Peptide Synthesis. Part 9.[1] Solid-phase Synthesis of Melanin Concentrating Hormone Using a Continuous-flow Polyamide Method

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Synthesis of the heptadecapeptide melanin concentrating hormone using a novel continuous-flow solid-phase method is described. A composite polydimethylacrylamide–macroporous kieselguhr support was used permitting low pressure flow operation. Fluorenylmethoxycarbonylamino acid anhydrides were used throughout for acylation reactions. The choice of compatible side-chain protecting groups is discussed. The synthetic product showed high melanin-concentrating properties *in vitro*.

The chemical synthesis of natural peptides presents two major problems. (i) Development of methods of exceptional chemical cleanliness, mildness, and efficiency for peptide-bond formation between amino and carboxy groups of α-amino acids (or peptides); and at the same time, (ii) development of methods for the protection or otherwise preventing modification of the many reactive groupings present in amino acid side chains. The need for the highest attainable reaction efficiency arises because of the large number of consecutive reaction steps commonly involved. These considerations are especially important in solid phase or other accelerated synthetic methods where the possibilities for intermediate purification are limited, though in any method the occurrence of significant side reactions may result in unacceptably low yields or in intractable mixtures which defy purification. Much progress has been made in establishing reaction conditions for peptide bond formation which meet these requirements. In our own laboratory, we have concentrated on development of the solid-phase method.[2] Recent papers have described the use of new polyamide resin supports,[1,3] fluorenylmethoxycarbonyl (Fmoc) amino protecting groups,[4] and symmetric anhydride[3] and pentafluorophenyl ester[5] coupling procedures which we believe provide a substantially optimised and efficient system. We have also described[1] a novel continuous-flow technique for carrying out solid-phase synthesis.

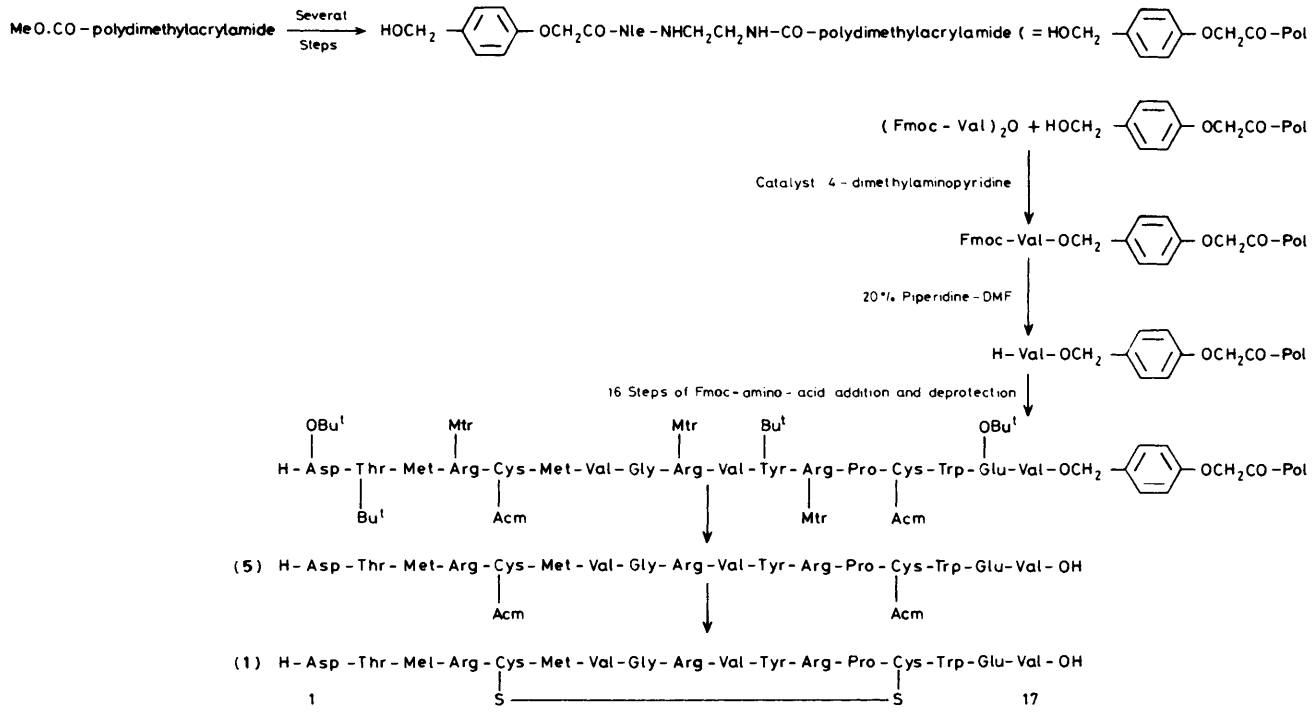
The situation is less satisfactory with regard to amino acid side chain reactivity. Of the 20 amino acids commonly occurring in proteins, 11 possess functional groups which are potentially troublesome in peptide bond-forming reactions. These range from simple hydroxy (serine, threonine, tyrosine), thiol and sulphide (cysteine, methionine), amino (lysine), and carboxyl groups (aspartic and glutamic acids), to the more complex guanidine (arginine), imidazole (histidine), and indole (tryptophan) derivatives. For many of these, simple protecting groups are available which fulfil the necessary criteria, *i.e.* ease of introduction, adequate protection, stability to the conditions of peptide bond formation, and ease of removal under mild reaction conditions. For others, notably arginine and histidine, the requirements vary with the application envisaged and completely satisfactory general procedures may not yet exist. For this reason, much of our current work[6–8] has centred around the problem of reactive side-chain protection, especially in relation to Fmoc-polyamide synthesis. This paper describes some experiences in the synthesis of the complex natural peptide, melanin-concentrating hormone (MCH) (**1**),[9] which includes among its 17 residues, 3 arginines, 2 methionines, a single tryptophan, and 2 cysteines linked by a disulphide bridge.

