## 778. *Synthesis of* L-*Aspartic* β-*Semialdehyde.*

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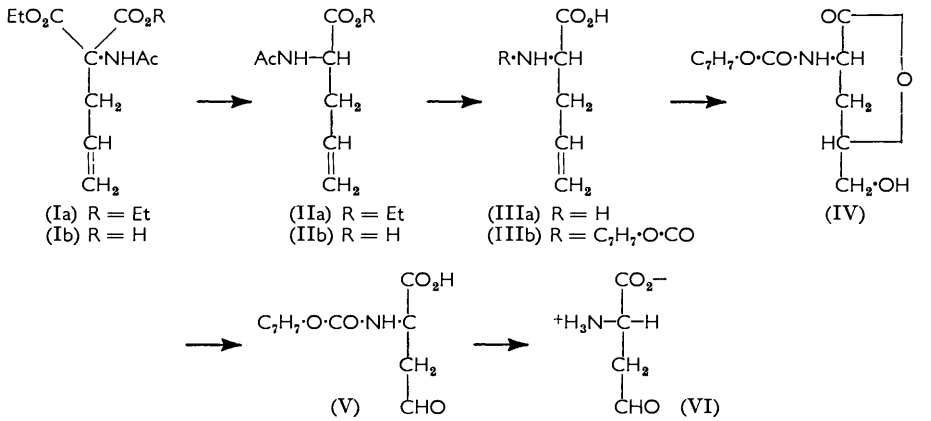
*N*-Benzyloxycarbonyl-L-aspartic β-semialdehyde has been prepared in crystalline form by oxidation of *N*-benzyloxycarbonyl-L-allylglycine with performic acid, followed by oxidation of the resulting γδ-dihydroxy-acid with sodium metaperiodate. The substituted amino-aldehyde (V), on hydrogenolysis in dilute acid, yielded free L-aspartic β-semialdehyde (VI), which however was only obtained in solution. The structure of the γδ-dihydroxy-acid prepared as an intermediate was shown by conversion of its γ-lactone into a toluenesulphonate, followed by hydrogenolysis and ring closure to yield a mixture of hydroxy-L-proline and allohydroxy-L-proline.

L-Aspartic β-semialdehyde (L-α-amino-γ-oxobutyric acid) (VI) has been shown to be an intermediate in the formation of L-threonine and L-methionine by *Neurospora crassa*, *Escherichia coli*, yeast, and higher plants (for review see Greenberg [1]). The biosynthetic pathway, which has been elucidated mainly by Black and Wright,[2] leads from aspartic acid to aspartyl β-phosphate; the latter is reduced to aspartic β-semialdehyde by a specific dehydrogenase, and the amino-aldehyde is then further reduced to homoserine by another enzyme, called homoserine dehydrogenase. In the last two steps reduced forms of pyridine nucleotides act as hydrogen donors. L-Homoserine is then converted into

[1] "Metabolic Pathways," Greenberg, Academic Press Inc., New York, 2nd edn., Vol. II, p. 186.
[2] Black and Wright, *J. Biol. Chem.*, 1955, **213**, 27, 39, 51.

L-threonine or L-methionine by known pathways. Aspartic semialdehyde was prepared [2] from L-allylglycine (L-2-aminopent-4-enoic acid) (IIIa) by oxidation with ozone; the ozonolysis product, formed in 95% yield, was absorbed on the cation-exchange resin Dowex 50 and the amino-aldehyde, which was not obtained in a solid form, was eluted with acid. Black and Wright found the aldehyde (VI) to be " reasonably stable " in acid solution, but to be largely decomposed within a few hours if stored at neutrality either in the dry state or in solution. No crystalline derivative of it was obtained and the identity of the material was based mainly on quantitative enzymic conversion by the reactions mentioned above into aspartyl β-phosphate and homoserine, respectively, together with formation of equivalent amounts of oxidised or reduced pyridine nucleotides.




