Peptide Synthesis. Part 3.† Comparative Solid-phase Syntheses of Human β-Endorphin on Polyamide Supports using t-Butoxycarbonyl and Fluorenylmethoxycarbonyl Protecting Groups

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Solid-phase syntheses of the 31 residue peptide human β-endorphin on polar polyamide supports are described. Minimisation of acidic reaction conditions by replacement of the customary t-butoxycarbonyl and benzyl-based protecting groups by fluorenylmethoxycarbonyl and t-butyl gave a higher yield of purer product.

Previous papers in this series have described new methods of solid-phase peptide synthesis based on polar polydimethylacrylamide supports [1a,b] and base-labile, fluorenylmethoxycarbonylamino-acids.[2] In the development and evaluation of these methods we have carried out exploratory syntheses of a number of substantial peptide sequences, selected both for their suitability as test cases and for their biological importance. This paper describes early experiences in the endorphin series; that following [3] deals with more recent experiments on the synthesis of gastrin peptides.

In essence the polyamide method [4] seeks to improve the efficiency of solid-phase synthesis, (i) by providing an environment within the polymer matrix which is substantially optimised for the chemical processes involved, and (ii) by reducing the extent of destructive side reactions. In (i) consideration has been given to solvation of both polymer and reactants by media appropriate to the various chemical steps in polypeptide (and oligonucleotide [5]) synthesis. The development of polar, polydimethylacylamide resins [1] freely permeated and solvated by dipolar, aprotic solvents of the dimethylformamide type has constituted a step forward in this direction. The choice of reaction medium need no longer be substantially dictated by the apolar character of previous supports (*e.g.* polystyrene). It is our belief that aggregation phenomena within the resin matrix may be correspondingly reduced by the enhanced solvation of both resin and reactants now possible. In (ii), we have sought to reduce the incidence of side reactions through mildening of reaction conditions. This has required reconsideration of overall protecting group strategy. Utilisation of base-labile, fluorenylmethoxycarbonyl (1) (Fmoc) amino-acids in place of the customary acid-labile t-butoxycarbonyl (Boc) derivatives (2) goes far to achieve this objective and is discussed below.

In our development of the polyamide method, we have preferred to meet these objectives one at a time. Thus the two syntheses of human β-endorphin described below utilised in the first [6] the polydimethylacylamide resin with a conventional Boc-benzyl combination of protecting groups, and in the second [7] with the newer Fmoc-t-butyl-*p*-alkoxybenzyl combination. It should be emphasised that all the synthetic work described was of an early, exploratory nature. The problems encountered and progress towards their solution are fully reported here. The Boc-benzyl synthesis was carried out in 1976/77,[6] before h.p.l.c. methods were routinely available in our laboratory. The Fmoc-t-butyl synthesis was completed in 1978.[7] The experience accumulated would doubtless enable new syntheses to be carried out with greater efficiency, as is demonstrated in the following paper.[3]

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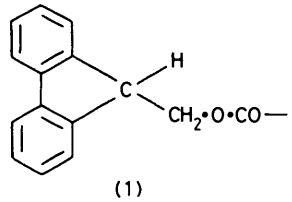


(1)

$Me_3C•O•CO—$

(2)

$CH_2=CH•CO•NMe_2$

(3)

$(CH_2=CH•CO•NH•CH_2—)_2$

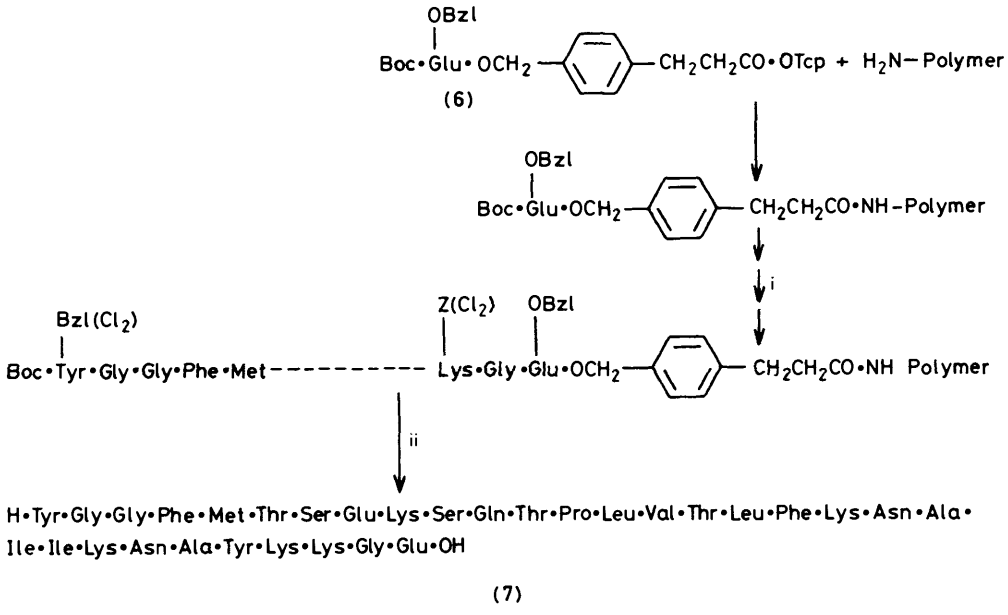
(4)

$Boc•NHCH_2CH_2CO•NH(CH_2)_6NH•CO•CH=CH_2$

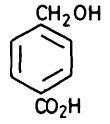
(5)

The resin used in the first synthesis was the original [1a] amorphous copolymer of *N,N*-dimethylacrylamide (3), *N,N'*-bisacryloylethylenediamine (4), and *N*-acryloyl-*N'*-t-butoxycarbonyl-β-alanylhexamethylene diamine (5). This polymer contained at the onset all the chemical features we considered desirable at that time for solid-phase peptide synthesis, *viz.*, a polar matrix, stable cross-links, a flexible spacer arm, an internal reference amino-acid (β-alanine), and a (protected) primary amino-group suitable for attachment of an appropriate peptide-resin linkage agent. The amorphous nature of this early polymer has been considered disadvantageous by others,[8] but in our hands it did not cause any insuperable difficulties. Blockage of the reaction vessel sintered glass filter often occurs once or twice in the early stages of a synthesis due to trapping of fine resin particles, but replacement of the vessel while the original is cleaned solves the problem which does not usually recur in the later stages. In any event, an alternative polydimethylacrylamide resin is now available [1b] in fully beaded form which is correspondingly easier to handle.

