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ASCORBALAMIC ACID: AN ASCORBIGEN-LIKE PLANT CONSTITUENT YIELDING IN HOT ACID 3-(2-FUROYL)ALANINE*

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Key Word Index—*Brassica oleracea;* Crucifereae; Brussels sprouts; ascorbalamic acid; ascorbic acid; ascorbigens; aspartic acid; 3-chloroalanine; 3-(2-furoyl)alanine; leucodrin; pyrrolidonecarboxylic acid.

Abstract—Ascorbalamic acid ($C_9H_{13}NO_8$) was isolated from *Brassica oleracea* L. MS study of various methylated derivatives suggested a structure (Ia) derivable by C–C coupling of C-3 of alanine with C-2 of ascorbic acid, followed by lactone → lactam rearrangement. Other derivatives provided supporting evidence, as did study of the reaction of L-3-chloroalanine with L-ascorbic acid *in vitro*. On treatment with hot 6 M HCl, ascorbalamic acid yielded L-aspartic acid and 3-(2-furoyl)alanine. For identification of the latter, DL-3-(2-furoyl)-alanine and its N-2,4-dinitrophenyl and N-acetyl methyl ester derivatives were synthesized. Unlike ascorbigens, ascorbalamic acid is probably present in the living plant. It seemed to be present in all crucifers examined, but to have a capricious distribution in other orders. During permethylation, rearrangements of ester groups were observed, both with ascorbalamic acid and with pyrrolidonecarboxylic acid as a model.

INTRODUCTION

A phenol–acetic acid–water extract of Savoy cabbage was fractionated by electrophoresis in the same solvent mixture.[1,2] The non-migrating fraction, comprizing most of the weakly acidic and neutral low-molecular constituents of the plant, was subjected to acid hydrolysis. On the Beckman–Spinco Amino Acid Analyzer, this hydrolysate showed a novel zone (brown (440 nm) with ninhydrin) emerging immediately after methionine.[3] Similar treatment of broad-bean leaves[1,2,4] and of chicory leaves[4] had yielded none of this, nor was the novel zone ever seen during analyses for the free amino acids of cabbage or of any other plant material. In this paper we describe our progress towards isolation, structural elucidation and synthesis of the precursor substance(s) (which we name 'ascorbalamic acid') and towards characterization of its acid-degradation products, particularly the novel amino acid 3-(2-furoyl)alanine.

RESULTS AND DISCUSSION

Isolation and Preliminary Characterization

Ascorbalamic acid, besides being extractable from the fresh plant material by cold phenol–acetic acid–water mixtures, could be extracted with cold 5% (w/v) aqueous tri-

* Additional experimental details are in Annexes 1–4, deposited as a Supplementary Publication [SUP 90003 (36 pp.)] at the National Lending Library, Boston Spa, Yorkshire, LS23 7BQ, England from whom copies can be obtained.

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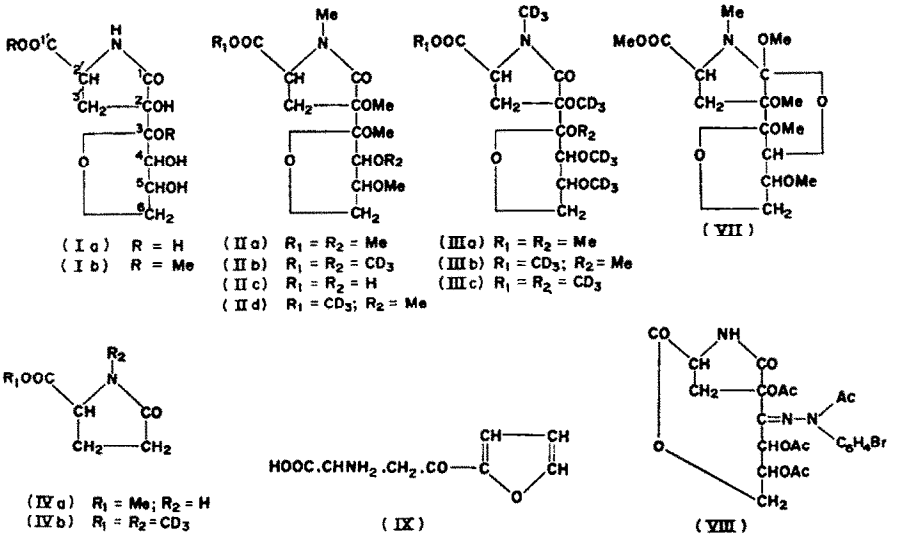
[1] A. C. JENNINGS, A. PUSZTAI, R. L. M. SYNGE and W. B. WATT, *J. Sci. Food Agric.* **19**, 203 (1968).
[2] E. M. W. CLARKE, G. M. ELLINGER and R. L. M. SYNGE, *J. Sci. Food Agric.* **19**, 214 (1968).
[3] D. H. SPACKMAN, W. H. STEIN and S. MOORE, *Analyt. Chem.* **30**, 1190 (1958).
[4] J. EAGLES, W. M. LAIRD, S. MATAI, R. SELF, R. L. M. SYNGE and A. F. DRAKE, *Biochem. J.* **121**, 425 (1971).

chloroacetic acid or with boiling 80 % (v/v) aqueous ethanol. Since all these treatments largely preclude action by enzymes, we infer that, unlike the ascorbigens (see below), ascorbalamic acid exists preformed in the living plant. Isolative purification from *Brassica* extracts was controlled by determining the novel amino acid after hot-acid treatment of fractions. During fractionation the precursor behaved at all stages as if it were a single substance. From the isolative procedure developed (Scheme 4), as well as from the properties and colour reactions of the colourless glassy product obtained (see Experimental), we could conclude that it was an acid (p*K ca.* 4), devoid of ionizing basic groups, MW (if monobasic) in the 200s and embodying the –NH– function (reaction of Rydon and Smith) and several vicinal –OH groups (electrophoresis in borate). It had no notable UV spectrum and several colour reactions for carbohydrate, amino-sugar and ureido derivatives were negative. It charred in the mass spectrometer without giving any spectrum.