MCH is a recently discovered hypothalamic hormone which causes melanin concentration in the melanophores of teleost fishes.[10,11] In these species but not in tetrapods[12] it also occurs in the pituitary gland whence it is released into the circulation. The hormone has been purified from the pituitaries of salmon and shown to have structure (**1**). A synthesis using classical solution procedures has been reported by Yajima and his colleagues,[13] and preparation by conventional solid-phase methods reported by Hruby[14] (but see below).

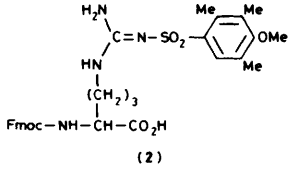
In contrast to other methods, the Fmoc-polyamide procedure uses only neutral or weakly basic reagents during peptide chain assembly. Treatment with acid is usually required only for detachment of the completed peptide from the resin support. This minimisation of contact with acidic reagents has important consequences. Where available, very acid labile t-butyl-based protecting groups may be used for side-chain protection (notably for serine, threonine, tyrosine, aspartic and glutamic acids, and lysine). Furthermore, intrinsically acid-sensitive side-chains such as the indole group of tryptophan may often be left unprotected. For cysteine, the only very acid labile group available is apparently the *S*-trityl derivative. This is highly lipophilic and conflicts with the generally polar character of the Fmoc-polyamide system.[15] We therefore selected the more polar *S*-acetamidomethyl derivative[16,6,17] for protection of this residue. This provides opportunity for disulphide bond formation simultaneously with protecting group cleavage.[18,19] For the three arginine residues, no very acid-labile protecting group is available.[8] Our search for a suitable acid-labile protected derivative had yielded only the N_G-methoxytrimethylbenzenesulphonyl compound (**2**) as the best candidate,[7] though this required conditions for its final cleavage more vigorous (several hours contact with trifluoroacetic acid) than were desirable.† It has proved useful in a number of cases (*e.g.* refs. 7,17) and was selected on this occasion though conflict with unprotected tryptophan was recognised as a significant possibility. The two methionine residues were left unprotected. Again, protection from oxidation and from alkylation during the final stages of protecting group removal was recognised, the latter requiring use of cation scavenging reagents.

Our recently described[1] continuous-flow solid-phase technique was used for the assembly. This technique has thus far been illustrated in detail only for a pentadecapeptide sequence (**3**) related to the adenovirus tail fibre protein. This particular example was chosen from a number of syntheses carried out recently because it allowed the efficiency of peptide bond formation to be seen clearly, relatively uncomplicated by

† More recent results have suggested that protonation of the guanidine function as in the 1-hydroxybenzotriazolide salt may provide an acceptable solution to this problem (see discussion in ref. 8).



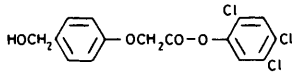
Scheme. Assembly of melanin concentrating hormone (**1**)



(**2**)

H-Lys-Leu-Ser-Val-Ala-Thr-Lys-Gly-ProLeu-Thr-Val-Ser-Asp-Gly-OH

(**3**)



(**4**)

interference from the reactive side chains of the 'difficult' amino acids. The present example has been selected because it is perhaps at the opposite extreme, illustrating problems remaining in side-chain protection (and incidentally progress in product purification). The flow technique allows spectrophotometric monitoring of both acylation and deprotection reactions (see discussion in ref. 1), but in this earlier application it was used only qualitatively.

The assembly was essentially straightforward (Scheme). A functionalised poly(dimethylacrylamide) gel resin supported in macroporous kieselguhr[1,20] was used in a semi-automatic microprocessor controlled synthesizer.[1,21] The preliminary treatments, *i.e.* conversion of the methoxycarbonyl functionalised resin into its amino form, acylation with Fmoc-norleucine anhydride to introduce an internal reference amino-acid for analytical purposes, and deprotection and introduction of the reversible peptide-resin linkage agent using the activated ester derivative (**4**) in the presence of hydroxybenzotriazole catalyst followed standard practice. Approximately two-fold excesses of acylating species were used and the reactions were complete at the time of the first ninhydrin[22] and trinitrobenzenesulphonic acid[23] colour tests for residual amine (25 min). The first amino acid of the sequence proper was esterified to the resin-bound linkage agent, again using the preformed symmetrical anhydride but in the presence of 4-dimethylaminopyridine catalyst. The reaction conditions were similar to those previously found to minimise racemisation[24] in this key step. No colour test is available for assaying completion of this reaction, but subsequent amino acid analysis indicated *ca.* 94% incorporation after 1 h. Residual hydroxy groups do not interfere with subsequent amino acylation reactions in the absence of basic catalysts (including the free imidazole group of histidine), and no blocking off procedure was necessary.