The first synthesis (Scheme 1) based on combination of Boc and benzyl protecting groups was commenced by addition of further spacer and internal reference β-alanine and norleucine residues. The latter gives a much higher ninhydrin colour yield in amino-acid analysis than does β-alanine, and allows greater analytical accuracy, particularly in assessing peptide loss from the resin. These and all subsequent amino-acid additions utilised symmetrical anhydride derivatives preformed with dicyclohexylcarbodi-imide in dichloromethane



Scheme 1. *Reagents*: i, 30 cycles of deprotection and amino-acid addition; ii, HF



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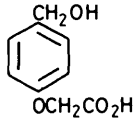


(9)

solution and added in dimethylformamide to the swollen resin.

The reversible peptide-resin linkage agent together with the first amino-acid (glutamic acid) of the endorphin sequence (7) were added together as the activated ester derivative (6). This procedure is one which we introduced early in our studies on solid-phase synthesis.[1a] Providing that the appropriate derivative analogous to (6) can be easily made, it offers a simple, effective, and general way of initiating the synthesis which avoids any problems of esterification of the first residue to the resin. However, the introduction of 4-dimethylaminopyridine as an efficiency catalyst for ester bond formation [9] and also the use of protected amino-acid caesium salts [10] provide alternative procedures of which the first is illustrated later.* The recent recognition that 4-dimethylaminopyridine is also an effective racemising agent for activated, urethane protected amino-acids indicates the need for great caution in its future use.[11]

The following 30 residues of the endorphin sequence (7) were added sequentially using the symmetrical anhydride procedure. Intermediate t-butoxycarbonyl derivatives were cleaved using 1M-HCl/AcOH and the resulting hydrochloride salts neutralised with di-isopropylethylamine. Side-chain protecting groups used were 2,4-dichlorobenzyloxycarbonyl (lysine),[12] 2,6-dichlorobenzyl (tyrosine),[13] benzyl (serine, threonine, and glutamic acid), and xanthydryl (asparagine and glutamine). The final two glycine residues were radiolabelled with tritium (3.75 mCi/mmol). A modified Beckman 990 Peptide Synthesiser was used for washing and deprotection steps with manual intervention for preparation and introduction of symmetrical anhydrides, and removal of resin samples for ninhydrin tests and amino-acid analysis. Ninhydrin tests were in all cases negative at the first determination (10—20 min), and no repeated acylations were necessary. Samples for amino-acid analysis and for solid-phase Edman degradation [14] studies were taken at appropriate intervals. The former enabled a nearly complete picture of amino-acid incorporation (Table 1, Experimental section) to be built up within the limits of the analytical method, but only 1—2 days after the relevant part of the sequence had been assembled. Likewise, solid-phase Edman degradation provided information about the course of the synthesis only after it had been completed. Neither technique was thus of particular value in a control sense, and qualitative ninhydrin colour reactions [15] provided the only reassurance that synthesis was proceeding efficiently.

The standard conditions of hydrolysis employed (6M-HCl, 110 °C, 18 h) gave incomplete cleavage of the Ile-Ile bond in the sequence (7), yielding at most only 1.5 residues of isoleucine. This result is regularly obtained for other sequences containing Ile-Ile bonds, *e.g.* pancreative trypsin inhibitor. Other residues, *e.g.* serine, were significantly destroyed during hydrolysis, but within these limitations and the overall accuracy expected for resin-bound peptide analysis, the results in this first assembly were good up to residues 29 and 30 (from the C-terminus) (Table 1). At this stage, the analytical figures indicated a substantial fall in amino-acid incorporation even though no residual amino-groups remained as indicated by the ninhydrin tests. The amino-acid residues in these positions are both glycine, significantly the amino-acid selected for introduction of the tritium radiolabel. Later results leave no doubt that the cause of this irreversible chain termination was use of impure radioactive Boc-glycine. In order to make efficient use of the costly tritiated glycine and to maintain a high level of radioactivity, conversion of the labelled amino-acid into its t-butoxycarbonyl derivative had been carried out on a very small scale and the product had not been purified by recrystallisation. The nature of the contaminant is not known, but its effect was disastrous. This assembly of the endorphin sequence was abandoned.

A second assembly was commenced immediately. With the experience gained, rapid progress was made, and addition of

* We have recently shown that the latter procedure is also applicable in the polyamide series using the benzyl chlorides corresponding to linkage agents (8) and (9).

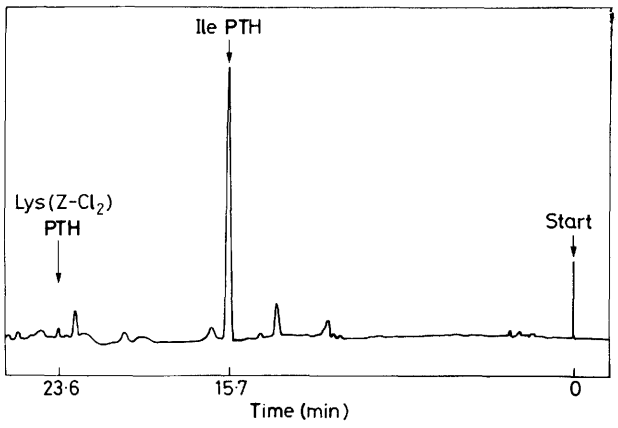
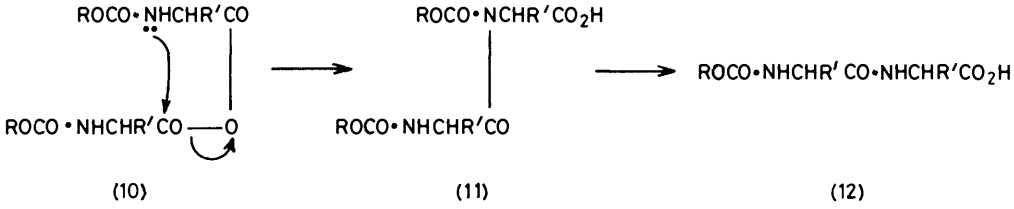


Figure 1. H.p.l.c. of Edman degradation product of peptide resin terminating in Ile-23 (Boc-benzyl synthesis). The elution position of the authentic phenylthiohydantoin of N_{ε}-dichlorobenzyloxycarbonyl lysine (residue 24) is shown

ated inadvertent introduction of multiple residues.* The latter could arise through premature deprotection of the chain terminus during the actual acylation reaction, or through the presence of dipeptide derivatives in activated protected amino-acid preparations. At residues 22 and 23 (Ile-Ile), t.l.c. showed an additional minor spot which could have indicated partial omission of isoleucine and preview of the phenylthiohydantoin of N_{ε}-dichlorobenzyloxycarbonyl-lysine. The degradation was repeated on a resin sample terminating in Ile-23 and the phenylthiohydantoins analysed by h.p.l.c. (Figure 1). A very minor peak coincident with the phenthiohydantoin of the lysine derivative was found in the first cycle of the degradation. The amount present was estimated to be less than 1%.

