Sulfur-Containing Polypeptides. XVI. Synthesis of the A\textsubscript{14-21} Fragment of Ovine Insulin\textsuperscript{1-3}

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The protected octapeptide N-2-(p-diphenyl)isoproplxycarbonyl-0-\textit{\textalpha};butyl-L-tyrosyl-L-glutamyl-L-leucyl-L-tyrosyl-L-glutamyl-L-asparaginyl-L-\textit{\textalpha};butyl-L-tyrosyl-L-trityl-L-cysteinyl-L-asparaginyl-2,4,6-trimethylbenzyl ester (III) has been synthesized. The route involves the use of the N-2-(p-diphenyl)isoproplxocarbonyl (DpOC) group as the principle amino protective group and N-hydroxysuccinimide and azide coupling methods.

In the accompanying report\textsuperscript{1} a synthetic route to a suitably blocked peptide containing the A\textsubscript{14-19} sequence of ovine insulin (I) was described. The present report concerns the development of a synthesis leading to the A\textsubscript{14-21} sequence (II) and describes our experience with the N-2-(p-diphenyl)isoproplxycarbonyl protective group developed by Sieber and Iselin\textsuperscript{5} for the elegant synthesis of thyrocalcitonin.\textsuperscript{6}

The preparation of the A\textsubscript{14-21} sequence was complicated by the presence of seven functional side chains in the octapeptide; four of these required protection. Since acid-labile protective groups were required and the presence of a cysteine residue ruled out the possibility of removal of groups by hydrogenolysis, it was clear that only protective groups of very specific acid lability could be utilized. The protective group of choice for the phenolic hydroxyl groups at A\textsubscript{1419} was the tert-butyl ether; the \textit{\textalpha};trityl group was required for the A\textsubscript{20} cysteine residue to permit selective formation of the two interchain disulfide bonds at A\textsubscript{7}B\textsubscript{7} and A\textsubscript{20}B\textsubscript{20}. The tert-butyl ester seemed to be suitable for the A\textsubscript{17} carboxyl group. The choice of the 2,4,6-trimethylbenzyl ester as the blocking group for the asparagine-21 residue was governed by the overall stability of this ester and the earlier use by Stewart\textsuperscript{7} in a synthesis of a modified sequence of the C-terminal portion of the A chain. Given these choices of ether and ester protective groups, relatively few possibilities were available for amino protective groups. The N-tert-butyloxycarbonyl group could not be used since O-tert-butyloxycarbonyl ethers and esters generally cleave at comparable rates\textsuperscript{1} and the presence of the cysteine residue prevented removal of the N-carbobenzyloxy group by hydrogenolysis. Thus the choices of amino protective groups were essentially limited to the N-trityl (Tr), the N-o-

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(3) The following abbreviations have been employed in the text: t-BOC = tert-butyloxycarbonyl; DpOC = 2-(p-diphenyl)isoproplxocarbonyl; o-NPS = o-nitrophenylsulfenyl; \textit{\textalpha};Bu = \textit{\textalpha};butyl; TMB = 2,4,6-trimethylbenzyl; Tr = trityl; Bzl = benzyl, Su = N-hydroxy succinimide; DCC = N,N'-dicyclohexylcarbodiimide; DME = 1,2-dimethoxystyrene; NMM = N-methylmorpholine; DMF = N,N-dimethylformamide; DMAc = N,N-dimethylacetamide.

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Scheme I
SYNTHESIS OF THE PROTECTED A_4_5_a PEPTIDE DERIVATIVE

L-Asparagine 2,4,6-trimethylbenzyl ester hydrochloride (IV) was coupled via the N-hydroxysuccinimide method to tert-butylxycarbonyl-S-trityl-l-cysteine dicyclohexylamylsine (V). The O-BOC group of the dipeptide VI was subsequently removed by the action of boron trifluoride in acetic acid, and S-trityl-l-cysteinyl-l-asparagine 2,4,6-trimethylbenzyl ester (VII) was obtained in 82% overall yield. The DpOC group was not employed at this point since a group of this lability was not required and since the preparation of this particular cysteine derivative has provided low-melting solids that are difficult to purify. In our early experiments VII was converted to the oxalate salt VIII for characterisation purposes; subsequently VII was used directly in the following coupling step.

