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The Benzyl and *p*-Nitrobenzyl Esters of L-Glutamine and L-Asparagine[1]

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The *p*-nitrobenzyl esters and benzyl esters of L-glutamine and L-asparagine have been prepared. The use of the benzyl esters in the synthesis of carboxyl-terminal glutamine and asparagine peptides is illustrated.

Normally, treatment of *N*-acylamino acid esters with alkali yields the alcohol and a salt of the acyl amino acid. However, the alkaline hydrolysis of *N*-acylasparagine and *N*-acylglutamine methyl esters under the conditions normally used in peptide synthesis is complicated by imide formation, isomerization due to hydrolysis of the imides, and in the case of the glutamine derivatives by partial racemization.[2] Therefore, the synthesis of peptides with carboxyl-terminal glutamine or asparagine groups requires methods in which these side reactions are avoided. One way of accomplishing this is to use methods that do not require a protected amino acid. Thus, Hoffman, Thompson, and E. T. Schwartz[3] prepared L-methionyl-L-glutamine by coupling carbobenzoxy-L-methionine and glutamine by the mixed anhydride method. Presumably this technique would also be suitable for the preparation of carboxyl-terminal asparagine peptides. This approach is limited to those coupling procedures which permit the use of unprotected amino acids and peptides with the component that has the activated carboxyl group. The availability of glutamine and asparagine derivatives with carboxyl-masking groups that can be removed innocuously, would greatly increase the number of applicable coupling procedures. Recently, Anderson and Callahan[4] prepared a number of *t*-butyl esters of amino acids, including that of L-glutamine. The chief advantages of these esters lie in the ease of deesterification by mild acid catalysts, and in their stability as the free bases. In this paper we wish to report the preparation of the benzyl and *p*-nitrobenzyl esters of glutamine and asparagine. The use of the benzyl esters in peptide syntheses is also demonstrated.

Schwarz and Arakawa[5] have described a procedure for the synthesis of amino acid *p*-nitrobenzyl esters in which a carbobenzoxy amino acid reacted with *p*-nitrobenzyl chloride in the presence of triethylamine. Subsequent treatment with hydrobromic acid removes the carbobenzoxy group. This method could be readily applied to the synthesis of the glutamine and asparagine esters. Since Schwarz and Arakawa have shown the general utility of several amino acid *p*-nitrobenzyl esters in peptide synthesis and have detected no racemization during the formation of their esters, the glutamine and asparagine *p*-nitrobenzyl esters should prove to be equally useful.

We have also prepared the benzyl esters of glutamine and asparagine by the use of diazotoluene. Diazotoluene is easily prepared by the oxidation of benzalhydrazone with yellow mercuric oxide[6] and is a dark brownish red liquid which can be distilled at reduced pressure. Attempts to benzylate glutamine and asparagine directly failed, possibly due to alkylation of the ammonium group.[7] However, the carbobenzoxy and formyl derivatives could be benzylated readily. The *N*-formyl group was tested as a blocking agent because Sheehan and Yang[8] have shown that its removal in acidic medium can be effected selectively. However, all our attempts to prepare glutamine and asparagine benzyl esters by this path failed. Under the conditions recommended by Sheehan and Yang, dilute hydrochloric acid in aqueous methanol, paper strip chromatography indicated the presence of six or more compounds from each ester. The spots obtained from *N*-formyl glutamine benzyl ester are in order of decreasing concentration: glutamine benzyl ester, *alpha*-benzyl *gamma* methyl glutamate (tentative), glutamine, *gamma*-methyl glutamate, glutamic acid and dimethyl glutamate. From this it is concluded that alcoholysis at the primary amide group is a serious side reaction while transesterification and hydrolysis are only of secondary significance. Numerous attempts to bring about deformylation by varying the solvents, reaction time, and temperature failed to give satisfactory results.

The carbobenzoxy benzyl esters could be readily converted to the corresponding benzyl esters by the use of hydrogen bromide in acetic acid.[9] The benzyl esters were noncrystalline, but the

(1) This investigation was supported in part by a research grant RG-5492 from the National Institutes of Health, Public Health Service.

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only contaminants detected by paper chromatography were traces of glutamine or asparagine. The use of these esters in peptide synthesis was demonstrated in the preparation of glycyl-L-glutamine and glycyl-L-asparagine. In neither case was there any evidence for isomerization or racemization. The carbobenzoxy benzyl esters of L-glutamine and L-asparagine have been prepared previously[10] by reaction of the carbobenzoxy anhydrides with benzyl alcohol, followed by conversion to the amides *via* the acid chlorides.

