Biochem. J. (1971) **121**, 425–430
Printed in Great Britain

N-Carbamoyl-2-(*p*-hydroxyphenyl)glycine from Leaves of Broad Bean (*Vicia faba* L.)

By J. EAGLES, W. M. LAIRD, S. MATAI,* R. SELF and R. L. M. SYNGE
Agricultural Research Council Food Research Institute, Colney Lane, Norwich NOR 70F, U.K.

and A. F. DRAKE†
School of Chemical Sciences, University of East Anglia, Norwich NOR 88C, U.K.

(*Received* 31 *July* 1970)

1. DL-2-(*p*-Hydroxyphenyl)glycine was resolved through the *bromocamphorsulphonate* to give its D-*isomer*. The N-*carbamoyl derivatives* of these amino acids were synthesized. Circular-dichroism studies on these and related compounds, reported in a deposited Annex, helped to establish the optical configuration. 2. *N*-Carbamoyl-DL-2-(*p*-hydroxyphenyl)glycine was isolated from broad-bean leaves. It amounted to about 0.1% of the leaf dry matter. Racemization may or may not have occurred during the isolation. There were indications of the same compound in chicory and in savoy cabbage. Under weakly acidic conditions it was converted gradually into 5-(*p*-hydroxyphenyl)hydantoin. Both these compounds yielded 2-(*p*-hydroxyphenyl)glycine on acid hydrolysis. 3. The occurrence is discussed of 2-phenylglycine derivatives in Nature and of *N*-carbamoyl-amino acids and hydantoins in plants. 4. Gradient elution from anion-exchange resin with acetic acid, besides proving useful for the present work, gave useful separations of pyrrolidonecarboxylic acid and of some *N*-acetyl-amino acids. 5. Supplementary material (Annex 1: details of experimental work other than ultraviolet and circular-dichroism spectra; Annex 2: ultraviolet absorption and circular dichroism of D-2-phenylglycine and some related compounds) has been deposited as Supplementary Publication SUP 50003 at the National Lending Library for Science and Technology, Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1971), **121**, 7.

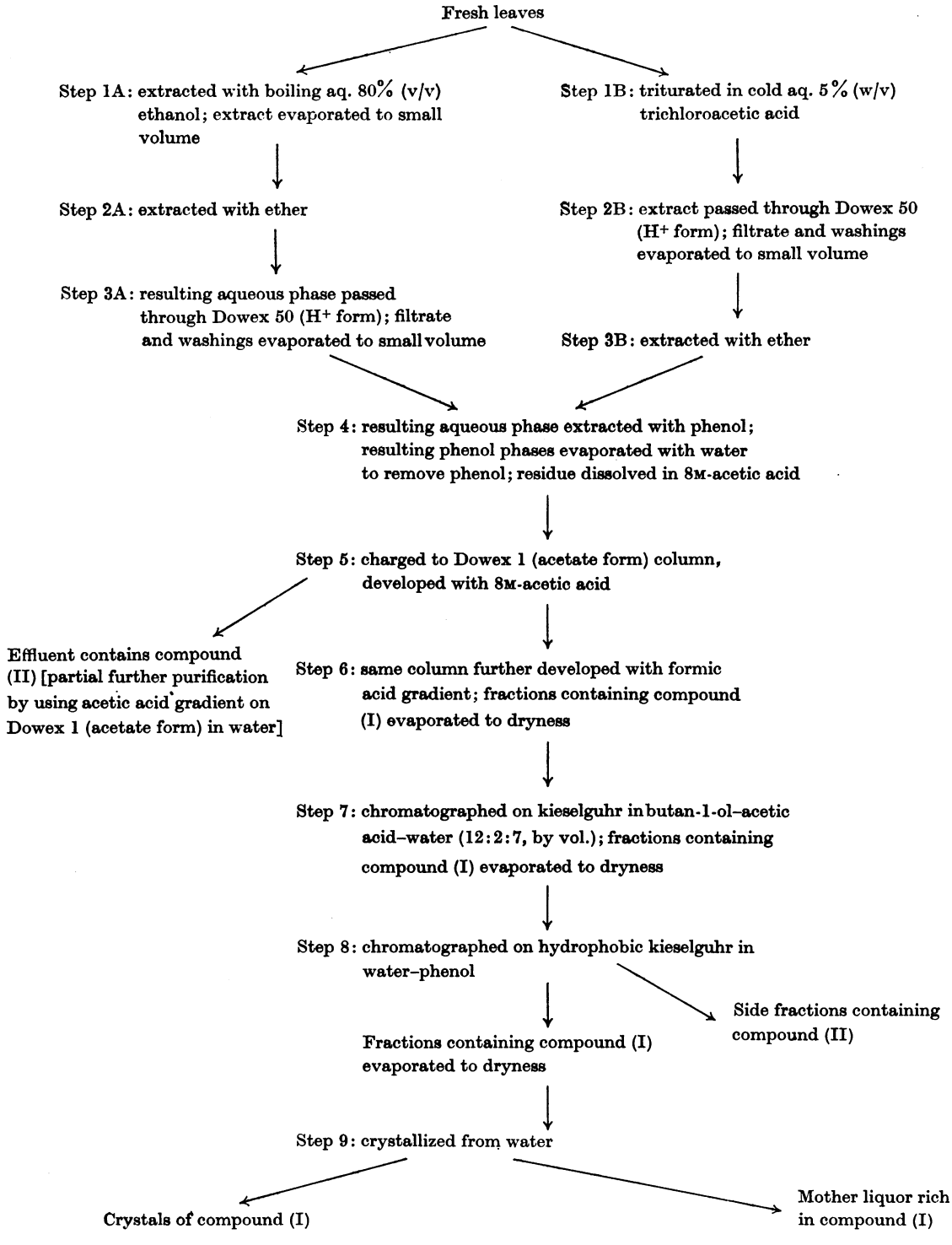
This work started from the observation at the Rowett Research Institute of an unusual zone, close to that of isoleucine, on amino acid analysis by the method of Moore & Stein (1951) of acid hydrolysates of a fraction from broad-bean leaves (footnote to Table II in Clarke, Ellinger & Synge, 1968). Such a component had not previously been recognized among the amino acids of *Vicia faba* L. (Petronici, 1956; Boulter & Barber, 1963; Clarke *et al.* 1968), and Dr G. M. Ellinger considered it to be a novel amino acid. When this experiment was repeated here, the zone lay between those of methionine and isoleucine, coinciding on our resin with the position of alloisoleucine, which misled our research for a considerable time. Dr Ellinger, however, found the zone to be eluted from her resin appreciably after that of alloisoleucine (for details see Annex 1).

