PEPTIDE SYNTHESES WITH THE 2,4,6-TRIMETHYLBENZYL ESTERS OF L-ASPARAGINE AND L-GLUTAMINE

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Summary

The 2,4,6-trimethylbenzyl esters of L-asparagine and L-glutamine have been obtained as the crystalline hydrochlorides by treatment of the *o*-nitrophenylsulphenyl amino acid esters with methanolic hydrogen chloride. These hydrochlorides were used in the synthesis of several benzyloxycarbonyl peptide 2,4,6-trimethylbenzyl esters, which were converted into the corresponding free peptides by the action of hydrogen bromide in acetic acid under mild conditions.

INTRODUCTION

In peptide syntheses it is often necessary to mask terminal carboxyl groups, and the methyl and ethyl esters of α-amino acids have been widely used as coupling components. With L-asparagine and L-glutamine, however, the use of alkyl esters for this purpose is unsatisfactory owing to the occurrence of various side reactions, such as cyclic imide formation and deamidation, during the conventional removal of the ester protecting group by alkaline hydrolysis.[1–4]

A common expedient in syntheses involving carboxyl-terminal asparaginyl or glutaminyl residues is to leave the carboxyl group unmasked, and perform the coupling reactions with the latter in the form of the carboxylate ion, generally in partly aqueous media for solubility reasons. This approach is not always satisfactory, but a notable success of the method was the synthesis of the asparaginyl-terminated 17–21 pentapeptide sequence of the A chain of insulin,[5] and many dipeptide derivatives have been obtained in this way.

Another possibility is the use of carboxyl-protecting groups which do not require alkaline hydrolysis for their removal. Sondheimer and Semeraro[6] prepared the benzyl and *p*-nitrobenzyl esters of L-asparagine and L-glutamine, and used the benzyl derivatives in the synthesis of some benzyloxycarbonyl dipeptide esters. Removal of the protecting groups from the products was effected by catalytic hydrogenation. Amiard and Heymès[7] used L-asparagine benzyl ester similarly. More recently, Fosker and Law[8] employed L-asparagine *p*-nitrobenzyl ester in a synthesis of the tripeptide

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[1] Sondheimer, E., and Holley, R. W., *J. Am. chem. Soc.*, 1954, **76**, 2467.

[2] Sondheimer, E., and Holley, R. W., *J. Am. chem. Soc.*, 1957, **79**, 3767.

[3] Roeske, R. W., *J. org. Chem.*, 1963, **28**, 1251.

[4] Leach, S. J., and Lindley, H., *Aust. J. Chem.*, 1954, **7**, 173.

[5] Zabel, R., and Zahn, H., *Z. Naturforsch.*, 1965, **20b**, 650.

[6] Sondheimer, E., and Semeraro, R. J., *J. org. Chem.*, 1961, **26**, 1847.

[7] Amiard, G., and Heymès, R., *Bull. Soc. chim. Fr.*, 1957, 1373.

[8] Fosker, A. P., and Law, H. D., *J. chem. Soc.*, 1965, 4922.

H-Ileu-Glu(NH_2)-Asp(NH_2)-OH. Hydrogenation was again used in the final cleavage step.

Use of the t-butyl esters of asparagine and glutamine has also been considered. t-Butyl esters are cleaved smoothly by mild acid treatment, and have been used extensively in the synthesis of large biologically active peptides. Schnabel and Schüssler[9] prepared the t-butyl esters of L-asparagine and L-glutamine as the crystalline *p*-toluenesulphonates by hydrogenation of the corresponding benzyloxycarbonyl derivatives. It is noteworthy that the direct t-butylation procedure of Roeske[3] cannot be applied to these two amino acids owing to their insolubility in the reaction medium used.

The application of 2,4,6-trimethylbenzyl esters as acid-sensitive derivatives for peptide synthesis has been described recently.[10,11] As far as behaviour toward acidic reagents is concerned, this carboxyl protecting group is very similar to t-butyl. Both types of ester are cleaved by hydrogen bromide in acetic acid under conditions which do not affect peptide bonds,[10] and also by cold trifluoroacetic acid.[11]

In the present work the possible use of 2,4,6-trimethylbenzyl esters in the synthesis of L-asparaginyl and L-glutaminyl peptides has been investigated.

Discussion

2,4,6-Trimethylbenzyl esters of *N*-substituted amino acids are conveniently prepared by condensation of the latter with chloromethylmesitylene in dimethylformamide in the presence of an equivalent of triethylamine.[10–13] The reaction proceeds rapidly at room temperature, and good yields are obtained. Furthermore, it was found that racemization did not occur with various optically active amino acid derivatives.

