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BIOPOLYMER SYNTHESIS ON SOLID SUPPORTS

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Abstract - The concept of solid phase synthesis began as a new approach to the preparation of peptides, but has been extended to the synthesis of polynucleotides, polysaccharides, polyamides and a variety of other classes of compounds. The solid support simplifies and accelerates the synthetic process, aids the separation of reagents and by-products, and reduces problems of insolubility of intermediates. The synthesis may involve a stepwise addition of monomer units or the condensation of preformed oligomer fragments. Large numbers of peptides and several small proteins have been synthesized by the solid phase method. To facilitate the synthesis we have developed a new, more acid-stable attachment of the peptide to the polystyrene support, in which the chain is anchored through a hydroxymethylphenylacetamidomethyl group. Alternatively, the peptide chain can be anchored through a more labile alkoxybenzyl ester linkage, if the very acid-sensitive Bpoc group is used for N^{α}-protection. The ideal design for a multi-step synthesis will depend on a combination of protecting groups that are removable by completely independent reaction mechanisms. Such a system is called orthogonal. A new amino protecting group, the dithiasuccinoyl (Dts) group, has been designed for that purpose. It is removed by reduction, but is stable to acids and photolysis and can be used in conjunction with these two orthogonal modes of deprotection. The synthesis of biopolymers on solid supports was illustrated by studies of the role of arginine in the neurotoxin, apamin, and of the substrate specificity of a histone deacetylase and by a recent synthesis of crystalline glucagon.

INTRODUCTION

Much is known about the major biopolymers, proteins, carbohydrates, and nucleic acids, from studies on the naturally occurring compounds themselves, but many questions remain unanswered. Some of these can be investigated better by means of a synthetic approach, and considerable progress in that direction has been achieved, particularly with the peptides and proteins. The ability to synthesize the parent molecule, and suitable analogs of it, provides a route to studies of its mechanism of action and the relation of its molecular structure to its biological activity. Synthesis has also provided peptide derivatives with enhanced potency and improved selectivity, and in some instances has yielded effective inhibitors. There is a great need for simple, efficient methods for the synthesis of pure peptides in high yield.

There are two general approaches to the synthesis of peptides, the classical method, in which all reactions are carried out in homogeneous solution, and the solid phase method, in which the reactions are heterogeneous ones between soluble reagents and an insoluble peptide chain that is attached to a solid support. Both approaches can be subdivided into stepwise methods, in which the chain is extended by one monomer unit at a time, and fragment methods, in which the peptide is enlarged by combining preformed oligomers. The purpose of this paper is to discuss some new developments in the methodology of solid phase peptide synthesis and some recent applications of the technique to the synthesis of representatives of this class of biopolymers.

SOLID PHASE PEPTIDE SYNTHESIS

The stepwise method of solid phase synthesis (1,2) consists of six kinds of reactions, three of which are carried out only once and three of which are repetitive and are performed at each cycle of a synthesis (Fig. 1).