EXPERIMENTAL[11]

Carbobenzoxy-L-glutamine p-nitrobenzyl ester. This compound was prepared from 1.4 g. of carbobenzoxy-L-glutamine[12] by the method of Schwarz and Arakawa in 79% yield; m.p. 135–136° after two recrystallizations from ethyl acetate and ligroin.

Anal. Calcd. for $C_{20}H_{21}N_3O_7$: C, 57.82; H, 5.10; N, 10.12. Found: C, 58.02; H, 5.31; N, 10.43.

Carbobenzoxy-L-asparagine p-nitrobenzyl ester. Since the esterification reaction did not give satisfactory yields in ethyl acetate, a better solvent was sought. A solution of 0.266 g. (1 mmole) of carbobenzoxy-L-asparagine,[13] 0.258 g. (1.5 mmoles) of *p*-nitrobenzyl chloride and 0.21 ml. (1.5 mmoles) of triethylamine in 5 ml. of dimethylformamide was heated at 75° for 5 hr. A precipitate appeared after 5 min. To the cooled mixture 20 ml. of water was added. The precipitate was filtered off, washed with 20 ml. of ether and dried in vacuum; yield 0.279 g., 70%, m.p. 158–165°, raised to m.p. 164–166° after two recrystallizations from ethanol.

Anal. Calcd. for $C_{19}H_{19}N_3O_7$: C, 56.85; H, 4.77; N, 10.47. Found: C, 56.93; H, 4.88; N, 10.26.

L-Glutamine p-nitrobenzyl ester hydrobromide. Carbobenzoxy-L-glutamine *p*-nitrobenzyl ester, 0.726 g., was dissolved in 2.5 ml. of 30% hydrobromic acid in glacial acetic acid. After the mixture had been held at room temperature for 15 min. it was cooled to −70° and evaporated by freeze-drying. The residue was washed with 50 ml. of ether and recrystallized twice from methanol and ether, yield 0.491 g., 77%, m.p. 175°.

Anal. Calcd. for $C_{12}H_{16}N_3O_5$.HBr: C, 39.68; H, 4.72; N, 11.57. Found: C, 39.30; H, 4.74; N, 12.18.

L-Asparagine p-nitrobenzyl ester hydrobromide. This compound was prepared by the same method as the glutamine ester; yield 70%, m.p. 170–171° after two recrystallizations from methanol and ether.

Anal. Calcd. for $C_{11}H_{14}N_3O_5$. HBr: C, 37.83; H, 4.33; N, 12.03. Found: C, 38.23; H, 4.02; N, 12.00.

N-Formyl-L-glutamine benzyl ester. Diazotoluene was obtained by the oxidation of benzalhydrazone with yellow mercuric oxide.[6] Benzaldehyde, 20 g., was added dropwise, with stirring, to 20 ml. of 95% hydrazine hydrate. After the mixture had cooled to room temperature it was extracted with two volumes of ether. The ethereal phase was dried overnight with potassium hydroxide and the ether evaporated. Distillation of the residue yielded benzalhydrazone as a colorless oil, b.p. 85° (2 mm.), which solidified on cooling in an ice bath. The benzalhydrazone should be used soon after preparation since it decomposes to benzalazine, yellow crystals, m.p. 93°, in the presence of traces of water. To 9 g. of benzalhydrazone suspended in 50 ml. of petroleum ether (b.p. 30–60°) was added at once 15 g. of yellow mercuric oxide. The mixture was shaken and the reaction rate moderated by occasional submersion of the mixture in an ice bath. After the reaction reached completion the mixture was filtered. This filtrate may be used directly, or the solvent evaporated and the residue dissolved in ether. The mixture can also be distilled at reduced pressure if desired. There appears to be no danger of explosions since the diazotoluene could be heated safely in a boiling water bath. The major advantage resulting from distillation is that the benzylated product will be free of yellow ether soluble contaminants.

To 3 g. of *N*-formyl-L-glutamine[14] in a minimum of ethanol was added slowly an ethereal solution of freshly prepared crude diazotoluene. The addition was continued until the vigorous reaction subsided and the reddish brown color of the diazotoluene persisted for 30 min. On decomposition of the excess diazotoluene with acetic acid and evaporation of the solvents a crystalline product was obtained, m.p. 118–134°. Two recrystallizations from ethanol and ether yielded 2.9 g., 64% m.p. 133–135°.

Anal. Calcd. for $C_{13}H_{16}N_2O_4$: C, 59.05; H, 6.10; N, 10.60. Found: C, 58.99; H, 6.06; N, 10.61.

N-Formyl-L-asparagine. This compound was prepared by Sheehan and Yang's method[8] for the preparation of optically active formyl derivatives. From 1.32 g. L-asparagine 1.14 g., 71% of product was obtained, m.p. 133–135°.

