159. *Polypeptides. Part IX.*[1] *Some Derivatives of Aspartylserine.*

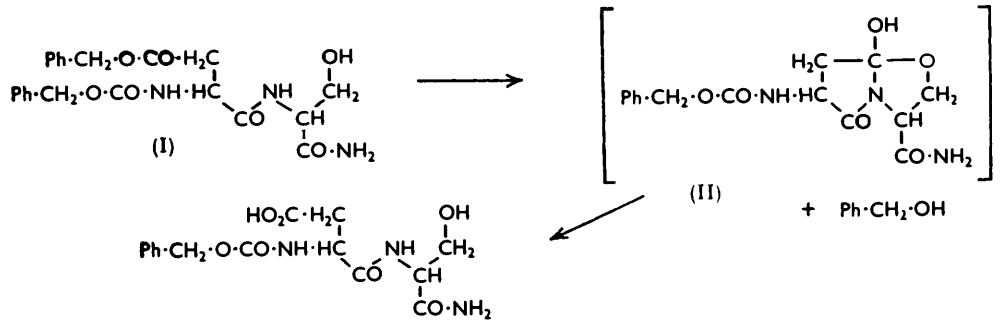
By R. W. Hanson and H. N. Rydon.

The actions of ammonia on (β-benzyl *N*-benzyloxycarbonylaspartyl)serine methyl ester (III) and of aqueous pyridine on (β-benzyl *N*-benzyloxycarbonylaspartyl)serine amide (I) have been investigated and are most simply interpreted as proceeding through the imides (IV) and (X). The intermediate in the second reaction is unreactive towards di-isopropyl phosphorofluoridate.

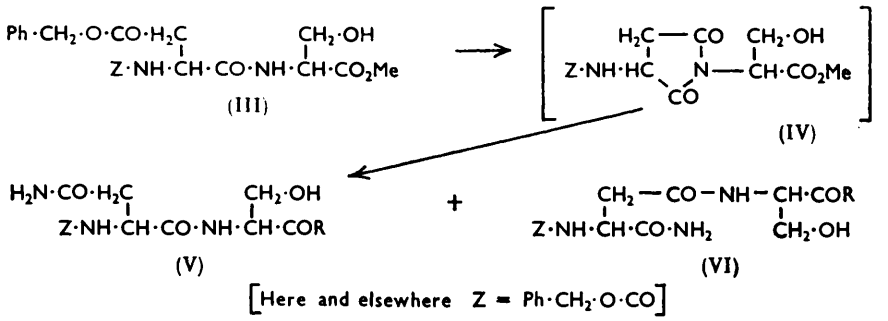
Bernhard,[2] in 1959, on the basis of the exceptionally rapid base-catalysed hydrolysis of the benzyl ester group in (β-benzyl *N*-benzyloxycarbonyl-L-aspartyl)-L-serine amide (I) and the changes in optical rotatory power which accompanied the reaction, made the interesting suggestion that the bicyclic compound (II) was an intermediate in this hydrolysis. He further suggested that a bicyclic structure similar to (II), involving the aspartic acid and the serine residue, was present in the active centres of enzymes such as α-chymotrypsin, trypsin, and cholinesterase, and accounted for the peculiar reactivity of the serine residue in such active centres. In view of our interest in this problem [3] we

[1] Part VIII, Benoiton, Hanson, and Rydon, preceding paper.
[2] Bernhard, *J. Cell. Comp. Physiol.*, 1959, **54**, Suppl. 1, 195.
[3] Cf. Porter, Rydon, and Schofield, *Nature*, 1958, **182**, 927; Rydon, *ibid.*, p. 928.

carried out experiments designed to investigate directly the reactivity towards di-isopropyl phosphorofluoridate (DFP) of the bicyclic compound (II) and the present paper describes these.[4]



