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CYCLOPROPANE AMINO ACIDS FROM *AESCULUS* AND *BLIGHIA*

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Abstract—Three compounds containing cyclopropane ring systems have been newly characterized from *Aesculus parviflora* and *Blighia sapida*. *Exo*-3,4-methanoproline was isolated together with its probable biogenetic precursor, *cis*-α-(carboxycyclopropyl)glycine, from seeds of *Aesculus*, whilst the structurally related *trans*-α-(carboxycyclopropyl)glycine was obtained from *Blighia* seed. The structures were assigned following the analysis of the hydrogenation products obtained from each substance and of the NMR and mass spectra of the compounds.

INTRODUCTION

THE CYCLOPROPYL group features in the structures of four known naturally occurring amino acids from among the large collection of non-protein amino acids now recognized as plant products. The simplest of these compounds, and the earliest to be characterized, is 1-amino-cyclopropyl-1-carboxylic acid, which occurs in fruit of the perry pear (*Pyrus communis*) and the cowberry (*Vaccinium vitis-idaea*).[1] In the three other examples, the cyclopropyl residue carries an exocyclic methylene group and is itself attached to a glycine or alanine-type moiety. The homologous compounds, α-(methylenecyclopropyl)glycine and β-(methylene-cyclopropyl)alanine (hypoglycin A), have been isolated from *Litchi chinensis*[2] and *Blighia sapida* (akee),[3] respectively. Both plants are assigned to the family Sapindaceae, and both amino acids cause hypoglycaemic symptoms in animals. The related compound, β-(methylenecyclopropyl)-β-methylalanine, formed one of a group of amino acids recently identified as components of the seed of *Aesculus californica* (Hippocastanaceae).[4]

Three further cyclopropane amino acids from plants are now described. Cis-α-(*carboxy-cyclopropyl*)*glycine* (I) is accompanied by exo(cis)-3,4-*methanoproline* (II) in seed of *A. parviflora*, suggesting a biogenetic relationship akin to that existing between glutamic acid and proline, while trans-α-(*carboxycyclopropyl*)*glycine* (III) is a constituent of akee seed. These newly characterized amino acids extend the phytochemical similarities existing between *Aesculus* and *Blighia*.

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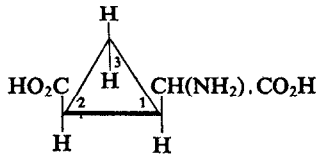
[3] E. V. ELLINGTON, C. H. HASSALL, J. R. PLIMMER and C. E. SEAFORTH, *J. Chem. Soc.* 89 (1959).
[4] L. FOWDEN and A. SMITH, *Phytochem.* **7**, 809 (1968).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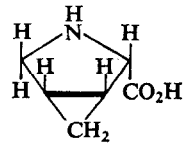
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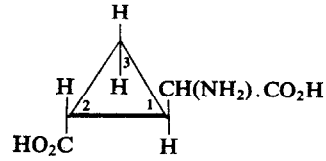
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RESULTS

The new compounds were observed first as unidentified spots on ninhydrin-treated chromatograms prepared from 75% (v/v) ethanolic extracts of mature seeds. Table 1 indicates the positions of the three substances on chromatograms developed in 75% (w/w) phenol, in the presence of ammonia, and in a butan-1-ol-acetic acid–water solvent: similar data are included for glutamic acid, proline and pipecolic acid to provide reference positions for comparison.

TABLE 1. R_f VALUES OF THE NEWLY CHARACTERIZED AMINO ACIDS AND SOME REFERENCE COMPOUNDS

Amino acid	R_f in phenol–NH_3	R_{Leu} in butanol–acetic acid–water	Ninhydrin colour
cis-α-(carboxycyclopropyl)-glycine (I)	0·36	0·39	red–purple → blue–purple (rapid)
trans-α-(carboxycyclopropyl)-glycine (III)	0·26	0·38	blue–purple
Glutamic acid	0·33	0·32	blue–purple
Exo-3,4-methanoproline (II)	0·82	0·53	bright yellow
Proline	0·87	0·43	yellow
Pipecolic acid	0·90	0·69	blue

Normal ion-exchange and preparative paper chromatographic procedures were employed to separate and isolate the amino acids from extracts of 380 g *A. parviflora* and 3·5 kg *B. sapida* seed.

