

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH 13, PENNA.]

Insulin Peptides IV. The Synthesis of a Protected Decapeptide Containing the C-Terminal Sequence of the A-Chain of InsulinBY PANAYOTIS G. KATSOYANNIS,¹ KENJI SUZUKI AND ANDREW TOMETSKO

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The synthesis of a protected decapeptide containing the C-terminal sequence of the A-chain of insulin is described. For its preparation, the product which was obtained by decarbobenzoylation of N-carbobenzoyl- γ -benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester was condensed with N-carbobenzoyl-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine azide. The synthesis of the former protected pentapeptide was reported previously, whereas the synthesis of the latter pentapeptide derivative is described in this report.

The amino acid sequence -seryl-leucyl-tyrosyl-glutamyl-L-leucyl-glutamyl-asparaginyl-tyrosyl-cysteinyl-asparagine represents the C-terminal region of the A-chain of insulin from various species.² In a previous communication we have reported the synthesis of a protected pentapeptide containing the sequence glutamyl-asparaginyl-tyrosyl-cysteinyl-asparagine which represents the carboxyl terminal portion of the aforementioned decapeptide sequence.³ In connection with our studies directed toward the synthesis of the insulin molecule,⁴ we have recently⁵ completed the preparation of the protected decapeptide N-carbobenzoyl-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucyl- γ -benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine-*p*-nitrobenzyl ester. This peptide derivative embodies within its structure the above-mentioned C-terminal region of the A-chain of insulin. For the synthesis of the protected decapeptide two pentapeptide subunits were first prepared and then condensed to give the final product. Thus interaction of the azide of N-carbobenzoyl-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine with γ -benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester yielded the protected decapeptide N-carbobenzoyl-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucyl- γ -benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzylester.

The synthesis of one of the peptide subunits in a fully protected form, namely, N-carbobenzoyl- γ -benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester, has been reported in an earlier communication.³ The synthesis of the other pentapeptide subunit also in a fully protected form, namely, N-carbobenzoyl-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine methyl ester, is described in this report.

Its construction was effected by the stepwise elongation of the peptide chain from the amino end. As the "carboxyl component" in each synthetic step the respective N-carbobenzoyl amino acids were used. Their activation was accomplished by conversion to the corresponding *p*-nitrophenyl esters with the exception of N-carbobenzoyl-L-serine which was activated by conversion to the respective azide.⁶

Deblocking of the intermediate carbobenzoyl peptides in each synthetic step was carried out by exposing

them to hydrogen bromide in acetic acid. The protected pentapeptide ester and several of the intermediate peptide derivatives were obtained in crystalline form. The chemical purity of all these compounds was established by elemental analysis and paper chromatography of the deblocked derivatives. In the latter case, formation of sharp single spots on paper chromatograms indicated the homogeneity of the pertinent peptide derivative. The stereochemical homogeneity of the protected pentapeptide was ascertained by decarbobenzoylation and digestion of the resulting product with leucine aminopeptidase (LAP) followed by paper chromatography of the digest. The chromatogram exhibited only ninhydrin-positive spots with R_f values corresponding to the constituent amino acids. Since no other ninhydrin-positive components were present, it was concluded that the digestion by LAP was complete and this was accepted as evidence for stereochemical homogeneity.⁷

The dipeptide N-carbobenzoyl-L-glutamyl-L-leucine methyl ester⁸ was obtained in crystalline form and in 80% yield by treating N-carbobenzoyl-L-glutamine *p*-nitrophenyl ester⁹ with leucine methyl ester. Coupling of N-carbobenzoyl-L-glutamine^{10,11} with leucine methyl ester through formation of the mixed anhydride with isobutyl chloroformate resulted also in the formation of the same dipeptide in 75% yield. Decarbobenzoylation of the protected dipeptide by hydrogen bromide in acetic acid and coupling of the ensuing product with N-carbobenzoyl-O-benzyl-L-tyrosine *p*-nitrophenyl ester⁹ yielded the crystalline tripeptide N-carbobenzoyl-O-benzyl-L-tyrosyl-L-glutamyl-L-leucine methyl ester in 85% yield. On exposure to hydrogen bromide in acetic acid the carbobenzoyl and benzyl groups of the protected tripeptide were removed. The amino free derivative thus formed was converted to the protected tetrapeptide N-carbobenzoyl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine methyl ester in 86% yield by condensation with N-carbobenzoyl-L-leucine *p*-nitrophenyl ester.⁹

For paper chromatography the protected di-, tri- and tetrapeptide derivatives were deblocked on treatment with hydrogen bromide in acetic acid. The resulting hydrobromides were chromatographed on Whatman No. 1 paper using the Partridge system.¹² All three chromatograms exhibited single ninhydrin positive spots with R_f values 0.70, 0.59 and 0.78, respectively.