Since the acid-labile tert-butyl ether was required for the protection of the phenolic hydroxyl of Tyr_134, clearly either the o-NPS, the DpOC, or the Tr group was necessary for amino protection. Despite the fact that a number of separate steps are required for the preparation of N-2-(p-diphenylaminoisopropylxycarbonyl-0-tert-butyl-l-tyrosine dicyclohexylamylsine salt (IX), this group was preferable to the o-NPS group since S-trityl cleavage can sometimes occur when the o-NPS group of an S-trityl-l-cysteine peptide is removed from the amino terminus^12 or to the N-trityl group which is known to give lowered yields in the coupling steps because of steric hindrance. In the preparation of IX, O-tert-butyl-l-tyrosine was cleanly acylated by the action of [2-(p-diphenylaminoisopropyl)phenyl carbonate; IX was obtained in 61% yield and could readily be converted into the crystalline N-hydroxysuccinimide ester derivative (X) in 66% yield. The coupling reaction between X and the crude free base VII proceeded smoothly and afforded the tripeptide derivative, N-2-(p-diphenylaminoisopropylxycarbonyl-O-tert-butyl-l-tyrosyl-S-trityl-l-cysteinyl-l-asparagine 2,4,6-trimethylbenzyl ester (XI) in 76% yield. Alternatively, XI could be prepared from the crystalline oxalate salt VIII and the active ester X by using 2 equiv of N-methylmorpholine. Although both preparations exhibited identical behavior on tlc and essentially the same melting point, the product obtained from 2 equiv of base showed a slightly lower specific rotation and hence subsequent preparations were conducted using VII. Removal of the N-DpOC group was accomplished using the conditions described by Sieber and Iselin. The free base XII was obtained as a ninhydrin-positive solid, homogeneous on tlc; cleavage over a 17-hr period gave better results than when shorter times were employed.

The choice of an amino protective group for asparagine-18 was complicated by the earlier observations of Sieber and Iselin concerning the DpOC derivative of L-asparagine. This derivative was obtained in rather low yield and exhibited low solubility in common solvents employed for coupling. Thus it appeared that o-nitrophenylisulfenyl-L-asparagine would provide better results despite the anticipated deblocking problems. N-0-Nitrophenyl-L-asparagine N-hydroxysuccinimide ester (XIV_a) was prepared by the procedure of Walter, et al., the coupling reaction between XII and XIV_a

proceeded readily in DME to provide N-o-nitrophenylsulfenyl-L-asparaginyl-O-tert-butyl-l-tyrosyl-S-trityl-L-cysteinyl-l-asparagine 2,4,6-trimethylbenzyl ester (XIIIa) in 88% yield.

Removal of the o-NPS group from XIIIa was studied rather carefully. Cleavage experiments in acetic acid, methanol-pyridine, or acetic acid-pyridine-DMF gave incomplete reaction. Treatment of XIIIa with thioglycolic acid in DMF gave no reaction; similar results were also obtained using o-nitrothiophenol. Complete cleavage was finally observed using exactly 1 equiv of o-nitrophenylsulfenyl chloride in the presence of β-mercaptethanol. This reagent generated 1 equiv of hydrogen chloride and produced the hydrochloride salt of the tetrapeptide XVIIa, in 91% crude yield. The product was homogeneous on tlc, and colored impurities due to S-trityl cleavage were not observed. The salt XVIIa was converted to the free base XVIIb and coupled with N-2-(p-diphenyl)isoproplyloxycarbonyl-γ-tert-butyl-l-glutamic acid N-hydroxysuccinimide ester (XVI), obtained in 83% yield from the corresponding acid XV. When the reaction was carried out on a small scale, a good yield of the pentapeptide N-2-(p-diphenyl)isoproplyloxycarbonyl-γ-tert-butyl-l-glutamyl-l-asparaginyl-O-tert-butyl-l-tyrosyl-S-trityl-l-cysteinyl-l-asparagine 2,4,6-trimethylbenzyl ester (XVIII) was obtained. However, when the conversion of the o-NPS peptide XIIIa to the hydrochloride salt XVIIa was performed on a large scale, the resulting salt XVIIa was not homogeneous and mobile colored impurities were detected on tlc. Since purification of XVIIa from this preparation was difficult, the use of the o-NPS group was abandoned in favor of the N-trityl group. N-Trityl-L-asparagine N-hydroxysuccinimide ester (XIVb) was prepared in 52% yield and was allowed to react with XII in dioxane solution. The coupling reaction appeared to proceed smoothly and N-trityl-L-asparaginyl-O-tert-butyl-l-tyrosyl-S-trityl-l-cysteinyl-l-asparagine 2,4,6-trimethylbenzyl ester (XIIIb) was obtained in 76% yield. Treatment of XIIIb with aqueous acetic acid at room temperature provided the free base XVIIIb in 78% crude yield. The product was homogeneous on tlc and was coupled directly with XVI without further purification. The pentapeptide XVIII was obtained in reasonable yield (80%) and high purity as indicated by tlc, elemental, and amino acid analysis. Thus, in subsequent experiments using larger quantities the route involving the N-trityl protective group proceeded smoothly and provided the crystalline hydrochloride of L-glutaminyl-L-leucine benzyl ester (XXII) in 92% yield. The dipeptide was then coupled with X to provide N-2-(p-diphenyl)isopropylloxycarbonyl-O-tert-l-tyrosyl-l-glutaminyl-l-leucine benzyl ester (XXII) in 97% yield. Treatment of either the methyl ester XXIII or the benzyl ester XXII with hydrazine provided the same hydrazide derivative, N-2-(p-diphenyl)isopropylloxycarbonyl-O-tert-l-tyrosyl-l-glutaminyl-l-leucine hydrazide (XXVI). The substance was obtained as a gel which could be solidified by crystallization from alcohol and was homogeneous on tlc.