Exo(cis)-3,4-*Methanoproline* (II)

This substance formed the predominant component of the soluble-nitrogen pool of *A. parviflora* seed and 1·8 g were eventually isolated.

Its ninhydrin chromophore was a brilliant yellow, easily distinguished from the dull yellow colour given by proline. When chromatograms were sprayed with isatin and heated at 100°, II caused a partial decolorization of the isatin: this behaviour resembled that observed with 4-methyleneproline,[5] but differed markedly from that of proline and pipecolic acid, which both react sensitively with isatin to yield blue chromophores.

Assignment of structure was based on the following considerations. Elementary analysis indicated the empirical formula $C_6H_9NO_2$. Hydrogenation using Adam's platinum catalyst

[5] D. O. GRAY and L. FOWDEN, *Nature* **193**, 1285 (1962).

under slightly acidic conditions gave *cis*-3-methylproline as the main product. A small amount of alloisoleucine was also produced by splitting of the pyrrolidine ring (compare the formation of leucine during hydrogenation of 4-methyleneproline[5]). These facts alone were consistent with a 3-methyleneproline structure, but the NMR spectrum of II gave no support for the presence of exocyclic methylene protons. The spectrum contained strong signals at high field indicative of cyclopropyl protons and this, together with doublet signals attributable to the α and C-5 protons of a proline ring, led to the adoption of the 3,4-methanoproline structure.

The mass spectrum of II gave the molecular ion at *m/e* 127 ($C_6H_9NO_2$) and indicated the loss of 45 mass units to give the base peak at *m/e* 82, a behaviour typical of an α-amino acid. The spectrum was in fact very similar to those of 4-methyleneproline and baikiain measured under the same operating conditions, indicating a close structural relationship between compound II and these isomers.

The formation of the *cis*-isomer of 3-methylproline during hydrogenation was consistent with cyclopropyl ring fission and indicated a *cis* relationship between the cyclopropylmethylene group and the carboxyl carbon. When hydrogenation was performed at pH 8·0, a second type of reductive fission occurred and pipecolic acid was produced in amounts approximately equal to those of *cis*-3-methylproline: under these conditions alloisoleucine was not detected. *Cis*-4-methylproline, the third possible product of cyclopropyl ring fission, was not found in any of the hydrogenation mixtures.

Specific optical rotation values measured for II in water and in 5 N-HCl showed the shift to more positive rotations in acid generally associated with an L-configuration at the α-carbon in amino and imino acids.

Cis-α-(Carboxycyclopropyl)glycine (I)

The isolation procedure involving an absorption step onto an anion-exchange resin indicated the acidic nature of I. Elementary analysis suggested the empirical formula $C_6H_9NO_4$.

The compound was stable to prolonged heating with strong mineral acid but readily formed a lactam when autoclaved at pH 3 at 120°, a behaviour characteristic of glutamic acid and certain of its substituted derivatives. The mass spectrum of compound I obtained at about 200° did not give a peak corresponding to the expected parent ion at *m/e* 159, but contained intense ions at *m/e* 141 (M-18) and *m/e* 114 (M-45). The ion at 114 decreased steadily with time as compared with that at 141, indicating that the latter is the parent ion of another molecular species, presumably the lactam of I, whose spectrum is superimposed on and increases in intensity relative to that of the parent acid. The NMR spectrum of I showed the absence of olefinic protons; in the high field region complex signals, indicative of a cyclopropyl ring, were present, and so the spectrum was consistent with the structure proposed. The crucial evidence in support of the proposed cyclopropyl formulation came from an analysis of the products obtained after hydrogenation of I under weakly acidic conditions. *Erythro*-γ-methylglutamic acid represented the major product (1,3-fission), while smaller amounts of α-aminoadipic acid (1,2-fission) were formed: this mixture of products could not have arisen from any alternative structure. The *erythro*-configuration of the γ-methylglutamate indicated that I is the *cis*-isomer of α-(carboxycyclopropyl)glycine (assuming an L-configuration at the α-carbon based on specific optical rotation values). The *cis* relationship of the carboxyl and glycine moiety in I is consistent with the observed lactam formation, and with the coexistence of I and II in the same species and the related proposal that I represents

the biogenetic precursor of II. Unequivocal confirmation of the *cis* configuration was provided by the degradation of I into *cis*-cyclopropane-1,2-dicarboxylic acid using *N*-bromosuccinimide and silver oxide as oxidants.

