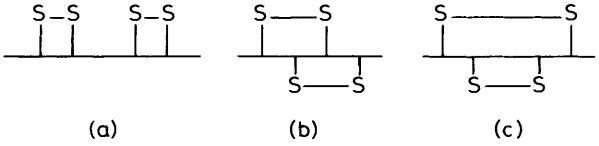
Peptide Synthesis. Part 7.[1] Solid-phase Synthesis of Conotoxin G1

Eric Atherton, Robert C. Sheppard,* and Peter Ward
Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH

The neurotoxin conotoxin G1 from *Conus geographus* has been synthesised by a solid-phase method using fluorenylmethoxycarbonylamino acids and a polar, poly(dimethylacrylamide)-based support. The two disulphide bridges were formed selectively by differential protection of cysteine residues using *S*-acetamidomethyl and *S*-t-butylsulphenyl groups.

Our attention was first drawn to the series of highly potent peptide neurotoxins from *Conus* sea snails when Dr W. Gray of the University of Utah visited our Laboratory in 1977. He brought with him the newly determined amino acid sequence (**1**) for conotoxin G1 from *Conus geographus*. The four cysteine residues in (**1**) were thought to be linked in pairs forming two intramolecular disulphide bonds in a bicyclic tridecapeptide amide structure of type a, b, or c. These structures were of interest to us because of their biological significance and novelty, and because total synthesis would provide a severe test for the new polyamide-based solid-phase methods which we were developing at that time.[2] Conotoxin G1 includes in its tridecapeptide amide sequence both arginine and histidine as well as the four cysteine residues; all three are recognised as difficult amino acids in peptide synthesis. Accordingly, synthetic studies were carried out on two (b, c) of the three possible disulphide isomers of (**1**), the third possibility (a) being considered less likely. These studies were apparently successful; the synthetic bicyclic tridecapeptide amides obtained were very similar to natural conotoxin in physical properties, but both were biologically inactive. We know now that the amino acid sequence (**1**) was incorrect. Details of this early work have been published elsewhere,[3] together with later synthetic and other studies establishing the correct disulphide pairing in conotoxin G1 as type b.

1 5 10 13
Glu.Cys.Cys.Asn.Pro.Ala.Cys.Gly.Arg.His.Tyr.Cys.Ser.NH_2
(1)



1 5 10
Glu.Cys.Cys.Asn.Pro.Ala.Cys.Gly.Arg.His.Tyr.Ser.Cys.NH_2
(2)

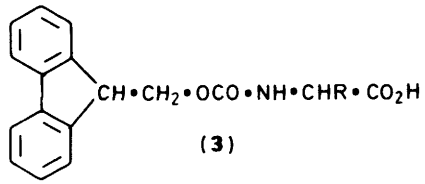
A revised amino acid sequence (**2**) for conotoxin G1 and sequences for two homologues were reported in 1981.[4] In the intervening period our solid-phase synthesis methods had undergone substantial further development.[5,6] The most notable change was the adoption of N_α-fluorenylmethoxycarbonyl (Fmoc) amino acids (**3**) in place of the customary t-butoxycarbonyl (Boc) derivatives (**4**). This single change has far reaching consequences. The high base-lability and nearly complete acid-stability of Fmoc-amino acids and peptides permit important concomitant changes in side-chain and C-terminal-protecting-group strategy. Use where possible of *O*-t-butyl (rather than benzyl)-based protecting groups for amino acid side-chains and a similarly acid-labile *p*-alkoxybenzyl alcohol linkage agent (**5**)[7] for carboxy-terminal protection and attachment to the solid support permits very substantially milder overall conditions of synthesis. The need for liquid hydrogen fluoride or other very strong acidic reagents is avoided with sometimes dramatic improvement in the yield and quality of synthesis.[8]

Some amino acids require special consideration in the light of this new protecting-group strategy. In the foregoing paper,[1] we reported a comparative study of *S*-t-butyl- (**6**), *S*-t-butylsulphenyl- (**7**), and *S*-acetamidomethyl- (**8**)-protected cysteines for the incorporation of this amino acid in Fmoc-polyamide solid-phase synthesis. All three groups proved suitable and when used in appropriate combination provide good opportunity for the selective formation of intramolecular disulphide bonds in polycysteinyl peptides. We have also briefly reported a study of arginine guanidino-group protection,[9,10] concluding that the N_G-4-methoxy-2,3,6-trimethylphenylsulphonyl derivative of N_α-Fmoc-arginine, (**9**),† is likely to be suitable for use in solid-phase synthesis, at least for objectives which are not excessively acid-labile. A study, currently in progress, of the blocking of the imidazole ring of N_α-Fmoc-histidine has shown, *inter alia*, that transitory protection by a second Fmoc group as in compound (**10**) may provide a simple and frequently applicable solution.[10] This is analogous to the common use of N_α,N_{im}-bis(Boc)-histidine in conventional solid-phase synthesis. In both cases the imidazole ring becomes free after the first deprotection cycle following its incorporation.‡ The revised structure for conotoxin G1 offered a demanding test of all these innovations.

The combination of *S*-t-butylsulphenyl (cleavable by reduction with tributylphosphine) and *S*-acetamidomethyl (cleavable by iodine oxidation with simultaneous disulphide-bond formation) was selected for cysteine protection.[1] This combination offered probably the best prospect for selective disulphide pairing without major disulphide interchange, although the latter was expected to be a significant problem. Substantial disulphide scrambling was observed during structural studies on conotoxin.[4] Early studies on re-formation of the correct disulphide configuration (b) when fully reduced conotoxin was oxidised were unsuccessful[4] and this was finally achieved only under conditions of extreme dilution, when other

† This protecting group may be cleaved by protracted (2—6 h) treatment with trifluoroacetic acid (TFA) in the presence of certain scavengers. Groups that are more labile are desirable and we hope to report shortly on an alternative solution to the arginine problem.

‡ The very recently described (R. Colombo, F. Colombo, and J. H. Jones, *J. Chem. Soc., Chem. Commun.*, 1984, 292) π-t-butoxymethyl derivative of N_α-Fmoc-histidine provides a more sophisticated alternative which merits careful examination.



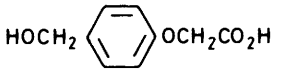
(3)

(4)

(6) = (3; R = CH_2SCMe_3)

(7) = (3; R = CH_2SSCMe_3)

(8) = (3; R = CH_2SCH_2NHAc)



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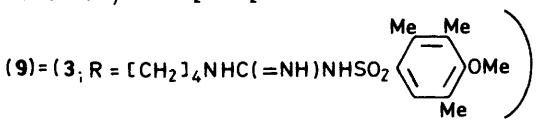
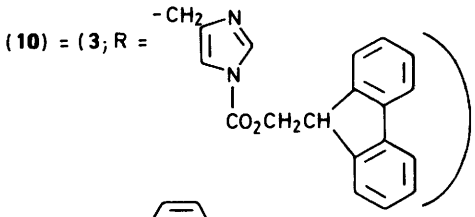
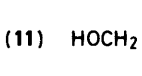






products were also formed.[3] Thus the final stages of the conotoxin synthesis were expected to be difficult.

