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The Polyamide Method of Solid Phase Peptide and Oligonucleotide Synthesis[1]

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A versatile system of solid-phase peptide synthesis based on polar polyamide resins, a range of reversible peptide-resin linkage agents, and *Nα*-*t*-butoxycarbonyl or fluorenylmethoxycarbonyl-amino acids has been developed. Principles used in the design of the method are discussed and illustrated by synthesis of a number of natural peptides. Application of these principles to oligodeoxyribonucleotide synthesis has provided for the first time a practical solid phase method in the nucleotide field.

INTRODUCTION

The solid phase method of peptide synthesis was introduced by Merrifield in 1962 (*1*) and has found wide application (*2*, *3*). Merrifield's 1964 procedure (Fig. 1) involves initial attachment of a *t*-butoxycarbonyl (Boc) amino acid to a lightly cross-linked polystyrene resin through a benzyl ester bond, cleavage of the amino-protecting group, and elaboration of the peptide chain through successive amino acid addition and deprotection reactions. Dicyclohexylcarbodiimide is used as the coupling agent in peptide bond-forming reactions, and intermediate Boc-derivatives are cleaved with trifluoroacetic acid or hydrogen chloride in an organic solvent. The peptide is finally detached from the solid support and all benzyl-derived side chain protecting groups cleaved by the action of a very strongly acidic reagent, usually liquid hydrogen fluoride or a solution of hydrogen bromide in trifluoroacetic acid. This basic procedure has been followed with seemingly only slight variation in the vast majority of solid phase syntheses reported (*2*, *3*).

Solid phase oligonucleotide synthesis was introduced by Letsinger in 1965 (*4*). In the original procedure (Fig. 2) a substituted polystyrene resin is again used with attachment of the first deoxyribonucleoside (A, G, or C) through its side chain amino-group. The internucleotide bond is formed in a two step process by phosphorylation of the free 3′-hydroxy group with an activated phosphomonoester, further activation of the

[1] The work reviewed in this paper was conceived while one of us (R.C.S.) was still a member of George Kenner's staff at the University of Liverpool. Until near the end of his life, Kenner was a staunch and exclusive advocate of the strictly classical approach to organic synthesis. It is a measure of his breadth of vision that nonetheless he gave the fullest support and encouragement to this program throughout its experimental realization in Cambridge. For this support and in so many other ways we shall always be greatly indebted to him. In 1977 he wrote in a memorandum to the Royal Society: "The methods of classical peptide synthesis have been notably successful, but they are not adequate for the future. The problem is to marry Merrifield's visionary concept of automation with the realities of organic chemistry." This paper summarizes just one approach towards a solution of Kenner's clear-sighted statement of the problem.

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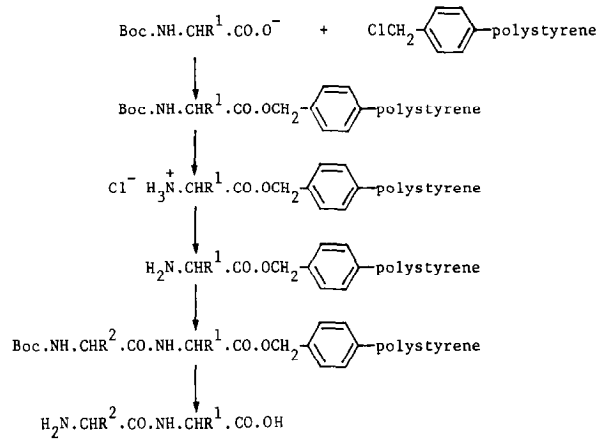


FIG. 1. Merrifield's scheme for solid phase peptide synthesis (*1*).

resulting resin-bound phosphodiester, and coupling with a second nucleoside component. Considerable variation on this approach is evident in subsequent procedures (*5*, *6*). The first nucleoside or nucleotide component may also be attached to the resin by its 3′ or 5′-oxygen atom through a variety of linkage groups. Nucleoside or nucleotide derivatives may also be used for chain extension, in the latter case with the phosphate linked initially to the 3′ or 5′ position. Both phosphodiester and phosphotriester routes have been used for formation of the internucleotide bond. Notwithstanding this wide search for an effective procedure, yields in coupling reactions have generally been so low that only recently has a practical method emerged.

In contrast, Merrifield's peptide synthesis was rapidly applied to a wide range of hormones and their analogs. An assessment of the method in 1971 (*7*) confirmed its utility but with important limitations. These arose from features intrinsic to the solid phase approach, particularly the lack of purification of resin-bound intermediate peptides apart from removal of excess soluble reagents. This methodology is contrary to a basic tenet of classical organic synthesis—that of full purification and characterization of intermediates—and in principle is acceptable only if all coupling and deprotection reactions are strictly quantitative. In practice it has been held to limit the size of *pure* peptides accessible by the solid phase technique to those in which failure

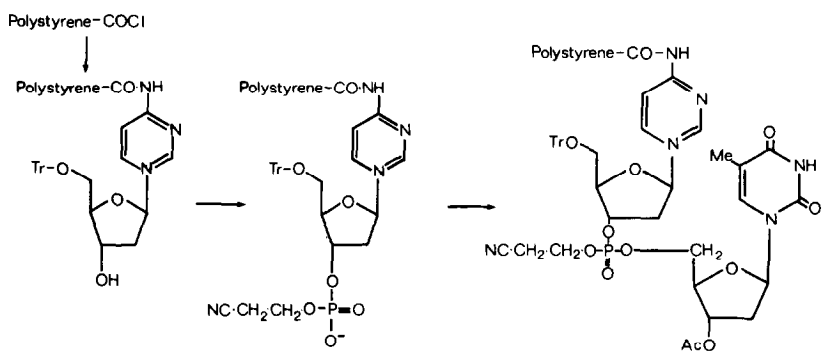


FIG. 2. Letsinger's scheme for solid phase oligonucleotide synthesis (*4*).