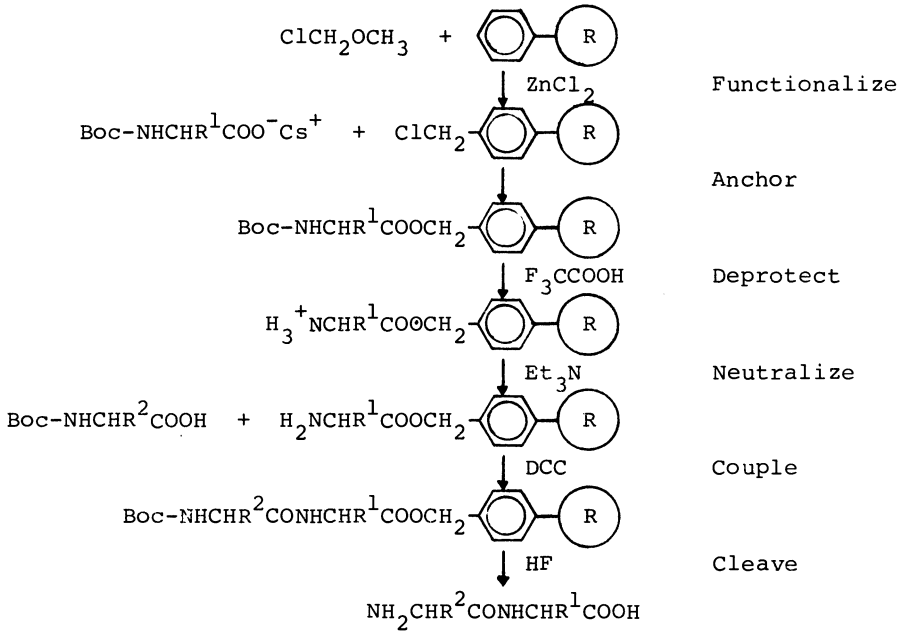


Fig. 1. A typical scheme for solid phase peptide synthesis

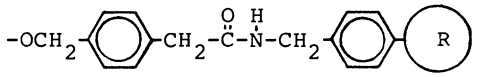
Because they must be repeated many times during the synthesis of a long peptide, the deprotection, neutralization and coupling steps must proceed rapidly, in high yield, and with a minimum of side reactions. The functionalization, anchoring and cleavage steps are required only once during the synthesis and speed and yield are less critical, although the absence of side reactions is still an important requirement. The stepwise method can be designed to give either protected or free peptides.

As an alternative to the usual stepwise technique, a variety of fragment condensation schemes can also be devised. The fragments can be prepared either by solution methods or by solid phase methods and can then be coupled either in solution or to a polymer-supported peptide chain. The advantage of this approach is that all the intermediates can be purified and analyzed before they are used in the next step of the synthesis, in order to minimize the accumulation of impurities. In addition, a long peptide chain that is missing a fragment of several residues will differ from the target molecule more than a deletion peptide missing only a single amino acid residue and should be more easily removed. In general it will be necessary to obtain the intermediate fragments in the protected form to enable them to be used for further condensation reactions. This requirement modifies the chemistry that can be used for their synthesis.

Attachment to the support

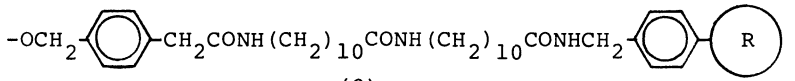
The most frequently used solid support is a suspension copolymer of styrene with 1% divinylbenzene. The original functionalization was achieved by chloromethylation of the resin, and the peptide chain was anchored to the resin beads by reaction of the first N^{α}-protected amino acid with the chloromethyl-resin to form a benzyl ester (1). This bond, which is relatively stable to mild acid but labile to strong acid, has been very useful for the synthesis of large numbers of peptides and even some proteins. For the larger molecules, however, there is a significant loss of peptide chains during the synthesis (1 or 2% per step) due to the repeated acidolytic conditions required for the removal of the N^{α}-Boc group at each step (3). To avoid these losses we have designed and synthesized an anchoring bond of increased acid stability.

Pam-resin. The new resin was prepared by inserting a phenylacetamidomethyl (Pam) group between the polystyrene aromatic rings and the peptide chain (4,5).



(1)

The observed rate of cleavage of a peptide from this resin by 50% trifluoroacetic acid in dichloromethane was only 1% as fast as that from the usual benzyl ester support. This improved stability also avoids the generation of hydroxymethyl groups that are formed when peptide chains are lost, and the accompanying side reactions that ensue. A phenylacetamido linkage was also present in a long extender arm which was inserted between the peptide and polystyrene support (6).



(2)

The improved yield of a synthetic 57-residue apolipoprotein made on this support (7) was attributed to the flexibility of the arm and to the removal of the peptide chain from the vicinity of the polystyrene backbone. Whether the improved syntheses with the new linkages are due to their increased stability or to their role as extenders, or both, is not yet certain.

Boc-Amino acyl-Pam-resin could be prepared in several ways (5). The most unambiguous way is shown in Fig. 2.

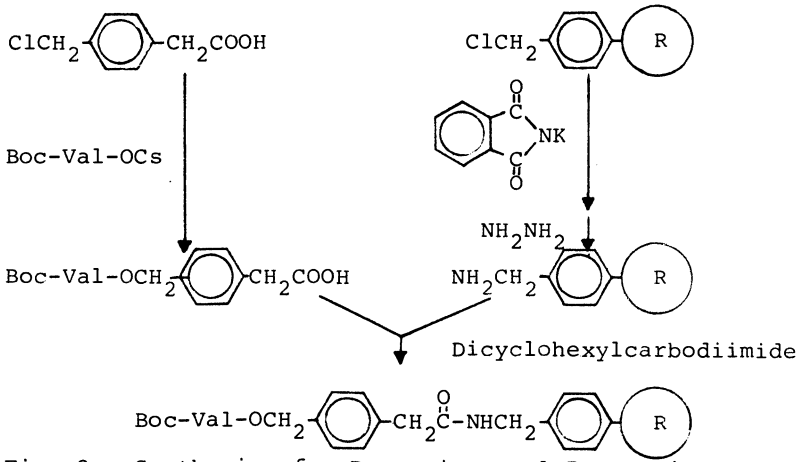


Fig. 2. Synthesis of a Boc-amino acyl-Pam-resin

Aminomethyl-resin. To facilitate the preparation of the Pam-resin a new synthesis of aminomethyl-copoly(styrene-divinylbenzene) Fig. 3 was developed,