Judged by the fractions in which it had been found, the precursor of this new substance seemed likely to be weakly acidic or neutral, devoid of basic ionizing groups. This was borne out by the procedures that we elaborated for concentrating it, which are shown diagrammatically in Scheme 1. Incidentally, the use of an acetic acid gradient on anion-exchange resin (Hulme & Wooltorton, 1958) proved useful for concentrating and separating pyrrolidonecarboxylic acid (pyroglutamic acid) and various *N*-acetyl-amino acids. Details of the procedures are in Annex 1.

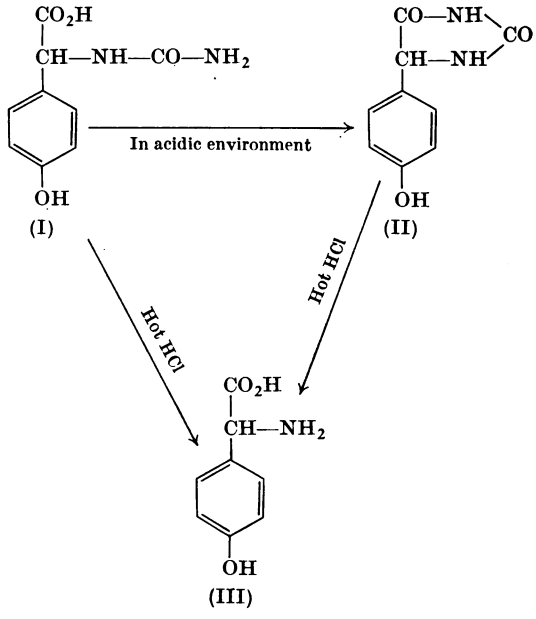
Clarification resulted when, on studying fractions from step 8 (Scheme 1), we obtained a crystalline side fraction that yielded the 'novel amino acid' on acid hydrolysis and, on high-resolution mass spectrometry, gave a molecular ion [*m/e* 192.0541 ($C_9H_8N_2O_3$)] with fragments suggestive of a

* Present address: Indian Statistical Institute, 203 Barrackpore Trunk Road, Calcutta-35, India.

