661. *Syntheses from Phthalimido-acids. Part III.* The Preparation of* DL- *and* L-*Asparagine from Phthalyl-*DL- *and* -L-*aspartic Anhydride.*

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It has been shown that the action of ammonia on phthalyl-DL- and -L-aspartic anhydride leads to the formation of β-amides, since on removal of the phthalyl groups with hydrazine DL- and L-asparagine are obtained. In this the phthalylaspartic anhydrides resemble the corresponding phthalylglutamic anhydrides which with suitable reagents afford solely γ-derivatives, whereas, in general, anhydro-compounds of the other known acyl-aspartic and -glutamic acids give predominantly or exclusively α-substituted products.

DURING an investigation which led to the first recorded application of the phthalyl group as a protective device in peptide synthesis (King and Kidd, *Nature*, 1948, **162**, 776; *J.*, 1949, 3315), it was observed that the phthalyl-DL- and -L-glutamic anhydrides gave γ-substituted products on reaction with various amino-compounds, in contrast to the anhydrides of most acylamido-acids, from which α-derivatives or mixtures containing a preponderance of the α-isomer are generally formed. Treatment of the products with hydrazine removed the phthalyl group and thus opened a direct route to glutamine and other simple peptides of glutamic acid. The exclusive formation of γ-derivatives from the phthalylglutamic anhydrides extends also to their reactions with alcohols (King, Jackson, and Kidd, *J.*, 1951, 243), and the circumstances naturally suggested an extension of the enquiry into the chemically and biologically related aspartic acid series.

The anhydrides of acylaspartic acids in general give rise on ring-scission to α-substituted products, although certain inconsistencies have been reported in their reactions with both alcohols and amines. Benzoyl-L-aspartic anhydride, for example, gives with methanol a pure α-methyl ester (Pauly and Weir, *Ber.*, 1910, **43**, 661), whereas the action of benzyl alcohol on the toluene-*p*-sulphonylaspartic anhydride leads to a mixture of α- and β-benzyl esters in the ratio of approximately 4 : 1 (Bovarnick, *J. Biol. Chem.*, 1943, **148**, 151). The product obtained from the benzoyl-L-aspartic anhydride and ammonia has usually been regarded as the α-amide since it was apparently not identical with benzoylasparagine (Pauly and Weir, *loc. cit.*); its melting point corresponds approximately with that of benzoyl*iso*asparagine later recorded by Akizuki (*J. Biochem., Japan*, 1937, **25**, 43). Unpublished work by one of us with R. J. S. Beer (Thesis, Oxford, 1945) has confirmed the earlier inference by demonstrating the identity of the products obtained by the action of ammonia on the authentic methyl benzoyl-DL-aspartate and on the corresponding anhydride.

From carbobenzyloxy-L-aspartic anhydride α-amides normally appear to be formed, *e.g.*, from ammonia (Bergmann and Zervas, *Ber.*, 1932, **65**, 1192) and glycine ethyl ester (Bergmann, Zervas, and Fruton, *J. Biol. Chem.*, 1935, **111**, 235), but the product obtained with L-tyrosine ethyl ester, initially described as the α-amide (Bergmann and Zervas, *loc. cit.*), was subsequently shown to be the β-isomer (Bergmann, Zervas, Salzmann, and Schleich, *Z. physiol. Chem.*, 1934, **224**, 17). Acetylaspartic acid can be dehydrated with acetic anhydride either to an oxazolone (Bergmann, Stern, and Witte, *Annalen*, 1926, **449**, 277) or to a normal dicarboxylic anhydride (Harington and Overhoff, *Biochem. J.*, 1933, **27**, 338). Presumably products arising from the former will be α-derivatives but the constitution of compounds which may be derived from the true anhydride has not been ascertained.

Phthalylaspartic acid has been prepared by Piutti (*Gazzetta*, 1885, **14**, 473; 1886, **16**, 1) from L-aspartic acid and also from asparagine by heating them with phthalic anhydride at 150°. The configuration of the product was not indicated but from experience in the preparation of phthalylglutamic acid (King and Kidd, *loc. cit.*) it seemed probable that under these conditions racemisation would occur. This was verified by fusing a mixture of L-asparagine and phthalic anhydride: when the product, presumably an ammonium salt, was dissolved in water and acidified, a feebly active substance was precipitated which after recrystallisation had a melting point (225°) identical with that determined by Piutti.