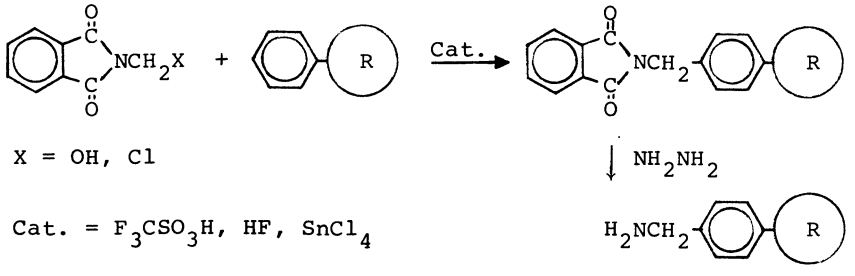
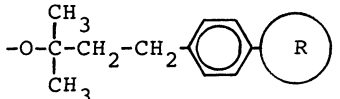


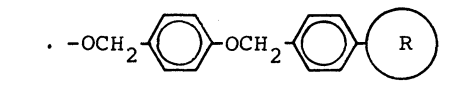
Fig. 3. The synthesis of aminomethyl-resin.

The reaction is easily controlled, and circumvents the need for chloromethylation of the polymer. In that way, small amounts of unreacted chloromethyl or hydroxymethyl groups can be prevented from accumulating and the use of the carcinogenic reagent, chloromethyl methyl ether can be avoided. We believe that the new route to aminomethyl-resin and the attachment of the peptide chain through a Pam-resin represent important improvements in the solid phase synthesis of free peptides.

Synthesis of protected peptides. For the preparation of the protected peptides needed for fragment syntheses, modified synthetic schemes are necessary. A useful one involves the combination of a very acid labile N^{α}-protection, such as the biphenylisopropyloxycarbonyl (Bpoc) group, with stable benzyl-based side chain protection and a C-terminal attachment through a linkage of intermediate acid stability. The tert-alkyl alcohol resin (3) and especially the 4-alkoxybenzyl alcohol resin (4) have been useful for the purpose (9,10).

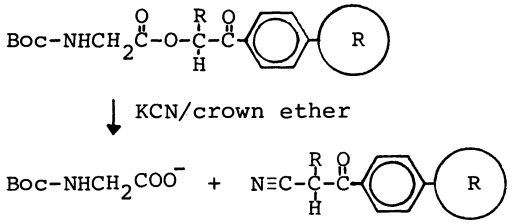


(3)



(4)

Another versatile route to protected peptides has combined N^{α}-Boc and side-chain benzyl protection with a C^{α}-phenacyl ester linkage to the resin. The latter can be cleaved by hydrazine, hydroxide or thiophenoxide (11,12) or, in the case of the α-methyl phenacyl ester, by photolysis (13). We have found recently (14) that these phenacyl esters can also be cleaved by a complex of potassium cyanide and 18-crown-6 ether.



R = H or CH_3 (1)

The crown ether (15) enhances the nucleophilicity of cyanide in an aprotic solvent such as DMF or acetonitrile sufficiently to split the phenacyl ester in high yield without affecting Boc or benzyl groups. This mild and selective procedure appears to be an improvement over the previous methods of cleaving phenacyl esters.

Orthogonal protection

Each of the protection schemes just described has relied in whole or in part on differential rates of acidolysis of the various groups. Although the relative rates of removal of Bpoc, alkoxybenzyl ester, and benzyl ester are approximately 1:3,000:1,000,000, there is, nevertheless, some loss of the more stable groups under the conditions that will remove all of the more labile groups. The combination is practical for peptides up to about 50 residues, but may become marginal beyong that range.

The ideal synthesis would depend on a set of completely independent reactions, and such a scheme of protection has been called orthogonal (16). This term means that members of each class of protecting groups are cleaved by a specific mechanism and that all other groups are stable to reaction by that mechanism. Therefore, any class of groups can be removed in any order without affecting any other class of groups. Since, in general, there will be at least three kinds of groups that must be distinguished, the α-amino-, α-carboxyl-, and side chain-, we need three compatible classes of protecting groups. If various side chain groups must also be distinguished, further subdivision will be required and additional classes of protecting groups will be needed.

A scheme of solid phase peptide synthesis that can be envisioned as meeting the basic requirements of orthogonal protection is illustrated in Fig. 4.

