilute sodium hydroxide and dried over anhydrous sodium sulphate. The solution was left at 0°; it deposited crystals of melacacidin tetramethyl ether, m.p. 138-141° (0.37g.). The mixed melting point with the tetramethyl ether of melacacidin from A. harpophylla (m.p. 144-145°) was 140-143°. A second crop (0.15g.) of crystals m.p. 136-138° was obtained by slight concentration of the mother liquors. (Total yield, 8%).

The acetone extracts (c) and (d) were concentrated to syrups (89, 11g. respectively) which were dissolved together with water (2l.). Next day, the mixture was filtered (the brown, amorphous residue weighed 4.8g.) and, after concentration under reduced pressure to 200ml., continuously extracted with ethyl acetate for 16 hours. The extract (15.6g.) appeared from paper chromatograms to consist almost entirely of mixed leucoanthocyanidins.

The aqueous solution contained 13.6g. of solids. Paper chromatography (BAW, 11 hours, 42cm.) showed that considerable polyol Rf 0.13 was present. When a strong solution inwater was allowed to evaporate slowly, much material crystallised. This product was washed with ethanol and acetone, and dissolved in water. The solution was treated with basic lead acetate until no precipitation occurred on further addition, with dilute sulphuric acid and then with barium hydroxide, so that the solution contained no lead or sulphate. This solution was concentrated to a syrup which crystallised, giving 4.9g. of crystals, m.p. 172-174°, $[\alpha]_D^{27}$ + 60.1° (c,3.3 in water). A mixed m.p. with pinitol from A. intertexta (m.p. 185°) was 176-182°. On chromatography in acetone:water 85:15 (177,178,179) (8 hours) and detection with periodate and starch-iodide sprays (180), the crude pinitol from A. melanoxylon gave a single spot, at the same distance from the origin (23cm.) as the spot from pinitol from A. intertexta.

The ethanol extract (fraction (f)) was processed in the same way as fractions (c) and (d); the ethyl acetate extract (5.2g.) was a mixture showing no major components on paper chromatography. The residual aqueous solution was examined by paper chromatography (BAW, 11 hours, 42 cm.). A small amount of pipecolic acid (Rf 0.30, same as a sample run on the

same sheet) was detected by ninhydrin.

The aqueous extract (fraction (g)) was filtered and concentrated to about 200ml. No amino-acids were detected by paper chromatography.

The fractions containing mixed leucoanthocyanidins were added to those of Extraction M for further processing.

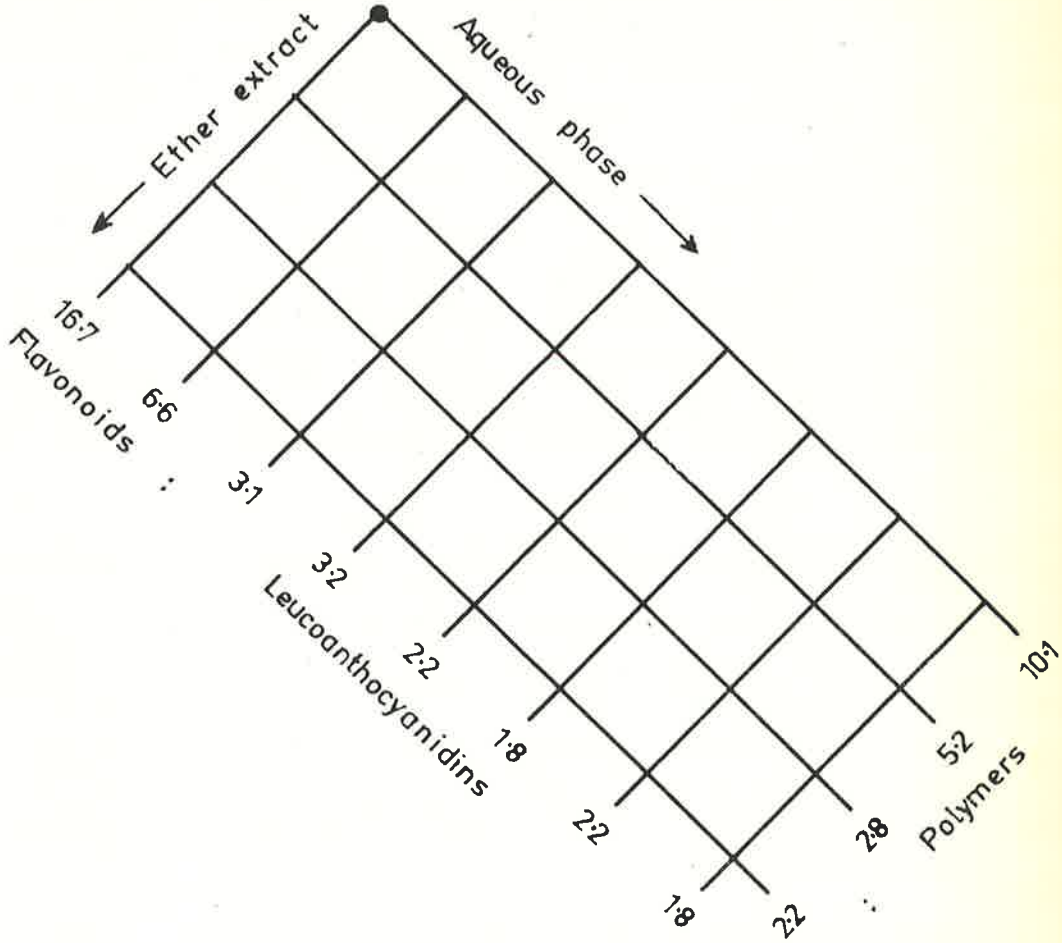
Extraction M: (See Figure 17). Milled heartwood (1562g.) was extracted with light petroleum (10 hours) yielding 2.26g. of wax. Acetone extraction (19 hours) gave a deposit (12.8g., fraction (a)) and a solution which was concentrated to a syrup (154g., fraction (b)). Acetone extraction for a further 24 hours gave no more deposit; the extract was concentrated to a syrup (10g. fraction (c)). The wood was then extracted with ethanol for 11 hours (fraction (d)).

The deposit obtained during acetone extraction (fraction (a)) had m.p. 182-4°, and mixed m.p. 183-184° on admixture with pinitol from A. intertexta (m.p. 185°). Paper chromatography (BAW) showed a single component of the same Rf as pinitol. After recrystallisation from water-ethanol it had $[\alpha]_D^{27}$ +60.5°. (C, 3.0 in water).

Fractions (b) and (c) were continued and processed as in extraction, yielding 78g. ethyl acetate extract. The ethanol extract was processed similarly, yielding only 2g. of ethyl acetate extract. Paper chromatography showed the presence of

melacacidin and O-ethyl isomelacacidin in the ethyl acetate extracts from (b) and (c); other polyphenols including much polymeric material were also present. The material from the ethanol extract appeared to contain little leucoanthocyanidin, and to be mainly polymeric material.

An attempt was made to isolate melacacidin by systematic continuous liquid/liquid extraction with ether, (see Figure 18). The combined ethyl acetate extracts in 200ml. of water were extracted with ether for eight 8-hour periods, and then with ethyl acetate. The first ether extract was dissolved in about 50ml. of water and extracted with ether for 8 hours. To the aqueous residue the second ether extract of the original solution was added, and ether extraction carried out for 8 hours. The process was repeated systematically so that the material was put through four ether extractions. A series of fractions was obtained; the first fraction contained material which had been extracted by ether within 8 hours on each of the four occasions, whereas the second fraction contained material which had been extracted by ether within 8 hours on only three of the four occasions, and on the other occasion had been extracted in a second 8 hour extraction. The material not extracted by ether during the eight 8-hour extractions on the first, second and third occasions (weighing 10.1, 5.2, 2.8g. respectively) proved to be mainly poly-



Each intersection represents 8 hours continuous ether extraction.

Weights of fractions are in grams.

Figure 18 : Systematic ether extraction of A. melanoxylon polyphenols, Extraction M.

meric material and was discarded. The first two fractions of ether extractions were found to contain little leucoanthocyanidin; they weighed 16.7 and 6.6g. respectively. The remaining fractions (16.8g.) contained almost exclusively leucoanthocyanidins. No deposit of melacacidin was obtained at any stage.

The fractions containing mixed leucoanthocyanidins, from this extraction (40g.), and from Extraction L (46g.) were dissolved in ethanol (400ml.) containing acetic acid (4ml.) The solution was boiled for 2 hours, the solvent was removed by distillation, and the residue was subjected to the usual Bush and Densen separation. The combined aqueous fractions were concentrated to 180ml. under reduced pressure, and continuously extracted with ether. No crystals were obtained during extraction, but the ether extract, on concentration, gave some cyrstalline material which was contaminated by polymeric material carried over as an emulsion in the ether, and could not be obtained pure. The crude leucoanthocyanidins weighed 9.8g.

The combined ethyl acetate phases contained 58g. of material. This was heated in water (400ml.) containing acetic acid (8ml.) in a boiling water bath for 2 hours. A deposit (2.2g.) formed; this was identified as 7,8,3',4'-tetrahydroxyflavonol by chromatography (BAW and 2% acetic acid). The solution was again subjected to the Bush and Densen

distribution. The ethyl acetate phases contained 25.8g. of material. The combined aqueous phases were added to the corresponding fraction from Extraction N and used for the preparation of O-ethyl isomelacacidin; this preparation is described in the section on O-ethyl isomelacacidin.

Extraction N: (See Figure 19). Milled heartwood (5,934g.) was extracted with light petroleum (12 hours), acetone (36 hours), ethanol (12 hours) and water (12 hours). The light petroleum extract contained 9.0g. of yellow wax.

The acetone extract deposited 55g. of crude pinitol which gave a single spot on paper chromatography (BAW) at the same Rf as authentic pinitol. After one recrystallisation from water-ethanol, it had m.p. 184° alone and when mixed with pinitol from A. intertexta, $[\alpha]_D^{16}$ + 66.2° (C,3.1 in water). The acetone solution was concentrated to 1 litre and then diluted with water to 8. Next day, the mixture was filtered, evaporated under reduced pressure to 700ml., and continuously extracted with ethyl acetate for 24 hours. The extract was withdrawn after 2 and 6 hours. The total extract weighed 286g. The residual aqueous solution contained some pinitol.

The ethanol extract was processed in the same way as the acetone extract. Ethyl acetate extraction of the aqueous solution yielded 18.0g. of extractives in 24 hours.

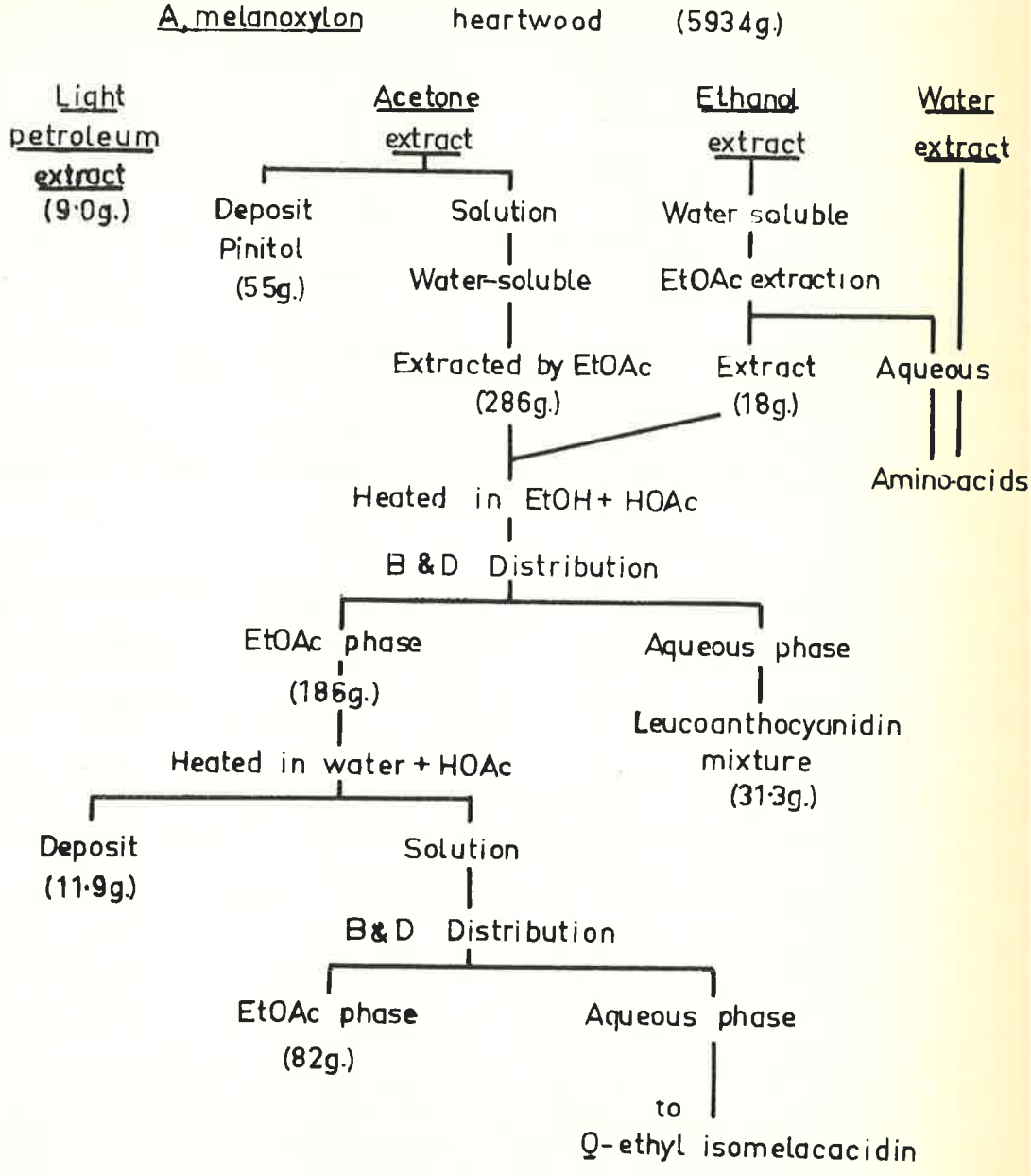


Figure 19 : Extraction N.

The residual aqueous solution from the ethanol extraction, and the water extract were combined, freed from acid-insoluble material, and the amino-acid fraction isolated by use of an ion-exchange resin column (Zeokarb 225). This yielded 0.20g. of black, non-crystalline material. Paper chromatography (BAW) showed the presence of a trace of pipecolic acid, and some α-amino-acids.

The material (304g.) extracted by ethyl acetate was boiled in ethanol containing 1% acetic acid, (in batches of 50g. of polyphenols and 250ml. of ethanol) for 2 hours, and then solvent was removed. The residue was dissolved in 800ml. of M/15 phosphate buffer pH7.0, and subjected, in two portions, to the usual Bush and Densen distribution. These distributions were attended with considerable difficulties due to emulsions, and the division between the phases was unusually difficult to distinguish. The aqueous phases were combined, concentrated under reduced pressure (45-50°C) to 500ml., and continuously extracted with ether. Although the extracts were seeded, no deposit of melacacidin was obtained. The amounts of ether extractives obtained in successive periods of extraction were: 12 hours, 8.5g.; 24 hours, 15.7g.; 12 hours, 3.2g.; 8 hours, 1.7g.; 12 hours, 2.2g.; total 31.3g. in 68 hours.

A large deposit of black material formed in the aqueous residue.

The combined ethyl acetate fractions from the distributions contained 186g. of material. This was dissolved in water (700ml.) containing acetic acid (14ml.) and heated in a boiling water-bath for 2 hours. On standing at room temperature, a grey powder deposited (11.9g.). This deposit was identified as 7,8,3',4'-tetrahydroxyflavonol by paper chromatography (BAW and 2% acetic acid). The solution was adjusted to a volume of 800ml. with water, and subjected, in two portions, to the usual Bush and Dencen distribution. The combined ethyl acetate phases contained 82g. of material. The combined aqueous phases were concentrated under reduced pressure, and, this fraction together with the corresponding fraction from Extraction M, was used for the preparation of O-ethyl isomelacacidin which is described in the section on O-ethyl isomelacacidin.

Extraction of A. melanoxylon SN5905 sapwood.

Extraction O: Milled sapwood containing no heartwood (991g.) was extracted with light petroleum (16 hours), acetone (41 hours), ethanol (7 hours) and water (7 hours).

The light petroleum extract contained 1.1g. of almost colourless, sticky wax.

The acetone and the ethanol extracts were con-

centrated to syrups (42,25g. respectively) and taken up together in water (400ml.) The mixture was filtered and the solution was continuously extracted with ethyl acetate, yielding in successive 8 hour periods, 1.9, 0.3 and 0.2g. of syrupy material. These extracts appeared to contain no polyphenols. The residual aqueous solution was concentrated to a syrup (5.1g.), in which crystals formed; these were collected after dilution of the syrup with ethanol (10ml.). Recrystallisation from aqueous ethanol gave D-mannitol as loose clusters of needles (90mg.), m.p. 163°, mixed m.p. with an authentic sample 164-165° (the sample had m.p. 166°). On paper chromatograms (BAW, 12.6cm.) it gave a single spot Rf 0.16 (same Rf as mannitol), which reduced alkaline silver nitrate slowly in the cold and readily at 100°. In BAW (20 hours), the mother liquor showed a spot of the same Rf as mannitol (11.0cm.) and two other polyol spots (13.0 and 15.3cm.); no reducing sugars were present. (On this chromatogram, glucose ran 10.5cm. and pinitol, 10.5cm.). In ethyl acetate;acetic acid: water, 6:3:2, the polyols ran 13.5cm. (mannitol), 16.3 and 19.0cm. Pinitol, which has a much lower Rf in this solvent than mannitol, was absent.

The above aqueous solution, and the water extract, were added to the corresponding fractions from Extraction P for the isolation of the amino-acids.

Extraction P: Milled sapwood (2,781g.) was extracted with acetone (24 hours), ethanol (12 hours) and water (12 hours). The acetone and ethanol extracts were concentrated to syrups which were diluted with water. The mixture was filtered and continuously extracted with ethyl acetate for 16 hours; emulsions occurred during the extraction. The extract was concentrated to a syrup (3.4g.) Polyphenols were not detected in this extract.

The aqueous solution remaining from the above extraction, and the water-extract, were combined with the corresponding fractions from Extraction O, and passed through a Zeokarb 225 column (200g.). The amino-acids were eluted with 0.1N-ammonia; no selective elution was apparent. This fraction weighed 1.28g. The following amino-acids were detected by paper chromatography (BAW, 15 hours, 28cm.): pipecolic acid (Rf 0.37), 5-hydroxypipecolic acid (?) (Rf 0.22), a trace of proline (Rf 0.28) and

α - amino-acids (Rf 0.06, 0.12, 0.18, 0.34, 0.44, 0.55, 0.61).

The amino-acids in dilute hydrochloric acid were treated with nitrous fumes for 1 hour, then left overnight. The solution was then boiled gently for 30 minutes, and evaporated to dryness. The residue, in water (50ml.), was added to Zeokarb 225 column (50g.). The imino-acids were eluted with the same

series of hydrochloric acid solutions as in Extraction I. After evaporation to dryness, the fractions were examined by chromatography (BAW); the following components were detected with ninhydrin: pipecolic acid Rf 0.35) in fractions 25-28; a component (Rf 0.14) giving a purple, non-fluorescent colour with ninhydrin in fractions 7-21; a major component (Rf 0.21) (5-hydroxypipecolic acid?) giving a blue purple colour with ninhydrin, which fluoresced bright red in UV light, in fractions 9-23; 4-hydroxypipecolic acid (Rf 0.19) appeared to be absent. Fractions 17-27 showed strong chloride areas on the chromatogram and were thought to contain a salt. The ammonia eluate contained a trace of pipecolic acid.

Fractions 13-23 were combined; the free amino-acids were obtained by adsorption on Zeokarb 225 and elution with ammonia, but attempts to obtain a crystalline product were unsuccessful. Fractions 25-28 were combined and evaporated to a syrup (0.24g.) which crystallised in part. After the syrup was diluted with a little ethanol, the crystals were collected and recrystallised from ethanol, giving 52mg., m.p. 228-235° decomp., then 25mg., m.p. 238° decomp. A chromatogram on the once-recrystallised hydrochloride showed that it contained no pipecolic acid. Dragendorff's reagent (181) detected a component giving a red-purple spot fading to an orange-red; this had Rf 0.24 in BAW, 0.71 in ethanol:water:con-

centrated ammonia 90:5:5, 0.36 in n - butanol: 10N-HCl:water 100:20:39, (Found: C,39.3; H,8.0; N,7.8). This material was not identified.

Extraction of A. mollissima heartwood.

Extraction Q: Milled heartwood (1,841g.) was extracted with light petroleum for 11 hours, yielding 3.59g. of a deep orange-coloured, low-melting wax. The wood was then extracted with acetone for two 24 hour periods; the extracts were concentrated to syrups (239 and 7g. respectively). The wood was finally extracted with ethanol for 8 hours.

The combined acetone extracts were taken up in water (2l.) and the mixture, after being left overnight was filtered from a brown powder (48g.) which appeared to be entirely polymeric material. The filtrate was concentrated under reduced pressure to 200ml. and continuously extracted with ethyl acetate. Successive 4 hour extractions yielded 47.2, 3.4, 0.6g. of material. The residual aqueous solution was evaporated to a syrup (41.5g.) which crystallised. Paper chromatography (BAW) showed the presence of compounds reducing alkaline silver nitrate at 1.5cm (trace amount only) and 5.0cm. from the origin (pinitol moved 5.0cm. from the origin). Amino-acids were absent.

The ethanol extract was concentrated under reduced pressure to a syrup which was diluted with

water (1l.). Next day the mixture was filtered; the filtrate was concentrated under reduced pressure and continuously extracted with ethyl acetate. Successive 4 hour extractions yielded 2.6, 0.1. 0.05g. of material. The aqueous residue was evaporated to a syrup (4.1g.) which crystallised. Paper chromatography (BAW) showed the presence of the same two polyols as in the acetone extract, and also three components detected with ninhydrin; pipecolic acid (9.8cm. from the origin), 4-hydroxypipecolic acid (5.5cm.) and a substance (3.7cm.) which gave a blue colour with ninhydrin. The last was present apparently in very small amount. This fraction was added to the corresponding fraction of Extration R.

The ethyl acetate extracts obtained above were examined by paper chromatography in BAW (12 hours, 23cm.) and 2% acetic acid (2¼ hours, 31cm.) All the fractions showed much polyphenolic material as streaks of no definite Rf: this reacted with the ferric-ferricyanide reagent, and gave a red colour with Roux's spray. Individual components (leucoanthocyanidins) seemed to be present at Rf 0.73-0.83 and 0.58-0.66 in BAW, 0.70-0.77 and 0.50-0.60 in 2% acetic acid. On a different chromatogram, gleditsin (= mollisacacidin) had Rf 0.57-0.65 in BAW.

The ethyl acetate extracts were combined (53g.).

Portion (25g.) was subjected to 50 tube counter-current distribution between ether and M/15 aqueous phosphate buffer pH7.0. The polyphenol mixture was added in one portion and much remained undissolved. After distribution, a pink colour was present in the aqueous phases of tubes 38-44. Samples from even numbered tubes were chromatographed in BAW (11½ hours at 0°C, 18.5cm.) and 2% acetic acid (2½ hours, 19cm.). The components detected with ferric-ferricyanide reagent are listed in Table 12. Components 1-4 were leucoanthocyanidins: component 5 had a yellow fluorescence in UV light. Component 1 had the same Rf in both solvents as a sample of gleditsin run simultaneously.

Table 12.

Counter-current distribution of A. mollissima polyphenols.

Component	Tube Nos.	Peak	Rf (BAW)	Rf (acetic acid)
1	2-18	10	0.56-0.73	0.59-0.69
2	30-42	36	0.80-0.94	0.43-0.51
3	34-46	41	0.80-0.94	0.71-0.79
4	46-50	50	?	0.26-0.35
5	46-50	50	about 0.7	0.0

The contents of tubes 1-5 gave 2.2g. of material when continuously extracted with ether. The contents of tubes 6-18 were concentrated under reduced pressure to about 100ml. and continuously extracted

with ethyl acetate (4 hours) to yield 4.04g. of material. From tubes 30-35, 1.22g. of material was obtained by the same method. Tube 36 yielded 0.32g. of material. The contents of tubes 37-40, by extraction several times with ether in a separatory funnel yielded 1.23g. of material. Tube 41 yielded 0.26g. of material by ethyl acetate extraction in a separatory funnel. The contents of tubes 41-47 were extracted several times with ether in a separatory funnel, and yielded 1.27g. of material. The contents of tubes 48-50 were extracted three times with ether and gave 0.50g. of yellow powder.

The substances isolated from tubes up to 41 were examined by chromatography in 2% acetic acid. All showed with Roux's spray, a major component Rf 0.52-0.60 and a minor component Rf 0.60-0.69. The fraction from tubes 6-18 did not yield any crystalline product from water, methanol-water, ethanol-water or methanol-ether; seeding with gleditsin had no effect.

A portion of the fraction from tubes 1-5 (54mg.) in water (2ml.) was heated in a boiling water bath. Samples were withdrawn at intervals and chromatographed in 2% acetic acid (21.5cm.). The solution initially showed three leucoanthocyanidin spots, Rf 0.59-0.71 (major), 0.45-0.54, 0.73-0.83, together with much polymeric material as a streak from

the origin to Rf 0.5. The spot Rf 0.73-0.83 disappeared during the heating, being reduced by about half at 10 minutes and very faint at 40 minutes.

Extraction R: The previous extraction (Extraction Q) was repeated three times. During extraction with acetone, a white crystalline deposit formed in the still (that from the third extraction was lost by charring). Details are given in Table 13.

Table 13.

Details of Extraction R.

Weight of wood	Light petroleum extract	Deposit from acetone.
1,462g.	2.6g.	19.9g.
1,430	2.6	25.0
3,896	6.9	lost

The deposit from the acetone extraction had m.p. 183-186°, alone and mixed with pinitol from A. intertexta. It had $[\alpha]_D^{26}$ +61.9° (C,3.2 in water).

The ethanol extracts, together with that from Extraction Q, were concentrated to syrups, then diluted with water. The filtered solution was continuously extracted with ethyl acetate to remove polyphenols. The residual aqueous phase was passed through a cation exchange column (Light's, 200g., 65 x 2.5cm.). The amino-acids were recovered from the column with 1N-ammonia and obtained as a black mass (1.80g.). This was dissolved in water (20ml.) and passed through a column of alumina (5g.) in an

unsuccessful attempt to remove the colour. The solution was diluted to 80ml. and was boiled with alumina (3g.); most of the colour remained on the alumina. The solution was evaporated and the residue (1.43g.) in water (5ml.) was diluted with ethanol (30ml.). The remaining colouring matter presipitated. Crystals formed slowly in the filtrate and were collected after 14 days. Fractional crystallisation from water-ethanol yielded 4-hydroxypipecolic acid (0.53g.) which contained a trace of pipecolic acid; the mother liquors contained pipecolic acid, proline and 4-hydroxypipecolic acid (0.79g).

Extraction S: This extraction was carried out to isolate the amino-acids; the wood used was the same as in the previous extractions, but had been in storage for about 15 months. Light petroleum extraction (8 hours) of the heartwood (6.25Kg) yielded 8.3g. of wax. Acetone extraction (two 12 hour periods) did not yield a deposit of pinitol. The acetone extract was concentrated, and a filtered solution of the residue in water was extracted. with ethyl acetate to remove polyphenols. Evaporation of the aqueous solution gave a dark dyrup (74g.) which crystallised. Paper chromatography (BAW) showed that this contained only one polyol constituent (of the same Rf as pinitol) besides some polymeric polyphenols.

The wood was extracted with ethanol (12 hours) and with water (12 hours). The ethanol extract was concentrated, then diluted with water. The filtered solution was continuously extracted with ethyl acetate to remove polyphenols, and then passed through a Zeokarb 225 column (259g.). The amino-acids were eluted with ammonia (1.05g.). The water extract from the wood contained much material insoluble in cold water; this appeared to be polymeric. The filtered extract was freed from acid-insoluble material, and was passed through the ion exchange resin. The crude amino-acid fraction eluted by ammonia weighed 0.96g. Paper chromatography (BAW) showed that these fractions contained pipecolic and 4-hydroxypipecolic acids. Proline was absent and no other ninhydrin-positive constituents were present in appreciable quantity.

The combined amino-acid fractions were added to a Zeokarb 225 column (50g.) and the amino-acids eluted with dilute hydrochloric acid. (The concentrations used for elution were the same as those given in Extraction I). Pipecolic acid was present in fractions 22-32, 4-hydroxypipecolic acid in fractions 9-23 and also in the ammonia eluates. The amount of pipecolic acid was very small. 4-hydroxypipecolic acid was obtained from fractions 10-22 (0.49g., m.p. 292-293° decomp.) and from the ammonia eluates,

fractions 38-39 (0.21g. m.p. 272-274° decomp.). The identity of these products was confirmed by paper chromatography (BAW).

Extraction of A. mollissima sapwood.

Extraction T: Milled sapwood (7.39Kg.) was extracted with acetone (12 hours). A syrupy deposit formed in the still (48g., fraction (a)). The acetone solution was concentrated to a syrup (299g., fraction (b)). The wood was extracted with ethanol (12 hours) and with water (12 hours).

No polyphenols appeared to be present in fraction (b). Extraction of the water-soluble portion with ethyl acetate yielded a syrupy deposit in the ethyl acetate (19.3g.). No individual component could be detected in this fraction by paper chromatography (BAW) and examination for polyols and polyphenols. The ethyl acetate solution contained 4.5g. of material, in which, apart from a trace of reducing sugar, possibly fructose, no component could be recognised.

The syrupy deposit from the acetone extraction (fraction (a)) formed cyrstals over a period of months. They were collected and washed with 60% and 80% ethanol, with considerable loss. Paper chromatography (BAW, 20 hours, 39.0cm.) showed that both the crystals and the mother liquors contained fructose which reduced alkaline silver

nitrate readily in the cold, and gave a pink colour with naphthoresorcinol (182). An authentic sample of fructose chromatographed on the same sheet had the same Rf. Chromatography in ethyl acetate:acetic acid:water 6:3:2 showed the presence of reducing sugars at 16.0 and 13.3cm. from the origin (glucose 13.3, fructose 16.0cm.) in the crystalline material; a component (9.5cm.) was present in small quantity in the mother liquors (sucrose, 8.9cm.). Chromatography in 85% acetone (8 hours) failed to separate the sugars, which all had Rf values similar to that of glucose. A spot corresponding to sucrose could not be detected with periodate and starch-iodide sprays (180). The crystalline mixture (2g.) was converted to osazone in standard conditions (180a), under which fructose yields 3.7g. of osazone and glucose, 3.0g. of osazone. The yield from the crystalline mixture was 1.55g., although the osazone formed readily. The m.p. of the osazone was 190-196°, and, after recrystallisation from ethanol containing a little water, 198°, alone and mixed with authentic glucosazone. The crystalline mixture therefore appeared to contain, besides glucose and fructose, some other component in large amount.

