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

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The protected octapeptide \( N\)-\( 2\)-(p-diphenyloxy)isopropyl-\( O\)-\( \alpha\)-butyl-L-tyrosyl-L-glutaminy-L-leucyly-L-tyrosyl-L-asparaginyl-L-cysteinyl-L-tyrosyl-3-asparaginyl-2,4,6-trimethylbenzyl ester (III) has been synthesized. The route involves the use of the \( N\)-2-(p-diphenyloxyl)isopropyl-oxycarbonyl (DpOC) group as the principle amino protective group and \( N\)-2-hydroxysuccinimide and azide coupling methods.

In the accompanying report\textsuperscript{1} a synthetic route to a suitably blocked peptide containing the \( A_{4-11} \) 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-diphenyloxy)isopropyl-oxycarbonyl protective group developed by Sieber and Iselin\textsuperscript{4} for the elegant synthesis of thyrocalcitonin.\textsuperscript{5}

\[ \text{t-BOC-Cys-Cys-Ala-Gly-Val-Cys-Ser-Leu-OH} \]

<table>
<thead>
<tr>
<th>6</th>
<th>13</th>
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<tbody>
<tr>
<td>I</td>
<td>Bu</td>
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<tr>
<td>H-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn-OH</td>
<td>14</td>
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(2) Supported by Grant AM-03416 from the Institutes of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.

(3) The following abbreviations have been employed in the text: t-BOC = tert-butyloxycarbonyl; DpOC = 2-(p-diphenyloxy)isopropyl-oxycarbonyl; o-NPS = \( o\)-nitrophenylsulfenyl; \( \alpha\)-Bu = \( \alpha\)-butyl; TMB = 2,4,6-trimethylbenzyl; Tr = trityl; Bzl = benzyl, Su = \( N\)-hydroxysuccinimide; DCC = \( N\),\( N\)'-dicyclohexylcarbodiimide; DMF = \( N\),\( N\)'-dimethylformamide; DMAc = \( N\),\( N\)'-dimethylacetamide.

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nitrophenylsulfonyl\(^{6}\) (o-NPS), and the \(N\)-2-(p-diphenyl-yl)isopropylxycarbonyl\(^{6}\) (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 \(A_{8}-E\) PEPTIDE DERIVATIVE**

\[
\begin{array}{cccccc}
\text{Bu} & \text{O} & \text{Bu} & \text{Bu} & \text{Tr} \\
\text{DpOC-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn-OTMB} & 14 & 15 & 16 & 17 & 18 & 19 & 20 & 21 \\
\text{III} \\
\end{array}
\]

L-Asparagine 2,4,6-trimethylbenzyl ester hydrochloride (IV) was coupled via the \(N\)-hydroxysuccinimide method to tert-butylxycarbonyl-S-trityl-l-cysteine dicyclohexylamylate (V). The \(\alpha\)-BOC group of the dipeptide VI was subsequently removed by the action of boron trifluoride in acetic acid, and \(S\)-trityl-l-cysteinyll-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 \(Tyr_{10}\), clearly either the \(\alpha\)-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-diphenylylisopropylxycarbonyl-\(O\)-tert-butyl-\(l\)-tyrosine dicyclohexylamylate salt (IX), this group was preferable to the \(\alpha\)-NPS group since \(S\)-trityl cleavage can sometimes occur when the \(\alpha\)-NPS group of an \(S\)-trityl-\(l\)-cysteine peptide is removed from the amino terminus\(^{10}\) 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-diphenylylisopropyl)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-diphenylylisopropylxycarbonyl-\(O\)-tert-butyl-\(l\)-tyrosyl-\(S\)-trityl-l-cysteinyll-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 tic 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.\(^ {6}\) The free base XII was obtained as a ninhydrin-positive solid, homogeneous on tic; 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 \(\alpha\)-nitrophenylsulfonyl-L-asparagine would provide better results despite the anticipated deblocking problems. \(N\)-\(\alpha\)-Nitrophenyl-1-asparagine \(N\)-hydroxysuccinimide ester (XIVa) was prepared by the procedure of Walter, et al.,\(^ {11}\) the coupling reaction between XII and XIVa.