Formation of the azide from the hydrazide XXVI was now considered. In order to avoid any cleavage of the DpOC group, our initial diazotization experiments utilized 1 equiv of hydrogen chloride in DMF. Treatment of the azide, generated by this method, with the free base of the pentapeptide XXVII, obtained by acetic acid cleavage of the DpOC group XVIIIa, gave low yields of the desired octapeptide derivative, N-2-(p-diphenyl)isopropylloxycarbonyl-O-tert-butyl-L-tyrosyl-L-glutaminyl-L-leucyl-γ-tert-butyl-L-glutamyl-l-asparaginyl-O-tert-butyl-l-tyrosyl-S-trityl-l-cysteinyl-l-asparagine 2,4,6-trimethylbenzyl ester (III). However, subsequent control experiments with XXVI established that the DpOC group was stable to excess hydrogen chloride in THF-DMF mixtures at low temperatures (−20 to −40°). The coupling between the azide, prepared by the Rudinger method,12 and the free base XXVII proceeded smoothly and provided good yields (75–85%) of the desired octapeptide III. The product was homogeneous on tlc and gave the expected elemental and amino acid analyses. Future experiments will deal with the formation of the fully blocked A chain and the combination of this material with an appropriate B chain.

**Experimental Section**

N-o-Nitrophenylsulfenyl-L-asparagine.—L-Asparagine (79.2 g, 0.6 mol) was dissolved in 750 ml of dioxane, cooled to 5°, and treated with 300 ml of 2 N sodium hydroxide solution. The clear solution was treated simultaneously with 126 g (10% excess) of nitrophenylsulfonyl chloride and 360 ml of cold 2 N sodium hydroxide solution. The pH of the solution was maintained at 9–10. Vigorous stirring was continued for 2 hr at room temperature and 600 ml of water was added, and the reaction mixture filtered. The filtrate was acidified with cold 2 N sodium hydroxide solution. The clear solution was washed with water to yield 154 g (92%) of yellow solid, mp 161-162°, homogeneous (system B) (lit.8 mp 165-166°). The substance was identified by mp, TLC, and amino acid analysis. Future experiments will deal with the formation of the fully blocked A chain and the combination of this material with an appropriate B chain.

ethylamine and 27.6 g (0.16 mol) of molten 2,4,6-trimethylbenzyl chloride. The clear solution was stirred 4 days at room temperature and diluted with cold 10% sodium bicarbonate solution. The crude product was filtered and washed with water; recrystallization from a cyclohexane–chloroform mixture provided 400.5 mg (82.8%) of white solid, homogeneous (system D), mp 173-174° (lit. 14 mp 173-174°).

L-Aspartic acid hydrochloride (IV) was prepared by the procedure of Stewart in 69% yield, homogeneous (system B), mp 198-199° (lit. 14 mp 194.5-195.5°).

