Sulfur-Containing Polypeptides. XVI. Synthesis of the A_{14-21} Fragment of Ovine Insulin

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The protected octapeptide N-2-(p-diphenylisopropyl)isopropylxycarbonyl-0-2-tert-butyl-L-tyrosyl-L-glutamyl-L-leucyl-γ-tert-butyl-L-glutamyl-L-asparaginyi-O-tert-butyl-γ-tyrosyl-S-trityl-L-cysteinyl-I-0-tert-butyl-L-asparagine, 2,4,6-trimethylbenzyl ester (III) has been synthesized. The route involves the use of the N-2-(p-diphenyl)isopropylxycarbonyl (DpOC) group as the principle amino protective group and N-hydroxysuccinimide and azide coupling methods.

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

The preparation of the A_{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 acidity could be utilized. The protective group of choice for the phenolic hydroxyl groups at A_{14,19} was the tert-butyl ether; the S-trityl group was required for the A_{20} cysteine residue to permit selective formation of the two interchain disulfide bonds at A_{7}B_{7} and A_{20}B_{20}.

The tert-butyl ester seemed to be suitable for the A_{19} 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 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-butyl ethers and esters generally cleave at comparable rates1 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-
nitrophenylsulfenyl (o-NPS), and the N-2-(p-diphenyl-yl)isoproproyloxy carbonyl (DpOC) groups. The synthetic goal was then the fully protected octapeptide derivative III; the route finally adopted is shown in Scheme I.

### Scheme I

#### SYNTHESIS OF THE PROTECTED AMINO PEPTIDE DERIVATIVE

- **Bu**
- **OHBu**
- **HOBu**
- **R**
- **Tr**
- **DpOC-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn-OTMB

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<thead>
<tr>
<th>Step</th>
<th>Reaction</th>
<th>Product</th>
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L-Asparagine 2,4,6-trimethylbenzyl ester hydrochloride (IV) was coupled via the N-hydroxysuccinimide method to tert-butylloxycarbonyl-S-trityl-L-cysteinyl-L-asparagine (V). The DpOC group of the dipeptide VI was subsequently removed by the action of boron trifluoride in acetic acid, and S-trityl-L-cysteinyl-L-asparagine derivative (X) 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 protection 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 characterization 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 Tyr19, 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-diphenyl-yl)isoproproyloxy carbonyl-O-tert-butyl-L-tyrosine dicyclohexylamine 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 terminus10 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-diphenyl)isopropyl]phenyl carbonate; IX was obtained in 61% yield and could readily be converted into the crystalline N-hydroxysuccinimide ester derivative (X) in 69% yield. The coupling reaction between X and the crude free base VII proceeded smoothly and afforded the tripeptide derivative, N-2-(p-diphenyl-yl)isoproproyloxy carbonyl-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-nitrophenylsulfenyl-L-asparagine would provide better results despite the anticipated deblocking problems. N-o-Nitrophenyl-L-asparagine N-hydroxysuccinimide ester (XIVa) was prepared by the procedure of Walter, et al.,11 the coupling reaction between XII and XIVa

![Diagram](image_url)
proceeded readily in DME to provide N-o-nitrophenylsulfenyl-L-asparaginyl-O-tert-butylic-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-nitrosothiophenol. Complete cleavage was finally observed using exactly 1 equiv of o-nitrophenylsulfenyl chloride in the presence of β-mercaptoethanol. 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 tlc of XVIIa was converted to the free base XVIIb and coupled with N-2-(p-diphenyl)isopropylxycarbonyl-γ-tert-Bu-L-glutamic acid N-hydroxy succinimide ester (XVI), obtained in 83% yield. When the reaction was carried out on a small scale, a good yield of the pentapeptide N-2-(p-diphenyl)isopropylxycarbonyl-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 N-trityl-L-asparagine has been the method of choice.

