Synthesis of the A_{14-21} Sequence of Ovine Insulin by the Solid-Phase Technique[1]

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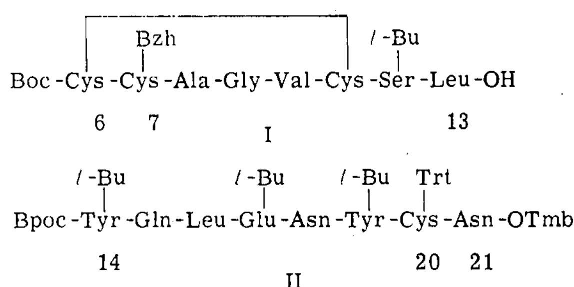
An improved synthesis of the *tert*-alkyloxycarbonylhydrazide resin (IX) is given. The resin has been used as the support in the solid-phase synthesis of an N-protected hexapeptide hydrazide (XIX) comprising the A_{14-19} fragment of insulin. Incorporation of the subsequent amino acid residues as their *N*-2-(*p*-biphenylyl)isopropyloxycarbonyl derivatives enabled us to determine the incorporation percentage in each coupling step and to find optimum conditions for the condensation reactions. Dicyclohexylcarbodiimide–*N*-hydroxybenzotriazole was used as the condensing agent for the coupling of asparagine and glutamine residues. Residual free amino groups were blocked by formylation. The N-terminal amino group was temporarily protected by the newly developed 2-(methylsulfonyl)ethyloxycarbonyl group. After cleavage from the resin the product has been coupled with *S*-trityl-L-cysteinyl-L-asparagine by the azide method, resulting in the formation of an *S*-trityl derivative of the C-terminal A_{14-21} sequence of insulin.

Although several useful laboratory syntheses of the A and B chains of insulin have been described,[2] the problem of the specific introduction of the four disulfide bonds has not yet been solved. An approach to overcome this problem has been described by Hiskey,[3] who demonstrated that selective bridging of *S*-trityl thioethers in the presence of an *S*-benzhydryl thioether is possible with thiocyanogen as the oxidizing agent. This method has been used[4] in the synthesis of a suitably protected octapeptide (I) comprising the A_{6-13} sequence of ovine insulin, in which the cysteinyl residue at A_7 is present as its *S*-benzhydryl derivative.



This technique can be applied to the ultimate bridging of the complete A and B chains of insulin. The peptide I has then to be incorporated in an A chain containing *S*-tritylcysteine at A_{20}. The synthesis of a proper A_{14-21} fragment (II), suitable for the desired extension of I at the C-terminal end, has already been described.[5]

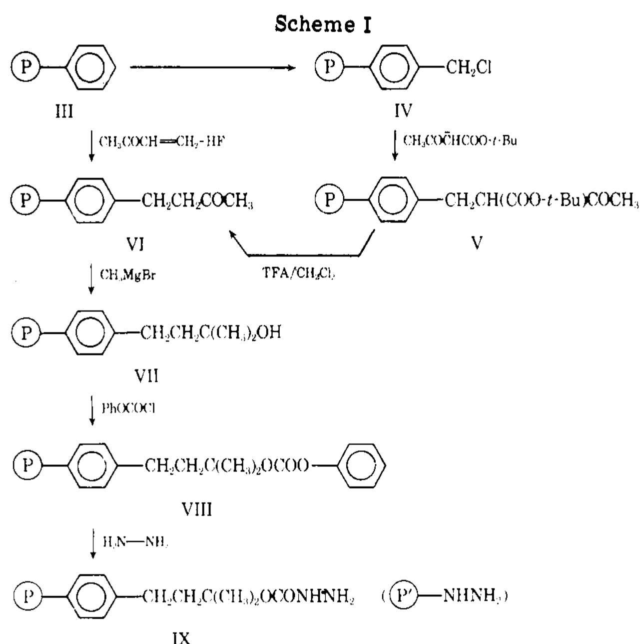
In view of this promising progress toward a useful synthesis of the complete insulin molecule we started investigations on the *solid-phase* synthesis of fragments of the A chain, fitting into the given overall strategy. The present paper concerns the development of a new synthesis of the A_{14-21} fragment which has largely been done on a solid support.

Since it was found in orienting experiments that acylation of free amine functions in solid-phase synthesis with *S*-trityl cysteine derivatives is always far from quantitative,[6] the synthesis of the desired octapeptide was conducted in two stages: a solid-phase synthesis of the hydrazide of an N-protected A_{14-19} sequence, followed by a condensation of the corresponding azide with *S*-tritylcysteinylasparagine in solution.

The obvious support for this investigation was the *tert*-alkyloxycarbonylhydrazide resin (IX), introduced by Merrifield and Wang.[7] The mild conditions needed to cleave a peptide from this resin facilitate isolation of a partly protected peptide hydrazide, suitable for further fragment condensations.