The crystalline protected pentapeptide N-carbobenzoyl-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine methyl ester was obtained in 52% yield by the interaction of N-carbobenzoyl-L-serine azide⁶ with

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the product formed by decarbobenzoylation of the protected tetrapeptide. Removal of the carbobenzyloxy group from this pentapeptide derivative was carried out on exposure to hydrogen bromide in trifluoroacetic acid. Use of this method for deblocking serine-containing peptides is preferable¹³ to hydrogen bromide in acetic acid since the latter reagent causes acetylation of the hydroxyl function of the serine.¹⁴

The decarbobenzoylated pentapeptide ester behaved as a single component when analyzed by paper chromatography exhibiting an R_f of 0.80 in the Partridge system. Leucine aminopeptidase converted the deblocked pentapeptide into a mixture of serine, leucine, tyrosine and glutamine. This was demonstrated by paper chromatography, thus indicating that no racemization had taken place during the various synthetic steps.

Conversion of the protected pentapeptide into the corresponding hydrazide was readily accomplished by refluxing a suspension of the peptide ester in methanol in the presence of hydrazine. The resulting hydrazide was converted to the azide in the usual manner. Coupling of this azide with the product obtained by decarbobenzoylation of N-carbobenzyloxy- γ -benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester³ yielded the desired protected decapeptide in 40% yield. The chemical purity of this compound was established by elemental analysis, paper chromatography of the decarbobenzoylated derivative and amino acid analysis of an acid hydrolysate. For paper chromatography, the protected decapeptide was deblocked by exposure to hydrogen bromide in nitromethane. The chromatogram, using the Partridge system, exhibited only a single spot (R_f 0.53) by ultraviolet quenching, ninhydrin reaction and reactions for tyrosine and methionine.¹⁵ Amino acid analysis of an acid hydrolysate of the protected decapeptide by a Beckman-Spinco analyzer showed the expected composition, expressed in molar ratios: asp_{1.1}glu_{1.0}ser_{0.45}leu_{1.0}tyr_{1.0}. S-*p*-Nitrobenzylcysteine present on a paper chromatogram (R_f in Partridge system 0.71) was not determined.

Incubation of the decarbobenzoylated decapeptide ester with leucine aminopeptidase caused complete degradation of the decapeptide ester to its constituent amino acids as demonstrated by paper chromatography of the digest.

Experimental

Capillary melting points were determined for all compounds and are corrected.

N-Carbobenzyloxy-L-glutamyl-L-leucine Methyl Ester. A. *p*-Nitrophenyl Ester Method.—To a solution of L-leucine methyl ester hydrochloride (11.8 g.) in dimethylformamide (40 ml.) and triethylamine (8.6 ml.), N-carbobenzyloxy-L-glutamine *p*-nitrophenyl ester (24.3 g.) was added. After 24 hours at room temperature the reaction mixture was diluted with 1 *N* NH₄OH (4 ml.), stirred for 1 hour and mixed with ethyl acetate. The resulting solution was washed successively with 1 *N* NH₄OH, water, 1 *N* HCl and water again. Concentration of the ethyl acetate to a small volume resulted in the precipitation of a crystalline product which was collected by filtration and recrystallized from aqueous methanol; wt. 19.7 g. (80%), m.p. 164°, [α]_D²⁰ -11.0° (lit.⁸ m.p. 163–164°), R_f (hydrobromide) 0.70, single ninhydrin-positive spot.