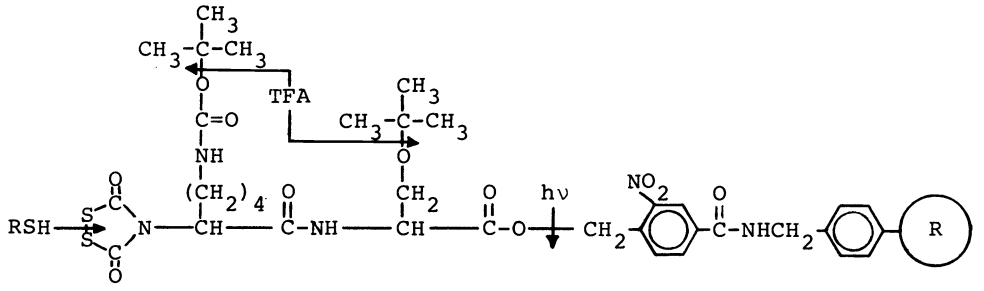


Fig. 4. An orthogonal protection scheme for solid phase synthesis.

This makes use of acid-labile side chain protection, a photolabile carboxyl group to anchor the peptide to the resin, and a new thiol-sensitive α-amino protecting group. Thus, there are three kinds of reactions and three classes of protecting groups, each of which is sensitive to one of the reactions and stable to the others.

The dithiasuccinoyl group. The new thiol-sensitive amino protecting group (16,17), dithiasuccinoyl (Dts), was developed specifically to meet the requirements of an orthogonal scheme. Several Dts-amino acids were prepared by the reactions shown in Fig. 5.

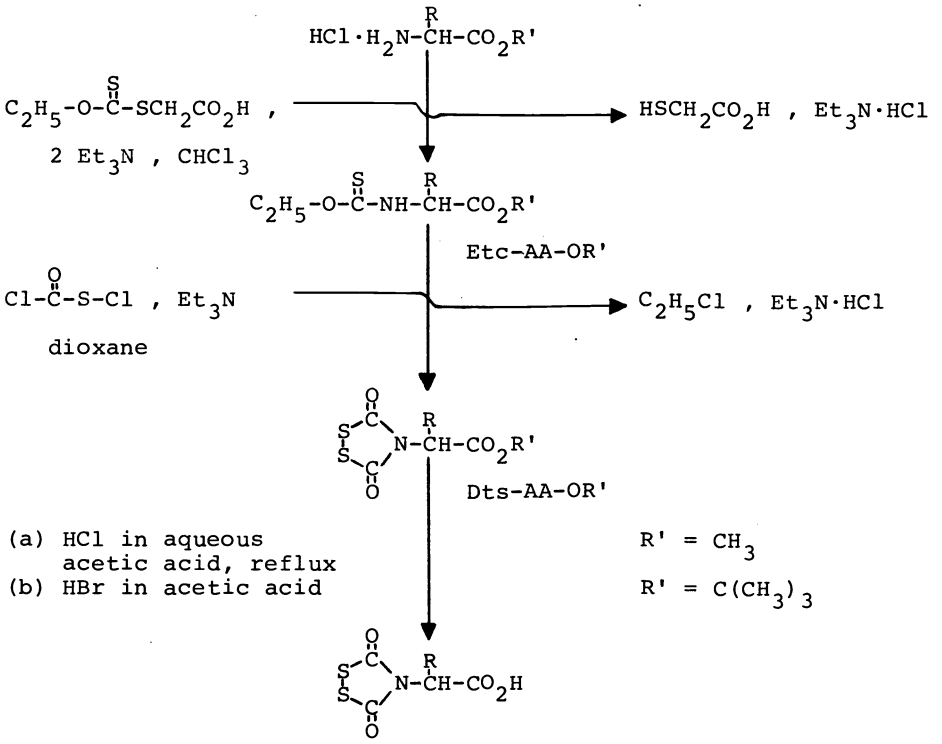


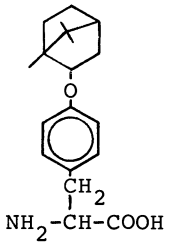
Fig. 5. The synthesis of Dts-amino acids.