sequences, i.e., peptides lacking one or more amino acid residues, could be detected and removed by available purification procedures. In 1971 it was thought (*7*) that in some cases this might be as low as 10 residues. For longer peptides incapable of complete separation from contaminating failure sequences, useful if not completely pure products might still be obtained providing sufficiently high yields in coupling and deprotection reactions were attainable. The demands, however, are very great. Thus stepwise assembly of a 30 residue polypeptide requires an average efficiency of better than 99% per cycle to give directly a crude product containing 75% of the desired sequence (*3*). At 98% efficiency the yield falls to about 56% and rapidly thereafter. The situation is exacerbated by the generally harsh conditions employed in solid-phase synthesis, especially in the cleavage of the resin linkage, which causes further attrition. Substantial purification of the crude products would of course be possible because several of the common amino acids carry charged or other distinctive side chain groups, omission of which gives rise to failure sequences differing significantly in physicochemical properties from those of the completed peptide. The development of high performance liquid chromatography and other advanced techniques has also extended substantially the scope of purification methods. However, separation of failure sequences lacking, say, single neutral amino acids will always be problematic, *and ultimately the success of extended solid phase synthesis will depend on the nearness to which quantitative reactions can be consistently achieved.* Perhaps in no other area of organic synthesis is this requirement so critical. It applies to all the chemical steps involved and has implications concerning choice of the solid support and reaction medium, resin linkage, coupling agent, and protecting groups. Reaction conditions must be chosen with great care so that not only are the desired changes effected nearly quantitatively, but are so mild that alterations elsewhere in sensitive molecules are minimized. At the outset of our work it seemed that scope remained in several areas for improvements which might extend the range of peptides accessible by the solid phase procedure. An additional objective was the development of more flexible systems which would allow, for example, use of fragment condensation strategies rather than simple sequential amino acid addition. This paper reviews progress made in one particular approach toward achieving these aims, and application of some of the principles developed to oligonucleotide synthesis.

THE NATURE OF THE SOLID SUPPORT

It is probable that most users of solid phase peptide synthesis have assigned a rather passive role to the solid support, and the readily available polystyrene has been the overwhelmingly popular choice without special regard to its complete suitability. Our interest in the function of the solid support arose initially from consideration of a number of reports in the literature (*8–10*) of the sudden onset of catastrophically low or variable amino acid incorporation during polystyrene-based syntheses. The outstanding example known to us is the decapeptide sequence residues 65–74 of acyl carrier protein (*11*), where incorporation fell to 50–60% after six residues, rose seemingly to 100% for the next two, and then fell again to 30%. A study of reaction conditions failed to raise the incorporations to acceptable levels (*11*). Events of this type

are difficult to explain except on the basis of cooperative effects in the delicately balanced dynamic situation involving the peptide–polymer combination as a whole.

Discussion of this problem has been presented elsewhere (*7*, *12*). We concluded that a possible solution lay in reducing the solvation differences between the resin matrix and the growing peptide chains coupled with the use of good solvating media for both at all times. Under these conditions both polymer and peptide chains should be well extended with minimization of steric interference and hindrance. It is evident that this requires a polymer support more polar in character than the hydrocarbon polystyrene, and experiments were begun with polyamides. Polyacrylamide itself is unsuitable for use in solid phase synthesis because strong internal hydrogen bonding causes the resin to be permeated and swollen only by powerful hydrogen bond-breaking solvents such as water (cf. *13*). In accord with expectation, however, when intrachain hydrogen bonding is reduced by *N*-methylation a polymer is obtained which swells well in dimethylformamide and other polar aprotic solvents well suited to peptide synthesis.

Such a polymer was first prepared (*14*) by replacement of a substantial proportion of the primary amide groups in a commercial polyacrylamide by hydrazine, conversion to the acid azide and thence to the dimethylamide. Sites for attachment of the peptide chain were also introduced during this last step. The resin was used in solid phase peptide synthesis under conditions not dissimilar to those of the conventional Merrifield technique, except for the use of more polar dimethylformamide in place of methylene chloride as the coupling reaction medium. Successful syntheses of the test tetrapeptide Leu · Ala · Gly · Val and of the nonapeptide bradykinin were carried out. More importantly, the acyl carrier protein decapeptide sequence mentioned above was assembled on the resin without the dramatic rise and fall in amino acid incorporation observed elsewhere using a polystyrene support (*11*). These early results established the general suitability of polyamide resins for solid phase synthesis, and encouraged the belief that difficulties encountered with polystyrene might be reduced with these polar supports. More defined polymers were subsequently prepared by direct polymerization.

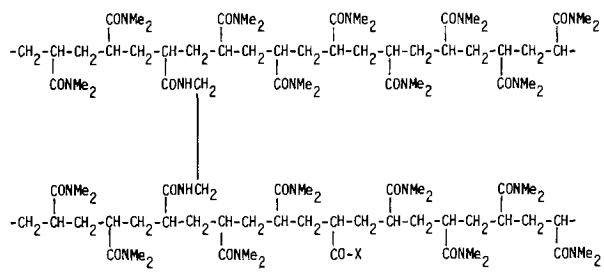
The initial experiments enabled us to define (*15*) some of the requirements for polyamide resins specifically designed for solid phase synthesis, viz. (i) minimization of intrachain hydrogen bonding by incorporation of a high proportion of tertiary amide groups; (ii) free permeation of the resin matrix by anhydrous polar aprotic solvents such as dimethylformamide known to disrupt secondary structure in polypeptides; (iii) cross-links stable to the full range of chemical reagents used in solid phase synthesis. Conventional peptide synthesis techniques utilize mostly acid reagents for deprotection steps (but see also below) but oligonucleotide synthesis also requires alkali stability. The formaldehyde-derived methylenebisacrylamide cross-links of commercial polyacrylamide were in our view unlikely to be adequately stable for extended syntheses; (iv) a suitable level of functionality for attachment of the first amino acid (or nucleotide) residue. Greater flexibility would be obtained if the reversible linkage group was not part of the basic polymer system. We therefore elected to functionalize the resin with easily acylable primary amino-groups to which a range of reversible linkage agents could be subsequently attached; (v) an internal reference amino acid directly attached to or part of the polymer system. Such a residue not removable by any of the synthetic (including peptide detachment) steps would provide a useful analytical reference point for monitoring the progress of the synthesis; (vi) minimum chemical manipulation of the

resin after polymerization. A number of observations in our early work suggested that postpolymerization side reactions, occurring during functionalization etc. of solid supports leading to further cross-linking and possibly the accumulation of unknown reactive groups might be responsible for some difficulties in solid phase synthesis. Ideally, therefore, all the necessary chemical features should be present at the monomer stage.

Most of these requirements were accommodated in a resin (**V**) prepared (*16*) by copolymerization of a mixture of dimethylacrylamide (**I**) (basic monomer), ethylene bisacrylamide (**II**) (cross-linking agent), and *N*-acryloyl-*N'*-(*t*-butoxycarbonyl-*β*-alanyl)hexamethylene diamine (**III**). The last monomer provided the internal reference amino acid (*β*-alanine) whose amino-group served, after deprotection, as attachment point for the linking agent and the first amino acid or nucleotide residue. A spacer arm

(I) $CH_2=CH.CO.NMe_2$ (II) $(CH_2=CH.CO.NH.CH_2-)_2$

(III) $CH_2=CH.CO.NH(CH_2)_6NH.CO.CH_2.CH_2.NH.Boc$ (IV) $CH_2=CH.CO.NMe.CO.OMe$



(V, X = $-NH(CH_2)_6NH.CO.CH_2.CH_2.NH.Boc$)

(VI, X = $-NMe.CH_2.CO.OMe$)

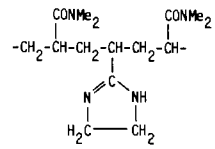
well separating the functional amino-group from the polymer backbone is also included. An emulsion polymerization technique was used which on a small scale provided an easily handled beaded product. Larger scale (50 g) preparations were usually amorphous but were chemically equally satisfactory and could be used in synthesis without undue difficulty.