The ethanol extract from the wood was concentrated to a syrup which was diluted with water. The mixture was filtered and continuously extracted with

ethyl acetate for 24 hours. The extract (4.7g.) appeared to be polymeric.

The aqueous extract from the wood contained a large quantity of grey insoluble material, apparently polysaccharide. The filtered extract was concentrated under reduced pressure to 600ml. Concentrated hydrochloric acid (20ml.) was added, and the solution was heated in a boiling water bath for 3 hours. No colour developed, but a considerable quantity of fawn-coloured material precipitated. This also appeared to be polysaccharide.

The water-soluble portions of the ethanol and water extracts were combined and passed through an ion exchange column (Zeobark 225, 259g.). Elution of the amino-acids was begun with 0.1N-ammonia but the flow rate diminished, and the flow almost ceased after addition of about 250ml. of eluant. Satisfactory elution was obtained with 1.5N-ammonia. The amino-acid fraction (15.2g.) contained pipecolic acid, 4-hydroxypipecolic acid, proline and several α-amino-acids. It was dissolved in water (100ml.), and hydrochloric acid (20ml. of 6N), the solution was cooled in ice and treated with seven portions of hydrochloric acid (10ml. of 6N) and sodium nitrite (10ml. of 6M) at hourly intervals. The solution was then continuously extracted with ether for 47 hours; water (50ml.) was added to the ether before

the extraction was started. The ethereal solution was strongly acidified with hydrochloric acid and concentrated to a syrup (15.0g.). The residual aqueous solution was twice treated with further portions of hydrochloric acid and sodium nitrite, followed by continuous ether extraction for 22 hours; this yielded 0.30 and 0.45g. of syrupy product.

The imino-acid fractions were added to the corresponding fraction of Extraction U.

Extraction U: This extraction was carried out on wood (10.51Kg.) which had been stored about 12 months; the wood was sapwood and some outer heartwood. Extraction was carried out to obtain the amino-acids; no other components were investigated.

Light petroleum extraction yielded 12.9g. of wax. The wood was extracted with acetone; no deposit formed in the still. The alcohol and water extracts were processed as described in the previous extraction. The water extract did not deposit much polysaccharide. The alcohol extract yielded 18.0g. of a crystalline mixture of amino-acids and the aqueous extract yielded 4.7g. of a very impure, dark mixture.

The amino-acids were dissolved in water (150ml.) and hydrochloric acid (40ml. of 10N) and the solution cooled in ice. Sodium nitrite solution (50ml. of 6M) was added during 55 minutes. The mixture frothed to about 500ml., although sprayed with alcohol at intervals. There was considerable loss of nitrous

fumes. The nitrosated mixture was continuously extracted with ether for 33 hours. The extract, with hydrochloric acid, was evaporated to a syrup (6.50g.). To the residual aqueous solution hydrochloric acid (10ml. of 10N) and sodium nitrite (10ml. of 6M) were added and the extraction was continued for 36 hours, which yielded, after evaporation with hydrochloric acid, 3.3g. of syrup. Repetitions using three times the above quantities gave, after 33 hours extraction, 7.4g., and 1.1g. of syrupy products. The last contained much pipecolic acid with some proline and 4-hydroxypipecolic acid, The total yield was 18.3g. of syrup.

The crude imino-acid fraction was combined with that from Extraction T, and was dissolved in water (200ml.). The solution was continually extracted with ether for 8 hours to remove hydroxy-acids. The ether extract, when concentrated gave crystals (1.4g.). (The mother liquor contained 3.3g. of material). Recrystallisation from ether (carbon) gave oxalic acid dihydrate as colourless needles, m.p. 100°, alone and mixed with authentic oxalic acid dihydrate.

The residual aqueous solution contained the imino-acids in dilute hydrochloric acid. It was passed through the ion-exchange column and the amino-acids were eluted with 1N-ammonia. After evaporation of the eluate, the residue was dissolved in hot water

(50ml.) and hot ethanol (150ml.) was added. The solution was filtered while hot to remove a brown flocculent precipitate. 4-Hydroxypipecolic (3.7g.) crystallised slowly. A second crop (2.62g.) was obtained after the addition of 300ml. of ethanol to the mother liquor. The first crop was of slightly brown crystals and was not decolourised completely by charcoal in water. Recrystallisation from aqueous ethanol yielded 2.0g. of 4-hydroxypipecolic acid, m.p.292-294° decomp. The mother liquors were used to recrystallise the second crop. A few drops of acetic acid were added before charcoaling, which resulted in almost complete decolourisation. The yield of 4-hydroxypipecolic acid from this second recrystallisation was 3.52g., m.p. 289-291° decomp. (Total yield, 5.52g.).

Acacia intertexta S.N.5891 heartwood.

Extraction V: Milled heartwood (579g.) was extracted with light petroleum for 10 hours, yielding 0.87g. of yellow wax. The wood was then extracted with wet ether (50ml. of water added to 2.6l of ether) for 43 hours. The still contents were changed after 10 hours (extract (a)), and at the end of the ether extraction, the still contained a deposit (1.1g., fraction (b)) (ether solution = fraction (c)). The wood was extracted with acetone for 20 hours (fraction (d)) and with ethanol for 10 hours (fraction (e)).

Fractions (a) and (c) were taken to dryness (9.1,

6.0g. respectively) and dissolved in ethanol. Some wax remained undissolved. Paper chromatography (BAW, 12 hours, 23.8cm.) showed the presence of two leucoanthocyanidins Rf 0.52-0.61 (major) and 0.63-0.69, a component Rf 0.80-0.90 giving a blue spot with hydrochloric acid, and components giving a yellow fluorescence in UV light Rf 0.47-0.68 (strong) and Rf 0.72-0.85. This solution was used for the isolation of teracacidin as described later.

Fraction (b) slowly gave crystals (0.42g.) from aqueous alcohol. After two recrystallisations from water-ethanol, it had m.p. 184-186°. This was added to a further portion of the same compound obtained from the acetone extract.

The acetone extract (fraction (d) contained 23.0g. of material. It was taken up in cold water (50ml.), the mixture was filtered, and the solution was continuously extracted with ether. This gave 1.5g. of material (fraction (da)) after 8 hours, and 0.2g. of material (fraction (db)) after a further 16 hours. Ethyl acetate extraction (24 hours) yielded 0.5g. of hygroscopic material. Fraction (da) was similar in composition to fraction (a) and (c); fractions (db) and the ethyl acetate extract did not show any major components on paper chromatography.

The aqueous residue from the ethyl acetate extraction of fraction (d) was concentrated to a syrup

under reduced pressure. The syrup was boiled in ethanol for 1 hour. Next day the crystals (2.5g.) m.p. 184-186° were collected. (The filtrate contained 0.9g. of material). Recrystallisation from aqueous ethanol (charcoal) gave colourless prisms (1.76g.) m.p. 184-185°, identified as (+) - pinitol (see below).

The ethanol extract of the wood (fraction (e)) contained 5.0g. of material, very little of which was soluble in water. The water-insoluble material was a black amorphouspowder.

Identification of (+) - pinitol.

In this work, pinitol was first isolated from A. intertexta in Extraction V. It was later obtained from the other heartwoods, and each newisolation was compared with that from A. intertexta by mixed melting point and paper chromatography.

The pinitol isolated from A. intertexta had m.p. 184-185°, $[\alpha]_D^{25}$ +64.4° (C,3.0 in water); lit. (40) m.p. 186-188°, $[\alpha]_D^{25}$ + 65.5° (water). (Found: C,43.0; H,7.3; OCH_3, 15.6. Calc. for $C_7H_{14}O_6$: C,43.3; H,7.3; OCH_3, 16.0%).

A crystalline product was not obtained from acetylations with acetic anhydride and sulphuric acid (183), acetic anhydride and pyridine (184), or acetic anhydride and sodium acetate.

Demethylation: During extraction G, it was

noticed that boiling 6N-hydrochloric acid converted pinitol to a compound of lower Rf in BAW.

Pinitol (0.5g.) was heated under reflux in 6N-hydrochloric acid (10ml.). Samples were withdrawn at the start, and at ½,1,2,4,8 hours after the start, and chromatographed (BAW, 24 hours, 46.5cm.). Polyols were detected with alkaline silver nitrate. The pinitol spot (Rf 0.23) rapidly decreased, being very faint at 4 hours, and absent at 8 hours. The spot corresponding to the product (Rf 0.14) was present at ½ hour, and increased steadily. No other polyols were detected. It was not possible to judge the time of 50% conversion by comparison of spot intensities because the product reduced alkaline silver nitrate much more actively than pinitol.

Pinitol (5g., from A. melanoxylon) was boiled in 6N-hydrochloric acid for 8 hours; the pale brown solution was then evaporated to dryness on a water-bath. To the crystalline residue, water was added and re-evaporated twice to remove hydrochloric acid. This was repeated with ethanol, leaving 4.75g. of crystals, m.p. 194-221°. Recrystallisation from aqueous ethanol gave (+)-inositol as colourless prisms, m.p. 236-238° (4.1g., 88%). After a second recrystallisation, the product had m.p. 239-240°, $[\alpha]_D^{16}$ + 63.8° (C, 1.17 in water). (Found: C,39.8; H,6.7. Calc. for $C_6H_{12}O_6$: C, 40.0;

H,6.7). Stephen (185) gives m.p. 244°; $[\alpha]_D^{28}$ + 66.2° (C, 1.21 in water) for (+)- inositol obtained by demethylation of pinitol with hydriodic acid.

Extraction of A. intertexta SN5977 heartwood.

Extraction W: Milled heartwood (3,132g.) was extracted with light petroleum for 12 hours, yielding 4.5g. of yellow wax, then with acetone for 24 hours (fraction (a)), ethanol for 8 hours (fraction (b)) and water for 12 hours (fraction (c)).

The acetone extract (fraction (a)) was concentrated to a syrup (312g.) which was taken up in water (2l.) and the mixture left overnight. It was then filtered from a brown amorphous residue, and the filtrate was concentrated under reduced pressure to 250ml. Continuous extraction with ethyl acetate yielded, in successive 4 hour periods, 22.0, 2.1 and 0.7g. of material. The residual aqueous solution was evaporated, leaving a crystalline residue (16.7g.) which was combined with the corresponding fractions from Extractions X, Y and Z (total of heartwood used, 11.5Kg.) in 500ml. water. The solution was treated while hot with basic lead acetate until no further precipitation occurred, then with dilute sulphuric acid to remove lead, with barium hydroxide to remove sulphate, and with carbon dioxide to remove the excess of barium as carbonate. The filtrate was concentrated to a syrup and ethanol was added. This gave 39,8g. of pinitol which was recrystallised from

aqueous ethanol; m.p. 182-183°, $[\alpha]_D^{16}$ + 64.8° (c, 2.76 in water). The mixed m.p. with (+)- pinitol from Extraction V was 183-184°. The mother liquors contained two other polyol components. When chromatographed in BAW (45 hours), these moved 8.5 and 1.9cm. (pinitol moved 15.0cm.)

The ethanol extract (fraction (b)) was concentrated and processed as described for fraction (a). The ethyl acetate extracts from successive 4 hour extractions of the water-soluble portion weighed 5.1, 0.7, 1.1g. The residual aqueous solution contained 4.25g. material.

The material remaining after ethyl acetate extraction of the water-soluble portion of fraction (b), and fraction (c) were combined with similar fractions from Extractions X, Y and Z. (total of heartwood used; 11.5Kg.), and freed from acid-insoluble material. Chromatograms (BAW, 11 hours, 11.5cm.) contained no ninhydrin-positive substances.

The ethyl acetate extracts from fractions (a) and (b) were used for the isolation of teracacidin by counter-current distribution.

Extraction X: Milled heartwood (2.9Kg.) was extracted with light petroleum for 8 hours, yielding 4.3g. of wax, then with acetone (24 hours), ethanol (12 hours), and water (12 hours).

The acetone extract was concentrated to a syrup

(235g.) which was dilted with water to 5l. Next day the brown supernatant liquid was decanted and concentrated to 180ml. under reduced pressure. This concentrate was extracted in a separatory funnel with four 180ml. portions of ethyl acetate, which extracted 18.1g. of material and left 17.6g. of material in the aqueous phase.

The ethanol extract was processed similarly; it yielded 4.4g. of ethyl acetate extract and 6.6g. of material remained in the aqueous phase.

The ethyl acetate extracts were used for the isolation of teracacidin by counter-current distribution.

Extraction Y: Milled heartwood (3.1Kg.) was extracted with light petroleum for 8 hours, yielding 3.6g. of wax, then with acetone (24 hours), with ethanol (12 hours) and with water (12 hours).

The acetone extract was concentrated to about 500ml. and poured into ether (4l.). The resulting mixture contained an aqueous layer (about 200ml.) and a brown deposit. The ethereal layer was decanted and the aqueous layer and deposit was shaken twice more with ether (4l.). The ethereal extracts contained 26, 13, 7.6g. of material respectively. The aqueous layer and deposit were then extracted four times with equal volumes of ethyl acetate, the last extraction being left overnight before separation.

These extractions gave 11, 4.4, 4.4, 13g. of extractives respectively. The aqueous layer was adjusted to 1l. with water and boiled together with the insoluble material to remove ethyl acetate, and then cooled and filtered. Evaporation on a water-bath left a residue (40g.) which contained much insoluble material.

The ethanol extract was concentrated to about 100ml. and poured into ether (4l.). No aqueous phase separated but a loose powdery deposit formed, which was collected and re-suspended in ether. The extracts so obtained weighed 9.3 and 1.3g. respectively. The ether-insoluble material was suspended in water (1l.): the water-soluble portion weighed 5.5g.

Paper chromatography (BAW, 15 hours, 27cm; 2% acetic acid, 3 hours, 23.6cm.) showed that all the ether and ethyl acetate extracts contained leucoanthocyanidins (teracacidin, Rf 0.41-0.52 in BAW, 0.47-0.54 in 2% acetic acid, and another leucoanthocyanidin, Rf 0.67-0.76 in BAW, 0.78-0.85 in 2% acetic acid). The leucoanthocyanidins were not prominent in the ether extracts from the acetone extract of the wood, but were prominent in the first ethyl acetate extract.

The ether and ethyl acetate extracts were combined (91g.) and used for the isolation of teracacidin.

Extraction Z: Milled heartwood (2.4Kg.) was extracted with light petroleum (10 hours) yielding 2.4g. of wax, then with wet ether (50 hours), acetone (24 hours) ethanol (12 hours) and water (12 hours).

The ether extract was concentrated, and taken up in water (final volume, 350ml.). A brown powdery deposit was removed, and the filtrate was continuously extracted with ether, giving in successive 6 hour periods, 8.6 and 8.3g. extractives. The residual aqueous solution contained 2.7g. of material; paper chromatography showed that pinitol was present in this fraction.

The acetone extract was concentrated to a syrup which was diluted with water (2l.). Next day the mixture was filtered and the filtrate was concentrated under reduced pressure to 150ml. This solution was continuously extracted with ether for 8 hours (3.9g. of extractives) and with ethyl acetate for 8 hours (3.0g. of extractives). The residual aqueous phase contained 18.6g. of material.

The ethanol extract was processed in the same way as the acetone extract, and gave with ether, 1.3g. of extractives and with ethyl acetate, 1.2g. of extractives: the residual aqueous phase contained 4.6g. of material.

Paper chromatography (BAW and 2% acetic acid)

showed that leucoanthocyanidins were present in each of the ether-extracted fractions and in the ethyl acetate extract from the water-soluble portion of the ether extract of the wood. The other ethyl acetate extracts contained only traces of leucoanthocyanidins. The fractions containing leucoanthocyanidins weighed 22.2g.

LEUCOANTHOCYANIDINS.

Attempted separation of leucoanthocyanidins by alumina chromatography of the methyl ethers.

Methylation of melacacidin with methyl sulphate :

(a). Methylation of melacacidin (2g.) with methyl sulphate gave 0.59g. (25%) of melacacidin tetramethyl ether m.p. 144-145°, as described above in the identification of melacacidin.

(b). Crystalline melacacidin from A. harpophylla (10g.) was boiled in acetone with methyl sulphate (20g.) and potassium carbonate (70g.) for 10 hours. After filtering and washing the potassium salts with

acetone, excess of methyl sulphate was destroyed with ammonia. Acetone was distilled from the solution and the residue was dissolved in ether with ethanol; the solution was washed with water and with dilute sodium hydroxide. The crude product (9.1g.) in ether (50ml.) and ethanol (20ml.) was charcoaled and left to crystallise at 0°. The first crop (2.1g.) collected the following day, had m.p. 132°; recrystallisation from ethanol-ether yielded 1.36g., m.p. 137-140°. Two subsequent crops were collected; 0.43g., m.p. 130-132° and 0.82g., m.p. 106-108°. This represents a total yield of 28% of melacacidin tetramethyl ether.

(c). A fraction, apparently crude melacacidin, from A. melanoxylon heartwood, when methylated as above, gave an 8% yield of melacacidin tetramethyl ether m.p. 138-141°. (See Extraction L).

(d). Crystalline melacacidin (from A. harpophylla) was methylated as in (b), in three 5g. portions. The mixtures were boiled 5, 7 and 10 hours. The yields of crystalline melacacidin tetramethyl ether were 0.51g. (8%); 0.45g. (7%); 0.51g. (8%).

Chromatography of melacacidin tetramethyl ether on alumina: The alumina used in this work was regenerated alumina of strength slightly less than I on the Brockman scale. The same batch was used throughout. It had been found that the methyl ether of Q-ethyl isomelacacidin was not absorbed from ether on this alumina.

(a). Crystalline melacacidn tetramethyl ether: Melacacidin tetramethyl ether m.p. 135-138° (0.51g.) was chromatographed on alumina (20g., 8.5 x 2.0cm.) using ether (150ml.) then ether-ethanol 9:1 (200ml.) The eluate was collected in 50ml. fractions; fractions 4 and 5 contained 0.18g. and 0.15g. of crystalline material, m.p. 142-144°.

(b) Methylated leucoanthocyanidin mixture: Leucoanthocyanidin mixture from A. harpophylla Extraction A, fraction (dd) (10g.) was methylated with methyl sulphate as above. The crude product (9.8g.) was chromatographed on alumina (50g., 21 x 2.0cm.) using ether (150ml.) then ether-ethanol 9:1. The crude methyl ether did not dissolve in the ether; it was therefore triturated with portions of ether and the residue remaining after 150ml. of ether had been used was dissolved in hot ethanol (5ml.) and ether (45ml. added.) Three such treatments were necessary before all the material had been added to the column. The eluate fractions (50ml.) were taken to dryness; the residues weighed nil, 1.53, 0.49, 0.16, 2.31, 1.34, 1.12, 0.63, 0.20g. respectively. Some fractions were slightly crystalline but most were non-crystalline. The fifth and sixth fractions were again chromatographed on alumina (50g.) using ether-ethanol 50:1 (350ml.) then ether-ethanol 20:1. No crystalline fractions were obtained.

(c). Impure melacacidin tetramethyl ether: Various samples of melacacidin tetramethyl ether m.p. ranging from 133° to 144° were combined (2.73g.) and chromatographed on alumina (100g., 40 x 2.0cm.) with the solvents: ether (100ml.), ether-ethanol 20:1 (100ml.), ether-ethanol 10:1. The melacacidin tetramethyl ether was triturated with the solvents until it had all been added to the column. The eluate was collected in 50ml. fractions and evaporated to dryness. The residues crystallised readily. Material from fraction 10 and following fractions had m.p. 138-144°. The product from some of these fractions showed a m.p. about 100°, e.g. fraction 13 had m.p. 100-102°, recrystallising and remelting 138-139°. After about 900ml. of solvents had been used, the flow-rate decreased markedly, apparently due to crystallisation of product within the column. Recrystallisation from ethanol-ether of material from the fractions of higher m.p. gave melacacidin tetramethyl ether m.p. 142-143° (1.6g.).

(d). Methylated mixed leucoanthocyanidins: The leucoanthocyanidin mixture used was the melacacidin-rich fraction obtained by counter-current distribution of *A. excelsa* heartwood flavanoids (Extraction H). Portions (2g.) were methylated by various methods and the crude methylation product chromatographed on alumina (50g., 18 x 2.0cm.) in ether -ethanol 20:1

(200ml.) and ether-ethanol 10:1 (300-500ml.). The eluate was collected in 50ml. fractions, most of the solvent removed by distillation, and the remainder allowed to evaporate at room temperature. There were in each case two major fractions: that eluted in the first 150-200ml. and a fraction slowly eluted by the second solvent. Although each fraction appeared to contain some crystalline material, no crystalline product could be obtained. The results are summarised in Table 14.

Table 14.

Fractions from methylation of mixed leucoanthocyanidins.

Methylation method	Technique	1st Fraction	2nd Fraction
1. Methyl sulphate	20 hours boil	0.74g.	0.84g.
2. " "	left at room temp. 24 hours, 16 hours boil	0.64	1.10
3. Methyl sulphate	No treatment	0.74	0.34
4. (20 hours boil)	Product 2 days in sodium ethoxide solution	0.40	0.48
5.	Product heated in 33% acetic acid (30ml.) 1 hour at 100°C.	0.41	0.32
6. Methyl sulphate	24 hours at room temp. only	--	--
7. " "	18 hours boil, ammonia treatment omitted.	0.65	0.70
8. Diazomethane	48 hours, room temperature.	0.48	0.66
9. Methyl iodide	--	0.34	0.52

The standard methyl sulphate method used leucoanthocyanidin mixture (2g.), methyl sulphate (4g.), and potassium carbonate (15g.) in acetone (50ml.). The acetone-soluble portion was left with excess ammonia for several hours before removal of solvent. Experiments 3, 4 and 5 were carried out on quarter aliquots from the methylation of 8g. of mixed leucoanthocyanidin. The other aliquot was used in an unsuccessful attempt to obtain a crystalline acetylation product (from acetic anhydride (5ml.) and pyridine (10ml.) at room temperature overnight). The aqueous acid treatment of the crude methylation product (experiment 5) gave much sticky red residue.

Methylation with methyl sulphate in acetone without heating (experiment 6) gave no non-phenolic product.

Methylation with diazomethane was carried out in ether, with an ethereal solution (200ml.) of diazomethane from 30g. of nitrosomethyl urea.

The methylation with methyl iodide was conducted in boiling acetone with potassium carbonate; a total of 24g. of methyl iodide was added in portions. The crude, alkali-insoluble product weighed 0.92g.

Methylation of melacacidin with diazomethane: Crystalline melacacidin (1g.) was heated with methanol (10ml.) and the suspension was cooled to 0° before adding a solution of diazomethane (from 10g. of

nitrosomethylurea) in ether (80ml.) in portions, with cooling and shaking, during 15 minutes. An immediate evolution of nitrogen occurred with the first additions. A small amount of material remained in suspension but complete dissolution of melacacidin appeared to have occurred. The suspended material was removed and the filtrate was left for 45 hours at room temperature. The solution was then concentrated (without addition of acid) to 5ml. on a water-bath at 50°. Melacacidin tetramethyl ether crystallised slowly in large clusters of colourless needles, collected after 2 days; 0.56g. (48%), m.p. 136-139°.

Repetitions on double the above scale gave 1.54g. (72%) m.p. 136-139° and 1.44g. (67%) m.p. 141-143°. These products were recrystallised from methanol, yielding 1.82g. m.p. 137-138°. Recrystallisation of 0.40g. of this low-melting product from ethyl acetate (1.5g.) gave, after 4 hours at 0°, 0.33g. m.p. 142-143°. Recrystallisation of the remainder from 6 parts of ethyl acetate yielded 0.98g., m.p. 143-143.5°.

Preparation of O-ethyl isomelacacidin.

Attempted separation of melacacidin and O-ethyl isomelacacidin on cellulose powder columns:

The Rf values of melacacidin and O-ethyl isomelacacidin on Whatman No.1 paper in various solvents were determined. Results are given in Table 15.

Table 15.

Rf values of melacacidin and O-ethyl isomelacacidin in various solvents.

Solvent mixture	Time (hours)	Distance (cm.)	Rf. melacacidin	Rf O-Ethyl isomelacacidin	Type of spot
2% acetic acid	2.5	17.4	0.40-0.54	0.64-0.79	Compact
20% acetic acid	5	20.3	0.54-0.67	0.74-0.87	Compact
70% ethanol	6	24.3	0.45-0.63	0.68-0.87	Streaky
EA:acetic: water 3:1:3	4	17.0	0.34-0.52	0.72-0.88	--
3:05:3	3	23.0	0.19.0.42	0.67-0.87	Streaky
3:0.25:3	3	28.9	0.04-0.35	0.73-0.82	"
Acetone-water 30:70	4	19.3	0.61.0.75	0.79-0.91	Compact
70:30	4	18.2	0.69-0.82	0.86-0.99	Compact
Methyl ethyl ketone: water 25:4	1.5	17.5	0.47-0.75	0.82-0.97	Streaky
EA:acetone: water 25:25:10	4	17.2	0.37-0.53	0.76-0.91	-

E.A. = Ethyl acetate

At the time this work was done, the existence of isomelacacidin as distinct from melacacidin was not certain. A sample of melacacidin was available

from Extraction A. As a sample of O-ethyl isomelacacidin, the leucoanthocyanidin mixture remaining in mother liquors from the isolation of melacacidin from fraction (dd) of Extraction A were used.

Cellulose powder (100g.) was wetted with the lower phase (25ml.) from a mixture ethyl acetate:acetone: water 5:5:3, and packed in a column (30 x 3.6cm.). Mixed leucoanthocyanidins (as above, 1.0g.) in solvent (5ml. of the upper phase) were added to the column. The eluate was collected in 50ml. fractions; the weights were: fractions 1-4, nil; 5, 0.23g.; 6, 0.49g.; 7, 0.17g.; 8, 0.02g.; 9, nil. Paper chromatograms (2% acetic acid) showed distinctly that three leucoanthocyanidins were present. All fractions 5-8 showed all three components, although slight variation in relative amounts was apparent.

The column was used again, without re-packing; the load was 2g. and 18ml. fractions were collected. The first fractions contained only O-ethyl isomelacacidin, and the last fractions, only melacacidin, but the proportion of the leucoanthocyanidin mixture thus separated was very small; most was in the intermediate mixed fractions.

The column was re-packed tightly (25 x 3.6cm.); 1g. of the mixture was added and eluted with 2% acetic acid. The flow rate was about 60ml./hour. The eluate was collected in 15ml. fractions, which

were examined by chromatography in 2% acetic acid. Fractions 12-15 contained O-ethyl isomelacacidin and isomelacacidin, and fractions 22-25, melacacidin only. Intermediate fractions contained all three leucoanthocyanidins.

The last experiment was repeated with 2g. of the mixture. The eluate was collected in 8ml. fractions. O-ethyl isomelacacidin was present in fractions 22-28, isomelacacidin in 24-30 and melacacidin in 26-35.

Preparation of O-ethyl isomelacacidin by counter-current distribution.

Partition distribution of melacacidin: Melacacidin (3.0g.) obtained from Extraction A was dissolved in hot water (25ml.) containing calcium carbonate. The brown solution was treated with carbon with negligable effect, and was left in a desiccator over phosphorus pentoxide for 8 days. The deposit which formed was collected and airdried; 1.17g. of fawn-coloured powder, decomposing at about 220°C. This product was chromatographically pure (BAW and 2% acetic acid) melacacidin. The U.V. spectrum was measured on a 0.0040% solution in 95% ethanol i.e. 1.313×10^{-4} M assuming that the melacacidin was anhydrous; the UV spectrum showed a maximum at 280mμ ($\log \varepsilon$, 3.50) and a minimum at 254 mμ ($\log \varepsilon$, 2.77).

Phosphate buffer solutions pH5.59, 6.64 and 7.38 were prepared according to Sorensen, and equilibrated with ethyl acetate. In stoppered test tubes,

1ml. of 1% melacacidin in ethanol was placed and the solvent removed under reduced pressure. Then 10ml. of each phase was added, and the mixture was shaken at room temperature (about 20°C.) The amount of melacacidin in the ethyl acetate phase was determined by evaporating 1ml., and measurement of the optical density at 280 mμ of a solution of the residue in 10ml. 95% ethanol. The results were calculated to the values of p, the proportion of melacacidin in the lighter phase. The results were: pH5.59, p=0.73; pH6.64, 0.47; pH7.38, 0.39.

Counter-current distribution of mixed leucoanthocyanidins:

(a) From the above determination of the distribution coefficients of melacacidin, it was expected that, in a 50 tube distribution, melacacidin would be present in peak concentration at about tube 20, if the buffer pH were 7.0.

The leucoanthocyanidin mixture (5.0g.) was distributed in the Towers 50 tube machine, between ethyl acetate and M/15 phosphate buffer pH 7.0. Samples of even-numbered tubes were diluted 1 to 100 (tubes 2-34, 1ml. of aqueous phase diluted with water; tubes 30-50, 0.1ml. of organic phase diluted in 95% ethanol), and the optical density at 280 m was measured. Tubes 1 and 2 contained much brown colouring matter, and the optical density of tube 2 was

very high. The density at 280 mμ reached peak intensity at tubes 16 and 44.

Samples of even numbered tubes (aqueous phase of tubes 2-26, organic phase of tubes 26-50) were chromatographed in 2% acetic acid (1½ hours, 17.7cm.). Leucoanthocyanidins were detected by Roux's spray. Melacacidin was present in tubes 10-28, reaching a maximum concentration at tube 17; isomelacacidin in tubes 8-20, peak at 14; O-ethyl isomelacacidin in tubes 38-46, peak at 42.

The contents of tubes 38-46 were collected and extracted with ethyl acetate in separatory funnels; the extracts were concentrated by distillation; and then taken to dryness under reduced pressure. The residue (1.6g.) was dissolved in ethanol from which crystals of O-ethyl isomelacacidin slowly formed (0.27g. after 4 days). The mother liquor was diluted with water (3 volumes) and when allowed to evaporate, gave more crystals, (plates) of O-ethyl isomelacacidin which were collected after 2 days (0.64g.).

(b) Three counter-current distributions were carried out as described above using (1) 10g. of leucoanthocyanidin mixture added in the first two tubes, (2) 15g. added in the first three tubes and (3) 9g. added in the first two tubes. The distributions were examined by paper chromatography (2% acetic acid); the results were similar to the

first distribution. The fractions containing O-ethyl isomelacacidin were collected and extracted with ethyl acetate in separatory funnels, yielding 3.3, 4.8 and 2.7g. respectively. These were combined.