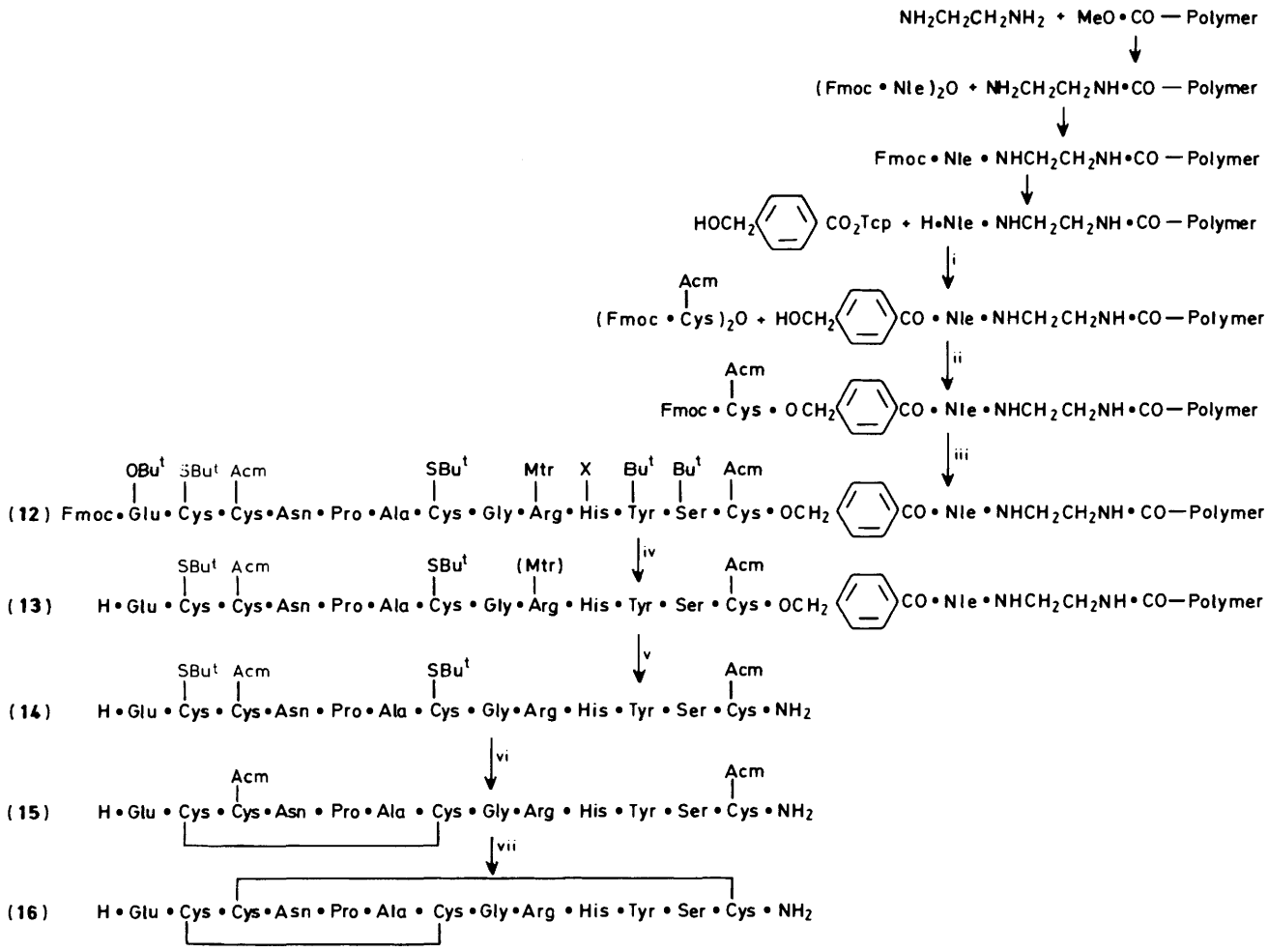
The amino-acid sequence (**2**) was assembled (Scheme) on cross-linked poly(dimethylacrylamide) resin[5] which had been functionalised with methyl ester groups by incorporation of acryloylsarcosine methyl ester into the original monomer mixture. The sarcosine content was 0.25 m equiv. g^{-1}. Ester groups were converted into amines by treatment with excess of ethylenediamine and a permanent internal reference amino acid was attached by acylation with the preformed symmetrical anhydride of Fmoc-norleucine. This and all subsequent acylation and deprotection reactions utilised dimethylformamide (DMF) as the sole solvent and swelling medium for the resin. Substantial excesses of acylating agent (7.2-fold based on the determined sarcosine content) were used throughout. This use of unusually large excess of reagent arose because of initial uncertainty about the level of functionalisation of the particular resin sample used. It is certainly unnecessary, and general experience has shown that equally satisfactory amino acid incorporation can usually be obtained with more modest (2—5-fold) excesses of anhydrides.

The Fmoc protecting group masking the norleucine residue was cleaved by brief (10 min) treatment with 20% piperidine, and the reversible peptide–resin linkage agent (**11**) was coupled as its 2,4,5-trichlorophenyl ester in the presence of catalyst (1-hydroxybenzotriazole). This special linkage agent[6] forms benzyl ester derivatives which are readily cleaved by ammonia and other simple nucleophiles (but not by anhydrous piperidine), and is thus particularly suitable for the preparation of peptide amides such as compound (**2**). It has the added advantage of forming a very acid-stable bond between the peptide and resin support, permitting t-butyl esters and other acid-labile side-chain-protecting groups to be cleaved before ammonolysis of the resin-bound peptide. This peptide–resin bond was also shown to be stable to the conditions required to cleave *S*-t-butylsulphenyl protecting groups from cysteine residues, thus allowing oxidative closure of at least one and possibly both disulphide bonds whilst the peptide is still attached to the resin. This additional flexibility was not in fact used in the synthesis to be described.

Esterification of the first amino acid to the hydroxy resin is usually carried out in the presence of 4-(dimethylamino)pyridine catalyst. We selected *S*-acetamidomethylcysteine rather than *S*-t-butylsulphenylcysteine as *C*-terminal residue on the expectation that carboxy-activated derivatives of the latter might be more prone to β-elimination under the basic conditions. The reaction conditions adopted were those found to minimise racemisation in an earlier study using Fmoc-isoleucine.[11] There is no simple analytical method for monitoring the incorporation of cysteine as *C*-terminal residue in solid-phase synthesis, in contrast to later peptide-bond forming steps. No sensitive colour tests (for residual hydroxy groups) are available and the amino acid itself is partly destroyed under the conditions of peptide hydrolysis and amino acid analysis. With the particular amino acid composition of the *C*-terminal region of conotoxin, reliable amino acid analysis of resin-bound peptides cannot be anticipated until after incorporation of glycine residue at step 6. Thus the efficiency of the initial esterification of the first Fmoc-amino acid to the resin support could not be determined in the early stages. Much previous experience[12] has shown that residual free hydroxy groups do not react significantly at subsequent acylation steps in the absence of basic catalysis. Special caution is indicated, however, in the case of histidine peptides. Use of the N_{α},N_{im}-bis(Fmoc) derivative (**10**) for the incorporation of this residue would introduce a free basic and potentially catalytic imidazole group into the resin environment after the subsequent deprotection cycle. It was therefore appropriate to block any remaining hydroxy groups before introduction of this residue. This was done after step 3 by the use of acetic anhydride in the presence of pyridine and 4-(dimethylamino)pyridine.