This procedure has been used to obtain several tritylamino acid 2,4,6-trimethylbenzyl esters, which were then converted into the corresponding amino acid ester hydrochlorides by selective cleavage of the trityl group on brief exposure to boiling methanolic hydrogen chloride.[10] The use of a three-stage synthesis of this type is necessary with amino acid 2,4,6-trimethylbenzyl esters as there is no practicable direct esterification method available at the present time for preparation of these compounds.[14]

In order to obtain the 2,4,6-trimethylbenzyl esters of L-asparagine and L-glutamine, however, it was considered that the *o*-nitrophenylsulphenylamino acids would be more suitable precursors than the trityl derivatives. In the first place, the *o*-nitrophenylsulphenyl group is cleaved more readily than trityl,[15] which permits the use of milder reaction conditions with less danger of any concomitant cleavage of the acid-sensitive 2,4,6-trimethylbenzyl group. Secondly, the *o*-nitrophenylsulphenyl

[9] Schnabel, E., and Schüssler, H., *Liebigs Ann.*, 1965, **686**, 229.
[10] Stewart, F. H. C., *Aust. J. Chem.*, 1966, **19**, 1067.
[11] Stewart, F. H. C., *Aust. J. Chem.*, 1966, **19**, 1511.
[12] Grummitt, O., and Buck, A., *Org. Synth.*, 1955, Coll. Vol. III, 195.
[13] Ledger, R., and Stewart, F. H. C., *Aust. J. Chem.*, 1965, **18**, 1477.
[14] Ledger, R., and Stewart, F. H. C., unpublished data.
[15] Zervas, L., Borovas, D., and Gazis, E., *J. Am. chem. Soc.*, 1963, **85**, 3660.

derivatives of L-asparagine and L-glutamine are prepared in a more convenient manner, and in considerably higher yield, than is the case with the trityl analogues.[15] Both compounds, moreover, have very favourable physical characteristics.

Condensation of the *o*-nitrophenylsulphenyl derivatives with chloromethylmesitylene in the manner used for the tritylamino acids[10] gave the corresponding 2,4,6-trimethylbenzyl esters, NPS-Asp(NH_2)-OTMB* and NPS-Glu(NH_2)-OTMB, which were purified by recrystallization. These intermediates were treated with slightly over two equivalents of 1N methanolic hydrogen chloride at room temperature. The crystalline ester hydrochlorides H-Asp(NH_2)-OTMB,HCl and H-Glu(NH_2)-OTMB,HCl, respectively, were obtained in this way. Thin-layer chromatography indicated that both compounds were homogeneous.

The fact that preparation of amino acid 2,4,6-trimethylbenzyl esters involves three steps would normally be a disadvantage. In the case of L-asparagine and L-glutamine, however, this is less important since the other potentially useful esters, t-butyl,[9] benzyl,[6,7] and *p*-nitrobenzyl,[6] are also obtained by multistage syntheses, which, for the most part, are rather less convenient than the procedure used for the 2,4,6-trimethylbenzyl derivatives.

The synthetic application of L-asparagine 2,4,6-trimethylbenzyl ester hydrochloride was illustrated by the preparation of the known free dipeptide L-glutaminyl-L-asparagine (I). This compound has been synthesized by several authors,[7,16,17] and has played an important part in syntheses of the pituitary hormones oxytocin and vasopressin, and many of their structural analogues.[18] The present synthesis of the dipeptide is shown in Figure 1.

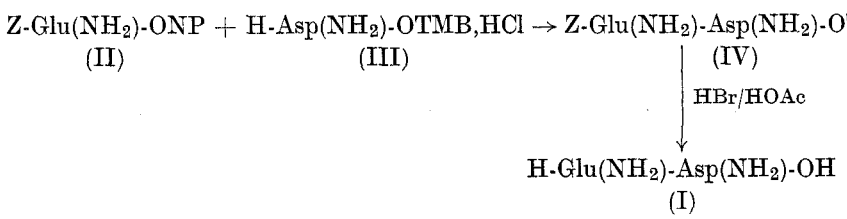

Fig. 1

Benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester (II)[19] was treated with L-asparagine 2,4,6-trimethylbenzyl ester, formed *in situ* from the hydrochloride (III). The protected dipeptide ester (IV) was obtained in 91% yield, and the protecting groups were then removed by treatment with 2N hydrogen bromide in acetic acid for 1 hr at room temperature. The free dipeptide (I) was chromatographically pure, and the physical constants corresponded to the values reported in the literature.

