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CYANOGENESIS IN MANIOC: CONCERNING LOTAUSTRALIN*

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Abstract—A glucoside of 2-hydroxy-2-methylbutyronitrile was shown to be present, in low concentration compared with linamarin, in three samples of relatively bitter tubers of manioc (*Manihot esculenta* Crantz). The glucoside was separated, as the trimethylsilyl derivative, by gas chromatography and identified by mass spectrometry. The β-D-glucopyranosides of the enantiomeric hydroxy nitriles were synthesized from acetobromoglucose and methyl ethyl ketone cyanohydrin, being separated by chromatography of the acetates. The less levorotatory of the synthetic glucosides, having the (*R*) configuration at the asymmetric center of the aglucone, was shown to be identical with the compound which occurs naturally in white clover (lotaustralin).

INTRODUCTION

LINAMARIN [2-(β-D-glucopyranosyloxy)isobutyronitrile] was shown to be the principal cyanogenetic glycoside of manioc (*Manihot esculenta* Crantz, Euphorbiaceae) by Dunstan, Henry and Auld.[1] We have recently described its isolation by column chromatography from manioc tubers with a relatively low hydrogen cyanide content.[2] Concurrently with the biosynthesis of linamarin from L-valine in plants, L-isoleucine is almost always transformed into a glucoside of 2-hydroxy-2-methylbutyronitrile.[3–5] This hydroxy nitrile, in contrast to the aglucone of linamarin, possesses a chiral center and corresponds to two diastereomeric β-D-glucopyranosides. These diastereomers have not been distinguished from each other, and all natural samples, whether isolated or detected chromatographically, have been given the name lotaustralin. We prefer, in accord with standard practice for cyanogenetic glycosides, to restrict the term lotaustralin to that member of the pair of 2-(β-D-glucopyranosyloxy)-2-methylbutyronitriles which is the predominant isomer in *Lotus australis* or a similar plant;[6] we shall call the pair collectively methyllinamarins. Methyllinamarins were not detected by thin-layer chromatography (TLC) in the tubers of "sweet" manoic used in our earlier work. However, we have now investigated several samples of manioc tubers with a higher hydrogen cyanide content and found the presence of small quantities of a methyllinamarin indicated by TLC. The separation of the glucoside by a combination of column chromatography and gas

* Part II in this series; for Part I, see Ref. 2.

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[1] W. R. DUNSTAN, T. A. HENRY and S. J. M. AULD, *Proc. R. Soc.* **78B**, 152 (1906).

[2] R. C. CLAPP, F. H. BISSETT, R. A. COBURN and L. LONG, JR., *Phytochem.* **5**, 1323 (1966); **6**, 623 [erratum] (1967).

[3] G. W. BUTLER and B. G. BUTLER, *Nature* **187**, 780 (1960).

[4] G. W. BUTLER and E. E. CONN, *J. Biol. Chem.* **239**, 1674 (1964).

[5] G. W. BUTLER, *Phytochem.* **4**, 127 (1965).

[6] H. FINNEMORE, J. M. COOPER, M. B. STANLEY and J. H. COBCROFT, *J. Soc. Chem. Ind. (London)* **57**, 162 (1938).

chromatography and its definite identification as a methyllinamarin are described in this paper. Butler[5] had reported a small amount of a methyllinamarin together with linamarin (ratio 4:96) in roots ascribed to another species of *Manihot*, *M. carthaginensis* (Jacq.) Muell. Arg. In a paper that appeared after completion of our work, Nartey[7] presents evidence for the occurrence of a methyllinamarin as well as linamarin (ratio 7:93) in manioc seedlings (named as *M. utilissima* Pohl, a synonym[8] of *M. esculenta.*

Synthesis of a glucoside of 2-hydroxy-2-methylbutyronitrile has never been reported. In the present paper, the synthesis of both methyllinamarins by a method that we have previously used[2] to synthesize linamarin as well as its anomer is described. The individual methyllinamarins have been characterized and configurations assigned to them, so their phytochemistry now stands on a more rational footing, as we discuss hereafter.

RESULTS AND DISCUSSION

Separation and Structural Identification of a Methyllinamarin from Manioc

Thin-layer chromatograms of ethanolic extracts of manioc tubers from Brazil, Colombia and Costa Rica, with hydrogen cyanide contents of approximately 40 mg/100 g, showed a weak carbohydrate spot that had a somewhat greater mobility than the strong linamarin spot. The mobility of this spot corresponded to that of known samples of lotaustralin (from white clover, see later). Except for strong, slow-moving spots that corresponded to sucrose and monosaccharides such as glucose, all other spots in the TLC that represented reducing sugars were very weak.

The presence of small quantities of a methyllinamarin in the alcoholic extracts of the manioc tubers was also indicated by paper chromatography. In a methyl ethyl ketone–acetone–water (30:10:4) system[4] the extracts gave a weak spot corresponding to lotaustralin at R_f 0·66 and a strong linamarin spot at R_f 0·52.

Column chromatography on silica gel with 5:1 chloroform–methanol as the eluting solvent had previously been used[2] for the isolation of linamarin in manioc extracts, and attempts were made to employ silica gel chromatography for the separation of methyllinamarins from these extracts. However, although some of the fractions collected from the column before the bulk of the linamarin may have been enriched in methyllinamarins, no methyllinamarin could be separated by this method from the large quantity of linamarin present.

Model experiments on mixtures of the trimethylsilyl (TMS) derivatives of known samples of linamarin and lotaustralin demonstrated that they could be separated efficiently on gas–liquid chromatographic columns by the method of Sweeley *et al.*[9] On 6-, 10- and 12-ft SE-30 (methyl silicone gum rubber) columns differences in retention times between the TMS derivatives of 2·5, 4·75 and 5·25 min could be obtained. When samples of the manioc extracts that had been partially purified by column chromatography on silica gel were trimethylsilylated and fractionated on SE-30 columns, a weak peak with a retention time equal to that of lotaustralin was obtained (Fig. 1, Chromatogram B). Initial attempts to trap this peak were hampered by the formation of aerosol fogs. However, by collecting the effluents in capillary tubes[10] and rechromatographing the collected fractions, a sample composed largely of the material corresponding to lotaustralin was obtained (Fig. 1, Chromatogram D).