Trans-α-(Carboxycyclopropyl)glycine (III)

The assignment of structure in this instance followed from evidence of the type obtained above for I. The mass spectrum (and elementary analysis) was in agreement with the molecular formula $C_6H_9NO_4$. The NMR spectrum was again complex, but it was very similar to the spectrum of I and consistent with that expected for a diastereoisomer.

A different pattern of cyclopropyl ring fission occurred when III was hydrogenated; no reductive 1,2-fission was observed, but about equal amounts of *threo*-γ-methylglutamic acid (a 1,3-split) and *erythro*-β-methylglutamic acid (a 2,3-split) were produced. The configurations of these methyl-substituted glutamic acids are those expected from a *trans*-L isomer of α-(carboxycyclopropyl)glycine. Specific optical rotation measurements, and the failure to produce a lactam from III, supported this isomeric assignment.

Oxidative degradation of III using *N*-bromosuccinimide followed by silver oxide gave *trans*-cyclopropane-1,2-dicarboxylic acid.

DISCUSSION

The confirmation that compounds I, II and III are further examples of cyclopropane amino acids has provided additional evidence of the close phytochemical relationship existing between the genus *Aesculus* and the members of the family Sapindaceae. The relationship is supported not only by the existence of diastereoisomers of α-(carboxycyclopropyl)glycine, i.e. compounds I and III, in *Aesculus* and *Blighia* respectively, but also by a structural comparison of these diastereoisomers with α-(methylenecyclopropyl)glycine isolated previously from *Litchi* seed.[2] These compounds differ only in the oxidation level of the C_1 α-substituent and may represent products arising from a common, but uncharacterized, biogenetic intermediate such as α-(hydroxymethylcyclopropyl)glycine. These structural comparisons are particularly pertinent when it is realized that, before *Aesculus* was established as the family Hippocastanaceae, the genus was placed within the Sapindaceae (see Bentham and Hooker's *Genera Plantarum*).

Our study of the amino acids of the *Aesculus* genus is being extended gradually to include other species. Preliminary chromatographic evidence indicates that the *cis*- and *trans*-isomers of α-(carboxycyclopropyl)glycine may co-exist in seed of *Aesculus glabra* and *A. octandra* but, although some samples of *A. glabra* contained appreciable concentrations of the *cis*-isomer, *exo*(*cis*)-3,4-methanoproline was not detected in the seed extracts. The mechanism governing the stereospecific synthesis of *cis*- and *trans*-isomers of α-(carboxycyclopropyl)glycine within a single species poses an intriguing problem, but their interconversion by an epimerase-type enzyme would seem feasible.

Cis-α-(carboxycyclopropyl)glycine is a potent inhibitor of the growth of mung bean seedlings, but attempts to reverse the inhibition by supplying either glutamic acid or proline to seedlings together with I were unsuccessful. Therefore it does not appear likely that I is acting as an inhibitory analogue by interfering with the conversion of glutamic acid into proline or with the direct utilization of glutamic acid for protein synthesis.

EXPERIMENTAL

Chromatographic and Electrophoretic Methods

Descending paper chromatography was performed on Whatman No. 3 MM filter paper using the following solvents: 1, 75 per cent (w/w) phenol in the presence of NH_3 vapour; 2, butan-1-ol–acetic acid–water (90:10:29, by vol.); and 3, *tert*-amyl alcohol–acetic acid–water (20:1:20, by vol., upper phase). High voltage paper electrophoresis was performed on Whatman 3 MM paper using a Locarte Co. (London) apparatus having 1 m plates. Separations were obtained either at pH 2·0 (formic acid–acetic acid–water, 61:97:1842, by vol.) or at pH 3·5 (acetic acid–pyridine–water, 10:1:190, by vol.)