Merrifield, Mitchell, and Clarke [16] have suggested that rearrangement of the symmetrical anhydrides of protected, unhindered amino-acids, *e.g.* as in (10) → (11) → (12), may be a significant side reaction in solid-phase peptide synthesis, leading through further activation of (12) to partial incorporation of multiple residues. If it occurred, this side reaction might be



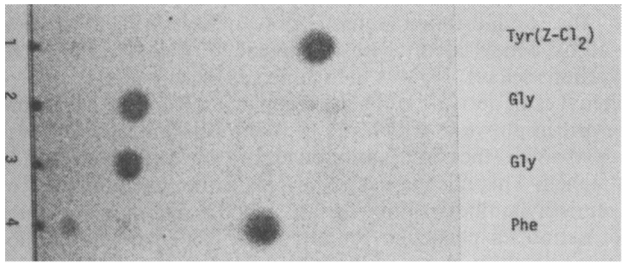


Figure 2. T.l.c. of the phenylthiohydantoins from four cycles of Edman degradation on the completed 31-residue peptide-resin (Boc-benzyl synthesis)

all 31 amino-acid residues was completed within five days. Resin samples for amino-acid analysis and Edman degradation were collected as before, but no analytical results were available during the course of the synthesis. Special care was taken with the preparation of the radiolabelled glycine which was now used only for the penultimate residue. The assembly proceeded smoothly with negative ninhydrin tests at every step. Efficient amino-acid incorporation throughout the entire sequence was later indicated by the amino-acid analysis results (Table 2, Experimental section), although the initial loading of the linkage agent and first amino-acid was less than usual.

Solid-phase Edman degradation was carried out on samples removed after addition of each ninth or tenth amino-acid residue and followed the technique already described [14] for the sequencing of natural peptides attached to the same polyamide resin. Liberated phenylthiohydantoins were examined by t.l.c. for evidence of premature release of the following residue in the sequence (' preview '), which would have indicated partial amino-acid omission, and for ' carry over ' of one amino-acid into the next position in the sequence which might have indicmore serious in the polar solvent and resin environment of the polyamide method. It would be seen most clearly in sequences of contiguous glycine residues. Such a sequence is present at the amino-terminus of β-endorphin, and the Edman degradation of the completed resin-bound peptide was examined carefully for this possibility. No evidence was found for release of more than the slightest trace of glycine at the fourth cycle (Figure 2), indicating the absence of significant contaminating sequences containing three or more consecutive glycine residues. It is possible that the earlier observations [16] may be ascribed in part or wholly to premature loss of the very acid-labile biphenylisopropoxycarbonyl group used rather than to rearrangement of the protected glycine anhydride. Such premature deprotection could easily occur through the presence of traces of acid in the dichloromethane used as solvent. In contrast, the dimethylformamide used in the present work has weakly basic properties.

Although interpretation of extended Edman degradation is often complicated by accumulation of artefacts from the degradation reactions themselves which give t.l.c. and h.p.l.c. spots and peaks not corresponding to the phenylhydantoins of any of the constituent amino-acids, the method provided in this case encouraging further support for efficient amino-acid incorporation throughout the entire assembly.

Eighty-five percent of the peptide chains were retained on the resin during the synthesis. Treatment with liquid hydrogen fluoride cleaved 85% of the peptide within 1 h at 0 °C. The total product was chromatographed on Sephadex G50 (Figure 3) (63% recovery) and the main peak on carboxymethylcellulose (Figure 4) (24% recovery), desalted on Sephadex G25 and chromatographed again on carboxymethylcellulose (85% recovery). It emerged from this last

* ' Carry over ' can also arise, in principle, from incompleteness of the previous degradation cycle.

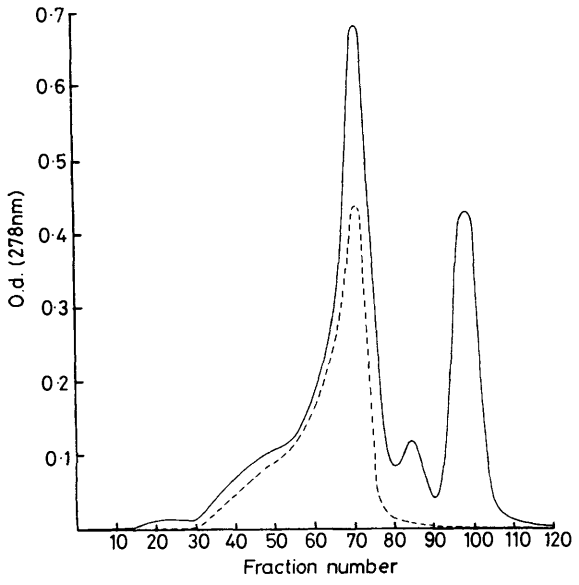


Figure 3. Gel filtration on Sephadex G50 of crude synthetic β-endorphin (Boc-benzyl assembly). Fractions 59—67 omitting aggregated high molecular weight material were collected for further purification. Solid line—optical density; broken line—radioactivity

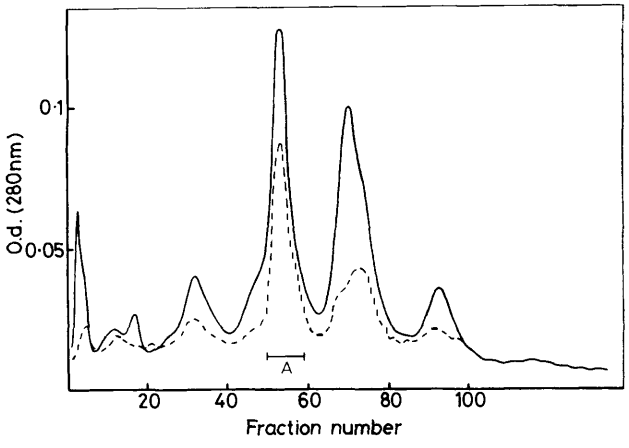


Figure 4. Anion-exchange chromatography of crude β-endorphin (Boc-benzyl assembly) on carboxymethylcellulose in the presence of urea. The fractions marked A were collected. Solid line—optical density; broken line—radioactivity

column essentially as a single peak and at this stage gave a single ninhydrin and fluorescamine reacting spot on both paper electrophoresis and thin layer chromatography. The chlorine-tolidine spray reagent revealed a second spot identified in three t.l.c. systems as urea. The use of urea in the chromatographic eluants was subsequently found to be unnecessary.