In connexion with experiments described elsewhere,[3] in which the effects of methionine and threonine on the formation of porphyrins and bacteriochlorophyll in *Rhodopseudomonas spheroides* were studied, we decided to investigate the various enzymic steps involved in the biosynthesis of these two amino-acids from aspartic acid. We therefore re-examined the synthesis of the aldehyde (VI) and in particular we wished to have available a crystalline and stable derivative from which the aldehyde itself could be easily obtained whenever needed. As in the work of Black and Wright the synthesis started from L-allylglycine. The racemic compound had been prepared by Sørensen [4] by the phthalimidomalonate method and by Locquin and Cerchez.[5] Albertson [6] later prepared the racemic acid (IIIa) by condensing allyl bromide with ethyl sodioacetamidomalonate, to give an ester (Ia), followed by hydrolysis with strong acid. However, treatment of this ester with boiling mineral acid was found to give, apart from the desired allylglycine, a salt of α-aminovalerolactone.[7] We therefore tried to obtain the desired *N*-acetyl derivative (IIb) by two successive treatments with alkali in the cold. The ester (Ia) was treated with one equivalent of cold alkali, to give the acid ester (Ib). This readily lost carbon dioxide at 130—132°. Further treatment of the resulting ester (IIa), which was not isolated, gave *N*-acetyl-DL-allylglycine (IIb) in 75—80% overall yield. Deacetylation of the L-isomer with the enzyme deacylase was carried out as described by Black and Wright.[2] The L-configuration of the product follows from the established stereochemical specificity of the enzyme and the direction of the shift of molecular rotation with ionisation of the carboxyl group.[2]

The amino-acid (IIIa) was treated under usual conditions with benzyl chloroformate,

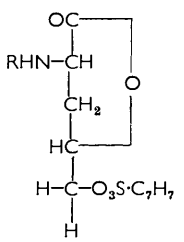
[3] Gibson, Neuberger, and Tait, *Biochem. J.*, 1962, **84**, 483.
[4] Sørensen, *Compt. rend. Trav. Lab. Carlsberg, Ser. chim.*, 1905, **6**, 187.
[5] Locquin and Cerchez, *Bull. Soc. chim. France*, 1928, **43**, 932.
[6] Albertson, *J. Amer. Chem. Soc.*, 1946, **68**, 450.
[7] Hillman and Albertson, *J. Amer. Chem. Soc.*, 1948, **70**, 1711; Goering, Cristol, and Dittmer, *ibid.*, p. 3310.

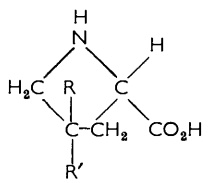
to give the derivative (IIIb) and this was oxidised with performic acid at 40°. The only compound which could be isolated in reasonable quantity was a neutral substance which from its analysis and behaviour on titration appeared to be a lactone (IV) of α-amino-γδ-dihydroxyvaleric acid. The size of the lactone ring and the configuration of the γ-carbon atom could be deduced from the conversion of the derived toluene-*p*-sulphonate into a mixture of allohydroxy-L-proline and hydroxy-L-proline to be described below. Small amounts of mixtures of isomeric lactones and possibly of corresponding acids were also isolated when the conditions of the oxidation and working-up were varied, but these substances were not further investigated. The lactone ring was readily opened by treatment with two equivalents of cold alkali; after adjustment of the pH to neutrality, the sodium salt of the acid was oxidised with periodate to give ultimately crystalline α-L-benzyloxycarbonylamino-γ-oxobutyric acid (V). The presence of a carbonyl group in this was shown by formation of a *p*-nitrophenylhydrazone. The material was also oxidised with alkaline permanganate and the product resulting after hydrogenolysis was shown chromatographically to be aspartic acid. The benzyloxycarbonyl derivative (V) of the aspartic β-semialdehyde was stable for at least 6 months if stored at room temperature as a solid.