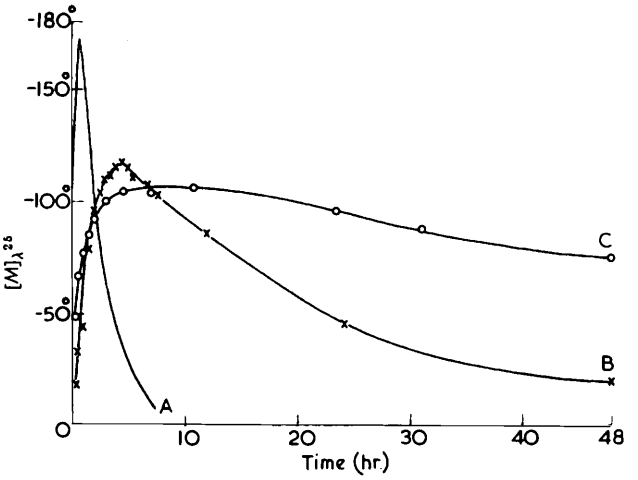
Bernhard [2] did not describe the synthesis of the dipeptide ester amide (I) and, since we required the corresponding benzyl methyl ester (III) for another purpose we synthesised that compound, by the carbodi-imide coupling of β-benzyl N-benzyloxycarbonylaspartate and serine methyl ester, in the hope that ammonia might attack the methoxycarbonyl group preferentially, leading to the desired ester amide (I). In fact, however, brief action of methanolic ammonia on the diester (III) gave a mixture of the two ester amides (V and VI; R = OMe), of which only the latter was isolated in a pure state and converted, by catalytic hydrogenolysis, into isoasparaginylserine methyl ester. Prolonged action of methanolic ammonia on the diester (III) likewise gave two products, namely, the asparaginylserine derivative (V; R = NH_2) and the isomeric isoasparaginyl compound (VI; R = NH_2); the latter gave isoasparaginylserine amide on hydrogenolysis, while the structure of the former was confirmed by direct comparison with material synthesised by coupling *N*-benzyloxycarbonylasparagine with serine amide. As with other reactions of β-esters of aspartic acid, these reactions are most simply interpreted [5] as proceeding by way of the imide (IV), ring-opening of which occurs in the two possible ways to give the two observed products.



In view of these results, the required ester amide (I) was prepared by direct carbodi-imide coupling of β-benzyl *N*-benzyloxycarbonylaspartate and serine amide. The changes in optical rotatory power which accompany the base-catalysed hydrolysis of this compound in various solvents (see Figure) show that the rates of both the formation and the decomposition of the intermediate of high rotatory power are less in buffered 50% aqueous pyridine than in buffered 50% dioxan,[6] presumably owing to the difference in dielectric

[4] Preliminary communication: Hanson and Rydon, *Nature*, 1962, **193**, 1182.
[5] Cf. Sondheimer and Holley, *J. Amer. Chem. Soc.*, 1954, **76**, 2467; Battersby and Robinson, *J.*, 1955, 259.
[6] Bernard, Berger, Carter, Katchalski, Sela, and Shalitin, *J. Amer. Chem. Soc.*, 1962, **84**, 2421.

constant of the two solvents, while in unbuffered 50% aqueous pyridine the decomposition of the intermediate is much slower, clearly owing to the fall in pH (9·6 ⟶ 6·9) which occurs during the experiment. Thin-layer chromatography showed that, during the reaction, the starting material, R_{FD} 0·89, passed successively into an intermediate, R_{FD} 0·85, and a final product, R_{FD} 0·63 (for solvent see below); the intermediate was

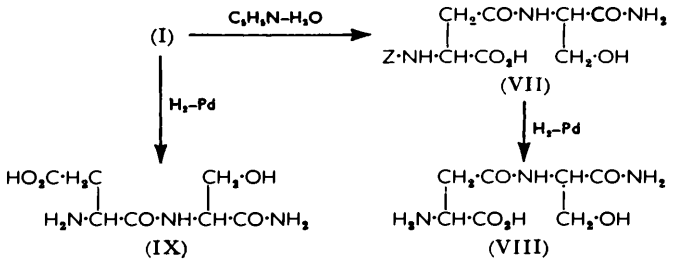


Changes in molecular rotation during hydrolysis of (β-benzyl *N*-benzyloxycarbonyl-L-aspartyl)-L-serine amide (I).