A portion of this product (1g.) was warmed in water (25ml.), charcoaled (0.5g.), and filtered. No noticeable decolourisation resulted. When chromatographed (BAW, 11 hours, 42cm.), this solution showed a spot corresponding to isomelacacidin (Rf 0.30 to 0.40); O-ethyl isomelacacidin was absent.

The remainder of the product (9.8g.) was dissolved in ethanol (15ml.), and the solution was left in an open beaker at room temperature (about 37°C). The solution was seeded with crystals from the first distribution. O-Ethyl isomelacacidin began to crystallise as brown plates after some hours, and was collected next day; yield 1.9g. The colour of the product was reduced by washing with cold water, which left 1.3g. of pale brown plates. By evaporation of the mother liquors at room temperature, and solution in water, a second crop (0.65g.) was obtained. The mother liquors were continuously extracted with ethyl acetate for 8 hours, and attempts were made to obtain crystals from the extract (7.7.g). From 0.20g. in water (1ml.), a small amount of crystalline product, together with much brown amorphous material was obtained at 0°C. A solution of commercial

detergent (0.05% in water) gave slightly better results. Methanol, and methanol-water 1:1 gave no crystals.

(c) Crude leucoanthocyanidin mixture from Extraction B (30g.) was subjected to counter-current distribution as above. Paper chromatography showed that O-ethyl isomelacacidin was present in tubes 34-46; the material from these tubes was recovered by extraction with three equal volumes of ethyl acetate (yield, 6.4g.). This was dissolved in ethanol (10ml.); crystals were not obtained until some evaporation had occurred (1 day in a petri dish). The crystals were collected after 3 days (0.8g.); the mother liquors did not give a second crop. After the investigations described below, the product was recrystallised from methanol (3.2ml.) and water (12.8ml.), yielding 0.64g. after 5 days at 0^{o}. A second recrystallisation gave 0.40g. of shining plates, and a third, 0.26g. of almost colourless plates of O-ethyl isomelacacidin, which were used for analysis.

Repititions, using the remainder of the leucoanthocyanidin mixture (30g. and 40g. lots) gave only 0.3g. of crude crystalline O-ethyl isomelacacidin (from the 30g. batch).

Recrystallisation of O-ethyl isomelacacidin:

(a) Water-washed crystalline O-ethyl isomelacacidin (22.4mg.) was dissolved in methanol (0.2ml.) and

water (0.8ml.) added. Crystals (plates) and a slight flocculent deposit formed. The solids were centrifuged; after drying *in vacuo* at 40° over P_2O_5, the loose crystals (13.7mg.) were easily separated from the amorphous material which adhered to the centrifuge tube.

(b) Crystalline O-ethyl isomelacacidin (62 to 111mg.) was dissolved in methanol (0.4ml.) with slight warming, water (1.6ml.) was added, and the solution was centrifuged free from a gelatinous deposit. Crystals were collected after 2 days at 0-4°. Recoveries were as follows: (1) from 62mg., 37mg. recovered (2) 89mg., 62mg. (3) 100mg., 69mg. (4) 102mg., 66mg. (5) 111mg. 66mg. From (4) and (5) second crops (16mg. and 26mg. respectively) were obtained after a further 5 days.

Interconversion of melacacidin, isomelacacidin and O-ethyl isomelacacidin:

(a) A sample of leucoanthocyanidin mixture (1g., from *A. excelsa*) containing melacacidin, isomelacacidin and a trace of O-ethyl isomelacacidin, was heated in ethanol (10ml.) with acetic acid (1ml.) in a water bath so that evaporation of the ethanol took place in about 30 minutes. A sample was then withdrawn, ethanol (10ml.) was added to the residue, and the slow evaporation repeated. Sampling, addition of ethanol, and evaporation was repeated so that a

total of five evaporations was made.

The samples were chromatographed, together with untreated mixture, in 2% acetic acid (4½ hours, 20.8cm.) and leucoanthocyanidins were detected with Roux's spray.

Melacacidin (Rf 0.38-0.50) was present in all samples, isomelacacidin (Rf 0.51-0.59) in untreated material only, and O-ethyl isomelacacidin (Rf 0.67-0.76) in untreated material (trace only) and in major amount in all other samples.

(b) Leucoanthocyanidin mixture (0.5g.) was dissolved in the following solvent mixtures: 5ml of ethanol, 0.2ml. of acetic acid; 5ml. of ethanol, 0.1ml. of acetic acid; 5ml. of ethanol; 4.5ml. of ethanol, 0.2ml. of acetic acid, 0.5ml. of water; 4.5ml. of ethanol, 0.5ml. of water. The solutions were placed on a boiling water-bath so that evaporation occurred over 30 minutes. The residues were then diluted in ethanol to the origianl weight of the solutions, and examined by paper chromatography as in the previous experiment. All samples showed conversion of isomelacacidin to O-ethyl isomelacacidin; this conversion appeared to be complete when absolute ethanol was used; when 90% ethanol was used it appeared that some isomelacacidin may have remained unconverted. Due to impurities in the leucoanthocyanidin mixture used, this point could not be determined definitely.

(c) A boiling mixture of ethanol (10ml.) and

acetic acid (0.2ml.) was added to 1g. of leucoanthocyanidin mixture in a flask on a boiling water-bath, and boiling under reflux maintained. Samples were withdrawn when the solvent was added, and after 5, 10, 15, 30 and 45 minutes. The samples were examined by paper chromatography (2% acetic acid, 20cm.).

Melacacidin was present in all samples apparently in unchanged amount. Isomelacacidin was present initially, and in decreasing amount in later samples, a trace being present at 45 minutes. O-Ethyl isomelacacidin was not detected in the sample taken at the start, but was present in later samples in continuously increasing amount. The amounts of isomelacacidin and O-ethyl isomelacacidin present were approximately equal at 15 minutes.

(d) The interconversion of the leucoanthocyanidins was investigated using crystalline melacacidin and O-ethyl isomelacacidin.

Leucoanthocyanidin (20mg.) was placed in a test tube in a boiling water-bath, and hot dilute acid (1ml.) added. The following dilute acid solutions were used; distilled water, 0.1N-acetic acid, 0.5N-acetic acid, 0.01N-hydrochloric acid, 0.05N-hydrochloric acid, 0.25N-hydrochloric acid. Samples were withdrawn by capillary as soon as the solid had dissolved (usually ½ to 1 minute after the addition) and after 10, 20, 30, 60 and 90 minutes, and spotted

directly on to chromatography paper. Chromatograms were run in 2% acetic acid (2-3½ hours) in duplicate. Leucoanthocyanidins and polyphenols were detected with Roux's spray and ferric-ferricyanide spray respectively. Representative Rf values were: melacacidin, 0.35-0.44; isomelacacidin, 0.47-0.57; O-ethyl isomelacacidin, 0.61-0.68.

The results with O-ethyl isomelacacidin were as follows:

In distilled water, slow conversion to isomelacacidin occurred, conversion being over 50% at 60 minutes.

In 0.1N-acetic acid, conversion to isomelacacidin was almost complete at 10 minutes. No other polyphenol appeared on the chromatograms.

In 0.5N-acetic acid, conversion to isomelacacidin was about 50% in the first sample (½ minute). A faint spot in the position of melacacidin was apparent at 10 minutes, and it increased to 60 minutes, but it was always much smaller than the isomelacacidin spot.

In 0.01N-hydrochloric acid, conversion to isomelacacidin was considerable in the first sample (1 minute). A spot in the position of melacacidin was apparent after 10 minutes, but after 20 minutes, polymeric material gave a streak which prevented clear recognition of the leucoanthocyanidins in this region of the chromatogram.

In 0.05N-hydrochloric acid, conversion to isomelacacidin was almost complete in the first sample. Chromatograms of subsequent samples were obscured by polymeric material; a spot in the position of melacacidin could be detected at 10 minutes but not later.

In 0.25N-hydrochloric acid, no Q-ethyl isomelacacidin was detected in the first sample which contained only isomelacacidin. At 20 minutes and later, neither isomelacacidin or melacacidin were apparent.

The results with melacacidin were as follows:

In distilled water, no other polyphenol appeared, even after 90 minutes heating.

In 0.1N-acetic acid, a spot in the position of isomelacacidin was apparent in the 60 and 90 minute samples.

In 0.5N-acetic acid, a spot in the position of isomelacacidin was apparent at 10 minutes, and the amount increased progressively. At 60 minutes, the quantities of melacacidin and isomelacacidin appeared to be approximately equal, and at 90 minutes, there appeared to be about twice as much isomelacacidin as melacacidin.

In 0.01N-hydrochloric acid, about 90% of the leucoanthocyanidin present at 10 minutes was isomelacacidin, and this proportion appeared to remain

unchanged by further heating. Polymeric material appeared in the chromatogram at 60 minutes.

In 0.05N-hydrochloric acid, conversion to isomelacacidin occurred as in 0.01N-hydrochloric acid. Polymeric material appeared in the chromatogram at 20 minutes.

In 0.25N-hydrochloric acid, some isomelacacidin was present in the first sample, and at 10 minutes, the amount present was much greater than the amount of melacacidin. The chromatograms of this and later samples was obscured by polymeric material.

(e) Crystalline melacacidin (0.48g.) was boiled in ethanol (5ml.). Much melacacidin remained undissolved. Acetic acid (0.1ml.) was added to the boiling solution, and samples were withdrawn at the time of the addition of acid, and 10, 20, 30, 30, 60 and 90 minutes later. These samples were chromatographed (2% acetic acid). A faint trace of O-ethyl isomelacacidin was present in each sample, the amount increasing very slightly. The ratio of melacacidin to O-ethyl isomelacacidin was approximately 50:1 after 90 minutes heating.

The reaction mixture was diluted with ethanol (about 45ml.) until the melacacidin had dissolved in the boiling solution. No crystals were obtained on cooling. The solution was concentrated at atmospheric pressure to about 5ml. at which stage crystals began to separate. The solution was left

at room temperature for 24 hours before the crystals (small prisms) were collected (0.30g.). The product was identified as melacacidin by paper chromatography.

(f) The previous experiment was repeated using 49mg. of crystalline melacacidin in 10ml. ethanol. The melacacidin dissolved on refluxing, but portion crystallised on the sides of the flask. Acetic acid (0.2ml.) was added. Samples were withdrawn before addition of acid, and 10, 20, 30, 60 and 90 minutes after. They were chromatographed in 2% acetic acid.

A spot in the position of Q-ethyl isomelacacidin (Rf 0.62-0.69; melacacidin Rf 0.35-0.46) was detectable with ferric-ferricyanide reagent at 30 minutes, and barely detectable with Roux's spray at 60 minutes. At 90 minutes the spot obtained with Roux's spray was very faint, and about 1/100 the amount of melacacidin present.

Melacacidin.

The identification of melacacidin is described at the end of Extraction A; the recrystallisation of this material from water is described above in the section on the partition distribution of the leucoanthocyanidins.

Recrystallisation of melacacidin from ethanol:

(a) During investigation of the effect of ethanol containing acetic acid on melacacidin, it was found possible to recrystallise melacacidin from ethanol (see above).

(b) Crystalline melacacidin (from Extraction I; 0.5g.) was dissolved in boiling ethanol (30ml.) and the solution was concentrated to 4ml. On seeding, melacacidin crystallised rapidly, (0.36g.). This was crystallised again from ethanol containing a trace of acetic acid. The solution was concentrated to 10ml. and cooled slowly; 0.18g. of opaque colourless prisms were obtained.

(c) Crystalline melacacidin (from Extraction C; 3.9g.) was dissolved in boiling ethanol containing 1% acetic acid (200ml. was used; slightly more than the minimum volume). The solution was charcoaled hot, and concentrated to 75ml. After 1 day, melacacidin crystallised from the orange-coloured solution; it was collected after 40 days as pale pink, very small prisms (2.8g.).

(d) Crystalline melacacidin (from Extraction D, 8.28g.) was dissolved in boiling ethanol (500ml.) with acetic acid (1ml.), the solution charcoaled, and boiled to evaporate ethanol until crystals began to appear (volume about 120ml.). After 9 days at room temperature, the small, slightly pink prisms were collected (6.13g.). Slow evaporation of the mother liquor to about 30ml. over a period of four summer months yielded a crop of less pure, crystalline melacacidin (0.56g.).

Properties of melacacidin: The material recrystallis-

ed from ethanol melted with decomposition at 229°, after becoming brown at 200°, and sintering over the range 200-225°.

An airdried sample was analysed. (Found: C,58.9; H,4.9; loss on drying, less than 0.1%. $C_{15}H_{18}O_9$ requires C,58.8; H,4.6%) $[\alpha]_D^{16}$ -74.8° (C,0.2 in ethanol, 4dm. tube).

$[\alpha]_D^{24}$ -85.0° (C, 1 in acetone-water, 1:1). To this solution (10ml.), hydrochloric acid (0.1ml. 10N) was added; after 43 hours at room temperature, the reading (initially -0.850°) was -0.825° (not corrected for dilution).

$[\alpha]_D^{22}$ -74.9° (C, 1 in dioxan-water, 1:1). To this solution (10ml.), hydrochloric acid (0.1ml. 10N) was added. After 24 hours at room temperature, the reading (initially -0.749°) was -0.735° (not corrected for dilution).

Pure melacacidin was almost insoluble in water. Its solubility in ethanol was less than 0.5% at room temperature, and about 2% at the boil. It was more readily soluble in acetone and dioxan.

A 1% solution in ethanol of melacacidin which had been recrystallised from water was left for 20 months without exclusion of light or air. The solution did not darken appreciably, the only polyphenol detectable by paper chromatography (BAW and 2% acetic acid, ferric-ferricyanide reagent) was melacacidin.

The anthocyanidin formed by heating melacacidin with 3N-hydrochloric acid for 15 minutes in a boiling water-bath was extracted with amyl alcohol and chromatographed in "Forestal" solvent. The anthocyanidin had Rf 0.58 compared with 0.55 cyanidin.

Acetylation of melacacidin: No crystalline product was obtained from the reaction of recrystallised melacacidin (0.5g.) with acetic anhydride (2ml.) in pyridine (10ml.) at room temperature overnight.

Tetramethyl ether: The preparation of the tetramethyl ether with methyl sulphate and with diazomethane has been described in the section on the attempts at the separation of leucoanthocyanidin methyl ethers by alumina chromatography.

Tetramethyl ether diacetate: This is described in the identification of melacacidin.

Attempted preparation of the 4, 7, 8, 3', 4'-pentamethyl ether: Melacacidin tetramethyl ether (0.5g., m.p. 142-143°) was boiled in methanol (50ml.) containing acetic acid (2.5ml.) for 20 hours. The solvent was removed under reduced pressure, and two further lots of methanol were added to the crystalline residue, and removed under reduced pressure, to remove remaining acetic acid. The crystalline residue had m.p. 143-144°, alone and mixed with starting material.

Effect of hot acetic acid on melacacidin tetramethyl ether: A solution of melacacidin tetra-

methyl ether (0.5g.) in acetic acid (10ml.) was heated on a boiling water-bath for 12 hours, and the acetic acid removed under reduced pressure. The residue was a brown resin from which crystalline material could not be obtained.

A second experiment, in which the product was boiled in methanol for 25 minutes, also gave no crystalline product.

O-Ethyl isomelacacidin.

The development of the preparation of O-ethyl isomelacacidin from leucoanthocyanidin mixtures has been described in a separate section above; that work dealt with the lability of the product, and the experiments on recrystallisation have therefore been included in the same section.

Some preparations of O-ethyl isomelacacidin from heartwood extractives were carried out in the course of extractions (see Extractions D and I). A similar preparation is described below.

Preparation of O-ethyl isomelacacidin from A. melanoxylon heartwood extractives: From Extractions L, M, and N, there was available a polyphenol fraction containing isomelacacidin from which melacacidin and ketonic flavonoids had been removed by a series of distributions. Crude isomelacacidin (106g.) was recovered from the aqueous solution by continuous extraction with ethyl acetate for 12 hours, and was boiled in ethanol (1l.) containing acetic acid (10ml.)

for 2 hours. The solvent was removed, and the residue was subjected to the usual Bush and Densen distribution. The combined aqueous phases were concentrated under reduced pressure to 100ml. and continuously extracted with ethyl acetate, yielding 40g. of material. The ethyl acetate phases, containing O-ethyl isomelacacidin, were dried (Na_2SO_4), and the solvent was removed, to leave 56g. of grey powder. This was dissolved in ethanol (112ml.), and the solution, after cooling in ice, was added to water (450ml.) at 5°C. Crystallisation began within an hour. After 4 days at 0°, the crystals of O-ethyl isomelacacidin (small brown plates) were collected (20.4g.). A second crop of 0.7g. was obtained after a further 6 days.

The above product was recrystallised from ethanol (40ml.) with water (160ml.) to give 11.5g. of pale brown plates.

Properties of O-ethyl isomelacacidin: An airdried sample was analysed. (Found: C,51,5; H,6.0; C-methyl, 3.7; O-alkyl as O-ethyl, 10.0; loss on drying, 15.9. $C_{17}H_{18}O_7 \cdot 3.5H_2O$ requires C,51.4; H,6.3; C-methyl, 3.8; O-ethyl 11.4; H_2O, 15.8%). A sample dried at 90° in vacuo over P_2O_5 was analysed. (Found: C,60.8; H,5.5; O-alkyl as O-ethyl, 11.7. $C_{17}H_{18}O_7$ requires, C,61.1; H,5.4; O-ethyl, 13.3%). O-Ethyl isomelacacidin, dried as above, showed on chromatography in BAW and in 2% acetic acid only one spot which had the same Rf as the undried material.

The Rf values in various solvents are quoted in Table 7. (BAW and 2% acetic acid) and Table 15. (other solvents).

The anthocyanidin formed by heating O-ethyl iso-melacacidin in 3N-hydrochloric acid for 15 minutes in a boiling water-bath was extracted with amyl alcohol, and chromatographed in "Forestal" solvent. It had the same Rf as the anthocyanidin from melacacidin, Rf 0.58 (cyanidin, Rf 0.55), and the same colour bath before and after being sprayed with alcoholic aluminium chloride.

Optical rotations: The material used was airdried; the amount of water remaining in it was determined by drying in vacuo over phosphorus pentoxide at 90° for 9 hours. The optical rotation in ethanol was determined on material of which 88.6mg. lost 8.8mg. on drying, i.e. 10%. The other optical rotations were determined on material of which 97.5mg. lost 3.7mg. on drying, i.e. 3.7%. Undried material was used for measurement of optical rotations; the specific rotations are calculated with respect to the anhydrous compound.

A solution of 100mg. of the first sample of 10ml. of ethanol at 22° had an optical rotation of -0.308°, i.e. $[\alpha]_D^{22}$ -33.9° (C, 0.9 in EtOH).

A solution of 0.625g. of the second sample in 25ml. acetone-water 1:1 at 23° in a 4dm. tube had

an optical rotation of -0.398°, i.e. $[\alpha]_D^{23}$ -41.3 (C, 2.40 in acetone-water 1:1). To this solution, 0.25ml. of 10N-hydrochloric acid was added. Single readings taken subsequently were: at 9 minutes, -0.58°; 26 minutes, -0.62°; 74 minutes, -0.68°; 136 minutes, -0.73°. At 6 hours, the optical rotation was -0.693°, and at 23 hours, -0.697° at 17°C. The latter figure corresponds to $[\alpha]_D^{17}$ -73.0° (corrected for dilution).

A solution of 0.250g. of the second sample in 10ml. of dioxan-water 1:1, kept at 25°C, had an optical rotation of -0.990° i.e. $[\alpha]_D^{25}$ -41.1° (C, 2.4 in dioxan-water 1:1). To this solution, 0.025ml. of 10N-hydrochloric acid was added: the solution immediately became too dark for further readings.

A solution of 100mg. of the second sample is 10ml. dioxan-water 1:1 at 24° had an optical rotation of -0.397° i.e. $[\alpha]_D^{23}$ -41.4° (C,1 in dioxan-water 1:1). To this solution, 0.10ml. of 10N-hydrochloric acid was added. The solution became red-brown, but the colour faded after 1 hour. After 17 hours, the solution had an optical rotation of -0.643° at 15°C, and after 24 hours, -0.608°C. These figures correspond to $[\alpha]_D$ -66.7° and -63.2°.

Derivatives of O-ethyl isomelacacidin: The methyl ether was prepared from O-ethyl isomelacacidin (1g.) dissolved in methanol (10ml.) with diazomethane

(from 15g. of nitrosomethylurea, in ether (150ml.). The solutions were kept at 0° during mixing, and then at room temperature for 40 hours. Addition of acetic acid then gave rise to vigorous evolution of gas. The solution was washed with water and with dilute sodium hydroxide and dried over sodium sulphate. The solvent was removed and left a thick oil which could not be obtained crystalline from ether, ethanol or methanol. It was chromatographed on alumina (20g.); there was apparently no adsorption, all material being eluted very quickly. The oil was distilled; b.p. 245° at 1mm. There was no apparent decomposition, and the product redistilled at the same temperature, to yield O-ethyl isomelacacidin tetramethyl ether? (0.44g.) pale yellow viscous oil, slowly thickening. The product gave a red colour when heated in ethanolic hydrochloric acid. (Found; C, 65.3; H,7.2; O-methyl, 35.3. $C_2H_{26}O_7$ requires C, 64.6; H,6.7; O-alkyl as O-methyl, 38.7%).

The methyl ether was prepared from other samples of O-ethyl isomelacacidin, but no sign of crystallisation was seen. The acetyl and p-nitrobenzoyl derivatives of the methyl ether also failed to crystallise.

The methyl ether toluenesulphonate was prepared from the methyl ether (from 1g. of O-ethyl isomelacacidin) in pyridine (10ml.) with toluene-p-

sulphonyl chloride (1.5g.) at room temperature overnight. On dilution with water, an oil was obtained, which crystallised. From ethanol, O-ethyl isomelacacidin tetramethyl ether p-toluenesulphonate crystallised as large colourless prisms, m.p. 125° (0.99g.; about 70%). It was recrystallised twice from five parts of ethanol; m.p. 125°. These clear crystals became opaque at 110-120°, and, over phosphorus pentoxide, crumbled to a powder. The analytical figure for loss on drying at 100° the airdried crystals was 0.64%. For analysis, a sample was dried over P_2O_5 for 7 days at room temperature. (Found: C, 61.5; H,6.1; S, 6.3; O-methyl, 27.6. $C_{28}H_{32}O_9S$ requires C, 61.75; H,5.9; S, 5.9; O-methyl, 28.5%). $[\alpha]_D^{23}$ -18.5° (C, 0.4 in EtOH).

O-Methyl isomelacacidin.

Preparation of O-methyl isomelacacidin: The isomelacacidin fraction from Extraction C (33.5g.) was boiled in methanol (300ml.) containing acetic acid (10ml.) for 2 hours. The methanol and as much acetic acid as possible was removed under reduced pressure. The residue was dissolved in warm methanol (70ml.) and the solution was cooled in ice water, and added to ice-cold water (140ml.). Seed crystals were obtained by evaporation of a portion overnight in a watch glass. For satisfactory crystallisation of the main portion, some evaporation was required.

After 15 days at 0°, the first crop (3.5g.) was collected, and after a further 10 days, a second crop (2.3g.). The first crop was dissolved, with slight warming, in methanol (15ml.), the solution was charcoaled heavily, and concentrated (to 9.6g.), cooled and added to ice-cold water (28ml.). The solution was seeded, and kept at 0°. A mat of colourless needles formed in 1 hour; these were filtered and washed with cold water; the product was a mixture of needles and plates (1.75g.). The mother liquor slowly deposited colourless plates, which were collected after 10 days (1.0g.). Recrystallisation of the first crop from methanol-water gave, without seeding, needles which changed into plates during 3 days (1.3g.). O-methyl isomelacacidin was obtained as light-fawn plates which, when heated, began to darken at 145°, sintered at 153-168°, and darkened appreciably at 175° becoming black at 200°, without melting. A sample dried over anhydrous calcium chloride for 3 days, to form a pink powder, was analysed. (Found: C, 58.4; H,5.4, OCH_3, 9.0. $C_{16}H_{16}O_7 \cdot 0.5H_2O$ requires C, 58.4; H,5.2; OCH_3 9.4%).

The following Rf values were observed: in BAW (10 hours,21cm.), 0.63-0.73 (melacacidin, 0.42-0.48; O-ethyl isomelacacidin, 0.73-0.82); in 2% acetic acid (2½ hours, 23cm.), 0.53-0.66 (melacacidin, 0.35-0.47; O-ethyl isomelacacidin, 0.63-0.74).

The anthocyanidin formed by heating O-methyl iso-melacacidin in 3N-hydrochloric acid in a boiling water-bath for 15 minutes was extracted with amyl alcohol and chromatographed in "Forestal" solvent. The anthocyanidin had Rf 0.58 (anthocyanidin from melacacidin, Rf 0.58; cyanidin Rf 0.55). The appearance of the chromatogram before and after being sprayed with ethanolic aluminium chloride was similar to the appearance of a chromatogram of the anthocyanidin from melacacidin.

Optical rotations: The material used was an airdried sample, of which 118.0mg. lost 12.3mg. on drying *in vacuo* at 90° for 9 hours i.e. 10.4%. The material dried in this way showed on paper chromatograms (BAW, 2% acetic acid) only one spot of the same Rf as airdried material. Airdried material was used for measurement of optical rotations; the specific rotations are calculated with respect to the anhydrous compound.

A solution of 100mg. in 10ml. of methanol at 22°C had an optical rotation of -0.563° i.e. $[\alpha]_D^{22}$ -62.8° (C, 0.9 in MeOH).

A solution of 100mg. in 10ml. of acetone-water 1:1 at 18° had an optical rotation of -0.678°, i.e. $[\alpha]_D^{22}$ -75.6° (C, 0.9 in acetone-water, 1:1). To this solution, 0.1ml. of 10N-hydrochloric acid was added; the solution was strongly acid to litmus. The

optical rotation was determined at intervals over 24 hours; there was no definite change, the reading after 24 hours being -0.660° i.e. $[\alpha]_D$ -74.3° (corrected for dilution).

A solution of 100mg. in 10ml. of dioxan-water 1:1 had an optical rotation of -0.513° at 25° i.e. $[\alpha]_D^{25}$ -57.2 (C, .09 in dioxan-water 1:1). To this solution, 0.1ml. of 10 N-hydrochloric acid was added. The optical rotation was -0.637° after 17 hours, and -0.616° after 24 hours. The latter reading corresponds to $[\alpha]_D^{18}$ -69.4° (corrected for dilution).

A solution of 500mg. in 25ml. of dioxan-water 1:1 was prepared, and kept at 25°C in a 4dm. polarimeter tube. The optical rotation was -4.197° ie. $[\alpha]_D^{25}$ -58.2° (C, 0.9 in dioxan-water 1:1). To this solution, 0.25ml. of 10N-hydrochloric acid was added. The solution immediately darkened, but readings became possible again after 4 hours. Readings were -4.77° at 4½ hours, -4.80° at 6 hours, -4.72° at 10 hours, -4.53° at 30 hours. The solution then again became too dark for the optical rotation to be determined.

Derivatives: The product of methylation of O-methyl isomelacacidin with diazomethane in ether was not obtained crystalline. Portions of this methylation product were converted to the acetyl and p-toluene-sulphonyl derivatives, but the products did not crystallise.

The methylation product from 0.5g. of O-methyl iso-

melacacidin was dissolved in pyridine (5ml.), and 0.75g. of p-nitrobenzoyl chloride added. The mixture was heated until it became homogeneous, and then left at room temperature. Next day, the mixture was diluted with water. The crude product gave, from methanol, a yellow solid (O-methyl isomelacacidin tetramethyl ether p-nitrobenzoate?) m.p. 117° (39mg.). The purity of this product was doubtful and it was not analysed. It gave a red colour when heated in ethanolic hydrochloric acid.

Isomelacacidin p-tolyl sulphone.

Preparation: exploratory work.

(a) The following mixtures were prepared:

(1) 50mg. of O-ethyl isomelacacidin in 0.5ml. of ethanol with 80mg. of sodium p-toluenesulphinate dihydrate (186) in 0.5ml. of water and 0.05ml. of acetic acid.

(2) As (1), but omitting the toluenesulphinate.

(3) As (1), but omitting the O-ethyl isomelacacidin.

After keeping the solutions at room temperature for about 3 weeks, samples were chromatographed (BAW, 14 hours, 29.4cm.). Mixture (2) contained isomelacacidin (Rf 0.31-0.41) and O-ethyl isomelacacidin (Rf 0.61-0.73). Mixture (1) showed traces of these leucoanthocyanidins, and a large spot (Rf 0.65-0.76) giving a brighter red with Roux's spray than did

the leucoanthocyanidins. This material also reduced the ferric-ferricyanide reagent more strongly.

The presence of a new compound in mixture (1) was confirmed by chromatography in 2% acetic acid (26.5cm); the new compound had Rf 0.34-0.47, isomelacacidin 0.51-0.64, O-ethyl isomelacacidin 0.64-0.74, and p-toluenesulphinic acid (detected by ferric-ferricyanide reagent) 0.82-0.88.

After slow evaporation for about 2 months, colourless prisms formed in mixture (1). The molar ratio of the reagents in mixture (1) was leucoanthocyanidin: sulphinate:acid 1:2.9:7.3.

(b) A solution of O-ethyl isomelacacidin (0.20g.), and sodium p-toluenesulphinate dihydrate (0.335g.) in water (4ml.) and acetic acid (0.2ml.) was prepared. The solution was heated in a test tube in a boiling water-bath, and samples were withdrawn at intervals and chromatographed in BAW and in 2% acetic acid. The components were detected with Roux's spray and with ferric-ferricyanide reagent. The results are given in Table 16.

Table 16.

Formation of sulphone from O-ethyl isomelacacidin.

	Rf values		Time (mins.)					
	BAW	2% acetic acid	3	10	20	30	60	120
O-Ethyl isomelacacidin	0.64-0.79	0.54-0.66	+++	++	+	+	—	—
Isomelacacidin	0.31-0.40	0.44-0.50	—	—	+	+	+	++
New compound	0.69-0.85	0.32-0.40	++	++	++	++	++	++

In a mixture made up as above, but omitting toluenesulphinate, 9-ethyl isomelacacidin was present after 3 minutes in the boiling water-bath, but not later. Traces of melacacidin appeared to be present after 10 minutes heating; the remaining polyphenol was present as isomelacacidin.