The remaining 16 amino acid residues were all added using preformed symmetrical anhydrides. The reactions appeared to slow quite early with marginally incomplete acylation after 25 min for the t-butyl glutamate (step 2) and acetamidomethyl cysteine (step 4) additions. At this stage the amount of Fmoc-amino-acid anhydride was increased to four-fold. The anhydride of Fmoc-tryptophan precipitated during its preparation and acylation was distinctly incomplete even after 50 min reaction. The reaction was repeated and gave negative colour tests after 25 min. Two arginine additions (steps 9 and 14) and the second cysteine (step 13) were also repeated. Probably the anhydrides of these side-chain protected derivatives are unstable,[25] and do not persist in the reaction medium long enough when the acylation reaction becomes sluggish for steric or other reasons. It is possible that incorporation of the third arginine residue step (6) was also incomplete. Later amino acid analysis of intermediate peptide-resins (Table 1) showed a marked discontinuity at this point, although in the careful purification studies described below, no evidence for a substantial proportion of truncated peptide was found. The amino terminal residue at this stage is proline for which the

ninhydrin test is markedly less sensitive and the trinitrobenzene sulphonic acid test inapplicable.

A number of pilot cleavage, characterisation, and purification experiments were carried out with the completed peptide resin. In the most significant of these, all the acid-labile protecting groups (*i.e.* all except the two *S*-acetamidomethyl derivatives) were cleaved by an extended (5 h) treatment with trifluoroacetic acid in the presence of anisole and ethanedithiol. These scavengers have previously been found effective in the presence of tryptophan and methionine.[26] Analysis of the residual resin indicated that peptide detachment was 90% complete. It should be noted that acid treatment of this severity would normally be expected to cleave the peptide-resin linkage agent quantitatively. Failure to do so on this occasion is almost certainly a result of back addition of the detached peptide to the resin. Presumably this new covalent bonding results from reaction of the benzyl cation derived from the resin-bound linkage agent with the tryptophan, acetamidomethylcysteine, or methionine residues. In other cases and in the absence of scavenging agents, back addition of tryptophan-containing peptides has occurred to the extent of 50%.

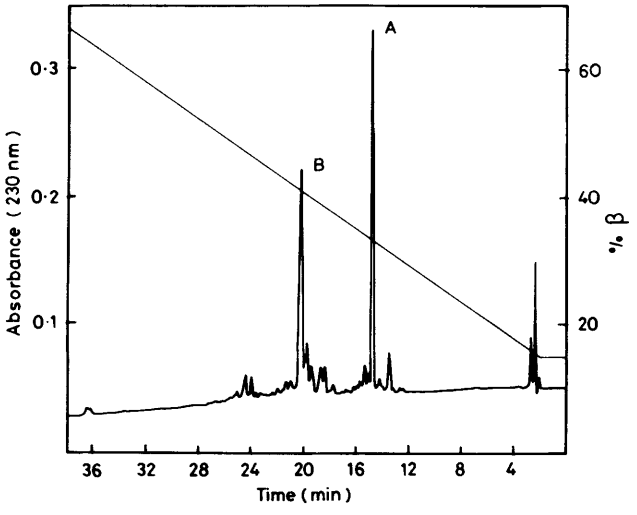


Figure 1. H.p.l.c. of total crude resin cleavage product. Conditions: analytical Aquapore RP 300 column; eluant A, 0.1% aqueous trifluoroacetic acid; B, 90% acetonitrile, 10% A. The column was eluted isocratically for 2 min with 15% B and then with a gradient of 15—100% B over 60 min. Flow rate 1.5 ml/min; chart speed 0.5 cm/min. Optical density was monitored at 230 nm, absorbance range 0.5, cell thickness 1 cm

The soluble part from the cleavage reaction amounted to *ca.* 60% of that expected and gave the h.p.l.c. profile of Figure 1. Although the two principal peaks A and B measured at 230 nm have approximately equal areas, it was shown later that MCH peptide corresponding to A was present to more than three times the extent of that corresponding to B. The latter therefore has anomalously high u.v. absorption at 278 nm. The ratio of the two peaks differs when measured at 250 nm, indicating the presence of an additional chromophore in B. Both gave an amino acid analysis (Table 2) consistent with the MCH sequence, though tryptophan and cysteine were not determined. The crude cleavage product was subjected to anion-exchange chromatography on carboxymethylcellulose. The elution profile (Figure 2) showed the presence of four distinct peaks. All were examined quantitatively for peptide content by total acidic hydrolysis and amino acid analysis (Table 2). Individual fractions from the peaks were characterised by h.p.l.c. (Figure 2, insets). The first eluted (peak C) contained no significant peptide material and is presumably derived from the cleaved protecting groups. Peak D gave the h.p.l.c. profile shown in the inset. It corresponds quite remarkably to almost all the minor impurities observed by h.p.l.c. (Figure 1) in the crude cleavage product. It amounts *in toto* to *ca.* 20% of the peptide material eluted from the ion-exchange column. Peak E contains 60% of the eluted material. On h.p.l.c. it corresponds to the major peak (Figure 1,A) of the initial crude product. Likewise, peak F (20%) corresponds on h.p.l.c. to the second major peak (Figure 1,B) in the crude product.