(A) $[M]_{436}^{25}$; c = 1·0 in 1 : 1 v/v dioxan–0·4M-" tris " buffer; pH 9·0 (recalculated from data of ref. 6). (B) $[M]_{589}^{25}$; c = 3·0 in 1 : 1 v/v pyridine–25% aqueous ammonium acetate; pH 8·0 ⟶ 7·6. (C) $[M]_{589}^{25}$; c = 3·0 in 1 : 1 v/v pyridine–water; pH 9·6 ⟶ 6·9.

present in maximal amount, and was the major component of the system, after about 6 hours in unbuffered 50% aqueous pyridine. Paper electrophoresis gave similar results but yielded the additional information that the intermediate was electrically neutral at pH 6·5, whereas the final product was negatively charged at this pH.

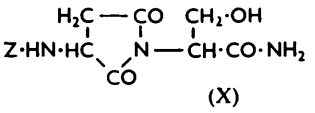
Direct isolation showed the major product of the reaction in unbuffered 50% aqueous pyridine to be the β-aspartyl-peptide (VII), which was obtained in 74% yield; Bernhard *et al.*[6] found the reaction in buffered 50% aqueous dioxan to yield almost equal amounts of α- and β-aspartyl-peptides, but this conclusion was based exclusively on chromatographic evidence. Hydrogenation of the β-aspartyl-peptide (VII) gave β-aspartylserine amide (VIII) which differed markedly in properties from the isomeric α-aspartylserine amide (IX), prepared by catalytic hydrogenolysis of the ester amide (I).



Chromatographic examination of the products obtained by treating solutions of the ester amide (I) with di-isopropyl phosphorofluoridate, (i) added at once in aqueous dioxan containing sodium hydrogen carbonate, (ii) added at once in 50% aqueous pyridine, and

(iii) added after 6 hours in 50% aqueous pyridine, failed to reveal any phosphorylated products and it is concluded that the intermediate, like the starting material and end product, in the base-catalysed hydrolysis of the ester-amide (I) is unreactive towards DFP.

Since the completion of our experimental work and the publication of our preliminary communication,[4] Bernhard and his co-workers [6] have published a full account of their very detailed study of the base-catalysed hydrolyses of a series of β-benzyl esters of *N*-benzyloxycarbonylaspartyl amides and peptides, including (I). Their mechanistic conclusions differ from those originally put forward by Bernhard [2] in the very important respect that the bicyclic structure (II) is no longer assigned to the intermediate in the hydrolysis of (I), which is now regarded as proceeding by way of the simple imide (X) as the only stable intermediate. This is consistent with our observations,[7] but, of course, renders our finding that the intermediate in the base-catalysed hydrolysis of (I) is unreactive towards DFP irrelevant to the question of the validity of Bernhard's hypothesis,[2] now entirely without experimental basis, that structures such as (II) are involved in the active centres of DFP-sensitive enzymes.



(X)

Experimental

The purity of most compounds was confirmed chromatographically. Descending chromatograms were run on Whatman No. 1 paper with butan-1-ol–acetic acid–water (100 : 17 : 37 v/v) (R_{FA}), butan-1-ol–pyridine–water (39 : 21 : 39) (R_{FB}), or phenol saturated with 10% aqueous trisodium citrate (R_{FC}); ascending, thin-layer chromatograms were run on Kieselgel-G with butan-1-ol–acetic acid–water (3 : 1 : 1) (R_{FD}) or propan-1-ol–aqueous ammonia (*d* 0·880) (R_{FE}). Spots were revealed with ninhydrin or by the chlorine–starch–iodide method.[8] Solutions were concentrated under reduced pressure.