The samples withdrawn from the complete reaction mixture at 30, 60 and 120 minutes formed crystals very quickly on cooling. The reaction mixture remaining after all the samples had been withdrawn was allowed to cool slowly; it deposited slightly coloured prisms (0.196g.; 79%). Recrystallisation from water, with slow cooling, gave the sulphone as colourless prisms (94mg.).

(c) Leucoanthocyanidin mixture, mainly melacacidin but dark in colour (4g.) was dissolved in boiling 0.01N-hydrochloric acid (60ml.), and the solution was heated under reflux in a boiling water-bath for 10 minutes. A hot solution of sodium toluenesulphinate dihydrate (12g.) in water (40ml.) and acetic acid (8ml.) was added, and heating continued. A 10ml. sample was withdrawn immediately, and further samples at intervals. The samples were left at room temperature for 3 days when the crystalline product from each sample was collected. The yields were as follows: (no heating) 0.090g.; (10 minutes) 0.189g.; (20 minutes) 0.214g.; (30 minutes) 0.223g.; (45 min-

utes) 0.234g.; (60 minutes) 0.241g.; (90 minutes) 0.253g.; (120 minutes) 0.266g.. The products after 45 and 60 minutes were dark, and those after 90 and 120 minutes were very dark. One recrystallisation from water (carbon) gave colourless crystals.

(d) Leucoanthocyanidin mixture (4.33g.) was dissolved in 0.01N-hydrochloric acid (65ml.) and the solution was heated in a boiling water-bath for 10 minutes. Aliquots of 20ml. (approximately 1.33g. of leucoanthocyanidin) were withdrawn and added to the following mixtures:

(1) 1.33g. of sodium p-toluenesulphinate dihydrate in 5ml. of water and 1.0ml. of acetic acid.

(2) 2.0g. of sulphinate in 5ml. of water and 1.5ml. of acetic acid.

(3) 3.0g. of sulphinate in 5ml. of water and 2.25ml. of acetic acid.

The quantities of sulphinate are approximately 1.5, 2.2, and 3.3 equiv. with respect to the leucoanthocyanidins. Each mixture was heated in the boiling water-bath for 30 minutes, then left 3 days at room temperature before the products were collected. The yields were (1) 0.40g., almost black, (2) 0.400g., brown and (3) 0.430g., pale. The products were readily decolourised by recrystallisation from water (carbon).

(e) Leucoanthocyanidin mixture, mainly isomelacacidin (3g.) was added to solutions of sodium toluene-sulphinate dihydrate (6g., about 2.9 equiv.) in water (40ml.) containing (1) 2.4ml., (2) 3.2ml., (3) 4.8ml., (4) 6.4ml., of acetic acid (i.e. 1.5, 2.0, 3.0 and 4.0 equiv. with respect to the sulphinate) and the solutions were heated for 30 minutes in a boiling water-bath. The crystalline products were collected after 5 days at room temperature, and again after 7 more days. Yields were; (1) first crop, 0.87g.; second crop, 0.24g. (total, 1.11g.); (2) 0.77, 0.29, (1.06); (3) 0.78, 0.34 (1.12); (4) 0.16, 0.86 (1.02).

Preparation from melacacidin: Recrystallised melacacidin (0.612g.) was heated in 0.01N-hydrochloric acid (10ml.) in a boiling water-bath for 20 minutes. The melacacidin dissolved completely after 3 minutes, and the final solution was pale red. Acetic acid (0.70ml.) and sodium p-toluenesulphinate dihydrate (1.27g.) were added (molar ratio, melacacidin: acetic acid :sulphinate 1:12:6) and the solution was heated in a boiling water bath for 30 minutes. After cooling and seeding small crystals were rapidly deposited and were collected after 2 days: 0.347g. (36%). Recrystallisation (carbon) from 5% acetic acid (20ml.) gave isomelacacidin p-tolyl sulphone in pale pink prisms

(0.273g.), m.p. 102-110°, $[\alpha]_D^{23}$ -24.3° (C, 1 in acetone).

Acetylation of the sulphone (100mg.) with acetic anhydride (0.4ml.) in pyridine (2ml.) overnight at room temperature gave, after recrystallisation from ethanol, the penta-acetyl derivative in fine needles, m.p. 191° (89mg.).

Preparation from O-ethyl isomelacacidin: Crystalline O-ethyl isomelacacidin (371mg. of a sample which lost 10% on drying) was dissolved in 0.01N-hydrochloric acid (5ml.) by heating in a boiling water bath for one minute, and acetic acid (0.35ml.) and sodium p-toluene-sulphinate dihydrate (0.643g.) were added. The solution was heated in a boiling water-bath for 30 minutes. Crystals formed quickly in the cooled and scratched solution, and were collected after 2 days; 0.39g. (81%). Recrystallisation (carbon) from 5% acetic acid (25ml.) gave isomelacacidin p-tolyl sulphone as colourless prisms (0.31g.) m.p. 103-111°. The melting point of a mixture with that prepared from melacacidin was 102-110°. $[\alpha]_D^{26}$ -24.5 (C, 1 in acetone).

Acetylation as described for the product from melacacidin gave, after recrystallisation, the penta-acetyl derivative, m.p. 192-193° (90mg.). The mixed melting point with that derived from melacacidin was 191-192°.

Preparation from leucoanthocyanidin mixtures:

(a) Leucoanthocyanidin mixture remaining after

crystallisation of O-ethyl isomelacacidin (9.4g.) was boiled for 10 minutes in 0.01N-hydrochloric acid (200ml.), acetic acid (20ml.) and sodium p-toluene-sulphinate dihydrate (25g.) were added and the mixture was heated in a boiling water-bath for 30 minutes, and then left at room temperature for 7 days. Yield, 4.54g. of sulphones.

(b) Leucoanthocyanidin mixture (4.73g.) was boiled in 0.01N-hydrochloric acid (60ml.) for 10 minutes, acetic acid (3.6ml.) and sodium p-toluenesulphinate dihydrate (9g.) were added, and the mixture was heated in a boiling water-bath for 30 minutes. The yield after 4 days at room temperature, was 1.47g. of sulphone.

(c) From 9.9g. of leucoanthocyanidin mixture, with 0.01N-hydrochloric acid (130ml.), acetic acid (8ml.) and sulphinate (20g.), 7.40g. of sulphone was obtained.

Isomelacacidin p-tolyl sulphone: The crystals (prisms) from water were colourless or faintly pink. The products melted slowly in the range 102-115° to a clear, viscous syrup which became less viscous at about 170°, and above that temperature became red at the surface exposed to air. The sample for analysis was dried over P_2O_5 for 1 day at atmospheric pressure and room temperature. (Found: C,54.8; H,5.0; S,6.6. $C_{22}H_{20}O_8S$. $2H_2O$ requires C,55.0; H,5.0; S,6.7%).

Stability in aqueous solutions: Sulphone (50mg.) was heated in test tubes in a boiling water-bath in 1ml. portions of water, aqueous 5% acetic acid, and aqueous 5% pyridine. Samples were withdrawn at intervals up to 60 minutes, and chromatographed (BAW, 12 hours). Leucoanthocyanidins and polyphenols were detected with Roux's spray and ferric-ferricyanide sprays respectively.

The sulphone did not dissolve completely in water; the chromatograms showed that apart from the sulphone, the only polyphenol present was isomelacacidin, in traces only. The sulphone dissolved completely in the 5% acetic acid solution within 20 minutes; the chromatograms showed traces of isomelacacidin, and at 45 and 60 minutes, possible traces of melacacidin. The sulphone dissolved in the 5% pyridine solution within 10 minutes; an oily suspension formed on slight cooling. The solution darkened slightly during the heating; the chromatograms showed mainly sulphone, with traces of isomelacacidin.

Stability in acetone: Sulphone (95mg.) was boiled for 60 minutes in 5ml. of acetone previously dried over potassium carbonate. Chromatograms (BAW, 2% acetic acid) showed that no other polyphenol was present. The acetone solution was allowed to evaporate at room temperature; crystals of sulphone remained.

Recrystallisation of crude sulphone: Crude sulphone (1g.) was recrystallised from (1) 50ml. of water and (2) 30ml. of 5% acetic acid. The solutions were decolourised while hot with carbon, and left at room temperature for 2 days. The yields were (1) 0.73g., (2) 0.77g. of almost colourless crystals.

Conversion to anthocyanidin: The sulphone was heated in 3N-hydrochloric acid in a boiling water-bath for 15 minutes, and the dark red solution was extracted with amyl alcohol. The extract was chromatographed in "Forestal" solvent. The single anthocyanidin formed had the same Rf as the anthocyanidin prepared similarly from melacacidin (Rf 0.58, compared with cyanidin Rf 0.55) and the appearance of the chromatograms of the two preparations was similar both before and after being sprayed with alcoholic aluminium chloride.

Penta-acetyl derivative: Recrystallised sulphone (0.50g.) was acetylated with acetic anhydride (2ml.) in pyridine (10ml.) at room temperature overnight, and the solution was then poured into water (80ml.); the aqueous layer became pink. A gummy deposit formed which crystallised during 24 hours, and was dissolved in methanol, charcoaled, and the solution was concentrated to about 10ml. The product crystallised as very pale pink needles, m.p. 191° (0.32g., 47%). Recrystallisation from ethanol gave

isomelacacidin p-tolyl sulphone penta-acetate in colourless, fine needles (0.26g.), m.p. 193° $[\alpha]_D^{23}$ -13.5° (C, 1 in acetone). (Found: C,59.0; H,5.0; S,5.3; O-acetyl, 30.3. $C_{32}H_{30}O_{13}S$ requires C, 58.7; H,4.6; S,4.9; O-acetyl 32.9%).

Tetramethyl ether: Recrystallised sulphone (1g). in methanol (10ml.) was treated at 0° with diazomethane (from 10g. of nitrosomethylurea) in ether (81ml.), added during 5 minutes. After 45 hours at room temperature, the solution was concentrated to about 5ml.; slow evaporation of the residue gave a clear gum which crystallised after three months. The product was triturated with ether which left 0.85g. of solid that could not be recrystallised from acetone-ether, but crystallised from a syrupy solution in a little acetone and was collected and washed with acetone-ether. Isomelacacidin p-tolyl sulphone tetramethyl ether was obtained as plates, m.p. 153-154°. The liquid formed on melting became dark red at the surface at about 200° $[\alpha]_D^{25}$ -43.5° (C, 1 in acetone). (Found: C,62.5; H,5.9; S,6.4; OCH_3,24.4. $C_{26}H_{28}O_8S$ requires C, 62.4; H,5.6; S,6.4; OCH_3, 24.8%.

Tetramethyl ether acetate: Sulphone (1.5g.) was methylated as above, and the crude methyl ether was acetylated with acetic anhydride (2ml.) and pyridine (10ml.) at room temperature overnight. Addition of

water (80ml.) caused the separation of a viscous oil. This was dissolved in ethanol, charcoaled, and the solution concentrated to about 10ml. before seeding. (A previous preparation had given no crystals from methanol, but had given crystals from a mixture of the gum with ethanol after about 7 days). Most of the product separated as a gum, then crystallised as bundles of needles during a week; 0.63g., 47%, m.p. 117-118°. Recrystallisation from methanol gave isomelacacidin p-tolyl sulphone tetramethyl ether acetate as fine needles (0.28g.), m.p. 119-122°. $[\alpha]_D^{23}$ -33.7° (C, 1 in acetone). (Found: C,60.8, 60.8; H,5.6, 5.9; S,5.7; O-methyl, 22.5; O-acetyl 8.7. $C_{20}H_{30}O_9S$. $0.5H_2O$ requires C,61.0; H,5.7; S,5.8; O-methyl, 22.3; O-acetyl, 7.8%).

Isolation of teracacidin and isoteracacidin p-tolyl sulphone.

Teracacidin from Extraction V: The ethanol-soluble portion of the ether extract from the wood (fractions (a) and (c)) was taken to dryness, and the residue was subjected to a 50 tube counter-current distribution between ethyl acetate and M/15 phosphate buffer, pH 7.7. The polyphenol mixture was added to the first tube only. The distribution was examined by paper chromatography (BAW, 20cm.) of samples from even-numbered tubes. The results are given in Table 17.

Table 17.

Counter-current distribution of polyphenols from Extraction V.

Component	Extent	Peak	Rf
Teracacidin	20-36	29	0.40-0.52
Isoteracacidin	24-32	indefinite	0.53-0.60
O-Ethyl isoteracacidin	34-42	38	0.63-0.72
Unidentified	42-50	48	0.75-0.85

The terminology of the leucoanthocyanidins is derived by analogy with the melacacidin series. The amount of isoteracacidin was very small compared with the amount of teracacidin.

The contents of tubes 20-32 were collected; the ethyl acetate phase was separated, and the solvent was removed to leave 1.58g. of residue. Continuous ethyl acetate extraction of the aqueous phase gave 1.63g. of material. Both these fractions were brown powders; they consisted of crude teracacidin. Recovery of material from the other tubes yielded the following; tubes 33-35, 0.46g.; 36-40, 0.59g. mainly O-ethyl isoteracacidin; 41-43, 0.66g.; 44-50, 3.8g., mainly wax.

Teracacidin from A. intertexta SN5977. The polyphenol fractions of Extraction W which were extracted from aqueous solution by ethyl acetate were combined (31g.) and subjected to 50 tube counter-current distribution between ethyl acetate and M/15 phosphate buffer,

pH7.0. The polyphenol mixture was added to the first two tubes.

Examination of the distribution by paper chromatography (2% acetic acid) showed the presence in tubes 24-36 (peak at 29) of teracacidin Rf 0.46-0.54 and isoteracacidin Rf 0.58-0.68. Other polyphenols were present in tubes 40-50.

The contents of tubes 24-36 were collected; the ethyl acetate phase was separated and the aqueous phase was extracted three times with an equal volume of ethyl acetate, the combined ethyl acetate extracts were dried with sodium sulphate and the solvent removed to leave 4.8g. of residue. Paper chromatography (BAW, 16 hours, 31.4cm.) showed that this product contained teracacidin Rf 0.46-0.54 and O-ethyl isoteracacidin Rf 0.75-0.87. A 2% solution of this material in water was heated in a boiling water-bath for 2 hours, and the solution was examined by chromatography (BAW, 15 hours, 27.0cm. and 2% acetic acid, 3 hours, 23.6cm.). The solution contained teracacidin and isoteracacidin; O-ethyl isoteracacidin was absent. The Rf values are given in Table 18.

Table 18.

Rf values of leucoanthocyanidins from A. intertexta.

	BAW	2% acetic acid
Teracacidin	0.47-0.54	0.41-0.52
Isoteracacidin	0.58-0.64	0.58-0.68
O-Ethyl isoteracacidin	0.78-0.85	0.67-0.76

The contents of tubes 37-50 were recovered (as described for tubes 24-36); 19.1g. This fraction contained much water-insoluble polymeric phenolics.

Much polymeric material remained in the first few tubes of the distribution.

This distribution was repeated on the polyphenolic extractives from Extractions X and Y. The results are summarised in Table 19.

Table 19.

Counter-current distributions of A. intertexta polyphenols.

	Polyphenols used	Products Tubes 24-36	Products Tubes 37-50
Extraction W	31g.	4.8g.	19.1
Extraction X	22.5	3.3	13.8
Extraction Y	30	3.6	--
	30	2.4	23.9
	31	2.7	22.4

When the polyphenol mixture from Extraction Y was added in the first two tubes, the ethyl acetate phase became the heavier, and the aqueous phase was transferred until the ethyl acetate phase had been sufficiently diluted with incoming ethyl acetate for the distribution to function normally. It was therefore necessary to add the mixture over the first three tubes. In these distributions, tubes 40-46 contained

O-ethyl isoteracacidin; the amount of polymeric material in these tubes was too high to permit individual detection of other components.

The fractions from tubes 24-36 of these distributions were collected (16.8g.) and boiled in ethanol (170ml.) with acetic acid (1.7ml.) for 2 hours. The solvent was removed, and the residue was submitted to counter-current distribution as above. Tubes 24-40 (peak at 32) contained teracacidin only (5.1g.). O-ethyl isoteracacidin was present in tubes 41-50 (peak 47); the fraction from these tubes (8.3g.) was very dark and contained much water-insoluble material.

Fractions from tubes 37-50 of the distributions of polyphenol mixture were collected (9.2g.) and heated in water (600ml.) containing acetic acid (12ml.) for 2 hours in a boiling water-bath. Much material did not dissolve. The mixture was cooled and the solution was decanted; the wet residue weighed 72g. The solution was subjected to a Bush and Densen distribution of the same pattern as that used to separate the leucoanthocyanidins of the melacacidin series (Figure 3); 200ml. of ethyl acetate and 600ml. of M/15 phosphate buffer ph7.0 were used in each vessel. The ethyl acetate phases from this distribution contained 21.4g. of material. The aqueous phases were combined and concentrated under reduced pressure to 200ml. The solution was filtered from some insoluble material, and continuously extracted with ether, which gave 11.8g.

of material.

The latter fraction was boiled in ethanol (100ml.) with acetic acid (1ml.) for 2 hours, the solvent was removed, and attempts were made to crystallise the residue from ethanol-water without success. The solution in ethanol (23ml.) and water (100ml.) deposited much amorphous material. The solution was filtered, and after the addition of hydrochloric acid (0.1ml. of 10N), was boiled for 10 minutes; a solution of sodium p-toluenesulphinate dihydrate (10g.) in water (20ml.) and acetic acid (6ml.) was added, and the mixture was heated in a boiling water-bath for 30 minutes. Isoteracacidin p-tolyl sulphone was deposited in small crystals when the solution was cooled, and was collected next day (3.4g.); no further deposition occurred.

Methylation and degradation of teracacidin.

Teracacidin was obtained above as a free-flowing brown powder. Tests on small portions showed that it would be possible to obtain it crystalline from ethanol.

Both teracacidin and isoteracacidin sulphone gave a red colour when heated in 3N-hydrochloric acid in a boiling water-bath for 15 minutes. The red material was extracted with amyl alcohol, and chromatographed in "Forestal" solvent (6 hours, 20cm.). The anthocyanidin from both teracacidin and isoteracacidin

sulphone was orange-red, Rf 0.74 (cyanidin Rf 0.55, melacacidin anthocyanidin, Rf 0.56).

Methylation of teracacidin.

(a) With methyl sulphate: Crude teracacidin (1.63g.) was boiled in acetone with methyl sulphate (3g.) and potassium carbonate (10g.) for 5 hours. The potassium salts were removed by filtration, and washed with acetone, and the combined acetone solutions were left overnight after addition of a few ml. of aqueous ammonia. The acetone was distilled, and the residue was dissolved in ether (the addition of some ethanol was necessary). The etheral solution was washed with water with dilute sodium hydroxide and again with water, then dried (Na_2SO_4), and concentrated. Teracacidin trimethyl ether crystallised in colourless plates (0.53g.). Recrystallisation from ethanol gave fine needles, m.p. 159° $[\alpha]_D^{18}$ -64.6° (C, 1 in EtOH). (Found: C, 64.9; H,6.0; OCH_3 28.0. $C_{18}H_{20}O_6$ requires C,65.0; H,6.1; OCH_3, 28.0).

(b) With diazomethane: Crude teracacidin (2g.) was dissolved in methanol (20ml.) and the solution was cooled to 0°. A cold (0°C) solution of diazomethane (from 20g. of nitrosomethylurea) in ether (150ml.) was added during 5 minutes. The temperature rose to about 10°. The mixture was left at 0° overnight, and then at room temperature for 2 days. Acetic acid was added to destroy remaining diazo-

methane, and the ethereal solution was washed with dilute sodium hydroxide solution, dried (sodium sulphate) and the solvent was removed. The residue (2.04g.) crystallised from ethanol-ether; 0.59g., m.p. 151-154°. Recrystallisation from ethanol gave 0.43g., m.p. 155-157°. The mixed melting point with teracacidin trimethyl ether from the previous preparation was 156-158°.

Repetitions on 1g. portions of teracacidin yielded 0.46g., m.p. 151-154° and 0.37g., m.p. 151-154°. Recrystallisation from methanol gave 0.37g. m.p. 158° and 0.22g. m.p. 158-159° respectively.

Oxidation of teracacidin trimethyl ether:

(a) Teracacidin trimethyl ether (107.7mg.) in dry acetone (50ml.) was heated on a water-bath while treated with potassium permanganate. After addition of 0.51g., the solution remained coloured. The deposit was collected, and was treated in sulphuric acid (20ml. of 5%), with sulphur dioxide to dissolve the manganese dioxide. The mixture was heated to boiling, and filtered while hot. On cooling, the solution deposited 24.3mg. of coloured crystals, m.p. 135-170°, which were purified by dissolution in 1ml. of 10% sodium bicarbonate (a few mg. remained undissolved), decolourisation with carbon, and addition of hydrochloric acid (0.2ml. of 10N). The precipitate was redissolved by heating, and cooling gave

16.6mg. of slightly yellow crystals, m.p. 160-177°. Sublimation under water-pump vacuum at 150° (bath) gave 12.1mg. of crystals, m.p. 173°. The mixed m.p. with p-anisic acid (m.p. 180-181°) was 130-181°. The mixed m.p. with veratric acid (m.p. 180°) was 142-145°. The yield was 24%.

(b) Teracacidin trimethyl ether (1.03g.) was boiled in acetone (50ml.) with potassium permanganate (1.5g.) for 4 hours. Acetone was then evaporated, water (50ml.) being added simultaneously. Sulphuric acid (5ml. of 10%) was added, and sulphur dioxide was passed in until the manganese dioxide had dissolved. Much organic material remained in suspension. The mixture was extracted with ether (100ml., then four 50ml. portions) and the combined ethereal solutions were washed with water (two 5ml. portions) and then extracted with 5% sodium carbonate solution (five 10ml. portions). The ether contained 0.14g. of material, which, when crystallised from benzene, gave teracacidin trimethyl ether (81mg.) as needles, m.p. 159°, alone and mixed with pure teracacidin trimethyl ether.

The sodium carbonate solutions were acidified with sulphuric acid and extracted with ether (20ml., then three 10ml. portions) to yield 0.67g. of material. This was dissolved in ether and diazomethane (from 4g. of nitrosomethylurea) in ether (16ml.) added. Af-

ter 5 minutes, the solution was evaporated; the residue was re-dissolved in ether and the solution was extracted with 2N-sodium hydroxide (two 5ml. portions), washed with water (two 5ml. portions), dried (sodium sulphate) and the ether removed, which left 0.34g. of crystalline residue. Recrystallisation from light petroleum gave methyl p-anisate as colourless flat plates, m.p. 48-49°, alone and mixed with an authentic sample of the same m.p.

The sodium hydroxide extracts were carbonated and extracted with ether (three 10ml. portions); the combined ether extracts were dried (sodium sulphate) and the solvent removed, leaving 0.29g. of crystalline residue. Recrystallisation from methanol-water gave methyl 3,4-dimethoxy-2-hydroxybenzoate as colourless prisms (0.25g.), m.p. 75-76°, alone and mixed with an authentic sample.

The yield of methyl 3,4-dimethoxy-2-hydroxybenzoate was 43%, and crude methyl anisate, 76%, calculated on trimethyl ether consumed.

Isoteracacidin p-tolyl sulphone.

The crude sulphone (3.4g.) was recrystallised from boiling 5% acetic acid (500ml.) (carbon), and gave small fawn-coloured crystals (2.16g.). Recrystallisation (of 1g.) from acetone-water 1:1 (20ml.) gave isoteracacidin p-tolyl sulphone as clusters of transparent plates becoming opaque on air-drying (0.61g.), m.p. 214° decomp. after becoming brown at 204°.

$[\alpha]_D^{24}$ -22.4° (C, 1 in acetone). (Found: C,60.6; H,5.1; S,7.3. $C_{22}H_{20}O_7S.\frac{1}{2}H_2O$ requires C, 60.4; H,4.8; S,7.3%).

Tetra-acetate: Acetylation of pure sulphone (0.1g.) with acetic anhydride (0.4ml.) and pyridine (2ml.) at room temperature overnight, gave, after addition to water (20ml.) a thick oil which was left in contact with water 24 hours. The oily product was dissolved in methanol (carbon), and the solution concentrated to 2-3ml., which gave small needles (85mg., 68%) m.p. 133-136°. Recrystallisation from ethanol gave woolly masses of very fine needles (67% recovery), m.p. 137-138° $[\alpha]_D^{24}$ -15.65° (C, 0.92 in acetone). (Found: C, 60.3; H,5.0; S, 5.35; O-acetyl, 27.4. $C_{30}H_{28}O_{11}S$ requires C, 60.4; H,4.7; S,5.4; O-acetyl, 28.8%).

Catalytic hydrogenation of leucoanthocyanidins.

Reduction of melacacidin and O-ethyl isomelacacidin.

In the series of experiments described here, the acetic acid used as solvent was either British Drug Houses' "Analar" or commercial acetic acid distilled from chromium trioxide until no further oxidation occurred. Test runs showed that the same results were obtained with each solvent. The catalyst used was 10% palladium on carbon from two different preparations, which behaved similarly.

(a) Crystalline melacacidin (0.49g.) suspended in acetic acid was shaken with catalyst (103mg.) and

and hydrogen at room temperature and pressure for 70 hours. There was no uptake of hydrogen.

The mixture was transferred to a pressure hydrogenator, and shaken at 65°C under about 70 atmospheres of hydrogen for 20 hours. Chromatography of the product (BAW and 2% acetic acid) showed that two polyphenols had been formed. The major product had Rf 0.52-0.61 in BAW, 0.41-0.50 in 2% acetic acid. The minor product had Rf 0.75-0.85 in BAW, 0.59-0.68 in 2% acetic acid. (Melacacidin had Rf 0.30-0.40 in BAW, 0.59-0.68 in 2% acetic acid on these chromatograms). The products gave a brown colour when heated after the application of Roux's spray, and reacted readily with the ferric-ferricyanide reagent. Melacacidin was still present in high proportion in the mixture.

(b) Crystalline melacacidin (0.52g.) in ethanol (10ml.) was shaken with catalyst (107mg.) and hydrogen at 65° and 65 atmospheres for 20 hours. Paper chromatography (BAW) showed that slight reduction had occurred. Acetic acid (2ml.) was added to the mixture which was shaken under hydrogen at 65° and 60 atmospheres for 13 hours. Paper chromatography showed that much melacacidin remained; hydrogenation had proceeded slightly after the addition of acetic acid. Fresh catalyst (100mg.) was added, and the mixture was shaken under hydrogen at 65°C and 60 atmospheres for 12 hours. Paper chromatography showed that much

melacacidin remained. It seemed that much less than half of the melacacidin had been reduced, although an accurate estimate could not be made through inadequate knowledge of the properties of the products.

(c) Crystalline melacacidin (0.49g.) in acetic acid (10ml.) was shaken with catalyst (107mg.) and hydrogen at 65°C and 65 atmospheres for 11 hours. Paper chromatography showed that very little melacacidin remained.

(d) Crystalline O-ethyl isomelacacidin (0.50g.) in acetic acid (10ml.) was shaken with catalyst (103mg.) under hydrogen in the same conditions as (c). The product was compared with that from melacacidin (experiment (c)) by paper chromatography (BAW, 10 hours, 25.8cm. and 2% acetic acid, 3 hours, 27.8cm.). The major reduction product from O-ethyl isomelacacidin had the same Rf as that from melacacidin in each solvent, and a single spot was obtained from a mixture in each solvent. Rf values: (BAW) melacacidin, 0.23-0.31; O-ethyl isomelacacidin, 0.67-0.75; major reduction product, 0.48-0.56; (2% acetic acid) melacacidin, 0.41-0.49; O-ethyl isomelacacidin, 0.66-0.75; major reduction product, 0.46-0.55.

(e) Leucoanthocyanidin mixture from A. excelsa Extraction I, which remained after melacacidin had crystallised during ether extraction, and which contained much melacacidin and apparently no poly-

phenols other than leucoanthocyanidin was hydrogenated in acetic acid at 65° and 55 atmospheres pressure. A preliminary experiment with 0.50g. of mixture in 10ml. of acetic acid with 100mg. of catalyst resulted in a product in which leucoanthocyanidins could not be detected by paper chromatography. Repetition on a larger scale (9.94g. of mixture and 0.40g. of catalyst in 50ml. of acetic acid) also gave a product free from leucoanthocyanidins.

The combined solutions were filtered and solvent was removed under reduced pressure. The residue was subjected to 50-tube counter-current distribution between ether and M/15 phosphate buffer pH7.0. Samples from even-numbered tubes were chromatographed in BAW (12 hours, 25.5cm.) and in 2% acetic acid (2¾ hours, 23cm.) and the constituents were revealed with Roux's spray and with ferric-ferricyanide reagent. The results are shown in Table 20. No leucoanthocyanidins were detected. The first two tubes contained much polymeric material.

The polyphenols were recovered by continuous ether extraction. The amounts were: tubes 1-7, 2.47g.; tubes 8-16, 2.30g.; tubes 17-22, 0.23g.; tubes 33-50, 0.80g. (Total recovery; 5.80g., 55%).

Table 20.

Counter-current distribution of hydrogenation mixture

Com-ponent	Extent	Peak	Rf BAW	Rf 2% acetic	Relative amount
1	2-6	2	0.46-0.54	0.42-0.51	Medium
2	4-20	12	0.53-0.61	0.45-0.55	Major
3	14-22	18	0.61-0.68	?	Minor
4	34-40	37		0.24-0.32	Minor
5	38-46	42	0.81-0.88	0.61-0.70	Medium
6	46-50	50		0.91-0.31	Major

The fraction from tubes 8-16 was chromatographically pure and corresponded in Rf values to the major product of hydrogenation of crystalline melacacidin and O-ethyl isomelacacidin. It was obtained as a brown powder. Attempts to obtain the polyphenol crystalline by slow evaporation of an aqueous syrup were unsuccessful. Acetylation of the phenol (0.50g.) with acetic anhydride (4ml.) in pyridine (10ml.) at room temperature overnight, gave after addition of water, an oil which did not crystallise, either alone or from ethanol or from benzene.

Portion of this fraction (0.5g.) was dissolved in methanol (3ml.) and the solution, cooled in ice, was treated with diazomethane (from 5g. of nitrosomethylurea) in ether (50ml.) After 3 days at room temperature, the solution was filtered from a slight

deposit and concentrated under reduced pressure to a small volume. The syrup was left at 0°C. Slow evaporation occurred, and crystals appeared in the glassy residue after 4 days. These were very soluble in ether, ethanol, methanol and benzene. Attempted crystallisation from benzene-light petroleum gave a resin. When again taken to dryness, and left at 0° with a few drops of ethanol, the material again crystallised. Although these crystals were stable at room temperature, they were not formed at room temperature.