This resin has been applied successfully in a number of extended syntheses in both the peptide and nucleotide fields (see below).

Recently a more easily prepared polyamide resin (**VI**) has been obtained (*17*) in which acryloylsarcosine methyl ester (**IV**) replaces the complex functionalizing agent (**III**). The latter unsymmetrically substituted diamine is a difficulty accessible compound and is also only sparingly souble in the two phase polymerization medium. Its replacement by (**IV**) leads reproducibly to a nicely beaded polymer functionalized with carbomethoxy groups. Furthermore, the new monomer (**IV**) is structurally much more similar to dimethylacrylamide (**I**) than is (**III**), and may therefore possess similar reactivity in polymerization and be incorporated more uniformly into the polymer matrix. Reaction of the new polymer with a large excess of ethylene diamine at room temperature smoothly converts the methyl ester groups into primary amino functions.

This departs from the principle of avoidance of postpolymerization chemical modification reactions and care must be taken not to cause additional cross-linking or formation of resin bound cyclic amidines (**VII**), but the resin appears to function equally satisfactorily in solid phase synthesis.

All the polydimethylacrylamide resin samples thus far prepared swelled dramatically in water and a wide range of organic solvents. Tenfold increase in volume (1 g swelling to ca. 20 ml) is typical in dimethylformamide, pyridine, acetic acid, methanol, and water. The methyl ester resin (**VI**) also swells to this extent in dichloromethane but only fivefold in dioxan. In the protonated form (e.g., after cleavage of *t*-butoxycarbonyl



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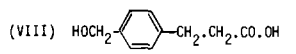

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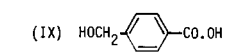

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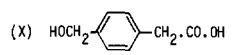

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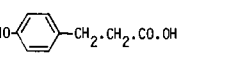

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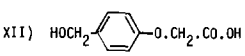

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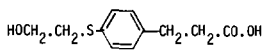

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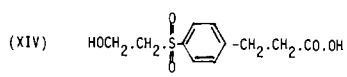

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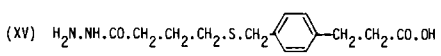

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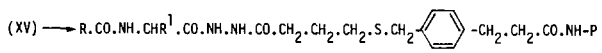

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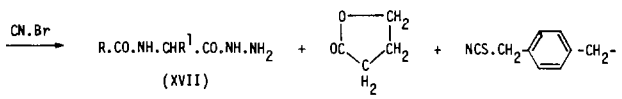


(XVII)

groups with hydrogen chloride in acetic acid) the resin shrinks but regains its original volume on neutralization. The polyamide resins are chemically and physically stable; usually no significant losses are observed after several days of agitation in a Merrifield-style glass reaction vessel. The degree of functionality is easily adjusted by varying the molecular proportion of (**III**) or (**IV**) in the polymerization mixture. The range 0.2–0.4 mEquiv/g appears to be generally suitable.

THE REVERSIBLE PEPTIDE-RESIN LINKAGE

Benzyl ester-type linkages have dominated polystyrene-based solid phase peptide synthesis since its inception. There has, however, been a growing realization that this

linkage is not ideal, and that the drastic acidic conditions necessary for the cleavage of simple benzyl esters may cause extensive degradation of sensitive peptides. More labile or specifically cleavable linkages are desirable (*7*) but these can be considered only in relation to the protecting group problem as a whole. At the outset, therefore, we adopted the benzyl ester for use with polyamide resins enabling direct comparison with the polystyrene system, but appreciating that a flexible approach was necessary. The latter was achieved by use of individual linkage agents rather than by direct incorporation of the benzyl alcohol unit into the resin structure.

A range of linkage agents of varying lability have now been prepared, each bearing a carboxy group for attachment to resin-bound primary amine. The simple benzyl alcohol derivative (**VIII**) gives, as expected, esters of stability comparable to those derived from chloromethylated polystyrene. Liquid hydrogen fluoride or hydrogen bromide in trifluoroacetic acid are necessary for their cleavage. Use of (**VIII**) in combination with α-*t*-butoxycarbonylamino acid derivatives and benzyl-based side chain protecting groups provides a polyamide solid phase system differing from Merrifield's only in the nature of the solid support and reaction medium. The same combination of protecting groups can be used with the *p*-carboxy-substituted linkage agent (**IX**) which has even greater acid stability.[2] Its esters are in fact almost totally unaffected by hydrogen fluoride—a property enabling selective cleavage of other benzyl-based protecting groups without detachment from the solid support. On the other hand, esters derived from (**IX**) are particularly labile to nucleophilic attack, e.g., by hydroxide ion, ammonia, and hydrazine. This linkage agent is thus particularly useful for the preparation of peptide amides and hydrazides. Greater reactivity toward nucleophilic reagents is shown by phenyl esters derived from the linkage agent (**XI**).

The most generally useful reagent at present is the *p*-alkoxy derivative (**XII**). *p*-Alkoxybenzyl esters are known from solution and solid phase peptide chemistry to have lability towards acids rather comparable to *t*-butyl derivatives. Peptide-resin linkages derived from (**XII**) are therefore cleavable under exceptionally mildly acidic conditions and are applicable to the synthesis of sensitive peptide sequences not easily accessible by more conventional means. Use of *p*-alkoxybenzyl esters for resin linkage requires an entirely different combination of $N\alpha$ and side chain protecting groups discussed in the following section.

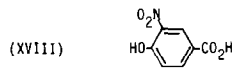
An interesting recent development is the recognition that polymer-bound benzyl esters can be cleaved by hydrogenolysis in the presence of palladium acetate (*19*). Evidently deposition of metal catalyst occurs within the polymer matrix. We have confirmed that this reaction is effective with polydimethylacrylamide resins. With appropriate combinations of protecting groups it offers another possibility for detachment of synthetic peptides under mild conditions.

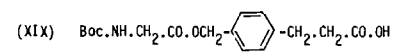
A number of more specialized linkage agents has also been examined. Esters from the β-thioethyl alcohol derivative (**XIII**) (*20*) are cleaved in a two stage process involving oxidation of the thioether to the sulfone (**XIV**) and base catalyzed β-elimination. This is of particular importance in oligonucleotide synthesis (see Oligonucleotide Synthesis) but may also have potential in special peptide situations. The thioether (**XV**) (*21*) gives the diacylhydrazine derivative (**XVI**) when incorporated into the polymer and coupled with the first protected amino acid. This is cleaved by cyanogen bromide in a manner

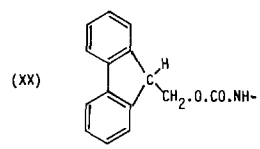
[2] Merrifield (*18*) has recently prepared the derivative (**X**) with the expected intermediate stability.