(*β-Benzyl* N-*Benzyloxycarbonyl-*L-*aspartyl*)-L-*serine Methyl Ester* (III).—(*a*) *Preparation.* Triethylamine (5·09 g.) was added to β-benzyl *N*-benzyloxycarbonyl-L-aspartate [9] (17·85 g.) and L-serine methyl ester hydrochloride [10] (7·77 g.) in a mixture of anhydrous chloroform (40 ml.) and anhydrous acetonitrile (40 ml.), and the solution was cooled to −10°. *NN'*-Dicyclohexylcarbodi-imide (11·34 g.) was added in small portions and the mixture was allowed to attain room temperature (2 hr.), kept overnight, filtered, and evaporated. The residue was dissolved in chloroform (200 ml.) and washed successively with water (50 ml.), saturated sodium hydrogen carbonate solution (15 ml.), 2N-hydrochloric acid (25 ml.), and water (50 ml.), dried ($CaSO_4$), and evaporated. The residue was treated with hot acetone (120 ml.), cooled to 4°, filtered, and evaporated, giving the *diester* (16·64 g., 73%), m. p. 124—126°, a sample recrystallised from benzene had m. p. 125—126° (lit.,[6] m. p. 121—123°), R_{FA} 0·90, R_{FB} 0·91, $[\alpha]_D^{20}$ 0·0° (*c* 1·45 in MeOH) (Found: C, 60·5; H, 6·0: N, 6·6. Calc. for $C_{23}H_{26}N_2O_8$: C, 60·2; H, 5·7; N, 6·1%). The purified ester (458·2 mg.) was heated at 100° with 5N-hydrochloric acid for 24 hr., cooled, diluted with 5N-hydrochloric acid, and extracted with ether (3 × 2 ml.); the solution, freed from ether by gentle warming and made up to 15·0 ml. with 5N-hydrochloric acid, had $[\alpha]_D^{19}$ +0·65°. A mixture of L-aspartic acid (132·8 mg.) and L-serine (105·6 mg.), similarly treated, gave a solution with $[\alpha]_D^{19}$ +0·65°.

(*b*) *Hydrogenolysis.* The diester (2·29 g.), in *t*-butyl alcohol (75 ml.) and water (25 ml.), was shaken in hydrogen with 5% palladised charcoal; approximately 2 mol. of hydrogen were absorbed. The mixture was heated to the b. p., and filtered hot and the spent catalyst was washed with boiling water (25 ml.). Evaporation of the filtrate and washings gave α-L-*aspartyl*-L-*serine methyl ester* (1·15 g., 91%), m. p. 174—176°, which crystallised from aqueous methanol as the *monohydrate*, m. p. 178—180°, R_{FA} 0·06, R_{FB} 0·25, R_{FC} 0·67, $[\alpha]_D^{20}$ 0·0° (*c* 3·0 in H_2O) (Found: C, 38·25; H, 6·4; N, 11·3. $C_8H_{14}N_2O_6,H_2O$ requires C, 38·1; H, 6·4; N, 11·1%). The acid hydrolysate of this ester (235·6 mg.) made up to 15 ml. with 6N-hydrochloric

[7] Cf. Hanson, Ph.D. Thesis, Exeter, 1961.
[8] Rydon and Smith, *Nature*, 1952, **69**, 922.
[9] Benoiton, *Canad. J. Chem.*, 1962, **40**, 570.
[10] Guttmann and Boissonnas, *Helv. Chim. Acta*, 1958, **41**, 1853.

acid had $[\alpha]_D^{20}$ +0·68°; a mixture of L-aspartic acid (132·7 mg.) and L-serine (106·2 mg.), similarly treated, gave a solution with $[\alpha]_D^{20}$ +0·66°.

(*c*) *Ammonolysis.* (i) The diester (4·0 g.) was dissolved in methanol (200 ml.), and saturated methanolic ammonia (200 ml.) was added. After 12·5 min. at room temperature, the solution was evaporated and the residue (3·22 g.; m. p. 100—120°) recrystallised from methanol and then from water, affording (N-*benzyloxycarbonyl*-L-*isoasparaginyl*)-L-*serine methyl ester monohydrate* (VI; R = OMe) (0·90 g., 28%), m. p. 192°, $[\alpha]_D^{19}$ +13·7° (*c* 3·0 in dimethylformamide), R_{FA} 0·61, R_{FB} 0·78 (Found: C, 49·8; H, 6·1; N, 11·3. $C_{16}H_{21}N_3O_7,H_2O$ requires C, 49·9; H, 6·0; N, 10·9%). Evaporation of the mother-liquors from the methanol recrystallisation gave gelatinous material (2·1 g.).