[7] F. NARTEY, *Phytochem.* **7**, 1307 (1968).
[8] D. J. ROGERS, *Econ. Botany* **19**, 369 (1965).
[9] C. C. SWEELEY, R. BENTLEY, M. MAKITA and W. W. WELLS, *J. Am. Chem. Soc.* **85**, 2497 (1963).
[10] S. C. BROOKS and V. C. GODEFROI, *Anal. Biochem.* **7**, 135 (1964).

Unequivocal identification of this sample as the TMS derivative of a methyllinamarin was demonstrated by the close correspondence (Fig. 2) of its mass spectrum to that of the TMS derivative of an authentic sample of natural lotaustralin, similarly collected from an SE-30 column. The spectra contained peaks at *m/e* 534 and 200 for the M-15 ion (534·256) and the ion $(CH_3)_3SiO—CH=\overset{+}{O}—C(CN)(CH_3)C_2H_5$ (200·111) that would be anticipated for the TMS derivative of a methyllinamarin from the work of Kochetkov *et al.*[11] on the mass

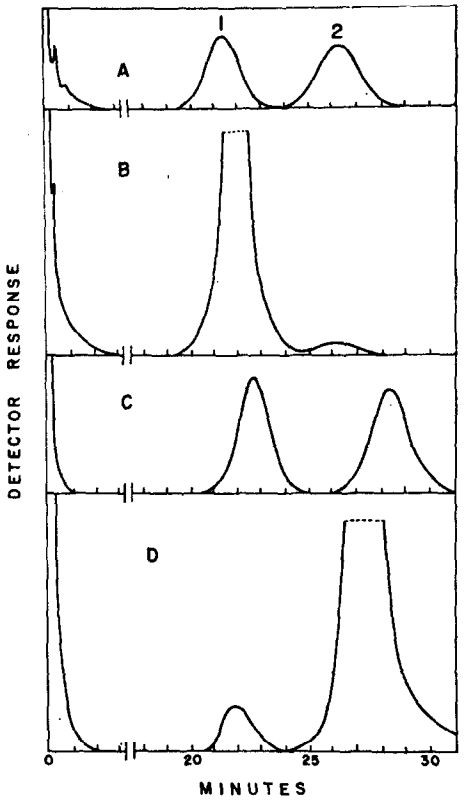


FIG. 1. GAS CHROMATOGRAPHY OF MANIOC EXTRACTS.

Chromatograms: A, mixture of known samples of linamarin (1) and lotaustralin (2); B, manioc extract after purification on silica gel column; C, samples collected from B; D, samples collected from C. A was obtained with a 10-ft, 20% SE-30 column at 210°; B, C and D with a 12-ft, 10% SE-30 column at 222°.

spectra of TMS derivatives of glycosides. High resolution calculations gave *m/e* values for the peaks of 534·248 and 200·111 and confirmed their assignments.

The mass spectrum of the TMS derivative of linamarin showed peaks for analogous ions at *m/e* 520 and 186. The spectra of the derivatives of both glucosides contained strong peaks at *m/e* 73, 103, 147, 204 and 217 that are characteristic of the trimethylsilyl ethers of glycosides.[11]

[11] O. S. CHIZOV, N. V. MOLODTSOV and N. K. KOCHETKOV, *Carbohyd. Res.* **4**, 273 (1967).

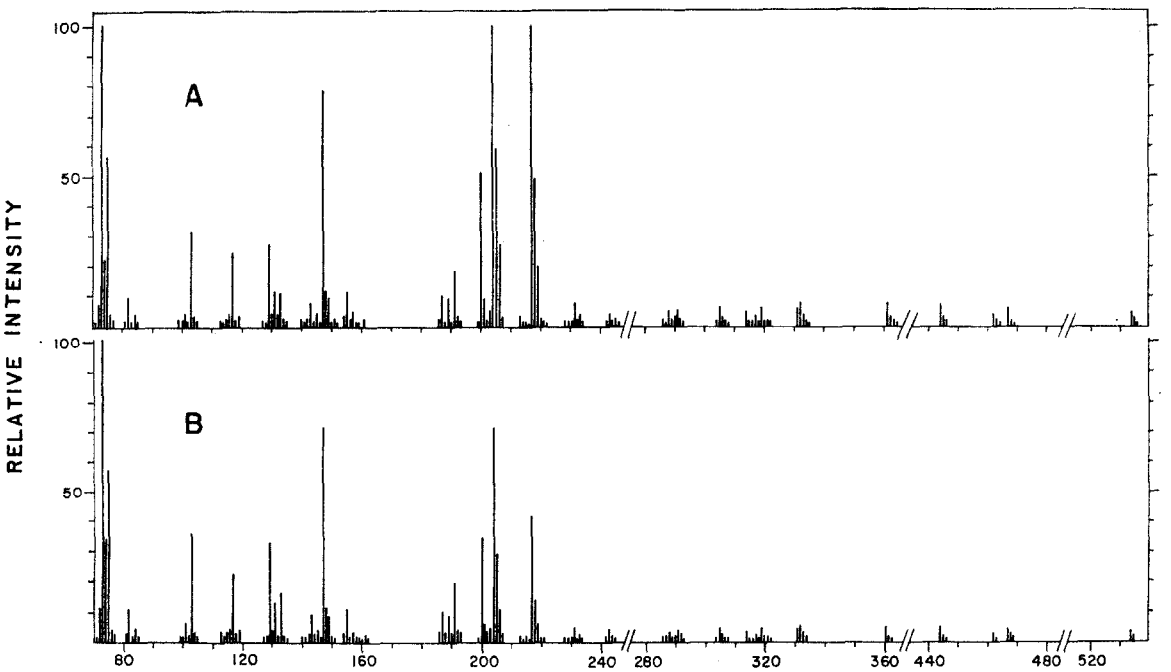


FIG. 2. MASS SPECTRA OF TMS DERIVATIVES.

Spectra: A, methyllinamarin from manioc; B, lotaustralin from white clover. The reduced relative intensity at higher mass numbers in B is due to the difficulty in controlling the temperature during the scan for samples introduced on a probe.