On heating a mixture of DL-aspartic acid and phthalic anhydride first in boiling pyridine and then briefly in acetic anhydride, phthalyl-DL-aspartic anhydride was synthesised. With hot water the anhydride gave the DL-acid, m. p. 225°. When treated with ammonia under anhydrous conditions, the phthalyl-DL-anhydride was almost quantitatively converted into

* Part II, *J.*, 1951, 243.

a single amide which when submitted to hydrolysis with aqueous hydrazine at room temperature yielded the β-derivative, DL-asparagine, thus establishing the intermediate as phthalyl-DL-asparagine. The identification of the isomeric DL-aspartic monoamides is impracticable from melting-point determinations, and Bovarnick (*J. Biol. Chem.*, 1943, **148**, 151) has distinguished the racemic isomers by the difference in their rates of hydrolysis and in their crystalline form, the α-amide occurring in needles and the β-amide in prisms. The product from the hydrolysis of the phthalyl-amide crystallised in well-defined prisms and was further identified as β-DL-asparagine by the ninhydrin analysis of van Slyke *et al.* (*J. Biol. Chem.*, 1941, **141**, 671), the liberation of 0·9 mol. of carbon dioxide demonstrating the presence of the free α-carboxyl group.

The preparation of the optically active L-series started from the more readily accessible L-asparagine which though more stable than glutamine is readily converted by refluxing alcoholic hydrogen chloride (Fischer and Koenigs, *Ber.*, 1904, **37**, 4599) into diethyl L-aspartate. Treatment of a solution of this ester in ether with phthalic anhydride gave what is presumed to be the phthalamic acid which with thionyl chloride was cyclised to the liquid diethyl phthalyl-L-aspartate. Hydrolysis of the ester without affecting the phthalimido-group proved difficult but was finally achieved by a boiling mixture of acetic acid and concentrated hydrochloric acid. The crude phthalyl-L-aspartic acid was briefly heated with acetic anhydride from which on cooling the phthalyl-L-aspartic anhydride then crystallised. Phthalyl-L-aspartic acid was obtained from it by crystallisation from hot water.

By a procedure similar to that used in the DL-series, the L-anhydride was converted into phthalyl-L-asparagine, from which L-asparagine was obtained by hydrolysis with hydrazine. The specific rotation of the product determined in N/10-hydrochloric acid was found to be comparable with the published values for natural asparagine. Moreover the R_F values determined in different media were identical. The absence of appreciable amounts of the α-amide was also demonstrated in a two-dimensional chromatogram according to the method of Crumpler and Dent (*Nature*, 1949, **164**, 441), the first run being carried out along a copper carbonate band, which immobilises α-amino-acids by complex formation. No evidence of a β-amino-carboxyl grouping, such as is present in *iso*asparagine, was obtained on subsequent development and treatment with ninhydrin.

The phthalyl-L-aspartic anhydride was also treated with aniline, giving an apparently homogeneous anilide, which from the results of the ammonia reaction is assumed to be the β-anilide. Hydrolysis as before removed the phthalyl group giving L-aspartic acid β-anilide, the presence of the free α-carboxyl group being confirmed by the ninhydrin reaction.

Experimental.

Phthalyl-DL-aspartic Acid (cf. Piutti, *loc. cit.*).—An intimate mixture of phthalic anhydride (2 g.) and L-asparagine (2 g.), when heated in an oil-bath at 140—145°, first melted with effervescence and then partly resolidified. After 20 minutes' heating, the cooled product was dissolved in water, and crude phthalyl-DL-aspartic acid (2·4 g., 68%), m. p. 211—213° with softening from 203°, was precipitated by the addition of hydrochloric acid. Repeated crystallisation from water raised the m. p. to 220—223° alone or mixed with the product prepared *via* the anhydride (below).

Phthalyl-DL-aspartic Anhydride.—A suspension of phthalic anhydride (4·3 g., 1 mol.) and DL-aspartic acid (3·9 g., 1 mol.) in pyridine (75 c.c.) was boiled under reflux for 1½ hours. When the solvent had been removed under reduced pressure, the residue was boiled with acetic anhydride (50 c.c.) for 5 minutes. After concentration and cooling, *phthalyl-DL-aspartic anhydride* (4·7 g., 66%) separated in prisms, m. p. 220—221° (decomp.); it was also conveniently recrystallised from anhydrous dioxan (Found: C, 58·9; H, 2·7; N, 5·8. $C_{12}H_7O_5N$ requires C, 58·8; H, 2·9; N, 5·7%). On cooling of a solution of the anhydride in boiling water, *phthalyl-DL-aspartic acid* separated in rosettes of small needles, m. p. 225°; Piutti (*loc. cit.*) gives m. p. 225° but does not indicate the optical configuration of the product (Found: C, 54·9; H, 3·3; N, 5·2. $C_{12}H_9O_6N$ requires C, 54·8; H, 3·4; N, 5·3%). Crude phthalyl-DL-aspartic acid prepared by the fusion method is best purified by dissolution in hot acetic anhydride. The phthalylaspartic anhydride, m. p. 219—220°, then crystallises, and is converted by hot water into the acid, m. p. 225°, having zero rotation in aqueous sodium carbonate solution.