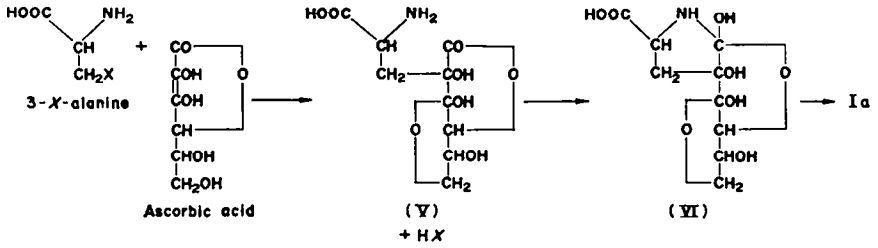


Substitution by Methylation and Deuteriomethylation

Positive evidence for the structure emerged when permethylation,[5] followed by high-resolution mass spectrometry (HRMS) suggested an atomic composition $C_{15}H_{25}NO_8$ for the proposed molecular ion at $m/e = 347{\cdot}1571$ (calc. value 347·1580). Low-resolution mass spectrometry (LRMS) of the perdeuteriomethylated substance indicated that six methyl groups had been introduced into the molecule, suggesting $C_9H_{13}NO_8$ for the parent acid. The fragmentation observed for the hexamethyl derivative (with molecular formulae by HRMS and with the number of extraneous methyl groups in each fragment) is given in Table 1 (II*a* and III*c*). By considering this evidence, and, in addition, the fact that hot HCl treatment gave aspartic acid in significant quantity besides the novel amino acid, we were led, by analogy with the ascorbigen studies of Kiss and Neukom[6–8] (see also review by Feldheim[9]), to consider structure Ia for ascorbalamic acid. This could arise by C–C

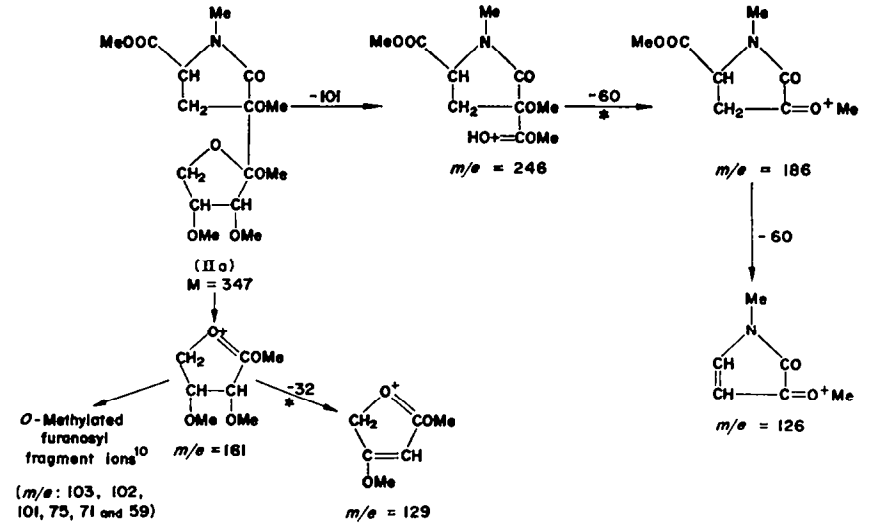
[5] B. C. DAS, S. D. GERO and E. LEDERER, *Biochem. Biophys. Res. Commun.* **29**, 211 (1967).
[6] G. KISS and H. NEUKOM, *Helv. Chim. Acta* **49**, 989 (1966).
[7] G. KISS, *Über die Struktur des Ascorbigens, Diss. No.* 4015, *Eidg. Tech. Hochschule.* Juris Druck & Verlag, Zürich (1967).
[8] G. KISS and H. NEUKOM, *Experientia* **24**, 326 (1968).
[9] W. FELDHEIM, *Industr. Obst.-Gemüseverwertung* **55**, 27 (1970).

coupling of C-3 of a substituted alanine with C-2 of ascorbic acid, with subsequent lactone → lactam rearrangement (Scheme 1). The hexamethyl derivative would then be IIa (all structural formulae in this paper disregard stereochemistry).



SCHEME 1. POSSIBLE BIOSYNTHETIC ROUTE TO ASCORBALAMIC ACID.

We were thus led to propose Scheme 2 for the MS fragmentation of the hexamethyl derivative. This shows two main pathways. The first pathway is initiated by cleavage of the inter-ring C–C bond, yielding an intense ion at $m/e = 161$ (base peak). Further fragmentation of this ion produced the common methylated-sugar fragment ions discussed by Kochetkov and Chizhov.[10] The second pathway involves a complex fission of the furanose ring, with loss of 101 m.u. from the molecular ion, giving rise to the substituted-pyrrolidone ion ($m/e = 246$) (25%). Further decomposition by two losses of methyl formate gave ions at $m/e = 186$ and 126.



*Metastable ion identified

SCHEME 2. FRAGMENTATION SCHEME FOR PERMETHYLATED ASCORBALAMIC ACID (SEE TABLE 1).

On this argument, C-3 of Ia is a potential carbonyl group. Treatment of ascorbalamic acid with methanolic HCl would be expected to give Ib, and this, on subsequent perdeuteriomethylation, to give IIIa. On carrying out these operations, the product gave a molecular ion (M = 362) with fragmentations corresponding to IIIb (Table 1: IIIb). A total of

[10] N. K. KOCHETKOV and O. S. CHIZHOV, *Adv. Carbohyd. Chem.* **21**, 39 (1966).

five deuteriomethyl groups were incorporated, three of which were situated on the pyrrolidone ring, and two on the furanose ring. Unfortunately, because of an anomalous deuterium exchange, the ion at $m/e = 257$ in this spectrum cannot be used to confirm unambiguously the presence of a glycosidic methyl. However, by comparing the spectrum of IIIc with that of IIIb and considering the chemical treatment, there is certainly an –OMe group on the furanose ring and it is most likely to be attached to C-3. Further studies of the deuterium-exchange processes operating here would be necessary to eliminate the ambiguity. The ester methyl group was thought to be rearranged during perdeuteriomethylation, and we got support for this view by showing mass spectrometrically that methyl L-pyrrolidone carboxylate (IVa) was rearranged to IVb on perdeuteriomethylation. Rearrangement of ester linkages under permethylation conditions seems to depend upon the neighbouring groups in the molecule; further study is needed to define the extent of its occurrence.