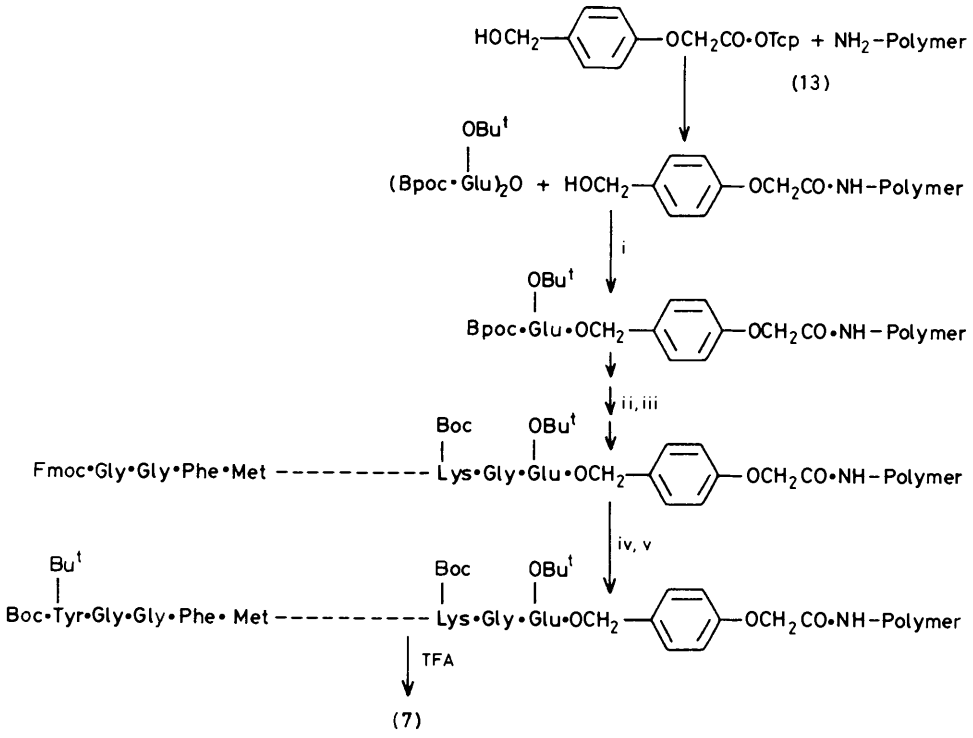
The overall yield was 9.2% (see below). The freshly prepared synthetic endorphin was judged by Dr. J. F. W. Deakin of the National Institute for Medical Research, London, to be nearly equiactive in the rat tail flick test with natural ovine endorphin (kindly provided by Dr. D. Smythe). Likewise Dr. R. Dingledine of the MRC Neurochemical Pharmacology Unit found the endorphin to be somewhat more active in binding studies (displacement of tritiated diprenorphine and etorphine) than was a synthetic sample purchased from a commercial laboratory. Some months later, however, bioassays by Dr. G. E. Metcalf of Reckitt and Coleman, Hull, gave much lower potencies. This decline in activity appears to be associated with post-synthesis oxidation of the methionine residue at position 5 in the enkephalin sequence, probably accelerated by the radiolabel at position 2. Hydrolysis of the peptide-resin five months after synthesis showed reduced methionine and the presence of a high proportion of methionine sulphone.

The same polydimethylacrylamide support was used in the second endorphin synthesis but the protecting group strategy was entirely different (Scheme 2). We have discussed in detail previously [2] the advantages (in our view) of the fluorenylmethoxycarbonyl (Fmoc) t-butyl-*p*-alkoxybenzyl protecting group system. Briefly, replacement of the customary acid-labile t-butoxycarbonylamino-acids by base-labile fluorenylmethoxycarbonyl derivatives removes the element of selectivity in protecting group removal at each deprotection step. It dispenses with the repeated acid treatments which characterise the conventional method and replaces them with weak base (secondary amine) treatments. Most importantly simultaneous replacement of benzyl-based side chain and carboxy terminal (resin linkage) protecting groups by t-butyl and/or *p*-alkoxybenzyl derivatives enables the vigour of the final deprotection and resin cleavage step to be much reduced. The brutal treatment with liquid hydrogen fluoride or hydrogen bromide in trifluoroacetic acid which causes many known side reactions and has long been a weakness of the conventional method, can be replaced by, *e.g.*, trifluoroacetic acid with substantial advantage. This second assembly of the endorphin sequence was carried out primarily to see how far these supposed advantages might be realised in practice.

Synthesis was commenced in the usual way by addition of a permanently bound internal reference amino-acid (norleucine) to the amino-functionalised polyamide resin. The new *p*-alkoxybenzyl alcohol linkage agent (13) (for an improved synthesis see ref. 17) was then added as its activated trichlorophenyl ester derivative. This is a departure from the procedure described above in which the peptide-resin linkage agent was added with the first amino-acid of the sequence already attached. This change has its origins in the introduction [9] of 4-dimethylaminopyridine as an effective catalyst for ester formation, in particular for attachment of protected amino-acids to resin supports.[18] It enabled this key step to be carried out efficiently under mild conditions and seemingly reduced the advantage of prior formation of the ester bond in solution which was a feature of our original polyamide method.[1a] More recently, however, a significant disadvantage of the use of 4-dimethylaminopyridine has emerged with the recognition that it is an effective racemising agent for activated acylamino-acids, even when the acyl residue is a urethane protecting group.[11] This may have significance in the present synthesis (see below). Fluorenylmethoxycarbonyl derivatives are appreciably labile to 4-dimethylaminopyridine [2] and it was therefore thought appropriate to use the base-stable, very acid-labile biphenylisopropoxycarbonyl (Bpoc) derivative of γ-t-butylglutamic acid for addition of the first residue only.*

After cleavage of the Bpoc protecting group using 0.09M-hydrogen chloride in acetic acid and neutralisation with tertiary base, the following 29 residues were added sequentially using Fmoc-amino-acids exclusively. Side chains were protected as the appropriate t-butyl derivatives, *viz.* t-butyl ethers (serine, threonine, and tyrosine), t-butyl ester (glutamic acid), or t-butoxycarbonyl derivatives (lysine).

* Subsequent experience has shown that Fmoc-amino-acids may in fact be used in the presence of 4-dimethylaminopyridine without detectable loss of the protecting group in the short acylation periods usually required.



Scheme 2. *Reagents*: i, 4-Dimethylaminopyridine; ii, HCl–AcOH; iii, Pr^i_2NEt; then 29 cycles of Fmoc-amino-acid addition and deprotection; iv, piperidine deprotection; v, Boc.Tyr(Bu^t) anhydride

Preformed symmetrical anhydrides of the Fmoc-amino-acids were used for acylation except for asparagine and glutamine. The latter were now introduced using *p*-nitrophenyl esters in the presence of catalyst, hydroxybenzotriazole. Intermediate Fmoc-peptide resins were deprotected with 20% piperidine following our standard ' Fmoc-t-butyl ' procedure.[2] Resin samples were removed regularly for ninhydrin tests and at intervals for amino-acid analysis, but not for solid-phase Edman degradation. The combination of protecting groups and resin linkage employed renders this last technique inapplicable since detachment from the resin would occur at the first cycle. On the other hand, the relative ease of detachment of intermediate peptides from the resin by trifluoroacetic acid (compared with liquid hydrogen fluoride in the ' Boc-benzyl ' procedure) allows t.l.c. and h.p.l.c. examination of side chain deprotected peptides to be made easily and rapidly. Extensive use was not made of this facility in the present synthesis, but more recent studies [19] have demonstrated its value.