† Present address: Department of Chemistry, King's College, Strand, London W.C.2, U.K.



Scheme 1. Fractionation of leaves for isolation of *N*-carbamoyl-2-(*p*-hydroxyphenyl)glycine (I) and 5-(*p*-hydroxyphenyl)hydantoin (II).



Scheme 2.

5-(hydroxyphenyl)hydantoin. Synthetic DL-5-(*p*-hydroxyphenyl)hydantoin (II) gave a closely similar mass spectrum. This suggested that the parent compound might be *N*-carbamoyl-2-(*p*-hydroxyphenyl)glycine (I) and that compounds (I) and (II) yielded 2-(*p*-hydroxyphenyl)glycine (III) on acid hydrolysis, as shown in Scheme 2.

We confirmed this supposition by completing the isolation of compound (I) from broad-bean leaves, by synthesizing compounds (I) and (III) and by comparison of isolated and synthetic materials, particularly by high-resolution mass spectrometry (unpublished work of J. Eagles & R. Self, with fragmentation schemes; low-resolution mass spectra have been deposited with the Mass Spectrometry Data Centre, Atomic Weapons Research Establishment, Aldermaston, Berks., U.K.). The *m*- and *o*-hydroxy isomers were excluded by the i.r. spectrum of compound (I) as well as by mass-spectrometric and chromatographic observations on DL-2-(*m*-hydroxyphenyl)glycine (see Annex 1).

We find compound (I) in amounts of the order of 0.1% of the dry matter of the leaves (see Annex 1). One difficulty in determining it is that compound (III) is completely destroyed when our acid hydrolysis conditions are applied to leaf extracts or concentrates of compound (I) before taking them through step 4 (Scheme 1) or phenol–acetic acid–water electrophoresis (Clarke *et al.* 1968; Jennings, Pusztai, Synge & Watt, 1968). This instability on acid hydrolysis in the presence of extraneous substances (cf. Brazhnikova & Kudinova, 1963), which is reminiscent of that of tryptophan, may account for compound (III) not having previously been noticed in plant material. The second difficulty is that we do not yet know in detail the conditions of conversion of compound (I) into compound (II) nor the yields of compound (III) to be expected from either compound (I) or compound (II) on acid hydrolysis. In order to isolate a fair yield of compound (I) it was necessary to work rather fast, by controlling the fractions by paper chromatography and by appropriate colour reactions, rather than by waiting for complete amino acid analyses.

Optical configuration and stability of 2-(p-*hydroxyphenyl*)*glycine and related compounds*

To complete the characterization of the compound (I) isolated from broad-bean leaves, it was necessary to establish its optical configuration. No published method for optical resolution of DL-compound (III) was available. Makleĭt, Starychkaĭ & Pushkash (1967) had stated that they had resolved synthetic DL-compound (III) through the tartrate of its ethyl ester, but gave neither results nor experimental details. They also stated that they had been unsuccessful in resolving DL-compound (III) by the bromocamphorsulphonate method of Betti & Mayer (1908*a*,*b*). We had no success with the camphorsulphonate method of Betti & Mayer (1908*a*,*b*), but were successful with their bromocamphorsulphonate method, and have resolved synthetic DL-compound (III) to give D-compound (III) and have prepared D-compound (I) therefrom. Since this work was completed, Long & Nayler (1970) have described the resolution of the *N*-benzyloxycarbonyl derivative of DL-compound (III) as its quinine salt, without assigning configurations. Our evidence for the configuration is: (i) that the rotations of compound (III) in water and M-hydrochloric acid have the same sense as (and are greater than) those found by Lomakina, Zenkova & Yurina (1969), who established the configurational relationship with D-2-cyclohexylglycine; (ii) that these rotations suggest a D-configuration according to the Clough–Lutz–Jirgensons Rule, which is obeyed by 2-phenylglycine and its known derivatives (Larsen, 1969; Lomakina *et al.* 1969); (iii) from the studies of circular dichroism reported and discussed in Annex 2 by A.F.D. It was found that the compound (I) from broad-bean leaves was completely racemic. The circular-dichroism procedure is very sensitive, and it would have detected 0.5% of optically active material in our final product. It could be used to establish the configuration of as little as 20μg of active compound (I). A different isolation procedure, involving less acidic conditions or blocking of reactive groups, is probably required for estab-

lishing whether or not compound (I) is racemic in the intact leaf. At present we can simply note the tendency of compound (I) to hydantoin formation during the (chiefly acidic) conditions of our isolation, the fact that hydantoin formation from most known *N*-carbamoyl-amino acids is accompanied by heavy racemization and the fact that 2-phenylglycine and its derivatives are much more readily racemized under acidic conditions than are the common amino acids. On the other hand, synthetic D-compound (I) was recovered during its preparation in good yield and with full optical activity, after the mother liquors from the crystalline product had been put through steps 5 and 6 (Scheme 1) for elimination of compound (II) and potassium chloride (see Annex 1).