On hydrogenolysis in acid solution, the derivative (V) gave the semialdehyde (VI) in practically quantitative yield, as shown by reaction with NADPH and homoserine dehydrogenase. The amino-aldehyde also reacted with aspartic semialdehyde dehydrogenase and $NADP^+$, provided that inorganic phosphate was present. This is in accordance with the findings of Black and Wright.[2] When catalytic reduction was carried out at a neutral pH only small amounts of material active in enzyme assays were obtained. In view of the instability of the free amino-aldehyde no effort was made to obtain it in solid form. It should be noted that the yield of semialdehyde obtained by the method described here is appreciably lower than that given by the more direct ozonolysis of Black and Wright.

Of the two centres of asymmetry in the lactone (IV) the α-carbon atom must have L-configuration, as the reactions leading to it from L-allylglycine are unlikely to invert this centre. However, the configuration of the γ-carbon atom was uncertain and in order to clarify this point the lactone was treated with toluene-*p*-sulphonyl chloride, giving the ester (VIIa); the benzyloxycarbonyl group was then removed, and the resulting free amino-lactone (VIIb), which was not further characterised, was heated at pH 10·6 in aqueous methanol. The product gave all the tests characteristic for hydroxyproline, but

(VIIa) R = $C_7H_7O{\cdot}CO$
(VIIb) R = H





(VIIIa) R = OH, R′ = H
(VIIIb) R = H, R′ = OH

the specific rotation (−66°) suggested that the material was a mixture of hydroxy-L-proline (VIIIa) and allohydroxy-L-proline (VIIIb). The latter is reported[8] to have $[\alpha]_D$ −58·1°, while $[\alpha]_D$ for hydroxy-L-proline[9] is −76°. This conclusion was confirmed by electrophoretic analysis on paper[10] at pH 1·9 with the two authentic acids as markers.

It appears, therefore, that the lactones (IV) and (VII) are also mixtures of γ-epimers.

[8] Neuberger, *J.*, 1945, 429.
[9] Greenstein and Winitz, " Chemistry of the Amino Acids," John Wiley, New York, 1961, Vol. III, pp. 2019—2026.
[10] Wieland and Wintermeyer, *Chem. Ber.*, 1957, **90**, 1721; Wieland and Pfleiderer, *Angew. Chem.*, 1957, **69**, 199.

As judged from the rotation of the hydroxyproline finally isolated and the size of the electrophoretic spots the mixture contained a slight excess of the allo-isomer.

## Experimental

*Ethyl Hydrogen α-Acetamidoallylmalonate.*—Ethyl α-acetamidoallylmalonate[6] (97·2 g., 0·38 mole) was dissolved in absolute alcohol (400 ml.). 5·95N-Sodium hydroxide (70·4 ml., 0·418 equiv.) was added and the solution left at room temperature for 2 hr. After addition of 6N-sulphuric acid (70 ml., 0·420 equiv.) and alcohol (100 ml.) and after 1 hr. at 0° the solution was filtered and concentrated under reduced pressure. The residue was crystallised from ethyl acetate–light petroleum (b. p. 60—80°), giving 87% of *ester* of m. p. 117—119°. On repeated recrystallisation from ethyl acetate–light petroleum the m. p. rose to 126—126·5° (Found: C, 52·3; H, 6·4; N, 6·0. $C_{10}H_{15}NO_5$ requires C, 52·5; H, 6·6; N, 6·1%).

N-*Acetyl*-DL-*allylglycine.*—The above acid ester (75·5 g., 0·33 mole) was decarboxylated at 130—132°. The product was dissolved in alcohol (100 ml.), and 5·95N-sodium hydroxide (61·0 ml., 0·36 equiv.) was added. After 2 hr. at room temperature and addition of 6N-sulphuric acid (61·0 ml., 0·37 equiv.), the solution was cooled to 0° and after a further 2 hr. was filtered and evaporated. The residue crystallised from acetone–light petroleum (b. p. 60—80°), giving 31·3 g. of material, m. p. 112·5—113·5° (Black and Wright record m. p. 114°). A further 14·6 g. of less pure material were recovered from the mother-liquor (total yield 89%).