The complete amino acid sequence of compound (**2**) was assembled straightforwardly. N_{α}-Fmoc derivatives with side-chains protected as indicated in the Scheme were used. In all cases negative ninhydrin[13] and trinitrobenzenesulphonic acid[14] colour reactions for residual amine were obtained at the first test (usually after 15—20 min), including Fmoc-Arg(Mtr) (*cf.* ref. 9). Resin samples were removed for analysis from step 2 onwards. The results are collected in Table 1. Some caution should be used in interpreting the data in this Table, especially as indicated above for residues in the carboxy-terminal region. The very low results recorded for serine, for example, are in agreement with earlier data obtained for serine or threonine adjacent to acetamidomethylcysteine in other instances.[15] Satisfactory analysis for this residue was not obtained until after cleavage of the *S*-acetamidomethyl group. The ratio of glycine to internal reference norleucine indicates that esterification of the first acetamidomethylcysteine residue to the resin probably occurred to the extent of about 80%, justifying the precautionary blocking of residual hydroxy groups by acetylation. An overall satisfactory assembly is indicated by an incorporation of the last residue (glutamic acid) equal to that of the earlier glycine. Although some degree of acylation of the imidazole ring is to be anticipated from addition of the arginine residue onwards, this is not reflected in the results in Table 1 because resin samples were deprotected with piperidine before total acid hydrolysis. This cleavage of the terminal Fmoc group is essential if a full yield of the amino-terminal residue is to be obtained, and simultaneously cleaves aminoacyl substituents on the imidazole ring.

The wide range of protecting groups present on the final fully



Scheme. Solid-phase assembly and disulphide-bond formation of conotoxin G1. The polymeric support used was a copolymer of dimethylacrylamide, ethylenebisacrylamide, and acryloylsarcosine methyl ester. *Reagents:* i, 1-Hydroxybenzotriazole; ii, 4-(dimethylamino)pyridine; iii, 11 cycles of deprotection (20% piperidine–DMF) and acylation; iv, (a) 20% piperidine–DMF; (b) 95% TFA; v, (a) saturated methanolic ammonia; (b) phenol–TFA; vi, (a) tri-*n*-butylphosphine; (b) air; vii, I_2

protected peptide–resin (**12**) indicated caution before we proceeded with the various selective cleavage steps. It is at this stage that major artefact formation is to be anticipated, and pilot and model studies were therefore carried out.

The 4-methoxy(trimethyl)phenylsulphonyl protecting group on the guanidine function of arginine requires prolonged acidic treatment for its removal,[9] and model studies using simple amino acid derivatives were made to determine the corresponding stability of the sulphur-protecting groups. Both *S*-acetamidomethyl and *S*-(t-butylsulphenyl)cysteine were unaffected by anhydrous TFA over 3 h, during which time 74% of the arginine protecting group was cleaved. On the other hand, *ca.* 13% of the *S*-acetamidocysteine and 54% of the *S*-t-butylsulphenyl derivative was destroyed when the same reaction was carried out in the presence of 5% thioanisole, the conditions recommended by Fujino and his colleagues for removal of this protecting group from arginine.[16] These results were obtained by direct amino acid analysis of the reaction mixture. Further studies were carried out using h.p.l.c. on partially deprotected conotoxin to establish alternative, more satisfactory cleavage conditions (*vide infra*). Likewise, small-scale pilot experiments on the peptide–resin were used to define conditions for ammonolysis. After cleavage of the terminal fluorenylmethoxycarbonyl and side-chain *O*-t-butyl groups with piperidine and aqueous TFA respectively, resin samples were subjected to ammonolysis in methanolic ammonia for periods up to 21 h. Cleavage was rapid, being 66% complete after 10 min and 94% after 1 h as determined by residual-resin analysis. H.p.l.c. of the soluble peptide showed substantial amounts (~30%) of a less polar contaminant in the 10-min reaction product, probably the corresponding methyl ester, which was absent in the 1-h product. Prolonged ammonolysis resulted in substantial degradation of the desired product.

With these preliminaries completed, the peptide resin (**12**) was successively deprotected in the following manner. The terminal fluorenylmethoxycarbonyl group was removed with piperidine as usual and all *O*-t-butyl groups (on glutamic acid, tyrosine, and serine) were cleaved with aqueous TFA to give the product (**13**). This treatment was also expected to effect limited cleavage of the arginine Mtr group. The partially deprotected peptide was then detached from the resin by ammonolysis with methanolic ammonia for 1 h. The yield of soluble peptide material was 95% (by amino acid analysis), and the h.p.l.c. profile of the total crude product is shown in Figure 1. The major contaminant, peak B, is coincident with the *S*-protected tridecapeptide amide (**14**) lacking the arginine Mtr group.

Table 1. Peptidyl resin hydrolysis results. Amino acids relative to Nle = 1.25 or Gly = 1.00

Step	2	3	4	5	6	7	8	9	10	11	12	13
Nle	1.25	1.25	1.25	1.25	1.26	1.25	1.25	1.29	1.31	1.28	1.28	1.30
Ser	0.57	0.56	0.59	0.55	0.62	0.69	0.72	0.71	0.72	0.66	0.70	0.70
Tyr		0.77	0.80	0.85	0.84	0.88	0.85	0.85	0.87	0.89	0.84	0.86
His			0.76	0.76	0.78	0.78	0.76	0.79	0.80	0.78	0.78	0.79
Arg				0.91	0.92	0.90	0.88	0.91	0.92	0.92	0.91	0.91
Gly					1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ala							0.93	0.94	0.96	0.96	0.96	0.95
Pro								0.98	1.01	1.09	1.02	1.15
Asp									1.02	1.03	1.02	1.02
Glu												1.05
Gly loading (mmol g^{-1})					0.16	0.15	0.16	0.15	0.13	0.14	0.13	0.12
Nle loading (mmol g^{-1})	0.23	0.22	0.19	0.18	0.20	0.18	0.19	0.19	0.17	0.17	0.16	0.16

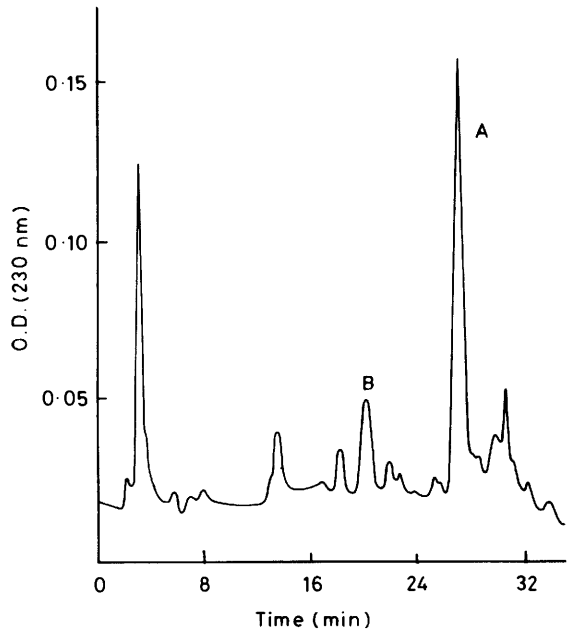


Figure 1. H.p.l.c. of total product (**13a**) from ammonolysis of the peptide resin (**13**). Linear gradient of 15—60% B during 40 min (see Experimental section)