A. Synthesis of the *tert*-Alkyloxycarbonylhydrazide Resin. An attempted preparation[7] of the resin was unsuccessful. Direct substitution of copolystyrene–divinylbenzene (III) with methyl vinyl ketone did not occur when the recommended HF was used, nor when other Friedel–Crafts catalysts ($AlCl_3$, $SnCl_4$, $BF_3 \cdot OEt_2$, H_2SO_4) were applied. This may have been due to the particular batch of the copolymer used. The resin VI could, however, be readily obtained by the substitution of an acetoacetate residue for chlorine in the chloromethyl resin (IV), followed by acid hydrolysis and decarboxylation with 50% TFA–CH_2Cl_2 (Scheme I). The intermediate V was completely free from chlorine and could be well characterized by ir spectroscopy. The product VI had an ir spectrum identical with that given by Merrifield.[7]

Scheme I



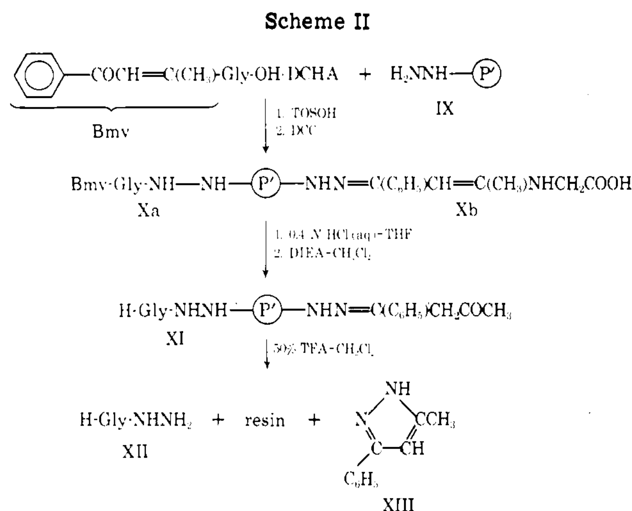
The further synthesis of IX proceeded smoothly as described in the literature and reproduced in Scheme I. From the nitrogen content it was calculated that the overall yield over five reaction steps was 71%.

The new route includes two important improvements: the percentage of anchoring side chains in the hydrazide resin can be controlled by the extent of chloromethylation in the synthesis of IV, and the use of the hazardous HF can be avoided.

B. Synthesis of the Hydrazide of an N-Protected A_{14-19} Fragment. Peptide syntheses on the resin IX can only be done with amino acid derivatives containing N_α-protecting groups which are more acid labile than the tertiary carbazate link between the peptide and the resin. Possible N-protecting functions seemed to be the Bpoc group[8] and the Bmv group[9] because of their high acid lability. Use of either group also permits measurement of the effectiveness of each coupling step. Cleavage of the Bpoc group with 0.5% TFA–CH_2Cl_2 proceeds quantitatively, yielding apart from carbon dioxide 2-(*p*-biphenylyl)propene and a trace of 2-(*p*-biphenylyl)propanol-2. Removal of the Bmv group under acidic conditions (0.4 *N* HCl in aqueous THF) gives benzoylacetone quantitatively. In both cases the amount of the cleavage products can be determined by uv spectrophotometry.

The Bmv group cannot be used, however, in the coupling of the first amino acid with IX. In a preliminary experiment with Bmv-glycine (Scheme II) it appeared that the Bmv amino acid is not only bound *via* the carboxyl group as a hydrazide (Xa) but also as a hydrazone by condensation of the hydrazide groups on the resin with the carbonyl function in the Bmv group (Xb). Subsequent cleavage of the Bmv group and liberation of the amino acid from the resin yielded apart from the expected glycylhydrazide (XII) substantial amounts of a side product which appeared to be 5-methyl-3-phenylpyrazole (XIII) by comparison with an authentic sample.

Scheme II



The Bpoc group appeared very attractive for N_α protection. It is stable under the experimental conditions of the synthetic procedures, and the conditions for its removal are milder than for the cleavage of Bmv residues.

Before cleavage of the A_{14-19} fragment from the resin the N-terminal Bpoc group was exchanged for the Msc group[10] which remains during this cleavage procedure. The Msc group could be removed at the octapeptide stage with dilute base and without interference with the *S*-trityl function at A_{20}.

For side-chain protection of the tyrosyl residues (A_{14} and A_{19}) and the glutamyl residue (A_{17}) *tert*-butyl groups were used. They are completely stable under the cleavage conditions for the Bpoc group but are eliminated during removal of the peptide from the resin, which was done with 50% TFA in CH_2Cl_2. The absence of side-chain protecting groups in the final product improves its solubility, which is of advantage in the subsequent azide coupling with the A_{20-21} fragment. The same is true when the resulting A_{14-21} fragment is to be extended at the N-terminal side *via* the mixed anhydride method or *via* an azide coupling.