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 thiglycic acid in DMF gave no reaction; similar results were also obtained using o-nitroethionol. 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 salt XVIIa was converted to the free base XVIIb and coupled with N-2-(p-diphenyl)isopropylxoycarbonyl-L-γ-tert-butyl-L-γ-glutamyl-L-asparginyl-O-tert-butyl-L-tyrosyl-S-trityl-L-cysteinyl-L-asparagine 2,4,6-trimethylbenzyl 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)isopropylxoycarbonyl-L-γ-tert-butyl-L-γ-glutamyl-L-asparginyl-O-tert-butyl-L-tyrosyl-S-trityl-L-cysteinyl-L-asparagine 2,4,6-trimethylbenzyl ester (XVII) 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. After 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-γ-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 XVIIb 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 installation of the azide, generated by this method, with the free base of the pentapeptide XVII, obtained by acetic acid 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 XVII, obtained by acetic acid cleavage of the DpOC group XVIIa, gave low yields of the desired octapeptide derivative, N-2-(p-diphenyl)isopropylxoycarbonyl-L-γ-tert-butyl-L-γ-tyrosyl-L-glutaminyl-L-leucyl-γ-tert-butyl-L-glutamyl-L-asparaginyl-O-tert-butyl-L-γ-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°C). 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°C, and treated with 300 ml of 2 N sodium hydroxide solution. The clear solution was treated simultaneously with 126 g (10% excess) of nitrophenylsulfenyl chloride and 300 ml of cold 2 N sodium hydroxide. 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 sulfuric acid and the product washed with water to yield 154 g (92%) of yellow solid, mp 161–162°C, homogeneous (system B) (lit.8 mp 165–166°C). N-o-Nitrophenylsulfenyl-L-asparagine 2,4,6-Trimethylbenzyl Ester.—A solution of 46 g (0.16 mol) of N-o-nitrophenylsulfenyl-L-asparagine in 80 ml of DMF was treated with 22.5 ml of tri-
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; recrystallisation from a cyclohexane–chloroform mixture provided 40.96 g (97.9%) of the tetrapeptide, mp 204-206°, homogeneous (system A), [α]D +20.6° (c 1.1, methanol).

N-2-(p-Diphenylyl)isopropoxy carbonyl-O-tert-buty1-L-tyrosyl-S-trityl-L-cysteiny1-L-asparagine 2,4,6-Trimethylbenzyl Ester (XII)—A solution containing 22.1 g (0.03 mol) of the S-trityl dicyclohexylamine salt (VII) in 40 ml of DMAc was dissolved in 20 ml of DME at 0° and treated with 1.1 ml (0.02 mol) of N-methylmorpholine and 5.72 g (0.01 mol) of DCC. 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 crystallised from a methanol–water mixture providing 3.87 g (77.5%) of the protected tripeptide, mp 189-190°, homogeneous (system A), [α]D +2.28° (c 1.25, DMF).

Anal. Calcd for C38H60N6O6S: C, 69.49; H, 6.69; N, 5.24; S, 3.00. Found: C, 69.83; H, 6.52; N, 5.24; S, 3.03.

N-2-(p-Diphenylyl)isopropoxy carbonyl-O-tert-buty1-L-tyrosyl-S-trityl-L-cysteiny1-L-asparagine 2,4,6-Trimethylbenzyl Ester (XIII)—A solution containing 6.32 g (0.026 mol) of the free base XII in 60 ml of 1,2-dimethoxyethane was treated, at room temperature, with 2.46 g (0.028 mol) of N-hydroxy succinimide ester. The tripeptide XI could also be obtained from the oxalate salt VII. To a solution of 6.99 g (0.01 mol) of VII 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 DCC 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 crystallised from a methanol–water mixture providing 6.0 g (77.5%) of XI, mp 187-188°, [α]D +1.53° (c 1.25, DMF), homogeneous (system A).
ether mixture to yield 12.0 g (65%) of the salt, mp 136-138", [a]D +12.9° (c 1.7, methanol).
Anal. Calcd for C6H9NO3S: C, 50.71; H, 5.30; N, 8.89. Found: C, 50.79; H, 5.47; N, 9.07.
N-Tryptyl-L-asparagine N-Hydroxy succinimide Ester (XIVb).—A solution containing 2.49 g (0.003 mol) of the free base XIV in 25 ml of dioxane was stirred at room temperature with 1.88 g (0.0077 mol) of N-hydroxysuccinimide and 1.6 g (0.0077 mol) of DCC. The solution was still stirred for 4 hr at 10° and was allowed to stand at 4° overnight. The dicyclohexylurea was filtered and washed with cold dioxane. The filtrate was concentrated in vacuo and the residue recrystallized from ethyl acetate-n-hexane provided 1.7 g (59%) of the active ester, mp 152-153°, [a]D +24.9° (c 1.55, dioxane).
Anal. Calcd for C6H9NO3S: C, 68.64; H, 6.92; N, 7.05; S, 2.09.