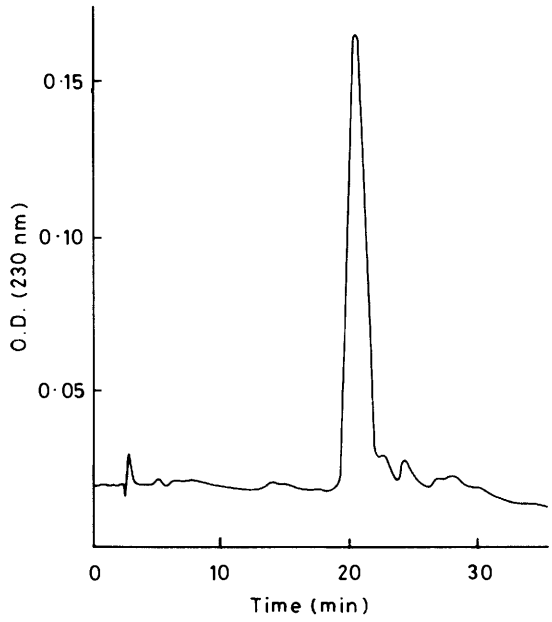


Figure 2. H.p.l.c. of *S*-protected tridecapeptide amide (**14**) after chromatography on (carboxymethyl)cellulose. Linear gradient of 15—60% B during 40 min (see Experimental section)

Small-scale pilot experiments were carried out using h.p.l.c. analysis to establish the best conditions for completing its removal. The desired cleavage was effected by prolonged (7 h) treatment with anhydrous TFA but serious side-reactions were evident, confirming the need for a scavenging reagent. Side-reactions were suppressed by addition of water to the reaction mixture, but the cleavage rate was considerably reduced. Phenol was effective in suppressing side-reactions but had little effect on cleavage rate. Rapid degradation was produced by addition of thioanisole, confirming the results of earlier model studies above.

On a preparative scale, the Mtr group was cleaved using TFA in the presence of phenol, h.p.l.c. being used to monitor the disappearance of peak A and appearance of peak B in Figure 1. After evaporation, the residue was partitioned between water and dichloromethane (to remove residual phenol). Remarkably, a greater quantity of the peptide (**14**) (55%) was subsequently recovered from the organic layer than from the aqueous (42%). Each was purified separately by chromatography on (carboxymethyl)cellulose to give a total recovery from the peptide resin of 41%. The h.p.l.c. profile of peptide recovered from the dichloromethane layer in this way is shown in Figure 2. Amino acid analyses are given in the Experimental section.

Selective formation of the two disulphide bridges was achieved as follows. Both *S*-t-butylsulphenyl groups were removed from the tetra-*S*-protected peptide by reduction with tributylphosphine. After dilution to approx 4×10^{-5}M under argon, excess of reductant was removed by extraction and the resulting solution was exposed to air until a negative Ellman test for free thiol groups was obtained. Gel filtration (Figure 3) was used to remove dimeric and higher molecular weight material and the product (58% recovery) was again purified by ion-exchange chromatography (Figure 4). The monocyclic disulphide (**15**) was recovered from the main peak in a yield of 84% giving an overall yield from peptide resin of 19%; h.p.l.c., Figure 5.

The second disulphide bridge was closed by direct iodine-oxidation of the bis(acetamidomethyl) peptide.[17] High mole-

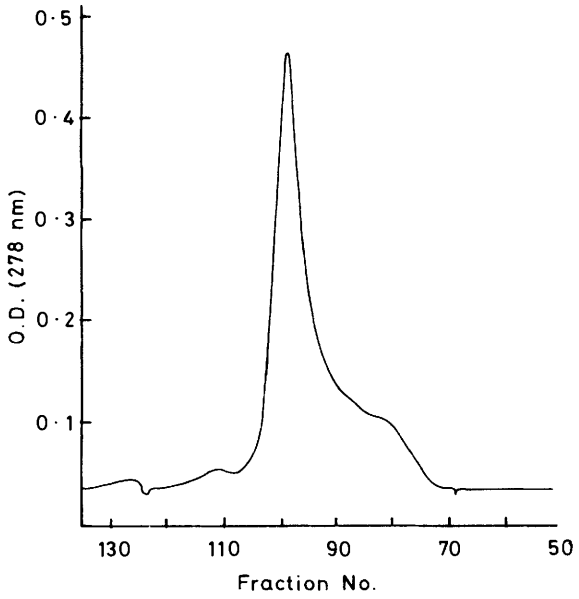


Figure 3. Gel filtration of crude monocyclic oxidation product (**15**). For conditions see Experimental section.

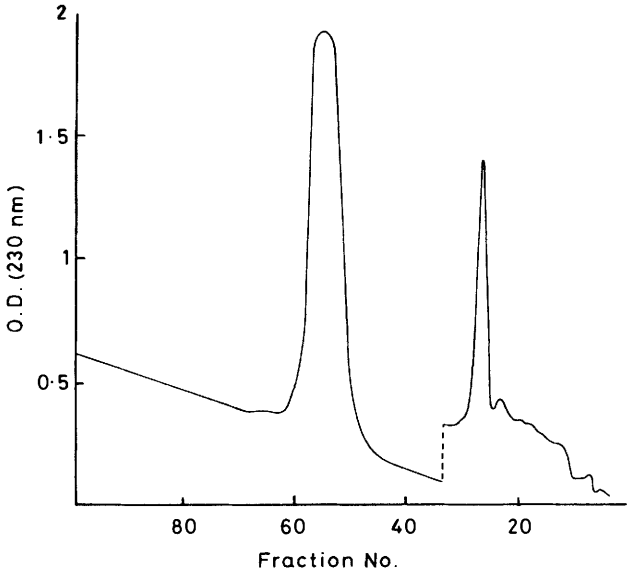


Figure 4. Cation-exchange chromatography of crude monocyclic tridecapeptide amide (**15**). For conditions see Experimental section.

cular weight material (*ca.* 10%) was again removed by gel filtration. The product was purified by ion-exchange chromatography which gave the elution profile of Figure 6. Material from the trailing edge of the main peak (C, Figure 6) gave nearly pure conotoxin (h.p.l.c., Figure 7, E), whereas the remainder of the peak (D, Figure 6) contained a larger proportion of the unidentified contaminant F (Figure 7). This mixture could be separated by semi-preparative h.p.l.c. The combined yield of conotoxin G1 (**16**) was 30%, giving an overall yield of 6% from peptide–resin.