The purification of the peptide products achieved in this single ion-exchange chromatography is quite remarkable. It is

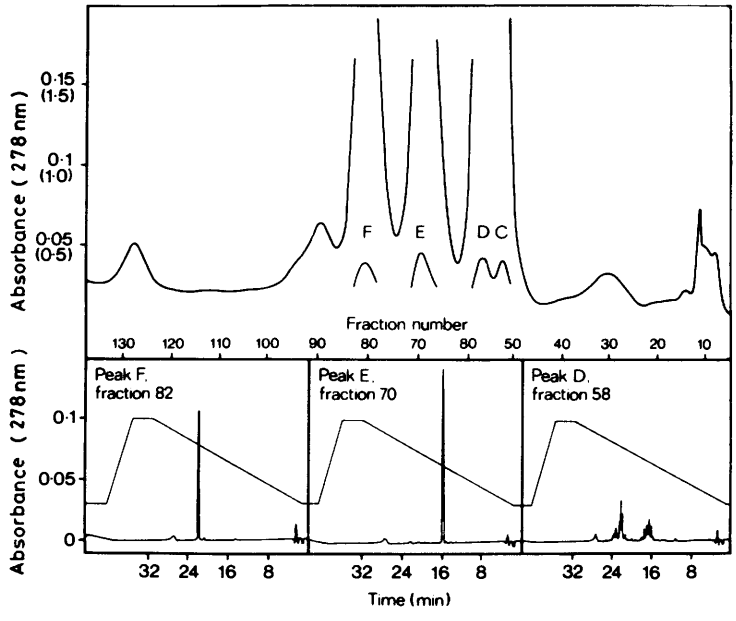


Figure 2. Column chromatography of crude cleavage product on carboxymethylcellulose. For conditions see Experimental section. Insets: h.p.l.c. of peak fractions indicated. H.p.l.c. conditions as in Figure 1 except that the gradient was from 15 to 50% B over 30 min. Optical density was measured at 278 nm, absorbance range 0.2

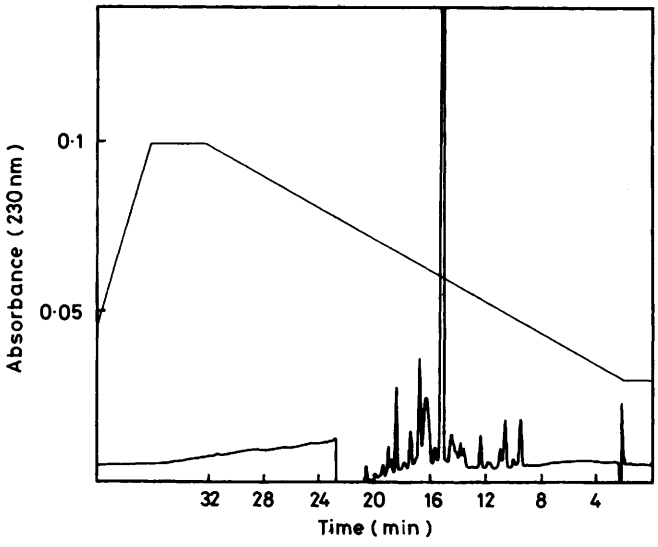


Figure 3. H.p.l.c. of crude oxidation product after desalting on Sephadex G 15. Conditions as in Figure 2 except that the optical density was measured at 230 nm. The baseline was adjusted manually at 23 min

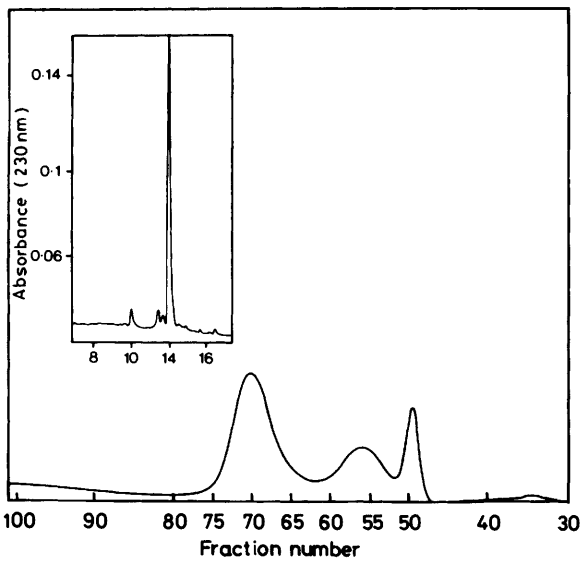


Figure 4. Column chromatography of crude MCH on carboxymethylcellulose. For conditions see Experimental section. Inset: h.p.l.c. of combined fractions 65—77. Conditions as in Figure 3

shown below that the major component peak E does indeed correspond to the linear, *S,S'*-bisacetamidomethyl derivative (**5**) of natural MCH. The order and position of elution of the contaminants from the anion-exchange column suggests that the main by-product (Figure 2,F; Figure 1,B) may bear one additional positive charge (or one less negative charge) than the expected product; alternatively, the clear-cut separation may be an effect of hydrophobicity. Its behaviour on reversed-phase h.p.l.c. is consistent with the latter. The anomalous u.v. absorption suggests it is a by-product of cleavage of the arginine Mtr-protecting groups and/or contains a modified tryptophan residue. In previous applications of the same arginine derivative,[7,17] no similar by-products were observed, though none of these examples contained tryptophan. The sample of protected amino acid used was also of different origin, and isomer formation in the preparation of similarly substituted arylsulphonyl derivatives has been observed elsewhere.[27] The minor impurities present in peak D (Figure 2, and inset) fall neatly into two groups, presumably related to peaks E and F but again probably differing in charge.