TABLE 1. ABBREVIATED MS RESULTS ON ASCORBALAMIC ACID DERIVATIVES

Permethylation (IIa)			Permethylation, saponification, perdeuteriomethylation (IId)			Methanolic-HCl treatment followed by perdeuteriomethylation (IIIb)			Perdeuteriomethylation (IIIc)		
m/e	%	Atomic formula	*m/e*	%	CD_3gps	*m/e*	%	CD_3gps	*m/e*	%	CD_3gps
347(M)	0·25	$C_{15}H_{25}NO_8$	350(M)	2·4	1	362(M)	3·5	5	365(M)	1·8	6
246	25·0	$C_{10}H_{16}NO_6$	249	31·8	1	257	24·2	*	258	18·8	4
186	21·2	$C_8H_{12}NO_4$	189	25·7	1	195	15·0	3	195	8·1	3
161	100	$C_7H_{13}O_4$	161	100	0	167	100	2	170	100	3
129	35·8	$C_6H_9O_3$	129	65·1	0	132	20·8	1	135	47·5	2
126	20·6	$C_6H_8NO_2$	126	25·7	0	132	20·8	2	132	6·5	2
103	20·0	$C_4H_7O_3$	103	36·4	0	109	9·1	2	109	13·1	2
102	18·7	$C_5H_{10}O_2$	102	28·8	0	108	18·3	2	108	12·3	2
101	25·9	$C_5H_9O_2$	101	39·4	0	107	15·8	2	107	74·5	2
75	22·8	$C_3H_7O_2$	75	33·3	0	81 78	19·2 15·8	2 1	81	68·8	2
71	45·3	C_4H_7O	71	81·7	0	74 71	25·0 37·5	1 0	74	67·1	1
59	5·3	C_3H_7O & $C_2H_3O_2$	59	17·6	0	62	13·3	1	62	10·6	1

Atomic formulae by HRMS (peak-matching technique). % = intensity relative to base peak (100) of spectrum. * This anomalous *m/e* value is discussed in text.

In the biosynthetic sequence proposed in Scheme 1, the mixed ortho-lactone-lactam VI is a probable intermediate.[11–15] It is isomeric with Ia and should give a different hexamethyl derivative (VII) on permethylation. VII should saponify with cold alkali[11–15] to give IIc, which on perdeuteriomethylation would give IIb. When these operations were carried out on permethylated ascorbalamic acid, IId resulted. The MS of this compound (Table 1: IId) shows a molecular ion at M = 350, consistent with only one deuteriomethyl

[11] M. BERGMANN, E. BRAND and F. WEINMANN, *Hoppe-Seyl. Z. Physiol. Chem.* **131**, 1 (1923).
[12] M. BERGMANN and E. BRAND, *Chem. Ber.* **56**, 1280 (1923).
[13] A. P. PHILLIPS and R. BALTZLY, *J. Am. Chem. Soc.* **69**, 200 (1947).
[14] A. STOLL, A. HOFMANN and TH. PETRZILKA, *Helv. Chim. Acta* **34**, 1544 (1951).
[15] A. HOFMANN, H. OTT, R. GRIOT, P. A. STADLER and A. J. FREY, *Helv. Chim. Acta* **46**, 2306 (1963).

substituent. The fragments displaying this substituent are *m/e* 249 and 189; this indicates that the substituent is on the pyrrolidone ring (Scheme 2). Again, although the MS data are not unambiguous, the observations with pyrrolidonecarboxylic acid (above) suggest that only the carboxyl group has become deuteriomethylated. This we take as evidence against the permethylated derivative having structure VII, which is also made unlikely by its fragmentation behaviour. Structure IIa can therefore be assigned with some confidence to the hexamethyl derivative. With ascorbalamic acid itself, while V is excluded because it has a free $-NH_2$ group, which would prevent its passage through Dowex 50 (H^+), we have as yet no grounds for distinguishing between Ia and VI, which could be in tautomeric equilibrium, each tautomer having the possibilities of stereochemical complexity discussed below.

All the above structural information was derived by MS study of the hexamethyl derivative, prepared and isotopically substituted in various ways, from a few mg of ascorbalamic acid isolated from cabbage. A larger preparation from purple sprouting broccoli yielded further supporting evidence and was used for studies of the acid-degradation products and of the stereochemistry, as follows.

Other Substitution Products

We have not yet obtained any satisfactorily crystalline derivatives of ascorbalamic acid, but two types of substitution have given further evidence in support of structure Ia.

Acetylation with acetic anhydride in pyridine gave a product showing on LRMS the molecular ion and fragment ions corresponding to a tetra-acetyl derivative (see Annex 2). The expected product would be O^{2-5}-tetraacetyl-Ia.

Reaction with p-*bromophenylhydrazine* in aqueous ethanol was sluggish, but tended towards an equilibrium mixture which, on electrophoresis at pH 6, gave an anionic product slower than the accompanying unchanged ascorbalamic acid, and a neutral product. We regard the slow-anionic product as a 'normal' *p*-bromophenylhydrazone of ascorbalamic acid. The neutral product, after acetylation, gave on LRMS a molecular-ion group (M = 581, 583) with peak intensities consistent with the presence of one Br atom. This may tentatively be assigned the lactonized structure VIII ($C_{23}H_{24}BrN_3O_{10}$) (cf.[16,17]). These substitutions with *p*-bromophenylhydrazine are further supporting evidence for the reducing sugar function in Ia.

Hot-acid Degradation Products

On refluxing overnight in 6 M HCl, ascorbalamic acid gave on amino acid analysis two major zones, one in the position of aspartic acid, the other the novel zone mentioned in the Introduction. Trace zones corresponding to glycine, alanine, serine, threonine and glutamic acid were also always present, and are easily conceived as breakdown products of Ia and/or furoylalanine (see Experimental).