The crude methyl ether, in pyridine (5ml.) with p-toluenesulphonyl chloride (0.5g.) was left overnight at room temperature, then the mixture was poured into water (40ml.). A viscous oil separated, and slowly deposited crystals. After 7 days in contact with water, the oil was dissolved in ethanol (carbon) and the solution was concentrated to 10ml. On seeding, rapid crystallisation occurred. After 1 day at 0°, the crystals were collected; 0.137g. (about 15%), m.p. 162-163°. Recrystallisation from ethanol gave a 7,8,3',4'-tetramethoxyflavan-3-ol p-toluenesulphonate as pale yellow blades, (81mg.), m.p. 165-166°. $[\alpha]_D^{24}$ -33.0 (C, 3.3 in $CHCl_3$). (Found: C, 62.4; H,6.1; S,6.5; OCH_3, 24.2. $C_{26}H_{28}O_8S$ requires C, 62.4; H5.6; S,6.4; OCH_3, 24.8%). These crystals became pink after a few weeks.

(f) Crystalline O-ethyl isomelacacidin (4.0g.) in acetic acid 20ml. was shaken with catalyst (0.11g.) and hydrogen at 70° and 70 atmospheres for 24 hours. The filtered solution became red-brown on exposure to air. Chromatograms (BAW, 14 hours) showed the absence of leucoanthocyanidins and the presence of the major product and two minor products. The solvent was removed under reduced pressure without an air inlet; traces of acetic acid were removed by repeated addition of water and re-evaporation. The product, as a thick aqueous syrup, was left at 0°, but had not crystallised after 4 weeks.

Water was removed from the syrup, the residue was dissolved in methanol (10ml.) and the solution, cooled in ice, treated with diazomethane (from 20g. of nitrosomethylurea) in ether. Two days later, the solution was decanted from a small residue and again treated with diazomethane. The solution was kept for 2 days and then allowed to evaporate, but no crystals were obtained.

The methylation product was divided into four aliquots.

One portion in pyridine (10ml.) with acetic anhydride (2ml.) was left overnight, and poured into water. The gummy product did not crystallise and could not be obtained crystalline from methanol or ethanol.

A second portion was heated in pyridine (10ml.) with p-nitrobenzoyl chloride (1.5.g) until the reagent had dissolved, and the mixture was left overnight and then poured into water. The product consisted of oily crystals (0.35g.), and gave from methanol yellow-prisms (90mg.), m.p. 231° decomp.

To a third portion in pyridine (10ml.), p-toluene-sulphonyl chloride (1.5g.) was added and the solution was left overnight before being poured into water. The oily product partly crystallised, and crystals also formed in the aqueous phase. The latter was decanted, and the crude product was recrystallised from ethanol in dense clusters of small needles (0.25g.) which turned pink on the outer surfaces when kept. This product melted slowly at 120-130° to a viscous liquid in which crystals formed at 150° and remelted at 155°. The product was recrystallised from methanol, and then ethanol. Neither solvent was satisfactory, recovery from methanol being very poor, and crystallisation from ethanol being very slow. The recrystallised product (67mg.) was again obtained as dense aggregates which turned pink when kept. These melted partly at 120-130° but part did not melt until 161°. The molten material was red. When mixed with the toluenesulphonate obtained in section (e) there was no marked change in melting behaviour, and melting was complete at 160°.

This hydrogenation was repeated twice with O-ethyl isomelacacidin (2g. each) and the product converted to the methyl ether toluenesulphonate. Attempted crystallisation from ethanol gave gummy mixtures (0.58g. and 0.76g.).

(g) Melacacidin (recrystallised from ethanol, 2g.) suspended in acetic acid (20ml.) was shaken with catalyst (0.21g.) in hydrogen at 70°C and 7 atmospheres for 90 minutes. Chromatography of the solution showed that melacacidin was still present. Fresh catalyst (95mg.) was added and the hydrogenation was continued under the above conditions for 60 minutes. Chromatography (BAW) showed the presence of the major product (Rf 0.58-0.65), some melacacidin (Rf 0.34-0.41) and other products. The solution was filtered and evaporated under reduced pressure, ethanol being added to the residue and evaporated to sweep out remaining acetic acid. The residue was methylated with diazomethane (from 20g. of nitrosomethylurea) in ether at room temperature for 2 days and, after removal of ether, the product was dissolved in pyridine (20ml.) with p-toluenesulphonyl chloride (3g.) and the mixture was next day poured into water (150ml.). After one day, the residue was dissolved in methanol; no crystalline product was obtained. The solution was allowed to evaporate and the residue, when triturated with ethanol, gave a yellow-orange powder

(1.35g.), m.p. 120-145°. This was dissolved in hot ethanol (carbon). The solution (50ml.) was decanted from a resin which deposited, and then slowly deposited a white powder, collected after 14 days at room temperature; 0.245g. m.p. 149-153 (after sintering at 147°). The mixed melting point with the toluene-sulphonate obtained in section (e) was 156-163°. The white powder slowly became pink.

(h) Crystalline O-ethyl isomelacacidin (50mg.) in acetic acid (1ml.) was shaken with catalyst (10mg.) and hydrogen at 65°C and 70 atmospheres for periods of 2, 4, 8 and 16 hours. Chromatograms (BAW) showed that in each case, no leucoanthocyanidin remained, and there appeared to be a single major product in each case.

A sample of O-ethyl melacacidin kept under the same conditions, but without catalyst, for 13 hours, had developed a red colour, and the solution had deposited purple material on the glass surfaces.

A similar series was run with crystalline melacacidin, but at 70°C and 40 atmospheres. The melacacidin did not dissolve under these conditions, and hydrogenation was incomplete even after 16 hours. A comparison run with O-ethyl isomelacacidin showed incomplete hydrogenation. A run in acetic acid containing 0.1ml. of 10N-hydrochloric acid to 100ml. (i.e. 0.01N) for 1½ hours, resulted in complete

reduction of melacacidin; there was a single major product (Rf 0.46-0.55 in BAW, melacacidin Rf 0.29-0.35).

A series of hydrogenations of melacacidin in acetic acid containing different amounts of added hydrochloric acid was carried out at 70°C and 70 atmospheres for 1½ hours. Hydrogenation with formation of only one product was obtained with 0.01N, 0.02N and 0.04N hydrochloric acid, but with 0.08N hydrochloric acid, much colour developed during hydrogenation.

Attempted reduction of melacacidin tetramethyl ether.

(a) In acetic acid. Melacacidin tetramethyl ether m.p. 136-139° (0.50g.) was dissolved in hot acetic acid (10ml.) and the solution was shaken with catalyst (56mg.) under hydrogen at room temperature and pressure. No hydrogen uptake occurred in 3 days. The mixture was then transferred to a pressure hydrogenator and shaken for 24 hours at 65°C and 60 atmospheres of hydrogen. Filtration, removal of solvent and crystallisation from methanol yielded 47mg. of melacacidin tetramethyl ether m.p. 136-138°, alone and mixed with a pure sample.

(b) In dioxan. Melacacidin tetramethyl ether m.p. 142-143.5° (0.48g.) in dioxan (purified by distillation from sodium, and redistillation; (10ml.)) was shaken with catalyst (83mg.) and hydrogen at 70°C and 70 atmospheres for 18 hours. Filtration and removal of solvent left a thick oil which crystall-

ised on the addition of a few drops of ethyl acetate; m.p. 137-140°, and, after admixture with melacacidin tetramethyl ether, 140-142°.

The recovered melacacidin tetramethyl ether was redissolved in dioxan (10ml.) and shaken with catalyst (95mg.) and hydrogen at 100°C and 70 atmospheres for 30 hours. The solution was worked up as above but a crystalline product could not be obtained. The crude product gave a red colour when heated with ethanolic hydrochloric acid.

4-HYDROXYPIPECOLIC ACID.

Isolation of the imino-acids from A. Oswaldii leaves.

Examination of single-tree samples: Single-tree samples were collected as follows: 7 December, 1958, 3 samples from Devlin's Pound (north bank of River

Murray, between Waikerie and Overland Corner), and 3 samples between Berri and Renmark; 8 August, 1959, samples from 16 miles west of Blanchetown, 5 miles west of Blanchetown and 10 miles east of River Murray at Blanchetown, and five samples from an area 5 miles south of River Murray, near Kingston.

The samples were extracted with hot ethanol and the extract was evaporated to dryness. The residue was dissolved in water and chromatographed (BAW, 18 hours). Examination of the second batch of samples was made semi-quantitative by extracting 5g. (wet weight) of leaf with a mixture of 50ml. of ethanol and 10ml. of water for 6 hours on a water bath; the evaporated extract was dissolved in 5ml. of water and the 4-hydroxypipecolic acid spot given by 0.010ml. was compared with those given by 20, 50 and 100 μg. 4-hydroxypipecolic acid run on the same chromatograms.

All samples showed spots corresponding to pipecolic acid, proline and 4-hydroxypipecolic acid, the last being very prominent. In the semiquantitative work, the 4-hydroxypipecolic acid spot appeared to be of the same intensity and size as that from 50 μg. of 4-hydroxypipecolic acid i.e. the leaf samples contained about 0.5% 4-hydroxypipecolic acid. Although the specimens sampled ranged from young bushes to old trees, there did not appear to be much variation in 4-hydroxypipecolic acid content.

Preparative isolations: These were carried out on leaf collected as follows (fresh leaf weights):

(a) 2,322g. collected Devlin's Pound, 21 December, 1958 (while in flower).

(b) 5,280g. collected Spring Cart Gully (between Berri and Kenmark) 12 April, 1959.

(c) 6,470g. collected Berri, 3 May, 1959.

(d) 5,540g. collected 24 May, 1959 from the same tree as (c).

The processing of batch (d) is described.

The leaf was homogenised in portions in a blender with 80% ethanol, and the homogenate was heated to the boiling point in a water bath, which appeared to coagulate some white flocculent material. About 18l. of ethanol was used. The homogenate was placed in a percolator (capacity 9l.). The solid residue was mainly fibrous material, and liquid readily drained from it. The residue was percolated with cold 80% ethanol until the extract was only very faintly green. The combined extracts were concentrated under slightly reduced pressure to a thin aqueous syrup containing much dark green water-insoluble semi-solid material. The concentrated extract was diluted with cold water to 5l., and filtered through kieselguhr. The pale-brown solution was diluted with an aqual volume of water. A small amount of sticky green sludge formed and was removed by gravity filtration through paper.

This solution, in two portions, was passed through a column of Zeokarb 225 resin (972g. drained weight, 60 x 5cm.), the column was washed each time with 5l. of water and the amino-acids were eluted with 3N-ammonia, about 4l. being used for each half of the extract. The column effluent was collected in 1l. fractions when the extract and washings were passing through, and the ammonia eluate was collected in 500ml. fractions. Each fraction was examined for amino-acids by paper chromatography (BAW, 12 hours). The column removed all the amino-acids from the extract, and these were obtained in four ammonia-eluate fractions. These fractions were evaporated to dryness in dishes on a boiling water-bath; total crude amino-acids, 95g. (1.65%).

A solution of the total crude amino-acids in 200ml. of 5N-hydrochloric acid was cooled in ice and additions of hydrochloric acid (12ml. of 10N) and sodium nitrite (20ml. of 6M) were made at 30 minute intervals, without stirring. Five such additions were made and the solution was left at room temperature overnight (nitrous acid was still present), and a sixth portion of acid and nitrite was added. The solution was then continuously extracted with ether. 2N-Hydrochloric acid (100ml. initially, then 50ml.) was placed in the ether-still. At intervals, the still contents were removed, the ether distilled, and the residue

evaporated on a water-bath. The weights of acidic syrup so obtained were: first 3 hours, 28.9g.; next 2 hours 22.8g.; 12 hours, 4.0g.; 24 hours, 2.2g. To the residual aqueous solution in the extractor, hydrochloric acid and sodium nitrite (above amounts) were added, and ether extraction was continued for 24 hours. This was repeated three times. The acidic syrups obtained on evaporation weighed 5.0, 3.95, 3.2 and 1.5g. respectively. The residual aqueous solution was combined with the corresponding material from extractions of leaf samples (b) and (c), and processed as described below.

The syrups containing the imino-acids and hydroxy-acids were diluted with water (200ml.) and boiled gently for 30 minutes, and then continuously extracted with ether for 48 hours. The ether extract weighed 10.2g., and after combination with the same fraction from the extraction of leaf sample (c), gave from about an equal weight of water, 5.5g. of crude oxalic acid dihydrate, m.p. 98-100°, mixed m.p. with an authentic sample, 99-100°.

The residual aqueous solution, containing the imino-acids, was evaporated to a syrup (59g.) which was dissolved in water (200ml.) and passed through a column of Zeokarb 225 resin (200g.). (A second passage through the regenerated resin was necessary in order to remove all the amino-acid). The column

was washed and the amino-acids were eluted with 3N-ammonia. The eluates containing amino-acids were evaporated to dryness on a water-bath; total crude imino-acids, 46g. (0.83%).

The imino-acids were dissolved in the minimum of water (58ml.) while heated in a boiling water bath, and boiling ethanol (400ml.) was added. The mixture was left to crystallise, first at room temperature, then at 0° for 6 days. Much brown pigment quickly separated as a loose deposit, and large crystals of 4-hydroxypipecolic acid formed slowly on the walls and bottom of the flask. The solution was decanted, and the residue was washed with ethanol; the crude 4-hydroxypipecolic acid weighed 10.7g. A second crop was obtained by evaporating the mother liquor, dissolving the residue (20.4g.) in hot water (15ml.) and adding hot ethanol (220ml.). After 7 days at 0°, 6.9g. of almost colourless crystalline 4-hydroxypipecolic acid was collected. The mother-liquors contained 11.45g. of material.

The first crop contained most of the brown pigment present in the crude imino-acids. It was dissolved in water (500ml.) containing acetic acid (2.5ml.) and the solution was left at 0°. During 3 days much of the pigment separated as a flocculent deposit. The solution was passed through a column (7 x 2cm.) of carbon (2g.) mixed with kieselguhr. The colour-

less eluant and washings were evaporated to dryness under reduced pressure, the residue was dissolved in a minimum of hot water and the solution was diluted with ethanol. After 5 days at 0°, 17.78g. of 4-hydroxypipecolic acid, m.p. 285-286° decomp. was collected. The second crop was decolourised and recrystallisation gave 5.25g. of 4-hydroxypipecolic acid, m.p. 187° decomp. (Total yield, 0.41%).

The residues from the first crystallisation of 4-hydroxypipecolic acid were dissolved in 5N-hydrochloric acid (25ml.) and the solution was evaporated on a boiling water-bath until crystals began to form (weight of mixture, 16.4g.). After 6 days, the crystals were collected and washed with ethanol; 1.26g. of crude (-)-pipecolic acid hydrochloride, m.p. 227-238° $[\alpha]_D^{24}$ -10.2° (c, 4.9 in water). A second crop (1.04g.) was obtained on repetition.

The residues from the crystallisation of 4-hydroxypipecolic acid from the four batches were combined and added to a Zeokarb 225 column. The amino-acids were eluted with dilute hydrochloric acid (0.1 to 0.8N) to separate 4-hydroxypipecolic acid from pipecolic acid and proline. Several repetitions were necessary as the last two tended to be eluted early from the overloaded column. In the final separation, hydrochloric acid up to 1.6N was used; the ammonia eluate then contained a single ninhydrin-positive compound which

crystallised from water-ethanol as small needles (1.66g.). Recrystallisation from water-ethanol gave 1.50g. m.p. 231-234° decomp. $[\alpha]_D^{24}$ +15.0° (C, 1 in water) $[\alpha]_D^{26}$ + 22.1° (C, 1 in 5N-HCl). (Found: C, 46.7; H,7.8; N,13.1. $(C_4H_8O_2N)_x$, requires C, 47.05; H, 7.9; N,13.7). This material, chromatographed in BAW (30 hours), moved 10.0cm., compared with pipecolic acid, 23.5cm., 4-hydroxypipecolic acid, 15.8cm., cis-4-hydroxypipecolic acid, 12.9cm. The colour with ninhydrin (at 100°) was a reddish grey, with a red fluorescence in UV light. This compound gave a green colour with isatin at 100°. A solution in 6N-hydrochloric acid was boiled for 16 hours; no other ninhydrin-positive component was detected in the solution.

The aqueous solutions containing the amino-acids not destroyed by nitrous acid or extracted as N-nitroso compounds during the ether extractions (batches (b), (c) and (d)) were evaporated to dryness on a water-bath, and the residues, which were mainly large crystals of sodium chloride, were extracted with boiling ethanol. The combined ethanol extracts were evaporated to a syrup in which crystals formed. These were collected and washed with ethanol (17.7g.), and were dissolved in hot ethanol, which left some sodium chloride undissolved, and deposited ammonium chloride (0.8g.) on cooling. The ethanol solution was concentrated until

crystallisation began (28g.), and left at 6° overnight, to give 6.5g. of crystals. Recrystallisation from water-ethanol gave (-)-pipecolic acid hydrochloride, m.p. 256 258° $[\alpha]_D^{18}$ -10.7° (c, 7.9 in water).

The mother liquor from the initial crop of crystals was added to a Zeokarb 225 column (200g.), the column was washed and the amino-acids were eluted with ammonia. The eluates were evaporated and the bases converted the hydrochlorides. This fraction, and the mother-liquors from recrystallisation of the (-)-pipecolic acid hydrochloride were evaporated to give crops of crystals which were successively re-crystallised twice. The following crops of pipecolic acid hydrochloride were obtained: 1.85g., $[\alpha]_D$ not determined; 4.3g., $[\alpha]_D^{22}$ -5.4° (c, 6.36 in water); 4.9g., $[\alpha]_D^{24}$ -6.1° (c, 6.9 in water); 3.2g., $[\alpha]_D^{17}$ -5.5 (c, 6.0 in water). These (and intermediate crops) appeared to be homogeneous; after the first recrystallisation, the only ninhydrin-positive component was pipecolic acid.

Various crops of pipecolic acid hydrochloride of specific rotation -5 to -10° were collected (17.66g.) and converted to free pipecolic acid by addition to a Zeokarb 225 column (200g.) and elution with ammonia. The eluate was evaporated under reduced pressure and the product, in dilute aqueous acetic acid, was de-colourised with carbon (0.3g.). The solution was

concentrated to a syrup. (-)-Pipecolic acid crystallised in small prisms; 5.0g., m.p. 272-273° decomp. $[\alpha]_D^{17}$ -25.5 (C, 2 in water) (1st crop) and 2.8g., m.p. 272-273° decomp., $[\alpha]_D^{17}$ -25.9° (C, 2 in water) (2nd crop).

The following points were observed during the isolation of 4-hydroxypipecolic acid from these leaves:

(a) The aqueous extract contains some material which, after removal of the amino-acids by the ion-exchange column, separated as a gel (about 200g. wet weight from a large batch). This material was soluble in alkali and reprecipitated by acids. In hot water it formed a clear sol which did not pass through filter paper; addition of ethanol to this sol gave a flocculent precipitate. The material had a very astringent taste and stained skin brown; the sol gave a blue precipitate on addition of ferric chloride.

If the aqueous extract were left on the Zeokarb 225 column overnight, the flow-rate was greatly diminished, and could be restored only by flushing with 3 to 5N-ammonia. In batches (b) and (c), the aqueous extract was acidified (to 0.2N-HCl) before removal of the amino-acids, but this did not eliminate the trouble.

It was therefore necessary to pass the extract through the column without delay, and to wash the column with water overnight.

(b) Addition of the nitrite and acid in portions at intervals gave much better results than slow continuous

addition with stirring.

(c) During extraction of the N-nitroso compounds, dilute hydrochloric acid was placed in the ether still in order to dilute the resulting solution of N-nitroso compounds, and to encourage their gradual decomposition. If it was omitted, and more than a few grams of N-nitrosoimino-acid were present, the residue on removal of ether decomposed exothermically with copious evolution of nitrous fumes.

(d) When ethanol was added to the strong aqueous syrup containing the imino-acids during crystallisation of 4-hydroxypipecolic acid, it was sometimes possible to remove the flocculent brown precipitate by filtration before crystallisation began. However, it was preferable to proceed as described.

(e) The crude 4-hydroxypipecolic acid was decolourised completely by carbon if the solution contained a trace of added acid e.g. 0.5. to 2% acetic acid. In its absence, carbon was not effective.

Up to this stage, vacuum distillation of aqueous solutions of the amino-acids was extremely difficult due to frothing. Evaporation in basins on a boiling water-bath, even of the ammonia eluates, apparently did not epimerise the 4-hydroxypipecolic acid.

(f) The first crop of 4-hydroxypipecolic acid was of satisfactory purity (m.p. in range 285-293°). Later crops sometimes had lower melting points, and

although they gave only a single spot (ninhydrin) on chromatography, recrystallisation from water-ethanol did not raise the melting point. One such crop had m.p. 276° decomp. $[\alpha]_D^{15}$ -11° (C, 1 in water).

4-Hydroxypipecolic acid: Properties and Derivatives.

4-Hydroxypipecolic acid: Repeated crystallisation from water-ethanol gave the pure acid as clusters of prisms, m.p. 294° decomp. The sample for analysis was dried at 90° over P_2O_5 *in vacuo* for 1½ hours. (Found: C, 49.7; H,7.7; N,9.8; C-methyl, 0.1; O-methyl, 0.2. Calc. for $C_6H_{11}O_3N$: C,49.6; H,7.6; N,9.65%). The IR absorption spectrum (nujol mull) showed major peaks as follows: 2.98vs, 3.13s, 3.35vs, 3.42m, 3.63s, 3.78w, 3.91m, 3.95m, 6.18vs, 6.75w, 6.85s, 6.97m, 7.03vs, 7.08m, 7.29vs, 7.39vs, 7.59vs, 7.75m, 8.24m, 8.61w, 8.72vs, 9.20w, 9.37s, 9.61s, 9.71w, 9.97s, 10.56s, 10.82m, 10.92w, 11.24s.

Different samples had the following specific rotations in water: $[\alpha]_D^{20}$ -13.4°, $[\alpha]_D^{27}$ -12.7°, $[\alpha]_D^{25}$ -12.5° (all C, 1 in water). In 5N-hydrochloric acid, it had $[\alpha]_D^{16}$ +2.7° (C,1). A solution in 1N-sodium hydroxide showed a possible slight change in optical rotation within 30 minutes of preparation (-0.45° to -0.60°) but then remained steady, and on paper chromatography (BAW, 36 hours) only, 4-hydroxypipecolic acid was detected. The final figure was $[\alpha]_D^{25}$ -6.0° (C, 1 in 1N-NaOH).

Colours with spray reagents: Chromatograms in BAW were normally sprayed with ninhydrin or isatin, and heated at 100-110° for 5-10 minutes. 4-Hydroxypipecolic acid gave with isatin a green colour, and with ninhydrin, a dull colour ranging from a greyish green to a brown-purple, with a dull red fluorescence in UV light.

The temperature at which the paper was heated after spraying had a marked effect on the colour. Papers spotted with 12.5, 25, and 50 µg. of 4-hydroxypipecolic acid and chromatographed in BAW 16 hours were sprayed with ninhydrin (0.2%)in water-saturated n-butanol) and with isatin (0.2% in acetone), and the papers were heated at 80°, 100° or 115°. Heating for 15 minutes gave the same results as heating for 5 minutes. The colours observed on removal of the paper from the oven are given in Table 21. The colour from ninhydrin heated at 80° changed to a grey-purple after 15 minutes at room temperature and then slowly deepened. The colour from ninhydrin heated at 100° changed to a brown-purple after 15 minutes at room temperature. The colours from isatin heated at 100° and 115° changed to green after 24 hours at room temperature. Other observations suggest that the isatin colour is dependent on the amount of acetic acid remaining on the paper.

Table 21.

Colours given by 4-hydroxypipecolic acid with ninhydrin and with isatin.

Temperature	Ninhydrin	Isatin
80°	Green-yellow-brown	No colour
100°	Yellow-brown	Faint purple
115°	Brown-purple	Purple

Two other spray reagents were used. Folin reagent (1, 2-naphthaquinone-4-sulphonic acid) was introduced as a spray reagent by Müting (187) and used by Harris and Pollock (169) and more recently by Linko (188). In this work, the paper was sprayed with 5% sodium carbonate, then with a saturated aqueous solution of the ammonium salt (189). p-Nitrobenzoyl chloride in the presence of a base reacts with N-monosubstituted amino-acids to give colours (190); this has been used for the detection of imino-acids by Sheehan (191). In Table 22, the colours obtained by spraying with p-nitrobenzoyl chloride in benzene, followed by pyridine in benzene are given. The use of 5% aqueous sodium carbonate as the base gave the same colours. Table 22 also includes the colours given by ninhydrin and by isatin (developed at 100-110°).

Table 22.

Colours with spray reagents.

	Ninhydrin	Fluorescence of ninhydrin colour	Isatin	Folin reagent	Sheehan's reagent
4-Hydroxypipecolic	Brown-purple	Dull red	Green	Dark red	Red
5-Hydroxypipecolic	Blue-purple	Bright red	Green	Dark red	Red
Baikiain	Grey-green	Dull red	Pink	--	Red
Pipecolic	Blue-purple	Bright red	Green	Dark red	Red
Nipecotic	Purple	Faint red	No colour	Light red	No colour
Isonipecotic	Purple	Faint red	No colour	Light red	No colour
Proline	Yellow-brown	--	Blue	Light red	No colour
Glycine	Violet	None	Pink	Green	Brown

Attempted preparation of hydrochloride:

(a) A sample of pure 4-hydroxypipecolic acid with an excess of hydrochloric acid was evaporated on a water-bath, but the syrupy residue could not be induced to crystallise.

In other work on 4-hydroxypipecolic acid, there were several occasions in which solutions in hydrochloric acid were concentrated, but crystals were not observed.

(b) 4-Hydroxypipecolic acid (138mg.) was dissolved in a minimum of water at room temperature (0.348g. of

water at 20°C) and hydrogen chloride was passed in with cooling in an ice-bath, until the solution was saturated (weight increase, 0.308g.). No crystals were obtained from the solution at 0°C.

Copper salt: 4-Hydroxypipecolic acid (0.725g.) was boiled in water (50ml.) with copper carbonate (from 2.5g. of copper sulphate pentahydrate; 300% excess) for 1 hour, the solution was filtered while hot and the residue was washed twice with hot water, the second wash being colourless. The deep blue solution was concentrated to 10g. but no crystals were obtained. The solution was then evaporated to a syrup (2.15g.) which gave crystals when rubbed with acetone (0.986g.; 92%). This product was soluble to the extent of 26.2mg. in 49.2mg. of water in a boiling water-bath, and in 173mg. of water at 20°C i.e. about 1:2 in hot and 1:7 at room temperature. The crude copper salt was recrystallised by dissolution in hot water, centrifuging a small amount of insoluble material and allowing the copper salt to crystallise at room temperature for 3 days. The product was washed with water-acetone mixture, then with acetone. When left over anhydrous calcium chloride, the crystals slowly lost weight. A sample, recrystallised from water, and airdried, was obtained as deep blue prisms, m.p. 229° decomp. (Found: N,6.5; CuO,17.2; loss on drying at 90°C., 16.2%. $C_{12}H_{20}O_6N_2Cu.\ 4H_2O$ requires

N,6.6; CuO, 18.7; H_2O, 17.0%).

Attempted preparation of N-acetyl derivative:

(a) 4-Hydroxypipecolic acid (0.36g.) was dissolved with slight warming in acetic acid (5ml.) and acetic anhydride (0.5ml.) was added. After shaking for 2 hours, volatile components were removed under reduced pressure. A partly crystalline syrup remained; this was diluted with ether and filtered. The residue (68mg.) was identified as 4-hydroxypipecolic acid by m.p. (289° decomp.) and mixed m.p. (291° decomp). No crystalline material was obtained from the ether-soluble portion.

(b) 4-Hydroxypipecolic acid (1.45g.) was dissolved in boiling acetic acid (20ml.) and acetic anhydride (1.05g., 1.03 equiv.) was added to the boiling solution. The solution was cooled quickly and evaporated under reduced pressure. The resulting gum crystallised on addition of acetone. The crystals were very soluble in water and were considered to be the amino-acid.

The acetone was removed and the residue was re-dissolved in boiling acetic acid (20ml.), and acetic anhydride (1.65g.) was added to the boiling solution. The solution was allowed to cool and was left for 18 hours at about 20°C before it was evaporated under reduced pressure. The residual syrup when triturated with acetone gave 0.33g. of crystals which were very soluble in water. The acetone-soluble portion

gave no crystals from ethanol-water or from ethyl acetate-acetone.

N-Benzoyl derivative: 4-Hydroxypipecolic acid (1.02g.) in water (10ml.) with sodium hydroxide (2.5ml. of 10N) was cooled in an ice-bath. Benzoyl chloride (1.08g., 1.1 equiv.) was added in portions during 30 minutes with occasional shaking; some yellow gummy material separated. The solution was charcoaled. Acidification with hydrochloric acid (2ml. of 10N) caused the separation of an oil. Light petroleum (about 20ml.) was added and the oil was rubbed with a glass rod to induce crystallisation (seeding was not very effective). The mixture was left at 0° overnight, and the product, N-benzoyl-trans-4-hydroxy-L-(-)-pipecolic acid, was collected; 1.15g. (66%), m.p. 170-171°. Recrystallisation from aqueous ethanol gave clusters of colourless thick needles, m.p. 174°. $[\alpha]_D^{15}$ -54.1° (C, 1 in EtOH). (Found: C,62.8; H,6.1; N,5.6. $C_{13}H_{15}O_4N$ requires C,62.6; H,6.1; N,5.6%).

This preparation was repeated several times, usually on 2g. of 4-hydroxypipecolic acid, and yields ranged from 60 to 77%. It was essential for good yields to allow crystallisation of the crude product to proceed at 0° for some time. The recovery on recrystallisation was low if much ethanol was used; it was desirable to evaporate as much ethanol as possible from the boiling solution after dilution with water.

Crystallisation from aqueous ethanol was often very slow, requiring several days. It was sometimes necessary to wash the crude product several times with light petroleum to remove benzoic acid.

Attempted preparation of the O,N-dibenzoyl derivative by benzoylation of 4-hydroxypipecolic acid with an excess of benzoyl chloride (5 equivalents) [cf. O,N-dibenzoylhydroxyproline(192)] gave only the N-benzoyl derivative (60% yield).

Attempted preparations of N-benzoyl-O-p-toluenesulphonyl-4-hydroxypipecolic acid gave no crystalline product. To N-benzoyl-4-hydroxypipecolic acid (0.249g.) in chloroform (5ml.) with pyridine (0.3ml.), p-toluenesulphonyl chloride (0.381g.) was added. Next day the solution was washed with water, solvents were removed and the residue was washed with light petroleum. The residue did not give a crystalline product; hydrolysis with 6N-hydrochloric acid for 24 hours at 100° and chromatography (BAW, 44 hours) showed 4-hydroxypipecolic acid (21.6cm.), cis-4-hydroxypipecolic acid (19.2cm.) but no baikiain (29.4cm.).