* Abbreviations used are: NPS, *o*-nitrophenylsulphenyl; TMB, 2,4,6-trimethylbenzyl; Z, benzyloxycarbonyl; DMF, dimethylformamide; DCA, dichloroacetic acid; M.A., mixed carbonic anhydride method.

[16] Swan, J. M., and du Vigneaud, V., *J. Am. chem. Soc.*, 1954, **76**, 3110.

[17] Rudinger, J., Honzl, J., and Zaoral, M., *Colln Czech. chem. Commun.*, 1956, **21**, 202.

[18] Schröder, E., and Lübke, K., "The Peptides." Vol. 2, p. 281. (Academic Press: New York 1966.)

[19] Bodanszky, M., and du Vigneaud, V., *J. Am. chem. Soc.*, 1959, **81**, 5688.

Similarly, the protected dipeptide ester Z-Leu-Asp(NH_2)-OTMB was prepared, and converted into the known dipeptide L-leucyl-L-asparagine. The use of L-glutamine 2,4,6-trimethylbenzyl ester hydrochloride was exemplified by the preparation of the new dipeptide L-asparaginyl-L-glutamine from the derivative Z-Asp(NH_2)-Glu(NH_2)-OTMB, which was also synthesized by the *p*-nitrophenyl ester method.

For peptide syntheses involving several successive coupling reactions with 2,4,6-trimethylbenzyl as the carboxyl-protecting group, it is necessary to employ an amino-protecting group which can be cleaved selectively at the intermediate stages. *o*-Nitrophenylsulphenyl has been found to serve admirably for this purpose.[10] Accordingly, the synthesis of a free tetrapeptide H-Glu(NH_2)-Glu(NH_2)-Asp(NH_2)-Asp(NH_2)-OH, containing L-glutaminyl and L-asparaginyl residues only, was carried out, and the details are shown in Figure 2.

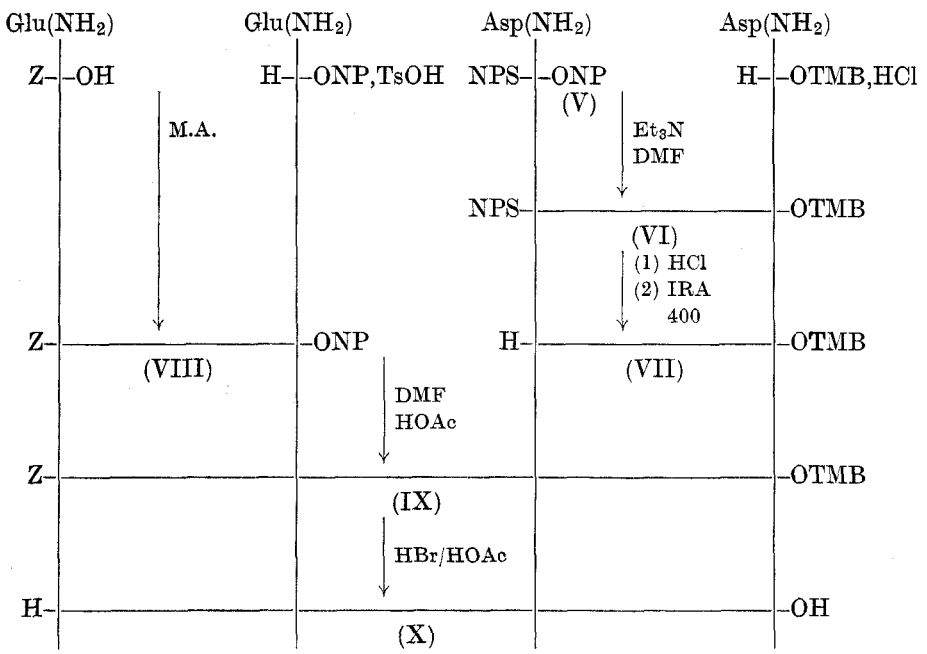

Fig. 2

o-Nitrophenylsulphenyl-L-asparagine was converted into the *p*-nitrophenyl ester (V) by the dicyclohexylcarbodiimide procedure described by Bodanszky and du Vigneaud[20] for the benzyloxycarbonyl analogue. In the latter case extensive dehydration of the amide side-chain occurred, with formation of a β-cyano-L-alanine derivative, which, however, was readily separated from the desired product by appropriate washing and recrystallization procedures. Formation of the *p*-nitrophenyl ester (V) was likewise accompanied by dehydration of the β-amide group, but the pure asparagine derivative could be obtained by careful recrystallization.