The material responsible for the novel zone was isolated by volatile-buffer ion-exchange chromatography.[18,19] HRMS of the molecular ion of the *N*-acetyl methyl ester suggested its derivation from $C_8H_9NO_4$. LRMS of this derivative (cf.[20]) and the UV spectrum of the

[16] A. Fóti, F. Ruff and Á. Gerecs, *Magyar Kém. Fol.* **70**, 314 (1964); *Acta Chim. Hung.* **43**, 439 (1965).
[17] Z. M. El-Shafei and S. H. El Ashry, *Carbohyd. Res.* **3**, 184 (1966).
[18] P. Padieu, N. Malekina and G. Schapira, *3rd Amino Acid Colloquium*, p. 71, Technicon Instruments, Chertsey (1965).
[19] M. Redford-Ellis and M. N. Kelson, *J. Chromatog.* **59**, 434 (1971).
[20] C.-O. Andersson, R. Ryhage and E. Stenhagen, *Ark. Kemi.* **19**, 417 (1962).

free amino acid (closely resembling that of furyl methyl ketone[21]) suggested that it was 3-(2-furoyl)alanine (IX). DL-IX was synthesized by reacting furacyl bromide with the Na derivative of diethyl acetamidomalonate, and agreed in all of the above-mentioned properties. The 'natural' and synthetic *N*-2,4-dinitrophenyl (DNP) derivatives likewise agreed in behaviour. The postulated structure was confirmed by NMR spectrometry. A brown colour with ninhydrin is not commonly given by primary amines in solution as well as when dried on cellulose. However, 'orange' and 'brown' colours with ninhydrin are given by the somewhat-related stizolobic and stizolobinic acids,[22] although, in studies giving revised structures and syntheses for these amino acids, a 'bluish-purple' colour with ninhydrin is also mentioned.[23] In any case, 3-(2-furoyl)alanine evolved N_2 normally during the Van Slyke amino-N determination with nitrous acid.[24]

The furoylalanine (IX) is plausibly derived from Ia by hydrolysis of the lactam ring, decarboxylation of C-1 and dehydrative cyclization (cf.[25,25a]). The process is closely analogous with the formation of furosine from fructose-lysine[26,27] and of *N*-furacylglycine, an isomer of furoylalanine, from fructose-glycine.[28–31] The aspartic acid is formed mainly more directly from Ia but in part from furoylalanine (see Experimental).

The aspartic acid was isolated as the *N*-DNP derivative,[32] identified mass spectrometrically (see Experimental) and shown by its circular dichroism[33] to have predominantly the L-configuration. This technique has general value for establishing configuration of amino acids on the micro scale.

Stereochemistry of the Ascorbigens and Related Compounds

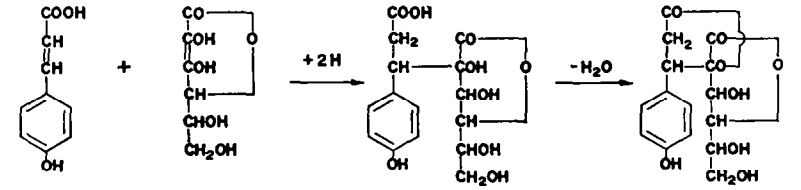
Our only firm stereochemical evidence so far is that C-2′ of ascorbalamic acid (Ia) has the configuration of the 'natural' series of L-amino acids. Even if, as seems probable, the configuration at C-4 and C-5 is determined by origination from L-ascorbic acid, both Ia and VI have possibilities of existing in α-, β- or open-chain forms at the reducing-sugar C-3, and of diastereoisomerism at C-2, where the C-C coupling introduces a new centre of asymmetry. This gives two possible families (based on the configuration at C-2), each containing six isomers possibly in equilibrium with one another (nine, if diastereoisomerism at the new asymmetric centre C-1 in VI is taken into account). We observed no mutarotation on dissolving the glassy ascorbalamic acid in water; no meaningful deduction from this is possible at present.

Kiss and Neukom[6,7] found their synthetically prepared skatyl ascorbigen to exist in two diastereoisomeric forms about C-2 of the ascorbic acid moiety, whereas only one of these

[21] R. F. RAFFAUF, *J. Am. Chem. Soc.* **72**, 753 (1950).
[22] S. HATTORI and A. KOMAMINE, *Nature, Lond.* **183**, 1116 (1959).
[23] S. SENOH, S. IMAMOTO, Y. MAENO, T. TOKUYAMA, T. SAKAN, A. KOMAMINE and S. HATTORI, *Tetrahedron Letters* 3431, 3439 (1964).
[24] J. P. PETERS and D. D. VAN SLYKE, *Quantitative Clinical Chemistry*, Vol. 2, p. 385, Baillière, Tindall & Cox, London (1932).
[25] K. TOKUYAMA, K. GOSHIMA, N. MAEZANO and T. MAEDA, *Tetrahedron Letters* 2503 (1971).
[25a] M. S. FEATHER, D. W. HARRIS and S. B. NICHOLS, *J. Org. Chem.* **37**, 1606 (1972).
[26] K. HEYNS, J. HEUKESHOVEN and K.-H. BROSE, *Angew. Chem.* **80**, 627; *Internat. Edn.* **7**, 628 (1968).
[27] P. A. FINOT, J. BRICOUT, R. VIANI and J. MAURON, *Experientia* **24**, 1097 (1968).
[28] S. H. LIPTON, H. FINEGOLD and R. C. DUTKY, *J. Agric. Food Chem.* **19**, 125 (1971).
[29] S. H. LIPTON and R. C. DUTKY, *Chem. & Ind.* 1099 (1971).
[30] S. H. LIPTON and C. E. BODWELL, *J. Agric. Food Chem.* **20**, 232 (1972).
[31] S. H. LIPTON and R. C. DUTKY, *J. Agric. Food Chem.* **20**, 235 (1972).
[32] A. V. PORTUGAL, R. GREEN and T. M. SUTHERLAND, *J. Chromatog.* **12**, 183 (1963).
[33] A. F. DRAKE, unpublished work.

forms could be demonstrated in crushed cabbage leaves. Two diastereoisomers resulted on synthesis of the *p*-hydroxybenzyl ascorbigen,[8] but one of these was present in overwhelming proportions. Elliott and Stowe[33a] reported no chromatographic heterogeneity in their supposed *N*-sulphonoskatyl ascorbigen from woad.