Apart from a single case where the anhydride of the Fmoc-amino-acid (glycine) precipitated during its preparation and was removed along with the dicyclohexylurea, ninhydrin tests were negative throughout and no repeated acylations were judged necessary. Amino-acid incorporation (Table 3, Experimental section) was comparable to that of the foregoing synthesis except for a 25% irreversible chain termination at step 4. Partial failures in couplings involving Fmoc-lys(Boc) have been observed in other sequences,[2] but their cause remains particularly obscure. They are not due solely to the presence of an impurity in the particular protected amino-acid preparation, for the same sample was used for all five lysine residues in the endorphin sequence and the problem occurred at only one. Later syntheses have avoided this problem by using (with advantage) Fmoc-lys(TFA) for the introduction of lysine,[20] and our colleagues [21] have found that use of Fmoc-Lys(Boc)-ONp also appears to overcome the difficulty. In the present synthesis, the short truncated peptide was easily removed during purification and presented no particular difficulty, but interpretation of the amino-acid analysis figures for subsequent resin-bound peptide (always problematic when multiple residues of the same amino-acid type are present) was made exceedingly difficult. The last amino-acid of the endorphin synthesis (tyrosine) was conveniently added as its N_α-Boc-*O*-t-butyl derivative, enabling detachment from the resin and cleavage of all protecting groups to be achieved in a single step.

Trial cleavages using aqueous trifluoroacetic acid in the presence of anisole with ion-exchange and then h.p.l.c. examination of the products revealed substantial formation of sulphoxide derivative. This was confirmed by hydrogen peroxide oxidation of the endorphin-endorphin sulphoxide mixture resulting in depletion of the h.p.l.c. peak assigned to the former and increase in the latter. Sulphoxide formation was much reduced in the presence of excess of methionine as scavenger, and these conditions were adopted preparatively. The cleavage yield was 90%. A much purer product was obtained than in the foregoing ' Boc-benzyl ' synthesis. A single carboxymethylcellulose chromatography gave the elution profile shown in Figure 5 which is to be compared with that of Figure 4 previously obtained. Material recovered from the major peak gave a single ninhydrin and fluorescamine-reacting spot on paper electrophoresis and t.l.c., and the amino-acid analysis was good. The h.p.l.c. is shown in Figure 5 (insert). The contaminant on the leading edge coincides in elution position with endorphin sulphoxide; the identity of that on the trailing edge has not been established but present knowledge [11] would suggest that partial racemisation (up to *ca.* 5%) of the carboxy terminal residue might be anticipated under the conditions used for its attachment to the resin. In the gastrin series we have observed similar separation on h.p.l.c. of main peak and carboxy terminal diastereisomeric contaminant. The overall yield at this stage of purity was 41%, disregarding chains terminated at step 4 in the synthesis.

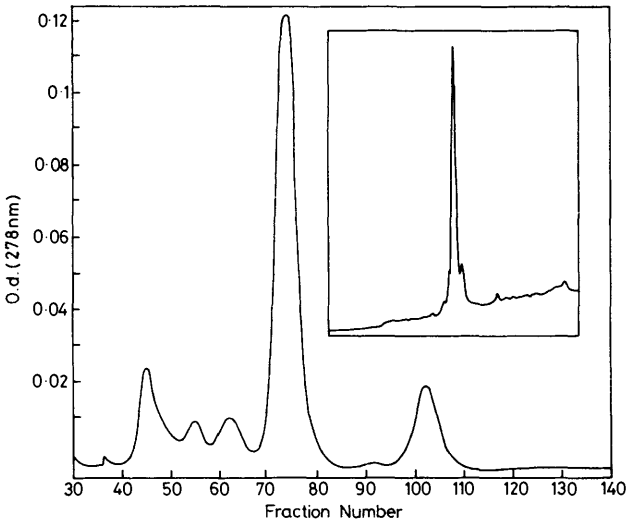


Figure 5. Chromatography of crude β-endorphin (Fmoc-t-butyl synthesis) on carboxymethylcellulose. Inset: reversed phase h.p.l.c. of main peak

The conclusions to be drawn from these two syntheses appear to be quite clear. That based on the Boc-benzyl protecting group combination gave a substantially lower yield than did the Fmoc-t-butyl synthesis. There is evidence of aggregation (Figure 3) and major by-product formation in the former (compare Figures 4 and 5). In both syntheses assembly of the polypeptide chain appeared to be efficient using the polar, polyamide method, apart from the instances described which are attributable to specific amino-acid residues or samples. Thus the differences in the two syntheses reflect largely the extensive and vigorous acidic treatments in the first compared with the milder basic and reduced acidic treatments in the second. In our view the single most deleterious factor in the first synthesis is likely to be the final cleavage of completed peptide from the resin support by liquid hydrogen fluoride. Supporting evidence comes from the more recent description [22] of a synthesis of β-endorphin using wholly solution methods, in which purified fully protected peptide was deprotected by liquid hydrogen fluoride. The yield in this step was only 13%. On the other hand, Li and his colleagues [23] have also reported an overall yield of 30% in a solid-phase assembly of human β-endorphin which included use of hydrogen fluoride. In the light of this, we carried out further hydrogen fluoride cleavage and peptide isolation experiments mimicking as closely as possible the described [23] procedures. The yield of endorphin was 13%.

Experimental

N_α-t-Butoxycarbonyl-N_γ-xanthydryl-L-asparagine.—t-Butoxycarbonyl-L-asparagine (4.65 g, 20 mmol) was dissolved in glacial acetic acid (120 ml) and xanthydrol (3.97 g, 20 mmol) added. The mixture was stirred for 63 h at room temperature, poured into stirred water (200 ml), and the precipitated product collected, washed thoroughly with water, and dried (P_2O_5) *in vacuo*. Crystallisation from ethyl acetate–light petroleum gave the xanthydryl derivative (3.1 g, 37%), m.p. 183—185 °C, $[\alpha]_D$ +4.4° (*c*, 1 in DMF) [lit.[24] m.p. 183—184 °C, $[\alpha]_D$ +2.1° (DMF)] (Found: C, 64.15; H, 6.01; N, 6.55. Calc. for $C_{22}H_{24}N_2O_6$: C, 64.07; H, 5.87; N, 6.79%).

N_α-t-Butoxycarbonyl-N_δ-xanthydryl-L-glutamine.—This compound was prepared in a similar manner to the foregoing in 39% yield, m.p. 152—154 °C, $[\alpha]_D$ −9.85° (Found: C, 64.55; H, 6.25; N, 6.45. $C_{23}H_{26}N_2O_6$ requires C, 64.78; H, 6.15; N, 6.57%).