Tyrosine hydantoin (Stark & Smyth, 1963) and compound (II) share the property of being retained on anion-exchange resins at low pH and of requiring rather high concentrations of acetic acid for their elution.

These peculiarities suggest the need for a thorough physicochemical study of ionization, racemization, cyclization and hydrolysis in this group of substances.

Preparation and properties of compounds studied

During this work the *new compounds* shown in Table 1 were prepared and characterized. Some supplementary information was also obtained about DL-compound (II), DL-compound (III), *N*-carbamoyl-D-2-phenylglycine (IV), D-2-phenylglycine (V), DL-5-phenylhydantoin and DL-2-(*m*-hydroxyphenyl)glycine. The preparative and analytical details, colour reactions, chromatographic and electrophoretic behaviour and i.r. spectra are in Annex 1, u.v. and circular-dichroism spectra in Annex 2.

2-Phenylglycine derivatives in Nature

Larsen (1969) (cf. also Yoshida, 1969) has reviewed the occurrence of substituted 2-phenylglycines in plants, as well as presenting new evidence. The D-forms of 2-(*m*-carboxyphenyl)glycine and of 2-(3-carboxy-4-hydroxyphenyl)glycine have been found in the free state. Müller & Schütte (1968) found 2-(*m*-hydroxyphenyl)glycine and 2-(3,5-dihydroxyphenyl)glycine free in the latex of *Euphorbia helioscopia*, but did not have enough material to recognize the optical configuration. A claim has been made (Dietrichs & Funke, 1967), though without adequate identification, for the presence of 2-phenylglycine in beech phloem sap.

Compound (III) seems not to have been found previously in plants. However, it has been isolated from acid hydrolysates of the glycopeptide antibiotic actinoidin (Bognar, Makleĭt, Starichkai, Lomakina & Yurina, 1964; Lomakina, Yurina &

Table 1. *New compounds synthesized and studied*

Compound	Formula deduced from elementary analyses	M.p. [°C (corr.)]	Optical rotation			
			$[\alpha]_D$	°C	Solvent	*c*
N-Carbamoyl-DL-2-(*p*-hydroxyphenyl)glycine [DL-compound (I)]	$C_9H_{10}N_2O_4,H_2O$	194–195 (with foaming)	—	—	—	—
D-2-(*p*-Hydroxyphenyl)glycine (+)-α-bromocamphor-π-sulphonate	$C_8H_9NO_3,C_{10}H_{15}BrO_4S$	—	+10.1°	26	Water	1.1
D-2-(*p*-Hydroxyphenyl)glycine [D-compound (III)]	$C_8H_9NO_3$	—	−105°	28	Water	0.75
			−154°	27.5	M-HCl	0.6
N-Carbamoyl-D-2-(*p*-hydroxyphenyl)glycine [D-compound (I)]	$C_9H_{10}N_2O_4,\frac{1}{2}H_2O$	169 (with foaming)	−175°*	22.7	Aq. 50% (v/v) ethanol	0.5*

* Calc. for the anhydrous compound.

Brazhnikova, 1964; Yurina, Lomakina, Murav'eva & Spiridonova, 1965; Yurina & Lomakina, 1967; Bognar, 1968). It also arose by reductive degradation of an amino acid obtained from the related glycopeptide ristomycin (Bognar, 1968; Lomakina *et al.* 1968). In both cases it was heavily racemized, but was shown (Lomakina *et al.* 1969) to possess the D-configuration by reductive conversion into D-2-cyclohexylglycine, for which the configuration was already established. Compound (III) has also been found in hydrolysates of the antibiotic peptide enduracidin. In this case optically active and racemic material was isolated. The reported optical in hydrochloric acid corresponded in sense to that of the L-isomer, but was more than double that found for the D-isomer in the present work (Asai *et al.* 1968).