The active ester (V) was coupled with L-asparagine 2,4,6-trimethylbenzyl ester to form the protected dipeptide ester NPS-Asp(NH_2)-Asp(NH_2)-OTMB (VI). Removal

[20] Bodanszky, M., and du Vigneaud, V., *Biochem. Prep.*, 1962, **10**, 122.

of the *o*-nitrophenylsulphenyl group with methanolic hydrogen chloride, and treatment of the resultant hydrochloride with a basic exchange resin, gave the dipeptide ester (VII). This material was then condensed with the protected dipeptide active ester Z-Glu(NH_2)-Glu(NH_2)-ONP (VIII) in dimethylformamide containing one equivalent of acetic acid to counteract base-catalysed racemization. The activated carboxyl component (VIII) was prepared by the mixed carbonic anhydride approach of Goodman and Stueben[21] using the recently described L-glutamine *p*-nitrophenyl ester *p*-toluenesulphonate[22] to provide the amino component.

In this way the protected tetrapeptide ester Z-Glu(NH_2)-Glu(NH_2)-Asp(NH_2)-Asp(NH_2)-OTMB (IX) was obtained. The product (IX) was a very sparingly soluble compound, which, however, reacted readily with 2N hydrogen bromide in acetic acid. The crystalline tetrapeptide H-Glu(NH_2)-Glu(NH_2)-Asp(NH_2)-Asp(NH_2)-OH (X) was isolated in high yield, and proved to be homogeneous by thin-layer chromatography in several solvent systems.

It is not always necessary to start from an amino acid 2,4,6-trimethylbenzyl ester in order to use this protecting group in peptide synthesis. The introduction of the ester group by condensation with chloromethylmesitylene can be applied to a preformed protected peptide, and an example of this variation has been reported.[10] Application of this approach to a peptide derivative with carboxyl-terminal L-asparagine is illustrated by the synthesis of NPS-Glu(NH_2)-Asp(NH_2)-OTMB as shown in Figure 3.

$$\text{NPS-Glu}(NH_2)\text{-OH} + \text{H-Asp}(NH_2)\text{-OH} \xrightarrow{\text{M.A.}} \underset{\text{(XI)}}{\text{NPS-Glu}(NH_2)\text{-Asp}(NH_2)\text{-OH}}$$

$$\xrightarrow[Et_3N]{\text{TMB-Cl}} \underset{\text{(XII)}}{\text{NPS-Glu}(NH_2)\text{-Asp}(NH_2)\text{-OTMB}} \xrightarrow{\text{HBr/HOAc}} \underset{\text{(I)}}{\text{H-Glu}(NH_2)\text{-Asp}(NH_2)\text{-OH}}$$

Fig. 3

o-Nitrophenylsulphenyl-L-glutamine[15] was coupled with free L-asparagine by the mixed carbonic anhydride method in aqueous dimethylformamide. The resultant protected dipeptide (XI) was converted into the 2,4,6-trimethylbenzyl ester (XII) in high yield. If required, this derivative could now serve to provide the amino component for further coupling reactions, in a manner analogous to that shown in Figure 2.

Removal of the protecting groups in (XII) by 2N hydrogen bromide in acetic acid gave L-glutaminyl-L-asparagine (I), which had the same specific rotation and chromatographic behaviour as the product obtained as in Figure 1. This result indicates that no significant racemization occurred during formation of the 2,4,6-trimethylbenzyl ester (XII) from the protected dipeptide (XI).

The standard conditions used for cleavage of the protecting groups in benzyloxycarbonyl peptide 2,4,6-trimethylbenzyl esters consist of treatment with 2N hydrogen bromide in acetic acid for 1 hr at room temperature.[10] This particular reaction period was chosen because Bláha and Rudinger[23] have shown that treatment for about 1 hr

[21] Goodman, M., and Stueben, K. C., *J. Am. chem. Soc.*, 1959, **81**, 3980.

[22] Stewart, F. H. C., *Aust. J. Chem.*, 1966, **19**, 2361.

[23] Bláha, K., and Rudinger, J., *Collect. Czech. chem. Commun.*, 1965, **30**, 585.

is necessary to ensure essentially complete removal of benzyloxycarbonyl. The *o*-nitrophenylsulphenyl group, on the other hand, is cleaved almost instantaneously by the hydrogen bromide reagent,[15] and in the case of NPS-Glu(NH_2)-Asp(NH_2)-OTMB (XII) it was found that a reaction time of 10 min was adequate for removal of the 2,4,6-trimethylbenzyl ester group.

Further applications of the 2,4,6-trimethylbenzyl derivatives of L-asparagine and L-glutamine are now being investigated.