N_α-Biphenylisopropoxycarbonyl-L-Glutamic Acid γ-t-Butyl Ester Dicyclohexylammonium Salt.—This compound was prepared by the method of Hiskey *et al.*[25] in 49% yield, m.p. 134—136 °C, $[\alpha]_D$ +14.2° (*c*, 1.03 in MeOH) [lit.,[25] m.p. 136—138 °C, $[\alpha]_D$ +12.9° (*c*, 1.7 in MeOH)].

N_α-t-Butoxycarbonyl-L-tyrosine t-Butyl Ether.—L-Tyrosine t-butyl ether (2 g, 8.5 mmol, obtained by hydrogenolysis of the N_α-benzyloxycarbonyl derivative) was dissolved in dioxan–water (2 : 1, 21 ml) and a solution of di-t-butoxycarbonyl carbonate (2.02 g, 9.25 mmol) added dropwise with stirring. The pH indicated by a glass electrode was maintained at 9.5 by concurrent addition of 1M-aqueous sodium hydroxide. After alkali uptake had ceased, the solution was extracted twice with ether, acidified (pH 2—3) with aqueous citric acid, and the precipitated oil extracted into ethyl acetate. The extracts were washed with water, dried (Na_2SO_4), and evaporated to give 2.8 g [oil, single spot on t.l.c. in $CHCl_3$–MeOH–AcOH (85 : 10 : 5) (CMA)]. Part (1.7 g) was crystallised from light petroleum (b.p. 40—60 °C) giving the t-butyl ether (1.3 g, 76%), m.p. 112—115 °C, $[\alpha]_D$ +15.86° (*c*, 1.03 in MeOH) (Found: C, 63.85; H, 8.1; N, 3.95. $C_{18}H_{27}NO_5$ requires C, 64.07; H, 8.06; N, 4.15).

Solid-phase Assembly of the Human β-Endorphin Sequence Using N-t-Butoxycarbonylamino-acids.—(1) General procedures for solid-phase synthesis using polydimethylacrylamide resins and Boc-amino-acids have been given previously.[1] All Boc-amino-acids were checked for purity and identity by melting point, optical rotation, and t.l.c. (silica, CMA) prior to use. Boc-Gly, Boc-Ala, Boc-Ile, Boc-Phe, Boc-Leu, Boc-Thr(Bzl), Boc-Val, Boc-Pro, Boc-Ser(Bzl), Boc-Glu(OBzl) DCHA salt, and Boc-Met DCHA salt were commercial samples (Fluka). Boc-Lys(Z-Cl_2) [12] and Boc-Tyr(Bzl-Cl_2) [13] were prepared by literature methods. Boc-Asn(Xan) and Boc-Gln(Xan) were prepared as described above. Boc-glycine prepared from tritiated glycine (3.75 Ci/mmol) was used for addition of the last two glycine residues (see text). Symmetrical anhydrides of the foregoing Boc-amino-acids were prepared as described previously,[1] except for Boc-Asn-(Xan) and Boc-Gln(Xan) which because of sparing solubility in dichloromethane were dissolved in DMF together with dicyclohexylcarbodi-imide (0.5 eq.) 5 min before addition of the whole reaction mixture to the drained peptide resin.

Synthesis was commenced on DMF-swollen Boc-β-alanine resin [1] (3 g dry resin, Found: β-alanine, 0.33 mequiv./g) by addition of three further β-alanine spacer residues and a norleucine internal reference amino-acid. The Boc-amino-acid anhydride (5.23 mmol dissolved in 35 ml DMF) was used in coupling, deprotection, and neutralisation procedures already described.[1] Part (equivalent to *ca.* 2 g dry resin) was then treated with 3-(γ-benzyl-t-butoxycarbonyl-L-glutamyl-4-oxymethylphenyl)propionic acid 2,4,5-trichlorophenyl ester (4 mmol) in the presence of hydroxybenzotriazole (4 mmol) in DMF (35 ml). Free amino-groups remained [15] after 7 h and this coupling was repeated for a further 6 h when a negative ninhydrin reaction was obtained. All subsequent acylation reactions utilised pre-formed symmetrical anhydrides (4 mmol) dissolved in DMF (35 ml) (apart from the radiolabelled glycine residues, 2 mmol) for periods of 80—120 min; acylation was judged complete [15] in all cases and no repeated acylations were necessary. Resin samples (2—4 mg) were removed for solid-phase Edman degradation after addition of residues 10, 19, 28, and 31 (from the C-terminus); similar

Table 1. Amino-acid analysis of resin-bound peptides referred to Glu = 1.00, 2.00 or 3.00 as appropriate.

Step	3	7	9	17	19	22	27	31
Residues	31—29	31—25	31—23	31—15	31—13	31—10	31—5	31—1
Nle	1.05	1.10	1.03	1.13	1.13	1.28	1.29	1.27
Glu	1.00	1.00	1.00	1.00	1.00	2.00	3.00	3.00
Gly	1.11	1.09	1.09	1.09	1.11	1.16	1.14	2.00
Lys	1.18	2.23	3.15	4.42	4.36	4.84	5.60	5.53
Tyr		1.06	0.91	1.02	1.04	0.90	1.06	1.62
Ala		1.05	1.01	2.06	2.03	1.97	2.17	2.15
Asp		0.99	0.99	1.99	2.08	2.12	2.08	2.06
Ile			0.86	1.53	1.55	1.64	1.53	1.49
Phe				0.99	1.00	0.96	0.97	1.93
Leu				0.96	1.94	2.12	2.06	1.97
Thr				0.97	1.10	1.96	2.80	2.82
Val				0.96	0.95	1.00	0.96	0.98
Pro					N.D.	N.D.	N.D.	N.D.
Ser						0.83	1.88	1.84
Met							1.18	1.00

Table 2. Amino-acid analysis of resin-bound peptides referred to glutamic acid = 1.00, 2.00, or 3.00 as appropriate.

Step	3	7	9	17	19	22	27	31
Residues	31—29	31—25	31—23	31—15	31—13	31—10	31—5	31—1
Nle	1.67	1.73	1.68	1.88	1.89	1.92	2.07	1.96
Glu	1.00	1.00	1.00	1.00	1.00	2.00	3.00	3.00
Gly	1.09	1.08	1.06	1.03	1.03	N.D.	1.12	3.25
Lys	1.07	2.19	3.20	4.24	4.13	3.92	4.91	5.25
Tyr		0.96	0.98	0.99	1.00	0.97	1.00	2.06
Ala		1.03	1.01	1.98	1.97	1.95	2.02	2.08
Asp		0.96	0.96	1.90	1.89	1.89	1.93	1.96
Ile			0.81	1.33	1.48	1.52	1.55	1.51
Phe				0.92	0.94	0.90	0.90	1.97
Leu				0.97	1.94	1.84	1.95	1.94
Thr				1.01	1.01	1.75	2.83	2.80
Val				0.92	0.94	0.85	0.92	0.95
Pro					0.71	0.93	0.75	1.05
Ser						0.95	1.76	1.81
Met							1.14	1.15

samples were removed after steps 3, 7, 9, 17, 19, 22, 27, and 31 for quantitative amino-acid analysis (Table 1). This synthesis was discontinued after the failure of the radiolabelled glycine couplings became evident.