L-*Allylglycine.*—The resolution was done as described by Black and Wright. An 85% yield of material having $[\alpha]_D^{24}$ −35·8° (*c* 4·37 in $H_2O$) was obtained. After recrystallisation $[\alpha]_D^{24}$ was −37·0° (*c* 3·95 in $H_2O$). Black and Wright report $[\alpha]_D^{20}$ −37·1° in $H_2O$.

N-*Benzyloxycarbonyl*-L-*allylglycine.*—L-Allylglycine (6·90 g., 60 mmoles; $[\alpha]_D^{24}$ −35·8°) was dissolved in 5N-sodium hydroxide (15 ml., 75 mmoles) and cooled to 0°. 5N-Sodium hydroxide (20 ml., 100 mmoles), and a 5N-solution of benzyl chloroformate in toluene (20 ml., 100 mmoles), were added in five equal portions during 90 min. The solution was shaken between additions, and shaking was continued for a further 16 hr. The mixture was diluted with water (75 ml.) and extracted with benzene (4 × 50 ml.), then with ether (2 × 50 ml.). To the aqueous solution was added concentrated hydrochloric acid (20 ml.), and the mixture was extracted with ether (2 × 75 ml.). The combined ether solutions were washed with water (25 ml.), and the product extracted with M-sodium hydrogen carbonate (70 ml., 10 ml.). The combined aqueous extracts were washed with ether, then with light petroleum (b. p. 40—60°). To the aqueous residue was added concentrated hydrochloric acid (10 ml.) until no further precipitation occurred. The almost colourless oily *derivative* crystallised spontaneously (90%; m. p. 65°). Recrystallisation from ethyl acetate–light petroleum (b. p. 60—80°) did not raise the m. p.; $[\alpha]_D^{20}$ was +17·6° ± 0·6° (*c* 5·0 in $CHCl_3$) (Found: C, 62·9; H, 5·9; N, 5·5. $C_{13}H_{15}NO_4$ requires C, 62·7; H, 6·0; N, 5·6%).

α-L$_S$-γ-DL-α-*Benzyloxycarbonylamino*-δ-*hydroxy*-γ-*valerolactone.*—The above benzyloxycarbonyl compound (10·5 g., 42·2 mmoles) was dissolved in 98—100% formic acid (84 ml.), and 9·1M-hydrogen peroxide (5·35 ml., 49·0 mmoles) was added. After 16 hr. at 40° the formic acid was removed under reduced pressure. To the residue was added water (100 ml.), and it also was removed under reduced pressure. Water (100 ml.) was again added and this time removed at atmospheric pressure. The oily residue was dissolved in ethyl acetate (100 ml.), which was extracted successively with M-sodium acetate (2 × 50 ml.) and water (10 ml.). After being dried ($Na_2SO_4$), the ethyl acetate was removed under reduced pressure and the residue crystallised from ethyl acetate–light petroleum (b. p. 60—80°) (35%; m. p. 85—86°). Recrystallisation gave a lactone mixture, m. p. 90·5—91·5°, $[\alpha]_D^{20}$ −21·0° ± 1° (*c* 2·7 in ethyl acetate) (Found: C, 58·6; H, 5·9; N, 5·1. Calc. for $C_{13}H_{15}NO_5$: C, 58·9; H, 5·7; N, 5·3%).

N-*Benzyloxycarbonyl*-L-*aspartic* β-*Semialdehyde.*—The above lactone mixture (0·70 g., 2·64 mmoles) was dissolved in ethanol (30 ml.) and water (20 ml.), giving an apparent pH of 5·1. 0·5N-Sodium hydroxide (10·0 ml., 5 milliequiv.) was added. After 2 hr. at room temperature the solution was titrated to pH 6·5 with 0·5N-sulphuric acid. It was found that 2·57 milliequiv. of alkali had been consumed (98% of that required to open the lactone ring). To this solution was added sodium metaperiodate (1·2 g. in 25 ml. of water; ~5·6 mmoles). The pH was adjusted to 8·5 with sodium hydrogen carbonate, and the solution diluted with water to 200 ml. After 2 hr. at room temperature the solution was left at 4° overnight. A large precipitate had formed but this dissolved on acidification with sulphuric acid (Congo Red). The acidified