Experimental

The microanalyses were carried out by the Australian Microanalytical Service, Melbourne. Melting points are uncorrected. Infrared spectra were obtained using KBr disks. Thin-layer chromatography was carried out on silica gel G, and the plates developed with ninhydrin. The following solvent systems were used: (A) n-butanol/acetic acid/water (3 : 1 : 1); (B) chloroform/methanol/34% ammonia (2 : 2 : 1); (C) s-butanol/formic acid/water (15 : 3 : 2); (D) n-propanol/34% ammonia (2 : 1).

(*a*) o-*Nitrophenylsulphenyl*-L-*asparagine 2,4,6-Trimethylbenzyl Ester*

o-Nitrophenylsulphenyl-L-asparagine[15] (4·0 g) and triethylamine (1·96 ml) were dissolved in dimethylformamide (7·0 ml), and molten 2,4,6-trimethylbenzyl chloride (chloromethylmesitylene) (2·4 g) added. After 1–2 days at room temperature, the mixture was diluted with 1N $NaHCO_3$. The dark oily product solidified on cooling, and was washed with water and dried *in vacuo*. The ester was recrystallized from chloroform/cyclohexane (4·0 g; 70%), m.p. 173–174°; $[\alpha]_D^{20\cdot5}$ −31·7° (*c*, 1·0 in DMF) (Found: N, 10·1. Calc. for $C_{20}H_{23}N_3O_5S$: N, 10·1%).

(*b*) o-*Nitrophenylsulphenyl*-L-*glutamine 2,4,6-Trimethylbenzyl Ester*

Similar treatment of *o*-nitrophenylsulphenyl-L-glutamine[15] gave the 2,4,6-trimethylbenzyl ester in 99% yield. The compound was recrystallized from chloroform/cyclohexane, m.p. 143·5–145·5°; $[\alpha]_D^{20}$ −5·2° (*c*, 1·0 in DMF) (Found: C, 58·3; H, 6·0; N, 9·6. Calc. for $C_{21}H_{25}N_3O_5S$: C, 58·5; H, 5·8; N, 9·7%).

(*c*) L-*Asparagine 2,4,6-Trimethylbenzyl Ester Hydrochloride*

The *o*-nitrophenylsulphenyl derivative (2·3 g) was suspended in methanol (6·9 ml) and 2N methanolic hydrogen chloride (6·9 ml) added. After a few minutes at room temperature a clear solution was obtained, from which the product began to crystallize. Excess ether was added, and the hydrochloride collected, washed with ether, and recrystallized from methanol/ethyl acetate (yield, 1·33 g; 80%), m.p. 194·5–195·5°; $[\alpha]_D^{21\cdot5}$ −3·6° (*c*, 5·0 in H_2O); ν_{max} 2050 (broad; α-amino ester hydrochloride), 1730 (ester CO), 1665 (amide I), 1610 cm^{-1} (amide II) (Found: C, 55·9; H, 7·3; N, 8·9. Calc. for $C_{14}H_{21}ClN_2O_3$: C, 55·9; H, 7·0; N, 9·3%). A single yellow spot was obtained on thin-layer chromatography, R_F 0·68 (A).

(*d*) L-*Glutamine 2,4,6-Trimethylbenzyl Ester Hydrochloride*

This compound was obtained as in (*c*), and recrystallized from methanol/ethyl acetate in 93% yield, m.p. 172·5–174°; $[\alpha]_D^{21}$ −2·8° (*c*, 2·0 in H_2O); ν_{max} 2005 (broad; α-amino ester hydrochloride), 1730 (ester CO), 1660 (amide I), 1615 cm^{-1} (amide II) (Found: C, 57·0; H, 7·2; N, 8·8. Calc. for $C_{15}H_{23}ClN_2O_3$: C, 57·2; H, 7·3; N, 8·9%). Thin-layer chromatography gave a single spot, R_F 0·61 (A).

The hydrochloride (90 mg) was treated with 2N hydrogen bromide in acetic acid (0·9 ml) for 15 min at room temperature. Ether was added, and the precipitate collected and dissolved in a little water. The solution was neutralized with aqueous ammonia and filtered. Ethanol was added, whereupon crystals of L-glutamine separated rapidly (33 mg; 80%), $[\alpha]_D^{22\cdot5}$ +31·3° (*c*, 1·0 in 1N HCl). The specific rotation of pure L-glutamine is +31·8°.