(2) Some minor changes were made in this second assembly. A single further β-alanine spacer amino-acid was added to the same [1] Boc-β-alanine resin (2 g) using Boc-β-alanine 2,4,5-trichlorophenyl ester (4 mmol) and hydroxybenzotriazole (4 mmol) in DMF (30 min). Boc-norleucine internal reference amino-acid was coupled as its symmetrical anhydride (4 mmol) as before, and only a single coupling of 3-(γ-benzyl-t-butoxycarbonyl-L-glutamyl-4-oxymethylphenyl)propionic acid 2,4,5-trichlorophenyl ester (4 mmol) in the presence of hydroxybenzotriazole (4 mmol) was carried out (3 h). Subsequent acylations utilised pre-formed symmetrical anhydrides (4 mmol) in DMF (30 ml) for 70—120 min. Negative ninhydrin reactions were obtained throughout, usually after 15 min acylation. Exceptionally, 2.3 mmol of the penultimate residue (radiolabelled glycine) dissolved in DMF (20 ml) was used. Resin samples were removed for solid-phase Edman degradation and for quantitative amino-acid analysis (Table 2) as above. A total of 67 analytical samples were removed during the assembly leaving 2.35 g of peptide resin [Found: 0.072 mequiv./g dry resin; Glu : Nle = 0.51 (85% of peptide chains retained)].

Cleavage and Isolation of Human β-Endorphin. (1) *From the Boc-Amino-acid Assembly.*—Part of the foregoing peptide resin (0.17 g) was treated with dry (CoF_3) distilled hydrogen fluoride and anisole (0.6 ml) in a closed polytetrafluoroethylene vessel, initially at −78 °C warming to 0 °C over 1 h. The hydrogen fluoride was evaporated under reduced pressure and the residue dried in a vacuum desiccator over KOH and P_2O_5 for 2 h. The solid was washed with ethyl acetate (100 ml) and extracted with 0.2M-acetic acid (25 ml). The extract was applied to a column of Sephadex G50 (70 × 2.5 cm diam.) and eluted with further 0.2M-acetic acid at a flow rate of 0.81 ml/min. The effluent was continuously monitored at 278 nm and individual fractions (4.9 ml) scintillation counted for tritium content. Fractions 59—67 (Figure 3) were combined and freeze dried giving 6.72 μmol (63% recovery) (Found: Asp, 2.06; Thr, 2.81; Ser, 1.79; Glu, 3.00; Pro, 1.07; Gly, 3.19; Ala, 2.15; Val, 0.98; Met, 0.92; Leu, 2.04; Ile, 1.19; Tyr, 2.05; Phe, 1.99; Lys, 5.08). The residual resin was washed thoroughly with DMF, 10% di-isopropylethylamine–DMF, DMF, and dichloromethane, and dried *in vacuo* (Found: Glu : Nle = 0.08 [15.5% of peptide chains retained]). Part (3.76 μmol) of the product was applied to a column of carboxymethylcellulose (Whatman CM52) (9 × 1 cm diam.) and eluted with a linear gradient of 0—0.1M-NaCl in 0.01M-phosphate buffer, 6M-urea, pH 6.0 (250 ml in each mixing chamber). Fractions (3 ml) were collected with a flow rate of 0.5 ml/min. Effluent was monitored at 278 nm and scintillation counted as before The main peak comprising fractions 36—46 was collected desalted on Sephadex G50 using 0.2M-acetic acid as eluant and freeze dried giving 0.90 μmol (24%) of peptide (Found'

Table 3. Amino-acid analysis of resin-bound peptide relative to glutamic acid. For ease of interpretation, the glutamic acid figures in analyses 22, 29, and 31 are adjusted to accommodate a 25% chain termination at step 4.

Step	3	7	9	17	19	22	29	31
Residues	31—29	31—25	31—23	31—15	31—13	31—10	31—3	31—1
Nle	0.97	1.03	0.98	0.96	0.95	0.97	1.02	1.03
Glu	1.00	1.00	1.00	1.00	1.00	1.75	2.50	2.50
Gly	0.95	0.94	0.92	0.96	0.99	0.93	1.59	2.20
Lys	0.96	1.74	2.38	3.58	3.61	3.53	4.59	4.55
Tyr		0.68	0.71	0.77	0.75	0.74	0.72	1.25
Ala		0.77	0.71	1.55	1.49	1.52	1.51	1.52
Asp		0.76	0.73	1.54	1.49	1.52	1.52	1.49
Ile			0.64	0.91	0.87	0.84	0.79	0.80
Phe				0.72	0.69	0.73	1.36	1.33
Leu				0.74	1.46	1.50	1.45	1.44
Thr				0.69	0.67	1.42	1.97	1.99
Val				0.66	0.62	0.67	0.63	0.66
Pro					0.99	0.78	0.75	0.86
Ser						0.63	1.16	1.17
Met							0.53	0.62

Asp, 1.99; Thr, 2.80; Ser, 1.71; Glu, 3.08; Pro, 1.00; Gly, 3.00; Ala, 1.99; Val, 1.03; Met, 0.95; Leu, 1.97; Ile, 1.33; Tyr, 1.93; Phe, 1.92; Lys, 4.70). In a second similar experiment using a gradient of 0—0.15M-NaCl in 0.01M-phosphate, 6M-urea, the elution profile shown in Figure 4 was obtained; the recovery (0.48 μmol from 1.92 μmol applied) was the same (24%). Rechromatography of the first product (0.85 μmol) on CM52 (11 × 1 cm diam.) using a linear gradient of 0.015—0.15M ammonium acetate pH 6.0 (25 ml in each mixing chamber) gave a single u.v. absorbing and radioactive peak containing 0.72 μmol (85% recovery, 9.2% overall yield) (Found: Asp, 1.95; Thr, 2.72; Ser, 1.80; Glu, 3.00; Pro, 0.99; Gly, 2.98; Ala, 2.00; Val, 0.99; Met, 0.94; Leu, 1.95; Ile, 1.43; Phe, 1.97; Tyr, 2.02; Lys, 4.75). Paper electrophoresis at pH 6.5 and 3 kV gave a single fluorescamine and ninhydrin reacting spot, R_{Lys} 0.38; t.l.c. (SiO_2) in butan-1-ol–pyridine–acetic acid–water (90 : 80 : 60 : 72) gave a single ninhydrin and chlorine/tolidine reacting spot R_F 0.63 with a second ninhydrin negative, chlorine/tolidine reacting spot R_F 0.56 coincident with urea in this and other solvent systems. The urea was removed by gel filtration on Sephadex G10. The major contaminant separated in the first carboxymethylcellulose chromatography (Figure 4) was collected and desalted on Sephadex G50 yielding 0.5 μmol (13.5%) (Found: Asp, 1.80; Thr, 2.53; Ser, 1.66; Glu, 2.50; Pro, 0.85; Gly, 3.00; Ala, 1.89; Val, 0.89; Met, 0.83; Leu, 2.01; Ile, 1.09; Tyr, 1.84; Phe, 1.75; Lys, 4.62). The first eluting peak was similarly recovered (4.3%) from two CM52 columns (Found: Asp, 2.19; Thr, 2.93; Ser, 1.64; Glu, 3.15; Pro, 1.20; Gly, 3.00; Ala, 2.53; Val, 1.22; Leu, 2.79; Ile, 1.55; Tyr, 1.93; Phe, 1.97; Lys, N.D.). In a later experiment, a sample (0.138 g) of peptide resin was cleaved with hydrogen fluoride and the endorphin isolated as described by Li *et al.*[23] The overall yield was 13.3%.