Anal. Calcd. for $C_5H_8N_2O_4$: C, 37.52; H, 5.03; N, 17.50. Found: C, 37.24; H, 5.18; N, 17.58.

N-Formyl-L-asparagine benzyl ester. An ethereal solution of crude diazotoluene was added to 2 g. of *N*-formyl-L-asparagine until a reddish color persisted in the mixture for 30 min. After the excess diazotoluene was decomposed with acetic acid and the solvent evaporated the product was obtained in the form of a gel. This was dissolved in 5 ml. of ethanol and precipitated with ether. The precipitate was washed twice with 75-ml. portions of ether, yielding 0.69 g., 22% of crystalline ester, m.p. 83–85°.

Anal. Calcd. for $C_{12}H_{14}N_2O_4$: C, 57.58; H, 5.64; N, 11.20. Found: C, 57.72; H, 5.63; N, 11.23.

Attempts to remove the formyl group from glutamine and asparagine derivatives. In the initial experiments Sheehan and Yang's procedure was used. To 0.16 g. of *N*-formyl-L-glutamine benzyl ester in 1 ml. of methanol, 0.66 ml. of 1*N* methanolic hydrochloric acid was added and the solution stored at room temperature. Periodic samples were removed and chromatogramed on Whatman number 1 filter paper with 1-butanol, acetic acid, and water (4:1:5) and the amino acids detected with ninhydrin. At least six spots were obtained, R_f 0.17, 0.28, 0.40, 0.63, 0.71, and 0.78. The largest spot, R_f 0.63, was identified as glutamine benzyl ester. This spot disappeared on hydrogenolysis and glutamine, R_f 0.17, was formed. The product obtained on hydrobromic acid treatment of carbobenzoxy-L-glutamine benzyl ester had the same R_f value. The second largest spot, R_f 0.78, is tentatively identified as *gamma*-methyl *alpha*-benzyl glutamate. The evidence for this assignment rests on the fact that after hydrogenolysis this spot is no longer detected and a new spot with the same R_f value, 0.40, as *gamma*-methyl glutamate appears. The third largest spot formed during the deformylation was glutamine, R_f 0.17. We also detected small amounts of glutamic acid, R_f 0.28, *gamma*-methyl glutamate and dimethyl glutamate, R_f 0.71. On several occasions a very small salmon colored spot, R_f 0.20, believed to be *alpha*-aminoglutarimide,[15] was detected.

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(10) M. Bergmann, L. Zervas, and L. Salzmann, *Ber.*, 66B, 1288 (1933).

(11) All melting points were determined on a Mel-temp heating block in capillary tubes and are uncorrected. Analyses are by Spang Microanalytical Laboratory, Ann Arbor, Mich., and George Robertson, Florham Park, N. J. All samples were dried at 60° in a vacuum over phosphorus pentoxide prior to analysis.

(12) R. A. Boissonas, S. Guttmann, P. Jaquenoud, and J. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).

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Attempts to isolate pure glutamine benzyl ester hydrochloride from this reaction mixture have been unsuccessful. The most serious side reaction appears to be alcoholysis on the *gamma* carbon. Transesterification and hydrolysis at the *alpha* carbon seem to be of secondary importance.

Comparable results were obtained with *N*-formylasparagine benzyl ester where again at least six spots were detected chromatographically. Numerous attempts to make the deformylation reactions more selective by changes in the solvents, acid concentrations, reaction temperature, and time were unsuccessfuls. Other solvent systems that were tried were water, *t*-butyl alcohol, aqueous tetrahydrofuran, aqueous acetic acid, aqueous acetonitrile, and aqueous trifluoroethanol. In these solvents the reaction rate was drastically reduced without apparent increase in the selectivity.

Carbobenzoxy-L-glutamine benzyl ester. To 500 mg. of carbobenzoxy-L-glutamine in 2 ml. of dimethylformamide a solution of crude diazotoluene in petroleum ether was added until a reddish brown color persisted for 30 min. The excess diazotoluene was decomposed with acetic acid and the petroleum ether removed under reduced pressure. The resulting solution was treated with 45 ml. of ether and 40 ml. of water and the mixture stored overnight at 0°. Filtration, washing of the precipitate with ether, and two recrystallizations from aqueous ethanol yielded 320 mg. of ester, 49%, m.p. 130–131°, reported[10] m.p. 123°.

Anal. Calcd. for $C_{20}H_{22}N_2O_5$: C, 64.85; H, 5.99; N, 7.57. Found: C, 64.69; H, 6.07; N, 7.44.