(*e*) *Benzylcarbonyl-*L*-glutaminyl-*L*-asparagine 2,4,6-Trimethylbenzyl Ester*

L-Asparagine 2,4,6-trimethylbenzyl ester hydrochloride (301 mg) was suspended in dimethylformamide (4 ml), and triethylamine (0·14 ml) added with shaking, followed by benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[19] (401 mg). The mixture was kept for 1–2 days at room temperature and diluted with ethanol. After several hours at 0° the precipitated dipeptide derivative was collected, and washed with ethanol and ether (yield, 481 mg; 91%). The product was precipitated from acetic acid/ethanol, m.p. 225–227°; $[\alpha]_D^{18\cdot5}$ −1·3° (*c*, 1·0 in DMF), $[\alpha]_D^{20}$ +6·2° (*c*, 1·0 in HOAc) (Found: C, 61·5; H, 6·7; N, 10·6. Calc. for $C_{27}H_{34}N_4O_7$: C, 61·6; H, 6·5; N, 10·6%).

(*f*) L-*Glutaminyl-*L*-asparagine*

The foregoing protected dipeptide ester (460 mg) was treated with 2N hydrogen bromide in acetic acid (3 ml) for 1 hr at room temperature. Dilution with ether precipitated the dipeptide hydrobromide, which was dissolved in water. The solution was brought to pH 7 with 1N ammonia, and ethanol added until just turbid. The crystalline dipeptide separated on keeping at 0° (174 mg; 77%), and was recrystallized from aqueous ethanol, m.p. 203–204° (dec., rapid heating); $[\alpha]_D^{21\cdot5}$ +19·5° (*c*, 1·0 in H_2O), $[\alpha]_D^{23}$ +20·2° (*c*, 1·0 in 0·5N HCl). Lit. values are m.p. 210–211° (corr.);[17] $[\alpha]_D^{21}$ +17·1° (*c*, 1·5 in H_2O), +20·8° (*c*, 2·7 in 0·5N HCl);[17] +24·0° (*c*, 1·0 in H_2O).[7] Thin-layer chromatography in several solvent systems gave one spot, R_F 0·26 (A), 0·58 (B), 0·25 (C).

(*g*) *Benzyloxycarbonyl-*L*-leucyl-*L*-asparagine 2,4,6-Trimethylbenzyl Ester*

Prepared as in (*e*) except that the reaction mixture was diluted with water instead of ethanol. The product was obtained in 89% yield, and recrystallized from aqueous ethanol, m.p. 174–175°; $[\alpha]_D^{21}$ −11·4° (*c*, 1·0 in DMF) (Found: C, 65·5; H, 7·1 N, 8·2. Calc. for $C_{28}H_{37}N_3O_6$: C, 65·8; H, 7·2; N, 8·2%).

(*h*) L-*Leucyl-*L*-asparagine*

The hygroscopic hydrobromide was obtained in quantitative yield as in (*f*). Isolation of the free dipeptide proceeded as in the case of L-glutaminyl-L-asparagine, and the product was recrystallized from aqueous ethanol, m.p. 215·5–221°; $[\alpha]_D^{21}$ +15·3° (*c*, 2·0 in H_2O). Lit. value $[\alpha]_D^{25}$ +15·7° (*c*, 5·0; H_2O).[24] R_F 0·51 (A), 0·53 (C), 0·60 (D).

(*i*) *Benzyloxycarbonyl-*L*-asparaginyl-*L*-glutamine 2,4,6-Trimethylbenzyl Ester*

This compound was obtained in 90% yield from benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester[20] and L-glutamine 2,4,6-trimethylbenzyl ester hydrochloride as in (*e*), and purified by precipitation from acetic acid/ethanol, m.p. 225–227° (dec.); $[\alpha]_D^{21}$ +11·4°; (*c*, 0·5 in HOAc) (Found: C, 61·7; H, 6·6; N, 10·6. Calc. for $C_{27}H_{34}N_4O_7$: C, 61·6; H, 6·5; N, 10·6%).

(*j*) L-*Asparaginyl-*L*-glutamine*

The protected dipeptide ester (400 mg) was dissolved in hot acetic acid (2 ml), and the cooled solution treated with 4N hydrogen bromide in acetic acid (2 ml). After 1 hr at room temperature, ether was added, and the product isolated as in (*f*) (143 mg; 73%). The dipeptide was recrystallized from aqueous ethanol, m.p. 217–219° (dec.); $[\alpha]_D^{22}$ +7·3° (*c*, 1·0 in 0·5N HCl). Thin-layer chromatography gave a single spot, R_F 0·26 (A), 0·58 (B) (Found: C, 41·6; H, 5·9; N, 21·5. Calc. for $C_9H_{16}N_4O_5$: C, 41·5; H, 6·2; N, 21·5%).