Quantitative Estimation of Linamarin and Methyllinamarin by Gas Chromatography

Aliquots of the extracts of the manioc tubers from the three sources were passed over a silica gel column, and after trimethylsilylation gas chromatograms of the purified mixtures were obtained. By comparison of the peak areas to those given by standard samples of linamarin and lotaustralin the quantities shown in Table 1 were calculated. In Table 2 the corresponding quantities of hydrogen cyanide are shown and compared with the values obtained for the hydrogen cyanide content of the manioc tubers by Winkler's method.[12] Although the correlation between the methods is not exceptional, the data indicate that, as expected, any additional cyanogenetic substances in the tubers are present in small quantity.

TABLE 1. QUANTITIES OF LINAMARIN AND METHYLLINAMARIN IN MANIOC TUBERS

Source	Concentration (mg/100 g)	
	Linamarin	Methyllinamarin
Brazil	394	8·0
Colombia	393	11·3
Costa Rica	332	10·6

[12] W. O. WINKLER, *J. Assoc. Offic. Agr. Chem.* **34**, 541 (1951).

TABLE 2. HYDROGEN CYANIDE CONTENT OF MANIOC TUBERS

Source	HCN content (mg/100 g)			
	From linamarin	From methyllinamarin	From linamarin and methyllinamarin	By total analysis
Brazil	43·0	0·8	43·8	40·1
Colombia	42·9	1·2	44·1	34·8
Costa Rica	36·2	1·1	37·3	39·9

Synthesis of the Methyllinamarins

The reaction of acetobromoglucose and acetone cyanohydrin without additional solvent, followed by deacetylation, had previously been used for the synthesis of both linamarin and its α-anomer, isolinamarin.[2] The analogous reaction of acetobromoglucose with the cyanohydrin of methyl ethyl ketone in nitromethane as solvent afforded, after column chromatography and deacetylation, a glucoside that had a melting point of 112–113°. The spectra of the synthetic glucoside demonstrated that it had the β-configuration, but the 2-hydroxy-2-methylbutyric acid that was obtained from it on hydrolysis did not show significant optical activity. The glucoside thus appeared to be a mixture of diastereomers.

Separation of the epimers was achieved by chromatography of the tetraacetates on a silica gel column with a benzene–ether solvent system. When the product from the reaction of acetobromoglucose and methyl ethyl ketone cyanohydrin was fractionated in this way, two crystalline tetraacetates of 2-(β-D-glucopyranosyloxy)-2-methylbutyronitrile were obtained. The acetates differed in melting point, optical rotation and chemical shift of the 2-methyl group in the aglucone residue. Deacetylation furnished the two crystalline, epimeric methyllinamarins, designated the less and the more levorotatory, whose physical properties are compared with those of linamarin[2, 13] in Table 3. The specific rotation calculated for linamarin from the average of the observed molecular rotations of the methyllinamarins is −28·4°, well within the range of the experimental values, as would be expected in the absence of conformational differences.

TABLE 3. PHYSICAL PROPERTIES OF LINAMARIN AND THE METHYLLINAMARINS

Compound	M.p. (deg.)	$[\alpha]_D$ in H_2O (temp., concn.)	M.p. of tetraacetate (deg.)
Linamarin[2, 13]	143–144	−27·5° (26, 8); −28·5° (32, 4); −29·1° (18, 3)	140–141
(−)Methyllinamarin (lotaustralin)	123·5–124·5	−19·15° (25, 1)	116–116·5
(− −)Methyllinamarin (epilotaustralin)	117·5–118·5	−34·6° (23,2)	121–121·5

The Identity of Lotaustralin

Three preparations of analyzed samples of a methyllinamarin have been described in the literature, all in 1938 and all from plants of the Leguminosae, subfamily Lotoideae. The first

[13] E. FISCHER and G. ANGER, *Ber.* **52**, 854 (1919).

source employed by Finnemore *et al.*,[6] which gave the accepted name to the glucoside, was called by them (with author citations added) *Lotus australis* Andr. var. *pubescens* Benth. The variety was described in 1848 but omitted from Bentham's *Flora Australiensis* in 1864, and according to Domin[14] the original specimen falls within the scope of typical *L. australis*. The *Lotus* studied by Finnemore *et al.*,[6, 15] however, probably was the same stock poison that was earlier[16, 17] called *L. australis* var. *Behrii*; and under either varietal name this plant is referable[14, 18] to the Australian taxon now recognized as the species *L. cruentus* Court (*L. coccineus* Schlechtendal). Finnemore *et al.*[6] also isolated a methyllinamarin sample from white clover, *Trifolium repens* L., and Henry[19] immediately afterwards reported a preparation from *L. arabicus* L. The physical properties of these natural methyllinamarin fractions are collected in Table 4. The following points should be noted when the data in Tables 3 and 4 are compared: (1) the coincidence in melting points of the acetates between the *Trifolium* glucoside and the more levorotatory synthetic methyllinamarin is deceptive, as we shall show, (2) the calculated elemental compositions of linamarin and methyllinamarin differ by only 2% in carbon content (1% for the acetates), for three out of the five samples of natural glucoside and acetate, analyses gave intermediate results; (3) the melting points of the *Trifolium* and *L. arabicus* glucosides were not lowered when the materials were admixed with

TABLE 4. PUBLISHED PHYSICAL PROPERTIES OF METHYLLINAMARIN (LOTAUSTRALIN) ISOLATES FROM PLANTS

Source	M.p.	$[\alpha]_D$ in H_2O (temp., concn.)	M.p. of tetraacetate
Lotus "australis"[6]	128–133°; 137–139°	−26·4° (20, 1)	127–128·5°
L. arabicus[19]	133°	−24·9° (—, 3)	—
Trifolium repens[6]	132–134°	—	121–124·5°

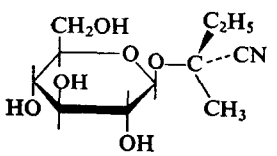
linamarin; (4) Finnemore *et al.*[6] recognized the presence of linamarin in their *Lotus* glucoside but hoped that their *Trifolium* sample was pure; however, Melville and Doak[20] found a similar isolate, m.p. 128–131°, from *T. repens* to contain 20–25 per cent of linamarin; (5) Butler's chromatographic analyses[5] confirm the occurrence of linamarin in *T. repens* and show, as does the work of Abrol and Conn,[21] substantial linamarin also in *L. arabicus*. The upshot of these considerations and the data in the tables is to demonstrate not only that all previously reported methyllinamarin specimens were seriously contaminated with linamarin but also that their melting points are useless as far as we know for determining which methyllinamarin was present. The fact that both *Lotus* glucosides were slightly less levorotatory than pure linamarin is the only available evidence and suggests that they contained the less levorotatory methyllinamarin.

