Easily Prepared Polar Support for Solid Phase Peptide and Oligonucleotide Synthesis. Preparation of Substance P and a Nonadeoxyribonucleotide

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Summary Persulphate initiated polymerisation of a mixture of dimethylacrylamide, ethylene bisacrylamide, and acryloylsarcosine methyl ester yields a beaded polymer which after reaction with ethylenediamine is a suitable support for both peptide and oligonucleotide synthesis.

POLAR supports derived from cross-linked polydimethylacrylamide resins have proved of value in both peptide[1] and oligonucleotide[2] synthesis. Our original resin was prepared[1a] by emulsion copolymerisation of a mixture of dimethylacrylamide (**1**), ethylene bisacrylamide (**2**), and *N*-acryloyl-*N'*-(t-butoxycarbonyl-β-alanyl)hexamethylenediamine (**3**). The last named monomer provided a spacer arm, internal reference amino-acid, and after cleavage of the Boc-protecting group, a primary amino function suitable for attachment of the first amino-acid or nucleotide unit through various reversible linkage agents.† The difficult preparation of asymmetrically substituted diamines of this type has impeded use of the resin in some laboratories. We have therefore developed a new polymeric support incorporating the ester-containing functional monomer (**4**) in place of (**3**).‡ Conversion of the ester groups in the new polymer into primary amino groups is easily achieved by treatment with excess of ethylenediamine. Furthermore, the close structural similarity of the new monomer (**4**) to dimethylacrylamide (**1**) might be expected to lead to a more homogeneous distribution of functional groups throughout the matrix. The new polymer is reproducibly obtained in an easily handled beaded form and is suitable for both peptide and oligonucleotide synthesis.

$CH_2{=}CH{\cdot}CO{\cdot}NMe_2$ (**1**)

$(CH_2{=}CH{\cdot}CO{\cdot}NH{\cdot}CH_2{-})_2$ (**2**)

$CH_2{=}CH{\cdot}CO{\cdot}NH[CH_2]_6NH{\cdot}CO{\cdot}CH_2{\cdot}CH_2{\cdot}NH{\cdot}Boc$ (**3**)

$CH_2{=}CH{\cdot}CO{\cdot}NMe{\cdot}CH_2{\cdot}CO_2{\cdot}Me$ (**4**)

Typically, a monomer mixture consisting of dimethylacrylamide (**1**) (15·0 g), ethylene bisacrylamide (**2**) (1·75 g), and the acryloylsarcosine methyl ester (**4**) (1·25 g) was dissolved in pre-cooled 66% aqueous dimethylformamide (150 ml) and ammonium persulphate (2·25 g) was added. This solution was introduced into a polymerisation vessel[3] containing cellulose acetate butyrate (12·5 g) dissolved in dichloroethane (300 ml) which had previously been warmed to 50 °C, purged with nitrogen for 10 min, and the stirring rate adjusted to 450 ± 20 r.p.m. Polymerisation was allowed to continue under a slow stream of nitrogen for 15 h at 50 °C. The beads were collected and washed thoroughly with aqueous acetone, acetone, and finally with ether. 1 g of the resin swelled to a volume of *ca.* 20 ml in a wide range of solvents (water, methanol, dimethylformamide, pyridine, or dichloromethane). Conversion of methoxycarbonyl groups into primary amino groups was complete (i.r. spectrum) after treatment with excess of ethylenediamine overnight at room temperature and thorough washing with 1·5% aq. KCl, water, and dioxan. The degree of functionalisation of this resin sample as judged by both sarcosine content and subsequent amino-acid incorporation was 0·35 mequiv./g.

The decapeptide amide (**5**) (substance P) was prepared using the previously described fluorenylmethoxycarbonyl (Fmoc) amino-acid anhydride procedure[4] with some variations. An internal reference-spacer amino-acid (Fmoc-norleucine) was first added followed by the reversible linkage agent (**6**).§ Formation of the benzyl ester peptide–resin linkage utilised Boc-methionine anhydride in the presence of *p*-dimethylaminopyridine. After de-protection with HCl–AcOH, the following eight residues were added using preformed Fmoc-amino-acid anhydrides except for glutamine (when *p*-nitrophenyl ester was used in the presence of 1-hydroxybenzotriazole). Intermediate resin-bound Fmoc-derivatives were cleaved with 20% piperidine in dimethylformamide. The terminal arginine residue was conveniently added as its simple N^{α}-Boc derivative using the preformed symmetrical anhydride procedure as before.