(*k*) *Benzyloxycarbonyl-*L*-glutaminyl-*L*-glutamine* p-*Nitrophenyl Ester*

Isobutyl chloroformate (0·14 ml) was added to a solution of benzyloxycarbonyl-L-glutamine (280 mg) and triethylamine (0·14 ml) in dimethylformamide (4 ml) at −15° with stirring. After 20 min at this temperature, a solution of L-glutamine *p*-nitrophenyl ester

[24] Miller, A., Neidle, A., and Waelsch, H., *Archs Biochem. Biophys.*, 1955, **56**, 11.

p-toluenesulphonate[22] (439 mg) in dimethyl sulphoxide (1·5 ml) was added, followed by triethylamine (0·14 ml) in dimethylformamide (0·5 ml). The mixture was allowed to attain room temperature over 1–2 hr, and then diluted with water. The solid product was collected, washed with water and ether, and recrystallized from acetic acid/acetone (319 mg; 60%), m.p. 180–181·5°; $[\alpha]_D^{19}$ −33·7° (*c*, 1·0 in DMF); ν_{max} 1765 (NP ester CO), 1690 (Z amide I), 1670 (primary amide I), 1650 (peptide amide I), 1625 cm^{-1} (primary amide II) (Found: C, 54·7; H, 5·4; N, 13·8. Calc. for $C_{24}H_{27}N_5O_9$: C, 54·5; H, 5·1; N, 13·2%).

(*l*) o-*Nitrophenylsulphenyl*-L-*asparagine* p-*Nitrophenyl Ester*

A solution of *o*-nitrophenylsulphenyl-L-asparagine[15] (4·29 g) and *p*-nitrophenol (2·52 g) in dimethylformamide (36 ml) was stirred at 0°, and dicyclohexylcarbodiimide (3·09 g) added. Stirring was continued for 30 min at 0°, and then for 1 hr at room temperature. Removal of the precipitated dicyclohexylurea, followed by dilution with water, gave the crude *p*-nitrophenyl ester (4·77 g). Although this material was contaminated with the β-cyano-L-alanine derivative resulting from dehydration of the amide side-chain, the infrared spectrum showed no absorption in the 2240 cm^{-1} nitrile region. The compound was recrystallized three times from dimethylformamide/ethanol *at room temperature*. Considerable loss of material occurred during these operations (yield, 1·35 g; 22%), m.p. 145·5–146·5°; $[\alpha]_D^{20}$ −111·2° (*c*, 1·0 in DMF); ν_{max} 1760 (NP ester CO), 1670 (primary amide I), 1620 cm^{-1} (primary amide II) (Found: C, 47·2; H, 3·8; N, 13·2. Calc. for $C_{16}H_{14}N_4O_7S$: C, 47·3; H, 3·4; N, 13·8%).

A crystalline material was obtained from the first mother liquor on storage at 0°, m.p. 122–125°; $[\alpha]_D^{21}$ −71·0° (*c*, 1·0 in DMF). The infrared spectrum had a weak but sharp maximum at 2260 cm^{-1}, and *two* strong bands in the ester carbonyl region (1765 and 1720 cm^{-1}). This product evidently contains a substantial amount of the β-cyano-L-alanine derivative, but was not investigated further.

(*m*) o-*Nitrophenylsulphenyl*-L-*asparaginyl*-L-*asparagine 2,4,6-Trimethylbenzyl Ester*

The compound was prepared in 76% yield from the foregoing *p*-nitrophenyl ester and L-asparagine 2,4,6-trimethylbenzyl ester hydrochloride as in (*e*). The product was recrystallized from dimethylformamide/ethanol, m.p. 230·5–232·5° (dec.); $[\alpha]_D^{22.5}$ −26·3° (*c*, 1·0 in DMF); ν_{max} 1735 (ester CO); 1685–1620 cm^{-1} (broad composite amide band) (Found: C, 54·0; H, 5·5; N, 13·1. Calc. for $C_{24}H_{29}N_5O_7S$: C, 54·2; H, 5·5; N, 13·2%).