The structure of these stable, readily crystallizable derivatives was established by elemental analysis, infrared and ultraviolet spectra, proton and carbon-13 nuclear magnetic resonance, electron impact and chemical ionization mass spectroscopy and chemical transformations. The 1,2,4-dithiazolidine-3,5-dione ring is readily opened upon mild reduction with a thiol, liberating two moles of carbonyl sulfide and generating the free amine. Nucleophilic attack at the carbonyl by the thiol to give a thiourethane does not seem to occur. Dts-compounds are resistant to strong acids, including HBr in acetic acid and hot 6 N HCl; and to weak bases, such as aqueous sodium bicarbonate and α-amino groups of peptides. Cleavage at the carbonyl occurs with strong bases,

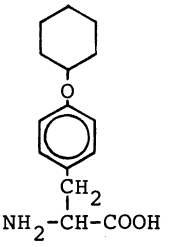
yielding the free amine with aqueous NaOH and the urea with benzylamine.

Side chain protecting groups

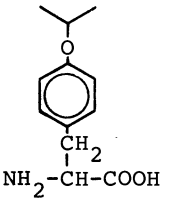
A problem of special interest to us has been the acid-catalyzed rearrangement of tyrosine alkyl ethers to stable ring-alkyl derivatives (18). It was shown that deprotection of O-benzyltyrosine, or of peptides containing this residue, by means of HF containing 10% anisole could give rise to 10-70% of 3-benzyltyrosine via an intramolecular route. O-(2,6-dichlorobenzyl)-tyrosine was shown to be much more acid stable and to reduce the rearrangement product several fold but not to zero. Three new tyrosine ethers have now been synthesized (19).



Tyr(iBor)



Tyr(cHex)



Tyr(iPr)

The isobornyl and cyclohexyl derivatives were prepared in good yield by the reaction of N-trifluoroacetyl tyrosine methyl ester with boron trifluoride and either camphene or cyclohexene. The isopropyl derivative was obtained in low yield from isopropylbromide and the copper complex tyrosine. Table 1 lists the loss per cycle of the protecting groups in 50% TFA/CH_2Cl_2 and the first order rate constants, together with the extent of rearrangement to the 3-alkyl derivatives occurring in HF/anisole.

Table 1. Acid stability of protected tyrosine

Compound	k_1 (sec^{-1})	Loss/cycle (50% TFA) (%)	3-Alkyl-Tyr (HF-anisole) (%)
Tyr(iBor)	2×10^{-2}	100	0
Tyr(Bzl)	6.4×10^{-6}	0.76	15
Tyr(cHex)	5.1×10^{-8}	0.006	0.5
Tyr(iPr)	1.5×10^{-9}	0.00017	3.5
Tyr(2,6-Cl_2Bzl)	1.2×10^{-9}	0.00014	5.0

For solid phase synthesis of tyrosine peptides the O-cyclohexyl derivative has the best combination of properties. Its acid stability is satisfactory for a multistep synthesis and, although the rearrangement can still be detected, it is acceptably low for many purposes. Boc-Tyr(cHex) was used for the solid phase synthesis of angiotensin II, and the unfractionated cleaved product was shown to contain only 0.3% of the 3-cHex-Tyr isomer. In this instance the by-product was readily removed chromatographically, and the pure angiotensin II was fully active in the pressor assay. Trudelle and Spach (20) observed a by-product when Tyr(Bzl) was deprotected in HF or HBr/TFA, but detected none when HBr/HOAc was the reagent. This finding was confirmed by Bell et al. (21). A careful examination of the reaction products shows, however, that low but significant levels are definitely formed. Tyr(Bzl) gave 1.0% of 3-Bzl-Tyr and Tyr(cHex) gave 0.3% of 3-cHex-Tyr after treatment with 6 N HBr in HOAc at 25° for 2 hr. The rearrangement can be avoided entirely by protection of tyrosine with benzyloxycarbonyl (18) or 2,4-dinitrophenyl

(22) groups, which can be removed by acid or by thiols respectively. Both groups, however, are sensitive to amine nucleophiles.

PEPTIDES SYNTHESIZED ON SOLID SUPPORTS

Since 1962, when solid phase peptide synthesis was first introduced, a large number of peptides have been synthesized by the new technique. These have included biologically important peptides such as angiotensin, bradykinin, oxytocin, ACTH, somatostatin, parathyroid hormone, insulin, β-endorphin and many of their analogs (see Ref. 2). The method has also been extended to the synthesis of several small proteins such as ribonuclease, lysozyme, acyl carrier protein and trypsin inhibitor. In the present paper three examples will be given of medium sized peptides that have recently been synthesized in our laboratories.

Histone fragments

Postsynthetic acetylation and deacetylation of the histones of the cell nucleus have been implicated in the control of chromatin structure and function (23). The principal sites of acetylation of calf thymus histone H4 are known to be at lysine residues 16, 12, 8 and 5, but the relative rates of addition and removal of the acetyl groups or the order in which it occurs is not known. To answer these questions and to learn about the substrate specificity of a histone deacetylase from calf thymus nuclei (24), a synthetic study was initiated using the synthetic methods that have been described here.