B. Mixed Anhydride Method.—A solution of N-carbobenzyloxy-L-glutamine (13.5 g.) and triethylamine (6.6 ml.) in tetrahydrofuran (140 ml.) was cooled to -10° and isobutyl chloroformate (6.4 ml.) was added with subsequent addition after 10 minutes of a cooled solution of L-leucine methyl ester in tetrahydrofuran prepared as noted: L-leucine methyl ester

hydrochloride (10 g.) was suspended in tetrahydrofuran (100 ml.) and triethylamine (7.8 ml.), stirred for 10 minutes and the precipitated triethylamine hydrochloride was filtered off and washed with tetrahydrofuran (30 ml.); the combined filtrates then were added to the mixed anhydride prepared as described above. The reaction mixture was stirred at -10° for 1 hour, at room temperature for 18 hours and then evaporated to dryness. The crystalline residue was extracted into ethyl acetate (500 ml.) and water (50 ml.). The organic layer was washed successively with water, 1 *N* HCl, water, 1 *N* KHCO₃ and water again. On removal of the solvent *in vacuo* a crystalline residue remained which was recrystallized from aqueous methanol; wt. 14.5 g. (75%), m.p. 164°, [α]_D²⁰ -11.0° (c 1.04, dimethylformamide), R_f (hydrobromide) 0.70.

N-Carbobenzyloxy-O-benzyl-L-tyrosyl-L-glutamyl-L-leucine Methyl Ester.—N-Carbobenzyloxy-L-glutamyl-L-leucine methyl ester (8 g.) was dissolved in 2 *N* HBr in acetic acid (70 ml.). After 1 hour at room temperature, dry ether (600 ml.) was added and the precipitated product was washed with ether, dried over KOH *in vacuo* and dissolved in dimethylformamide (60 ml.). To this solution triethylamine (5 ml.) was added followed by N-carbobenzyloxy-O-benzyl-L-tyrosine *p*-nitrophenyl ester (9.5 g.). After 24 hours the reaction mixture was diluted with 1 *N* NH₄OH (5 ml.), stirred for 1 hour and poured into ethyl acetate (800 ml.). The resulting solution was washed successively with water, 1 *N* NH₄OH, water, 1 *N* HCl and water. (In order to prevent precipitation of the product during the washing, methanol was added to the ethyl acetate solution.) Concentration of the solvent to one-third of its volume and cooling caused the product to crystallize out; wt. 11 g. (85%), m.p. 219–221°. A sample for analysis was recrystallized from aqueous acetic acid; m.p. 222–224°, [α]_D²⁰ -6.5° (c 1.08, dimethylformamide), R_f (hydrobromide) 0.59, single ninhydrin- and tyrosine-positive spot.

Anal. Calcd. for C₃₆H₄₄N₄O₈: C, 65.4; H, 6.71; N, 8.5. Found: C, 65.4; H, 6.84; N, 8.7.

N-Carbobenzyloxy-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine Methyl Ester.—N-Carbobenzyloxy-O-benzyl-L-tyrosyl-L-glutamyl-L-leucine methyl ester (6.6 g.) was suspended in acetic acid (30 ml.) and treated with 4 *N* HBr in acetic acid (30 ml.). After 1 hour, dry ether (400 ml.) was added and the precipitate which was formed was collected by filtration, washed with ether and dried over KOH *in vacuo*. To a solution of this solid in dimethylformamide (30 ml.), triethylamine (3.6 ml.) was added followed by N-carbobenzyloxy-L-leucine *p*-nitrophenyl ester (3.6 g.). After 24 hours at room temperature, the reaction mixture was diluted with 1 *N* NH₄OH (3 ml.), stirred for 1 hour and poured into ice-cold 1 *N* NH₄OH (300 ml.). The precipitated product was isolated by filtration, washed with 1 *N* NH₄OH, water, 1 *N* HCl and water again. The crude product was purified by reprecipitation from aqueous ethanol or aqueous acetic acid; wt. 5.85 g. (86%), m.p. 226–229°, [α]_D²⁰ -31.2° (c 0.99, dimethylformamide), R_f (hydrobromide) 0.78, single ninhydrin- and tyrosine-positive spot.

Anal. Calcd. for C₃₅H₄₉N₅O₉: C, 61.5; H, 7.22; N, 10.2. Found: C, 61.0; H, 7.14; N, 10.5.