There seem to be no previous reports of natural occurrence of compound (I), although an acidic compound $C_9H_{10}N_2O_4,H_2O$, which was optically inactive and may have been a hydrate of DL-compound (I), was isolated by Fromherz (1911) from the urine of a dog to which he had administered DL-compound (III).

Compound (I) may turn out to have a fairly wide distribution in plants, as we have seen minor zones corresponding to compound (III) on Moore & Stein (1951) chromatography of hydrolysates of corresponding fractions from savoy (*Brassica oleracea* L.) and from chicory leaves (*Cichorium intybus* L.) (Annex 1).

N-*Carbamoyl-amino acids, hydantoins and other unrecognized non-protein nitrogenous compounds as plant constituents*

Apart from citrulline and albizziine (which give colours with ninhydrin), few if any *N*-carbamoyl-amino acids have yet been isolated from plant material. The occurrence of ureido compounds in plants was reviewed by Tracey (1955). *N*-Carbamoylaspartic acid presumably occurs as a normal intermediate of pyrimidine biosynthesis (cf. Buchowicz, Reifer & Makowski, 1961). We have seen a number of zones staining yellow with Ehrlich's reagent on paper chromatography of material after step 4 of our purification procedure, which justifies a wider search for carbamoyl derivatives. In this connexion we recall the isolation by von Lippmann (1896), on a single occasion, of hydantoin itself from etiolated sugar-beet sprouts. As he gave few details of the initial steps in his isolation procedure, or of yield, it is difficult to judge whether or not the hydantoin arose as an artifact from *N*-carbamoylglycine (hydantoic acid). van der Drift & Vogels (1966) noticed none of this last in *Phaseolus hysterinus*. Our failure to find evidence for compound (I) in summer broad-bean leaves and in early-autumn cabbage (Annex 1) might suggest an influence of lighting on its occurrence, but we are insufficiently sure of our analytical procedures to stress this possibility. The involvement of substituted 2-phenylglycine in recent semisynthetic penicillins and in the antibiotics mentioned above, all of which inhibit bacterial cell-wall biosynthesis (cf. Bognar, 1968), suggests that possible antibiotic functions of compound (I) and related compounds in the plant should be considered.

Earlier (Crokaert, 1961*a*,*b*; Stark & Smyth, 1963) and present work shows that *N*-carbamoyl-monoamino acids pass fairly readily through cation-exchange resins, but can be isolated, like the polycarboxylic 'plant acids' (Hulme & Wooltorton, 1958), by the use of formic acid gradients on anion-exchange resins. They may thus contribute appreciably to the largely acidic 'bound amino acid' category of the plant non-protein N [reviewed by Synge (1968); cf. also Synge & Wood (1958), Rosa & Neish (1968), Jennings *et al.* (1968) and Clarke *et al.* (1968)]. In all studies of this group of substances very complicated mixtures of acidic or neutral 'bound' forms of the common amino acids were found. In the present work it was notable, on our anion-exchange columns eluted with acetic acid and with formic acid gradients, that, although the only derivatives of compound (III) to be found emerged at definite positions on the gradients [compound (II) on the acetic acid gradient and compound (I) on the formic acid gradient], yet there was a continuous background all along both gradients of compounds of most of the common amino acids. Among these, aspartic acid, glutamic acid, glycine, alanine and serine predominated. Any thorough elucidation of the nature of these amino acid derivatives (of which the amino acid moieties often amount to 2–3% of the non-protein N) is likely to be complicated and difficult. It remains unexplained how Karasek (1963), in a search for *N*-acetyl-amino acids in tobacco leaves, in which he used methods essentially similar to those of Rosa & Neish (1968) and of the present work, failed completely to find any conjugated amino acids.

We thank Dr Gabrielle M. Ellinger for much advice and help, especially with chromatographic identification of amino acids, Dr A. C. Hulme, Dr P. O. Larsen and Dr H. R. Schütte for advice and specimens, Dr M. J. Fishwick for i.r. observations, Professor S. F. Mason for his interest and discussions of the work on optical configuration, Dr H. E. Davenport and Mr W. B. Watt for growing the chicory and savoys, Mr M. Smith and Mrs Julia Levett for amino acid determinations and Mr K. Parsley and Miss Janice J. Payne for skilful technical assistance. The Director of the Rowett Research Institute kindly permitted some of the experiments to be done there. S. M. acknowledges maintenance allowances under the Colombo Plan from the U.K. Ministry of Overseas Development and the Government of India.