Phthalyl-DL-asparagine.—A supercooled solution of phthalyl-DL-aspartic anhydride (4 g.) in dioxan (75 c.c.) was treated with excess of anhydrous ethereal ammonia, and the bulky white ammonium salt collected, washed with ether, and rapidly dissolved in a little water. Acidification to Congo-red with 5N-hydrochloric acid precipitated *phthalyl-DL-asparagine* (3·9 g., 91%), m. p. 217° (decomp.), which crystallised from aqueous ethanol in minute prisms, m. p. 217—218° (decomp.) (Found: C, 55·0; H, 3·5; N, 10·1. $C_{12}H_{10}O_5N_2$ requires C, 55·0; H, 3·8; N, 10·7%).

DL-*Asparagine*.—A solution of the phthalyl-DL-asparagine (4 g.) in aqueous sodium carbonate (0·82 g. in 50 c.c.) was treated with hydrazine hydrate (1·7 g. of 50%) and set aside for 12 hours at room temperature. Acidification with dilute hydrochloric acid precipitated phthalylhydrazide which was removed by filtration, the filtrate then being rendered neutral to litmus with aqueous ammonia, and evaporated below 45° to 10 c.c. When the residue was treated with ethanol (10 c.c.) and set aside in the refrigerator, DL-asparagine slowly separated. It crystallised from aqueous ethanol in hydrated

rhombic prisms and decomposed at 280—290° after sintering from 210° (Found: Loss in wt. on drying at 110° in a high vacuum, 12·5. Calc. for $C_4H_8O_3N_2,H_2O$: H_2O, 12·0%. Found, in dried sample: C, 36·6; H, 6·0; N, 20·8. Calc. for $C_4H_8O_3N_2$: C, 36·4; H, 6·1; N, 21·2%). In a ninhydrin estimation (van Slyke *et al.*, *loc. cit.*), 5-c.c. portions of a 25-c.c. aqueous solution containing 205·6 mg. of synthetic DL-asparagine gave α-carboxy-amino-nitrogen, 3·40, 3·55 mg. (Calc.: 3·8 mg.).

Diethyl Phthalyl-L-aspartate.—Diethyl L-aspartate was obtained from the ester hydrochloride (Fischer and Koenigs, *loc. cit.*) by covering it with benzene and stirring it with diethylamine (1·2 mols.). Diethylamine hydrochloride was precipitated by dry ether, and the aspartic ester obtained by evaporating the filtrate (yield from L-aspartic acid, 76%).

A solution of diethyl L-aspartate (14·3 g.) in ether was treated with portions of powdered phthalic anhydride (11 g.) and set aside overnight. The mixture was then twice extracted with aqueous sodium hydrogen carbonate, the combined extracts were acidified, and the precipitated phthalamic acid was isolated with ethyl acetate. The crude compound, which did not crystallise, reacted vigorously at room temperature with thionyl chloride (35 c.c.). When the reaction was complete, excess of thionyl chloride was removed in a vacuum, and benzene distilled off from the residue, which was then taken up in ether and washed with water, aqueous sodium hydrogen carbonate, and water. Evaporation of the ether afforded *diethyl phthalyl-L-aspartate* (16·4 g., 78%), which was purified for analysis by distillation, the pure ester being collected as a colourless syrup, b. p. 180—190° (bath-temp.)/0·05 mm., n_D^{17} 1·5263 (Found: C, 60·3; H, 5·1; N, 4·4. $C_{16}H_{17}O_6N$ requires C, 60·2; H, 5·3; N, 4·4%).