N-2-(p-Diphenylyl)isopropoxycarbonyl-O-tert-buty1-L-tyrosyl-S-trityl-L-cysteinyl-L-asparagine (XIIIa).—A solution containing 4.52 g (0.0074 mol) of the N-succinimidyl ester was dissolved in 30 ml of DMAC. The stirrer was removed and the mixture was allowed to stand at room temperature for 1 hr. Evaporation of the filtrate provided a solid which was crystallized twice from isopropyl alcohol to yield 8.0 g (61.5%) of the protected tripeptide, mp 189-190°, homogeneous (system A), [α]D +2.08 (c 1.25, DMF).

Anal. Calcd for C2H4O3N5S: C, 69.92; H, 6.92; N, 5.24; S, 3.04. Found: C, 70.09; H, 6.92; N, 5.24; S, 3.04. The tripeptide XI could also be obtained from the oxalate salt VIII. To a solution of 6.99 g (0.01 mol) of VIII in 20 ml of DME at 0° was added 1.1 ml (0.02 mol) of N-methylmorpholine and 5.72 g (0.01 mol) of X. After 3 hr of stirring at 20° the oxalate salt of N-methylmorpholine was precipitated with water and the aqueous layer was extracted with chloroform. Evaporation of the solvent provided an oil which could be crystallized from methanol-water mixture (70:30) to yield 6.7 g (75%) of XI, mp 187-188°, [α]D +1.53 (c 1.25, DMF), homogeneous (system A).

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ether mixture to yield 12.0 g (65%) of the salt, mp 136–138°, [α]D +12.9° (c 1.7, methanol).

Anal. Caled for C14H25ON2S: C, 64.10; H, 7.87; N, 10.78; S, 6.46. Found: C, 64.17; H, 7.64; N, 10.58; S, 6.55.

N-2-(p-Diphenylamino)isopropylxyloxy carbonyl-L-tert-butyl-L-glutamyl-O-tert-butyl-L-tyrosyl-S-trityl-L-cysteinyl-L-asparagine 2,4,6-Trimethylbenzyl Ester (XVI).—A solution containing 1.0 g of the protected tetrapeptide in 80 ml of chloroform, containing 1 ml of β-mercaptoethanol, was stirred vigorously and treated with 0.668 g (3 mmol) of n-tert-butylsulfenyl chloride in 40 ml of chloroform, containing 0.5 ml of β-mercaptoethanol. The suspension was stirred 30 min at room temperature, filtered, and evaporated.

The residue was triturated with ether and the resulting hydrochloride salt (1.8 g), homogeneous in system A, was used directly in the following coupling reaction.

N-2-(p-Diphenylamino)isopropylxyloxy carbonyl-L-tert-butyl-L-glutamyl-O-tert-butyl-L-tyrosyl-S-trityl-L-cysteinyl-L-asparagine 2,4,6-Trimethylbenzyl Ester Hydrochloride Salt (XVII).—A suspension of 2.2 g (0.004 mol) of the salt XV was partitioned between a 10% aqueous citric acid solution and ethyl acetate at 0°. The layers were separated and the aqueous phase extracted again with ethyl acetate. The organic layers were washed with 10% citric acid solution, water, and brine. The dried extract was concentrated to a small volume and divided in 20 ml of 1,2-dimethoxyethane and treated with 1.30 g (0.012 mol) of N-hydroxy succinimide and 2.5 g (0.012 mol) of DCC at 0°. The mixture was stirred for 12 hr in an ice bath and stored overnight at 0°. Evaporation and filtration of the filtrate yielded an oil which crystallized on standing, dried with isopropyl alcohol, and extracted with 1.7 g (92%) of the active ester, mp 152–153°, [α]D +73.1° (c 1, methanol).

Anal. Caled for C34H45O8N8S: C, 57.82; H, 6.01; N, 11.16; S, 5.46. Found: C, 57.82; H, 6.07; N, 11.07; S, 5.40.