solution was extracted with ethyl acetate (2 × 100 ml.). The combined ethyl acetate extracts were washed with water (30 ml.), dried ($Na_2SO_4$), and evaporated under reduced pressure. The crystalline residue was recrystallised from ethyl acetate–light petroleum (b. p. 60—80°), giving the *semialdehyde derivative*, (46·1 g.) m. p. 135—137°, $[\alpha]_D^{21}$ −19·8° ± 1° (*c* 1·7 in ethyl acetate) (Found: C, 57·0; H, 5·4; N, 5·5. $C_{12}H_{13}NO_5$ requires C, 57·4; H, 5·2; N, 5·6%).

The *p*-nitrophenylhydrazone of the aldehyde was prepared as follows. The aldehyde (25 mg., 100 μmoles) was dissolved in methanol (~1 ml.), and *p*-nitrophenylhydrazine (15 mg., 100 μmoles) in methanol (1 ml.) was added. The solution was heated for a few minutes. On cooling and addition of 20% (v/v) aqueous acetic acid yellow crystals separated. The yield of *hydrazone* was 35 mg., and the m. p. 162·5—163·5°. Recrystallisation from methanol–20% aqueous acetic acid did not raise the m. p. (Found: C, 55·5; H, 4·8; N, 14·7. $C_{18}H_{18}N_4O_6$ requires C, 56·0; H, 4·7; N, 14·5%).

L-*Aspartic* β-*Semialdehyde.*—*N*-Benzyloxycarbonyl-L-aspartic β-semialdehyde (50 mg., 200 μmoles) was hydrogenated in ethyl acetate (15 ml.) in the presence of dilute hydrochloric acid (10 ml. containing 400 μmoles) over 10% palladium–charcoal (~10 mg.) for 1 hr. at room temperature. The mixture was filtered, the ethyl acetate layer removed, and the aqueous layer freed from ethyl acetate by evaporation *in vacuo*. By using an enzyme extract from *Rhodopseudomonas spheroides* containing homoserine dehydrogenase the product of the hydrogenation was found to be active in oxidising NADPH.[3] By this assay 190 μmoles of L-aspartic β-semialdehyde were obtained (95% calc. on the benzyloxycarbonyl compound used). The solution contained 191 μ-atom-equiv. of total nitrogen. The solution also reduced $NADP^+$ in the presence of *Rps. spheroides* extract containing aspartic β-semialdehyde dehydrogenase only if inorganic phosphate was also present. This is expected on the basis of the results of Black and Wright.

*Conversion of* N-*Benzyloxycarbonyl*-L-*aspartic* β-*Semialdehyde into Aspartic Acid.*—The benzyloxycarbonyl compound (V) (25 mg., 100 μmoles) was dissolved in M-sodium carbonate (5 ml.) and potassium permanaganate solution (100 μ-equiv., 33·3 μmoles) was added during 10—15 min. The solid manganese dioxide was removed by centrifugation, and the colourless supernatant layer was acidified with hydrochloric acid (Congo Red) and extracted with ethyl acetate. The ethyl acetate extract was dried ($MgSO_4$) and taken to dryness under reduced pressure. The oily residue was hydrogenated in ethyl acetate in the presence of palladium–charcoal for 1 hr. at room temperature. After filtration, the ethyl acetate solution was extracted with N-hydrochloric acid. The aqueous solution was taken to dryness and the residue dissolved in a small volume of water. Samples were spotted on Whatman No. 1 paper and chromatographed along with aspartic acid markers in butan-1-ol–acetic acid–water (60 : 15 : 25 by vol.) and also in phenol–water (4 : 1 w/v). Spraying with ninhydrin solution gave a single purple spot in both solvents with $R_F$ values of 0·11 and 0·09, respectively. Authentic aspartic acid was found to have $R_F$ of 0·10 in both solvents.