Hydrogenation of the ester amide (1·0 g.) over 5% palladised charcoal in 67% aqueous methanol (60 ml.) gave L-*isoasparaginyl*-L-*serine methyl ester* which, recrystallised from ethanol containing a little water, had m. p. 140—141°, $[\alpha]_D^{20}$ +22·2° (*c* 2·0 in acetic acid) (420 mg., 66%), R_{FA} 0·03, R_{FB} 0·40 (Found: C, 41·8; H, 6·8; N, 17·6. $C_8H_{15}N_3O_5$ requires C, 41·2; H, 6·5; N, 18·0%).

(ii) The diester (5·0 g.) was kept overnight with methanol (125 ml.) previously saturated with ammonia at 0°. Next day the mixture was filtered and the insoluble portion (1·87 g., m. p. 223—225°) recrystallised from 75% aqueous methanol, affording (N-*benzyloxycarbonyl*-L-*isoasparaginyl*)-L-*serine amide* (VI; R = NH_2) (1·61 g., 42%), m. p. 230°, $[\alpha]_D^{24}$ +10·2° (*c* 3·0 in dimethylformamide), R_{FB} 0·61 (Found: C, 51·0; H, 5·9; N, 15·6. $C_{15}H_{20}N_4O_6$ requires C, 51·1; H, 5·7; N, 15·9%); the same compound was obtained, in 74% yield, by similar treatment of (N-benzyloxycarbonyl-L-isoasparaginyl)-L-serine methyl ester. The filtrate from the ammonolysis was evaporated to dryness and the thoroughly dried gelatinous product (1·86 g.) repeatedly recrystallised from slightly aqueous methanol; the product had m. p. 192—194°, $[\alpha]_D^{23}$ +9·2° (*c* 2·2 in dimethylformamide) (Found: C, 50·5; H, 5·7; N, 15·9. $C_{15}H_{20}N_4O_6$ requires C, 51·1; H, 5·7; N, 15·9%), and was identified as (*N*-benzyloxycarbonyl-L-asparaginyl)-L-serine amide (V; R = NH_2) (see below) by mixed m. p., chromatography (R_{FA} 0·30; R_{FB} 0·61), and identity of the infrared absorption spectra.

Hydrogenolysis of (*N*-benzyloxycarbonyl-L-isoasparaginyl)-L-serine amide (1·00 g.) over 5% palladised charcoal in water (150 ml.) gave a glass. This was dissolved in a little hot acetic acid and the cooled solution saturated with hydrogen chloride; the solid which separated was collected by centrifugation, washed thrice with ether, dried, and twice recrystallised from aqueous ethanol, to give L-*isoasparaginyl*-L-*serine amide hydrochloride monohydrate* (440 mg. 57%), m. p. 150—152° (decomp.), $[\alpha]_D^{20}$ +6·5° (*c* 1·9 in H_2O), R_{FA} 0·01, R_{FB} 0·19 (Found: C, 31·3; H, 6·3; Cl, 12·5; N, 20·55. $C_7H_{14}N_4O_4,HCl,H_2O$ requires C, 30·8; H, 6·3; Cl, 13·0; N, 20·5%).

N-*Benzyloxycarbonyl*-L-*serine Amide.*—*N*-Benzyloxycarbonyl-L-serine [10] (11·95 g.) was dissolved in anhydrous chloroform (100 ml.) and dioxan (100 ml.), containing triethylamine (5·06 g.), and the solution cooled to −5°, treated with ethyl chloroformate (5·43 g.), and kept at −5° for 25 min. Anhydrous ammonia was bubbled through the solution for 15 min. and the mixture kept at room temperature for 3 hr. The solution was filtered and evaporated to dryness. Addition of chloroform (100 ml.) caused the oily residue to crystallise; recrystallisation of the solid (6·36 g.; m. p. 118—120°) from ethyl acetate containing *ca.* 2% of methanol gave the amide (5·34 g., 45%), m. p. 132°, $[\alpha]_D^{24}$ +14·9° (*c* 5·0 in EtOH) (lit.,[11] m. p. 132—133°, $[\alpha]_D^{26}$ +14·4°).