Methylation of N-benzoyl-4-hydroxypipecolic acid with diazomethane gave a product which could not be obtained crystalline. Subsequent reaction with p-toluenesulphonyl chloride in pyridine, at room temperature overnight, gave only a trace of water-insoluble product.

N-p-Toluenesulphonyl derivative:

(a) 4-Hydroxypipecolic acid (0.44g.) in 2.5N-sodium hydroxide (3ml.) was shaken at room temperature with p-toluenesulphonyl chloride (0.57g.) Reaction was very slow and 60 hours shaking was required. The solution was charcoaled, acidified (1ml. of 10N-hydrochloric acid) and the product collected; 0.43g. (47%) pale brown and partly crystalline. This material was very soluble in ethanol, and crystallised from ethanol-benzene in pale brown prisms (0.17g.), m.p. 153-154°.

(b) To a solution of 4-hydroxypipecolic acid (0.58g.) in water (5ml.) with sodium hydroxide (1.2ml. of 10N), cooled in an ice-bath, a solution of p-toluenesulphonyl chloride (0.95g., 1.2 equiv.) in acetone (5ml.) was added. Toluenesulphonyl chloride crystallised from the mixture in fine crystals which redissolved after about 5 minutes shaking. Shaking was continued intermittently for 10 minutes, then the clear solution was acidified (1ml. of 10N-hydrochloric acid). No deposit formed. The solution was extracted with ethyl acetate (five 10ml. portions), the combined extracts were dried (sodium sulphate) and most of the ethyl acetate was distilled. Ether was added to the residue and the turbid solution was filtered. The filtrate did not cyrstallise and was therefore concentrated, and benzene was added. Crystallisation did not occur until most of the ethyl

acetate had been removed, when a pink oil separated, and gave crystals when scratched. Further crystallisation occurred on standing. Yield 0.70g. (58%), m.p. 153-154° with slight sintering at 149°, alone and when mixed with the previous preparation. This crude product was dissolved in hot ethyl acetate, benzene was added and the hot solution filtered from a brown deposit. Crystallisation gave trans-4-hydroxy-N-p-toluenesulphonyl-L-(-)-pipecolic acid (0.40g.) as almost colourless prisms m.p. 162° after slight sintering at 149°. $[\alpha]_D^{19}$ -16.25° (C, 1 in EtOH). The sample for analysis was dried at 100° for 2 hours in vacuo.. (Found: C,51.8; H,5.5; N,4.5; S,10.7. $C_{13}H_{17}O_5NS$ requires C,52.1; H,5.7; N,4.7; S,10.7%).

N-Phenylcarbamoyl derivative: To a cold solution of 4-hydroxypipecolic acid (0.58g.) in 1N-sodium hydroxide (4ml.), phenyl isocyanate (0.6ml., 1.25 equiv.) was added in portions over 10 minutes, with intermittent shaking. Diphenylurea precipitated after the last addition. Acidification gave a thick oil which solidified (0.72g., 68%) and was dissolved in cold sodium carbonate solution (2-3%); re-acidification gave N-phenylcarbamoyl-trans-4-hydroxy-L-(-)-pipecolic acid as very small white crystals (0.48g.) m.p. 181-197° after sintering at 176°. $[\alpha]_D^{26}$ -24.5° (C, 1 in EtOH) unchanged after 24 hours. (Found: C,59.0; H,6.2; N,10.5. $C_{17}H_{16}O_4N_2$ requires C,59.1; H,6.1; N,10.6%).

Phenylhydantoin: The phenylurea (1.49g.) prepared from 4-hydroxypipecolic acid, was dissolved in boiling water and the filtered solution was concentrated on a hot plate (to 53g.). On cooling, (-)-trans-4-hydroxy-3'-phenylpiperidino-(1,2:1',5')-hydantoin crystallised as colourless small prisms (1.05g.) m.p. 204-205° $[\alpha]_D^{23}$ -53.2° (C, 1 in EtOH). (Found: C,63.5; H,5.6; N,11.5. $C_{13}H_{14}O_3N_2$ requires C,63.4; H,5.7; N,11.4%). In a nujol mull (CaF_2 prism) this material showed peaks at 2.88μ (-OH), 5.63μ (w) and 5.85μ (s). The latter peaks may be attributed to the hydantoin carbonyl groups as 4, 3'-dimethylquinoxalino-(1,2:1',5)- hydantoin shows peaks at 5.65μ and 5.85μ (Dr. J. W. Clark-Lewis).

The optical rotation in aqueous alkali was measured. The hydantoin (0.510g.) was dissolved in 4.63ml. of N-sodium hydroxide at room temperature - dissolution was slow and required about 1 hour. The solution was diluted to 10ml. with water. The first observed optical rotation was -1.04°, after 3 hours it was -2.45°, after 24 hours -2.77° at which it remained constant for another 24 hours. The equilibrium reading corresponds to $[\alpha]_D$ -45.4° (C, 6.1 in 2 equiv. of NaOH in water). The acidified solution slowly deposited colourless prisms which were collected after 18 hours at 0° C; 0.34g. m.p.

169-170°. Recrystallisation from ethanol gave the hydantoin m.p. 106°; the mixed mp. was 205-206°.

A solution of the hydantoin (100mg.) in N-sodium hydroxide (10ml.) was prepared at 25°. Dissolution required almost one hour. This solution was kept at 25°C; the optical rotation changed from -0.17° (read 1 hour after the hydantoin was added to the alkali) to -0.30° (1½ hours), -0.325° (2 hours), -0.42° (3½ hours), -0.43° (6½ hours), reaching -0.435° after 23 hours. This corresponds to $[\alpha]_D^{25}$ -43.5° (c, 1 in N-NaOH). The acidified solution very slowly deposited prisms (47mg. after several days), m.p. 205-206°, alone and mixed with the hydantoin.

Derivative with phenyl isothiocyanate: This was prepared by the procedure of Edman (193) but the reaction was carried out at room temperature (194).

A solution of 4-hydroxypipecolic acid (0.725g.) in water (12.5ml.) and pyridine (12.5ml.) was adjusted to pH10 (indicator papers) with N-sodium hydroxide (1.4ml.). Phenyl isothiocyanate (1.2ml.) was added in one portion and the mixture was shaken vigorously. Sodium hydroxide solution was added as required to maintain the pH at 9. No alkali was required after 15 minutes, and the solution was washed with benzene (five times, equal volumes). An emulsion was ob-

tained during the first washing, but later washings gave no difficulty. The aqueous solution was filtered through Kieselguhr to remove a sticky yellow deposit, and hydrochloric acid (4ml. of 1.25N) was added to the filtrate. The granular yellow precipitate was collected, washed with cold water and suspended in 2N-hydrochloric acid (15ml.). The suspension was boiled for 2 hours, and then taken to dryness under reduced pressure. Water was added and removed three times to remove remaining acid. The grey residue gave a purple solution in boiling acetic acid (20ml.); the hot solution was diluted with water until crystals formed. The product was collected next day as brown crystals (0.56g.), m.p. 201-2°. These were dissolved in boiling ethanol (33g.), and the solution was charcoaled and concentrated (to 11g.). Next day, the yellow plates were collected; 0.45g., m.p. 213-214°, raised to m.p. 214° by a second recrystallisation. $[\alpha]_D^{22}$ + 74.0° (C, 0.2 in EtOH, 4dm. tube) (Found: C,60.45; H,4.9; N, 9.6; S,15.6. $C_{20}H_{19}O_2N_3S_2$ requires C,60.4; H,4.8; N,10.6; S,16.1%). The analysis corresponds to the O-phenylthiocarbamate of the phenylthiohydantoin of 4-hydroxypipecolic acid i.e. L-(+)-3'-phenyl-trans-4-phenylthiocarbamoyloxy-2'-thio-piperidino-(1,2:1',5')- hydantoin.

(A trace of the epimer was produced in a neutral solution, but in N-hydrochloric acid, there was no change).

(b) The amino-acid (5mg.) in barium hydroxide solution (0.3ml., saturated at room temperature) was heated in a sealed tube at 155° for 12 hours.

5-Hydroxypipecolic acid mixed with other amino-acids was obtained from dates as decribed by Witkop and Foltz (122). The water-soluble portion of a 70% ethanolic extract of the dates was passed through a cation exchange column and the amino-acids were eluted with 2N-ammonia. The mixture, containing 5-hydroxypipecolic acid, obtained by evaporation, was used without further purification.

Samples of synthetic "allo" (i.e. cis-) 4-hydroxypipecolic acid and cis-3-hydroxypipecolic acid (197) were supplied by Professor H. Vanderhaeghe of Louvain and Dr. H. Plieninger of Heidelberg respectively.

A sample of the hydroxypipecolic acid from Armeria maritima (123) was supplied by Dr. L. Fowden.

Comparison with 5-hydroxypipecolic acid.

(a) In BAW: Chromatograms were run in BAW for 28, 48 and 84 hours. The distances the amino-acids moved were as follows: amino-acid from Acacia, 12.0, 19.1, and 35.5cm.; 5-hydroxypipecolic acid, the same in each case; epimer of the amino-acid

from Acacia, 8.7, 16.6 and 31.3cm.; pipecolic acid, 20.8, 32.2cm. and off the paper.

5-Hydroxypipecolic acid gave a blue-purple colour with ninhydrin and the spot had a bright red fluroescence in UV light; it could be readily distinguished in this way from the amino-acid from Acacia (see Table 22).

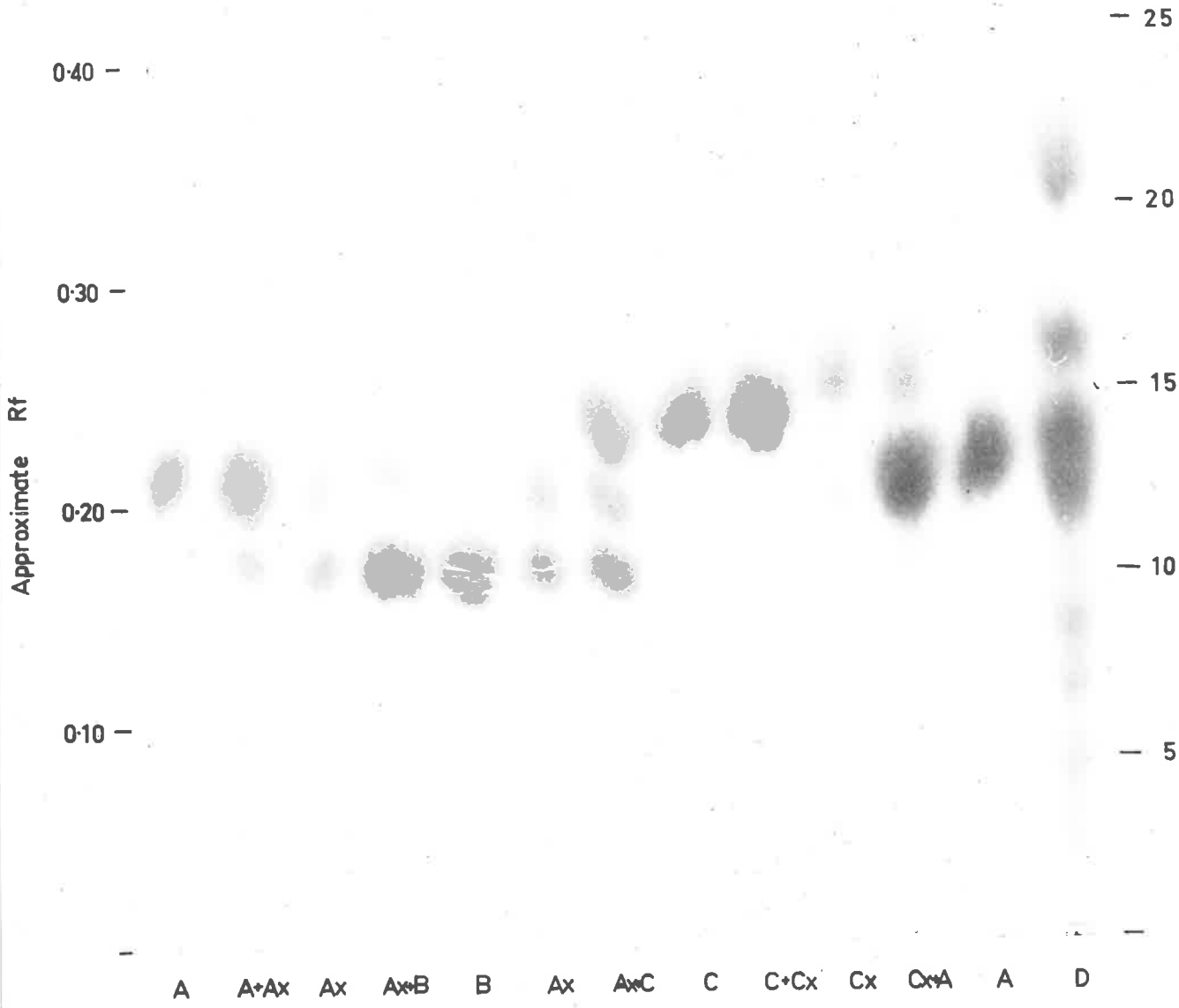
(b) In phenol-water: Chromatograms were run in phenol-water, in an atmosphere containing ammonia and hydrogen cyanide (198) for 12 and 24 hours (27.0 and 45.0cm.). Rf values were as follows: amino-acid from Acacia, 0.55 and 0.47; epimer, identical values; 5-hydroxypipecolic acid, 0.53 and 0.45; pipecolic acid, 0.33 and 0.27.

Comparison with synthetic 3-and 4-hydroxypipecolic acids: Samples of the synthetic 3-and 4-hydroxypipecolic acids were heated in barium hydroxide as for 4-hydroxypipecolic acid from Acacia (above). Chromatograms of the resulting mixtures were run, together with the untreated samples, and mixtures of both with the amino-acid from Acacia and its epimeric mixture. The results of one chromatogram are shown in the photograph (next page).

3-Hydroxypipecolic acid gave a red-purple colour with ninhydrin, but the colour given with ninhydrin

n-BuOH : HOAc : H_2O 4:1:5

36 Hours at 19–21°C



A Aminoacid from Acacia spp.

B cis-4-Hydroxypipecolic acid (Vanderhaeghe)

C cis-3-Hydroxypipecolic acid (Plieninger)

D Aminoacids from dates

x Heated in aqueous $Ba(OH)_2$ 12 hours at 155°C

by synthetic 4-hydroxypipecolic acid and its epimer were identical with that from the amino-acid from Acacia, both in day-light and in UV light. The 4-hydroxypipecolic acids also gave the same green colour with isatin. The synthetic 4-hydroxypipecolic acid gave on the chromatogram a major spot with the same Rf as the epimer of the amino-acid from Acacia from which it was not separated, and a very faint minor spot with the same Rf as the natural amino-acid. After epimerisation of the synthetic acid, the two components had the same Rf as the components of the epimeric mixture from the natural amino-acid, and were not separated from them.

The product from the barium hydroxide treatment of the 3-hydroxypipecolic acid showed several ninhydrin-positive components, together with some unchanged 3-hydroxypipecolic acid.

Comparison with the amino-acid from Armeria maritima: The amino-acid from Armeria maritima (0.8mg.) in barium hydroxide solution (48 µl) was heated in a sealed tube at 155° for 12 hours. The product was chromatographed, together with the epimeric mixture from the amino-acid from Acacia, and the untreated amino-acids, separately and mixed (BAW, 40 hours). In both cases, the natural amino-acids ran 14.5cm., and the epimers, 12.5cm. No separation was apparent in the mixtures. The colours with nin-

hydrin (in daylight and in UV light) and with isatin were identical.

Attempted separation of 4-hydroxy-L-pipecolic acid and cis-4-hydroxy-D-pipecolic acid as the copper salts.

It had been found that a sample of N-benzoyl-4-hydroxypipecolic acid accidentally heated at 150° for 3-4 hours during drying, gave on hydrolysis (24 hours in boiling 6N-hydrochloric acid) a mixture of epimeric 4-hydroxypipecolic acids. To examine this epimerisation further, samples of N-benzoyl-4-hydroxypipecolic acid (about 2mg.) were heated at 200° for 5, 10 and 40 minutes, and then hydrolysed in 6N-hydrochloric acid (0.5ml.) in sealed tubes at 100° for 26 hours. The solutions obtained were evaporated to remove hydrochloric acid, the residue was dissolved in water and chromatographed (BAW, 18 and 60 hours). A sample of unheated N-benzoyl-4-hydroxypipecolic acid, hydrolysed as above, showed only a single spot (4-hydroxypipecolic acid). The samples heated for 5 and 10 minutes gave two spots of the same Rf and ninhydrin-colours as those obtained from alkali-epimerised 4-hydroxypipecolic acid. The sample heated for 40 minutes showed other ninhydrin-positive products.

N-Benzoyl-4-hydroxypipecolic acid (2.49g.) was heated for 5 minutes in an oil-bath at 200° and the residue was boiled in 6N-hydrochloric acid (100ml.)

for 6½ hours. Benzoic acid was removed by extraction with light petroleum, and the aqueous solution was evaporated to dryness under reduced pressure. The residue was dissolved in water and added to a Zeokarb 225 column (50g.) and the amino-acids were eluted with 2N-ammonia. The eluates were evaporated to dryness to remove ammonia, and the residue in water (50ml.) was boiled with copper carbonate (from 3.75g. of copper sulphate pentahydrate; 200% excess) for 1 hour. The solution was filtered while hot, and the residue was washed three times with hot water. The filtrate and washings were concentrated (to 68g.). Deep-blue prisms formed slowly in the cold solution; these were collected and recrystallised from 60ml. of water and then from 30ml. of water. The mother-liquors were concentrated and subsequent crops were recrystallised as shown in Figure 20. The solutions were adjusted to the weights shown, and crystallisation was allowed to proceed for 6 or 7 days at each stage. There were thus obtained four crops of crystals; (a), (b), (c) and (d), and the residue (e). The weights of each crop (airdried) are given in Figure 20.

These crops were examined by suspending a portion (10mg.) in water (1ml.) and passing hydrogen sulphide through the hot suspension. The copper-free

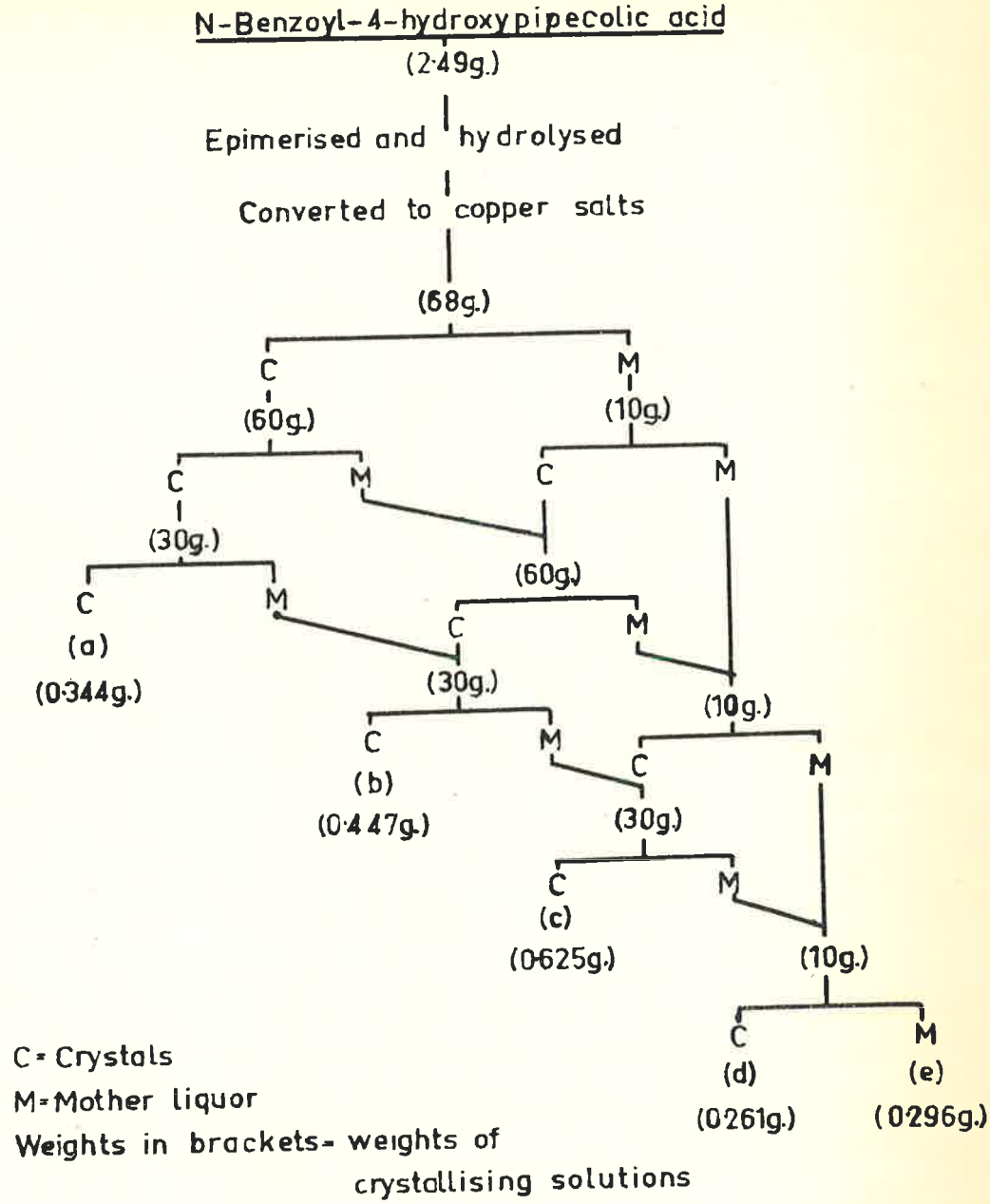


Figure 20 : Attempted separation of natural 4-hydroxypipecolic acid and its C_2-epimer as the copper salts.

solutions were chromatographed (BAW, 36 hours). All crops showed spots of the same Rf as 4-hydroxypipecolic acid (20.5cm.) and its epimer (18.1cm.) in approximately equal quantities.

The first crop of crystals, fraction (a) was analysed. (Found: N,6.7; CuO17.0. $C_{12}H_{20}O_6N_2Cu.4H_2O$ requires N,6.6; CuO, 18.7%). These crystals did not lose weight when left over anhydrous calcium chloride.

Action of hydriodic acid and red phosphorus on 4-hydroxypipecolic acid.

(a) 4-Hydroxypipecolic acid (51mg.) was heated in a sealed tube with red phosphorus (23mg.) and hydriodic acid (1ml. of S.G.1.94) at 143° for 6 hours. The colourless solution was evaporated under reduced pressure to a thick syrup and then chromatographed (BAW). A strong pipecolic acid spot was present, together with other ninhydrin-positive components. For estimation of the amount, the reduction mixture was diluted to 5ml. in water, and 5, 10, 15, 20 and 25 μl samples were chromatographed, together with the same amounts of 1% pipecolic acid.hydrochloride. The pipecolic acid spot from 5 μl. of standard solution had an intensity and size similar to that from 25 μl of the reduction product. The reduction product therefore contained about 7.6mg. of pipecolic acid (16% yield).

(b) 4-Hydroxypipecolic acid (19.3mg.) was heated in a sealed tube with red phosphorus (3.5mg.) and hydriodic acid (0.2ml. of S.G.1.94) at 145° for 9 hours. The clear solution was evaporated with added water on a water-bath and left over sodium hydroxide overnight. The residue was dissolved in water (1ml.) and treated with freshly-prepared silver carbonate until no further reaction occurred; the silver salts were separated at the centrifuge. Half the resulting solution was hydrogenated at room temperature and pressure for 3 hours with platinum (freshly-prepared from platinum oxide).

Chromatograms (BAW, 19 hours, 35cm.) showed the following components in the solution not hydrogenated: Rf 0.37, brown with ninhydrin, pink changing to silver with isatin; Rf 0.30, green with ninhydrin, and a pink colour, very faint, with isatin; Rf 0.16, 4-hydroxypipecolic acid (trace) and in the hydrogenated solution: Rf 0.49, purple with ninhydrin, not fluorescing in UV light, pink with isatin; Rf 0.33, blue-purple with ninhydrin, fluorescing bright red in UV light (pipecolic acid); Rf 0.16, 4-hydroxypipecolic acid; Rf 0.10, purple with ninhydrin, not fluorescing in UV light, pink with isatin (minor component). On the same chromatogram, 4-hydroxypipecolic acid had Rf 0.16, pipecolic acid Rf 0.33

and baikiain Rf 0.28.

The spot at Rf 0.49 in the chromatogram of the hydrogenated solution was very strong. It appeared from its Rf value, its ninhydrin colour and its isatin colour, to be an α-amino-acid of about 6 carbon atoms. A chromatogram (BAW, 15 hours) gave the following Rf values: unknown, 0.45; pipecolic acid, 0.33; α-aminobutryic acid, 0.29; leucine, 0.54; isoleucine, 0.54. A chromatogram run on paper buffered with M/15 phosphate buffer pH7.5, run in benzyl alcohol: n-butanol 1:1 (199,200) gave the following Rf values (14 hours run): unknown, 0.13; pipecolic acid, 0.15; leucine, 0.24; isoleucine, 0.21: valine, 0.10; 4-hydroxypipecolic acid, 0.04. When run from the same spot, the unknown was separated from valine. A chromatogram run in the same system as the previous one, for 30 hours, gave incomplete separation of valine and 2-aminopentanoic acid ("norvaline") (valine, 6.7 to 10.1cm; 2-aminopentanoic acid, 6.0 to 9.2cm.). The unknown amino-acid gave a single spot when run with 2-aminopentanoic acid. The identity of the unknown as 2-aminopentanoic acid was confirmed by a chromatogram in BAW (20 hours, 31.4cm.) which gave the following Rf values: unknown, 0.45; 2-aminopentanoic acid, 0.45; valine, 0.39. Mixtures of the unknown and 2-aminopentanoic acid gave

a single spot, Rf 0.39 while mixutes of valine and 2-aminopentanoic acid were almost completely separated into 2 spots.

(c) Isolation of pipecolic acid: Four sealed tubes, each containing 4-hydroxypipecolic acid (0.5g.), red phosphorus (80mg.) and hydriodic acid (5ml. of S.G.1.94) were heated at 150° for 12 hours. Two tubes were completely clear, but two contained brown insoluble material. Chromatography (BAW, 12 hours, 24cm.) showed major spots at Rf 0.37 (pipecolic acid) and at 0.48 and 0.56. The last two gave with ninhydrin, brown colours slowly changing to purple. A small portion of the reaction product left in contact with excess of ammonia for 30 minutes gave a similar chromatogram. The solutions were evaporated to remove hydriodic acid, and passed through a Zeokarb 225 column (50g.). The amino-acids were eluted with ammonia; the compounds of high Rf were not detected in the product, which contained pipecolic acid and several ninhydrin-positive components of lower Rf.

The crude amino-acid mixture (2.26g.) in water (10ml.) and hydrochloric acid (1.5ml. of 10N) was treated with hydrochloric acid (1.5ml. of 10N) and sodium nitrite (2.5ml. of 6M). An immediate copious precipitate of iodine formed. Subsequent additions of acid and nitrite (3 lots) gave no further precipi-

tate of iodine. The nitrosated solution was continuously extracted with ether for 72 hours, the extract was heated in dilute hydrochloric acid to decompose N-nitroso compounds, and the solution, after excess acid had been removed by evaporation, was added to the Zeokarb 225 column. The imino-acids were eluted with ammonia; 0.77g. A chromatogram showed, apart from pipecolic acid, only traces of ninhydrin-positive substances. Attempts to crystallise the free amino-acid failed. The mixture was acidified with hydrochloric acid, and, after evaporation to dryness, gave from aqueous acetone, 0.26g. of crystals m.p. 183-192°. The mother liquors contained 0.56g. of material, which contained much pipecolic acid. The crystals and residue were combined, and added to the Zeokarb 225 column, which was eluted as follows: 500ml. of 0.5N-HCl, 250ml. of N-HCl, 250ml. of water, 500ml. of 2N-ammonia. Fractions of about 70ml. were collected and evaporated to dryness and the residues were examined by chromatography (BAW). Pipecolic acid was present in the later 0.5N-HCl eluates, the N-HCl eluates and the initial ammonia eluates. Fractions which contained other ninhydrin-positive components were returned to the column and eluted with a more gradual increase in acid strength. The combined pipecolic acid fractions were evaporated under reduced pressure to dryness, and the crystalline

residue was recrystallised from a small amount of ethanol. This gave 0.22g. (8.5%) of pipecolic acid hydrochloride, m.p. 256-258° $[\alpha]_D$ +0.3° (c, 5.7 in water).

(d) Identification of baikiain and 2-aminopent-4-enoic acid: Sealed tubes containing 4-hydroxypipecolic acid (20mg.), red phosphorus (7mg.), hydriodic acid (0.20ml. of S.G.1.94) and various quantities of water, were heated at 145° for 12 hours. The resulting colourless solutions were evaporated under reduced pressure, and the residues were taken up in water (2ml.) and chromatographed (BAW, 16 hours, 37cm.), with the results given in Table 23. On this chromatogram, pipecolic acid had Rf 0.36 and 4-hydroxypipecolic acid, Rf 0.20. The identity of these compounds in the reaction mixtures was confirmed by their ninhydrin and isatin colours: the components Rf 0.38 and 0.52 gave purple colours with ninhydrin, and with isatin a faint pink and a slightly blue green colour respectively.

Reaction mixture 2 was treated with an excess of fresh silver carbonate and centrifuged. Chromatography (BAW, 15 hours, 39cm.) showed the following components: Rf 0.57, colours as before, in traces only; Rf 0.37, purple colour with ninhydrin (α-amino-acid colour, not pipecolic acid colour), and

a purple fading to pink with isatin; Rf 0.31, a grey-green with ninhydrin and pink with isatin, Some minor components of Rf 0.19 and less were present both before and after the silver carbonate treatment. No pipecolic acid was detected after treatment with silver carbonate, apparently being obscured by the component Rf 0.37.

Table 23.

Reaction of 4-hydroxypipecolic acid with red phosphorus and hydriodic acids of various strengths.

Mixture No.	Added water (ml).	Calculated % of HI	Appearance after heating	Chromatography results Rf0.36	Rf0.20	Rf0.58	Rf0.52
1	nil	66	Colourless crystals	++	nil	++	+++
2	0.20	63	Colourless solution	++	trace	++	+++
3	0.50	59	Some phosphorus	+	++	++	++
4	0.100	53	Much phosphorus	trace	+++	trace	trace
5	0.200	44	Much phosphorus	trace	+++	nil	nil

When colour development was carried out at 80°, the component of Rf 0.37 gave with ninhydrin a yellow-brown colour slowly changing to purple, and with isatin, a purple colour fading to pink, and the component of Rf 0.31, purple with ninhydrin and no colour with isatin, except in the presence of much acetic

acid (10% in the spray) when it gave a pink colour.