Synthetic conotoxin G1 (fraction C, Figure 6) was further characterised by amino acid analysis, t.l.c. (two systems), electrophoresis (Experimental section), and biological activity.* When assayed for toxicity in mice of the CRH strain (body

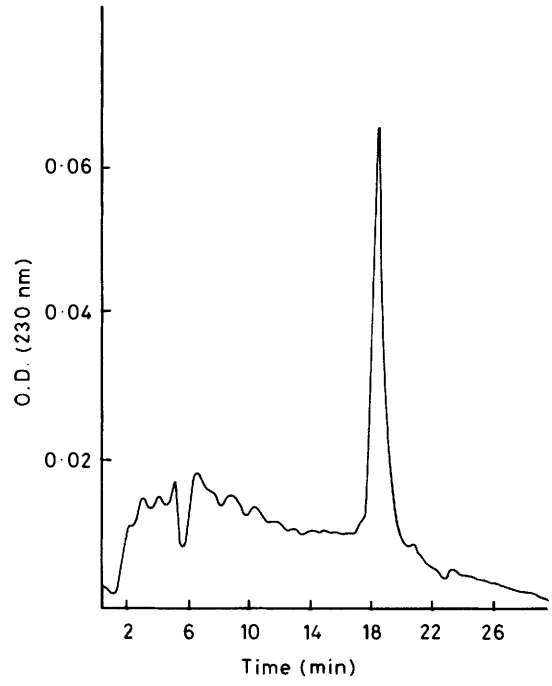


Figure 5. H.p.l.c. of ion-exchange purified (**15**). The initial fluctuation of the elution profile is attributable largely to malfunction of the h.p.l.c. system (*cf.* Figure 7). Linear gradient 5—30% B during 30 min (see Experimental section).

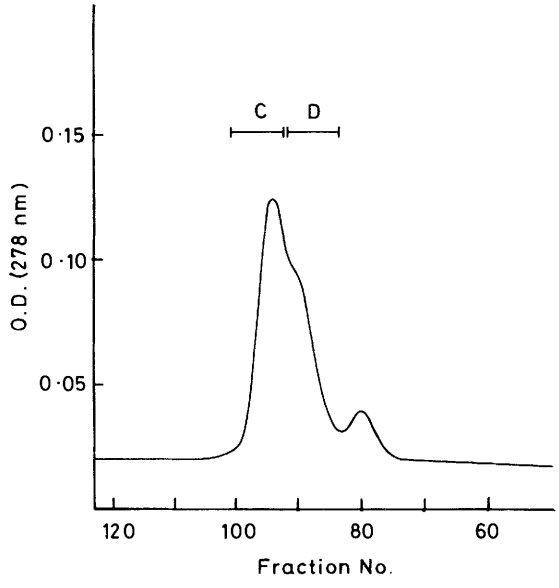


Figure 6. Cation-exchange purification of crude conotoxin G1. Fractions comprising C furnished nearly pure conotoxin. Fractions D were further purified by preparative h.p.l.c. For conditions see Experimental section.

weight 20—25 g) the synthetic toxin had a potency of 3.05 'mouse units' per nmol (*i.e.*, 1/3.05 nmol was required to kill a 20-g mouse in 20 min) and is therefore approximately twice as active as the natural toxin for which a value of 1.48 units per nmol has been quoted.[4] It is possible that this discrepancy is due

* We thank Dr D. J. Twissell of Glaxo Group Research, Greenford, Middx, who carried out these assays.

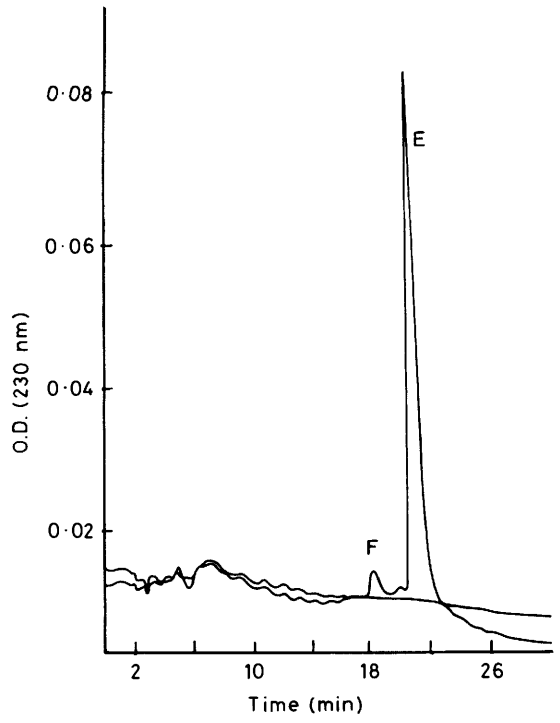


Figure 7. H.p.l.c. of fraction C (Figure 6) from ion-exchange purification of synthetic conotoxin. The second trace is a solvent blank. Linear gradient 5—30% B during 30 min (see Experimental section).

to a strain difference and does not necessarily reflect greater purity of the synthetic product or a systematic error in the bioassay.

This synthesis constitutes an application of the Fmoc-polyamide solid-phase method to a particularly complex and challenging structure. As anticipated, the major problems appear to arise not from the exceptionally mild reaction conditions of the Fmoc-polyamide peptide bond-forming steps, but from side-chain side-reactions, especially during final deprotection and disulphide-bond formation. For several amino acids (notably histidine and arginine) the need remains for readily accessible side-chain-protected derivatives unaffected by the conditions of synthesis and yet cleavable under such mild conditions as to leave the liberated, reactive groupings unaffected. This is a continuing challenge to peptide chemistry.

Experimental

General procedures for solid-phase peptide synthesis using poly(dimethylacrylamide) supports[5] and fluorenylmethoxycarbonylamino acids[6] have been described previously. Fmoc·Cys(SBu^t)·OH was prepared as described in the preceding paper.[1] Fmoc·Cys(Acm)·OH and Fmoc·Arg(Mtr)·OH were gifts from Dr J. Wade.

Ion-exchange chromatography was carried out on (carboxymethyl)cellulose (Whatman CM52; 12—14 cm × 1 cm columns) pre-equilibrated with 0.01M ammonium acetate buffer, pH 6.0, and eluted with gradients up to 0.15M or 0.25M of the same buffer. Peptide solutions were loaded onto the columns after adjustment of the pH to 6.0 and dilution to an ionic strength below that of starting buffer.

T.l.c. was performed on precoated silica gel plates (Merck D C Fertigplatten Kieselgel 60F_{254}) with butan-1-ol–acetic acid–water (66:12:66) or butan-1-ol–pyridine–acetic acid–water (90:18:60:72) as developer. Spots were visualised by spraying with ninhydrin (1% in acetone), or by chlorination followed by spraying with *o*-tolidine reagent.

High-performance liquid chromatography was carried out on μ-Bondapak C_{18} columns (0.39 cm × 30 cm, analytical; 0.79 cm × 30 cm, semi-preparative) using gradient elution of increasing percentage B in A + B, where A = 0.01 ammonium acetate, pH 4.5 and B = 90% acetonitrile:10% A.

Electrophoresis was carried out on 20 cm × 20 cm precoated cellulose t.l.c. plates in pH 6.5 buffer for 40 min at 400 V. The plates were developed by immersion in a solution containing equal volumes of 0.5 mg ml^{-1} fluorescamine and 1% pyridine in acetone and by immersion in a mixture of 1% ninhydrin in acetone (85 ml) and 15 ml of a solution of cadmium(II) acetate (0.75 g) in a mixture of water (75 ml) and glacial acetic acid (37.5 ml).

Preparation of the Fmoc-amino Acid Symmetrical Anhydrides.—A solution of the Fmoc-amino acid (1.80 mmol) in dichloromethane (10 ml) containing sufficient DMF (drops from a measured 7.5 ml) to ensure complete dissolution was stirred at room temperature and treated with a solution of dicyclohexylcarbodi-imide (DCC) (0.176 g, 0.855 mmol) in dichloromethane (2 ml). With the exception of the dichloromethane-insoluble anhydrides specified below, a rapid formation of the characteristic fine precipitate of dicyclohexylurea (DCU) ensued and after 10 min was removed by filtration. The filtrate was evaporated under reduced pressure (25 °C) and the residue was dissolved in the remaining DMF and added immediately to the reaction vessel at the appropriate step in the solid-phase synthesis cycle.