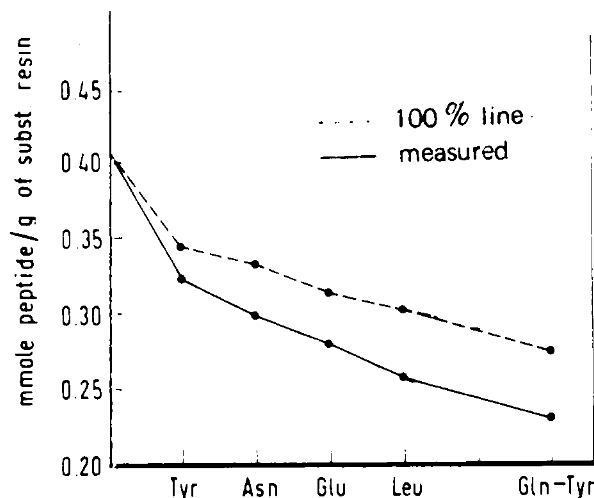


Figure 1. Relative incorporation of the subsequent amino acid residues in the solid-phase synthesis of the A_{14-19} fragment of insulin.

The introduction of asparagine (A_{18}) and glutamine (A_{15}) required special attention because condensing agents such as DCC can cause dehydration of amides to nitriles. Recent investigations[11] have revealed that *p*-nitrophenyl ester couplings proceed rather slowly in solid-phase syntheses, giving incomplete acylations even after prolonged reaction times. An attempt to use Mitin's method, previously applied to the incorporation of amide-containing amino acid residues,[12] had to be abandoned because it also led to incomplete condensations. Finally, a clean and rapid introduction of these amino acids appeared to be possible by condensation with DCC–HOBt, as was found by König and Geiger[13] in syntheses in solution and was recently applied to a solid-phase synthesis.[14] In preliminary experiments thin layer chromatograms of asparagine-containing peptides obtained thus showed that no appreciable formation of the corresponding β-cyanoalanyl derivative had occurred, and the complete absence of dehydration products could be deduced from the absence of any nitrile absorption at 2260 cm^{-1} in the ir spectra of the products.

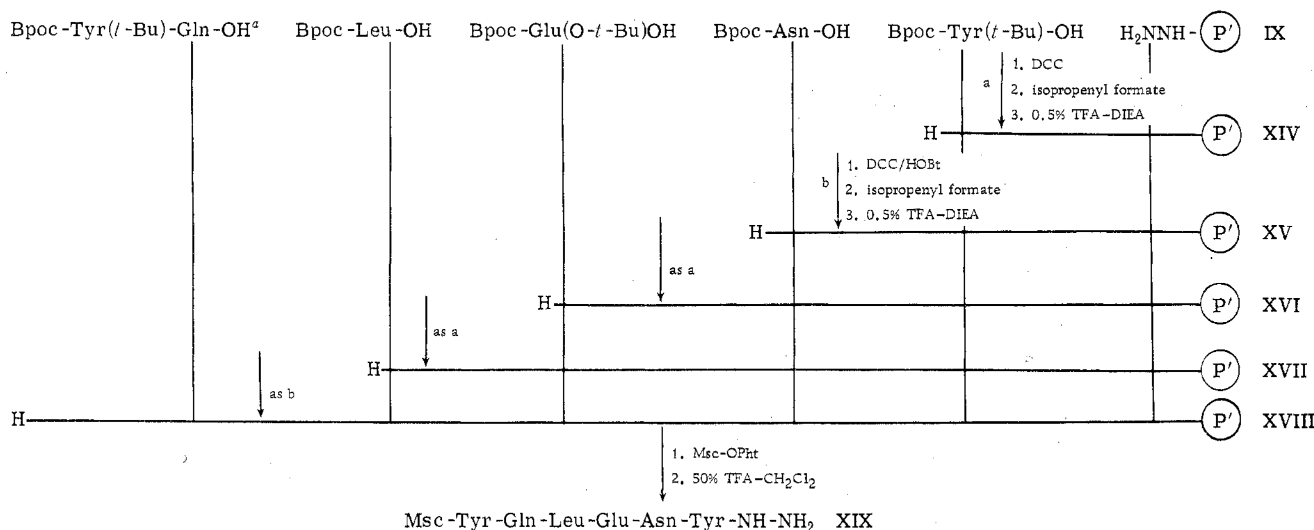
On account of these results DCC–HOBt was selected as the condensing reagent in the coupling of the asparagine and glutamine derivatives, and DCC alone in all other coupling steps. After each coupling residual free amino groups were formylated by treatment of the peptide resin with isopropenyl formate.[15] Omission of this treatment in a parallel synthesis led to an impure product difficult to purify.