Phthalyl-L-aspartic Anhydride.—Undistilled diethyl phthalyl-L-aspartate (16·4 g.) was heated under reflux in a mixture of acetic acid (145 c.c.) and concentrated hydrochloric acid (48 c.c.) for 1 hour. The solvents were then at once removed by evaporation at low pressure from a bath at 40—45°, and the residue was dried in a desiccator over sodium hydroxide. The product was swirled with acetic anhydride (35 c.c.) on the steam-bath and, as soon as a clear solution was obtained (2—3 minutes), it was rapidly cooled, thus causing *phthalyl-L-aspartic anhydride* to crystallise. When collected after a few hours at 2°, washed with ether, and dried over sodium hydroxide, the compound had m. p. 209—211°, unchanged by recrystallisation from ethyl acetate, from which it separated in needles (Found: C, 58·6; H, 3·2; N, 5·4. $C_{12}H_7O_5N$ requires C, 58·8; H, 2·9; N, 5·7%). The anhydride was dissolved in hot water; *phthalyl-L-aspartic acid* separated at 2° in tiny needles. After recrystallisation from water, the acid has m. p. 193°, $[\alpha]_D^{20}$ −59·5° (in ethanol) (Found: C, 55·0; H, 3·4; N, 5·2. $C_{12}H_9O_6N$ requires C, 54·8; H, 3·4; N, 5·3%).

Phthalyl-L-asparagine.—By a procedure similar to that described for the DL-isomer, phthalyl-L-aspartic anhydride was converted into phthalyl-L-asparagine. The L-amide (yield, 75%) crystallised from aqueous ethanol in rectangular prisms, m. p. 200—201° after sintering at 172—173° (Found: N, 10·8. $C_{12}H_{10}O_5N_2$ requires N, 10·7%).

L-Asparagine.—The phthalylasparagine (1 g.) was dissolved in aqueous hydrazine hydrate (2 c.c. of 20%) and set aside at room temperature for 48 hours, whereupon crystals of the phthalylhydrazide salt separated. The whole was then acidified with acetic acid, and the phthalylhydrazide removed by filtration. Acetone was added to the filtrate from which L-asparagine hydrate (0·2 g.) separated at 2°. Once recrystallised from aqueous acetone, it formed long needles, or rectangular prisms from water (Found, after drying at 110° under reduced pressure: C, 36·3; H, 5·9; N, 21·4. Calc. for $C_4H_8O_3N_2$: C, 36·4; H, 6·1; N, 21·2%); the monohydrate has $[\alpha]_D^{20}$ +32·6° in N/10-hydrochloric acid (1·1 mols.) (equiv. to +37·1° for the anhydrous compound). Recorded values show considerable variations with the concentration and quantity of mineral acid, but Champion and Pellet (*Compt. rend.*, 1876, **82**, 819) found $[\alpha]_D$ 37·27° in N/10-hydrochloric acid, and "Merck Index," 1940, p. 59, gives $[\alpha]_D^{20}$ +31° for the hydrate in N/10-hydrochloric acid.

The synthetic and natural asparagine (B.D.H.), when compared by partition chromatography, using *iso*amyl alcohol–pyridine (Edman, *Ark. Kemi, Min., Geol.*, 1945, *A*, **22**, No. 3) or phenol–ammonia and collidine (Dent, *Biochem. J.*, 1948, **43**, 169), showed identical R_F values, and were not separated by two-dimensional chromatography if the last two solvents (Dent, *loc. cit.*) were used.

Phthalyl-L-aspartic Acid β-Anilide.—Phthalyl-L-aspartic anhydride (0·5 g.) was shaken in ethereal suspension with aniline (0·3 c.c.), whereupon the solid material soon became pasty. The whole was extracted with aqueous sodium hydrogen carbonate, and the extract acidified with hydrochloric acid which precipitated the *β-anilide* (0·25 g.). Recrystallised from water, it separated as the *monohydrate* in microscopic rectangular prisms, m. p. 98—99° (Found: loss on drying in a vacuum at 65°, 5·0. $C_{18}H_{14}O_5N_2,H_2O$ requires H_2O, 5·1%. Found, in dried material: N, 8·4. $C_{18}H_{14}O_5N_2$ requires N, 8·3%).

L-Aspartic Acid β-Anilide.—The phthalyl-anilide (0·72 g.) was dissolved in aqueous hydrazine (2·2 c.c. of 10%) and after 48 hours the resulting partly crystallised mixture was acidified with acetic acid, stirred, and filtered from phthalylhydrazide. Addition of acetone to the filtrate precipitated a flocculent solid, which was separated centrifugally and washed with acetone. When its aqueous solution was diluted to turbidity with acetone and set aside at 2°, prisms of the *L-β-anilide*, m. p. 251—252° (decomp.), separated (Found: C, 57·2; H, 5·9; N, 13·4. $C_{10}H_{12}O_3N_2$ requires C, 57·6; H, 5·8; N, 13·5%). The anilide rapidly gave a deep colour with ninhydrin solution which imparted a mauve-purple stain to filter paper.

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