The nature of the components Rf 0.58 and 0.51 of the reduction mixture was examined further: when sprayed with neutral silver nitrate, the two spots gave yellow-brown colours within about 5 minutes in the cold, that with Rf0.56 being the more intense. An area of Rf 0.11-0.16 also slowly darkened. With a starch spray no components were detected, but after a subsequent spray with nitrous acid, an area of Rf 0.10 to 0.20 turned blue.

The silver carbonate-treated reaction mixture was chromatographed (BAW, 44 hours) together with baikiain and 2-aminopent-4-enoic acid ("allyl-glycine"), both alone and mixed with the reaction mixture, and with pipecolic acid as standard. The chromatogram was sprayed with ninhydrin and heated at 80-90°. Pipecolic acid moved 34.2cm. Baikiain ran 29.4cm., the same distance as a component (of Rf 0.31) in the reaction mixture; baikiain was not separated from this chromatogram in a mixture, and both gave the same green colour with ninhydrin, and a whitish-pink with isatin. 2-Aminopentenoic acid ran 33.8cm., and under these conditions gave a yellow-green with ninhydrin. A component (of Rf 0.37) in the reaction mixture ran the same distance, and was not separated from 2-aminopentenoic acid when mixed; it also

gave a yellow-green colour with ninhydrin. (It has been reported that the ninhydrin colour of 2-aminopent-4-enoic acid is a yellow-green changing to a violet (204)). Repetition of this chromatogram gave similar results.

The same samples were chromatographed with benzyl alcohol:n-butanol 1:1 on paper buffered with M/15 phosphate buffer pH7.5 (as in section (b) above) for 40 hours. The amino-acids were detected with ninhydrin and isatin sprays containing much acetic acid, followed by heating at 110°. The very distinctive colours obtained, and the distances the components moved, are given in Table 24. (Pipecolic acid has Rf 0.16 in this system).

Table 24.

Results of chromatography in benzyl alcohol:butanol.

Component	Distance	Ninhydrin colour	Isatin colour
Pipecolic acid	15.0cm.	Bright blue-violet	Green
Baikiain	10.7	Bright yellow	Brick red
2-Amino-pent-4-enoic acid	7.7	Brown (grey edges)	White
Proline	6.0	Orange	Blue
4-Hydroxy-pipecolic	3.7	Grey-brown	Green
5-Hydroxy-pipecolic	3.5	Yellow (grey edges)	Green

The compounds present in the reaction mixture being examined (namely pipecolic acid, baikiain, 2-aminopent-4-enoic acid and 4-hydroxypipecolic acid)

had the same colours as the reference samples, and ran the same distances. When mixed with authentic samples of baikiain and of 2-amino-pent-4-enoic acid, there was no separation from the components originally present in the reaction mixture.

Decarboxylation of 4-hydroxypipecolic acid.

Powdered 4-hydroxypipecolic acid (2g.) in redistilled acetophenone (8ml.) was stirred and heated at 150° (oil bath) for 1½ hours under nitrogen. There was no apparent reaction. The temperature was then raised to 190°. After 35 minutes, almost all of the amino-acid had disappeared. A few crystals remained after 1 hour. Heating was discontinued after 1½ hours (total). The mixture was cooled, diluted with ether (50ml.), and extracted with 2N-hydrochloric acid (one 10ml., two 5ml. portions.). The acid extracts were washed with ether (20ml.) and evaporated under reduced pressure, which left a thick syrup (2.5g.). To this syrup, water (2ml.) and potassium hydroxide pellets (2g.) were added, and the mixture was extracted with ether (20ml., then 10ml.). Ether was removed from the extracts, and the residue was distilled in a small still. Distillate first appeared above the oil bath at the bath temperature 219° (772mm). The distillate (0.52g, 37%) crystallised almost completely, and a portion washed

with dry ether showed m.p. 55-65° in a capillary. On a hot stage under a microscope, it was observed to undergo a change of crystalline form at 57-60°, forming needles melting at 85-87°. A mixed m.p. (hot stage) with synthetic piperidin-4-ol was 85°. (The synthetic sample showed, on a hot stage, a change of crystalline form at 59-60°, and melted at 85°). The IR spectrum of the decarboxylation product coincided with that of synthetic piperidin-4-ol except that a small peak at 6.3 μ was absent.

A portion of the decarboxylation product was converted into the N-p-toluenesulphonyl derivative, as described below for synthetic N-p-toluenesulphonylpiperidin-4-ol. The product was obtained as white needles m.p. 114-115° (capillary) after one recrystallisation from ethanol-water; mixed m.p. with authentic N-p-toluenesulphonylpiperidin-4-ol, 115-116°.

Piperidin-4-ol: Epichlorohydrin was converted into 1,3-dichloropropan-2-ol with concentrated hydrochloric acid, as described by Hill and Fischer (202). From 300g. of commercial epichlorohydrin, 315g. (75%) of 1,3-dichloropropan-2-ol b.p. 175-180° (mainly 177-178°) was obtained. 1,3-Dicyanopropan-2-ol was prepared from this as described by Bowden and Green (203) i.e. by reaction with aqueous sodium

cyanide at 50-55°. Two batches of 65g. of dichloropropanol yielded a total of 43.0g. of redistilled dicyanopropanol b.p. 131-135° at 0.05mm (39% yield). This boiling point is much lower than that quoted by Bowden and Green (145-155° at 0.02 to 0.05mm). The crude dicyanopropanol distilled at 0.20mm., without decomposition (b.p. about 150°) and left a considerable residue, which decomposed when heated to about 190°. No decomposition occurred during redistillation of the dicyanopropanol, and very little residue remained.

Hydrogenation of the dicyanopropanol was carried out as described by Bowden and Green (203) with Raney nickel in ethanol. At 50 atmospheres there was no uptake of hydrogen at room temperature, but hydrogenation proceeded readily on warming. The product (from 43g. of dicyanopropanol) was distilled and gave 19.0g. of product, b.p. 106° at 14mm. to 138° at 13mm.. This material crystallised almost completely, and redistillation gave 10.1g. (27%) of colourless crystalline piperidin-4-ol, b.p. 114-118° at 13mm.

The N-p-toluenesulphonyl derivative was prepared from piperidin-4-ol (0.194g.) in water (5ml.) and sodium hydroxide (0.5ml. of 10N) with p-toluenesulphonyl chloride (0.42g., 10% excess), added in acetone (2ml.). The mixture was boiled gently for

30 minutes and cooled. The oil which had separated then solidified (0.428g.). It crystallised very slowly as needles from ethanol-water, and the product had m.p. 123-124°, after sintering at 115° (capillary). Recrystallisation gave a product m.p. 125-126° with a change of crystalline form at 117° (hot stage). Another recrystallisation gave white needles, m.p.115-116° (capillary), sometimes re-solidifying and melting again at 125°. Lit.(204), m.p. 131-132° from benzene.

Reaction of periodate with pipecolic and 4-hydroxy-pipecolic acids.

(a) In unbuffered solution: To 0.15 millimol. of amino-acid in water, 25ml. of potassium periodate (approx. 0.02M) was added, the solution was diluted to 50ml. and left in the dark at room temperature (22-23°C). Five ml. aliquots were withdrawn at intervals and added to a mixture of 5.0ml. of standard 0.1N-arsenite (accurately measured) and about 10ml. of 10% sodium bicarbonate. After an interval of 10-15 minutes, 1ml. of 20% potassium iodide in 10% sodium bicarbonate solution, was added, and the solution was titrated with standard 0.1N-iodine and starch as indicator.

Pipecolic, nipecotic and isonipecotic acids were added as their hydrochlorides, together with 1 equivalent of sodium hydroxide. The final pH of the

pipecolic acid solution was 3.8 compared with 6.0 for 4-hydroxypipecolic acid.

Aliquots were titrated after 30 minutes, 2 hours, 8 hours and 24 hours. The following compounds gave titres not significantly different from the blanks run simultaneously (i.e. less than 0.1mol. of periodate per mol. of amino-acid): glycine, pipecolic, nipecotic isonipecotic and 4-hydroxypipecolic acids. Serine consumed periodate as follows: 30 minutes, 1.5mol. of periodate per mol. of serine; 2 hours, 1.8; 8 hours, 1.8; 24 hours, 1.8.

(b) In alkaline solution: A 0.05M sodium borate solution was prepared (standard pH9.18 at 25°C). To 50ml. portions, N-sodium hydroxide was added, and the pH measured, with the following results: 1.25ml. of alkali, pH9.42; 2.5ml., 9.65; 5.0ml., 10.23.

The above titration was repeated, using in each reaction mixture, 20ml. of a solution prepared by mixing 10 volumes of 0.05M-borate with 1 volume of N-sodium hydroxide. The following pH values were obtained at the end of the 24-hour reaction period: blank, pH9.77; pipecolic acid solution, 9.62; 4-hydroxypipecolic acid solution, 9.70. The reaction temperature was 19-21°.

The following compounds gave titres not differing significantly from the blanks in 24 hours; glycine,

pipecolic, nipecotic, isonipecotic and 4-hydroxypipecolic acids. Serine consumed periodate as follows: 30 minutes, 0.93 mol. of periodate per mol. of amino-acid; 2 hours, 1.1; 8 hours, 1.2; 24 hours, 1.5.

(c) Proline and pipecolic acid: The reactions of proline and pipecolic acid with periodate in acid, neutral and alkaline solutions were compared. Proline (about 0.15 millimol.) was used as the free amino-acid; an acidic solution was obtained by adding 1 equivalent of sulphuric acid. Pipecolic acid was used as the hydrochloride; an attempt was made to obtain a neutral solution by adding 1 equivalent of standard sodium hydroxide. Alkaline solutions were obtained with the borate-hydroxide mixture as in (b). Blanks were run in neutral and alkaline solutions. The pH of each solution was measured at the end of the 24 hour reaction period.

The results are shown in Table 25. The blanks used had pH6.8 and 11.3. The titre for pipecolic acid at pH2.9 after 24 hours differed from the blank by 0.08ml.; titres were reproducible to within about 0.03ml.

The acidic proline solution had a strong amine odour at the end of the 24 hour period.

Table 25.

Reaction of periodate with proline and pipecolic acid. (mol. periodate consumed per mol. amino-acid).

	Final pH	2 hours	8 hours	24 hours
Proline	3.9	0.2	0.6	0.8
	6.8	0.2	0.7	1.3
	11.3	0.1	0.1	0.2
Pipecolic acid	2.9	0	0	0.2
	4.3	0	0	0.1
	11.5	0	0	0.1

The reaction of proline and pipecolic acid with periodate in the presence of acetaldehyde (1 and 5 equivalents), was investigated; the acetaldhyde had no influence on the consumption of periodate over 24 hours.

(d) Reaction over 8 days: Solutions of about 0.6 millimol. of pipecolic and 4-hydroxypipecolic acid in 100ml. of 0.04M-periodate were preapred. The solutions of pipecolic acid hydrochloride contained 1 equivalent of sodium hydroxide (initial pH, 3.2), no addition (pH2.5) and 1 equivalent of sulphuric acid (pH2.2). The 4-hydroxypipecolic acid solutions contained no addition (pH6.2) and 1 equivalent of of sulphuric acid (pH2.3). Blanks run simultaneously contained 0.5 and 1.0ml. of 0.1N-sulphuric acid.

The mixtures were left in the dark at room temperature, which varied from 10 to 20°C. Aliquots (5ml.) were titrated after 1, 2, 3, 4 and 8 days. No significant reaction was detected after 4 days. The results after 8 days are given in Table 26.

Table 26.

Reaction of periodate with pipecolic acid and 4-hydroxypipecolic acid in 8 days.

	pH	Concentration	Titre	Consumption
Pipecolic acid	3.2	0.56	3.55ml.	0.8
	2.5	0.60	3.48	0.5
	2.2	0.63	3.45	0.4
4-Hydroxypipecolic	6.2	0.54	3.65	1.1
	2.3	0.53	3.52	0.7

Concentrations given as millimol. per 100ml.

Titre given as ml. 0.1N-iodine per 5ml. aliquot (blank, 3.33ml.)

Consumption given as mol. periodate per mol. amino-acid.

Oxidation of 4-hydroxypipecolic acid to 4-oxopipecolic acid.

An 8N solution of chromic acid in dilute sulphuric acid was prepared according to Djerassi, Engle and Bowers (205) i.e. 26.72g. of chromium trioxide and 23ml. of concentrated sulphuric acid diluted to 100ml. with water. This solution was used throughout the following work. The acetic acid used was purified

over chromium trioxide.

The 4-hydroxypipecolic acid used in this series of experiments contained between 2 and 4% of pipecolic acid.

(a) Preliminary investigations: It was found that oxidation of 4-hydroxypipecolic acid in water with chromium trioxide was very slow at 15°, and at 100°, oxidation was too general, e.g. although reduction of 4 equivalents of chromium trioxide was almost complete in 4 hours, much 4-hydroxypipecolic acid remained. In acetic acid at 100°, oxidation of 4-hydroxypipecolic acid was rapid, none being detectable after 5 minutes with 4 equivalents of chromium trioxide. In acetic acid at room temperature (about 20°), oxidation with 4 equivalents of chromium trioxide for 1 hour resulted in oxidation of all of the 4-hydroxypipecolic acid; with 2 equivalents of oxidant, some 4-hydroxypipecolic acid remained after 12 hours.

The oxidation product was detected on chromatograms (BAW) as a spot of slightly lower Rf than 4-hydroxypipecolic acid which gave a yellow colour with ninhydrin; reduction at pH9 with excess of sodium borohydride resulted in the removal of this component and the formation of cis-4-hydroxypipecolic acid. (As the solution of keto-acid contained some 4-hydroxypipecolic acid, it was not possible to determine if the reaction also gave the natural epimer).

(b) Small preparative oxidation: To a solution of 4-hydroxypipecolic acid (0.29g.) in acetic acid (20ml.), prepared by warming to about 50°C, 1ml. of 8N-chromium trioxide (4 equiv.) was added. The temperature, initially 20°, rose to 30.5° in 10 minutes, then slowly fell. After 90 minutes, methanol (0.2ml.) was added. Next day, the mixture was filtered and the chromium salts were washed with acetic acid; the solution and washings were evaporated under reduced pressure and left a clear residue (0.30g.).

In order to remove acidic oxidation products, (c.f.206), the crude product was added, in water, to a column of Permutit Deacidite FF (34g. wet weight; 28 x 1.2cm.) in the acetate form, and washed through with water. Paper chromatograms of the eluate fractions showed the presence of 4-oxo-pipecolic acid and a trace of pipecolic acid, but no 4-hydroxypipecolic acid; no other ninhydrin-positive substances were present. Subsequent elution of the column with 0.5%, 1% and 2% aqueous acetic acid gave no further material.

The purified product was evaporated under reduced pressure to a syrup which crystallised on the addition of dioxan. Attempts were made to recrystallise this product from water-dioxan, but it was difficult to obtain the right proportions with the small amount of product (50-100mg.).

(c) Preparative scale. Isolation of the free keto-acid: 4-Hydroxypipecolic acid (2.18g.) was dissolved in acetic acid (150ml.) by warming to 45°, and the solution was cooled to 20° before addition of chromium trioxide (7.5ml. of 8N; 4 equiv.). The temperature was controlled by cooling in an ice-bath so that it did not exceed 22°C. After 1½ hours, the solution was decanted from the precipitate of chromium salts, and methanol (3ml.) was added. After 1 hour (which proved insufficient for complete reduction of the remaining chromium trioxide), the solution was filtered and evaporated under reduced pressure to a syrup (1.9g.), which was dissolved in water (50ml.) and added to the Deacidite FF column (as in (b)) in the acetate form, and washed through with water. The first 150ml. of effluent contained the keto-acid; this slightly purple solution was evaporated under reduced pressure to a foam (0.79g.) which crystallised on the addition of a few drops of water. The product crystallised from water-isopropanol in small almost colourless needles (0.41g.). A slightly acidic aqueous solution was not decolourised by carbon. Recrystallisation from an aqueous syrup gave small needles (0.134g.) which, on heating began to develop a brown colour at 145°, sintered to 155° and foamed at 247°. A sample dried at 60° over P_2O_5 at

atmospheric pressure for 4 hours gave an unsatisfactory analysis. (Found: C,43.8; H,6.9; N,8.3. $C_6H_9NO_3.H_2O$ requires C,44.7; H,6.9; N,8.7%).

An aqueous solution of this product was optically active; the optical rotation of a 1% solution at 15° was - 0.097°.

(d) Preparative scale. Investigation of isolation procedure: The oxidation was carried out as in (c); the temperature was allowed to reach 26°C. The reaction mixture was left overnight after the addition of methanol, filtered and evaporated under reduced pressure. The residue was dissolved in water (200ml.) and, while boiling, treated with an excess of barium carbonate. The filtered solution was paseed through the Deacidite FF column (as in (b)) in the acetate form. The first portion of the effluent, which contained the keto-acid, was evaporated under reduced preasure to a syrup (4.7g.). This contained much chromium; in an attempt to remove this, the product was dissolved in hot acetic acid (15ml.) and concentrated sulphuric acid (1ml.) was added. Crystals formed slowly over a period of days and the solution became almost completely solid; filtration was impracticable. The mixture was redissolved in water and passed through.the Deacidite FF column (in acetate form) to remove sulphuric acid, and the eluate was evaporated with several additions of

water to remove as much acetic acid as possible.

The residue, in water (50ml.), was made slightly alkaline with sodium carbonate (2.65g.) and a solution of sodium borohydride (0.85g.) in water added in portions over 15 minutes; the mixture was left overnight. An attempt was made to obtain a copper salt of cis-4-hydroxypipecolic acid by adjusting the solution to pH5 with acetic acid and adding copper acetate (4g.). No product was obtained.

The solution was strongly acidified with hydrochloric acid and evaporated. The residue was extracted with ethanol. The green extract was evaporated, and the residue, in water, was treated with hydrogen sulphide until no further precipitation of copper sulphide was obtained; the solution was green.

The solution was added to a Zeokarb 225 column (50g.) in the acid form, the column was washed with water, and then the amino-acid was eluted with ammonia. The first fractions contained chromium, but the amino-acid remained on the column and was eluted by the ammonia. At this stage it had been observed (next experiment) that the keto-acid could be eluted from Zeokarb 225 with dilute hydrochloric acid. The amino-acid fraction was returned to the column, and eluted as follows: 1l. of 0.1N-HCl, 250ml. of 0.2N-HCl, 250ml. of 0.4N-HCl, 250ml. of 0.8N-HCl, 250ml. of water, 500ml. of 3N-ammonia.

Fractions (about 70ml.) were collected and evaporated to dryness, and the residues were taken up in 1-2 ml. of water. Paper chromatography (BAW, 40 hours) showed that both epimers of 4-hydroxypipecolic acid were present in the earlier fractions (0.1 to 0.2N-HCl), and later fractions (0.4-0.8N-HCl) also contained traces of pipecolic acid. The hydroxypipecolic-acid fractions gave a crystalline residue (0.81g.) on evaporation. This was crystallised from water-ethanol, but only sodium chloride was obtained (0.31g.). The mother liquors were treated with dinitrofluoro-benzene (0.50g.) in water (5ml.) and ethanol (5ml.) with sodium bicarbonate (1g.), and the N-2,4-dinitro-phenyl derivative isolated in the usual manner (195). The crystalline product was recrystallised from ethanol-water as small yellow prisms (0.27g.) m.p. 134°, alone and mixed with N-2,4-dinitrophenyl-cis-4-hydroxy-L-pipecolic acid (see later).

(e) Preparative scale. Isolation as the hydro-chloride: 4-Hydroxypipecolic acid (2.18g.) was dissolved in a mixture of acetic acid (150ml.), concentrated sulphuric acid (1.73ml.) and water (5ml.). Dissolution was rapid at room temperature. Chromium trioxide (7.5ml. of 8N) was added and the temperature was kept below 20° by cooling at intervals during the first 20 minutes. After 1½ hours, methanol (5ml.)

was added and the mixture was left overnight. The solution was filtered, diluted with water (500ml.), and, while boiling, treated with barium carbonate. This solution was filtered, and freed from excess barium with sulphuric acid. The solution was then evaporated under reduced pressure to a syrup; and passed through a column of Deacidite FF (as in (b)) in the acetate form. The initial eluates, containing the keto-acid, were evaporated under reduced pressure to a syrup (4.7g.) which did not crystallise from water-isopropanol.

The crude product was added to a Zeokarb 225 column (50g.) and eluted with dilute hydrochloric acid. Two passages were required to obtain a chromatographically pure keto-acid fraction. The more successful series of eluants was as follows: 1l. of 0.1N-HCl, 250ml. of 0.2N-HCl, 250ml. of 0.4N-HCl, 250ml. of 0.8N-HCl, 250ml. of water, 500ml. of 3N-ammonia. Some chromium passed through the solution without much retardation, but some was eluted by 0.4N-HCl. The keto-acid was eluted by 0.1N-HCl, with traces in later fractions. A ninhydrin-positive component, Rf 0.29 in BAW (pipecolic acid, Rf 0.38; keto-acid, 0.23; 4-hydroxypipecolic acid, 0.24) was eluted by 0.2N-HCl, and pipecolic acid partly by 0.4N-HCl with some remaining, to be removed by the ammonia.

The keto-acid fractions crystallised on evaporation; these fractions were collected and the keto-acid hydrochloride was crystallised from water-isopropanol in prisms (0.41g.) which became brown at 150° and foamed at 195°. Recrystallisation from water-isopropanol gave 4-oxo-L-pipecolic acid hydrochloride as prisms which turned brown at 195° and foamed at 204°.

The other ninhydrin-positive product was identified as β-alanine by paper chromatography (BAW, 36 hours). The unknown was not separated from a sample of β-alanine, and the colours given by β-alanine with ninhydrin (blue-violet) and with isatin (light-blue) were identical with the colours given by the unknown.

(f) Large preparative oxidation: Oxidation of 4-hydroxypipecolic acid (8.72g.) was carried out in four batches of the same size and under the same conditions as in (e). After reduction of the excess of chromium trioxide with methanol, the solutions were decanted from the chromium salts (which were examined separately) and evaporated under reduced pressure to about 40ml. and, after dilution with water, treated with barium carbonate to remove sulphate; excess barium was removed with sulphuric acid.

The solution was added to a Zeokarb 225 column

(200g., 53 x 2.5cm.) in the acid form, and eluted with dilute hydrochloric acid. The eluate was collected in 250ml. fractions. The following series of eluants was used: fraction 1, feed solution; 2, 250ml. of water; 3-10, 2l. of 0.1N-HCl; 11-12, 500ml. of 0.2N-HCl; 13-16, 1l. of 0.4N-HCl; 17-20, 1l. of 0.8N-HCl; 21-22, 500ml. of water; 23-27, 1l. of 3N-ammonia. The fractions were evaporated on a water bath, and the residues were redissolved in water and examined by chromatography (BAW, 12 hours, 27.4cm.). Chromium was present in fractions 1-6 and 17-20; the keto acid which gave an elongated spot Rf 0.20, was present in fractions 11-16; β-alanine, Rf 0.24, was present in fractions 16-20; pipecolic acid, Rf 0.35, was present in fractions 19-20. The first ammoniacal eluate (fraction 24) contained some of each amino-acid, especially pipecolic acid. The mixtures of amino-acids which occurred in some fractions were separated on a 50g. Zeokarb 225 column. The keto-acid fractions were combined and evaporated to a thin syrup under reduced pressure. The cooled syrup gave 4-oxo-L-pipecolic acid hydrochloride monohydrate as small white needles (1.28g.) foaming at 203° $[\alpha]_D^{21}$ + 3.8° (C, 2 in water). An airdried sample had a loss on drying at 80° of 8.0%. $C_6H_9O_3N.HCl.H_2O$ requires H_2O, 9.1%. A sample dried for 6 hours in vacuo was analysed.

(Found: C,39.9; H,5.65; N,7.3; Cl,19.4. $C_6H_9O_3N.HCl$ requires C,40.1; H,5.6; N,7.8; Cl, 19.7%).

Addition of isopropanol to the mother liquors gave further crops: 2.35g. of pink needles, foaming at 203° and 0.29g. of brown crystals, foaming at 198°. The mother liquors on evaporation gave brown crystals (0.85g.). The yield of pure hydrochloride was 32%.

The β-alanine fractions from the above isolation gave on evaporation a crystalline residue (0.59g.), which was dissolved in water and sodium hydroxide (9.6ml. of 1N), and the cooled solution was treated with phenyl isocyanate (about 0.75g.) until diphenylurea was precipitated. The filtered solution gave, when acidified, crystals (0.27g.), m.p. 146-157°. These were suspended in 5% sodium carbonate solution. (The insoluble portion was recrystallised from water to give pipecolic acid phenylhydantoin (17mg.), m.p. 155-157°, identified by mixed m.p. with an authentic sample.). The sodium carbonate solution was acidified, and gave N-phenylcarbamoyl-β-alanine (0.17g.), m.p. 164-169°, mixed m.p, with a pure sample, 170-172°.

N-Phenylcarbamoyl-β-alanine was prepared from β-alanine (0.356g.) in 0.5N-sodium hydroxide (8ml.) with phenylisocyanate (0.60g., 25% excess); 0.460g. (55%), m.p. 171-172°. Recrystallisation

from water gave feathery crystals, changing in 24 hours to large blades, m.p. 173-174°. Lit., m.p. 171° (207).

The chromium salts deposited from the acetic acid solution during the oxidation were dissolved in water, and the keto-acid and β-alanine were isolated as above. Most of the chromium was eluted from the Zeokarb 225 column after the β-alanine fraction. The large amount of chromium present made it necessary to repeat the separation with the ion-exchange column five times. The keto-acid fraction yielded 0.53g. (4%) of hydrochloride foaming at 203°; the mother liquors contained a mixture of componants (0.36g.).

The β-alanine fractions gave a crystalline residue (1.04g.) which when treated with phenyl isocyanate as above gave a product (0.24g.) m.p. 121°, separated with sodium carbonate solution into phenylurea (27mg.) m.p. 146-147° alone and mixed with an authentic sample, and N-phenylcarbamoyl-β-alanine (0.11g.) m.p. 162-168°, mixed m.p. with a pure sample, 169-171°.

The acidified mother liquors from the phenyl isocyanate reaction were investigated. They were heated on a water-bath for 8 hours, which gave no precipitate, and evaporated on a water-bath. Extraction with ethanol gave crude aniline hydrochlor-

ide.. (It is possible that the β-alanine fraction from the columns contained some ammonium chloride, which would lead to this result).

The total yield of crystalline keto-acid hydrochloride was 36%, and the N-phenylcarbamoyl-β-alanine obtained corresponded to a 2.2% yield.

4-oxo-L-pipecolic acid and derivatives.

4-oxo-L-pipecolic acid: The hydrochloride (0.4g.) was added to Zeokarb 225 column (28g.) and the amino-acid was eluted with N-ammonia. The amino-acid fraction was evaporated under reduced pressure to a syrup, and isopropenol was added. Crystallisation gave 4-oxo-L-pipecolic acid monohydrate as colourless prisms (0.19g.) which darkened slightly at 130-140° and fosmed at 240°. $[\alpha]_D^{23}$ -14.8° (C, 1 in water). An air-dried sample was analysed. (Found: C,44.3; H,7.0; N,8.5; $C_6H_9O_3N.H_2O$ requires C,44.7; H,6.9; N,8.7%).

The N-2,4-dinitrophenyl derivative, prepared in the usual way (195) was a thick oil which darkened, apparently undergoing aerial oxidation.

Phenylhydantoin: To a solution of 4-oxo-L-pipecolic acid hydrochloride (0.40g.) in water (4ml.) and sodium hydroxide (4ml. of N), cooled in an ice-bath, phenyl-isocyanate (0.30g., 25% excess) was added with shaking during 15 minutes. Diphenylurea was removed and the filtrate was acidified. Crystals began to form after about 10 minutes and were collected next

day, as colourless needles, m.p. 187°, insoluble in dilute sodium carbonate solution. (Found: C,63.8; H,5.05; N,11.3. $C_{13}H_{12}N_2O_3$ requires C,63.9; H,4.95; N,11.5%). The product was the phenylhydantoin of 4-oxo-L-pipecolic acid i.e. L-3'-phenyl-4-oxopiperidino - (1,2:1,5')-hydantoin.

A solution in ethanol exhibited mutarotation, apparently due to hemi-acetal formation. A solution of 100mg. in 25ml. of ethanol, prepared by warming, had an optical rotation which changed from -0.940° to 23°C to -0.437° after 23 hours. The solution was boiled for 6 hours, and then concentrated. Crystallisation was very slow and occurred only after the volume had been reduced to about 1ml.; 50mg., m.p. 187° alone and mixed with the original material. The product was still optically active; a 0.16% solution in ethanol, prepared using heat, had $[\alpha]_D^{23}$ -55°.

Recrystallisation of the original sample (150mg.) from ethanol (10ml.) as quickly as possible, gave clusters of needles, (102mg.) m.p. 187°. A 0.366% solution of this material was prepared in ethanol without the use of heat; 10 minutes after addition of solvent, the solution had an optical rotation of -0.318° at 23°C, i.e. $[\alpha]_D^{23}$ -87.0 (c, 0.366 in EtOH).

Reduction of 4-oxo-L-pipecolic acid with sodium borohydride: cis-4-hydroxy-L-pipecolic acid and derivatives.

A solution of 4-oxo-L-pipecolic acid hydrochloride monohydrate (2g.) in water (20ml.) was made alkaline (about pH9.0) with N-potassium hydroxide (16.6ml.), cooled to 20°, and a solution of sodium borohydride (112mg., 20% excess) in water was added in portions. After each addition there was an immediate temperature rise, but no colour. The solution was left at room temperature for 1 hour, and then acidified slightly with acetic acid. The solution was added to a Zeokarb 225 column (25g., 27.0 x 1.2cm., acid form), and the amino-acids were recovered with N-ammonia. This fraction was evaporated to a thin syrup, which gave crystals on the addition of ethanol; 1.05g., m.p. 265° decomp.. A second crop (0.23g.) had m.p. 250-263° decomp., and the mother liquor contained 0.27g. of material. Paper chromatography (BA., 40 hours) showed that the crystalline fractions contained mainly cis-4-hydroxypipecolic acid with a trace of the natural isomer, but, in the mother liquor, the natural isomer predominated. The yield of crystalline cis-epimer was 78% (calculated as monohydrate).