N-Trimethyl-L-asparaginyl-0-tert-buty-L-tyrosyl-S-trityl-L-cysteinyl-S-trityl-L-glutaminyl-L-leucine Benzyl Ester (XXI).—A solution of 5.5 g (0.011 mol) of the dipeptide derivative (XXI) in 20 ml of DMAc was treated at 0° with 1.9 g (0.004 mol) of N-phenylsulfonyl-L-glutaminyl-L-leucine benzyl ester (XXVII). The mixture was stirred 3 hr at room temperature 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 20 ml of glacial acetic acid and after 24 hr diluted with 2.2 ml of water, and treated with 2.2 ml of N-phenylsulfonyl chloride in 40 ml of chloroform. The reaction mixture was filtered and the product evaporated. Evaporation of the filtrate yielded an oil which crystallized on trituration with cold dioxane. The solid was washed with water and then tritured with ether to yield a gelatinous solid, 2.5 g (93%), mp 160° (recrystallized from chloroform-n-hexane), homogeneous (system A), [a]D +7.3° (c 1, methanol).
Anal. Calcd for C30H33ClNO9S: C, 57.82; H, 6.01; N, 11.16; S, 6.38. Found: C, 57.82; H, 6.07; N, 10.78; S, 6.34.
L-Glutaminyl-L-leucine Benzyl Ester Hydrochloride Salt (XXII).—A solution containing 7.92 g (0.02 mol) of the active ester and 7.86 g (0.02 mol) of L-leucine benzyl ester p-toluene sulfonate salt in 20 ml of 1,2-dimethoxyethane was stirred for 60 min at 0°, filtered, and treated with 2.2 ml of N-methylmorpholine. The reaction mixture was stirred overnight at room temperature, the solvent removed, and the residue dissolved in ethanol. The reaction mixture was washed with water, 10% sodium bicarbonate, and water. Removal of the solvent and trituration of the residue with cold ether provided 8.1 g (80.5%) of product, mp 110-120°, [a]D +54.6° (c 1, methanol).
Anal. Calcd for C24H30ClNO8S: C, 57.82; H, 6.01; N, 11.16; S, 6.38. Found: C, 57.82; H, 6.07; N, 10.78; S, 6.34.
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
chloroform. The chloroform was removed and the bicarbonate
semisolid dissolved in 5 ml of H20, the remaining oil on trituration with ether provided a white
evaporation of the solvent gave a crude product which on recrystallization from methanol-ether yielded 2.5 g (73%) of product, mp 124—125°C, [α]D +8.35 (c 2, DMF), homogeneous (system A).

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°C (recrystallized from methanol-chloroform).
Anal. Caled for C10H14N2O4: C, 54.77; H, 7.88; N, 17.49. Found: C, 54.43; H, 7.87; N, 17.45.

N-2-(p-Diphenylisopropylcarbonyl-O-tet-butyl-L-tyrosyl-L-glutaminyl-L-leucine Methyl Ester (XXVI).—The tripeptide benzyl ester XXII (0.8 g, 1.0 mmol) was dissolved in 10 ml of dry methanol and treated with 1.3 ml of hydrazine monohydrate (90%). The solution was stirred for 5 days at room temperature and diluted with ether and the resulting solid collected. The product was washed with ether and recrystallized from methanol-ether to yield 0.67 g (85%) of solid, mp 183—184°C (recrystallized from methanol-chloroform).
Found: C, 65.73; H, 7.45; N, 11.50. Found: C, 65.27; H, 7.30; N, 11.31.

N-2-(p-Diphenylisopropylcarbonyl-O-tet-butyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-tet-butyl-L-glutamyl-L- asparaginyl-O-tet-butyl-L-tyrosyl-L-tryptophyl-L-cysteinyl-L-asparagine 2,4,6-Tri-nitrophenylhydrazine (XXVI).—A solution of the hydrazide (0.6 g, 0.81 mmol) in 30 ml of DMF was cooled to —20°C and treated with 2.2 ml of 3 N hydrogen chloride in tetrahydrofuran solution. The temperature was lowered to —40°C and 0.11 ml of n-butyl nitrite was added dropwise. The reaction mixture was stirred at —20 to —25°C for 40 min, cooled to —60°C, and treated with 0.8 ml of N-methylmorpholine. The solution of the azide at —40°C was treated with a precooled (—40°C) solution of the penta-
peptide (0.9 g, 0.8 mmol) in 10 ml of DMF. The stirring was continued for 1 hr at —30 to —20°C and in an ice bath (0—2°C) for 3.5 days.
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 —13.2° (c 0.5, DMF).
Anal. Caled for C40H54O7N6: C, 65.73; H, 7.45; N, 11.50.
Anal. Caled for C40H54O7N6: C, 65.73; H, 7.45; N, 11.50.

Registry No.—III, 33608-46-7; VI, 30068-18-9; VIII, 30068-19-0; IX, 33527-10-4; 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.