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A General Strategy for the Synthesis of Large Peptides: The Combined Solid-Phase and Solution Approach.

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Dedicated to Professor Carl Djerassi on the occasion of his 70th birthday

Abstract: In the synthesis of large peptides, the yield and purity of the end-products will be greatly improved when smaller segments are purified prior to their use for fragment coupling either on a solid-phase resin or in solution. The N^{α}–Fmoc(9-fluorenylmethoxycarbonyl) method allows the selective acidolytic cleavage of fully protected peptides with a free α-carboxyl group from the solid-phase resin. For this cleavage, the highly acid-labile HMPB linker, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid, has been developed. The lipophilic protecting groups, in particular Trt on asparagine, glutamine, and histidine, as well as Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl) on arginine, confer a good solubility on most protected peptide segments in organic solution and enable their purification by silicagel chromatography. Whereas the addition of segments on solid-phase resins is often difficult, they can as a rule be coupled easily in solution to give products in high yield and purity. The combined solid-phase and solution strategy is illustrated by the syntheses of human calcitonin-(1-33), human neuropeptide Y, and the sequence 230-249 of mitogen-activated 70K S6 kinase.

INTRODUCTION

This paper describes experiences and contributions to the field of solid-phase peptide synthesis (SPS)[1] of our group at Ciba-Geigy during the last 6 years, including some historical and general comments on SPS.

In 1963, Merrifield[2] introduced the SPS on polystyrene beads as an addition to the "classical" synthesis, in which fragments are built up and coupled in solution. The past 30 years have witnessed continuous developments and improvements of SPS. This, together with a parallel refinement of analytical and purification techniques, is the reason why presently the vast majority of all peptides are made by SPS.

The introduction by Carpino[3] of the Fmoc group for the N^{α}-protection opened up new ways for SPS as well as for solution methods. Since Fmoc is stable to acids and cleavable by bases, its use in SPS is highly convenient and continually increasing. It allows the selective N^{α}-deprotection and the final acidolytic cleavage from the resin and of the side-chain protecting groups under very mild conditions.

In recent applications of the stepwise automated SPS, the isolation of large peptides in pure form has been possible[4]. Such syntheses become feasible if several critical questions, such as secondary structure formation, side-reactions and the stability of the peptide in the deprotection steps, all can be solved in a positive way. However, it would be misleading to believe that most large peptides or small proteins can now be obtained safely from automatic synthesizers with standardized reaction protocols. We may also assume that many syntheses have been attempted and, due to failure or inconclusive results, have not been published. In addition, there is a problem with an increasing number of companies offering custom-synthesized peptides made in

multiple-syntheses machines for competitive prices. Such peptides are often not under adequate analytical control. They may contain undetected faults or be grossly contaminated with byproducts. In this regard, two recent investigations[5,6] have revealed a rather disillusioning situation.

The accumulation of byproducts - mainly amino acid deletion and insertion peptides - during long stepwise syntheses can be avoided when the target peptides are subdivided into several segments, which are synthesized by SPS, detached from the resin in the protected form and, after purification, used for the assemblage of the complete sequence. In this respect, the Fmoc-method is best suited, since linkers to the solid-phase resins have been developed, which allow the extremely mild, selective acidolytic cleavage of the peptide-linker bond.

SIDE-CHAIN PROTECTING GROUPS FOR THE N^{α}-FMOC-METHOD

The principle of maximal protection of the amino acid side-chains is the best prerequisite for a clean build-up of peptides on a solid phase resin, as well as for the coupling of large peptide segments. Ideally, there should exist a collection of protecting groups for all the functional side-chains with the following properties: complete stability during the chain assemblage, avoidance of racemization, stability during the cleavage of the protected peptide from the carrier resin with diluted TFA, removal with strong TFA at room temperature, and no formation of byproducts in any of these steps. Although at present not all of these points are realized, some important steps in the right direction have been made in recent years. Whereas the tert.-butyl-type groups for the side-chain hydroxy, amino, and carboxy functions are well-established with a long tradition, the most important newer developments are illustrated in Fig. 1.