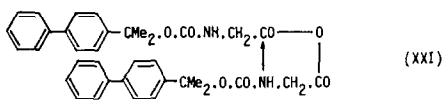
analogous to the cleavage of methionine peptides, but with liberation of an acyl hydrazide (**XVII**). Its application to the preparation of peptide hydrazides is limited at present by irreversible reaction of (**XVII**) with excess cyanogen bromide, but preliminary experiments have indicated that other cleavage reagents, e.g., *o*-nitrophenylsulfenyl chloride may be applicable. The *o*-nitrophenyl esters derived from resin bound (**XVIII**) (*22*) are potentially useful in reversed solid phase synthesis.

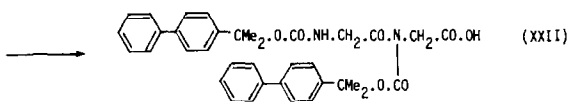
The linkage agent may be coupled to the polydimethylacrylamide resin either alone or with the first protected amino acid already attached. The latter procedure is

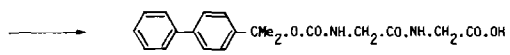












attractive because the combined amino acid-linkage reagent (e.g., **XIX**) contains the performed benzyl ester function, establishment of which is difficult to monitor when carried out in the solid phase. However, derivatives of type (**XIX**) are not easy to prepare for all amino acid residues, and the introduction of efficient catalysts for ester formation, especially *p*-dimethylaminopyridine (*23*), renders their synthesis unnecessary. Simple sequential addition of linkage agent and first amino acid is satisfactory. Activated esters (2,4,5-trichlorophenyl or pentachlorophenyl) are convenient for introduction of the linkage agents, usually in the presence of catalyst 1-hydroxybenzotriazole. Formation of the ester bond is rapid when the symmetrical anhydride of the protected amino acid is used as the activated species in the presence of *p*-dimethylaminopyridine. All coupling reactions are carried out in the polar aprotic solvents dimethylformamide or dimethylacetamide.

PROTECTING GROUPS

Although initial studies on the polyamide method of peptide synthesis utilized the conventional benzyl, *t*-butyl combination of protecting groups, it was recognized from the beginning that this imposed its own limitations (*7*). In particular, the acidic conditions required for the repetitive cleavage of *Nα-t*-butoxycarbonyl groups and especially the treatment with very strong acid necessary for cleavage of benzyl derivatives were expected to cause serious degradation of long and sensitive peptide sequences (*7*). These expectations were fulfilled (see Syntheses Achieved) and attention was turned to the development of new protecting group combinations.

The weakness in the conventional methodology lies essentially in the use of protecting groups of graded lability to the same reagent type. This results in the need for very vigorous conditions for cleavage of the more stable (benzyl-based) groups and often to inadequate selectivity in the cleavage of the more labile (*t*-butyl-based) derivatives. Consideration of the latter problem had led (*24*) to the introduction of even more acid stable nuclear substituted benzyl derivatives, presumably with worsening of the former difficulty. Another consequence of the use of groups of graded lability is the lack of flexibility it imposes. The conventional protecting group combination is not easily applicable to the preparation of protected peptide derivatives suitable for fragment condensation studies. This problem requires the use of α-amino and side chain protecting groups and a resin linkage cleavable as far as possible by individually specific reagents.

Our current methodology employs base-sensitive fluorenylmethoxycarbonyl (Fmoc) derivatives (**XX**) for α-amino protection (*25*). Carpino and Han (*26*) have already noted the special lability of Fmoc-derivatives to secondary amines; in our hands simple Fmoc-amino acids have cleaved within seconds by piperidine (20% in dimethylformamide). No other base sensitive protecting group of several considered [methylsulfonylethoxycarbonyl (*27*), α-cyano-*t*-butoxycarbonyl (*28*), 5-benzisoxazolylmethoxycarbonyl (*29*)] show comparable base-lability. Tertiary amines were relatively ineffective in cleaving Fmoc groups, and the low and rapidly declining concentration of resin bound primary amine does not cause detectable cleavage during coupling reactions. Benzyl and *t*-butyl esters (including the sensitive Asp(OBu^t) Gly sequence) were unaffected by long (15 hr) treatment with 20% piperidine permitting use of *t*-butyl derivatives for side chain protection and the various benzyl ester-type reagents (**VIII–XI**) for peptide-resin linkage. The *p*-alkoxy derivative (**XI**) was particularly appropriate for use in the synthesis of free (unprotected) peptides since it could be cleaved under mildly acidic conditions simultaneously with all the side chain protecting groups. Alternatively, use of the more acid stable linkage agent (**IX**) enabled acidic cleavage of side chain protecting groups and the detachment from the resin by treatment with ammonia. This procedure avoids risk of ammonolysis of side chain esters. The complete stability of fluorenylmethoxycarbonyl groups to hydrogenolysis reported by Carpino and Han (*26*) provides a potential cleavage for the resin linkage independent of the other protecting groups present. Thus with some limitations the *Nα*-fluorenylmethoxycarbonyl/*t*-butyl-side chain/benzyl ester resin linkage combination may also provide a useful system for the preparation and assembly of protected peptides in fragment condensation strategies. This aspect is under investigation.

The most important feature of the present system is its mildness. The acidic conditons of conventional solid phase synthesis typically involve repeated 30 min treatments with trifluoroacetic acid and then 30–60 min with liquid hydrogen fluoride. The present procedure replaces each of the trifluoroacetic treatments by 20% piperidine (10 min) and the very damaging hydrogen fluoride by a single acidic (trifluoroacetic or HCl–AcOH) treatment. The effect of these changes can be seen clearly in comparative syntheses of the 31-residue peptide β-endorphin (see Syntheses Achieved).

COUPLING AGENTS

The need for quantitative acylation is paramount in the choice of coupling method. From the beginning we were attracted to the use of preformed symmetrical anhydrides (*30*) which seemed to have the following favourable features: (i) Symmetrical anhydrides of, e.g., Boc-amino acids are usually stable crystalline solids yet react rapidly with primary and secondary amines. (ii) They possess only one site for nucleophilic attack. This is in contrast to many other activated carboxylic acid derivatives which in principle at least may react at more than one electrophilic centre. Reaction of amine at the "wrong" centre, e.g., in unsymmetrical anhydrides and possibly even in the dicyclohexylcarbodiimidecarboxylic acid adduct results in blocking of the amine and reduction in yield. (iii) No other chemical species which could participate in side reactions are present in the reaction mixture, other than the protected amino acid itself.

