Application of Polyamide Resins to Polypeptide Synthesis: Human β-Endorphin

By ERIC ATHERTON, MARIO CAVIEZEL, HILLARY OVER, and ROBERT C. SHEPPARD
(*Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge* CB2 2QH)

Summary The 31-residue, opiate-like polypeptide β-endorphin has been synthesised on a polydimethylacrylamide-based solid support with direct solid-phase Edman degradation for analytical control.

WE have previously reported that polar polyamide resins may be useful for solid phase reactions involving polar reactants or intermediates.[1] Illustrative applications include peptide[1,2] and oligonucleotide[3] synthesis and protein sequencing.[4] In all cases, a cross linked polydimethylacrylamide was used as the solid support. The recent discovery that part sequences of the protein β-lipotropin have strong affinity for opiate receptors in the brain[5] and that the longer sequences, notably the C-terminal 31 residue fragment (β-endorphin), show powerful and long-lasting analgesic effects,[6] provided an opportunity to test further the value of polyamide resins by synthesis of a substantial and important sequence. Furthermore, the suitability of the same resin for both synthetic and degradative operations enables the two techniques to be used in combination, the correct assembly of the polypeptide chain being monitored by solid-phase Edman degradation.[7] We now report an

efficient synthesis of human β-endorphin, H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH (I),† which illustrates the strength and current practice of the polyamide method.

The solid support was an amorphous copolymer of dimethylacrylamide, *NN'*-bisacryloylethylene diamine, and *N*-acryloyl-*N'*-t-butoxycarbonyl-β-alanylhexamethylenediamine.[1,2] Before start of the synthesis proper, additional residues of β-alanine and norleucine were added to act as spacer and internal reference amino-acids. The first residue of the β-endorphin sequence was then introduced as the activated ester derivative Boc·NH·CH(CH_2·CH_2·CO·O·CH_2Ph)·CO·O·CH_2·C_6H_4·CH_2·CH_2·CO·O·C_6H_2-2,4,5-Cl_3 (II), m.p. 84—85 °C, prepared by acylation of the 2,4,5-trichlorophenyl ester of *p*-hydroxymethylphenylpropionic acid with Boc-γ-benzyl glutamic anhydride, incorporating the reversible benzyl ester-type linkage to the resin (Boc = t-butoxycarbonyl). The initial loading was 0·14 mequiv. g^{-1}. Side-chain protecting groups used for other amino-acids were 2,4-dichlorobenzyloxycarbonyl (lysine), 2,6-dichlorobenzyl (tyrosine), benzyl (serine, threonine, and glutamic acid), and xanthydryl (asparagine and glutamine). The penultimate glycine (residue 3) was radiolabelled with tritium to the extent of 3·75 mCi $mmol^{-1}$.‡ All coupling reactions other than that with (II) were carried out using six-fold excesses of symmetrical Boc-amino-acid anhydrides prepared immediately beforehand by reaction between the protected amino-acid (2 equiv.) and dicylohexylcarbodiimide (1 equiv.) in dichloromethane. The anhydrides were dissolved in purified dimethylformamide and introduced manually into a Beckman Model 990 Peptide Synthesiser which had been modified to incorporate an all glass, internally totally wetted reaction vessel. Coupling reactions were rapid and in all cases were judged complete at the time of the first ninhydrin test[8] (10—20 min). They were allowed to continue for *ca.* 1·5 h. Deprotection, neutralisation, and washing procedures were as described previously,[2] except that di-isopropylethylamine was used in place of triethylamine. No repeated acylation or deprotection steps were necessary. Assembly of the 31 residue sequence was complete in 10 days.

Samples for amino-acid analysis were removed at strategic points during the synthesis (Table). Samples were also removed after each ninth or tenth residue and subjected to complete solid phase Edman degradation.[4] T.l.c. of the phenylthiohydantoins confirmed the overall correctness of the assembly. At residues 22 and 23 (-Ile-Ile-), Edman degradation gave an additional minor t.l.c. spot which might have indicated significant preview of the following

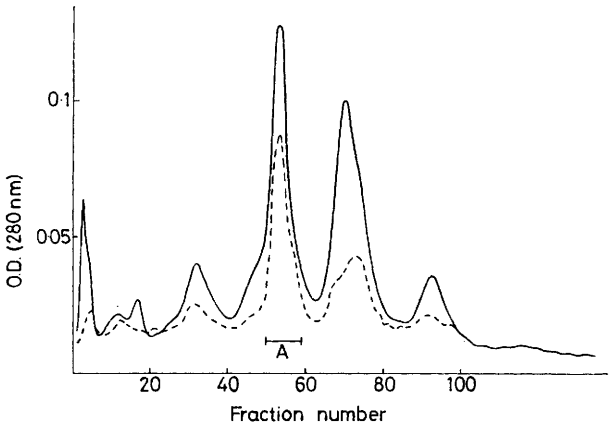

FIGURE. Chromatography of synthetic product on carboxymethylcellulose CM52. Conditions used are as described in text. Solid line: optical density; broken line: radioactivity. The second major peak on the chromatogram has an amino-acid analysis nearly identical to that of the principal product but an anomalously high optical density: radioactivity ratio. This high u.v. absorption has previously been observed in other peptides cleaved from supports by hydrogen fluoride [*e.g.*, G. W. Tregear, 'Peptides 1974,' Halstead Press, 1975, p. 177 (Proc. 13th European Peptide Symposium, Kiryat Anavim, Israel, 1974)].

TABLE. Amino-acid analysis data for resin-bound and free synthetic β-endorphin. All ratios are referred to glutamic acid = 1, 2, or 3 as appropriate and are uncorrected for destruction or incomplete release. The two isoleucine residues are present in an -Ile-Ile- sequence which is incompletely hydrolysed under the standard conditions (6N HCl, 110 °C, 18 h).