Isolation of New Compounds

(*a*) *From* A. parviflora *seed.* Fresh seed (380 g) was macerated and extracted by continuous shaking for 48 hr in 75% (v/v) ethanol (4 l.). The residue was re-extracted twice with 75% ethanol and finally with boiling water (2 l.). The filtrates were combined, evaporated to small volume at 40° *in vacuo* to remove ethanol, and decolorized with charcoal. The clarified extract (2 l., pH 4·0) was applied to a Zeokarb 225 (× 8) column (H^+ form, mesh 52–100, 105 × 3·5 cm) to absorb amino acids: after thoroughly washing with water to remove non-cationic materials, the amino acids were displaced with 0·2 N-NH_3 and fractions (10 ml.) were collected. Compounds I and II were present in the first thirty-five fractions, together with acidic and hydroxy amino acids and proline: these fractions were concentrated, evaporated to about 100 ml and adjusted to pH 7.

The next fractionation step employed Dowex-1 as an anion-exchanger. The solution of amino acids (pH 7) was run through a Dowex-1 (× 10) column (acetate form, mesh 100–200, 70 × 1·5 cm): compound II was washed through with water together with hydroxy amino acids and proline. The combined eluate was concentrated and retained. Compound I was retained by the column at this stage, but was eluted subsequently by displacement with 0·3 N-acetic acid. I was present together with glutamic acid in fractions (10 ml) No. 7–20 collected after amino acid breakthrough. Aspartic acid was eluted later in fractions 21–28. The residue (1·27 g) from the combined fractions containing I and glutamic acid was streaked as a concentrated solution of ammonium salts across ten sheets of Whatman No. 3 MM paper, which were developed for 4 days in phenol–NH_3 (solvent 1). I was recovered by eluting appropriate bands of the chromatogram with hot water and, after decolorizing and concentrating the extract, crystals (225 mg) of I separated.

Compound II was separated from other amino acids not held by the Dowex-1 column by a second cation-exchange fractionation. The mixture of compounds was applied to a Dowex-50 (× 8) column (H^+ form, mesh 100–200, 70 × 0·9 cm), which was eluted with 0·2 N-NH_3. Fractions (5 ml) were collected immediately amino acids appeared in the eluate and I was present in fractions 1–16, together with small amounts of serine and threonine. The residue from these combined fractions was recrystallized from an ethanol–acetone–water mixture (3:2:3, by vol.) to yield pure I (1·82 g).

(*b*) *From* B. sapida *seed.* Fresh seed (3·5 kg) was macerated and extracted for 5 days with occasional stirring using 75% (v/v) ethanol (15 l.): the residue was re-extracted using chloroform-saturated water (15 l.) for a further 5 days. The extract was concentrated, adjusted to pH 4, warmed to 60° to coagulate any soluble protein and finally decolorized with charcoal. The extract (3 l.) was applied to a Zeokarb 225 (× 8) column (H^+ form, mesh 52–100, 100 × 5·5 cm) and, after washing, the amino acids were eluted using 0·25 N-NH_3. Compound III was present in the early fractions together with other acidic amino acids and smaller amounts of some neutral amino acids. These fractions were combined and reduced in volume to 400 ml. After adjusting to pH 7 with ammonia, the solution was applied to a Dowex-1 (× 10) column (acetate form, mesh 100–200, 130 × 2·5 cm). After washing, the acidic amino acids were displaced by 0·3 N-acetic acid and fractions (25 ml) collected. Numbers 1–19 contained about equal amounts of glutamic acid and III, but No. 20–26 contained III with only traces of impurities: crystals of pure III (1·5 g) separated after concentration of these latter fractions. Aspartic acid and hypoglycin B (the γ-glutamyl peptide of hypoglycin A) separated cleanly in later fractions.

Properties of the New Amino Acids

Exo(cis)-3,4-*methanoproline* (*II*). The recrystallized material had the following analysis: C, 56·8; H, 7·2; N, 10·9. $C_6H_9NO_2$ required: C, 56·7; H, 7·1; N, 11·0 per cent. The $[\alpha]_D^{20}$ values were −132° (*c*, 2 in water) and −104° (*c*, 1 in 5 N-HCl). The NMR spectrum (in D_2O) at 60 Mc/s showed resonances at 5·7τ (single-proton doublet, J=5 c/s, α-proton) and 6·5τ (two-proton doublet, J=2·5 c/s, C-5 protons of proline ring). In addition, complex multiplets centred at 8·0τ and 9·3τ were present. At 220 Mc/s, these were resolved into four single-proton signals at 7·95τ (multiplet), 8·15τ (multiplet), 9·25τ (quartet), and 9·55τ (doublet of triplets). The bands at 7·95τ and 8·15τ were shown by spin decoupling experiments at 100 Mc/s to be coupled respectively to the α and C-5 protons of the proline ring, and were therefore assigned to the single C-3 and C-4 protons. The remaining bands at 9·25τ and 9·55τ are assigned to the geminal protons of the cyclopropane ring.