There is one report (*31*) of double amino acid incorporation during solid phase synthesis which has been ascribed to rearrangement of the glycine anhydride (**XXI**) to the corresponding protected diglycine (**XXII**). Activation of (**XXII**) or of an intermediate could then result in addition of two glycine residues to the growing peptide chain. This is a potentially serious side reaction which might have special significance in the polar reaction media used in the polyamide method. If it occurs its effect should be most easily detected in multiple sequences of the same residue where the effects would be cumulative. A favorable sequence is the *N*-terminal region of β-endorphin (**XXV**).

(XXIII) Val.Gln.Ala.Ala.Ile.Asp.Tyr.Ile.Asn.Gly

(XXIV) Trp.Leu.Glu.Glu.Glu.Glu.Glu.Ala.Tyr.Gly.Trp.Leu.Asp.Phe.NH_2

(XXV) 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

Careful Edman degradation (*32*) of this synthetic peptide while still attached to the polyamide resin failed to reveal any triglycine sequence ascribable to this rearrangement.

PROCEDURES

Two protocols for the use of polydimethylacrylamide resins in peptide synthesis are given below Fig. 3. Protocol A utilizing Boc-amino acids is analogous to conventional

polystyrene-based synthesis except for the use of dipolar aprotic solvents (dimethylformamide or dimethylacetamide) wherever possible. Acidic cleavage of Boc-groups is very slow in these media and best results for the deprotection step have been obtained

		A α-Boc-ω-Benzyl	B α-Fmoc-ω-Butyl
	t-AmOH	5 x 2 min	
	AcOH	5 x 2	
De-protection	HCl-AcOH	5 + 25	
	AcOH	5 x 2	
	t-AmOH	5 x 2	
	DMF	5 x 2	5 x 1 min
Neutralisation	Dipea-DMF	3 x 2	3 + 7 (Piperidine-DMF)
	DMF	5 x 2	10 x 1
Coupling	Boc.AA.anhydride	1 x 60-120	1 x 60-120
	DMF	5 x 2	5 x 1
	Total operations	41	23
	Total cycle time	5 - 6 hr	2 ~ 3 hr

FIG. 3. Protocols for solid phase peptide synthesis using polydimethylacrylamide supports and (A) *t*-butoxycarbonylamino acids, and (B) fluorenylmethoxycarbonylamino acids.

using hydrogen chloride in acetic acid. Neutralization of the resulting hydrochloride salt is by the hindered tertiary base diisopropylethylamine, again in dimethylformamide or dimethylacetamide. Because of the exothermic mixing of acetic acid with the amide solvents, washing with neutral *t*-amyl alcohol is interspersed.

Protocol B utilized fluorenylmethoxycarbonyl amino acids. Deprotection is with 20% piperidine in dimethylformamide or dimethylacetamide and no neutralization step is required. Because of this all reactions and washing procedures can now be carried out using a single solvent with halving of the number of steps and total time involved. This procedure appears to us to be the method of choice for the immediate future, although experience in its application is thus far limited (see following section).

Synthesis usually commences with the introduction of an internal reference-spacer amino acid. The β-alanine residue present in the early polydimethylacrylamide resin can serve this function, but its ninhydrin color is low in amino acid analysis reducing the accuracy of the determination. This is followed by the appropriate reversible linkage. Activated esters (trichlorophenyl or pentachlorophenyl) are the reagents of choice for introduction of the linkage agents bearing free hydroxyl groups. An equivalent amount of catalyst 1-hydroxybenzotriazole is added to speed the active ester reaction. Formation of the benzyl ester bond is carried out using the protected amino acid symmetrical anhydride in the presence of catalyst *p*-dimethylaminopyridine. The use of Fmoc-amino acids in the presence of a basic catalyst was initially a cause of concern, and for this first residue they were originally replaced by Boc or Bpoc derivatives (*25*, *33*). However, cleavage of Fmoc-derivatives by dimethylaminopyridine proved to be very slow ($t_{\frac{1}{2}} \sim 85$ min for a 10% solution in dimethylformamide; concentration of the

catalyst in the ester forming reaction is actually about 1.5%), and the acylation reaction is so rapid (complete within 5 min in a single case measured using linkage agent **IX**) that this is probably not a significant problem.

Further elaboration of the desired sequence follows protocols A or B. Substantial excesses (five- to sixfold) of the preformed protected amino acid anhydrides are used in order to achieve rapid acylation and minimize chain-terminating side reactions. Reaction is usually complete within a few minutes as judged by the sensitive ninhydrin test (*34*). For the introduction of asparagine and glutamine, *p*-nitrophenyl esters are preferred to anhydrides; addition of an equivalent amount of 1-hydroxybenzotriazole again gives very rapid reaction.

It should be noted that the success of solid phase synthesis in general and in our experience particularly with fluorenylmethoxycarbonyl derivatives and in the nucleotide field (see Oligonucleotide Synthesis) is critically dependent on the use of solvents and reagents of the highest purity attainable. The volumes of solvents involved are inordinately large compared with the reacting groups and traces of impurities can have serious effects. Likewise the protected amino acids (or nucleotides) must be absolutely free from contaminants which, when activated, might complete effectively in acylation of free amino (or hydroxy) groups.

SYNTHESES ACHIEVED

Peptides prepared thus far by the Boc or Fmoc variants of the polyamide method include the Merrifield–Dorman test-tetrapeptide (*14*), bradykinin (*14*), acyl carrier protein decapeptide residues 65–74 (*15*, *16*), undeca and octadecapeptide sequences from the *C*-terminus of the nuclease barnase (*16*), substance P (*17*) and some analogs (*35*), two disulfide-containing tridecapeptides related to the toxin from *Conus geographicus* (*36*, *37*), 12-leucine human minigastrin I and its des-Trp^1-derivative (*38*), human β-endorphin (*33*, *39*), and pancreatic trypsin inhibitor (*12*, *40*). Brief discussion is given here of some aspects of these syntheses which illustrate the high efficiency attainable in assembly of peptide sequences using the polyamide method, increased yield and purity obtained by the use of fluorenylmethoxycarbonylamino acids, and flexibility in synthesis permitting, for example, specific formation of individual disulfide bonds.