The symmetrical anhydrides from Fmoc·Cys(Acm)·OH and Fmoc·Ala·OH separated as flocculent precipitates during the activation reaction. In these cases, the reaction mixture was evaporated under reduced pressure, the residue was dried briefly *in vacuo* to ensure maximum removal of dichloromethane and was then shaken with DMF (7.5 ml) and filtered directly into the reaction vessel. Previous experience had shown that the Fmoc·Gly·OH symmetrical anhydride was also prone to co-precipitation, but this did not occur in the present synthesis. Nevertheless, the above procedure was adopted. Fmoc·His(Fmoc)·OH is too insoluble for activation in dichloromethane. This derivative (1.8 mmol) was therefore treated with DCC (0.855 mmol) in DMF solution (7.5 ml) and the total reaction mixture was added to the reaction vessel (without filtration) after 10 min.

Assembly of the Peptidyl-resin Sequence: Fmoc·Glu(OBu^t)·Cys(SBu^t)·Cys(Acm)·Asn·Pro·Ala·Cys(SBu^t)·Gly·Arg(Mtr)·His·Tyr(Bu^t)·Ser(Bu^t)·Cys(Acm)·$OCH_2C_6H_4$(1,4)CO·Nle·$NHCH_2CH_2NHCO$–*Resin* (**12**).—Dimethylacrylamide–ethylenebisacrylamide–acryloylsarcosine methyl ester co-polymer[5] (0.5 g), nominal loading 0.25 mmol g^{-1}, was shaken with redistilled ethylenediamine (16 ml) overnight at room temperature. The derivatised resin was washed successively with DMF (15 × 1 min), 10% ethyldi-isopropylamine in DMF (3 × 1 min), and DMF (5 × 1 min). The amino-resin was acylated with the symmetrical anhydride prepared from Fmoc·Nle·OH (0.636 g, 1.80 mmol). A 7.2-fold excess (based on the nominal loading) of acylating species in DMF (7.5 ml) was used in this and all subsequent acylations. Kaiser[13] and trinitrobenzenesulphonic acid (TNBSA)[14] tests showed the reaction to be complete after 15 min, but the mixture was shaken for 1 h. After the mixture had been washed with DMF (10 × 1 min) the Fmoc group was cleaved with 20% piperidine–DMF (3 min, 7 min) and the resin was washed again with DMF (10 × 1 min). A solution of 1-hydroxybenzotriazole (0.138 g, 0.9 mmol) in DMF (3 ml) was added to the amino-resin, followed after 1 min by a solution of

2,4,5-trichlorophenyl *p*-(hydroxymethyl)benzoate[6] (0.298 g, 0.9 mmol) in DMF (4.5 ml). Insufficient solvent was present in the mixture to produce a mobile slurry and therefore after 50 min a further quantity of DMF (3 ml) was added. Kaiser and TNBSA tests were distinctly positive after 1 h and slightly less so after 3 h. The mixture was therefore shaken overnight, after which the reaction was complete as indicated by the colour tests. The hydroxymethyl resin was washed successively with DMF (10 × 1 min), 10% ethyldi-isopropylamine in DMF (3 × 1 min), and DMF (5 × 1 min) and treated with a solution of the symmetrical anhydride from Fmoc·Cys(Acm)·OH (0.746 g, 1.8 mmol) in DMF (5.5 ml), followed after 1 min by a solution of 4-(dimethylamino)pyridine (11 mg, 0.09 mmol) and *N*-methylmorpholine (0.1 ml, 0.9 mmol) in DMF (2 ml). After the mixture had been shaken 1 h for completion of the acylation, the Fmoc group was cleaved and the assembly was continued by hour-long acylations with the symmetrical anhydrides from Fmoc·Ser(Bu^t)·OH (0.690 g, 1.8 mmol) and Fmoc·Tyr(Bu^t)·OH (0.827 g, 1.8 mmol). In both cases, reaction was complete after 15 min as judged by the Kaiser and TNBSA colour tests. A sample of the resin (*ca.* 5 mg) was removed and deprotected on a small sintered glass funnel by treatment with 20% piperidine–DMF, washed, dried, and hydrolysed with 6M-HCl at 110 °C for 18 h. The tyrosine content was determined as 0.14 mmol g^{-1} compared with a norleucine content of 0.22 mmol g^{-1}. Assuming complete acylation where indicated by colour tests, the low tyrosine figure suggested only *ca.* 64% incorporation of the first residue, Cys(Acm). As a precaution, the presumed unchanged hydroxy group on the peptide-resin linkage agent was blocked by acetylation. Thus the Fmoc-tripeptidyl-resin was treated with a solution of acetic anhydride (85 µl, 0.9 mmol) in DMF (4 ml), followed by a solution of 4-(dimethylamino)pyridine (11 mg, 0.09 mmol) and pyridine (73 µl, 0.9 mmol) in DMF (3.5 ml) and the mixture was shaken for 1 h. After the usual wash and deprotection cycles the assembly was continued with hour-long acylations using pre-formed symmetrical anhydrides (0.9 mmol) of the following protected amino acids or their *p*-nitrophenyl esters (0.9 mmol) in the presence of hydroxybenzotriazole (0.9 mmol). Negative Kaiser and TNBSA tests were obtained on resin samples removed at times indicated: Fmoc·His(Fmoc)·OH (30 min); Fmoc·Arg(Mtr)·OH (15 min); Fmoc·Gly·OH (15 min); Fmoc·Cys(SBu^t)·OH (15 min); Fmoc·Ala·OH (20 min); Fmoc·Pro·OH (20 min); Fmoc·Asn·ONp (colour tests ineffective); Fmoc·Cys(Acm)·OH (15 min); Fmoc·Cys(SBu^t)·OH (15 min); and Fmoc·Glu(OBu^t)·OH (40 min). Samples for hydrolysis and amino acid analysis were removed after addition and deprotection of each residue except the first. The ratios in Table 1 are expressed relative to norleucine = 1.25 up to and including residue 5 (arginine) and relative to glycine = 1.00 thereafter. After the final coupling, the resin was washed with DMF (10 × 1 min) and then transferred to a sintered glass funnel and washed with 1,1-diemthylpropyl alcohol (× 5), acetic acid (× 2), 1,1-dimethylpropyl alcohol (× 5), and ether (× 5). The resin was then dried for 2 h *in vacuo* over phosphorus pentaoxide (yield 0.661 g).

The Stability of t-*Butylthio- and Acetamidomethyl-cysteine Derivatives under Acidolysis Conditions Required to Cleave the Arg(Mtr) Group.*—A 10^{-3}M stock solution was prepared by dissolving the stated amounts of the following derivatives in water (50 ml): H·Cys(Acm)·OH (11.4 mg); H·Cys(SBu^t)·OH (10.5 mg); H·Gly·OH (3.8 mg); and H·Arg(Mtr)·OH (19.3 mg). Aliquots (0.75 ml) of the solution were evaporated to dryness and the residues were treated with TFA (1 ml) or TFA–thioanisole (95:5) (1 ml) for the times indicated (Table 2). The solutions were evaporated to dryness and the residue was partitioned between chloroform and citrate buffer, pH 2.2 (5.0 ml). The aqueous layers were examined by amino-acid analysis; except for H·Arg(Mtr)·OH, ratios were determined using K_F values obtained from an untreated aliquot of stock solution.