The purified linear heptadecapeptide (**5**) comprising peak E was dissolved in glacial acetic acid and oxidised with iodine. These reaction conditions are different from those normally employed for conversion of *S*-acetamidomethylcysteine derivatives into cystine peptides.[18] There were indications in the literature[18] that an anhydrous medium was likely to be favourable in the presence of tryptophan, though side products from oxidation of both this residue and of methionine were to be expected.[19] The crude product was desalted on Sephadex G15 and then examined by h.p.l.c. The profile (Figure 3) showed one very major product with a plethora of very minor impurities. The appearance is reminiscent of that around each of the major peaks in Figure 1. The total product was chromatographed on carboxymethylcellulose and again a substantial purification was achieved (Figure 4). The major peak [synthetic salmon melanin-concentrating hormone (**1**)] gave the h.p.l.c. profile shown in the inset.

The product was identified with natural material at this stage. A very small sample of the natural hormone was generously provided by Dr. Bridget Baker. This was part of the original isolate[9] and had evidently deteriorated substantially on storage. It gave the h.p.l.c. profile of Figure 5(a). The peak indicated corresponded in elution position exactly with that of the synthetic product. We considered it likely that the impurities in the natural material were products of oxidation at one, the other, or both methionine residues, and therefore subjected synthetic MCH to oxidation with hydrogen peroxide. When this oxidation reaction was carried out for 30 min, the h.p.l.c. profile of the product (Figure 5b) coincided in the position of every peak with that of the crude natural hormone. Prolonged oxidation resulted in relative increase of the earliest eluting peak, presumably the disulphoxide. At this stage, preliminary bioassays showed that the synthetic MCH was highly active in causing melanin concentration in rainbow trout scales.

The isolation of MCH was repeated on a modest preparative scale using two batches of 0.5 g of peptide resin. Some modifications were made to the cleavage procedure (see Experimental section) based on small scale h.p.l.c.-monitored experiments, but the results were very similar to those illustrated in Figure 1. Likewise, chromatographic purification of the linear bisacetamidomethylheptadecapeptide (**5**) on carboxymethylcellulose gave an elution profile almost identical with that of Figure 2. Further small-scale experiments indicated that methionine was a useful additive in the oxidative cleavage of the *S*-protecting groups. However on this occasion, it was found that the gel filtration and ion-exchange purification of the final cyclised product could be replaced by simple LH 20 chromatography to give a product of h.p.l.c. quality (Figure 6a) comparable to that previously obtained. The overall yield determined on a weight basis at this stage was *ca.* 26%. The product gave single spots both on the t.l.c. and on electrophoresis and was used for biological evaluation as described below. Further purification could be achieved by reversed-phase h.p.l.c. (Figure 6b).

The melanin-concentrating activity of MCH was tested with an *in vitro* bioassay using scales from the Chinese grass carp (*Ctenopharyngodon idellus*).[28] In this assay which has so far proved to be the most sensitive for MCH, the ED_{50} (*i.e.* half-maximal pigment aggregation) for synthetic (cyclic) MCH was 6×10^{-11} M whereas for linear, Acm-protected MCH the ED_{50} was 2×10^{-8} M. Thus the effect can also be elicited with the linear peptide, but only at a 300 times higher concentration. Since in a recent publication[14] it has been reported that

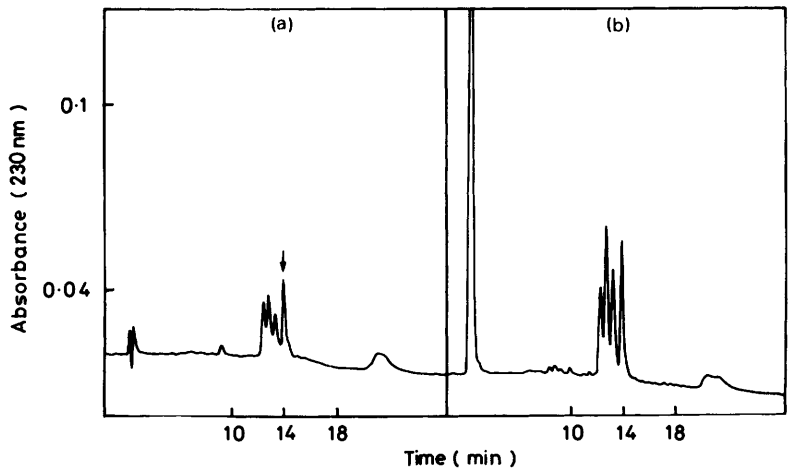


Figure 5. H.p.l.c. of (a) a sample of natural MCH, and (b) synthetic MCH after oxidation with hydrogen peroxide. Conditions as in Figure 3