A heptapeptide, H-Ala-Lys([^{14}C]Ac)-Arg-His-Arg-Lys-Val-OH, representing the short 15-21 segment of histone H4 and including the major site of acetylation, was prepared by the standard methods of solid phase peptide synthesis (25). The peptide was cleaved at its benzyl ester linkage to the resin support with HF, and simultaneously the other protecting groups (N^{α}-Boc, N^{ε}-Z, N^{G}-Tos, N^{im}-Tos) were also removed. The product was homogeneous after purification by ion-exchange chromatography on carboxymethyl cellulose and gave a good amino acid analysis.

It was shown that this small acetylated histone fragment was not a substrate for the deacetylase and no ^{14}C was released. This result was in agreement with the data of Horiuchi and Fujimoto (26) who found that 12,16-diacetyl-histone H4-(10-21) derived from the native protein was also not a substrate. We found, however, that histone H4-(1-37), which could be isolated from tritium-labeled acetylated calf thymus nuclei by chymotryptic digestion, was a good substrate for the enzyme. From this experiment it was not clear whether the acetyl groups could be removed independently from any of the acetyllysine residues or whether there might be a sequential mechanism for their release. Peptide synthesis provides a unique way in which to study this problem because molecules with individual or multiple labels at specific locations can be prepared. We began with the synthesis (27) of [Lys([^{14}C]Ac)^{12}, Lys([^{3}H]Ac)^{16}]histone H4-(1-37), Fig. 6. The differential labeling of the two acetyl groups could then be used to determine whether they were released selectively or randomly.

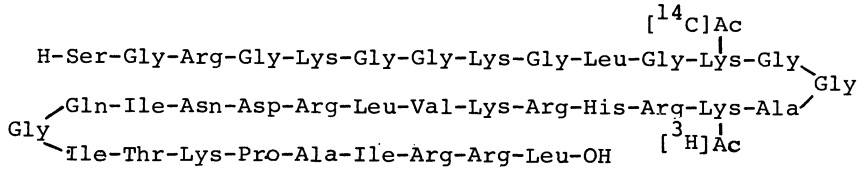


Fig. 6. Structure of the diacetyl-histone H4-(1-37) fragment.

This synthesis also followed the standard solid phase methodology, but was designed to take advantage of a new affinity chromatography method of purification (28). Thus, the chain was assembled stepwise on a benzyl ester-linked support. At the appropriate positions the radiolabeled acetyllysine residues were introduced. The synthesis was monitored by the automated picrate method (29) to determine the completion of the deprotection and coupling reactions and the extent of chain loss and chain termination that occurred at each cycle of the synthesis. Following the completion of the histone sequence at

serine-1, two more residues, Met and S-methoxybenzyl-Cys, were added. The peptide was cleaved and deprotected and then purified on a chloromercuribenzoyl-Sepharose column. Only those chains containing the cysteine sulfhydryl group were retained on the column, while all incomplete terminated chains passed through and were cleanly separated from the parent compound. Elution with cysteine, and cleavage of Cys-Met by cyanogen bromide regenerated the purified histone H4-(1-37) peptide.

When this 37-residue peptide was assayed for release of labeled acetyl groups by the nuclear deacetylase it was found that ^{3}H and ^{14}C were removed at exactly the same rate for at least the first 60% of the reaction. In addition, the rate of appearance of acetyl was the same as for [Lys([^{3}H]Ac)^{16}]histone H4-(1-37) mono-acetylated peptide. From these preliminary data we conclude that either the two acetyls are removed completely independently, or the acetyl is first removed from lysine-16 in a rate limiting step, followed by a very rapid removal of acetyl from lysine-12. The problem should be resolved by a careful product analysis and by separate syntheses of the two monolabeled peptides.

Apamin analogs

Apamin is an octadecapeptide neurotoxin derived from bee venom (30).

H-Cys-Asn-Cys-Lys-Ala-Pro-Gly-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His-NH_2

Its synthesis by the solid phase method has been described by Van Rietschoten et al. (31) and Sandberg and Ragnarsson (32). Chemical data suggested that the two arginines at positions 13 and 14 might be required for activity (33). We have conducted a synthetic study of this question which was designed to contain an internal structural control (34). The plan was to synthesize the molecule with N^{δ}-trifluoroacetylornithine residues in place arginine at positions 13 and 14. This protecting group could be removed to give the di-ornithine derivative, which in turn could be guanidinated to yield the di-arginine peptide. The latter would also contain homoarginine (Har) at position 4, but that was acceptable because it was already known that [Har^{4}]apamin is fully active (33). If either of the two analogs were to prove to be inactive but could be converted into active [Har^{4}]apamin we would have provided the desired internal control for the correctness of the amino acid sequence of the synthetic peptide. With the added assurance that the composition of the peptide chain was correct, more reliable conclusions about these replacements of arginine could be drawn.