To estimate the yields of the subsequent coupling steps the number of millimoles of the peptide present in 1 g of the resin was determined after each coupling by spectrophotometric determination of 2-(*p*-biphenylyl)propene, formed by removal of the terminal Bpoc group. The number was compared with that calculated for quantitative incorporation of the relevant amino acid residue (Figure 1).

The overall synthetic procedure is given in Scheme III. The synthesis was performed with a Schwarz Bio Research peptide synthesizer. After cleavage from the resin the Msc-hexapeptide hydrazide (XIX) was dissolved in DMF and precipitated with ethanol–ether. It was obtained in 62% yield, based on the Bpoc content at the hexapeptide level. It was chromatographically pure in a number of solvent systems and gave a correct elemental analysis.

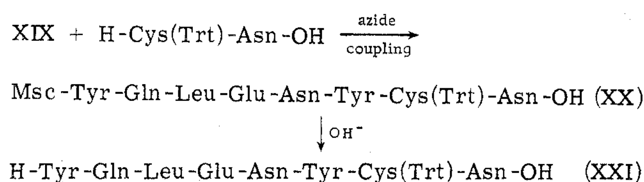
C. Synthesis of the *S*-Trityl A_{14-21} Sequence. The A_{14-19} fragment (XIX) was coupled with a slight excess of *S*-trityl-L-cysteinyl-L-asparagine *via* the azide method (Scheme IV). The remaining dipeptide was removed from the reaction mixture by filtration through an AG1-X2 ion

Scheme III
Synthesis of the Protected A_{14-19} Peptide Derivative



[a] In the automated synthesis coupling with a dipeptide was chosen in this stage because tlc of a pentapeptide derivative obtained from XVII and Bpoc-Gln-OH showed in addition to the ninhydrin-positive pentapeptide hydrazide a small spot which was Reindel–Hoppe positive but ninhydrin negative. This might have been a pyroglutamyl derivative. When the synthesis was completed *via* this pentapeptide stage by a coupling with Msc-Try(*t*-Bu)-OH the hexapeptide hydrazide (XIX) was, however, obtained in the same yield and as pure as *via* the given scheme.

Scheme IV



exchange column, which proved to be very useful for the selective separation of peptides differing in net negative charge. The N,S-protected octapeptide (XX) was obtained in 58% yield. Cleavage of the Msc group was done with dilute base in 1-butanol–methanol–water. The *S*-trityl substituted A_{14-21} fragment was isolated in 90.5% yield. It was homogeneous without purification and analyzed correctly.

Experimental Section

Melting points are uncorrected. Optical rotations were measured with a Zeiss 366343 polarimeter. Infrared spectra were taken on a Perkin-Elmer 257 infrared spectrophotometer with KBr pellets. For the automated solid-phase synthesis a Schwarz Bio Research instrument was used.

Thin layer chromatography was performed on precoated silica gel GF_{254} plates (Merck). The following solvent systems were used: heptane–*tert*-butyl alcohol–acetic acid–water–pyridine, 25:70:6:24:20 (system A); 1-butanol–acetic acid–water–pyridine, 4:1:2:1 (system B); chloroform–methanol, 3:1 (system C); *n*-heptane–1-butanol–acetic acid, 3:1:1 (system D); 1-butanol–acetic acid–water, 4:1:1 (system E); benzene–acetone, 1:1 (system F); 1-butanol–acetic acid–water–pyridine, 30:6:24:20 (system G).

For column chromatography AG1-X2 (chloride form, 200–400 mesh, Bio-Rad Laboratories) was washed twice with 2 *N* KOH, then with water until neutral, twice with 10% acetic acid, and again with water until neutral. Columns were equilibrated with the appropriate solvent before use.

Samples for elemental analysis were dried *in vacuo* over P_2O_5 at 50–60°.

***tert*-Butyl Acetoacetate Adduct of Chloromethylated Polystyrene (V).** *tert*-Butyl acetoacetate (18.5 ml, 0.113 mol) was added to a freshly prepared solution of 2.6 g (0.113 mol) of sodium in 60 ml of ethanol, and the mixture was supplied with more of the solvent to a total volume of 100 ml. Chloromethylated polystyrene (20.0 g, 2.26% Cl) was added to 40 ml of the solution and the solvent was evaporated *in vacuo*. DMF (15 ml) was then added and the mixture was concentrated *in vacuo* to a small volume. Another 25 ml of DMF was added and the reaction was allowed to proceed for 2 hr at 70° under stirring. The resin was filtered and washed several times with DMF, dioxane, dioxane-water, dioxane, CH_2Cl_2, and ethanol. An ir spectrum showed characteristic absorptions at 1735, 1715, and 1255 cm^{-1}. A Beilstein test for chlorine appeared to be negative.