[14] K. DOMIN, *Beiträge zur Flora und Pflanzengeographie Australiens*, pp. 736–738 (*Bibliotheca Botan.* **22**, Heft 89[II]), E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart (1925).
[15] H. FINNEMORE and J. M. COOPER, *Australian Vet. J.* **14**, 153 (1938).
[16] S. DIXON, *Trans. Proc. R. Soc. S. Australia* **8**, 14 (1886).
[17] F. B. GUTHRIE, *Agr. Gaz. N. S. Wales* **23**, 811 (1912).
[18] J. M. BLACK, *Flora of South Australia*, 2nd edition, p. 461, and *Supplement*, by H. EICHLER, p. 190, Govt. Printer, Adelaide (1948, 1965).
[19] T. A. HENRY, *J. Soc. Chem. Ind. (London)* **57**, 248 (1938).
[20] J. MELVILLE and B. W. DOAK, *New Zealand J. Sci. Technol.* **22B**, 67 (1940).
[21] Y. P. ABROL and E. E. CONN, *Phytochem.* **5**, 237 (1966).

As mentioned previously, we received through the kindness of Dr. B. A. Tapper and Dr. G. W. Butler a sample of a pure methyllinamarin isolated from *T. repens*, which we understand to have been chromatographically purified from the known[20,22] crystalline mixed glucosides. The physical properties of the sample (m.p. 124–125°, $[\alpha]_D - 19{\cdot}6°$) demonstrated and spectral comparisons confirmed that it was identical with our less levorotatory synthetic methyllinamarin. In view of the uncertainties about the occurrence of a particular methyllinamarin in *Lotus*, we here redefine lotaustralin as the less levorotatory 2-(β-D-glucopyranosyloxy)-2-methylbutyronitrile, which is proved to occur in *T. repens*. To the more levorotatory diastereomer, which is not known to occur in nature, we give the new name *epilotaustralin*.

The Configuration of Lotaustralin

Finnemore *et al.*[6] reported that their *Lotus* methyllinamarin was degraded by successive treatments with aqueous barium hydroxide (which converted the cyano group to carboxyl) and dilute hydrochloric acid to 2-hydroxy-2-methylbutyric acid. They isolated the hydroxy acid in crystalline form, but unfortunately did not measure its optical activity. When our synthetic methyllinamarins were hydrolyzed similarly, lotaustralin yielded (−)2-hydroxy-2-methylbutyric acid of good optical purity, and epilotaustralin the (+) acid. Assume for the moment that the intermediate carboxy glucosides were hydrolyzed mainly with glucosyl–oxygen cleavage. Then since Christensen and Kjær[23] (cf. also Nyburg *et al.*[24]) have shown that (−)2-hydroxy-2-methylbutyric acid has the (*R*) configuration, the equivalent (*R*) configuration is established for the asymmetric center in the aglucone of lotaustralin (I).



(I)

The accepted mechanism of acid-catalyzed hydrolysis of β-glucopyranosides is direct. Reversible protonation of the oxygen atom linking sugar and aglucone is followed by solvolysis of one of the bonds to carbon, predominantly the bond to the glucosyl residue for primary and secondary alkyl glucosides, to the aglucone portion for tertiary alkyl glucosides.[25] The net rate of hydrolysis by glucosyl–oxygen fission is subject to mild steric acceleration and is little affected by polar substituents on the aglucone[26] but highly sensitive to polar alterations in the sugar at the 2 and 6 positions;[25,27] in other words, the transition state resembles in its behavior the sum of the products of the rate-determining step, aglucone and a glucopyranosyl cation. The rate constants[26] for hydrolysis of *n*-alkyl β-glucopyranosides in 0·5 M sulfuric acid at 80° are near $3{\cdot}10^{-5}$ sec^{-1}. Significant aglucone–oxygen cleavage, competitive with glucosyl–oxygen cleavage, of the cation from a 2-glucosyloxy-2-methylbutyric acid can be ruled out, for both nucleophilic displacement and development of a positive charge at the

[22] J. Melville and J. G. Fraser, *New Zealand J. Sci. Technol.* **33A** (1), 56 (1951).
[23] B. W. Christensen and A. Kjær, *Acta Chem. Scand.* **16**, 2466 (1962).
[24] S. C. Nyburg, G. L. Walford and P. Yates, *Chem. Commun.* 203 (1965).
[25] C. Armour, C. Bunton, S. Patai, L. H. Selman and C. A. Vernon, *J. Chem. Soc.* 412 (1961).
[26] T. E. Timell, *Can. J. Chem.* **42**, 1456 (1964).
[27] B. Capon and B. C. Ghosh, *Chem. Commun.* 586 (1965).

fully substituted carbon atom next the carboxyl group are disfavored. However, acid-catalyzed solvolysis of the carboxylate ion, in which the aglucone residue can better give up electrons, must be considered too.[27] The steric course of replacement reactions at the α position of dialkylacetic acids seems to be unknown, and it is conceivable that ejection of glucose from our substrate upon ionization of the carboxyl group and protonation of the adjacent oxygen atom would give a real, inverted α-lactone, which might be hydrated with retention of configuration in the same way as β-butyrolactone in strong acid. Hence a way is needed of judging how fast this mode of decomposition of the glucoside can be. We have assembled in Table 5 hydrolysis rates *in water* of halides and corresponding glucosides and oxygen exchange rates (adapted to our standard conditions) in water of alcohols (*t*-butyl,[28,29] 1-phenylethyl[28] and benzyl[30] bromides; 1-phenylethyl chloride;[28] α-chloro[31] and α-bromo[32–35] phenylacetates; α-bromoisobutyrate;[36] other halides;[37] *t*-butyl,[38,39] *t*-pentyl,[39] 1-phenylethyl[40] and *sec*-butyl[41,42] alcohols; *t*-pentyl[43] and other[26] β-glucopyranosides). The α-halophenylacetic acids,[32,44,45] like α-bromobutyric acid,[46] are hydrolyzed at least two orders of magnitude more slowly than the corresponding anions. The closeness between the hydrolysis rate constants of α-bromopropionate and α-bromobutyrate ions,[47] respectively $2{\cdot}65.10^{-7}$ and $3{\cdot}3.10^{-7}$ sec^{-1} at 25°, as well as comparisons among the data for isopropyl,