Solid-phase Assembly of the Human β-Endorphin Sequence using N_α-Fluorenylmethoxycarbonylamino-acids.—General procedures for solid-phase synthesis using Fmoc-amino-acids on polydimethylacrylamide resins have been given previously.[2] Fmoc-amino-acids were prepared by published procedures [26,27] and were checked for purity before use as described above. H.p.l.c. was performed on μ-Bondapak C_{18} reversed-phase or Partisil SCX ion-exchange columns on a Waters pumping and gradient system. For reversed-phase chromatography, reservoir A contained 0.01M-ammonium acetate pH 4.5 and reservoir B acetonitrile. Elution was by a linear gradient from 5—60% over 40 min at a flow rate of 1.5 ml/min. For cation-exchange chromatography, reservoir A contained 0.5M-ammonium acetate pH 6 and B 1M-ammonium acetate pH 6, both containing 10% MeCN. Linear gradient of 1—100% B over 40 min was used. Effluent was monitored at 278 nm and/or 230 nm using a Cecil 212 monitor and 1-cm path-length cell.

The same Boc-β-alanyl-polydimethylacrylamide resin [1b] (1 g, 0.33 mequiv.) was used as above; after deprotection and neutralisation an internal reference Boc-Nle residue was added directly using the preformed symmetrical anhydride (2 mmol) in DMF (15 ml) for 1 h. After deprotection and neutralisation, *p*-hydroxymethylphenoxyacetic acid 2,4,5-trichlorophenyl ester (2 mmol) and hydroxybenzotriazole (2 mmol) in DMF (15 ml) was added for 1 h, and the resin then washed for 1 min with DMF (10 × 15 ml), 10% di-isopropylethylamine in DMF (3 × 15 ml), and DMF (5 × 15 ml). The C-terminal residue Bpoc-Glu(OBut) was coupled as the symmetrical anhydride (2 mmol) in the presence of 4-dimethylaminopyridine (2 mmol) in DMF (15 ml) for 12 h. The washed resin was divided at this stage and one half stored at 0 °C under N_2.

The Bpoc group was cleaved using 0.09M-HCl–AcOH (30 min) and the resin washed and neutralised as with Boc-derivatives. The following 29 residues were added as preformed Fmoc-amino-acid anhydrides (1 mmol) or *p*-nitrophenyl esters (asparagine and glutamine) (1 mmol) in the presence of hydroxybenzotriazole (1 mmol) in DMF (7.5 ml). The synthesis was terminated with Boc-Tyr(OBut). Ninhydrin tests indicated that acylation was usually complete at the time of the first test (10—20 min), but reactions were allowed to continue for 60—100 min. Precipitation of Fmoc-glycine anhydride occurred during its first preparation and was inadvertently removed by filtration. This coupling was the only one which was repeated. Fmoc groups were cleaved with 20% piperidine–DMF.[2] Samples for amino-acid analysis were removed at residues 3, 7, 9, 17, 19, 22, 29, and 31; the analytical results (Table 3) are expressed relative to glutamic acid = 1.00, 1.75, or 2.50 as appropriate to assist interpretation after partial chain termination at step 4 (see text). The final peptide resin contained 0.095 mmol/g.

The assembly was repeated with the remaining stored resin with essentially identical results.

Cleavage and Isolation of Human β-Endorphin. (2) *From the Fmoc-Amino-acid Assembly.*—Dried resin (0.06 g) was stirred with trifluoroacetic acid (10 ml) and methionine (0.134 g) for 2 h at room temperature. The resin was collected on a sintered funnel, washed further with trifluoroacetic acid and the com-

bined filtrate and washings evaporated. The residue was dissolved in water (10 ml) and freeze dried giving 5.14 μmol (90%) of crude peptide. The residual resin was thoroughly washed and dried (Found: Glu : Nle = 0.10, 10.3% peptide remaining). The crude product (5.03 μmol) was applied in water (5 ml) to a column of carboxymethylcellulose (CM52, 11 × 1 cm diam.) and eluted with a linear gradient of 0.015—0.15M-ammonium acetate pH 6.0 (250 ml in each mixing chamber) at a flow rate of 0.77 ml/min (Figure 5). The main peak (fractions 70—80) was collected and freeze-dried yielding 2.31 μmol (45.9% recovery, 41.3% overall yield) (Found: Asp, 2.00; Thr, 2.84; Ser, 1.73; Glu, 3.14; Pro, 1.14; Gly, 2.96; Ala, 2.00; Val, 0.89; Met, 1.04; Leu, 2.00; Ile, 1.11; Tyr, 1.96; Phe, 1.92; Lys, 5.35). Peaks comprising fractions 44—50, 54—57, 60—75, and 99—107 were also collected and contained *ca.* 0.45, 0.036, 0.068, and 0.47 μmol of peptide respectively. The synthetic endorphin gave a single ninhydrin and fluorescamine-reacting spot (R_{Lys} 0.45) on paper electrophoresis at pH 6.5 and 3 kV and on t.l.c. in butan-1-ol–pyridine–acetic acid–water (90 : 80 : 60 : 72) (R_F 0.67). 1-Butanol–acetic acid–water (60 : 12 : 26) R_F 0.1 (trailing) visualised by ninhydrin and by chlorine–tolidine). H.p.l.c. (of 0.025 μmol on μ-Bondapak C_{18} gave the elution profile shown (Figure 5) at 278 nm; a similar profile was observed at 230 nm. On Partisil SCX a single broad peak emerged at 27.7 min.

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