Table 2. Amino acid ratios after TFA treatment

(a) TFA alone

Reaction time (h)	1	2	3
H·Cys(Acm)·OH	1.00	0.94	0.99
H·Gly·OH	1.00	1.00	1.00
H·Cys(SBu^t)·OH	1.00	0.94	1.01
H·Arg·OH[a]	0.43	0.62	0.74

(b) TFA–thioanisole (95:5)

Reaction time (h)	1	2	3
H·Cys(Acm)·OH	0.86	0.84	0.87
H·Gly·OH	1.00	1.00	1.00
H·Cys(SBu^t)·OH	0.51	0.45	0.46
H·Arg·OH	0.68	0.85	0.72

[a] Acidolysis product; ratio relative to glycine was taken directly from integrator printout.

Cleavage of the Peptide–resin by Ammonolysis (Small-scale Time–Course Experiments.—A sample of the Fmoc-peptidyl resin (**12**) (10 mg) was treated with 20% piperidine in DMF and then with TFA–water (95:5), as described in the succeeding large-scale experiment, to afford the partially deprotected H-peptidyl resin. Portions (2.5 mg) of this material were separately treated with anhydrous ammonia in methanol (saturated at 0 °C) for 10 min, 1 h, and 21 h at room temperature. After filtration and evaporation, the crude peptide residues were dissolved in 0.2M acetic acid (0.2 ml) and examined by h.p.l.c. (gradient: 15—60% B during 40 min). The residual resins were washed, dried, and assayed for peptide content by hydrolysis and amino acid analysis (see text).

Selection of Phenol as a Scavenger during TFA-cleavage of the Mtr Group.—A crude sample of the Mtr-protected peptide (3.6 µmol) was prepared by TFA-deprotection and ammonolysis of the peptidyl resin (**13**) (30 mg) as described in detail later. Portions (0.36 µmol) of the peptide were treated with TFA alone (100 µl), or in the presence of 5% water, phenol, or thioanisole as scavengers. The reactions were monitored by evaporating aliquots (20 µl) during a period up to 7 h. The residues were dissolved in 1% aqueous acetic acid (20 µl), extracted with ether if necessary (to remove phenol or thioanisole), and examined by h.p.l.c. (gradient: 15—60% B during 40 min) (see text).

H.Glu·Cys(SBu^t)·Cys(Acm)·Asn·Pro·Ala·Cys(SBu^t)·Gly·Arg·His·Tyr·Ser·Cys(Acm)·NH_2 (**14**).—The Fmoc-peptidyl resin (**12**) (0.40 g) was treated twice (5 min and 9 min) with 20% piperidine in DMF (20-ml portions). The resin was washed (3 × 15 ml) successively with each of the following: DMF; 1,1-dimethylpropyl alcohol; acetic acid; 1,1-dimethylpropyl alcohol; DMF; and ether. The resin was dried *in vacuo* over P_2O_5 for 40 min. The peptide–resin was treated three times (20 min each) with portions (15 ml) of TFA–water (95:5), and was then washed (3 × 15 min) successively with each of the following: 1,1-dimethylpropyl alcohol; DMF; 10% ethyldi-isopropylamine in DMF; DMF; 1,1-dimethylpropyl alcohol; DMF; and ether. The product was dried *in vacuo* over P_2O_5 for 1 h to afford the partially protected peptide resin (**13**) (0.42 g). The glycine loading was 0.123 mmol g^{-1}, indicating a potential yield of 52 µmol of peptide.

The resin (0.418 g) was swollen in DMF (2 ml) for 5 min and

Table 3. Hydrolysis results for peptide intermediates

Compound / Degree of purification	(13a) Crude	(14) Crude	(14) After CM52 *ex* aqueous	(14) After CM52 *ex* CH_2Cl_2	(15) After CM52 and G-25
Cys	2.47	2.48	2.57	3.12	0.94
Ser	0.68	0.78	0.61	0.70	0.57
Tyr	0.90	1.04	0.89	0.86	0.93
His	0.74	0.88	0.84	0.81	0.93
Arg	0.87	0.92	0.91	0.94	0.94
Gly	1.00	1.00	1.00	1.00	1.00
Ala	0.96	0.93	0.95	0.94	0.97
Pro	1.03	0.90	1.06	0.97	1.16
Asp	0.98	0.99	0.96	0.97	0.94
Glu	1.02	0.97	1.08	1.05	1.13
Amount (μmol)	53.2	22.4	10.57	11.8	4.39

suspended in ice-cold methanolic ammonia (saturated; *ca.* 60 ml) in a stoppered flask. After being gently stirred at 20 °C for 1 h, the resin was filtered off and washed with methanol (× 5). The residual resin was washed successively with 1,1-dimethylpropyl alcohol, acetic acid, 1,1-dimethylpropyl alcohol, DMF, and ether and was dried (yield 0.273 g); glycine loading 0.007 mmol g^{-1}, corresponding to 1.9 μmol (3.7%) residual peptide or 96.3% cleavage. The ammoniacal filtrate and methanol washings were immediately evaporated to dryness. The residue was dissolved in 0.2M aqueous acetic acid (10 ml) and the solution was freeze-dried to afford the crude peptide (**13a**) (53 μmol); hydrolysis results are shown in Table 3; h.p.l.c., Figure 1.

The crude freeze-dried peptide (53 μmol) was dissolved in a solution of phenol (0.68 g) in TFA (13.6 ml). Progress of the cleavage of the Mtr group was monitored by h.p.l.c. After 7.5 h only traces of starting peptide remained and the solution was evaporated to dryness. The residue was partitioned between water and dichloromethane. The aqueous layer was washed with dichloromethane (× 4) and freeze-dried to afford the crude peptide (**14**) (22 μmol, 42%); hydrolysis results are shown in Table 3. The low recovery prompted further work-up of the dichloromethane layer, which was very cloudy. The combined dichloromethane layers were evaporated to dryness and the residue was partitioned between water (25 ml) and ether (25 ml). The aqueous layer was washed with ether (× 3) and freeze-dried to afford further quantities of peptide (**14**) (30 μmol, 55% recovery). Both crude peptide samples were separately purified by chromatography on (carboxymethyl)cellulose CM52. The sample originating from the aqueous lower layer (22 μmol) was eluted with a linear gradient of 0.01—0.15M ammonium acetate, pH 6.0 (250 ml of each), at a flow rate of 0.53 ml min^{-1}. Peak fractions were combined and freeze-dried to afford peptide (**14**) (11 μmol); hydrolysis results are shown in Table 3. The sample originating from the dichloromethane layer (29 μmol) was eluted with a linear gradient of 0.01—0.15M ammonium acetate, pH 6.0 to afford a second sample of the purified peptide (**14**) (12 μmol); h.p.l.c., Figure 2. The combined yield of purified peptide was 23 μmol (42.5%) (overall yield 96.3% × 42.5% = 40.9%).