The peptides were synthesized by the stepwise solid phase method using the protecting groups shown in Fig. 7.

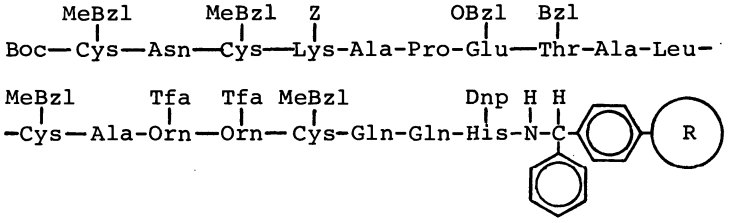


Fig. 7. Protected [Orn^{13,14}]apamin-benzhydrylamine-resin.

The support was a benzhydrylamine resin, coupling was with DCC alone except with Boc-Asn and Boc-Gln where hydroxybenzotriazole was added. The Dnp group was removed from His with 2.5% thiophenol in DMF, and the cleavage and deprotection of side chains was with HF/10% anisole, for 30 min, at 0°. The [Orn(Tfa)^{13,14}]apamin and [Orn^{13,14}]apamin were each reduced and then oxidized in air and purified chromatographically. The [Orn^{13,14}]apamin was guanidinated with O-methylisourea and purified by Sephadex G-25 and carboxymethylcellulose chromatography. The work-up of the three peptides is outlined in Fig. 8.

The coupling, cleavage, deprotection, and guanidination steps all proceeded in good yields. The largest losses were at the oxidation steps and during the several chromatographic steps, giving an overall yield of 14%. The synthetic peptides gave good amino acid analyses and were homogeneous by ion exchange

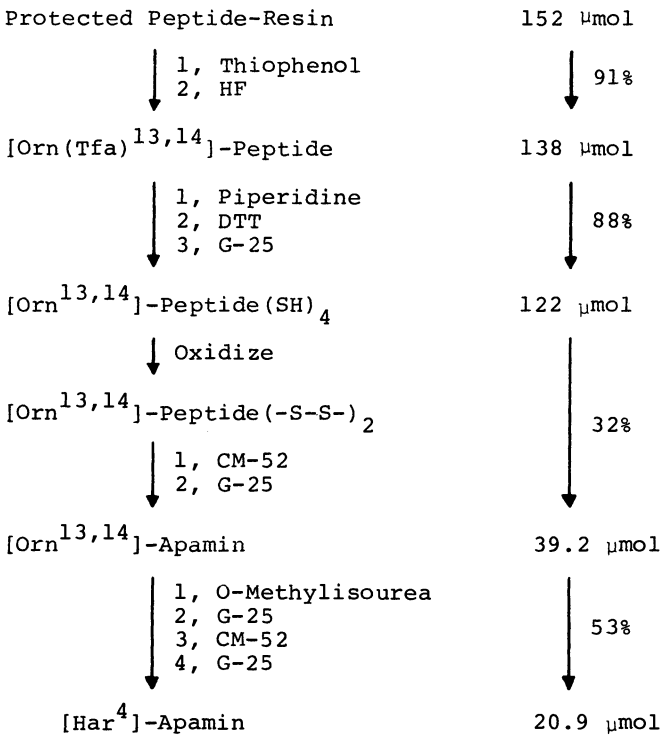


Fig. 8. Work-up of synthetic apamin analogs.

chromatography and polyacrylamide gel electrophoresis.

Neither the [Orn(Tfa)^{13,14}]apamin nor [Orn^{13,14}]apamin produced any symptoms of neurotoxicity whatever upon intravenous injection into mice at the highest levels that could be tested. Therefore, they were below 5% and 1% active, respectively. As expected, the synthetic [Har^4]apamin was fully active compared with native apamin and [Har^4]apamin derived from it. The data are summarized in Table 2.

TABLE 2. Neurotoxic activity of natural and synthetic apamin analogs

Compound	LD_{50}* (nmol)	Relative activity
Natural apamin	47	1.00
Natural [Har^4]apamin		1.00‡
Synthetic [Har^4]apamin	44	
Synthetic [Orn^{13,14}]apamin	>1200†	No activity (<1%)
Synthetic [Orn(Tfa)^{13,14}]apamin	>280†	No activity (<5%)

* Calculated from data at six concentrations on groups of six mice each.