Carbobenzoxy-L-asparagine benzyl ester. This compound was prepared from 650 mg. of carbobenzoxy-L-asparagine by the same procedure as that used for the glutamine derivative. A gel was obtained which crystallized slowly at 50° from ethyl acetate; yield 0.391 g., 45%, m.p. 128.5°, reported[10] m.p. 132°.

Anal. Calcd. for $C_{19}H_{20}N_2O_5$; C, 64.03; H, 5.66; N, 7.86. Found C, 64.03; H, 5.81; N, 7.81.

Carbobenzoxy-glycyl-L-glutamine benzyl ester. To 0.74 g., 2 mmoles, of carbobenzoxy-L-glutamine benzyl ester was added 3 ml. of 30% hydrogen bromide in acetic acid. After storage at room temperature for 15 min. the mixture was cooled in a Dry Ice-acetone bath and freeze-dried. The residue was washed with 30 ml. of ether and then dissolved in a minimum of dimethylformamide and reprecipitated with ether. The residue dissolved in ethanol was treated with 0.28 ml. of triethylamine and the mixture filtered. Evaporation of the filtrate yielded an oil and a further quantity of triethylamine hydrobromide. This residue was treated with 2 ml. of dimethylformamide, the mixture filtered, the precipitate washed with an additional 1 ml. of dimethylformamide and the combined solution stored at 0° for coupling with the azide.

An ethereal solution of carbobenzoxy-glycylazide was prepared in the usual manner from 223 mg., 1 mmole, of carbobenzoxy-glycyl hydrazide. The azide was combined with the benzyl ester and the solution stored overnight at 0°, then 4 hr. at room temperature. The addition of water caused precipitation of an oil which crystallized on storage at 0°. The precipitate was collected, washed with dilute hydrochloric acid, sodium bicarbonate and water, dried *in vacuo* over phosphorus pentoxide; yield 0.28 g., 67% based on the quantity of azide, m.p. 134–135°. Recrystallization from aqueous ethanol yielded 0.20 g. of product, m.p. unchanged.

Anal. Calcd. for $C_{22}H_{25}N_3O_6$: C, 61.80; H, 5.89; N, 9.83. Found: C, 61.73; H, 5.87; N, 10.10.

Carbobenzoxy-glycyl-L-asparagine benzyl ester. This compound was prepared by the same procedure as the glutamine analog in 58% yield, m.p. 135–136°.

Anal. Calcd. for $C_{21}H_{23}N_3O_6$: C, 61.00; H, 5.62; N, 10.19. Found: C, 60.87; H, 5.64; N, 10.46.

Test for racemization and chromatographic purity of the peptides. Hydrogenolysis of the carbobenzoxy-benzyl esters in the presence of palladium black at room temperature and atmospheric pressure yielded gycyl-L-glutamine, m.p. 202–203° reported[16] m.p. 199–200°, R_f 0.055, 1-butanol, acetic acid, water (4:1:5); and glycyl-L-asparagine, m.p. 214°, reported[17] m.p. 216°, R_f 0.052 in 1-butanol, acetic acid and water (4:1:5) and R_f 0.32 in water saturated phenol. Each peptide gave only one spot with ninhydrin. To check for possible racemization the peptides were refluxed 2 hr. in 6*N* hydrochloric acid and $[\alpha]_D^{25}$ of the hydrolyzate compared with $[\alpha]_D^{25}$ for L-glutamic acid and L-aspartic acid containing equivalent quantities of glycine and ammonium chloride. The hydrolyzate from glycylglutamine gave $[\alpha]_D^{25}$ +28.9° (*c* 3.02, 6*N* hydrochloric acid); L-glutamic acid, $[\alpha]_D^{25}$ +28.9°. The hydrolyzate from glycyl-L-asparagine gave $[\alpha]_D^{25}$ +22.2° (*c* 1.57, 6*N* hydrochloric acid); L-aspartic acid $[\alpha]_D^{25}$ +22.4°.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Synthesis of L-Leucyl-L-alanyl-L-valyl-L-glutamic Acid and Intermediate Peptides[1]

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The syntheses of the tetrapeptide, L-leucyl-L-alanyl-L-valyl-L-glutamic acid, the tripeptide, L-leucyl-L-alanyl-L-valine and the dipeptides, L-leucyl-L-alanine and L-valyl-L-glutamic acid, are described. The *p*-nitrobenzyloxycarbonyl group was employed to protect the amino groups during peptide bond formation by either the azide, acid chloride, or acid anhydride methods.

In connection with studies being performed in this laboratory on the oxidative cleavage of peptide bonds[1b,3] some model compounds were needed. It was desirable to obtain a series of peptides up to a tetrapeptide sequence in which the constituent

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