REFERENCES

Asai, M., Muroi, M., Sugita, N., Kawashima, H., Mizuno, K. & Miyake, A. (1968). *J. Antibiot. Tokyo*, **21**, 138.

Betti, M. & Mayer, M. (1908*a*). *Gazz. chim. ital.* **38**, II, 571.

Betti, M. & Mayer, M. (1908*b*). *Ber. dt. chem. Ges.* **41**, 2071.

Bognar, R. (1968). *Acta Univ. debrecen., Ser. Phys. Chem.*, **14**, 185.

Bognar, R., Makleĭt, Sh., Starichkai, F., Lomakina, N. N. & Yurina, M. S. (1964). *Antibiotiki*, **9**, 875.

Boulter, D. & Barber, J. T. (1963). *New Phytol.* **62**, 301.

Brazhnikova, M. G. & Kudinova, M. K. (1963). *Nature, Lond.*, **200**, 167.

Buchowicz, J., Reifer, I. & Makowski, J. (1961). *Acta biochim. pol., Engl. Trans.*, **8**, 377.

Clarke, E. M. W., Ellinger, G. M. & Synge, R. L. M. (1968). *J. Sci. Fd Agric.* **19**, 214.

Crokaert, R. (1961*a*). *Bull. Soc. Chim. biol.* **43**, 1317.

Crokaert, R. (1961*b*). *Bull. Soc. Chim. biol.* **43**, 1331.

Dietrichs, H. H. & Funke, H. (1967). *Holzforschung*, **21**, 102.

Fromherz, K. (1911). *Hoppe-Seyler's Z. physiol. Chem.* **70**, 351.

Hulme, A. C. & Wooltorton, L. S. C. (1958). *J. Sci. Fd Agric.* **9**, 150.

Jennings, A. C., Pusztai, A., Synge, R. L. M. & Watt, W. B. (1968). *J. Sci. Fd Agric.* **19**, 203.

Karasek, M. (1963). *Biochim. biophys. Acta*, **78**, 644.

Larsen, P. O. (1969). *Free Amino Acids in Cruciferae and Resedaceae.* Danish Atomic Energy Commission: Risö Report no. 189.

Lomakina, N. N., Yurina, M. S. & Brazhnikova, M. G. (1964). *Antibiotiki*, **9**, 880.

Lomakina, N. N., Zenkova, V. A., Bognar, R., Starichkai, F., Sheĭnker, Yu. N. & Turchin, K. F. (1968). *Antibiotiki*, **13**, 675.

Lomakina, N. N., Zenkova, V. A. & Yurina, M. S. (1969). *Khim. prirodn. Soedin.* **5**, 43.

Long, A. A. W. & Nayler, J. H. C. (1970). *W. Ger. Patent* 1942693.

Makleĭt, Sh., Starychkaĭ, F. & Pushkash, M. (1967). *Visn. kyyvs'k. Univ. Ser. Fiz. Khim.* Part 7, p. 155.

Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.

Müller, P. & Schütte, H. R. (1968). *Z. Naturf.* **23**B, 659.

Petronici, C. (1956). *Annali Sper. agr.* **10**, 841.

Rosa, N. & Neish, A. C. (1968). *Can. J. Biochem.* **46**, 797.

Stark, G. R. & Smyth, D. G. (1963). *J. biol. Chem.* **238**, 214.

Synge, R. L. M. (1968). *A. Rev. Pl. Physiol.* **19**, 113.

Synge, R. L. M. & Wood, J. C. (1958). *Biochem. J.* **70**, 321.

Tracey, M. V. (1955). In *Moderne Methoden der Pflanzenanalyse*, vol. 4, p. 119. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.

van der Drift, C. & Vogels, G. D. (1966). *Acta bot. neerl.* **15**, 209.

von Lippmann, E. O. (1896). *Ber. dt. chem. Ges.* **29**, 2645.

Yoshida, S. (1969). *A. Rev. Pl. Physiol.* **20**, 41.

Yurina, M. S. & Lomakina, N. N. (1967). *Antibiotiki*, **12**, 279.

Yurina, M. S., Lomakina, N. N., Murav'eva, L. I. & Spiridonova, I. A. (1965). *Antibiotiki*, **10**, 1090.