TABLE 5. SOLVOLYSIS RATES OF GLUCOSIDES, ALCOHOLS AND HALIDES

	Pseudo-first-order rate constants, sec^{-1}			
R-	Bromides RBr H_2O, 25°	Chlorides RCl H_2O, 50°	Alcohols ROH 0·5 M H_2SO_4, 80°	β-D-Glucopyranosides $ROC_6H_{11}O_5$ 0·5 M H_2SO_4, 80°
$C_2H_5(CH_3)_2C$-	—	1·1	9.10^{-3}	$3{\cdot}5.10^{-2}$
$(CH_3)_3C$-	$7{\cdot}5.10^{-1}$	$5{\cdot}4.10^{-1}$	5.10^{-3}	$1{\cdot}0.10^{-2}$
$C_6H_5(CH_3)CH$-	$5{\cdot}5.10^{-1}$	$6{\cdot}7.10^{-1}$	6.10^{-3}	—
$C_6H_5CH(CO_2^-)$-	$2{\cdot}8.10^{-4}$	7.10^{-4}	—	—
$(CH_3)_2C(CO_2^-)$-	$1{\cdot}2.10^{-4}$	—	—	—
$C_6H_5CH_2$-	$1{\cdot}5.10^{-4}$	$2{\cdot}2.10^{-4}$	—	$2{\cdot}8.10^{-5}$ (glucosyl-O)
$(CH_3)_2CH$-	$3{\cdot}8.10^{-6}$	$6{\cdot}3.10^{-6}$	4.10^{-7} [R-=$C_2H_5(CH_3)CH$-]	$4{\cdot}5.10^{-5}$ (glucosyl-O)

[28] A. H. FAINBERG and S. WINSTEIN, *J. Am. Chem. Soc.* **79**, 1597, 1602 (1957).
[29] E. A. MOELWYN-HUGHES, *J. Chem. Soc.* 4301 (1962).
[30] P. M. LAUGHTON and R. E. ROBERTSON, *Can. J. Chem.* **34**, 1714 (1956).
[31] G. SENTER, *J. Chem. Soc.* **107**, 908 (1915).
[32] A. M. WARD, *J. Chem. Soc.* 1184 (1926).
[33] W. R. BULCRAIG and H. M. DAWSON, *J. Chem. Soc.* 80 (1943).
[34] S. WIDEQVIST, *Ark. Kem.* **19**, 551 (1962).
[35] K. C. KEMP and D. METZGER, *J. Org. Chem.* **33**, 4165 (1968).
[36] B. N. HENDY, W. A. REDMOND and R. E. ROBERTSON, *Can. J. Chem.* **45**, 2071 (1967).
[37] R. E. ROBERTSON, *Progr. Phys. Org. Chem.* **4**, 213 (1967).
[38] I. DOSTROVSKY and F. S. KLEIN, *J. Chem. Soc.* 791 (1955).
[39] R. H. BOYD, R. W. TAFT, JR., A. P. WOLF and D. R. CHRISTMAN, *J. Am. Chem. Soc.* **82**, 4729 (1960).
[40] E. GRUNWALD, A. HELLER and F. S. KLEIN, *J. Chem. Soc.* 2604 (1957).
[41] J. MANASSEN and F. S. KLEIN, *J. Chem. Soc.* 4203 (1960).
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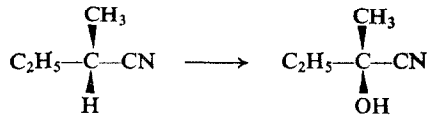
[43] S. VEIBEL and E. FREDERIKSEN, *Kgl. Danske Videnskab. Selskab, Matfys. Medd.* **19**, 1 (1941).
[44] M. J. YOUNG and A. R. OLSON, *J. Am. Chem. Soc.* **58**, 1157 (1936).
[45] H. M. DAWSON, W. R. BULCRAIG and G. F. SMITH, *J, Chem. Soc.* 90 (1944).
[46] J. ELORANTA, *Suomen Kemistilehti* **30B**, 225 (1957).
[47] J. ELORANTA, *Ann. Acad. Sci. Fenn., Ser. A II*, No. 70 (1956).

t-butyl and *t*-pentyl derivatives in Table 5 suggests that the solvolysis rates of the 2-bromo-2-methylpropionate and 2-bromo-2-methylbutryate ions are unlikely to differ from each other by more than a factor of 2. The oxygen exchange reaction of *t*-butyl alcohol in aqueous acid is an excellent match for the hydrolysis of *t*-butyl β-glucopyranoside, not only in the rates, which differ by a factor of 2 (Armour *et al.*[25] give a factor of nearly 6, apparently because they forgot to allow for their acid concentration in calculating the expected exchange rate), but also in activation energies[26, 38, 39, 43] and dependence on the acidity function.[25, 39] Thus we see from the oxygen exchange rate of *sec*-butyl alcohol that aglucone–oxygen fission can contribute at most a few per cent to hydrolysis of the isopropyl or *sec*-butyl glucosides. Benzyl β-glucopyranoside, which is hydrolyzed at the same speed as the ethyl derivative, evidently also reacts mainly by glucosyl–oxygen cleavage. The composite[27] rate constant[48] for hydrolysis of benzyl β-D-glucuronide in 0·5 M sulfuric acid at 80°, $8{\cdot}5.10^{-6}$ sec^{-1}, marks a sharper upper limit for the rate of benzyl–oxygen cleavage in benzyl glycosides under the specified conditions; a reasonable guess at an average constant for the latter process might be around 2.10^{-6} sec^{-1}.