Arg·Pro·Lys·Pro·Gln·Gln·Phe·Phe·Gly·Leu·Met·NH_2 (**5**)

4-HO·CH_2·C_6H_4·CO·$OC_6H_2Cl_3$-2,4,5 (**6**)

d(pG–A–T–C–C–C–C–C–C) (**7**)

4-HO·CH_2·CH_2·S·$C_6H_4CH_2$·CH_2·CO·OC_6Cl_5 (**8**)

† *Added in proof*: Another polyamide resin also using a monoacryloyl derivative of hexamethylenediamine as functionalising agent has just been described (C. W. Smith, G. L. Stahl, and R. Walter, *Internat. J. Peptide Protein Res.*, 1979, **13**, 109).

‡ Full details of the monomer and polymer preparations may be obtained from the authors prior to detailed publication.

§ Benzyl esters derived from this alcohol are particularly susceptible to cleavage by nucleophiles (ammonia or hydroxide ion) but are resistant to acids.

Assembly of the decapeptide sequence was completed in two days. Amino-acid incorporation as judged by analysis of intermediate resin samples was complete throughout, except for a 30% irreversible chain termination at the Fmoc-Lys(Boc) step. This phenomenon was also encountered[1b] in our recent synthesis of β-endorphin using the same amino-acid derivative and is under investigation.¶ There was no significant loss of peptide from the resin throughout the assembly.

97% of the total peptide content was detached from the resin by methanolic ammonia (room temp.; 160 min). Terminal and side chain t-butoxycarbonyl protecting groups were removed with 90% trifluoroacetic acid (room temp.; 30 min) and the total product chromatographed on carboxymethylcellulose CMC-52 using a gradient of 0·03—0·3 M ammonium acetate, pH 6·6. The main peak (56%) (found: Met, 0·88; Leu, 1·00; Gly, 1·03; Phe, 2·08; Glu, 2·09; Pro, 2·07; Lys, 1·00; Arg, 1·00) was shown by h.p.l.c. on μ-Bondapak C_{18} to consist of a mixture of substance P and its sulphoxide. Reduction with dithiothreitol and chromatography on Sephadex G-25 furnished substance P equipotent with an authentic sample in radioimmunoassay.[5] The overall yield was 47%; found: Met, 0·95; Leu, 1·00; Gly, 1·04; Phe, 2·07; Glu, 2·04; Pro, 2·06; Lys, 0·97; Arg, 0·97. The analytical h.p.l.c. profile at 230 nm is shown in Figure 1. Authentic substance P emerged at the same position.

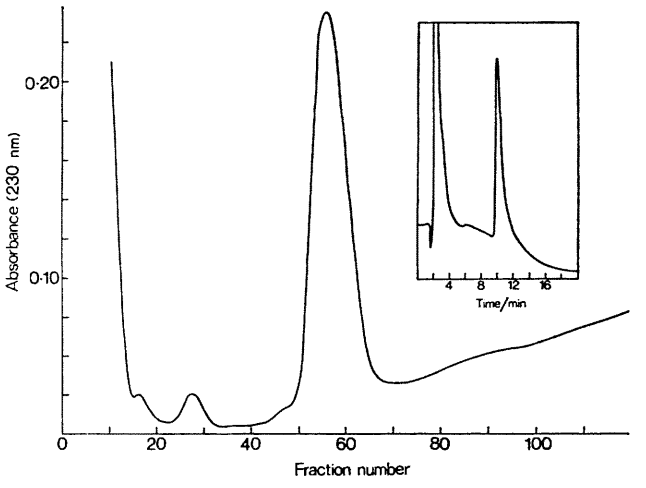

FIGURE 1. Cation-exchange chromatography of crude synthetic substance P on carboxymethylcellulose CMC-52. Linear gradient of 0·03—0·3 M ammonium acetate, pH 6·6. Flow rate of 0·6 ml/min; 6 ml fractions collected. Inset: h.p.l.c. on μ-Bondapak C_{18} of main peak after reduction with dithiothreitol and gel filtration on Sephadex G-25. Linear gradient of 25—50% MeCN in 0·01 M ammonium acetate, pH 4·5, over 20 min. The leading peak is due to solvent injection. All column effluents were monitored at 230 nm.