H·Glu·Cys·Cys(Acm)·Asn·Pro·Ala·Cys·Gly·Arg·His·Tyr·Ser·Cys(Acm)·NH_2 (**15**).—The remaining stages of the synthesis were carried out using the CM52-purified material derived from the aqueous layer in the previous experiment. A solution of this peptide (**14**) (10.2 μmol) in 0.01M aqueous acetic acid (11.8 ml) was basified to pH 7.8 with dil. aqueous ammonia and diluted with propan-1-ol (12 ml). After the mixture had been stirred under argon for 5 min, 0.6M tri-n-butylphosphine in propan-1-ol (1.6 ml; 0.96 mmol) was added. H.p.l.c. analysis of the solution after 30 min indicated complete conversion into a unique product. After 1 h of reaction, the solution was poured into water (216 ml) under a rapid stream of argon. The mixture was extracted with dichloromethane (6 × 40 ml) under argon to afford a clear aqueous solution. This was assayed using Ellman's reagent and the yield of free thiol groups was determined as 20.1 μmol (100%). The solution (total volume with rinsings, 252 ml) was transferred to a 500 ml beaker, basified to pH 8.3, and stirred gently while exposed to air. The progress of the oxidation was monitored by the Ellman test. After 66 h, 20% of the thiol groups remained unchanged. (In a previous small-scale experiment oxidation had been complete after 39 h) The pH, which had fallen to 7.5, was raised to 8.0 and air was passed into the solution for 24 h. The Ellman assay still indicated a slow conversion rate and it was therefore decided to re-extract the solution with dichloromethane (5 × 50 ml). The aqueous fraction (volume 212 ml) was buffered with ammonium acetate (0.01M) at pH 8.0 and stirred under oxygen saturation for 7 h. A negative Ellman test was then obtained, and the solution was freeze-dried to afford the crude monocyclic disulphide (**15**) (9.35 μmol, 95.5% allowing for Ellman test samples).

A solution of the crude peptide (9.2 μmol) in 0.2M aqueous acetic acid (3 ml) was applied to a column of Sephadex G-25 (83 × 2.5 cm) which was eluted with 0.2M aqueous acetic acid at a flow rate of 0.5 ml min^{-1}, and fractions were collected every 6 min. The absorbance of the eluate was monitored at 278 nm to give the profile shown in Figure 3. Fractions 90—105, corresponding to the major peak, were combined and freeze-

Table 4. Hydrolysis results for conotoxin G1 samples

Compound / Degree of purification	G1 After G-25	G1 CM52 fractions 93—102	G1 After preparative h.p.l.c.	By-product from preparative h.p.l.c.
Cys	2.00	3.80	3.30	3.53
Ser	1.09	0.83	0.83	0.80
Tyr	0.95	1.00	0.97	0.94
His	0.94	1.00	1.01	0.93
Arg	0.89	0.99	0.96	0.95
Gly	1.27	1.00	1.00	1.00
Ala	1.00[a]	0.97	1.15	1.02
Pro	0.89	1.00	1.03	1.02
Asp	1.01	0.99	1.02	0.97
Glu	0.97	1.06	1.08	1.08
Amount (μmol)	2.50	0.937	0.162	0.258

[a] Alanine used as a reference amino acid.

dried (5.3 μmol, 58% recovery). [A forerunning peak, fractions 73—89, presumably contained dimers and/or polymeric peptide material (1.2 μmol).]

The monomeric peptide (**15**) (5.24 μmol) was chromatographed on (carboxymethyl)cellulose CM52, 13 cm × 1 cm column, and was eluted with a linear gradient of 0.01—0.25M ammonium acetate, pH 6.0 (250 ml of each) at a flow rate of 0.5 ml min^{-1} with fractions collected every 6 min. The absorbance of the eluate was monitored at 230 nm to give the profile in Figure 4. Fractions 49—61, corresponding to the major peak, were combined and freeze-dried to afford the *title peptide* (**15**) (4.4 μmol, 84% recovery); hydrolysis results are shown in Table 3; h.p.l.c., Figure 5.

Overall yield at this stage = 95.5% × 58% × 84% = 46.5%.
Combined overall yield = 40.9% × 46.5% = 19%.

Conotoxin G1 (**16**).—A solution of the purified peptide (**15**) (4.21 μmol) in 50% aqueous acetic acid (15 ml) was added during 1 min to a rapidly stirred, freshly prepared mixture of 0.1M iodine in acetic acid (6.7 ml), 50% aqueous acetic acid (8.3 ml), and 1M aqueous hydrochloric acid (0.4 ml). The resulting solution was stirred for a further 10 min before the excess of iodine was quenched by dropwise addition of 1M aqueous sodium thiosulphate. After being concentrated under reduced pressure to *ca.* 6 ml, the solution was applied to a column of Sephadex G-25 (83 cm × 2.5 cm) which was eluted with 0.2M aqueous acetic acid at a flow rate of 0.5 ml/min^{-1}, with fractions collected every 6 min. The absorbance of the eluate was monitored at 278 nm. Fractions 95—110, corresponding to the major peak other than that due to salts, were combined and freeze-dried to afford the crude monomeric peptide (2.50 μmol, 59% recovery); hydrolysis results are shown in Table 4.

The monomeric peptide material (2.42 μmol) was applied to a (carboxymethyl)cellulose CM52 column (14 cm × 1 cm) which was eluted with a linear salt gradient of 0.01—0.15M ammonium acetate at pH 5.9 (150 ml of each) at a flow rate of 0.5 ml/min^{-1}, with fractions collected every 6 min. The eluate absorbance was monitored at 278 nm to give the profile in Figure 6. The major peak had a well-defined shoulder on the leading edge. Fractions before and after the point of inflection were therefore pooled separately. Fractions 93—102 were freeze-dried to afford a sample of nearly pure conotoxin G1 (0.94 μmol, 39% recovery); hydrolysis results are shown in Table 4; h.p.l.c. profile, Figure 7. Fractions 84—92, corresponding to the peak shoulder, were freeze-dried to afford a crude peptide mixture (0.658 μmol, 27% recovery) containing conotoxin G1 and a major by-product in approximately equal amounts. A portion of this crude mixture (0.619 μmol) was dissolved in water (1.5 ml) and separated by preparative h.p.l.c. (Waters semi-preparative μ-Bondapak column; 6 × 25 μl injections to afford a further sample of conotoxin G1 (0.162 μmol, 26% recovery; hydrolysis results are shown in Table 4) and a sample of the unidentified by-product (0.26 μmol, 42% recovery; hydrolysis results are shown in Table 4.

Overall yield at this stage = 59% × 38.7% + 27% × 26%
= 30%
Combined overall yield = 19% × 30% = 5.7%

Both conotoxin G1 samples appeared homogeneous on silica gel t.l.c.; R_F in butan-1-ol–acetic acid–water (66:12:26) = 0.056; R_F in butan-1-ol–pyridine–acetic acid–water (90:18:60:72) = 0.58. The conotoxin G1 sample obtained directly from the CM52 column showed a single spot with fluorescamine or ninhydrin *R*(Lys) = 0.37, on electrophoresis (cellulose plate; pH 6.5; 500 V; 40 min). The sample obtained by preparative h.p.l.c. contained anode-migrating impurities on electrophoresis.

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