Kiss[7] has pointed out that leucodrin, known since 1886 from the South African proteaceous tree *Leucadendron* but only structurally elucidated in recent years, has a relationship with the ascorbigens. This has not been discussed by those involved in studies of the leucodrin group. Scheme 3 shows how leucodrin (XI) could arise by reductive C–C coupling of *p*-coumaric acid with ascorbic acid to give X, with subsequent cross-lactonization. Configurations at C-4 and C-5 of the 'ascorbic' moiety are consistent with origination from L-ascorbic acid.[34,35] Conocarpin, from *Leucospermum* (also Proteaceae), is a diastereoisomer of leucodrin, whose configuration is established and awaits publication.[36,*] Perold and colleagues also expect to publish information about two further members of this group, lyrigin and reflexin.[35,*]



SCHEME 3. POSSIBLE BIOSYNTHETIC ROUTE TO LEUCODRIN, ETC.

Synthesis

By reacting L-3-chloroalanine with excess ascorbic acid in aqueous solution at pH 4·2 and 100° we have obtained molar yields of ascorbalamic acid of *ca.* 3% (based on the chloroalanine taken). The identification was based simply on the furoylalanine resulting from hot-acid treatment, but it is likely that Ia and/or VI had been formed, as the reaction mixture had been passed through (H^+)-sulphonated-polystyrene resin; this should have held back V. *S*-Methyl-L-cysteine (±)-*S*-oxide gave a significant but smaller yield (see below); L-cysteine, *S*-methyl-L-cysteine, L-serine and *O*-phosphoryl-L-serine gave none. We do not give experimental details here; it is hoped to publish these details later.[37]

Distribution in Plant Kingdom, Biosynthesis and Function

Table 2 shows the distribution of ascorbalamic acid in various plant materials, mostly common fruits and vegetables, so far studied. The figures are to be regarded as lower limits and are entirely based on yields of furoylalanine (see Experimental); actual isolations of ascorbalamic acid have so far been only from cabbage and purple sprouting broccoli.

The sporadic but wide occurrence is perplexing. Within the Solanaceae, potato is positive but tomato and sweet pepper negative; within the Liliaceae, *Asparagus* is positive but *Allium* negative; within the Leguminosae, *Phaseolus* is positive but others negative.

As we had first found ascorbalamic acid in a crucifer, and continued to find it in all

* G. W. PEROLD *et al.*, *J. Chem. Soc. Perkin I*, 2450, 2457 (1972).

[33a] M. C. ELLIOTT and B. B. STOWE, *Plant Physiol.* **47**, 366 (1971).
[34] R. D. DIAMAND and D. ROGERS, *Proc. Chem. Soc.* 63 (1964).
[35] G. W. PEROLD and H. K. L. HUNDT, *Chem. Commun.* 712 (1970).
[36] P. E. J. KRUGER and G. W. PEROLD, *J. Chem. Soc.* C, 2127 (1970).
[37] W. M. LAIRD, J. J. SEXTON and R. L. M. SYNGE, unpublished work.

TABLE 2. NATURAL DISTRIBUTION OF ASCORBALAMIC ACID

Family	Species	Common name	mg ascorbalamic acid/ 100 g fr. wt
Aizoaceae	*Tetragonia expansa* Murr.	New Zealand spinach	0·0
Bromeliaceae	*Ananas comosus* Merr.	Pineapple	0·0
Capparidaceae	*Capparis spinosa* L.	Caper	0·0
Chenopodiaceae	*Spinacia oleracea* L.	Spinach	0·0
Compositae	*Cichorium intybus* L.	Chicory	0·0
	Lactuca sativa L.	Lettuce	0·0
Cruciferae	*Brassica napus* L.	Swede	3·2
	Brassica oleracea L.	Brussels sprouts	10·8
		Cabbage (various)	4·1–12·7
		Cauliflower	8·2
		Purple sprouting broccoli	3·9–12·0
	Brassica rapa L.	Turnip	2·1
	Crambe maritima L.	Seakale	2·5
	Lepidium sativum L.	Cress	0·9
	Nasturtium officinale R.Br.	Watercress	2·1
	Raphanus sativus L.	Black Spanish radish	3·0
Cucurbitaceae	*Cucumis sativus* L.	Cucumber	0·0
Filicales	*Pteridium aquilinum* Kuhn	Bracken	0·0
Gramineae	*Zea mays* L.	Sweetcorn	0·0
Leguminosae	*Astragalus baeticus* L.	(Se non-accumulator)	?trace
	Astragalus bisulcatus A. Gray	(Se accumulator)	0·0
	Cicer arietinum L.	Chickpea	0·0
	Phaseolus coccineus L.	Runner bean	4·5
	Phaseolus lunatus L.	Lima bean	2·5
	Vicia faba L.	Broad bean	0·0
Liliaceae	*Allium porrum* L.	Leek	0·0
	Allium cepa L.	Onion	0·0
	Allium sativum L.	Garlic	0·0
	Asparagus officinalis L.	Asparagus	5·7
Papaveraceae	*Papaver rhoeas* L.	Field poppy	2·9
Polygonaceae	*Rheum rhaponticum* L.	Rhubarb	1·2
Rosaceae	*Fragaria virginiana x chiloensis* Duchesne	Strawberry	0·0
	Malus sylvestris Mill.	Apple	0·0
Rutaceae	*Citrus limon* Burm.f.	Lemon	4·5
Solanaceae	*Capsicum annuum* L.	Sweet pepper	0·0
	Lycopersicon esculentum Mill.	Tomato	0·0
	Solanum tuberosum L.	Potato	1·0–3·2
Tropaeolaceae	*Tropaeolum majus* L.	Garden nasturtium	0·0
Umbelliferae	*Apium graveolens* L.	Celery	0·0
	Petroselinum crispum Nym.	Parsley	0·0
Urticaceae	*Humulus lupulus* L.	Hop	3·6

For details see Annex 4.

crucifers which we examined, we thought that it might be involved in glucosinolate biosynthesis. It is an attractive idea that the S mobilized from cysteine might be enzymically transferred to an aldoxime,[38–40] while the C_3 moiety of the cysteine is coupled to ascorbic acid. Artefactual functioning of this same enzyme as a glucosinolase, when the tissues of the plant are damaged, could then explain: (a) the coupling of glucosinolate catabolites with