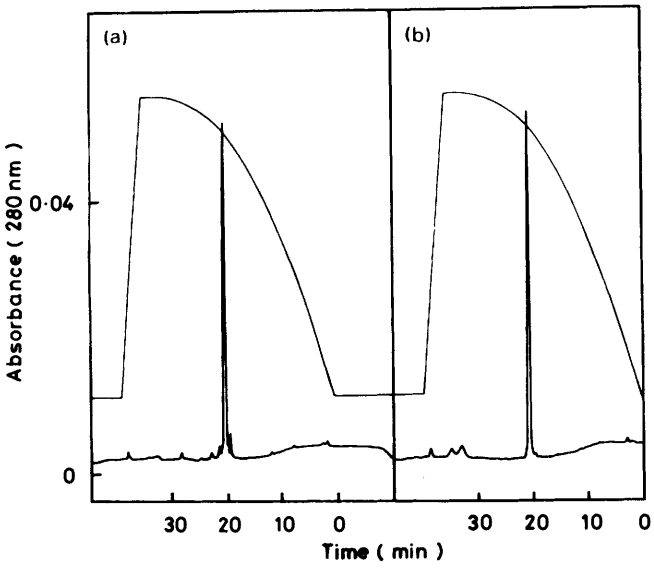


Figure 6. H.p.l.c. of synthetic MCH (a) after purification by chromatography on Sephadex LH20; (b) after purification by h.p.l.c. Conditions: Column analytical Shandon ODS C_{18}. Reservoir A contained 0.1% aqueous trifluoroacetic acid; B contained 70% acetonitrile, 30% A. After isocratic elution with 15% B for 2 min, the convex gradient depicted* (15—70%B over 30 min) was established. Flow rate 1.2 ml/min. Optical density was measured at 280 nm, absorbance range 0.08. * The elution profile shown lags by 2 min from the gradient tracing

synthetic MCH is a full melanophore-stimulating hormone (MSH) agonist in tetrapod melanophores with a potency *ca.* 600 times lower than that of α-MSH, we have also investigated the melanophore-stimulating activity of MCH using the *in vitro* frog skin (*Rana pipiens*)[29] and lizard skin (*Anolis carolinensis*) assays.[30] In contrast to the reported values, we found only a very slight effect at doses of 10^{-5}M in both assays. This means that in our hands the MSH activity of MCH in these two species is at least 10^5 times lower than that of α-MSH. Heating of MCH at 60 °C in 0.1M NaOH for 30 min completely destroyed the melanin-concentrating activity of the peptide in teleost melanophores whereas the MSH activity in tetrapods was 30 times higher.[28]

We conclude that the Fmoc-polyamide solid-phase method can be used effectively for the synthesis of complex peptides containing many 'difficult' amino acid side chains. In the present case, the principal by-product appears to be derived from the arylsulphonyl group protecting the arginine side chains, possibly involving also the tryptophan residue. Previous syntheses including tryptophan or arginine residues but not both have not shown this problem. It is probable that in common with many other side chain-protected arginine derivatives, activated Fmoc.Arg(Mtr) is incompletely stable towards lactam formation and needs to be used with care in acylation reactions. The general problem of arginine incorporation in solid-phase synthesis will be discussed further in a future publication.

Experimental

General procedures for solvent and reagent purification, symmetrical anhydride formation, and for continuous-flow solid-phase synthesis have been given previously.[1]

Solid-phase Synthesis of Melanin-concentrating Hormone.—Kieselguhr-supported poly(dimethylacrylamide) resin[1] (*ca.* 2.5 g, 0.11 m equiv. sarcosine/g) was treated overnight with an excess of ethylenediamine (30 ml), washed briefly with dimethylformamide (DMF) by swirling and decantation, and most transferred to a glass column (*ca.* 44 mm × 15 mm i.d.).[1] The column was attached to the semi-automatic synthesizer previously described[1] and washing with DMF continued until the effluent gave no colour with ninhydrin.[22] Fmoc-norleucine anhydride (0.5 mmol), and *p*-hydroxymethylphenoxyacetic acid trichlorophenyl ester (0.5 mmol) in the presence of hydroxybenzotriazole (0.5 mmol) were coupled successively to the resin as described previously,[1] the intermediate Fmoc derivative being cleaved with 20% piperidine–DMF. Both acylation reactions were complete within 25 min as judged by ninhydrin[22] and trinitrobenzenesulphonic acid[23] colour tests. 4-Dimethylaminopyridine (0.25 mmol) dissolved in DMF (0.5 ml) was added to the top of resin bed, rinsed on with DMF (0.5 ml), and then a solution of Fmoc-valine anhydride (1.25 mmol) in DMF (3 ml) added. The synthesizer was put into recirculation mode and the esterification reaction allowed to proceed for 1 h. Excess of reagents were then washed out with DMF (12 min). The Fmoc group was cleaved with 20% piperidine–DMF (11 min) (this time was used throughout) and washed with DMF (24 min). The following 16 Fmoc-amino-acids were coupled and deprotected using the amounts of anhydride and the coupling

Table 1. Amino acid analysis of intermediate peptide resins

	17	13—17	10—17	5—17	1—17
Nle	1.07	1.20	1.20	1.22	1.19
Val	1.00*	0.93	1.77	2.86	2.61
Glu		1.00*	1.00*	1.00*	1.00*
Pro		1.01	1.07	1.05	1.00
Arg			0.88	1.75	2.51
Tyr			0.81	0.84	0.80
Gly				0.90	0.86
Met				0.82	1.58
Thr					0.79
Asp					0.77

* Values are relative to the amino acid marked set equal to 1.00.