Crystallisation from aqueous ethanol gave pure cis-4-hydroxy-L-pipecolic acid monohydrate as colourless plates, m.p. 265° decomp. $[\alpha]_D^{23}$ -19.3° (C,1.124 in water) $[\alpha]_D^{26}$ +7.9 (C,1.124 in 5N-HCl). (Found: C, 43.6; H,8.1; N,8.4. $C_6H_{11}O_3N.H_2O$ requires C,44.2; H,8.0; N,8.6%).

Copper salt: **Cis-4-hydroxypipecolic acid** (163mg.) was boiled in water (20ml.) with copper carbonate (from 300mg. of copper sulphate pentahydrate), for 1 hour, the solution was filtered and the residue was washed with boiling water. The filtrate and washings were concentrated to a weight of 8g. Crystals of the copper salt began to form after a few hours at room temperature, and were collected after 2 days; shining, deep blue plates (54mg.), m.p.245° decomp.. The mother liquors were concentrated to 2.1g. and the crystals which formed were collected after 1 week (33mg.). The copper salt did not lose any weight when kept over anhydrous calcium chloride. An air-dried sample was analysed. (Found: N,6.6; CuO,16.9: $C_{12}H_{20}N_2O_6Cu.\ 4H_2O$ requires N,6.6; CuO, 18.7%).

N-2,4-Dinitrophenyl derivative: This was prepared in the same way as the derivative of 4-hydroxypipecolic acid. It was obtained in 62% yield as crystals, m.p. 132°. Recrystallisation from ethanol-water gave N-2,4-dinitrophenyl-cis-4-hydroxy-L-pipecolic acid as small yellow prisms, m.p. 134°. (Found: C,45.0; H,4.2; N,13.05. $C_{12}H_{13}O_7N_3.\ 0.5H_2O$ requires C,45.0; H,4.4; N,13.1%).

N-Benzoyl derivative: A solution of cis-4-hydroxy-L-pipecolic acid monohydrate (163mg.) in water (1ml.) and sodium hydroxide (2.2ml. of N) was cooled in ice

and benzoyl chloride (0.15g., 1.1 equiv.) was added in portions with shaking. The solution was filtered and acidified with hydrochloric acid. A layer of light petroleum was added, and the mixture was left at 0° overnight. N-Benzoyl-cis-4-hydroxypipecolic acid monohydrate crystallised as loose clusters of colourless blades, which were collected after 2 days; 119mg., m.p. 104°. $[\alpha]_D^{23}$ -39.5° (C, 1 in EtOH) (Found: C,58.45; H,6.45; N,5.2. $C_{13}H_{15}O_4N.H_2O$ requires C,58.4; H,6.4; N,5.2%).

The same product was obtained when 2.2 equiv. of benzoyl chloride was used in the benzoylation.

The solution of the above product (100mg.) in ethanol (10ml.) used for the measurement of the optical rotation was concentrated with the addition of water. Crystals (blades) formed slowly from the cold solution: 76mg., m.p. 191° $[\alpha]_D^{26}$ -38.5° (C, 1 in EtOH) (Found: C,58.65; H,6.4; N,5.2. $C_{13}H_{15}O_4N.H_2O$ requires C,58.4; H,6.4; N,5.2%). This appears to be a second form of N-benzoyl-cis-4-hydroxy-L-pipecolic acid monohydrate. A mixed m.p. with the first form was 191°. The IR spectra of the two forms (in chloroform solution, CaF_2 prism) was similar over the region 2.5 to 6.5 μ., showing peaks at 2.93 (w), 3.42(m), incompletely separated peaks at 5.73 and 5.81(s) and a peak at

6.15(s) with shoulders at 6.23 and 6.35. Both forms dissolved readily in cold sodium bicarbonate solution with evolution of carbon dioxide.

A crystalline product was not obtained from the reaction of N-benzoyl-cis-4-hydroxypipecolic acid with acetic anhydride in the cold.

Synthesis of cis-4-hydroxypipecolic acid by catalytic hydrogenation.

Methyl 4-chloropicolinate: This was prepared as described by Mosher and Look (155) by the action of thionyl chloride on picolinic acid, followed by esterification. Crude methyl 4-chloropicolinate was isolated by fractional distillation, b.p. 98-103° at 2mm., and recrystallised from an equal volume of light petroleum; a 37% yield of product, m.p. 48-52° was obtained (Mosher and Look quote a 71% yield, m.p. 57-58°).

4-Benzyloxypicolinic acid: Reaction of methyl 4-chloropicolinate (1.72g.) with sodium benzyloxide (from 0.3g. sodium) in benzyl alcohol (12ml.) gave, after hydrolysis with sodium hydroxide in 80% ethanol, 4-chloropicolinic acid (0.59g.), m.p. 189° decomp.; lit., (154), 180-181°, (155), 181-182°, (208), 183°, (209), 184-195°.

The following procedure was found to give reproducible results: methyl 4-chloropicolinate

(3.43g.) was dissolved in benzyl alcohol by slight heating, and a cold solution of sodium (1g.) in freshly distilled benzyl alcohol (30ml.) (prepared with the benzyl alcohol almost boiling to reduce the viscosity of the solution) was added in portions with stirring. The mixture was boiled gently for 45 minutes. The solution was cooled, becoming gelatinous, and water (50ml.), ether (100ml.) and hydrochloric acid (50ml. of 2N) were added in that order. The mixture was shaken, and after separation, the ether layer was washed twice with 2N-hydrochloric acid, and discarded. The acidic extracts were combined and washed with ether. Sodium hydroxide (50ml. of 5N) was added; a crystalline precipitate formed. The mixture was boiled to remove ether, and was left at 0° overnight. The crystalline product consisted of the sodium salt of 4-benzyloxypicolinic acid (3.65g.) and was collected, dissolved in hot water (20ml.) and acetic acid (1.3ml., slight excess) was added. Small white prisms (2.95g.) separated slowly and were collected after 2 days at 0°. Recrystallisation from ethanol (carbon) gave 4-benzyloxypicolinic acid (2.40 g., 52%), prisms m.p. 172° (Found: C,68.4; H,5.0; N,5.9. $C_{13}H_{11}O_3N$ requires C,69.1; H,4.8; N,6.1%).

The hydrochloride, prepared from the acid (0.58g.) crystallised in needles (0.56g., 83%), m.p. 158°

raised to 162° by recrystallisation from 5N-hydrochloric acid. (Found: C,55.2; H,5.0, N,4.6; Cl, 13.2 . $C_{13}H_{11}O_3N$. HCl.H_2O requires C,55.0; H,5.0; N, 4.9; Cl, 13.5%).

When the hydrochloride was heated above its melting point, crystals formed in the liquid (at about 205°) and a second m.p. 238-250° decomp. occurred.

The hydrochloride (0.39g.) was heated in an oil bath at 200°. It melted and resolidified; a liquid which distilled had the odour of benzyl chloride. The solid product was washed with light petroleum and from water gave 4-hydroxypicolinic acid (0.15g., 73%) as colourless prisms, m.p. 258° decomp., alone or mixed with 4-hydroxypicolinic acid obtained by hydrogenolysis of 4-benzyloxypicolinic acid (see below).

Catalytic hydrogenation of 4-benzyloxypicolinic acid: Hydrogenation of 4-benzyloxypicolinic acid (1g.) in 5N-hydrochloric acid (20ml.) at room temperature and pressure in the presence of platinum (from 0.1g. of oxide) proceeded rapidly, the material being completely dissolved after 2 hours. Uptake of hydrogen ceased after 29 hours. Paper chromatography showed that pipecolic acid was present - no other compound was detected by ninhydrin or by isatin. The filtered and concentrated solution deposited 4-hydroxypicolinic acid (0.32g., 35%), which was recrystallised from water as small prisms. The m.p. varied from

one determination to another, being in the range 255 to 258° decomp. Lit., (210), m.p. 257-258°, (211), 254-255°. (Found: C,51.7; H,3.6; N,10.1. Calc. for $C_6H_5NO_3$: C,51.8; H,3.6; N,10.1%).

4-Benzyloxypicolinic acid (50mg.) in solution (1ml.) was shaken for 24 hours with hydrogen and platinum at various temperatures, and the products were examined by paper chromatography (BAW, 24 hours). The experimental details and results are given in Table 27.

Table 27.

Products of hydrogenation of 4-benzyloxypicolinic acid.

Platinum (mg.)	Solvent	Temperature	Products. Pipecolic	4-hydroxypipecolic	cis-4-hydroxypipecolic
5.5	5N-HCl	65°C	+	Nil	Nil
7.4	Acetic	65	+	"	+
9.5	Acetic	105	+	"	+
13.1	Water	105	++	+	++
9.1	1.5N-NH_3	105	Trace	Nil	Nil

The 4-hydroxypipecolic acids obtained in the hydrogenations gave the same colours with ninhydrin and isatin as natural 4-hydroxypipecolic acid. The synthetic isomer of higher Rf (in BAW) had the same Rf as natural 4-hydroxypipecolic acid, and was not separated from it. The isomer of lower Rf similarly

had the same Rf as cis-4-hydroxypipecolic acid obtained by heating natural 4-hydroxypipecolic acid in barium hydroxide.

Hydrogenation of 4-benzyloxypicolinic acid (135mg.) in water (1ml.) with platinum (14mg.) for 4 hours at 105° and 70 atmospheres gave a product containing much pipecolic and cis-4-hydroxypipecolic acid, and some 4-hydroxypipecolic acid. The solution deposited crystals (36.6mg.) identified as 4-hydroxypicolinic acid by m.p. and mixed m.p..

4-Hydroxypicolinic acid hydrogenated under the same conditions gave the same products, and some 4-hydroxypicolinic acid was recovered.

cis-4-Hydroxypipecolic acid hydrochloride: 4-Benzyloxypicolinic acid (6.46g.) was hydrogenated in water (50ml.) with platinum (0.255g.) at 105° and 70 atmospheres. The pressure fell to 55 atmospheres over 18 hours; it was then raised to 70 atmospheres but no further change took place in 24 hours. The product had a strong odour of toluene. After filtration, the initially pale solution darkened considerably, depositing much dark brown material. The solution was concentrated and again filtered. The mixed 4-hydroxypipecolic acids were isolated from the soluble material (1.91g.) by preparative chromatography on Whatman seed-test paper (212) with the upper phase of the mixture ethyl acetate:ethanol:

acetic acid: water 6:1:2:1. The material, in 12ml. of water, was placed in a strip on a total width of 150cm. of paper. The use of solvent from 300ml. of the mixture for each 15cm. paper gave bands of pipecolic acid at 40-48cm. and the 4-hydroxypipecolic acids at 22-34cm. after about 24 hours. The bands were located by spraying the papers lightly with ninhydrin and heating. The 4-hydroxypipecolic acids were extracted with 90% ethanol in a soxhlet apparatus for 12 hours. The crude 4-hydroxypipecolic acid fraction weighed 0.32g.; it could not be induced to crystallise.

The crude fraction was added to a Zeokarb 225 column (50g.), which was eluted with dilute hydrochloric acid as follows: 250ml. of 0.1N; 250ml. of 0.2N; 250ml. of 0.4N; 250ml. of 0.8N. The 4-hydroxypipecolic acids were completely eluted in the last two solvents. The product (0.29g.) was charcoaled in dilute hydrochloric acid, and the solution was concentrated to a syrup, which deposited colourless prisms of a hydrochloride; (75mg., 1.4%), m.p. 253-255° decomp.. Paper chromatography (BAW, 36 hours) showed that this material contained cis-4-hydroxypipecolic acid as the only ninhydrin-positive constituent.

Attempted synthesis of 4-hydroxypipecolic acid by Dieckmann cyclisation.

Ethyl 2(N-ethoxycarbonylamino)-propionate was prepared from β-alanine by the method of Braun and Looker (213); β-alanine was converted into its N-ethoxycarbonyl derivative by reaction with ethyl chloroformate in aqueous alkali, and the product was esterified with ethanol containing hydrogen chloride. From 53.4g. of β-alanine, 65.5g. of 2(N-ethoxycarbonylamino)-propionic acid, m.p. 59-61° was obtained (67% yield). Another batch, double this size, gave 136g. of product (70% yield), m.p. 61°. Esterification was carried out on 50g. lots; the ethyl ester, b.p. 109° at 0.7mm., 112° at 1.5mm., 120° at 2mm. was obtained in 89-90% yield in each of three batches.

Ethyl fumarate: The esterification method of Micovic (214) was used. The toluene specified for azeotropic distillation was replaced by a high boiling petroleum fraction but extensive fractionation was required to purify the ester. From two batches of 156g. of fumaric acid, 640ml. of ethanol, 320ml. of "Shell X7" petroleum fraction and 2.2ml. of concentrated sulphuric acid, 350g. (77%) of ethyl fumarate, b.p. 213-215° at 764mm. was obtained.

Ethyl 2(N-ethoxycarbonyl-3-oxo-pyrrolidyl)-acetate (XL1X): To dry benzene (350ml.) containing sodium wire (4.6g., 0.20mol.), there was added successively

with continuous stirring, ethyl 2(N-ethoxycarbonylamino)-propionate (38.1g., 0.20mol.) and ethyl fumarate (34.4g., 0.20mol.). After the mixture had been stirred at room temperature for 15 minutes, reaction began slowly, then became more vigorous so that the mixture boiled 45 minutes after the addition. Stirring was then stopped for 30 minutes, during which the last pieces of sodium wire reacted, and the red-brown solution was then boiled and stirred for 30 minutes. The solution was cooled, ether (100ml.) was added and the solution was shaken with ice-water (300ml.). The almost colourless organic phase was extracted with ice water (two 75ml. portions) and the combined aqueous phases were washed with ether (75ml.), before being poured into a mixture of sulphuric acid (6.5ml.) and ice (75g.). An oil separated; the strongly acidic solution was saturated with sodium chloride before extraction with ethyl acetate (three 100ml. portions). The extracts were washed with saturated sodium chloride solution (50ml.) containing some sodium bicarbonate, and dried (Na_2SO_4). Removal of ethyl acetate by distillation at atmospheric pressure and finally under reduced pressure, left a thick dark oil (53.5g.).

The crude β-keto-ester was dissolved in 150ml. of 10N-hydrochloric acid and kept at room temperature. Next day hydrochloric acid was removed under reduced

pressure, and ethanol (100ml.) was added to the residue and distilled twice. A solution of the residue in ethanol (150ml.) previously saturated with hydrogen chloride was boiled for 4½ hours and hydrogen chloride and ethanol was then removed under reduced pressure, and the residue (37g.) was fractionated. (The purification by chloroform extraction and washing which Kuhn and Osswald (144) used at this stage, seemed unnecessary and was omitted). After a small fore-run, an almost colourless main fraction (24.2g.), b.p.122-128° (mainly 125°) at 0.3mm., and a small fraction (2.9g.), b.p.128-136° at 0.3mm were collected. The second fraction gave the same semicarbazone as the main fraction, which consisted of ethyl 2(N-ethoxycarbonyl-3-oxo-pyrrolidyl)-acetate (49%). (Found: C,53.9; H,7.0; N,6.0. $C_{11}H_{17}O_5N$ requires C,54.3; H,7.0; N,5.8). The infrared spectrum showed carbonyl bands at 5.68, 5.77 and 5.86μ in nujol mull and in CCl_4 solution.

Semicarbazone: From the keto-ester (0.79g.), semicarbazide hydrochloride (1.2g.) and sodium acetate (1.8g. of the trihydrate) in aqueous ethanol, there was obtained after 7 days at room temperature, the semicarbazone of (XLIX) (0.56g.), m.p. 124° unchanged by two recrystallisations from ethanol (colourless prisms). (Found: N,18.9. $C_{12}H_{20}O_4N_5$ requires N,18.7%).

2,4-Dinitrophenylhydrazones: From the keto-ester (0.5g.) in ethanol, and 2,4-dinitrophenylhydrazine (0.4g.) in aqueous-ethanolic sulphuric acid, there was obtained, after 3 days, 0.38g. of a 2,4-dinitrophenylhydrazone as orange plates, m.p. 112-114°. (A second preparation gave 0.52g. after a longer period). Recrystallisation from ethanol gave orange plates, m.p. 112-113°, and then, from a second recrystallisation, yellow prisms, m.p. 135°. It was found that either form could be obtained from a supersaturated ethanolic solution by appropriate seeding; the form m.p. 113-114° was obtained from a solution of the higher-melting form, and the form m.p. 135° was obtained from a solution of the lower-melting form. Sometimes the orange form, after melting at 113-114°, recrystallised in the capillary and then melted again at 133-135°. (Found: (orange form) N,16.6; (yellow form) N,16.4. $C_{17}H_{21}O_8N_5$ requires N,16.5%).

Two further Dieckmann condensations were carried out. The esters were added to sodium in boiling benzene, and a 10% excess of ethyl fumarate was used. From the first of these batches, an attempt was made to isolate the β-keto-ester by fractionation at 1mm pressure, but no definite fraction was obtained. The second gave a yield of 5.5g. of the keto-ester (XLIX), b.p. 145-153° at 2.0mm.

Catalytic hydrogenation of the keto-ester (XL1X):
(a) Keto-ester (67mg.) in ethanol (3ml.) was shaken with platinum (from 19.7mg. ofoxide) and hydrogen at room temperature and 70 atmospheres for 24 hours. The solution was filtered, barium hydroxide (0.5g. of octahydrate) was added, and the solution adjusted to be about 10ml. of 50% ethanol. After heating for 5 hours in a boiling water-bath, the solution was cooled and barium was removed as carbonate. Paper chromatography (BAW, 14 hours) showed the presence of a component, Rf 0.24, giving a yellow-orange colour with ninhydrin, and a component, Rf 0.12, giving a purple colour with ninhydrin (pipecolic acid, Rf 0.37; natural 4-hydroxypipecolic acid, Rf 0.22; cis-4-hydroxypipecolic acid, Rf 0.19). Hydrolysis of the keto-ester with barium hydroxide gave a component, Rf 0.26, giving a purple with ninhydrin.
(b) The component of Rf 0.24 in BAW, giving a yellow-orange colour with ninhydrin, was also obtained by hydrolysis (with barium hydroxide as above) of the products of hydrogenation of the keto-ester in acetic acid (with platinum as in (a)), in methanol with Raney nickel W7 catalyst at room temperature and 85 atmospheres for 24 hours, and in methanol at 105° and 35 atmospheres for 24 hours.
2(3-hydroxypyrrolidyl)-acetic acid (L): To a

solution of the keto-ester (XLIX) (4.86g.) in ethanol (50ml.) at 15°, sodium borohydride (0.38g., 2 equiv.) in water (1ml.) was added dropwise during 10 minutes. Initially each drop of borohydride gave an immediate temperature rise of about 0.5°; the temperature was kept below 20°C. After 40 minutes at room temperature, 10% sulphuric acid was added until all the borohydride was decomposed. (It would seem better to use acetone). Barium hydroxide (19.0g. of octahydrate) and water (50ml.) were added, and the mixture was heated in a boiling water bath for 16 hours. The mixture was diluted with water (100ml.) and filtered when cold. The solution showed on chromatograms (BAW, 15 hours) the same two components as were obtained by catalytic hydrogenation. The solution was acidified with 10% sulphuric acid, and the filtrate was continuously extracted with ether for 16 hours, to give 73mg. of boric acid. The residual aqueous solution was made alkaline with barium hydroxide, and the filtered solution was continuously extracted with ether for 30 hours, which gave 0.20g. of brown, non-crystalline resin. Barium was removed from the residual aqueous solution with carbon dioxide, and the filtrate was evaporated to dryness. The crystalline residue was digested with ethanol containing a few drops of acetic acid, filtered and

the residue was washed with 90% ethanol (5ml.). The filtrate and washings contained at least seven ninhydrin-positive components and were discarded. The residue was apparently a single component, Rf 0.27 in BAW (19 hours, 48cm.) compared with 4-hydroxypipecolic acid Rf 0.20 and pipecolic acid Rf 0.36, and gave with ninhydrin, a yellow-orange colour, and with isatin, a white spot, detectable as a non-absorbing spot under UV light. The crude product (1.11g.,38%) had m.p. 192-198° decomp.. It was recrystallised from aqueous ethanol (carbon); three recrystallisations gave 2(3-hydroxypyrrolidyl)-ecetic acid monohydrate (0.51g.) as colourless prisms, m.p. 215-216° decomp. For analysis, the sample was dried at 100° in vacuo for 2 hours. (Found: C,44.5; H,8.0; N,8.6. $C_6H_{11}O_3N.H_2O$ requires C,44.2; H,8.0; N,8.6%).

The N-2,4-dinitrophenyl derivative was prepared as described for the dinitrophenyl derivative of 4-hydroxypipecolic acid, and was obtained in 83% yield as yellow prisms, m.p. 203°, raised to 205° by recrystallisation from aqueous ethanol. (Found, on an air-dried sample: C,45.2; H,4.6; N,12.9. $C_{12}H_{13}O_7N_3.0.5H_2O$ requires C,45.0; H,4.4; N,13.1%).

To demonstrate that the amino group was secondary, a mixture of the amino-acid (L), pipecolic acid, glycine, and β-alanine in 2N-HCl was treated with

nitrous fumes for 10 minutes, left 8 hours, and then boiled. Paper chromatography (BAW, 12 hours) showed that pipecolic acid and the amino-acid (L) were the only ninhydrin-positive components present.

From the amino-acid (0.163g.) in water with sodium hydroxide (1ml. of 1N) and phenyl iso-cyanate (0.15g., 25%) there was obtained on acidification a sticky gum which crystallised on standing; 0.217g. (88%), m.p. 161°. Recrystallisation from water (14ml.) gave an N-phenylcarbamoyl derivative as colourless prisms, readily soluble in cold sodium bicarbonate solution with the evolution of carbon dioxide. The analysis corresponded to the N-phenylcarbamoyl derivative of the amino-acid (L) less one molecule of water. (Found: C,63.3; H,5.6; N,11.4. $C_{13}H_{14}O_3N$ requires C,63.4; H,5.7; N,11.4%). The structure of this compound is not known. It was recovered after being heated in 3N-hydrochloric acid on a boiling water-bath for 8 hours.

L-Pipecolic acid and derivatives.

L-Pipecolic acid was obtained from A. excelsa heartwood (Extraction G) as small prisms, m.p. 273-275° decomp.; lit., (215), m.p. 274°, (147), 268° $[\alpha]_D^{23}$-25.2° (C, 2.2 in water); lit., (216) $[\alpha]_D$-25.2°. (Found: C,56.2; H,8.6; N,11.0. Calc. for $C_6H_{11}O_2N$: C,55.8; H,8.6; N,10.85%). L-Pipecolic acid was also

isolated from A. Oswaldii leaves; m.p.272-273° decomp. $[\alpha]_D^{17}$ -25.5° and -25.9° (C, 2 in water).

The hydrochloride was isolated from various heartwoods. It was prepared from pure (-)-pipecolic acid: m.p. 256-258° decomp.; lit., (147), m.p. 256°, (217), 258-259°. There was no melting-point depression on admixture of L-pipecolic acid hydrochloride with (±)-pipecolic acid hydrochloride (m.p. 258-260° decomp.). The hydrochloride prepared from pure (-)-pipecolic acid had $[\alpha]_D^{22}$ -10.50° (C, 6 in water); Lit., (216), $[\alpha]_D$ -5 to -10.3°.

In 5N-hydrochloric acid, (-)-pipecolic acid had the following optical rotations: $[\alpha]_D^{23}$ -10.45° (C, 2), -10.01° (C, 6), -9.42° (C, 10).

N-Benzoyl-L-pipecolic acid: This was prepared from (-)-pipecolic acid hydrochloride $[\alpha]_D^{18}$ -10.7° (C, 7.9 in water) (0.79g.) with benzoyl chloride in alkaline solution. The product was obtained as a thick oil which crystallised after 7 days; 0.78g. (70%), m.p. 126-127°. Recrystallisation from ethanol-light petroleum gave N-benzoyl-L-pipecolic acid as large clear prisms, m.p. 133°; lit., (147), m.p. 145°. The mixed m.p. with N-benzoyl-(±)-pipecolic acid (m.p. 131°) was 119-122°. Recrystallisation from water gave N-benzoyl-L-pipecolic acid as opaque, colourless needles, m.p. 132-133°. $[\alpha]_D^{22}$ -72.0

(C, 1 in EtOH). Lit., $[\alpha]_D$ -72.8° (174).

Derivatives with phenyl isocyanate: From pure (-)-pipecolic acid (0.516g.) in sodium hydroxide solution (4ml. of N) with phenyl isocyanate (0.59g., 1.25 equiv.), there was obtained on acidification, small white crystals; 0.80g. (80%). Half of this product was dissolved in 5% sodium carbonate solution and reprecipitated with acid, to give N-phenylcarbamoyl-L-pipecolic acid as small white prisms, m.p. 178°. $[\alpha]_D^{20}$ -39.0 (C, 1 in EtOH). (Found: C, 63.4; H,6.4; N,11.4. $C_{13}H_{16}O_3N_2$ requires C,62.9; H,6.5; N,11.3%).

The other half of the crude product crystallised from boiling water (40ml.) in loose needles $[\alpha]_D^{24}$ ±0.6 (C, 1 in EtOH), m.p. 159-160° alone and when mixed with (±)-pipecolic acid phenylhydantoin.

Reference amino-acids and derivatives.

The piperidinecarboxylic acids were prepared by catalytic hydrogenation of the pyridinecarboxylic acids (5g.) in 5N-hydrochloric acid (20ml.), with platinum (0.2g.) at 60° and 15-35 atmospheres of hydrogen for 18-24 hours. Filtration and concentration gave the crystalline hydrochlorides, recrystallised from aqueous ethanol.

(±)-Pipecolic acid hydrochloride, m.p. 258-260° decomp. (91% yield).

(±)-Nipecotic acid hydrochloride, m.p. 235-238° decomp.; lit., (218), 233-234°, (219), 239°, (220), 240-241° (53% yield).

Isonipecotic acid hydrochloride, m.p. 299° decomp.; lit., (219), 287°, (221), 290-293°, (222) 295°, 300°, (220), 304-306° (68% yield).

Isonicotinic acid was recovered as the hydrochloride m.p. 278° (authentic hydrochloride, m.p. 279-283°) from the first attempted hydrogenation. This product (5.28g.) was reduced when the hydrogenation was repeated.

N-Benzoyl-(±)-pipecolic acid, m.p. 131°, prisms from aqueous ethanol, was prepared with benzoyl chloride in aqueous alkali; lit., (223), m.p. 126-127°.

N-p-Toluenesulphonyl-(±)-nipecotic acid was not obtained when Freudenberg's directions (220) were followed. The procedure of Beecham (224) gave a 63% yield of this compound, obtained as small opaque colourless prisms from ethanol, m.p. 151°. Freudenberg quotes m.p. 167°.

N-p-Toluenesulphonylisonipecotic acid was prepared by Beecham's procedure, and obtained as very small crystals (prisms?) from chloroform-benzene, m.p. 170°; lit., (220, 221, 222), m.p. 170°.

Derivatives of (±)-pipecolic acid with phenyl isocyanate: The reaction of (±)-pipecolic acid

hydrochloride with phenyl isocyanate was carried out as described for (-)-pipecolic acid; an extra equivalent of alkali was used. The product was obtained on acidification as small colourless crystals; 0.81g. from 0.66g. of hydrochloride (81%). Half of this was dissolved in cold 5% sodium carbonate solution and reprecipitated by acidification, to give N-phenylcarbamoyl-(±)-pipecolic acid, m.p. 138°, recrystallising and melting again at 156-158°. (Found: C,63.4; H,6.8; N,11.4. $C_{13}H_{16}O_3N_2$ requires C,62.9; H,6.5; N,11.3%).

The other half of the crude product was boiled in water (50ml.) for one hour, and crystallisation (from 40ml.) gave the phenylhydantoin of (±)-pipecolic acid, 3'-phenylpiperidino-(1,2:1',5')-hydantoin as loose needles, m.p. 158-159°. (Found: C,67.8; H,6.2; N,12.2. $C_{13}H_{14}O_2N_2$ requires C,67.8; H,6.1; N,12.2%).

N-2,4-Dinitrophenyl-β-alanine was prepared to examine its usefulness for recovery of β-alanine from the oxidation of 4-hydroxypipecolic acid. The method of Rao and Sober (195) gave a 44% yield of product crystallising from ethanol-water in yellow needles, m.p. 145°; lit., (225), m.p. 121-125°.

2-Aminopent-4-enoic acid ("allylglycine") was prepared from ethyl acetamidomalonate and allyl bromide by the procedure of Gaudry (226); m.p.

255-257° decomp.; lit., (226), m.p. 246-248°, (201), 250-255°, (227), 255-258°. On chromatograms it showed a single spot, free from glycine. The phenyl isocyanate derivative had m.p. 159-160°; lit., (201), m.p. 159-161°. This compound is the N-phenyl-carbamoyl-amino-acid as it is readily soluble in dilute sodium carbonate solution.

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SUMMARY.

The flavan-3,4-diol melacacidin, which had been obtained in a non-crystalline form from *Acacia melanoxylon* heartwood, was isolated in crystalline form from *A. harpophylla* and *A. excelsa* heartwoods. In these species it is accompanied by its 4-epimer, isomelacacidin. When heated in acidified ethanol, the latter formed a 4-*O*-ethyl derivative, which permitted the separation of the epimers. *O*-Ethyl melacacidin, which was obtained in crystalline form, was readily hydrolysed to isomelacacidin, this solvolysis being acid-catalysed. The acid-catalysed inter-conversion of melacacidin and isomelacacidin was studied. From these leucoanthocyanidins, isomelacacidin *p*-tolyl sulphone was obtained by reaction with *p*-toluenesulphinic acid.

From *A. intertexta* heartwood, a new leucoanthocyanidin, teracacidin, was obtained; it was shown to be a 7,8,4'-trihydroxyflavan-3,4-diol. This is the third hydroxylation pattern to be found to occur among the leucoanthocyanidins of *Acacia* heartwoods. The extract of *A. intertexta* heartwood gave isoteracacidin *p*-tolyl sulphone when treated with *p*-toluenesulphinic acid.

The heartwoods of the above species also yielded ( )-pinitol; this was found to be readily demethylated

by boiling hydrochloric acid. 7,8,3',4'-Tetrahydroxyflavonol and okanin were isolated from A. harpophylla heartwood.

4-Hydroxypipecolic acid, which was discovered in Acacia species by Virtanen, and L-pipecolic acid have been isolated from various Acacia samples. The structure of the former which had been in doubt has been firmly established, and evidence that it is trans-4-hydroxy-L-pipecolic acid is presented. Various aspects of the chemistry of 4-hydroxypipecolic acid, and its synthesis, have been investigated.