(*n*) *Benzyloxycarbonyl*-L-*glutaminyl*-L-*glutaminyl*-L-*asparaginyl*-L-*asparagine 2,4,6-Trimethylbenzyl Ester*

o-Nitrophenylsulphenyl-L-asparaginyl-L-asparagine 2,4,6-trimethylbenzyl ester (300 mg) was suspended in methanol (1 ml), and 2N methanolic hydrogen chloride (1 ml) added. A clear solution was obtained in a few minutes, and was diluted with ether. The precipitated dipeptide ester hydrochloride was dissolved in methanol, and the solution passed through a short column of IRA-400(OH) resin. The solvent was removed *in vacuo* below 30°, and the residue dissolved in dimethylformamide (3·5 ml) containing glacial acetic acid (0·035 ml). Benzyloxycarbonyl-L-glutaminyl-L-glutamine *p*-nitrophenyl ester (238 mg) was added. This quantity of the active ester corresponded to a 20% excess of the dipeptide 2,4,6-trimethylbenzyl ester, assuming quantitative isolation of the latter from the *o*-nitrophenylsulphenyl derivative. The solution was kept at room temperature for 3 days, whereupon complete solidification ensured. The mixture was triturated with ethanol, filtered, and the product boiled with ethanol, and finally washed with hot ethanol and ether (yield, 291 mg; 84%), m.p. 248–249·5° (dec.); $[\alpha]_D^{21.5}$ +0·8° (*c*, 0·5 in DCA) (Found: N, 14·6. Calc. for $C_{36}H_{48}N_8O_{11}$: N, 14·6%). The compound was virtually insoluble in all the usual solvents, including acetic acid and dimethylformamide, and dissolved with difficulty in dichloroacetic acid. Hydrolysis of a sample with hot 1N NaOH gave a colourless solution, indicating the absence of *p*-nitrophenol and unchanged active ester.

(*o*) L-*Glutaminyl*-L-*glutaminyl*-L-*asparaginyl*-L-*asparagine*

The foregoing protected tetrapeptide ester (200 mg) was shaken with 2N hydrogen bromide in acetic acid (2 ml) for 1 hr at room temperature, and ether then added to the resultant clear solution. The precipitated hydrobromide was treated as in (*f*). The free tetrapeptide was obtained as an amorphous solid (115 mg; 89%), which formed needles on recrystallization from aqueous ethanol at room temperature, m.p. 205·5–207·5° (dec.), $[\alpha]_D^{21}$ −25·4° (*c*, 1·0 in H_2O) (Found: C, 43·2; H, 6·2. Calc. for $C_{18}H_{30}N_8O_9$: C, 43·0; H, 6·0%). The infrared spectrum had no bands in the 1700–1800 cm^{-1} ester carbonyl region. Thin-layer chromatography in several solvent systems indicated that the compound was homogeneous, R_F 0·12 (A), 0·33 (B), 0·05 (C).

(*p*) o-*Nitrophenylsulphenyl*-L-*glutaminyl*-L-*asparagine*

o-Nitrophenylsulphenyl-L-glutamine[15] (897 mg) in dimethylformamide (12 ml) was converted into the mixed anhydride as in (*k*), and a solution of L-asparagine monohydrate (450 mg) and triethylamine (0·42 ml) in water (4 ml) added in one portion. The solution was stirred for 1–2 hr, then acidified with 1N H_2SO_4, and diluted with water. On storage at 0° overnight the product crystallized out, and was washed with water and ether (yield 676 mg; 54%). The compound was recrystallized from dimethylformamide/ethyl acetate, m.p. 150–151·5°; $[\alpha]_D^{20·5}$ −13·2° (*c*, 1·0 in DMF) (Found: N, 17·0. Calc. for $C_{15}H_{19}N_5O_7S$: N, 16·9%).

(*q*) o-*Nitrophenylsulphenyl*-L-*glutaminyl*-L-*asparagine 2,4,6-Trimethylbenzyl Ester*

The protected dipeptide (413 mg) was suspended in dimethylformamide (1 ml). Addition of triethylamine (0·14 ml) gave a clear solution, which was treated with chloromethylmesitylene[12,13] (170 mg). After 1–2 days at room temperature, the mixture was diluted with 1N $NaHCO_3$ and the precipitated solid collected, washed with water, and dried *in vacuo* (453 mg; 83%). The product was reprecipitated from dimethylformamide by the addition of ethyl acetate, m.p. 211·5–212·5° (dec.); $[\alpha]_D^{23}$ −13·6° (*c*, 1·0 in DMF) (Found: C, 54·8; H, 6·0; N, 12·7. Calc. for $C_{25}H_{31}N_5O_7S$: C, 55·0; H, 5·7; N, 12·8%).

The protected dipeptide ester (250 mg) was treated with 2N hydrogen bromide in acetic acid (2 ml) for 10 min at room temperature, and the mixture worked up as described in (*f*). L-Glutaminyl-L-asparagine (97 mg; 83%) was obtained, and was identical with the product prepared in (*f*), $[\alpha]_D^{21}$ +20·4° (*c*, 1·0 in 0·5N HCl).

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