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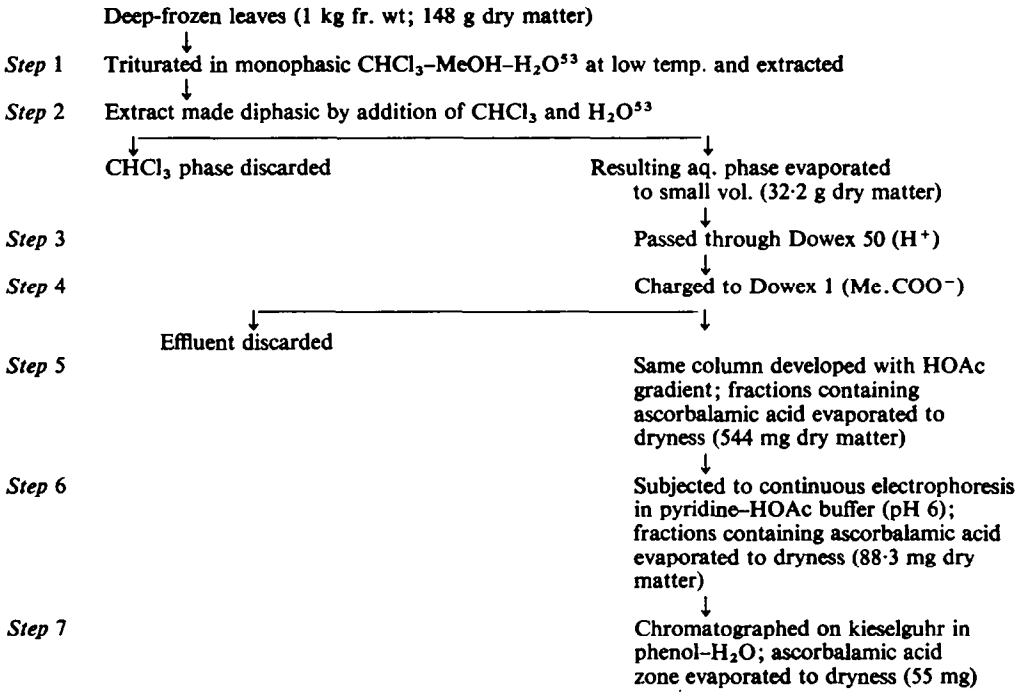
ascorbic acid to give 'ascorbigens'; and (b) the role of ascorbic acid as a coenzyme for some (but not all) glucosinolases.[41,42] However, when we examined glucosinolate-containing non-crucifers, we failed to find ascorbalamic acid either in *Tropaeolum* or *Capparis*.[38]



SCHEME 4. ISOLATION OF ASCORBALAMIC ACID FROM PURPLE SPROUTING BROCCOLI (FOR DETAILS SEE ANNEX 1).

Our next idea was that ascorbalamic acid might result from the transfer of the MeSO-group from *S*-methylcysteine *S*-oxide,[43] a major component of many crucifers, to some intermediate in the biosynthesis of those glucosinolates which contain this or the $MeSO_2$-group,[38] or to methylsulphinic acid.[44] Sulphoxide amino acids also occur in *Phaseolus*,[45] where we find ascorbalamic acid, but they are particularly notable in *Allium* spp.,[43] in which we have so far failed to find it. Ascorbalamic acid was absent both from the non-selenium-accumulating *Astragalus baeticus* and from the selenium-accumulating *Astragalus bisulcatus*, which contains *S*-methylcysteine.[46–48] The only generalization so far justified is that there may be some correlation between the occurrences of ascorbalamic acid and of flavoursome volatile S compounds.

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EXPERIMENTAL

General. TLC was on Eastman Chromagram Sheet 6064 (cellulose). High-voltage filter-paper electrophoresis was on Whatman No. 1 paper on a Gross-Locarte one-dimensional apparatus.[49] Continuous free-solution high-voltage electrophoresis was in the vertical apparatus of Hannig[50] (Elphor VaP2). Quantitative ion-exchange chromatography of amino acids was on the Beckman-Spinco Amino Acid Analyzer.[3] MS were obtained on a MS 902 mass spectrometer at a source temperature of 200° and an ionization energy of 70 eV. Both the direct-insertion probe and the coupled gas-chromatograph inlet with separator[51] were used (Pye 104 apparatus). HRMS measurements were made manually, using the peak-matching method, and also using an off-line data-processing system.[52] Evaporations were *in vacuo* below 40°. Elementary analyses were by Drs. F. Pascher and E. Pascher, Bonn.

Extraction and purification of ascorbalamic acid. Ascorbalamic acid could be extracted from fresh plant materials: (a) by trituration in cold 5% (w/v) trichloroacetic acid; (b) by trituration in phenol–HOAc–H_2O (see Introduction); (c) by the $CHCl_3$–MeOH–H_2O procedure of Bligh and Dyer[53] (cf.[54]); (d) by boiling 80% (v/v) aq. EtOH. Details are given in Annex 1 deposited at the NLL (see later). Starting with purple sprouting broccoli leaves, we isolated our best preparation of ascorbalamic acid by the procedure outlined in Scheme 4. Full experimental details are given in Annex 1. A previous preparation from 100 g fr. wt of cabbage (Flower of Spring), omitting Step 7, had yielded 4·2 mg of less pure material.

Physical properties and colour reactions of ascorbalamic acid. The colourless, glassy product was freely soluble in H_2O: $[\alpha]_D^{26.5°}$ + 24° (H_2O *c* 1·25). No mutarotation was noticed. It was a weak acid, and could be revealed on filter-paper or TLCs by spraying with bromophenol blue; it also gave a positive Rydon and Smith[55] reaction. Orcinol–H_2SO_4,[56] bound sialic acid[57] and Ehrlich[58] tests were negative. On filter-paper electrophoresis it had 75% of the anionic migration rate of pyrrolidonecarboxylic acid in pyridine–acetic acid buffer at pH 6; in borax buffer (pH 9·2), the rate was 115% of that of pyrrolidonecarboxylic acid. The partition coefficient in phenol–H_2O was 3·25 in favour of the aq. phase (Annex 1); on TLCs with the same solvent system R_fs for ascorbic (cf.[59]) and ascorbalamic acids were 0·44 and 0·34 respectively. On TLC with *n*-BuOH–HCO_2H–H_2O (4:1:5) ascorbalamic acid had R_f 0·25; R_fs for pyrrolidonecarboxylic, shikimic, ascorbic and quinic acids were 0·75, 0·47, 0·42 and 0·38 respectively (cf.[60]).