The principal features of the mass spectrum of II and of the closely related isomers 4-methyleneproline

and baikiain are listed below. Figures in parentheses give the relative abundances of the parent ion and those fragment ions above *m/e* 40 whose intensities are more than 5 per cent of the base peak (100) in each case. *Compound II:* 127 (1·5), 82 (100), 80 (23), 67 (13), 65 (6), 55 (21), 54 (7), 53 (14), 42 (7), 41 (9). *4-Methyleneproline:* 127 (2·5), 82 (100), 80 (18), 67 (17), 55 (13), 54 (6), 53 (15), 41 (7). *Baikiain:* 127 (8), 82 (100), 80 (34), 67 (7), 55 (24), 54 (26), 53 (16), 41 (7).

The products obtained after hydrogenation of II in dilute acetic acid using Adam's platinum catalyst were co-chromatographed with various methylprolines and isoleucines and leucine using solvent 3. The main product appeared as a yellow-coloured spot after ninhydrin treatment and was inseparable from *cis*-3-methylproline (kindly supplied by Dr. J. Kollonitsch, Merck & Co., Rahway, N.J.). This solvent resolved four isomeric methylprolines as follows (distances in cm moved by imino acids in 24 hr): *trans*-4, 18·4; *cis*-4, 17·2; *trans*-3, 15·4; and *cis*-3, 14·0. The two diastereoisomer 4-methylprolines produced blue chromophores when chromatograms were developed with isatin, but the 3-methylprolines gave no colour: this distinction was useful in confirming the nature of the hydrogenation product. The minor hydrogenation product was inseparable from alloisoleucine: this characterization was that expected if hydrogenation caused fission of the pyrrolidine ring in *cis*-3-methylproline between the N and C-5 atoms.

The products formed after hydrogenation at pH 8 were separated from unchanged II by chromatography in solvent 2. Pipecolic acid was readily distinguished as a product, together with *cis*-3-methylproline, by co-chromatography with authentic material and by the formation of blue chromophores with both ninhydrin and isatin.

Cis-α-(*carboxycyclopropyl*)*glycine* (*I*). Elementary analysis of pure I indicated: C, 45·1; H, 5·6; N, 8·3. $C_6H_9NO_4$ required: C, 45·2; H, 5·7; N, 8·8 per cent. $[\alpha]_D^{20}$ values were calculated as +25° (*c*, 1 in water) and +58° (*c*, 0·5 in 5 N-HCl).

The NMR spectrum (in $NaOD/D_2O$) showed the α-proton as a doublet at 6·9τ ($J = 10$ c/s) and the remaining four protons as broad multiplet at 8·2–9·2τ. In DCl/D_2O, the α-proton appeared at 5·8τ and the high field protons between 7·9 and 8·8τ.

The mass spectrum (at 200°) was time-dependent owing to the steady thermal transformation of the compound into a lactam. Ions at *m/e* 141, 114, 96, 87, 78, 68 and 41 were prominent in the early running of the spectrum, but after some time the ions at *m/e* 141, 96, 78, 68 and 41 tended to be dominant.

I was converted into a lactam (R_{Leu} 1·05 in solvent 2) when a solution was autoclaved at pH 3 for 4 hr. The lactam (a pyrrolidone carboxylic acid derivative) gave both the aniline–xylose reaction,[6] characteristic of an organic acid, and the starch–chloroimide reaction[7] associated with the presence of a peptide (lactam) bond.

The cyclopropane ring of I was cleaved slowly during hydrogenation in dilute acetic acid at laboratory temperature and pressure using Pt catalyst. The products were separated on chromatograms developed in solvent 3 and tentatively identified as α-aminoadipic acid and *erythro*-γ-methylglutamic acid by co-chromatography with authentic materials. The solvent effectively separated the following substances (distances in cm moved in 40 hr): *threo*-γ-, 27·7; *erythro*-γ-, 24·6; *erythro*-β, 22·2; *threo*-β-methylglutamic acid, 20·8; α-aminoadipic acid, 19·6. After separation the products were eluted and run on a paper electrophoretogram at pH 3·5 (60 v/cm, 40 mA, 3 hr) against marker compounds when their behaviour was again identical with α-aminoadipic acid and *erythro*-γ-methylglutamic acid (moving 1·1 and 8·0 cm towards the anode respectively).