L-*Serine Amide.*—*N*-Benzyloxycarbonyl-L-serine amide (1·06 g.) was hydrogenated over 5% palladised charcoal in 50% aqueous ethanol (20 ml.). The reaction mixture was heated to the b. p., filtered, and evaporated. Recrystallisation of the residue from aqueous ethanol gave the *amide* (0·35 g., 75%), m. p. 103°, $[\alpha]_D^{24}$ +24·6° (*c* 3·0 in acetic acid), R_{FA} 0·03, R_{FB} 0·37 (Found: N, 26·7, 27·0. $C_3H_6N_2O_2$ requires N, 26·9%).

(N-*Benzyloxycarbonyl*-L-*asparaginyl*)-L-*serine Amide* (V; R = NH_2).—*NN'*-Dicyclohexylcarbodi-imide (1·20 g.) was added to L-serine amide (0·60 g.) and *N*-benzyloxycarbonyl-L-asparagine [12] (1·54 g.) in anhydrous *NN*-dimethylformamide (10 ml.). Next day one drop of acetic acid was added and the mixture filtered. The filtrate was evaporated and the oily residue extracted with warm water (20 ml.). The extract was filtered and evaporated and the

[11] Fruton, *J. Biol. Chem.*, 1942, **146**, 463.
[12] Boissonnas, Guttmann, Jaquenoud, and Waller, *Helv. Chim. Acta*, 1955, **38**, 1491.

semi-solid residue (1·1 g.) recrystallised four times from methanol, to give the *diamide* (0·37 g., 18%), m. p. 220—222°, $[\alpha]_D^{23}$ +9·6° (*c* 2·2 in dimethylformamide), R_{FA} 0·30, R_{FB} 0·62 (Found: N, 16·2, 15·9. $C_{15}H_{20}N_4O_6$ requires N, 15·9%).

(β-*Benzyl* N-*Benzyloxycarbonyl*-L-*aspartyl*)-L-*serine Amide* (I).—(*a*) *Preparation.* *NN'*-Dicyclohexylcarbodi-imide (5·12 g.) was added to β-benzyl *N*-benzyloxycarbonyl-L-aspartate [9] (8·19 g.) and L-serine amide (2·38 g.) in anhydrous chloroform. Next day the gelatinous product was collected by filtration on a large sintered-glass funnel and thoroughly dried in a vacuum-desiccator. The dry product was extracted with boiling acetone (100 ml.), and the cooled extract was filtered to remove dicyclohexylurea. Evaporation of the filtrate and two recrystallisations of the residue from isobutyl alcohol gave the ester amide (7·11 g., 70%), m. p. 162° (Kofler block) after drying at 100°/2 mm., $[\alpha]_D^{21}$ +1·5° (*c* 3·0 in pyridine) (lit.,[6] m. p. 157°, $[\alpha]_D^{20}$ +1·7° in dioxan), R_{FA} 0·78, R_{FB} 0·90 (Found: C, 59·2; H, 5·7; N, 9·4. Calc. for $C_{22}H_{25}N_3O_7$: C, 59·6; H, 5·7; N, 9·5%).

(*b*) *Hydrogenolysis.* The ester amide (1·00 g.) was hydrogenated over 5% palladised charcoal in methanol (30 ml.). The product, isolated in the usual manner, was recrystallised from aqueous methanol, affording α-L-*aspartyl*-L-*serine amide* (IX) (0·45 g., 91%), m. p. 208° (decomp.), $[\alpha]_D^{20}$ +15·1° (*c* 3·0 in H_2O), R_{FA} 0·01, R_{FB} 0·16 (Found: C, 38·2; H, 6·0; N, 19·35. $C_7H_{13}N_3O_5$ requires C, 38·4; H, 6·0; N, 19·2%).