The correlational implications of Table 5 can be used in two overlapping ways to help settle the configuration of lotaustralin. The single, sufficient primary datum is the hydrolysis rate of the α-bromoisobutyrate ion. We note the consistent similarity between the reactivities of corresponding *t*-butyl and l-phenylethyl derivatives in Table 5, a similarity that extends also to carbon–sulfur bond cleavage[49] (solvolysis rates of the dimethylsulfonium ions in water differ by a factor of 1·5), and affirm that the narrow range of hydrolysis rates of the α-bromophenylacetate, α-bromoisobutyrate and (by inference) α-bromo-α-methylbutyrate ions implies a like resemblance in rates of carbon–oxygen cleavage among derivatives of the corresponding α-hydroxy carboxylates. Experimentally, Emil Fischer reported in one of his last papers[50] that the β-D-glucopyranoside of (*S*)-mandelic acid, which Karrer *et al.*[51] synthesized from acetobromoglucose and silver (*S*)-mandelate, and whose methyl ester was hydrolyzed by emulsin to methyl (*S*)-mandelate,[50] furnished on hydrolysis in dilute acid (*S*)-mandelic acid of 95% optical purity. Since solvolysis of the α-halophenylacetate ions in water leads to almost complete racemization (see Ward[32] for summary and references), retention of configuration in Fischer's experiment is incompatible with aglucone–oxygen cleavage. Hydrolysis of the glucoside of 2-hydroxy-2-methylbutyric acid therefore should also preserve the aglucone–oxygen bond. Alternatively, we can argue that since the pK_a's of glucosides of these α-hydroxy acids can hardly be less than 3, in order for aglucone–oxygen fission to dominate during hydrolysis in 1 M or more concentrated acid the rate constant for acid-catalyzed aglucone–oxygen fission of the carboxylate ion must exceed the rate constant for acid-catalyzed glucosyl–oxygen fission of the carboxylic acid by a factor of 10^3. Since the latter rate constant must be at least 10^{-5} sec^{-1} under the standard conditions of Table 5, the former would be at least 10^{-2} sec^{-1}. The spread in reactivity both of halides and of glucosides (aglucone–oxygen cleavage) between *t*-butyl and benzyl derivatives is a good three orders of magnitude; it is unreasonable to suppose that a halo carboxylate would be near the bottom of this range and the corresponding glucoside at the top. This view subsumes the known result for the glucoside of mandelic acid and parallel prediction for 2-hydroxy-2-methylbutyric acid. Hence we regard the configuration of lotaustralin as proved.

[48] T. E. TIMELL, W. ENTERMAN, F. SPENCER and E. J. SOLTES, *Can. J. Chem.* **43**, 2296 (1965).
[49] J. B. HYNE and H. S. GOLINKIN, *Can. J. Chem.* **41**, 3139 (1963).
[50] E. FISCHER, *Hoppe-Seyler's Z. Physiol. Chem.* **107**, 176 (1919).
[51] P. KARRER, C. NAGELI and H. WEIDMANN, *Helv. Chim. Acta* **2**, 242, 425 (1919).

The biosynthesis of lotaustralin from L-isoleucine in *T. repens*[3] involves hydroxylation of the β carbon of the amino acid, whose configuration at that center is (*S*). According to the current view of the biosynthesis of cyanogenetic glycosides,[52] the actual substrate of the oxygenation would be (*S*)-2-methylbutyronitrile (II) and the product the aglucone of lotaustralin. (The fact[3] that radioactivity from generally labeled isoleucine-^{14}C given to *T. repens* does not enter into linamarin argues against extensive reversible dissociation of acetone and methyl ethyl ketone cyanohydrins during synthesis of their glucosides in the plant and hence suggests that the latter cyanohydrin maintains its original configuration. Also, the metabolism of hydrogen cyanide in *T. repens* has been looked at,[53] and no significant incorporation into cyanogenetic glucosides has been reported.[4]) Since the aglucone of lotaustralin is (*R*)-2-hydroxy-2-methylbutyronitrile (III), the hydroxylation occurs with retention of configuration. Retention indeed is the established stereochemical course of numerous steroid hydroxylations[54, 55] and the conversion of proline to hydroxyproline[56] in nature.



(II) (III)

The occurrence in the plant kingdom of epimeric pairs of cyanohydrin β-glucosides, prunasin and sambunigrin, dhurrin and taxiphyllin, does not necessarily tell us to expect epilotaustralin also, for the steric outcome of hydroxylations of benzyl[52] and *p*-hydroxybenzyl cyanides depends not only on whether retention or inversion takes place but also on which of the enantiotopic[57] hydrogen atoms is replaced. However, it would be rash to assume that a methyllinamarin in plants or fungi[58] always is lotaustralin. Compounds with the same basic stereochemistry as that assigned to epilotaustralin are known from plants: (*S*)-2-hydroxy-2-methylbutyric acid[23] occurs esterified in *Veratrum* (Liliaceae) and *Simarouba* (Simaroubaceae), 2(*S*)-hydroxy-2-methylbutylglycosinolate[59] is found in Capparidaceae and *Putranjiva* (Euphorbiaceae). In *Putranjiva* the hydroxy glucosinolate accompanies[60] the 2(*S*)-methylbutyl and 2(*S*)-butyl glucosinolates, belonging to the L-isoleucine series. Here the tertiary hydroxy compound is opposite in configuration to its deoxy counterpart; but the pathway by which the hydroxyl group enters the glucosinolate is unknown and may involve, for example, hydration of an unsaturated intermediate.

We have not determined whether the methyllinamarin in manioc is lotaustralin or epilotaustralin. Techniques for identification of the individual diastereomers on a micro scale remain to be tested in the future.

[52] K. HAHLBROCK, B. A. TAPPER, G. W. BUTLER and E. E. CONN, *Archs Biochem. Biophys.* **125**, 1013 (1968).
[53] S. BLUMENTHAL-GOLDSCHMIDT, G. W. BUTLER and E. E. CONN, *Nature* **197**, 718 (1963).
[54] M. HAYANO, *Oxygenases* (edited by O. HAYAISHI), Ch. 5, Academic Press, New York and London (1962).
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[59] B. W. CHRISTENSEN and A. KJÆR, *Acta Chem. Scand.* **17**, 279 (1963).
[60] A. KJÆR and P. FRIIS, *Acta Chem. Scand.* **16**, 936 (1962).

EXPERIMENTAL

Preparation of Manioc Extracts

Samples of manioc tubers were received from Mr. G. F. J. Pabst of the Herbarium Bradeanum, Rio de Janeiro, Brazil, from Dr. U. J. Grant of the Columbia Office of the Rockefeller Foundation, Bogotá, Colombia, and from Dr. Lino Vicarioli of the Ministry of Agriculture, San José, Costa Rica. The samples were shipped by air and were analyzed and extracted upon arrival at this Laboratory. Analyses for HCN content[12] gave the results in the last column of Table 2. The tubers were extracted by the procedure previously used.[2] Portions of 600 g were slurried with 1500 ml of 90% EtOH in a Waring Blendor. After 10 min of boiling, the mixture was filtered, and the filtrate was concentrated at 35–40° in a rotary vacuum evaporator to 20–25 g of viscous sirup.