Mention has already been made (Introduction) of the use of the acyl carrier protein residues 65–74 decapeptide sequence (**XXIII**) as a stringent test case in solid-phase synthesis. Syntheses of this sequence on polydimethylacrylamide have been recorded using both Boc (*16*) and Fmoc (*25*) amino acids, the latter in 74% yield. No satisfactory polystyrene-based synthesis of this sequence has yet been reported. More recent demonstration of the efficiency of peptide assembly on polyamide supports comes from a preparation of Leu^{12}-human minigastrin I (**XXIV**)[3] and its des-Trp^1-analog (*38*). As far as we are aware, there has been no previous substantial solid-phase synthesis in the gastrin field, but a solution synthesis (*42*) of the tridecapeptide des-Trp^1

[3] Some early confusion regarding the structure of human minigastrin has now been clarified (*41*). The synthesis of "11-leucine human minigastrin I" reported by Wünsch and his collaborators is actually of the des-Trp^1-analc

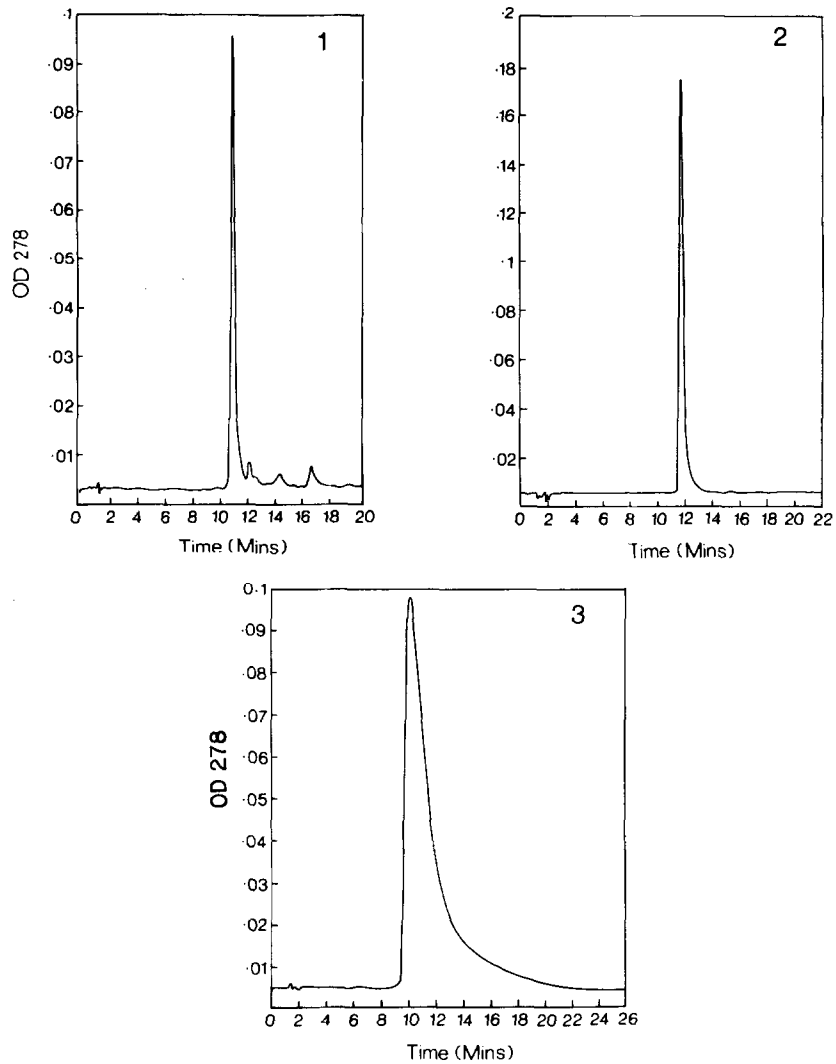


FIG. 4. Isolation of des-Trp[1]-Leu[12]-human minigastrin I. (1) Total crude product from ammonolysis of the peptide-resin. The peak at 14 min is the corresponding peptide methyl ester. Conditions: μ-Bondapak C_{18} column, linear gradient of 18–36% acetonitrile in 0.01 *M* ammonium acetate pH 4.5 over 20 min. Flow rate 2 ml/min. (2) Tridecapeptide after purification by preparative hplc. Conditions as in (1). (3) Analytical anion-exchange hplc of purified tridecapeptide. Conditions: Nucleosil 10 $N(CH_3)_2$ column, isocratic elution with 10% acetonitrile in 0.5 *M* ammonium acetate pH 8.1. Flow rate 2 ml/min.

analog has enabled a direct comparison with classically prepared material.[4] The polyamide synthesis was initiated by addition of Fmoc-norleucine anhydride to ethylene diamine treated resin (**VI**), followed by the trichlorophenyl ester of the linkage agent (**IX**) and the first residue (Boc-Phe anhydride). Thereafter rigorously purified Fmoc-amino acids were used in protocol B and the synthesis terminated with the appropriate

[4] We are grateful to Professor Wünsch for a generous gift of des-Trp[1]-leu[12]-human minigastrin.

Boc-amino acid. Side chains of aspartic and glutamic acids were protected as *t*-butyl esters; that of tyrosine as the *t*-butyl ether. Tryptophan was unprotected. After completion of the assembly, *t*-butyl groups were removed from the resin bound trideca and tetradecapeptides with 90% aqueous trifluoroacetic acid and the peptide amide detached by treatment with methanolic ammonia for 16 hr. (Cleavage from the support is actually achieved with equal efficiency within 2 hr, but the peptide amide is then contaminated with a substantial proportion of the corresponding methyl ester.) hplc analysis of the total crude tridecapeptide product is shown in Fig. 4 together with analytical chromatograms on μ-Bondapak C_{18} and Nucleosil 10 dimethylamino anion exchange columns of the preparative hplc-purified material. In these last analytical chromatograms, as well as on tlc and paper electrophoresis, the product is identical with the tridecapeptide amide prepared by solution synthesis.[4] The overall yield of seemingly completely pure solid-phase synthesized product was 59%. Similar results were obtained for leu^{12}-human minigastrin I itself. Excellent analytical data were obtained in both cases.

The enhancement in yield obtained using Fmoc-amino acids (presumably largely attributable to omission of the hydrogen fluoride treatment) is shown forcefully in two syntheses of the 31-residue opiate β-endorphin (**XXV**) (*33*, *39*). Assembly by protocol A (39)[5] with cleavage from the resin by hydrogen fluoride/anisole gave the elution profile shown in Fig. 5a on chromatography on carboxymethylcellulose. A second synthesis (*33*) using protocol B with cleavage by trifluoroacetic acid/methionine gave the elution profile of Fig. 5b. The very large impurity peak eluting after β-endorphin (A) in the first synthesis was almost entirely absent in the second, and the yield of purified β-endorphin increased correspondingly from 10 to 41%.