The nonanucleotide (**7**) was synthesised by our rapid solid-phase method[2] starting from the ethylenediamine functionalised resin. The sulphide reversible linkage agent (**8**) was used. Nucleotide additions were performed by the phosphodiester method using 10 equivalents of the appropriate 3′-*O*-acetyl-2′-deoxyribonucleotide preactivated with tri-isopropylbenzenesulphonyl chloride. After the mixture had been quenched with water, the resin was dried with excess of phenyl isocyanate and acetyl groups were removed with sodium methoxide in methanol–pyridine (1 : 1). Nucleotide additions were performed at the rate of one a day, either manually or using a modified Beckman 990 Peptide Synthesiser. H.p.l.c. analysis of oligonucleotide cleaved from resin samples taken after the second, third, and fourth cycles indicated coupling yields of 75, 78, and 83%, respectively.

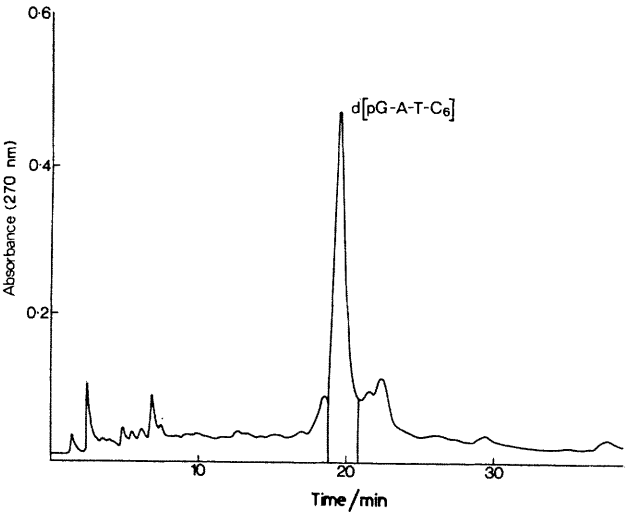

FIGURE 2. H.p.l.c. of the nonanucleotide d(pG-A-T-C_6) on μ-Bondapak C_{18}. Convex gradient of 5—9% MeCN in 0·1 M ammonium acetate over 60 min; see H.-J. Fritz, R. Belagaje, E. L. Brown, R. H. Fritz, R. A. Jones, R. G. Lees, and H. G. Khorana, *Biochemistry*, 1978, **17**, 1257.

After nine cycles, the total oligonucleotide was detached by oxidation of the sulphide linkage to sulphone and base catalysed elimination. The yield from 0·35 g of resin (0·1 mequiv. of original methoxycarbonyl groups) was 1300 A_{270} units. The product was fractionated in seven portions by h.p.l.c. on Partisil 10SAX using a linear gradient (0·025—0·2 M) of KCl and KH_2PO_4 (pH 6·8) in 5% aqueous ethanol.[2] The product in the major peak (98 A_{270} units) was desalted on Biogel P2 and deprotected with concentrated ammonia (50 °C; 5·5 h). The deacylated oligonucleotide (60 A_{270} units) was freed from non-nucleotidic material on Biogel P2 and again fractionated in seven portions by h.p.l.c. on μ-Bondapak C_{18}. A typical elution profile is shown in Figure 2; the total yield from six chromatograms was 10·5 A_{270} units of d(pG–A–T–C_6), λ_{max} 267 nm ($\epsilon_{260}/\epsilon_{280}$ 1·30, calc. 1·32).

¶ This chain termination has not been observed in other more recent syntheses using a new preparation of the protected amino-acid. Nor was it observed in a repetition of the synthesis using N^{α}-Fmoc-N^{ε}-trifluoroacetyl-lysine (D. Jarvis, unpublished results).

The structure and purity (>90%) of the nonanucleotide was confirmed by full sequence analysis of a ^{32}P-labelled sample.

We thank Dr. L. Iversen and his staff in the M.R.C. Neurochemical Pharmacology Unit for the radioimmunoassay.

(*Received, 8th February* 1979; *Com.* 130.)

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