Step	3	7	9	17	19	22	27	31	A	B	C	
Residues	31–29	31–25	31–23	31–15	31–13	31–10	31–5	31–1	HF-G50	CM 52-urea G50	2nd CM52	Theory
Glu	1·00	1·00	1·00	1·00	1·00	2·00	2·00	3·00	3·00	3·00	3·00	3
Gly	1·09	1·08	1·06	1·03	1·06		1·12	3·25	3·19	2·92	2·89	3
Lys	1·07	2·19	3·20	4·24	4·13	3·92	4·91	5·25	5·08	4·58	4·75	5
Tyr		0·96	0·98	0·99	1·00	0·97	1·00	2·06	2·05	1·88	2·02	2
Ala		1·03	1·01	1·98	1·97	1·95	2·02	2·08	2·15	1·94	2·00	2
Asn		0·96	0·96	1·90	1·89	1·89	1·93	1·96	2·06	1·94	1·95	2
Ile			0·83	1·33	1·48	1·52	1·55	1·51	1·19	1·30	1·43	2
Phe				0·92	0·94	0·90	0·90	1·97	1·99	1·87	1·97	2
Leu				0·97	1·94	1·84	1·95	1·94	2·04	1·92	1·95	2
Thr				1·01	1·01	1·75	2·83	2·80	2·81	2·73	2·72	3
Val				0·92	0·94	0·85	0·92	0·95	0·98	1·00	0·99	1
Pro					0·71	0·93	0·75	1·05	1·07	0·97	0·99	1
Ser						0·95	1·76	1·81	1·79	1·67	1·80	2
Met							1·14	1·15	0·92	0·93	0·94	1

† This work was initiated before the isolation of human β-endorphin had been described (C. H. Li, D. Chung, and B. A. Doneen, *Biochem. Biophys. Res. Comm.*, 1976, **72**, 1542). The target sequence was derived from that of the C-terminal 31 residues of human β-lipotropin (G. Cseh, E. Barát, A. Patthy, and L. Gráf, *FEBS Letters*, 1972, **21**, 344; C. H. Li and D. Chung, *Nature*, 1976, **260**, 622.)

‡ This level of radioactivity in an amino-acid close to the single methionine (residue 5) may have been unwise. The final product has shown a steady decline in biological potency which may be associated with oxidation of this methionine. The resin-bound peptide liberated more than 50% methionine sulphone on acidic hydrolysis five months after synthesis.

amino-acid residue (lysine), *i.e.* partial omission of isoleucine during assembly. The degradation was repeated on a resin sample terminating in Ile-23, and the phenylhydantoins were analysed by high-pressure liquid chromatography. A very minor peak coincident with the phenylthiohydantoin of Lys(Z-Cl_2) was found in the first cycle of this degradation; the maximum content was estimated to be <1%. The *N*-terminal sequence (Tyr·Gly·Gly·Phe) was also examined carefully for the presence of additional glycine residues which could arise through rearrangement[9] of Boc-glycine anhydride prior to incorporation. None was found.

Sixty-seven analytical samples were removed during the course of the synthesis leaving 2·35 g of completed peptide-polymer (from 2 g of starting resin). The final loading was 0·072 mequiv. g^{-1} corresponding to retention of 91% of the initial peptide chains. Treatment of this resin (173 mg) with liquid HF (5—8 ml) and anisole (0·6 ml) for 1 h at 0 °C cleaved 85% (10·69 μM) of the peptide from the resin. Chromatography of the 0·2 M acetic acid-soluble fraction on Sephadex G50 gave in the main peak 6·72 μM (63%) of peptide (analysis, column A in Table), of which an aliquot portion corresponding to 3·76 μM was rechromatographed on carboxymethylcellulose CM52 using a gradient of 0—0·1 M NaCl in 6 M urea—0·01 M phosphate (pH 6·0). The main peak (A, Figure) (0·90 μM, 24%, column B in Table) was collected, desalted on Sephadex G50, and again chromatographed on CM52 using a gradient of 0·015—0·15 M ammonium acetate (pH 6·0). A single peak was obtained with a recovery of 85% (amino-acid analysis, column C in Table). This product gave a single fluorescamine- and ninhydrin-reacting spot on paper electrophoresis at pH 6·5 (R_{lys} = 0·38) and on t.l.c. in butan-1-ol–acetic acid–water–pyridine (90 : 18 : 60 : 72), R_F 0·63. The only contaminant detectable was urea, identified in three t.l.c. systems (chlorine–tolidine spray) (use of urea in the initial ion-exchange chromatography has now been found unnecessary). The overall yield based on the first amino-acid attached to the resin was 10%.§

In the rat tail flick test, the synthetic β-endorphin exhibited analgesic properties apparently identical to the natural ovine peptide.¶ Details of this and opiate receptor binding assays will be reported separately.

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§ The overall yield is much less than the 30% recently reported by Li and his colleagues (C. H. Li, D. Yamashiro, L.-F. Tseng, and H. H. Loh, *J. Medicin. Chem.*, 1977, **20**, 325). The difference between the two syntheses most probably lies in the efficiency of the hydrogen fluoride cleavage step, rather than in the solid-phase assembly. In our case very substantial losses were observed due to aggregation and modification reactions at this stage in contrast to the published results of Li *et al.*

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[1] For a recent discussion, see E. Atherton, D. L. J. Clive, D. A. East, and R. C. Sheppard, in 'Peptides 1976,' Proceedings of the 14th European Peptide Symposium, Wepion, Belgium, 1976 (Edition de l'Universite Bruxelles, 1976, p. 291).
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