† No observable symptoms at this dose.

‡ Taken from Vincent et al. (33).

These results provide the necessary internal control and strengthen the conclusion that analogs of the correct amino acid sequence were synthesized. We

can then conclude that the observed inactivity of the ornithine derivative was due to the intended sequence and can infer that at least one of the arginines at positions 13 and 14 plays an important role in the action of apamin. The level at which arginine functions is not established, but it may provide electrostatic, steric, or conformation properties that are necessary for proper folding, stability, transport, or action at the receptor site.

Glucagon

Glucagon is 29-residue peptide produced by the α cells of the pancreas. It is intimately involved in the maintenance of carbohydrate balance, although its detailed mechanism of action is not fully understood. The chemical synthesis of this hormone and of suitably designed analogs should be of considerable value in future studies on its structure and function. The synthesis of this molecule has proven to be a difficult task, but two syntheses have been achieved. After extensive efforts Wünsch (35) successfully prepared crystalline glucagon by classical fragment methods. The product was shown to be pure and fully active. In 1975 the Protein Synthesis Group of the Shanghai Institute of Biochemistry reported a new synthesis of glucagon by fragment condensations on a solid support (36). They followed the basic Wünsch scheme of protection, coupling and fragment division rather closely. The first three residues at the carboxyl terminus were added stepwise to a pellicular poly(styrene-divinylbenzene) resin, which then served as the starting point for the coupling of the remaining fragments that had been synthesized by solution methods. The couplings were reported to be essentially quantitative. The completed protected peptide-resin was cleaved and fully deprotected in HF/anisole. Two peaks were obtained by gel filtration, and the glucagon fraction was further purified by DEAE cellulose chromatography. The yield from the first amino acyl-resin was about 17%. The final preparation (3.4 mg) was homogeneous, indistinguishable from natural glucagon by polyacrylamide electrophoresis, and gave typical rhombic dodecahedral crystals. It was essentially fully active in the rabbit blood sugar assay.

We have recently succeeded in synthesizing glucagon by a stepwise solid phase procedure (37) using the protecting groups shown in Fig. 9.

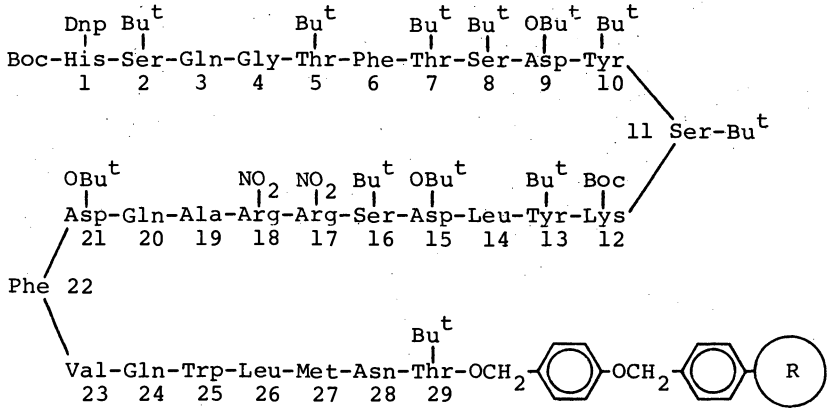


Fig. 9. The structure of synthetic protected glucagon

The support was the alkoxybenzyl alcohol resin of Wang (10). The α-amino protecting group was Bpoc in all but the last step where Boc was used. The couplings were with DCC (3 equivalents, double coupling). The Dnp group was removed from histidine by thiophenol and the remaining side chain groups, except Arg(NO_2), were removed during the cleavage step with 50% TFA in CH_2Cl_2 (45 min, 25°). The mild acid procedure was used to minimize the possibility of α→β rearrangement of the Asp^{15}-Ser^{16} sequence. The nitro groups could then be removed by HF/anisole or by catalytic hydrogenation. The crude free peptide was purified by gel filtration and by DEAE-cellulose chromatography in 6 M urea. The main peak (2.4% yield from the first amino acid residue on the resin) eluted at the position of native glucagon. The isolated product was readily crystallized from water at pH 8.5. Preliminary data indicate good purity and close identity with natural glucagon. The hyperglycemia assay showed essentially full activity compared with native glucagon and the product was also active in an adenyl cyclase assay. We conclude that

glucagon can be synthesized by classical methods, by the solid phase-fragment method, or by the stepwise solid phase method. The purified products appear to be homogeneous, fully active and indistinguishable from native glucagon, and it has been possible to obtain crystalline hormone from all three syntheses.

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