Methylation treatments of ascorbalamic acid and pyrrolidonecarboxylic acid. Permethylation. Ascorbalamic acid (0·45 mg) was dissolved in a drop of dry dimethyl sulphoxide and then treated with 0·1 ml of a 5% (w/v) solution of NaH in dimethyl sulphoxide; MeI (0·02 ml) was immediately added.[61] The mixture was kept for 1 hr at room temp., 0·5 M HOAc (2 ml) was then added and the mixture extracted with an equal vol. of $CHCl_3$. The $CHCl_3$ phase was washed 4× with H_2O and evaporated to give 0·4 mg colourless syrupy residue. This was transferred for further study in MeOH or CH_2Cl_2. For direct-insertion MS, see Table 1. For GLC, 1 μl was injected on to a 152 cm × 4 mm glass column with 2% SE 30 as stationary phase. The column was programmed from 150–230° at 3°/min, and a fraction of the effluent at the main peak (emerging at oven temp. *ca.* 220°) was transferred to the MS via a double-stage silicone-rubber membrane separator.[51] LRMS gave a picture scarcely distinguishable from that obtained by direct insertion (see Annex 2). IIIc was prepared as above, but using CD_3I (see Table 1).

Methanolic HCl *treatment.* Ascorbalamic acid (0·5 mg) was dissolved in 0·25 ml anhy. methanolic M HCl and kept at room temp. for 64 hr. The preparation was then evaporated to dryness and perdeuteriomethylated as above to give 0·42 mg residue (LRMS, Table 1, IIIb).

Alkali treatment. Ascorbalamic acid permethylated as above (0·5 mg) was kept overnight at room temp. in 0·05 M NaOH in 50% (v/v) aq. MeOH (0·2 ml). HOAc (0·1 M, 0·2 ml) was then added, the mixture was evaporated to dryness and perdeuteriomethylated as above (LRMS, Table 1, IId).

L-*Pyrrolidonecarboxylic acid* (for LRMS see Annex 2) was treated with methanolic HCl for 18 hr as above. An aliquot of the evaporated reaction mixture gave LRMS (Annex 2, IVa). The remainder was subjected to perdeuteriomethylation as above (cf.[62]) (for LRMS see Annex 2, IVb).

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Acetylation of ascorbalamic acid. Ascorbalamic acid (5·3 mg) was kept overnight at room temp. in Ac_2O (0·5 ml) and pyridine (0·5 ml). H_2O (2 ml) was added with cooling, and the mixture kept for 30 min at room temp. before repeated evaporation with H_2O. The part-crystalline residue was examined by direct-insertion LRMS (Annex 2).

Reaction of ascorbalamic acid with p-*bromophenylhydrazine.* Ascorbalamic acid (5·0 mg) was reacted in H_2O with 1 mol prop. of rubidium acetate; HOAc was removed by repeated evaporation and the product dissolved in 0·05 ml H_2O. *p*-Bromophenylhydrazine hydrochloride (1 mol prop.) was then added in 0·13 ml H_2O. EtOH (0.1 ml) was added, and the reaction mixture was kept at 25° in the dark for 10 days. On filter-paper electrophoresis in pyridine–HOAc buffer (pH 6) a sample of the reaction mixture showed,[55] besides unchanged ascorbalamic acid: (a) an anionic zone having 35% of its migration rate; (b) a zone coinciding in position with that of the neutral marker substance (*N*-2,4-dinitrophenylethanolamine). Zones (a) and (b) were isolated preparatively in the Hannig[50] apparatus (see Scheme 4, Step 6) (2·4 and 1·9 mg respectively). However, after keeping in 50% (v/v) aq. EtOH for 3 weeks at 4°, examination of aliquots by filter-paper electrophoresis showed both fractions to have partly reverted towards the composition of the original reaction mixture. The material from zone (a) was acetylated as above, and the partly crystalline residue was examined by direct-insertion LRMS (see above).

Hot-acid treatment of ascorbalamic acid: configuration of resulting aspartic acid. Ascorbalamic acid (5 mg) was refluxed 24 hr in 6 M HCl and evaporated to dryness. Amino acid analysis of a small sample of the hydrolysate revealed 0·46 mol prop. of furoylalanine (see below), 0·18 mol prop. of aspartic acid, 0·03 mol prop. of glycine, 0·02 mol prop. of serine and of glutamic acid, 0·01 mol prop. of alanine and 0·007 mol prop. of threonine. The rest of the sample was dinitrophenylated,[32] and the *N*-2,4-dinitrophenyl (DNP) derivatives subjected to continuous electrophoresis in pyridine (0·16% v/v)–HOAc (2·4% v/v) (pH 3·4). Some crystals of DNP-furoylalanine remained undissolved. A fast anionic zone containing DNP-aspartic acid was collected, free from the more slowly migrating 2,4-dinitrophenol but imperfectly resolved from DNP-furoylalanine. This mixture was further resolved by column chromatography,[32] to give the DNP-furoylalanine as a fast-moving zone (see below) and the DNP-aspartic acid as a slower zone (same R_f as authentic material). LRMS (cf. [63]) demonstrated the identity of the isolated product with authentic *N*-DNP-aspartic acid (Annex 2). The remainder was examined by Dr. A. F. Drake for circular dichroism (cf.[64,65]). He found it to have 83% of the activity of an authentic specimen of DNP-L-aspartic acid (Annex 3).

3-(2-*Furoyl*)*alanine from ascorbalamic acid.* A preparation of ascorbalamic acid from Brussels sprouts was taken as far as Step 5 (Scheme 4), except that three bed volumes of 8 M HOAc rather than an HOAc gradient was used for the elution. The evaporated residue was refluxed overnight in 6 M HCl. Dr. M. Redford-Ellis examined a small sample of this hydrolysate by her volatile-buffer ion-exchange procedure for amino acids,[19] and established that the novel amino acid was resolved from and eluted between valine and methionine. Our own attempts at isolation on a preparative scale did not give clear resolution from neighbouring amino acids, probably because of unsuitable resin. However, we isolated a few mg of product in which the novel amino acid predominated, with aliphatic amino acids as major contaminants. The UV spectrum was observed in H_2O and the *N*-acetyl methyl ester was prepared for MS study (see below).