3-Oxobutyl Resin (VI). The β-keto acid ester resin (V, 20 g) was suspended in 50 ml of 50% TFA–CH_2Cl_2, and the suspension was stirred for 1 hr. The resin was then filtered and thoroughly washed with CH_2Cl_2, 10% DIEA in CH_2Cl_2, CH_2Cl_2, and ethanol. The ir spectrum showed a strong absorption at 1715 cm^{-1} (keto C=O), but no absorptions at 1735 (ester C=O) and 1255 cm^{-1} (ester CO).

***tert*-Alkyloxycarbonyl Hydrazide Resin (IX).** The resin was prepared from VI as described by Wang and Merrifield.[7] The product contained 1.21% nitrogen, indicating 0.43 mmol of hydrazide/g of resin. The overall yield was therefore 71%, based on the chlorine content of the starting chloromethyl resin (2.26%).

Measurement of the Incorporation of Bpoc Amino Acid Residues. An accurately weighed sample of the dried *N*-Bpoc-peptide resin (4–7 mg) was suspended in 50 ml of a 0.5% TFA solution in CH_2Cl_2. After 10 min the optical density of the solution below the floating particles was measured at 274 nm. From the molar extinction of 2-(*p*-biphenylyl)propene in the solvent used (ϵ 20,400) the incorporation was calcualted as follows: milliequivalents of Bpoc residues/gram of substituted resin = $2.45\ d/x$, when d = optical density measured and x = milligrams of resin used.[16] The incorporation expressed in milliequivalents per gram of unsubstituted resin (C) can then be found from $C = x/[(1 - x) \cdot M \cdot 10^{-3}]$ in which M = equivalent weight of the newly introduced residue. Bpoc determinations carried out in duplicate never varied by more than 2–3%.

Methylsulfonylethyl Phthalimidocarbonate (Msc-OPht). 2-Methylmercaptoethanol was oxidized by treatment with the calculated amount of 30% hydrogen peroxide in a sodium tungstate catalyzed reaction. After evaporation of the reaction mixture with ethanol the remaining methylsulfonylethanol was crystallized from isopropyl alcohol (mp 29°). It was dissolved in a cooled tetrahydrofuran solution containing a twofold excess of phosgene, and the reaction flask was left for some hours at room temperature. The solution was then concentrated *in vacuo*, whereupon the residue crystallized as large crystals. The methylsulfonylethyl chloroformate was purified by crystallization from tetrahydrofuran–ether (mp 49.0–49.5°).

A solution of 2.44 g (13.1 mmol) of Msc chloride in 5 ml of acetone was added dropwise to a cooled solution (0°) of 2.14 g (13.1 mmol) of *N*-hydroxyphthalimide and 1.83 ml (13.1 mmol) of triethylamine in 10 ml of acetone. The addition was stopped as soon as the red color disappeared. Water was then added, and the pre-

Table I
DCC Mediated Coupling of Bpoc Amino Acids

Step	Reagent	Vol, ml	Time, min
1	4 equiv of Bpoc amino acid in CH_2Cl_2 or CH_2Cl_2–DMF[a]	10	2
2	4 equiv of DCC in CH_2Cl_2	9	90
3, 4, 5	CH_2Cl_2 (washing)	25	5, 2, 2
6, 7, 8	DMF (washing)	25	5, 2, 2
9, 10, 11	EtOH (washing)	25	5, 2, 2[b]
12, 13, 14	CH_2Cl_2 (washing)	25	5, 2, 2
15	10% isopropenyl formate solution in CH_2Cl_2	18	60
16, 17, 18, 19	CH_2Cl_2 (washing)	25	5, 2, 2, 2[c]

[a] In the couplings with Bpoc-Asn-OH and Bpoc-Tyr-(*t*-Bu)-Gln-OH 1 equiv of HOBt dissolved in DMF was added. [b] At this stage samples were taken for qualitative analysis; samples were treated with 50% TFA–CH_2Cl_2 to cleave the peptide from the resin. After filtration the solvent was evaporated, and DMF was added to get a 1% peptide solution which was applied to tlc plates. [c] At this stage samples were taken for measurement of the incorporation yield by the procedure given above.

cipitate was collected and washed with water, yielding 3.84 g (93%) of the carbonate, mp 160–163°.

Anal. Calcd for $C_{12}H_{11}NO_7S$: C, 46.01; H, 3.54; N, 4.47. Found: C, 46.2; H, 3.6; N, 4.4.

***N*-Methylsulfonylethyloxycarbonyl-*O*-*tert*-butyltyrosine.** *p*-Nitrophenyl chloroformate was dissolved in dry pyridine (2 ml/g) containing a slight excess of methylsulfonylethanol, with stirring at 0°. After 4 hr at room temperature the reaction mixture was concentrated to a thick sirup and poured into a hydrochloric acid solution (1 *N*). Msc-ONp separated as an oil which rapidly solidified. The tan-colored crystals were recrystallized from ethyl acetate–methanol, yield 73%, mp 102°.