N-Carbobenzyloxy-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine Methyl Ester Hydrate.—N-Carbobenzyloxy-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine methyl ester (2.23 g.) was suspended in acetic acid (10 ml.) and treated with 4 *N* HBr in acetic acid (10 ml.). After 1 hour at room temperature, dry ether (100 ml.) was added and the precipitated product was filtered off, washed with ether and dried over KOH *in vacuo*. To a cold solution of this product in dimethylformamide (5 ml.) containing triethylamine (0.7 ml.) was added an ethyl acetate solution (25 ml.) of N-carbobenzyloxy-L-serine azide⁶ (prepared from 1.3 g. of the hydrazide and 0.26 g. of sodium nitrite in 18 ml. of 1 *N* HCl at 0°). The reaction mixture was stirred at 5° for 20 hours and at room temperature for 1 hour. The mixture was diluted with ethyl acetate (200 ml.) and washed successively with water, 1 *N* KHCO₃, water, 1 *N* HCl and water. Concentration of the solvent to a small volume and addition of petroleum ether afforded a solid which was crystallized from aqueous acetic acid; wt. 1.2 g. (52%), m.p. 232° [α]_D²⁰ -27.5° (c 1.03, dimethylformamide).

Anal. Calcd. for C₃₈H₅₄N₆O₁₁·H₂O: C, 57.9; H, 7.17; N, 10.6. Found: C, 57.5; H, 7.04; N, 10.5.

For paper chromatography and enzymatic analysis the protected pentapeptide was decarbobenzoylated on exposure to HBr in trifluoroacetic acid; R_f (hydrobromide) 0.79. The deblocked pentapeptide ester was completely hydrolyzed to the constituent amino acids on incubation with LAP as was demonstrated by paper chromatography of the digest.

N-Carbobenzyloxy-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine Hydrazide.—A suspension of N-carbobenzyloxy-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine methyl ester (0.38 g.) in methanol (10 ml.) containing hydrazine (0.12 ml.) was refluxed for 8 hours and then allowed to stand at room temperature overnight. The precipitated product was then filtered off and washed with methanol and ether; wt. 0.2 g. (53%), m.p.

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(15) We have observed that S-acylated cysteine derivatives give a positive reaction with platinum iodide reagent routinely used for methionine detection on paper chromatograms.

236–238°. A sample for analysis was precipitated from a dimethylformamide solution with the addition of ethanol and ether; m.p. 240–243°, $[\alpha]^{25}_D -19.6^\circ$ (c 0.96, dimethylformamide).

Anal. Calcd. for $C_{87}H_{84}N_8O_{10}$: C, 57.5; H, 7.07; N, 14.5. Found: C, 57.2; H, 7.18; N, 14.3.

N-Carbobenzoxy-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucyl-L-benzyl-L-glutamyl-L-asparagyl-L-tyrosyl-S-p-nitrobenzyl-L-cysteinyl-L-asparagine p-Nitrobenzyl Ester Tetrahydrate.—N-Carbobenzoxy- γ -benzyl-L-glutamyl-L-asparagyl-L-tyrosyl-S-p-nitrobenzyl-L-cysteinyl-L-asparagine p-nitrobenzyl ester (3.25 g.) was dissolved in 2 N HBr in acetic acid (60 ml.). After 1 hour at room temperature, dry ether (500 ml.) was added and the precipitated product was isolated and reprecipitated from methanol-ether. To a cold solution of this product in dimethylformamide (50 ml.) and triethylamine (0.45 ml.) was added N-carbobenzoxy-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine azide prepared as noted: N-carbobenzoxy-L-seryl-L-leucyl-L-tyrosyl-L-glutamyl-L-leucine hydrazide (2.2 g.) was dissolved in a mixture of dimethylformamide (15 ml.), acetic acid (30 ml.) and 1 N HCl (80 ml.); the solution was cooled in an ice-bath and sodium nitrite (0.2 g.) in water (5 ml.) was added; the mixture was kept at 0° for 20 minutes and ice-cold water (50 ml.) was added; the precipitated solid was isolated by filtration, washed with ice-cold water, 1 N HCl and water and dried *in vacuo* for 30 minutes over phosphorus pentoxide at a temperature of 0°; this

azide was then added to the solution of the amino component prepared as described above. The reaction mixture was kept at 5° for 24 hours and at room temperature for 1 hour and then poured into ice-cold 1 N HCl (200 ml.). The precipitated product was collected by filtration and washed with water, 1 N NH_4OH and water again; wt. 2 g. (40%), m.p. 228–230° dec. A sample for analysis was reprecipitated from dimethylformamide-water; m.p. 231–233° dec., $[\alpha]^{25}_D -36.3^\circ$ (c 1.1, dimethylformamide).