time indicated in parentheses: Fmoc.Glu(OBu^t) (0.5 mmol, 50 min); Fmoc.Trp (0.5 mmol, 50 min; 0.5 mmol, 50 min); Fmoc.Cys(Acm) (0.5 mmol, 60 min); Fmoc.Pro (1 mmol, 50 min); Fmoc.Arg(Mtr) (1 mmol, 60 min); Fmoc.Tyr(Bu^t) (1 mmol, 50 min); Fmoc.Val (1 mmol, 50 min); Fmoc.Arg(Mtr) (1 mmol, 90 min; 0.5 mmol added directly, 110 min); Fmoc.Gly (1 mmol, 80 min); Fmoc.Val (1 mmol, 80 min); Fmoc.Met (1 mmol, 60 min); Fmoc.Cys(Acm) (1 mmol, 90 min; 0.5 mmol, 60 min); Fmoc.Arg(Mtr) (1 mmol, 50 min; 0.5 mmol added directly, 120 min); Fmoc.Met (1 mmol, 90 min); Fmoc.Thr(Bu^t) (1.5 mmol, 90 min); Fmoc.Asp(OBu^t) (1 mmol, 110 min; 0.5 mmol, 75 min). Samples were removed for colour tests after each acylation and deprotection reaction, and for amino acid analysis after the addition of Val-17, Pro-13, Val-10, Cys-5, and Asp-1 (Table 1). At the completion of the synthesis, the resin was removed from the column, washed thoroughly with DMF, 2-methylbutan-2-ol, acetic acid, 2-methylbutan-2-ol, DMF, and ether, and dried *in vacuo* (2.12 g).

A sample of the dried peptide resin (0.202 g) was treated with a mixture of trifluoroacetic acid, anisole, and ethanedithiol (30:1:0.3) for 350 min at room temp. The filtered resin was washed with trifluoroacetic acid and the filtrate and washings evaporated under reduced pressure. The residue was partitioned between water (60 ml) and ether (60 ml), the aqueous layer washed further with ether (4 × 60 ml), the ether extracts back washed once with water and the combined aqueous solution lyophilised. The residual resin was washed thoroughly as above and a sample taken for amino acid analysis (Glu:Nle, 0.08, 91% cleavage). The crude, freeze-dried peptide was dissolved in water (5 ml). Samples (0.1 ml) were withdrawn for amino acid analysis (Table 2) and h.p.l.c. (Figure 1). The residual solution was adjusted to pH 6.0 with dilute ammonium hydroxide and the ionic strength checked as below that of the starting buffer (0.01M-ammonium acetate, pH 6.0). The solution was centrifuged from some insoluble material and applied to a freshly prepared column of carboxymethylcellulose CM 52 (36 cm × 1 cm diam.) which had been equilibrated with 0.01M-ammonium acetate pH 6.0 at 5 °C. The column was developed with a linear gradient of the starting buffer to 0.15M-ammonium acetate, pH 6.0 (260 ml in each reservoir) at 5 °C. Fractions (2.9 ml) were collected every 6 min. The effluent was monitored continuously at 278 nm using a 5 mm silica flow cell and dual absorbance ranges of 0.2 and 2 max. The chart speed was 0.05 cm/min (Figure 2). Fractions from the four peaks were examined by h.p.l.c. (Figure 2, insets). Fractions 62—72, 76—84, 49—54, and 56—60 were separately combined and freeze dried (amino acid analysis, Table 2).

Material (1.22 μmol) from fractions 62—72 was dissolved in glacial acetic acid (4.35 ml), a sample (0.05 ml) removed for h.p.l.c, and the remainder added dropwise over 1 min at room temp to a briskly stirred solution of iodine (0.1M in acetic acid, 1.94 ml, diluted with further acetic acid, 2.52 ml). The mixture was stirred for a further 10 min and then quenched with M aqueous sodium thiosulphate. The mixture was concentrated under reduced pressure to *ca.* 3 ml and then diluted to *ca.* 5 ml with acetic acid to dissolve the precipitate which had formed. The solution was added to a column of Sephadex G15 (25 cm × 2.5 cm diam.) (some further precipitation occurred at this stage and addition of further acetic acid was necessary). The column was developed with 2M-aqueous acetic acid, fractions (3.6 ml) being collected every 6 min. The effluent was monitored at 230 nm with a dual absorbance ranges (0.2 and 2 O.D. units max). The first peak (fractions 19—27) was collected and freeze dried (amino acid analysis, Table 2; h.p.l.c., Figure 3) and then chromatographed on a freshly prepared column (33 cm × 1 cm diam.) of carboxymethylcellulose CM52 under the same conditions as previously. The effluent was monitored at 278 nm (Figure 4). Fractions 65—77 were combined and freeze dried. Amino acid analysis, Table 2; h.p.l.c., Figure 4 (inset).

Table 2. Amino acid analysis of intermediate fractions in the purification of bisacetamidomethyl-MCH and MCH. Columns: (1) crude cleavage product; (2) 1st carboxymethylcellulose chromatography, fractions 56—60 (Figure 2 peak D); (3) fractions 62—72 (Figure 2 peak E, bisacetamidomethyl-MCH); (4) fractions 76—84 (Figure 2 peak F); (5) crude oxidation product after G 15 chromatography (Figure 3); (6) 2nd carboxymethyl cellulose chromatography, fractions 65—77 (MCH) (Figure 4)

	(1)	(2)	(3)	(4)	(5)	(6)
Val	2.57	2.69	3.06	2.95	2.27	2.83
Glu*	1.00	1.00	1.00	1.00	1.00	1.00
Pro	1.08	0.93	1.15	1.11	nd	0.77
Arg	2.47	2.14	2.85	2.74	2.54	2.86
Tyr	0.74	0.76	0.77	0.95	0.76	0.87
Gly	0.83	1.00	1.00	1.00	0.89	1.09
Met	1.58	2.77	1.83	1.75	2.27	0.59
Thr	0.91	0.72	0.96	0.95	0.79	0.99
Asp	0.82	0.78	0.96	0.95	0.93	1.02
Met(O)						0.98†

* All values are based on Glu = 1.00. † The colour constant of aspartic acid was used. The very high proportion of sulphoxide and the general difficulty in obtaining acceptable analyses for methionine may be due to metal ion contamination.