Equimolecular amounts of this product and of *O*-*tert*-butyltyrosine were stirred together in 80% aqueous acetonitrile (10 ml/g of tyrosine) at room temperature. An equivalent amount of triethylamine was then added. After 1 hr the reaction mixture was concentrated, acidified to pH 5, and extracted with ether. The ether extracts were discarded. Acidification of the water phase with potassium hydrogen sulfate (2 *N*) yielded an oil which was extracted into ethyl acetate. Evaporation of the dried extract gave a foam which crystallized rapidly on trituration with ether, giving 2.9 g (75%) of the product, mp 130°, $[\alpha]^{24}D$ +4.0° (*c* 1.05, MeOH), $[\alpha]^{25}D$ +10.3° (*c* 1.01, 90% AcOH).

Anal. Calcd for $C_{17}H_{25}NO_7S$: C, 52.70; H, 6.50; N, 3.62; S, 8.28. Found: C, 52.8; H, 6.5; N, 3.6; S, 8.3.

***N*-2-(*p*-Biphenylyl)isopropyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-glutamine.** Bpoc-Tyr(*t*-Bu)-ONSu[5] (mp 139–141°, 2.9 g, 5 mmol) was dissolved in 15 ml of dimethoxyethane, and the solution was added to a solution of 0.73 g (5 mmol) of glutamine and 0.84 g (10 mmol) of sodium bicarbonate in 15 ml of water. To obtain a clear solution 10 ml of dimethylethane was added. After 4 hr the organic solvent was evaporated and a 10% citric acid solution was added at 0°. The precipitate was extracted into ethyl acetate, and the extract was washed with water, dried, and concentrated to a small volume. By addition of ether and diisopropylether 2.34 g (78%) of the product precipitated, mp 132–134°, $[\alpha]^{23}D$ +13.5° (*c* 1, DMF), homogeneous (system G).

Anal. Calcd for $C_{34}H_{41}N_3O_7 \cdot 0.25H_2O$: C, 67.14; H, 6.88; N, 6.91. Found: C, 67.2; H, 7.0; N, 6.85.

***N*-Methylsulfonylethyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-glutaminyl-L-leucyl-γ-*tert*-butyl-L-glutamyl-L-asparaginyl-*O*-*tert*-butyl-L-tyrosyl Hydrazide Resin (XVIII).** The necessary Bpoc amino acid derivatives were prepared according to Schnabel[17] by treatment of 2-(*p*-biphenylyl)isopropyl *p*-biphenylyl carbonate with a solution of the Triton B salt of the appropriate amino acid derivative. The compounds were isolated as DCHA salts. Bpoc amino acid solutions were prepared just before use. The acids were liberated from their salts with 2 *N* $KHSO_4$ at 0°.

The alternating coupling and deprotection steps given in Scheme III were performed with samples (1.5 g) of the resin (0.6–0.9 mmol) according to standard procedures which are given in Tables I and II. After the final deprotection step the N-terminal

Table II
Cleavage of Bpoc Groups

Step	Reagent	Vol, ml	Time, min
1, 2	0.5% TFA–CH_2Cl_2	44	2, 10
3, 4, 5	CH_2Cl_2 (washing)	22	2, 2, 2
6	10% DIEA–CH_2Cl_2	25	2
7, 8, 9	CH_2Cl_2 (washing)	25	2, 2, 2
10, 11, 12	EtOH (washing)	25	2, 2, 2
13, 14, 15	CH_2Cl_2 (washing)	25	2, 2, 2
16	10% DIEA–CH_2Cl_2	25	10
17, 18, 19, 20	CH_2Cl_2 (washing)	25	2, 2, 2, 2

amino groups were protected by treatment of the peptide resin with a tenfold excess of Msc-OPht, dissolved in DMF–CH_2Cl_2 for 16 hr at 20°.

***N*-Methylsulfonylethyloxycarbonyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl Hydrazide (XIX).** The peptide resin XVIII (1.2 g) was treated with 25 ml of 50% TFA–CH_2Cl_2 for 30 min at 20°. The remaining polymer was filtered and washed twice with 5 ml of CH_2Cl_2. From the absence of carbonyl absorptions in the ir spectrum of the resin it appeared that the peptide as well as the formyl residues had been completely cleaved from the resin.

The filtrate was evaporated, and the oily residue was dissolved in 12.5 ml of DMF. By addition of 50 ml of ethanol and 40 ml of ether a precipitate was formed, which was collected and dried over KOH. Tlc (solvents A and B) showed it to be homogeneous: yield 62% (based on the last Bpoc content measured); mp 225–227°; $[\alpha]^{23}D$ −28.3° (*c* 1, DMF).

Anal. Calcd for $C_{42}H_{60}N_{10}O_{16}S$: C, 50.80; H, 6.09; N, 14.10. Found: C, 50.7; H, 6.0; N, 14.1.