The sea snail toxin (*36*) [possible part structure (**XXVI**); disulphide linkages not established by degradation] provides a good example of the flexibility of polyamide-based methodology. Assembly of the 13-residue sequence using the combination of protecting groups and resin linkage shown in Fig. 6 proceeded smoothly by protocol A. Hydrogen fluoride treatment cleaved all the protecting groups except for the acid-resistant resin linkage and the two *S*-acetamidomethyl residues. The first disulfide bond was then established by air oxidation of the resin-bound peptide. Some dimer was also formed in this step—a further identification of the lack of rigidity in the polyamide matrix. The peptide was then cleaved from the resin by brief ammonolysis and the second disulfide formed in free solution by iodine oxidation. An identical procedure with interchange of acetamidomethyl and *p*-methylbenzyl protecting groups on two of the cysteine residues provided one of the two remaining disulphide isomers.

OLIGONUCLEOTIDE SYNTHESIS

Solid-phase oligonucleotide synthesis has been reviewed by Kössell and Seliger in 1975 (*5*) and more recently by Gait (*6*). The earlier review lists 32 investigations into

[5] This synthesis utilized xanthydryl side chain protected derivatives of asparagine and glutamine incorporated by symmetrical anhydrides. These derivatives are unsuitable for use with $N\alpha$-Fmoc protection, and the second synthesis by protocol B used the side chain unprotected amino acid *p*-nitrophenyl esters.

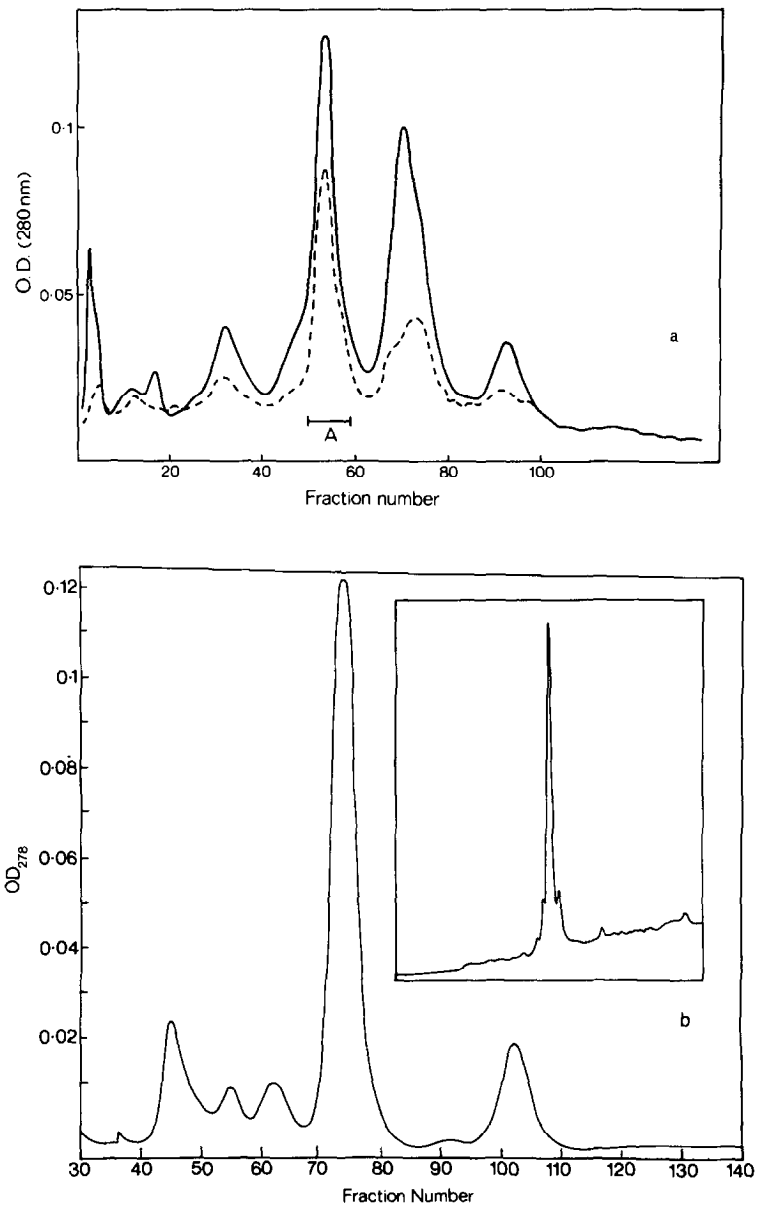
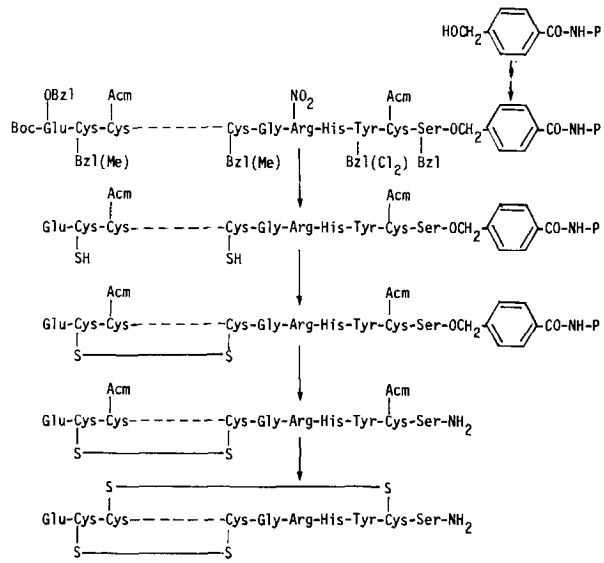


FIG. 5. Isolation of synthetic human β-endorphin. (a) Total reaction product from hydrogen fluoride-treated peptide-resin prepared by protocol A. Conditions: carboxymethylcellulose CM52 column, linear gradient of 0–0.1 *M* sodium chloride in 6 *M* urea–0.01 *M* phosphate pH 6.0. Solid line, optical density; dashed line, radioactivity. Further purification of the synthetic β-endorphin was by rechromatography of peak A under the conditions of (b) below. (b) Total reaction product from trifluoroacetic acid-treated peptide-resin prepared by protocol B. Conditions: carboxymethylcellulose CM52 column, linear gradient of 0.015–0.15 *M* ammonium acetate pH 6.0. Inset: analytical hplc of main peak on μ-Bondapak C_{18}.



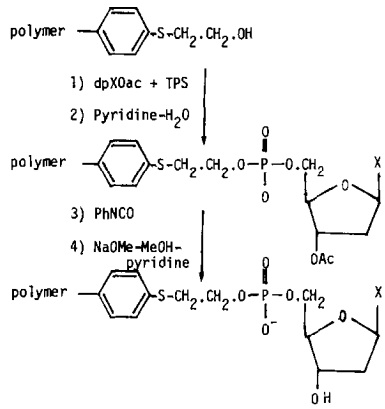
(XXVI)

FIG. 6. Scheme for synthesis of toxin from *Conus geographicus*.

the synthesis of oligonucleotides on polymeric supports which had appeared in the literature up to 1974. None of these has seemingly found useful application by subsequent synthesis of specific, biologically useful oligonucleotide sequences.