At this point a second fragment corresponding to the A14-18 portion of III was prepared and coupled to the free base XXVII by the azide method. Initially N-2-(p-diphenyl)isopropylxycarbonyl-O-tert-butyl-L-tyrosyl-L-glutamyl-L-tyrosyl-L-leucine methyl ester (XXII) was prepared from the N-hydroxysuccinimide ester (X) and N-o-nitrophenylsulfenyl-L-glutaminyl-L-leucine methyl ester (XXIV). Although the resulting tripeptide was obtained in fair yield and pure condition, the procedure was complicated by the hygroscopic nature of the dipeptide hydrochloride XXVII resulting from the removal of the o-NPS group from XXIV with hydrogen chloride. Attempts to obtain a crystalline free base invariably led to diketopiperazine formation. More satisfactory results were obtained using the corresponding benzyl ester. Treatment of L-leucine benzyl ester p-toluencesulfonate salt with N-o-nitrophenylsulfenyl-L-glutamine N-hydroxysuccinimide ester provided N-o-nitrophenylsulfenyl-L-glutaminyl-L-leucine benzyl ester (XX) in 80% yield. Removal of the amino protective group proceeded smoothly and provided the crystalline hydrochloride of L-glutaminyl-L-leucine benzyl ester (XXI) in 92% yield. The dipeptide was then coupled with X to provide N-2-(p-dimethoxy)isopropylxycarbonyl-O-tert-butyl-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-dimethoxy)isopropylxycarbonyl-O-tert-butyl-L-tyrosyl-L-glutaminyl-L-leucine benzyl ester (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 XVII, gave low yields of the desired octapeptide derivative, N-2-(p-dimethoxy)isopropylxycarbonyl-O-tert-butyl-L-tyrosyl-L-glutaminyl-L-leucyl-γ-tert-butyl-L-glutaminyl-L-asparaginyl-O-tert-butyl-L-tyrosyl-S-trityl-L-cysteinyl-L-asparagine 2,4,6-trimethylbenzyl ester (III). However, subsequent control experiments with XXXI 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, 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 N-o-Nitrophenylsulfenyl chloride and 360 ml of cold 2 N sodium hydroxide solution. The clear solution of the free base material washed with water to yield 154 g (92%) of yellow solid, mp 161-162°, homogeneous (system B) (lit. mp 165-166°). Vigorous stirring was continued for 2 hr at room temperature and the pH of the solution was maintained at 9-10. Nitrophenylsulfenyl chloride and 360 ml of cold 2 N sodium hydroxide solution. The clear solution was treated with 300 ml of 2 N sodium hydroxide solution. The clear solution was transferred to a separating funnel and extracted with 300 ml of diethyl ether. The ether layer was washed with water and dried over sodium sulfate; the ether was removed in vacuo. The residue was purified by column chromatography (tic) conducted on silica gel GF254. The following solvent systems were employed: (A) chloroform-methanol (9:1); (B) chloroform-methanol-acetic acid (8:1:1); (C) 1-butanol-acetic acid-water (10:1:3); (D) 1-heptanethiol-butyric acid-acetic acid-water-pyridine (25:70:6:24:20); (E) ace-butyl alcohol-3% ammonium hydroxide (7:8). A waste bottle containing a mixture of the products (triplet) was dried in vacuo over phosphorus pentoxide and sodium hydroxide pellets.


(13) Melting points are uncorrected. Combustion analyses were performed in the Micro-Tech Laboratories, Skokie, Ill. Amino acid analyses were performed on a Beckman Model 116 amino acid analyzer and have not been corrected for destruction during hydrolysis. Thin layer chromatography (tlc) was conducted on silica gel GF254 with the following solvent systems: (A) chloroform-methanol (9:1); (B) chloroform-methanol-acetic acid (8:1:1); (C) 1-butanol-acetic acid-water (10:1:3); (D) 1-heptanethiol-butyric acid-acetic acid-water-pyridine (25:70:6:24:20); (E) ace-butyl alcohol-3% ammonium hydroxide (7:8).
ethylamine and 27.6 g (0.16 mol) of molten 2,4,6-trimethylbenzoyl chloride. The clear solution was stirred 4 days at room temperature and diluted with cold 10% sodium bicarbonate solution. The crude product was washed with three washings of 500 ml each with 10% sodium bicarbonate solution and 10% acetic acid mixture to yield 12.0 g (84.6%) of white solid, mp 179–180°,[α]D +20.6° (c 1.4, methanol).