Synthesis of DL-3-(2-*furoyl*)*alanine.* Furacyl bromide[66] (3·7 g) was added to the Na-derivative of diethyl acetamidomalonate (4·25 g) in 39 ml dry EtOH, following a procedure recommended for synthesis of phenylalanine.[67] The mixture was refluxed, with stirring, for 2 hr, in the course of which time there was separation of some solid and much darkening. After evaporation of most of the alcohol, the residue was partitioned between H_2O (15 ml) and $CHCl_3$ (15 ml), and the aqueous phase was washed with $CHCl_3$ (2 × 15 ml). The dark $CHCl_3$ extract was evaporated to dryness, but the residue did not crystallize. It was refluxed overnight in 625 ml 5 M HCl. Amino acid analysis on a portion of hydrolysate showed the presence of substantial amounts of glycine, suggesting that the initial reaction time may have been inadequate. The mixture was evaporated to dryness, taken up in H_2O, filtered from much black precipitate, and charged to a 36-ml bed of Dowex 50W X8 (H^+ form). The column was washed with 360 ml H_2O and the washings discarded. It was then eluted with 200 ml M NH_3, and the brown eluate evaporated to dryness (residue 2·3 g). This was crystallized from H_2O to give 0·63 g yellowish crystals which, on recrystallization from H_2O (charcoal), gave a substantially colourless and pure product.

Properties of 3-(2-*furoyl*)*alanine and its derivatives. Synthetic* DL-3-(2-*furoyl*)*alanine* crystallized as a monohydrate, which did not lose H_2O in a vacuum desiccator over H_2SO_4 at room temp. (Found: C, 48·15; H, 5·56; N, 6·87; O, 39·46%. $C_8H_9NO_4H_2O$ requires: C, 47·76; H, 5·52; N, 6·96; O, 39·76%.) UV absorption in H_2O showed maxima at 278 nm (ε 15 300) and 227 nm (ε 3038) minimum at 241 nm (ε 1997). (Detail in Annex 2; cf.[21,26–28].) PMR spectrometry at 100 MHz (on the hydrochloride in D_2O, exchanged by repeated

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evaporation to dryness in D_2O), using sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DDS) as an internal reference, showed the protons on the furan ring as an AMX system with signals at δ 7·89 (H_A-proton at C-5), δ 7·58 (H_M-proton at C-3) and δ 6·75 (H_X-proton at C-4) with the coupling constants J_{AM} 0·6 Hz, J_{AX} 1·85 Hz and J_{MX} 3·8 Hz. The protons on the side chain appeared as a triplet ($J_{\alpha\beta}$ 5·5 Hz) at δ 4·55 (α-proton of alanine moiety) and a doublet at δ 3·74 (β-protons) (detail in Annex 2). 3-(2-Furoyl)alanine gave an orange–yellow colour with ninhydrin as a spot on cellulose, and likewise, on the Beckman–Spinco Amino Acid Analyzer, absorbance after reaction with ninhydrin was 8·85 × greater at 440 nm than at 570 nm. As a zone, it emerged at 25% of the time between the methionine and isoleucine peaks,[3] being imperfectly resolved from methionine. It had a colour factor C (440 nm) 2·4 × that for proline. R_fs on TLC were 0·33 (*n*-BuOH–HOAc–H_2O, 9:1:2·5) and 0·45 (*n*-ProH–H_2O–*n*PrOAc–HOAc–pyridine, 120:60:20:4:1),[54] more or less coinciding with proline in both solvents. On paper electrophoresis at pH 6 (pyridine–HOAc, very slow migration) and pH 1·8 (HOAc–HCO_2H) it had cationic migration rates 75 and 92 % of those of phenylalanine respectively. In the Van Slyke amino N determination (4 min) yield was 0·80 mol prop. N_2.[24] Recovery (Amino Acid Analyzer) after treating with 6 N HCl for 18 hr at the boiling point was somewhat variable. Our best yield (in a sealed evacuated tube) was 84%. At the same time there resulted 0·05 mol prop. of aspartic acid. The furoylalanine isolated from plant material was chromatographically and electrophoretically indistinguishable from the synthetic product. N-*Acetyl*-3-(2-*furoyl*)*alanine methyl ester* was prepared from the synthetic and 'natural' amino acids on the microscale.[68] The products were compared by GLC–MS and gave main zones indistinguishable from one another in both respects (for details see Annex 2). DL-N-2,4-*Dinitrophenyl*-3-(2-*furoyl*)*alanine* was prepared[32] from the synthetic amino acid in good yield and recrystallized as yellow needles from EtOH, m.p. 189–190° (uncorr.). (Found: C, 48·09; H, 3·11; N, 12·12; O, 36·79%. $C_{14}H_{11}N_3O_8$ requires: C, 48·15; H, 3·18; N, 12·02; O, 36·64%.) For HRMS see Annex 2. On paper electrophoresis at pH 3·4 (pyridine–HOAc), the compound had an anionic net migration rate 0·74 of that of DNP–aspartic acid, and at pH 6·0 (pyridine–HOAc) 0·39. PC in the pH 3·4 buffer showed the compound to be retarded relative to DNP–aspartic acid sufficiently to explain their overlap in free-solution electrophoresis at pH 3·4 (see above). The 'natural' DNP derivative (see above) agreed in mass-spectrometric, electrophoretic and chromatographic properties.

Natural distribution of ascorbalamic acid. Fresh plant material was usually extracted either in $CHCl_3$–MeOH–H_2O in the cold[54] or in boiling 80% (v/v) aq. EtOH. The extract, in H_2O and freed from lipids, was passed through a bed of Dowex 50W X8 (H^+ form); the effluent was hydrolysed by refluxing for 18 hr in 6 M HCl and subjected to amino acid analysis.[3] In calculating yields, it was assumed that 0·45 moles of furoylalanine resulted from each mole of ascorbalamic acid (see above); these values take no account of possible influences of other plant constituents on the degradation. The results are summarized in Table 2; full details of materials and procedures are in Annex 4 (by Janice J. Sexton).

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Supplementary Publication Scheme

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