I gave *cis*-cyclopropane-1,2-dicarboxylic acid when oxidized following the procedure of Black and Landor.[8] I (4 mg) was shaken with *N*-bromosuccinimide (4 mg) in 0·4 ml water at 30° for 3 hr. Then freshly prepared Ag_2O was added (2 ml of a suspension prepared by adding 3·2 ml N-NaOH to 0·32 g $AgNO_3$ in 0·5 ml 50% ethanol) and shaking was continued for a further 3 hr. After removal of excess Ag_2O by centrifuging, the supernatant was acidified with HCl and evaporated to dryness. The residue was extracted with hot acetone and aliquots of the solution were chromatographed against the *cis*- and *trans*-isomers of cyclopropane-1,2-dicarboxylic acid in solvent 3. Aniline–xylose reagent[7] was used for detection and showed good resolution of the diastereoisomers (R_f values of 0·65 and 0·85 for *cis*- and *trans*- forms): the oxidation product of I behaved identically with the *cis*-isomer. A small amount of succinic acid (R_f 0·76) was present on the chromatogram, and presumably arose from *N*-bromosuccinimide.

Trans-α-(*carboxycyclopropyl*)*glycine* (*III*). Found: C, 45·4; H, 5·9; N, 8·6; $C_6H_9NO_4$ required: C, 45·2; H, 5·7; N, 8·8 per cent. $[\alpha]_D^{20}$ values were +107° (*c*, 2 in water) and +146° (*c*, 1 in 5 N-HCl). The NMR spectrum (in $NaOD/D_2O$) at 60 Mc/s showed the α-proton as a doublet at 7·3τ ($J = 8$ c/s) and four protons as two overlapping multiplets centred at *ca.* 8·6 and 9·0τ. At 220 Mc/s, these were well resolved into two separate bands containing 14 and 13 discrete lines respectively.

In contrast to the behaviour of compound I, III showed no tendency to form a lactam at 200° in the mass spectrometer and thus gave a much simpler spectrum. The parent ion was again unobservable, the ion of highest mass being that at *m/e* 114, which was also the base peak. Other prominent ions were at *m/e* 96, 69, 68 and 41.

It is noteworthy that there is no prominent ion in the spectrum of either I or III at *m/e* 74 normally observed

[6] I. SMITH, *Chromatographic and Electrophoretic Techniques*, Vol. 1, p. 279, Heinemann, London (1960).
[7] H. N. RYDON and P. W. G. SMITH, *Nature* **169**, 922 (1952).
[8] D. K. BLACK and S. R. LANDOR, *J. Chem. Soc.* (*C*) 288 (1968).

for amino acids containing the moiety $NH_2—\underset{|}{C}H—CO_2H$. This is consistent with the general observation that electron-impact induced fission of alkyl chains does not usually occur α to an unsaturated linkage or to a cyclopropyl ring. III was stable to hot mineral acid and did not yield a lactam.

After hydrogenation using Pt catalyst, the reduction products were examined by chromatography and electrophoresis as for I above. Chromatography using solvent 3 indicated that, in addition to unchanged III, the reaction mixture contained *erythro*-β- and *threo*-γ-methylglutamic acids in approximately equal amounts. These identifications were confirmed by elution and subsequent electrophoresis at pH 3·5 against authentic materials. (The sample of β-methylglutamic acid available[9] contained both diastereoisomers, which separated on electrophoresis either at pH 1·9 (see Kagan and Meister[10] for mobilities of the two forms) or pH 3·5.)

Oxidative degradation of III using *N*-bromosuccinimide and Ag_2O proceeded smoothly to yield *trans*-cyclopropane-1,2-dicarboxylic acid.

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[9] J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. 3, p. 2428, Wiley, New York (1961).
[10] H. M. Kagan and A. Meister, *Biochemistry* **5**, 725 (1966).