Anal. Calcd. for $C_{88}H_{101}N_{15}O_{25}S \cdot 4H_2O$: C, 55.0; H, 6.06; N, 11.6. Found: C, 54.7; H, 6.06; N, 12.1.

Amino acid analysis of an acid hydrolysate by a Beckman-Spinco analyzer gave the expected amino acid composition as discussed previously. For paper chromatography and enzymatic analysis a sample of the protected decapeptide was decarboxylated on exposure to HBr in nitromethane; R_f (hydrobromide) 0.53; single ninhydrin, methionine and tyrosine positive spot and single spot as revealed by ultraviolet quenching. The decarboxylated decapeptide was completely digested by LAP as demonstrated by paper chromatography of the digest.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY, LAFAYETTE, IND.]

Solvation as a Factor in the Alkylation of Ambident Anions: The Importance of the Hydrogen Bonding Capacity of the Solvent^{1,2}

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When solutions of the salts of phenol or *p*-alkylphenols in a wide variety of solvents are alkylated with allyl or benzyl halides the ether (oxygen alkylation) is the sole product. However, when these reactions are conducted in water, phenol or fluorinated alcohols, substantial amounts of *o*- and *p*-alkylated products result. Neither carbon nor oxygen alkylation is a carbonium ion process but, rather, these are second-order nucleophilic displacements. An explanation for the fact that water, phenol and fluorinated alcohols foster carbon alkylation, which invokes their strong hydrogen bonding capabilities, is proposed.

Introduction

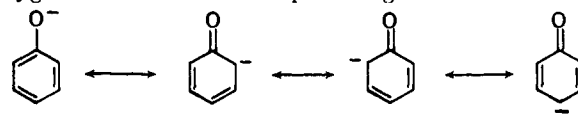
As part of a study of the factors which control the reactions of ambident anions,⁴ *i.e.*, anions possessing capability for covalent bond formation at either of two alternative positions, the role of the solvent has been investigated. The present paper describes experiments which employ salts of phenol and *p*-cresol and the one immediately following⁵ deals with the alkylation of β -naphthoxide salts. These studies clearly demonstrate that the solvent in which an ambident anion reaction is conducted may well decide the reaction course. As an example, when sodium β -naphthoxide is treated with benzyl bromide in dimethyl sulfoxide solution a 95% yield of benzyl β -naphthyl ether results, but in trifluoroethanol the major product is 1-benzyl-2-naphthol (85% yield).

In order to account for the ability of solvents to control the course of ambident anion alkylations we invoke two properties of solvents: their capacity for solvating ions and their dielectric constants. In so doing, we hark back to Hammett⁶ who, in 1940, pointed out that solvents which are effective at dissolving salts have two attributes: (1) a high dielectric constant and,

(2) a considerable tendency on the part of the solvent molecules to attach themselves to ions, *i.e.*, to solvate them. In particular, Hammett noted that such solvation often depends upon hydrogen bonding between the solvent molecules and the unshared electrons of the anion of the salt.

In the interest of orderly development, the present paper pays particular attention to the hydrogen bonding capacity of the solvent while the one which follows emphasizes the role of the dielectric factor. But in order to provide perspective it should be stated at the outset that, in our view, when an ambident anion reaction is conducted in protic solvents the free energies of the two possible transition states are influenced by a combination of the hydrogen bonding and dielectric factors. In aprotic solvents the dielectric factor and whatever capability the solvent molecules possess for solvating ions combine to influence the free energies of the two possible transition states. It is now clear, especially because of studies by Zook⁷ and by Zaugg,⁸ that aprotic solvents may possess significant capabilities for solvating cations; the consequences of cationic solvation for the reactions of ambident anions are discussed in the second of these two papers.⁵

The phenoxide ion is capable of bond formation at oxygen or at the *ortho* and *para* ring carbon atoms



(1) Paper V in the series, "The Chemistry of Ambident Anions." A preliminary account of this work appeared in *J. Am. Chem. Soc.*, **82**, 1257 (1960).

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