(*c*) *Action of aqueous pyridine.* The ester amide (800 mg.) was dissolved in 50% aqueous pyridine (26·5 ml.) and the solution kept in a thermostat-bath for 21 days at 25°. From time to time portions were removed for determination of optical rotatory power (see Figure) and chromatography; chromatography showed the starting material (R_{FD} 0·89) to be replaced successively by an intermediate product of R_{FD} 0·85 and finally by a material of R_{FD} 0·63, the latter being almost the only substance present at the end of the experiment. The solution was then evaporated and the residue rubbed with a little ether; the product [590 mg.; m. p. 185—187° (decomp.)] was recrystallised once from water and once from dilute ethanol, giving chromatographically and electrophoretically homogeneous (*N*-*benzyloxycarbonyl*-β-L-*aspartyl*)-L-*serine amide* (VII) (470 mg., 74%), m. p. 197—199° (decomp.), $[\alpha]_D^{19}$ −2·3° (*c* 3·0 in 50% aqueous pyridine), R_{FA} 0·39, R_{FB} 0·50, R_{FD} 0·63, R_{FE} 0·62 (Found: C, 51·2; H, 6·0; N, 11·8. $C_{15}H_{19}N_3O_7$ requires C, 51·0; H, 5·4; N, 11·9%).

This compound (350 mg.) was hydrogenated over 5% palladised charcoal in water (75 ml.). The product was heated to the b. p. and filtered. Evaporation of the filtrate gave a gum which, on trituration with ethanol, solidified to a slightly hygroscopic powder (220 mg., 97%). β-L-*Aspartyl*-L-*serine amide* (VIII), so obtained, had m. p. 80—90° (decomp.), R_{FA} 0·01, R_{FB} 0·16 (Found: C, 37·3; H, 6·5; N, 18·1. $C_7H_{13}N_3O_5,0{\cdot}5H_2O$ requires C, 36·8; H, 6·2; N, 18·4%). Paper electrophoresis of this material, which contained a trace of the isomeric α-aspartylserine amide, at 10 v/cm., showed it to differ from α-aspartylserine amide, the migration rates (cm./hr.) being:

pH 3·65 (+ve charge),	α-peptide 2·2,	β-peptide 2·0
pH 9·2 (−ve charge),	,, 3·7	,, 1·7

Furthermore, the β-peptide gave a blue-grey colour with ninhydrin, in contrast with the normal purple colour given by the α-peptide.[13]

(*d*) *Reactions with di-isopropyl phosphorofluoridate.* (i) The ester amide (87 mg.) and the phosphorofluoridate (110 mg.) were dissolved in dioxan (4·0 ml.); water (6·0 ml.) and sodium hydrogen bicarbonate (168 mg.) were added and the mixture was gently shaken until dissolution was complete, and was then kept at 25°. A control experiment, without the phosphorofluoridate, was also performed. Chromatography after 72 hr. showed both solutions to contain only (*N*-benzyloxycarbonyl-β-L-aspartyl)-L-serine amide, R_{FA} 0·39, R_{FB} 0·50. R_{FD} 0·63, R_{FE} 0·62.

(ii) The ester amide (445 mg.) was dissolved in 50% aqueous pyridine (10 ml.); 2 ml. of this solution was added to the phosphorofluoridate (110 mg.) and kept at 25°; a further 2 ml., without the phosphorofluoridate, was used as a control. Chromatography after 72 hr. showed the presence in both solutions of some starting material (R_{FA} 0·78; R_{FD} 0·89), the intermediate imide (X) (R_{FA} 0·68; R_{FB} 0·90; R_{FD} 0·82), and (*N*-benzyloxycarbonyl-β-L-aspartyl)-L-serine amide (R_{FA} 0·39; R_{FB} 0·50; R_{FD} 0·63; R_{FE} 0·62).

[13] Le Quesne and Young, *J.*, 1952, 24; Bryant, Moore, Pimlott, and Young, *J.*, 1959, 3868.

(iii) Experiment (ii) was repeated, the phosphorofluoridate being added after 6 hr. The chromatographic results were similar except that much less starting material was present.

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