α-L$_S$-γ-DL-α-*Benzyloxycarbonylamino*-δ-*toluene*-p-*sulphonyloxy*-γ-*valerolactone.*—L$_S$-γ-DL-α-Benzyloxycarbonylamino-δ-hydroxy-γ-valerolactone (2·65 g., 10 mmoles) was dissolved in dry pyridine (8 ml., 10 mmoles) contained in a 3-necked round-bottomed flask fitted with a calcium chloride tube, a thermometer, and a magnetic stirrer. The contents were cooled to 0° in an ice-bath. Toluene-*p*-sulphonyl chloride (2·10 g., 11 mmoles) was added in portions during 15 min., at <5°. After 3 hr. at 0—5° concentrated hydrochloric acid (10 ml.), ice and water (50 ml.) were added. A white oil separated and this was dissolved in ethyl acetate (60 ml.), washed with 3N-hydrochloric acid (25 ml.) and with water (2 × 30 ml.), dried ($Na_2SO_4$), and recovered under reduced pressure. The residue crystallised from ethyl acetate–light petroleum (b. p. 60—80°) (yield 84%; m. p. 95—103°). Recrystallisation from methanol–water gave an ester mixture of m. p. 101—102° (softening at 93°), $[\alpha]_D^{20}$ −8·9° ± 0·5° (*c* 2·36 in ethyl acetate) (Found: C, 57·0; H, 5·1; N, 3·3. Calc. for $C_{20}H_{21}NO_7S$: C, 57·4; H, 5·0; N, 3·3%).

*Hydroxy*-L-*proline* + *Allohydroxy*-L-*proline.*—The toluene sulphonate compound (VIIa) (1·05 g., 2·5 mmoles) was dissolved in 80% methanol (50 ml.), and glacial acetic acid (0·30 g., 5·0 mmoles) was added. Hydrogenation was performed at atmospheric pressure for 1½ hr. in the presence of 10% palladium–charcoal (~100 mg.). The catalyst was removed and the solution concentrated under reduced pressure, leaving a crystalline residue which was hygroscopic and was probably α-L$_S$-γ-DL-α-amino-δ-toluene-*p*-sulphonyl-γ-valerolactone acetate. This material was dissolved in methanol (10 ml.), and 0·5M-carbonate–bicarbonate buffer (pH 10·6; 30 ml.) was added. The solution was refluxed on a boiling-water bath for 2 hr., then neutralised with

hydrochloric acid and evaporated under reduced pressure. The solid residue was extracted with acetone containing 5% of 6N-hydrochloric acid (3 × 10 ml.). This extract was taken to dryness under reduced pressure and the excess of acid was removed by addition of water and evaporation again. The residue was dissolved in water (50 ml.), and Deacidite G (50-μ particle size; $OH^-$ form) added until the pH rose to 3·5. The solution was filtered and the resin washed with water. The combined filtrates were taken to dryness. The residue was dissolved in a small volume of water and crystallised on addition of ethanol (yield 220 mg.). On further recrystallisation the material had $[\alpha]_D^{20} -66° \pm 1°$ (*c* 0·70 in $H_2O$).

The material gave on paper a yellow colour with ninhydrin, a blue colour with isatin, and a purple colour with Ehrlich's *p*-dimethylaminobenzaldehyde reagent after the paper had been first treated with isatin. All these reactions are characteristic of both allohydroxy-L-proline and hydroxy-L-proline and were given by authentic specimens (Smith [11]).

On high-voltage electrophoresis on Whatman 3 MM paper in 0·2M-acetic acid–1·5M-formic acid (pH 1·85) at ~60 v/cm. for 1 hr., authentic hydroxy-L-proline ran 26·4 cm. and authentic allohydroxy-L-proline ran 31·5 cm. from the origin. The experimental material gave two spots, one at 26·8 cm. from the origin and the other at 31·4 cm. under the same conditions.

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[11] Smith, " Chromatographic and Electrophoretic Techniques," William Heinemann Medical Books, Ltd., London, 1960, Vol. I, p. 95.