An aliquot (100 μl) from the foregoing MCH preparation (fractions 65—77 dissolved in 3 ml) was treated with hydrogen peroxide (50%, 2 μl). After 30 min at room temp. part (50 μl) was removed for h.p.l.c. (Figure 5b). A second h.p.l.c. carried out after 80 min showed little unchanged starting material remaining and a preponderance of the earliest eluting product, presumably the disulphoxide.

The foregoing isolation and oxidation experiments were repeated using the same peptide resin starting material. Two batches of resin (0.5 g each) were treated with a mixture of trifluoroacetic acid (60 ml), anisole (2 ml), and ethanedithiol (0.6 ml) for 1 h at room temperature with gentle agitation every 3 min. The solutions were filtered and the filtrate evaporated under reduced pressure and the residues re-exposed to a mixture of trifluoroacetic acid (45 ml), anisole (1.5 ml), and ethanedithiol (0.45 ml) for 6, 6.5 h. The solutions were evaporated, re-evaporated with methanol until a dry residue was obtained, dissolved in water (50 ml), and extracted with ether (40 ml), one sample twice and the other five times. Lyophilisation of the aqueous solutions gave 75 mg and 63 mg of crude product respectively. Both preparations gave h.p.l.c. profiles very similar

Table 3. Amino acid analysis of intermediate fractions in the larger scale purification of bisacetamidomethyl-MCH and MCH. Columns: A, crude cleavage product; B, bisacetamidomethyl-MCH after carboxymethylcellulose chromatography; C, MCH after chromatography on LH 20; D, h.p.l.c. purified MCH

	A	B	C	D
Val	2.86, 2.56	2.58	2.31, 2.73	2.60
Glu*	1.00, 1.00	1.00, 1.00	1.00, 1.00	1.00
Pro	— 0.91	— 0.99	0.91, 0.99	0.90
Arg	2.68, 2.51	3.17, 2.82	2.26, 2.77	2.55
Tyr	0.84, 0.84	0.88, 0.96	0.80, 0.97	0.84
Gly	1.02, 0.95	1.04, 1.08	1.00, 1.10	0.99
Met	0.18, 0.19	1.94, 1.78	1.51, 1.55	0.77
Thr	0.82, 0.79	0.95, 0.72	0.86, 0.73	0.98
Asp	1.03, 0.81	1.00, 0.93	0.83, 0.83	0.93
Met(O)†	1.66, 1.25	— —	0.10 —	1.08

* All values are based on Glu = 1.00. † The colour constant for aspartic acid was used; see footnote to Table 2.

to that of the crude bis(acetamidomethyl) linear heptadecapeptide (**5**) shown in Figure 1. Amino acid analysis, Table 3.

The two samples were chromatographed individually on columns (35 cm × 1 cm diam.) of carboxymethylcellulose CM 52 using the same gradient of aqueous ammonium acetate as before. The elution profiles were very similar to that of Figure 2. The appropriate peaks were collected and lyophilised yielding 20.5 and 19.5 mg of purified heptadecapeptide derivative (**5**), amino acid analysis, Table 3.

After a number of small-scale preliminary experiments, the following procedure was adopted for oxidative closure of the disulphide ring. A solution of the linear heptadecapeptide (**5**) (4.9 mg, 2 μmol) in glacial acetic acid (2 ml) containing L-methionine (1.49 mg, 10 μmol) was added dropwise over 5 min under a nitrogen atmosphere to a vigorously stirred solution of iodine (101.6 mg, 400 μmol) in acetic acid (20 ml). The mixture was stirred for a further 25 min and concentrated aqueous ascorbic acid solution added until colourless (*ca.* 6 ml). The solution was concentrated under reduced pressure to not less than 5 ml and applied directly to a column of Sephadex LH 20 (140 cm × 3.5 cm diam.) packed in 1% acetic acid and eluted with the same solvent. The effluent was monitored at 280 nm. Fractions (10 ml) were collected every 10 min. Selected individual fractions were examined by h.p.l.c. before being combined and lyophilised. Fractions 53—57 furnished 2.95 mg, h.p.l.c. Figure 6a. Amino acid analysis Table 3. Fractions 51,52 and 58—62 were pooled, lyophilised, and further purified by h.p.l.c. to give a further 0.95 mg, h.p.l.c. Figure 6b. The total yield was thus 3.9 mg (80%). A second similar experiment gave 3.8 mg. The synthetic melanin-concentrating hormone gave single spots on t.l.c., developed with ninhydrin and the Reindel-Hoppe reagent[31] in butan-1-ol–acetic acid–pyridine–water (50:12.5:12.5:25), R_F 0.35, and butan-1-ol–acetic acid–pyridine–water (42:4:24:30), R_F 0.27. Biological activity is discussed in the text.

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