***N*-*tert*-Butyloxycarbonyl-*S*-trityl-L-cysteinyl-L-asparagine.** DCC (2.45 g, 11.8 mmol) was added to a stirred solution of 5.45 g (11.8 mmol) of *S*-tritylcysteine[18] and 1.36 g (11.8 mmol) of *N*-hydroxysuccinimide in 15 ml of 1,2-dimethoxyethane at −10°. Stirring was continued for 2 hr at 0° and 1 hr at 20°. The mixture was filtered, and the precipitate was washed with two 7.5-ml portions of dimethoxyethane. The combined filtrates were then added to a solution of 1.74 g (11.8 mmol) of asparagine hydrate and 1.63 g (11.8 mmol) of potassium carbonate in 20 ml of water. To obtain a clear solution 20 ml of dimethoxyethane was added. After 90 min this solvent was evaporated, and the residue was acidified with a $KHSO_4$ solution. The separated oil was extracted into ethyl acetate–ether (2:1), the extract was washed with water, and the oil was extracted again with a 10% *N*-methylmorpholine solution in water. The combined extracts were acidified with a 2 *N* H_2SO_4 solution and then extracted with ethyl acetate. Finally the organic layer was washed with water until neutral, dried, and evaporated, giving a foam which solidified on addition of petroleum ether. Tlc (systems C and D) showed that the N,S-protected dipeptide (5.97 g, 88%) was still contaminated with some impurities. Therefore, 0.9 g of the product was dissolved in 100 ml of 1-butanol–methanol–water (1:1:1) and applied to an AG1-X2 column (12 × 1.5 cm) which was washed and equilibrated previously with the same solvent system. On elution with 1-butanol–methanol–1.5% acetic acid (1:1:1) the chromatographically homogeneous fractions containing the protected dipeptide were collected and evaporated. Trituration of the residue with petroleum ether yielded 660 mg of a pure product, $[\alpha]^{22}D$ +29.0° (*c* 1, MeOH).

Anal. Calcd for $C_{31}H_{35}N_3O_6S$: C, 64.45; H, 6.11; N, 7.27. Found: C, 64.4; H, 5.9; N, 7.2.

***S*-Trityl-L-cysteinyl-L-asparagine.** Boron trifluoride etherate (0.26 ml, 1.8 mmol) was added to a stirred solution of 355 mg (0.615 mmol) of Boc-Cys(Trt)-Asn-OH in 5 ml of acetic acid. After 30 min the solution was poured into a solution of sodium acetate. The separated oil was extracted into 1-butanol, previously saturated with water, and the extract was washed with the corresponding water phase. The solvent was evaporated, and the oily residue was triturated with ether, yielding 285 mg (97.5%) of the product, homogeneous on tlc (system E), $[\alpha]^{23}D$ +27.7° (*c* 1, DMF).

Anal. Calcd for $C_{26}H_{27}N_3O_4S \cdot 0.5\ H_2O$: C, 64.18; H, 5.80; N, 8.64. Found: C, 63.9; H, 5.6; N, 8.55.

***N*-Methylsulfonylethyloxycarbonyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-*S*-trityl-L-cysteinyl-L-asparagine (XX).** A solution of XIX)286 mg, 0.288 mmol) in 8 ml of DMF and 1 ml of DMSO was cooled to −20° and

treated with 0.105 ml of a 7.5 *N* HCl solution in dimethoxyethane (0.79 mmol) and 0.043 ml (0.375 mmol) of *tert*-butyl nitrite. The mixture was stirred at −15° for 20 min, then cooled to −30° and treated with 0.11 ml (0.79 mmol) of triethylamine.

A cooled solution of 167 mg (0.35 mmol) of H-Cys(Trt)-Asn-OH in 3 ml of DMF was added to this azide solution. The pH was adjusted to 7 with 10% Et_3N–DMF, and the solution was left for 3 days at 4°. The mixture was then diluted with 200 ml of 1-butanol–methanol–water (1:1:1) and added to an AG1-X2 column (1.6 × 22 cm). The column was eluted with 1-butanol–methanol–dilute acetic acid (1:1:1) mixtures in which the concentration of the acetic acid used was gradually raised: 0% (100 ml), 0.3% (300 ml), 1% (100 ml), 3% (200 ml), 15% (400 ml). Absorbancy measurements (254 nm) and tlc revealed that the excess of dipeptide was eluted with the 0.3% acetic acid mixture, while the desired product was located in the 15% acetic acid eluate.

Fractions containing the product were collected and evaporated. The residue was dissolved in 5 ml of DMF and precipitated with methanol–water, yielding 242 mg (58%) of XX, mp 253° dec, $[\alpha]^{23}D$ −20.2° (*c* 1, DMF), homogeneous (systems B and F).