It seemed to us that application of the polyamide method to oligonucleotide synthesis would provide a particularly powerful test of the principles on which it was conceived. Oligonucleotides are highly polar molecules—in the backbone unprotected phosphodiester form they bear multiple ionized or ionizable groups—and inadequate compatibility with polystyrene and other nonpolar supports should be more marked than in the peptide series. In a practical sense, development of a useful rapid solid-phase method for oligonucleotide synthesis was of great importance in view of the explosive growth in demand for specific synthetic oligonucleotides. This has arisen because of rapid developments in nucleic acid sequencing methodology and in genetic engineering; it cannot easily be met by the slow and laborious methods of solution synthesis.

Some differences in objectives between solid phase oligonucleotide and peptide synthesis should be noted. Internucleotide bond-forming reactions rarely go to completion when carried out in free solution and there is no reason to suppose that this will be improved in the solid phase. Accumulation of failure sequences and of by-products produced from the complex reactants is bound to occur in extended synthesis. On the other hand, the purification problems may be less formidable than in peptide series. Simple failure sequences will inevitably differ in net charge at neutral or alkaline pH from the completed oligonucleotide, offering good prospects for separation. Development of powerful purification methods must nevertheless be an integral part of improved solid phase synthesis methodology. Because of the significantly non-quantitative nature of coupling reactions, overall yields will be low. Fortunately this is

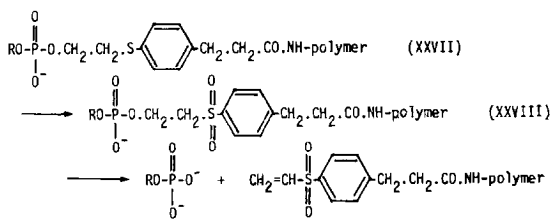


(X = T, bzA, anC, or ibG; TPS = triisopropylbenzene sulphonyl chloride)

FIG. 7. Scheme for solid phase oligodeoxyribonucleotide synthesis on polydimethylacrylamide supports.

counterbalanced by the very small quantities of material required in nearly all biological experiments. Optical density quantities (~100 μg) are often more than adequate, again facilitating purification.

Glycosidic linkages (especially those derived from the purine-containing nucleosides) are notably acid-labile, and acidic treatments were therefore to be avoided in any repetitive solid phase method. We therefore elected to use simple alkali-labile acetyl protecting groups for the 3′-hydroxy groups of the growing nucleotide chain as in established solution methods (*5*), and an alkali-labile "safety-catch" resin linkage (*43*). This utilizes the β-thioether reagent (**XIII**) (*20*) which gives a base-stable phosphodiester (**XXVII**) when combined with the first nucleotide unit labilized to base catalyzed elimination by oxidation to the sulfone (**XXVIII**). Protecting groups for the purine and pyrimidine amino-groups were as used in conventional solution methodology (*5*).



A phosphodiester strategy was adopted for elaboration of the oligonucleotide chain. This was chosen primarily because of the greater in-laboratory experience in application of this well-tested method, but the more recent phosphotriester approach (*5*) is also attractive and is under current investigation. The overall scheme of synthesis is shown in Fig. 7 (*44*). The experimental protocol is shown in Fig. 8.

Because of the nonquantitative nature of the internucleotide coupling reaction, treatment with phenyl isocyanate was included in order to block off unphosphorylated 3′-hydroxy groups (cf. *45*). This should minimize the formation of failure as opposed to truncated oligonucleotide sequences. It is now evident, however, that hydroxy groups not converted to internucleotide bonds in the coupling reaction are largely (temporarily)

masked in an unknown manner. The isocyanate reagent is therefore ineffective in its original purpose but is retained because it serves efficiently the equally important function of removing water from the resin matrix. This treatment replaces the conventional multiple azeotropic evaporation steps conventionally employed in solution synthesis, and enables solid phase synthesis to be conducted with the aid of a modified Beckman 990 Peptide Synthesizer (*44*).

Yields for one cycle of nucleotide addition and deprotection are usually within the range 70–90%. The key factors appear to be (i) maintenance of strictly anhydrous conditions throughout, and (ii) maximum purity of individual 3′-*O*-acetyl-nucleotides. Rigorous repurification of nucleotide starting materials has in several instances improved coupling yields significantly. In separate experiments it has been shown that added carboxylic acids reduce yields by an amount far greater than their relative molar proportions would suggest (*46*).

	Pyridine	5 x 2 min
Drying	PhNCO-pyridine	2 x 30 + 1 x 240
	Pyridine	5 x 2
	Pyridine-MeOH	5 x 2
De-protection	NaOMe-pyridine-MeOH	2 x 5
	Pyridine-MeOH	5 x 2
	Dimethylformamide	5 x 2
	Pyridine	10 x 2
Coupling	Activated nucleotide-pyridine	1 x 240
	Pyridine	5 x 2
	Pyridine-water	2 x 5 + overnight
	Pyridine	5 x 2

FIG. 8. Protocol for solid phase oligodeoxyribonucleotide synthesis.

The method has been used successfully (*20*, *44*) in the preparation of a wide range of oligodeoxyribonucleotides for biological studies, thus far up to 12 residues in length. Improved hplc fractionation procedures utilizing a microparticulate silica-based anion-exchange resins (Partisil 10 Sax) as well as a reversed phase μ-Bondapak C_{18} support have been of importance in isolating and purifying the synthetic products.

CONCLUSIONS

Bamford and Ledwith (*47*) have recently reemphasized "the need for polymer backbones that are carefully designed with the particular requirements of the desired (solid phase) synthesis in mind." The resins described above were designed specifically as supports for the synthesis of peptides and other polar molecules, and they function well. Time and much more experience will tell whether their use will contribute to the ultimate goal, the rational synthesis of proteins (*7*). It is certain even now, however, that their use has greatly extended the scope of solid phase oligonucleotide synthesis, for which seemingly no practical method hitherto existed. The compatability of the polydimethyl-

acrylamide supports with the very polar nucleotide reactants seems to us strong confirmation of the principles on which the resins were designed.

The introduction of fluorenylmethoxycarbonylamino acids, both in the present work and simultaneously into polystyrene-based synthesis (*25*), has enabled reaction conditions to be made much milder than formerly. The increased yields obtained are gratifying and auger well for the synthesis of sensitive molecules. Their use may also contribute to what we regard as the next step in solid phase peptide synthesis—development of true solid phase fragment condensation strategies. Realization of this last objective may in fact be the crucial step for solid phase protein synthesis.

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