N-2-O-Nitrophenylsulfenyl-L-glutamyl-N-2-tert-butyl-L-tyrosyl-S-trityl-L-cysteinyl-L-asparaginyl 2,4,6-Trimethylbenzyl Ester (XXV).—A solution containing 0.25 ml of N,N-dimethylmorpholine was stirred vigorously and treated with 0.568 g (0.012 mol) of N-o-nitrophenyl-sulfenyl-L-glutaminyl-2,4,6-Trimethylbenzyl Ester (XX).—The blocked pentapeptide XVIIa (2.3 g, 1.68 mmol) was dissolved in 20 ml of glacial acetic acid and after 24 hr diluted with 3 ml of water. The reaction mixture was stirred 3 hr at room temperature, and diluted with 200 ml of brine; the resulting semisolid was isolated by decantation. The product was dissolved in chloroform, washed with 2 N sodium bicarbonate and water, and dried. Evaporation and crystallization of the solid with ether provided 1.8 g (95%) of the crude deblocked pentapeptide. The crude solid was used directly in the subsequent coupling reaction with the azide generated from XXV.

N-2-O-Nitrophenylsulfenyl-L-glutamyl-N-2-tert-butyl-L-tyrosyl-S-trityl-L-cysteinyl-L-asparaginyl 2,4,6-Trimethylbenzyl Ester (XXVI).—A solution containing 7.92 g (0.02 mol) of the active ester and 7.86 g (0.02 mol) of L-tyrosyl benzyl ester p-toluene sulfonate salt in 20 ml of 1,2-dimethoxyethane was treated with 2.2 g (0.01 mol) of N-2-o-nitrophenyl-sulfenyl-L-glutaminyl-2,4,6-Trimethylbenzyl Ester (XXV). The reaction mixture was stirred 3 hr at room temperature, and diluted with 200 ml of isopropyl alcohol. Recrystallization of the solid from isopropyl alcohol and acetic acid in 3.2 g (79%) of the active ester, mp 148–149°, [α]D +55.6° (c 2.0, DMF). [lit. 148°–149°].

N-2-O-Nitrophenylsulfenyl-L-glutamyl-L-leucine Benzyl Ester (XXII).—A solution of the active ester (1.8 g, 0.003 mol) of N-2-o-nitrophenyl-sulfenyl-L-glutaminyl-2,4,6-Trimethylbenzyl Ester (XXV) in 20 ml of DMF was treated with 2.2 g (0.01 mol) of N-2-o-nitrophenyl-sulfenyl-L-glutaminyl-2,4,6-Trimethylbenzyl Ester (XXV) in 20 ml of DMF, and treated with 2.99 g (0.01 mol) of N-2-o-nitrophenyl-sulfenyl-L-glutaminyl-2,4,6-Trimethylbenzyl Ester (XXV). The reaction mixture was stirred 3 hr at room temperature, and diluted with 200 ml of isopropyl alcohol. Recrystallization of the solid from isopropyl alcohol and acetic acid in 3.2 g (79%) of the active ester, mp 148–149°, [α]D +55.6° (c 2.0, DMF) (lit. 148°–149°).
solution cooled to yield the diketopiperazine derivative, 0.73 g absolute methanol. The reaction mixture was stirred for 3 min.

XXIV in 5 ml of MeOH was treated with 2.5 ml of 4 M HCl in methanol and treated with 1.3 ml of hydrazine monohydrate at 50° for 1 hr and an additional 2 hr at 30°. Dilution with ether afforded 0.67 g of the octapeptide derivative which on recrystallization from methanol-chloroform. The reaction mixture was diluted with ice water and saturated with sodium chloride. The separated product was washed with water, dried, and triturated with ether. A chloroform solution of the octapeptide derivative was applied to a silica gel column and eluted with chloroform-methanol (98:2). The product was collected and recrystallized from chloroform-petroleum ether to yield 1.2 g (82%) of white solid, [α]D 0° +6.35 (c 2, DMF).

The amino acid analysis of a performic acid oxidized, acid hydrolyzate was Asp2, CysSO3H, Glu3, Leu1. The amino acid analysis of an acid hydrolyzate in the presence of phenol was Asp2, Glu5, Leu1, Tyr3.

Registry No.—III, 33608-46-7; VI, 30506-18-9; VIII, 30506-19-0; IX, 33522-10-4; X, 33527-03-6; XI, 33527-04-7; XII, 33527-05-8; XIIIa, 33608-48-9; XIIIb, 33527-06-9; XIVa, 21753-83-3; XIVb, 33527-08-1; XV, 25461-15-8; XVI, 33527-10-5; XVIII, 33527-11-6; XX, 33527-12-7; XXI, 33527-13-8; XXII, 33527-14-9; XXIII, 33527-15-0; XXIV, 33527-16-1; XXV diketopiperazine, 33527-17-2; XXVI, 33527-18-3.