Thin-Layer and Paper Chromatography

Solutions were prepared for chromatography by treating 0·5 g of concentrate with 6 ml MeOH. After 1–2 hr of mixing, a small quantity of flocculent particles was filtered off. Plates of silica gel G were used for the TLC, which were developed with 5:1 $CHCl_3$–MeOH and visualized by spraying with 2% α-naphthol in EtOH and then with conc. H_2SO_4 and heating. The ratio of the R_f value of lotaustralin to that of linamarin with 5:1 $CHCl_3$–MeOH was *ca.* 1·2.

Paper (Whatman No. 1) chromatograms were developed by descending chromatography and visualized by dipping in a solution of $AgNO_3$ in aqueous acetone and spraying with NaOH in aqueous EtOH[61].

Gas Chromatography

Gas chromatograms were run on 6-mm stainless-steel columns packed with 10% or 20% SE-30 on silanized Chromosorb W (80–100 mesh) in an Aerograph "Autoprep", Model A-700, with a hydrogen flame ionization detector. In trial experiments on known samples of linamarin and lotaustralin, 10-mg portions of the glucosides in 0·4 ml of dry pyridine were treated with 0·2 ml of hexamethyldisilazane and 0·1 ml of trimethylchlorosilane.[9] When mixtures of these preparations were placed on a 3 m column at 210°, well separated peaks were obtained for the TMS ethers (Fig. 1, Chromatogram A). The TMS derivatives were collected as viscous liquids in 1 mm capillary tubes inserted at the exit port of the column. Even when capillary tubes were used, there was evidence of incomplete condensation due to fogging. However, when a sample of the TMS preparation equivalent to 2·3 mg of linamarin was placed on a 3 m SE-30 column in six 25-μl portions, 88% of the TMS derivative was collected. Lengths of capillary tubing of 25–30 cm (containing a right angle bend to prevent flow down the tubing during successive collections) were used, but condensation appeared to take place largely in the first 1–2 cm.

In a preliminary experiment, a portion of the manioc extract was chromatographed, after trimethylsilylation, on a 1·8 m SE-30 column. The chromatogram contained a strong peak for linamarin, in addition to peaks for α- and β-glucose and fructose, but no indication of a methyllinamarin peak was observed. A 2-g sample of the manioc extract was treated with 25 ml MeOH, and after 2 hr of mixing the undissolved flocculent material was filtered off and washed with 5 ml of MeOH. A 2-ml portion of the resulting 30 ml of solution was placed on a column of 35 g of silica gel (Merck-Darmstadt). The column was eluted with 5:1 $CHCl_3$–MeOH and concentration of all the fractions containing linamarin and methyllinamarin afforded 19·4 mg of residue. After trimethylsilylation of this material in 0·8 ml of dry pyridine with 0·4 ml of hexamethyldisilazane and 0·2 ml trimethylchlorosilane, gas chromatography on a 3·6 m column at 222° showed a weak peak for methyllinamarin, in addition to the strong linamarin peak (Fig. 1, Chromatogram B).

10-μl portions of a trimethylsilylated mixture prepared in this way from Colombian manioc were injected into the 3·6 m column, and the effluents corresponding to the methyllinamarin peak were collected in a capillary tube. Rechromatography of the condensate from *ca.* fifty such collections, washed from the capillary tube with hexane, demonstrated that it contained approximately equal quantities of the linamarin and methyllinamarin derivatives (Fig. 1, Chromatogram C). Collection of the condensate corresponding to the methyllinamarin peak from samples of this mixture injected into the column in hexane yielded a product that contained a high proportion of the TMS ether of a methyllinamarin (Fig. 1, Chromatogram D).

The mass spectra of the TMS derivatives were obtained by introducing the samples in hexane solution into a Consolidated Electrodynamics Corporation CEC 21-110 mass spectrometer. The high resolution data were obtained by peak matching, with perfluorokerosene as a reference.

Samples were prepared for the quantitative determinations (Tables 1 and 2) by chromatography of 2-g aliquots of the manioc extracts on 35 g of silica gel, as above.

Preparation of Isomers of 2-(2,3,4,6-*Tetra*-O-*acetyl*-β-D-*glucopyranosyloxy*)-2-*methylbutyronitrile*

A mixture of 4·93g (0·012 mole) of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (acetobromoglucose), 4·69 g (0·048 mole) of 2-hydroxy-2-methylbutyronitrile (methyl ethyl ketone cyanohydrin), 3·12 g

[61] R. J. BLOCK, E. L. DURRUM and G. ZWEIG, *Paper Chromatography and Paper Electrophoresis*, 2nd edition, p. 178, Academic Press, New York (1958).

(0·012 mole) of $Hg(CN)_2$ and 25 ml CH_3NO_2 was stirred at 45° for 20 hr. TLC on silica gel G (3:1 $CHCl_3$–EtOAc) indicated that little, if any, acetobromoglucose remained. After filtration, the solution was concentrated under reduced pressure, and the residue was stirred with 50 ml H_2O for 15 min. The aqueous layer was separated, and the sirup remaining was again stirred with 50 ml of H_2O. The combined aqueous washings were extracted with CCl_4, and the CCl_4 extract was added to the sirup. After the resulting solution had been dried (Na_2SO_4), concentration afforded 6·3 g of yellow sirup.

The sirup was fractionated on a column of 500 g of silica gel (Merck-Darmstadt). The column was initially eluted with 20% ether–benzene, and the ether concentration was gradually increased to 35% during the fractionation. The fractions from the column were monitored by recording the NMR spectra of the fractions in CCl_4 at 60 and 100 MHz. By combining appropriate fractions, two pure acetates, representing the epimeric forms of the tetraacetyl β-glucoside, were obtained. The singlet for the 2-methyl group in the NMR spectrum of the more levorotatory epimer, which separated from the column first, appeared at 8·38τ. Concentration of the fractions containing this isomer yielded 378 mg of crystalline (− −)2-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyloxy*)-2-*methylbutryronitrile* (epilotaustralin tetraacetate), which could be recrystallized from MeOH as colorless needles; m.p. 121–121·5°; $[\alpha]_D^{28}$ −6·75° (*c* = 2·96, $CHCl_3$). (Found: C, 52·90; H, 5·96; N, 3·25. $C_{19}H_{27}O_{10}N$ required: C, 53·14; H, 6·34; N, 3·26%.)