Anal. Calcd for $C_{68}H_{83}N_{11}O_{20}S_2 \cdot H_2O$: C, 56.07; H, 5.88; N, 10.58. Found: C, 55.9; H, 5.8; N, 10.5.

Amino acid ratios in acid hydrolysate (in the presence of phenol): Asp, 1.9; Glu, 2.0; Leu, 1.1; Tyr, 1.95.

L-Tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-*S*-trityl-L-cysteinylasparagine (XXI). A suspension of 105 mg (0.073 mmol) of XX in water was treated with 0.35 ml (0.14 mmol) of 4 *N* NaOH. After 4 min the clear solution was acidified with a few drops of 2 *N* $KHSO_4$ solution and water was added. The precipitate was filtered, washed with water, and dried, yielding 85 mg (90.5%) of XXI, mp ~300° dec, $[\alpha]^{23}D$ −15.0° (*c* 1, DMF), homogeneous (system B).

Anal. Calcd for $C_{64}H_{77}N_{11}O_{16}S \cdot 2H_2O$: C, 58.04; H, 6.16; N, 11.63. Found: C, 57.9; H, 5.9; N, 11.5.

Amino acid analysis in acid hydrolysate, oxidized with performic acid: Asp, 2.0; Cys(SO_3H), 1.0; Glu, 1.9; Leu, 1.0; Tyr, 1.5.

Registry No.—XIX, 52278-85-0; XX, 52278-86-1; XXI, 52278-87-2; methylsulfonylethyl phthalimidocarbonate, 52278-88-3; 2-methylmercaptoethanol 5271-38-5; *N*-methylsulfonylethyloxycarbonyl-*O*-*tert*-butyltyrosine, 52278-89-4; *p*-nitrophenyl chloroformate, 7693-46-1; *N*-2-(*p*-biphenylyl)isopropyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-glutamine, 52278-90-7; Bpoc-Tyr(*t*-Bu)-ONSu, 33527-03-6; *N*-*tert*-butyloxycarbonyl-*S*-trityl-L-cysteinyl-L-asparagine, 52278-91-8; *S*-trityl-L-cysteinyl-L-asparagine, 52278-92-9.

References and Notes

(1) The following abbreviations have been employed in the text: DCC = *N,N*-dicyclohexylcarbodiimide; HOBt = *N*-hydroxybenzotriazole; TFA = trifluoroacetic acid; THF = tetrahydrofuran; DCHA = dicyclohexylamine; TOSOH = *p*-toluenesulfonic acid; DIEA = *N*-ethyldiisopropylamine; Im = imidazole; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; BOC = *tert*-butyloxycarbonyl; Bzh = benzhydryl; *t*-Bu = *tert*-butyl; Bpoc = 2-(*p*-biphenylyl)isopropyloxycarbonyl; Trt = triphenylmethyl; Tmb = 2,4,6-trimethylbenzyl; Bmv = 2-benzoyl-1-methylvinyl; Msc = 2-(methylsulfonyl)ethyloxycarbonyl; Pht = phthalimido; NSu = succinimido.

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Isolation and Structure Determination of One of the Toxic Constituents from *Tetradymia glabrata*

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Tetradymol (I) isolated from *Tetradymia glabrata*, has been shown to be 4β,5β-dimethyl-10β-hydroxyfuranoeremophilane. The combined results and relationships of chemical, nmr, and X-ray analyses of I are discussed. The mercuric chloride derivative of tetradymol crystallized in a space group $P2_12_12_1$ with cell dimensions a = 7.371 (5) Å, b = 10.304 (9) Å, c = 19.759 (17) Å with Z = 4. Counter data were refined by full-matrix least-squares to a residual of 5.4%. Tetradymol has been shown to effect hepatodysfunction and has an LD_{50} (mice) of 250 mg/kg.

Investigations reported in the literature on the components of *Tetradymia glabrata* are both long-standing and limited. The presented paper deals with the isolation of one of its toxic components, tetradymol (I),[2] and proof of its stereochemical structure.

Because of the work of Fleming,[3] Clawson and Huffman[4] prior to 1937, this plant was known to contain toxic components fatal to sheep and was further suspected to contain a component causing the maladay "bighead" in the same animal. These investigators suspected that at least two toxic compounds were present, one apparently effecting heptodysfunction, and the other effecting cardiac failure. We have not been successful in repeating the conditions necessary for the development of the "bighead" symptom,[5] and have found that the plant extract fraction reportedly containing the cardiac toxin actually contains another hepatotoxin which will be reported later. Tetradymol (I) has been shown to be a moderate hepatotoxin in several animals including sheep, mice, rats, rabbits, guinea pigs, and gerbils [oral LD_{50} (mice) is 250 mg/kg].

Results and Discussion

Isolation of Tetradymol. During the isolation procedure (Figure 1) we concluded that the toxin was located near the surface of the plant as we obtained similar amounts of I from either ground or unground plant. To