Anal. Calcd for C60H68N2O8S: C, 73.07; H, 6.69; N, 5.24; S, 3.15. Found: C, 70.90; H, 6.92; N, 5.84; S, 3.21.


Anal. Calcd for C60H68N2O8S: C, 73.07; H, 6.69; N, 5.24; S, 3.15. Found: C, 70.90; H, 6.92; N, 5.84; S, 3.21.

ether mixture to yield 12.0 g (65% of the salt, mp 136–138°, 
\[\alpha]_D^20 +12.9^\circ (c 1.7, methanol). 

**Anal.** Caled for C\textsubscript{16}H\textsubscript{18}O\textsubscript{3}N\textsubscript{2}S: C, 57.82; H, 6.01; S, 5.04. 

Found: C, 57.8; H, 6.1; S, 5.04. 

**N-2-(p-Diphenyl)isopropoxyoxycarbonyl-L-tert-butyl-L-glutamyl-L-asparaginyl-0- tert-butyl-L-tyrosyl-S-trityl-L-cysteyl-L-asparaginyl-2,4,6-Trimethylbenzyl Ester (XV).—A solution containing 0.87 g (80% of the crude product, mp 148–149°, \[\alpha]_D^20 +54.6^\circ (c 1, methanol). 

**Anal.** Caled for C\textsubscript{16}H\textsubscript{18}O\textsubscript{3}N\textsubscript{2}S: C, 57.82; H, 6.01; S, 5.04. 

Found: C, 57.8; H, 6.1; S, 5.04. 

**Amino acid analysis after performic acid oxidation and acid hydrolysis showed Asp\textsubscript{m}Cys\textsubscript{m}O\textsubscript{m}H\textsubscript{m}Glu\textsubscript{m}. Amino acid analysis of an acid hydrolysate in the presence of phenol showed Asp\textsubscript{m}Glu\textsubscript{m} -Tyri.o. (16) L. Zervas and D. M. Theodoropoulos, J. Amer. Chem. Soc., 78, 1339 (1956). 

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solution cooled to yield the diketopiperazine derivative, 0.73 g absolute methanol. The reaction mixture was stirred for 3 min

N XXIV in 5 ml of MeOH was treated with 2.5 ml of 40 3 extracted with chloroform and washed with 10% NaHC03 solution, and extracted with sulfuric acid, and H2O. Evaporation of the solvent gave a crude product which on recrystallization from methanol–ether yielded 2.5 g (73%) of product. mp 124–125°, [a]+6.3° (c 2, DMF), homogeneous (system A).

Anal. Calcd for C18H2606N4: C, 50.70; H, 6.10; N, 13.14; S, 7.60. Found: C, 50.48; H, 6.02; N, 13.03; S, 7.47.

Diketopiperazine of XXIV.—A solution of 2.13 g (0.005 mol) of XXIV in 5 ml of MeOH was treated with 2.5 ml of 4 N HCl in absolute methanol. The reaction mixture was stirred for 5 min and diluted with ether. The clear solution was decanted, and the remaining oil on trituration with ether provided a white hygroscopic solid. The semisolid was dissolved in 5 ml of H2O, neutralized with saturated NaHCO3 solution, and extracted with chloroform. The chloroform was removed and the bicarbonate solution cooled to yield the diketopiperazine derivative, 0.73 g (44%), mp 239–240° (recrystallized from methanol–chloroform).


The reaction mixture was diluted with water and the product was washed with ether and recrystallized from a methanol–chloroform mixture to yield 0.62 g (85%) of solid, mp 183–184°,

Registry No.—III, 33608-46-7; VI, 30806-18-9; VIII, 30806-19-0; IX, 33552-10-4; X, 33552-03-6; XI, 33567-04-7; XII, 33567-05-8; XIIIa, 33608-48-9; XIIIb, 33527-06-9; XIVa, 21753-83-3; XIVb, 33527-08-1; XV, 25461-15-8; XVI, 33527-10-5; XVII, 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.