The signal for the 2-methyl group in the NMR spectrum of the less levorotatory epimer was located at 8·47τ. From the fractions containing this isomer there was obtained 67 mg of crystalline (−)2-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyloxy*)-2-*methylbutryonitrile* (lotaustralin tetraacetate), which crystallized from aqueous MeOH as colorless needles; m.p. 116–116·5°; $[\alpha]_D^{28}$ −2·88° (*c* = 2·08, $CHCl_3$). (Found: C, 53·00; H, 6·00; N, 3·25. $C_{19}H_{27}O_{10}N$ required: C, 53·14; H, 6·34; N, 3·26%.)

Intermediate fractions from the silica gel column contained mixtures of the isomeric tetraacetates, and additional quantities of the pure isomers could be obtained by refractionation. A crystalline carbohydrate (985 mg) was obtained from fractions collected from the column before the tetraacetate fractions. Its melting point and spectra indicated that it was the 2-cyano-1,3-dioxolane derivative,3,4,6-tri-*O*-acetyl-1,2-*O*-(1-cyanoethylidene)-α-D-glucopyranose, which has been shown to form from acetobromoglucose and $Hg(CN)_2$ in CH_3NO_2.[62]

In another preparation with similar quantities, but in which less $Hg(CN)_2$ (0·008 mole) was used, 500 mg of the more levorotatory tetraacetate and 284 mg of its epimer were obtained.

Deacetylation of Glucosides

To a solution of 500 mg of the more levorotatory 2-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyloxy)-2-methylbutyronitrile in 15 ml of absolute MeOH were added 10 drops of a solution of $Ba(OMe)_2$, prepared by dissolving 3·8 g anhydrous BaO in 100 ml MeOH. After the solution had stood 18 hr at room temperature, TLC (silica gel G; 10:1 benzene–MeOH) indicated complete reaction. A small quantity of MeOH-washed Dowex 50W-X8 cation exchange resin was added to remove excess Ba^{2+}. Concentration of the filtered solution and crystallization of the resulting sirup (358 mg) from ethyl acetate gave 151 mg (50% yield) of (− −)2-(β-D-*glucopyranosyloxy*)-2-*methylbutyronitrile* (epilotaustralin) as colorless crystals; m.p. 117·5–118·5°; $[\alpha]_D^{23}$ −34·59° (*c* = 2·07, H_2O). (Found: C, 50·55; H, 7·29; N, 5·47. $C_{11}H_{19}O_6N$ required: C, 50·57; H, 7·33; N, 5·36%.)

To a solution of 284 mg of the less levorotatory tetraacetate in 5 ml of absolute methanol were added 7 drops of the $Ba(OMe)_2$ solution. After 24 hr at 5°, the product was isolated by the procedure described above. From the EtOAc solution of the concentrate there was obtained 124·5 mg (72% yield) of crystalline (−)2-(β-D-*glucopyranosyloxy*)-2-*methylbutryonitrile* (lotaustralin). Recrystallization from EtOH–hexane afforded fine, colorless needles; m.p. 123·5–124·5°; $[\alpha]_D^{25}$ −19·15° (*c* = 1·07, water). (Found: C, 50·58; H, 7·55; N, 5·37. $C_{11}H_{19}O_6N$ required: C, 50·57; H, 7·33; N, 5·36.%) A sample of natural lotaustralin from white clover gave m.p. 124–125° and $[\alpha]_D^{23}$ −19·59° (*c* = 0·98, water). The i.r. and NMR spectra of the natural and synthetic materials were identical.

The NMR spectra of both isomers contained a doublet for the anomeric proton at 5·25τ with a splitting of 7 Hz, characteristic of β-glucosides.[2]

Hydrolysis of Glucosides to 2-Hydroxy-2-methylbutyric Acid

A solution of 140 mg of (− −)2-(β-D-glucopyranosyloxy)-2-methylbutyronitrile in 4 ml of sat $Ba(OH)_2$ was heated at 100° for 3 hr. CO_2 was bubbled through the cooled solution, and the $BaCO_3$ removed. Conc. HCl (1 ml) was added to the filtrate, and after the solution had been heated at 100° for a further 2·5 hr, it was cooled and extracted with Et_2O. Crystallization of the concentrate from the Et_2O extracts from hexane yielded fine, colorless needles of (*S*)-2-hydroxy-2-methylbutyric acid; 26·4 mg, m.p. 73–74·5°, $[\alpha]_D^{28}$ +8·29° (*c* = 1·69, $CHCl_3$).

A sample of (*S*)-2-hydroxy-2-methylbutyric acid obtained from the hydrolysis of glaucarubin[63] gave

[62] B. COXON and H. G. FLETCHER, JR., *J. Am. Chem. Soc.* **85**, 2637 (1963).
[63] E. A. HAM, H. M. SCHAFER, R. G. DENKEWALTER and N. G. BRINK, *J. Am. Chem. Soc.* **76**, 6066 (1954).

m.p. 73–74° and $[\alpha]_D^{27}$ +8·78° (c=1·89, $CHCl_3$). The glaucarubin was extracted from press cake of *Simarouba glauca* seeds.

(−)2-(β-D-Glucopyranosyloxy)-2-methylbutyronitrile (82·5 mg) was hydrolyzed with $Ba(OH)_2$ and HCl by the procedure used above. Purification of the concentrate from the Et_2O extracts by sublimation gave 10·2 mg of (*R*)-2-hydroxy-2-methylbutyric acid as white crystals; m.p. 74–74·8°, $[\alpha]_D^{25}$ −6·2° (c=1·02, $CHCl_3$). Christensen and Kjær[23] reported m.p. 73·5–74·5° and $[\alpha]_D^{25}$ −8·5° (c=3·0, $CHCl_3$).

The identities of the (*S*)- and (*R*)-2-hydroxy-2-methylbutyric acids from the glucosides were confirmed by the congruence of their i.r. spectra to the spectrum of the acid from glaucarubin.

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