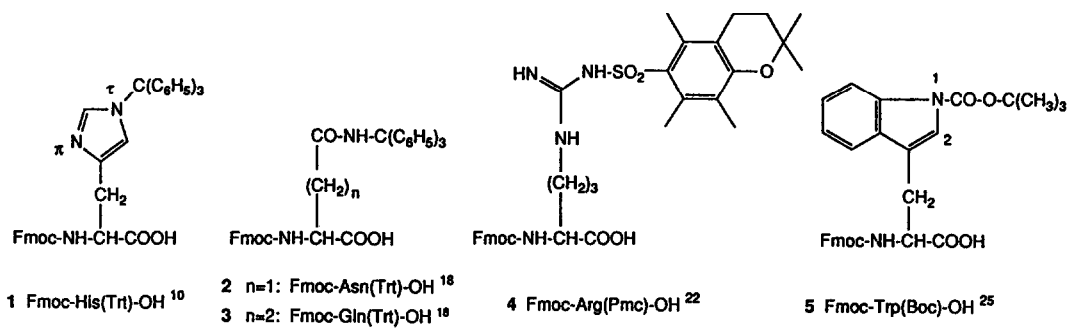

Fig. 1 Side-chain protections for the N^{α}-Fmoc method

Histidine. The imidazole group of His has attracted much attention, but the question of its protection has still not been answered in a totally satisfactory way. The tert.-butyloxymethyl (Bum) group has been introduced to the $N(\pi)$ position in order to eliminate racemization by intramolecular base catalysis[7]. Even though π-Bum is completely stable to nucleophiles and easily cleavable by TFA - producing formaldehyde as a byproduct - it has not found a wide acceptance in Fmoc peptide syntheses. On the other hand, the τ-Boc-group has the disadvantage of being gradually lost during the Fmoc cleavage steps, thus leading to byproducts due to the presence of an unprotected imidazole ring. In this context, we were tempted to re-evaluate the long-known Trt protecting group[8,9] for a new mode of application in Fmoc syntheses[10]. It can be introduced in good yield in the less hindered τ position of the imidazole ring (**1**, Fig. 1) and is, like π-Bum, completely stable to

nucleophiles and bases. The Trt-substituted histidine side-chain is less basic than the unsubstituted one, which is also reflected in a lower tendency of the former for racemization. However, under unfavorable conditions, Fmoc-His(Trt)-OH is still prone to partial racemization, as e.g. during the anchoring onto to a solid-phase resin[11] or upon activation with a carbodiimide, in the absence of HOBt and of the amino component. Whereas towards HCl in glacial acetic acid the Trt-group (due to complete protonation of the imidazole ring) is remarkably stable[12], it is rapidly cleaved off (t1/2 ca. 4 min) by 95% TFA. Under weakly acid conditions, Trt in the side-chain of His is more easily lost than any other protecting group mentioned in this paper, and, if desired, this amino acid of a fully protected peptide can selectively be deprotected[13]. The τ-Trt group is presently the most convenient protection for His in spite of its high acid-lability and the possibility of racemization under certain unfavourable conditions.

Asparagine, Glutamine. Syntheses with unprotected side-chains of Asn and, although to a lesser extent, also of Gln, are always exposed to a risk of byproduct formation, mainly resulting from attack of an activated α-carboxyl species to the unprotected side-chain amide function. For this reason, unprotected Asn as a carboxyl component in SPS often results in slow and incomplete coupling reactions. In addition, peptides with several Asn and Gln tend to be sparingly soluble and to form aggregates. For the protection of the ω-carboxamide function, Tmob[14] and Mbh[15] have been developed. Indeed, both groups prevent side-reactions involving the carboxamide function, but a serious problem arises during the cleavage of Tmob or Mbh by TFA from a peptide containing Trp. The indole moiety of this amino acid is sensitive to alkylations by various carbocations which are present in the acidolytic conditions required for the removal of protecting groups. Upon deprotection of Tmob and Mbh, an immediate formation of alkylation products in position 2 of indole takes place, even in the presence of large amounts of cation scavengers[16]. In contrast, we never observed alkylation by the Trt cation, when this group was used for the protection of His[17].

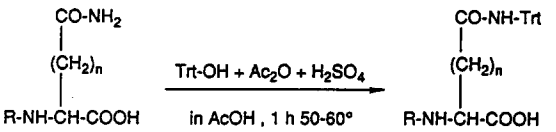

Fig. 2. Side-chain tritylation of Asn and Gln

This prompted us to investigate the suitability of Trt as a protecting group for side-chain amide functions. In fact, the ω-tritylamides of Asn and Gln (Fig.1, 2,3) turned out to be accessible in good yield by acid-catalyzed reaction of Trt-OH and Ac_2O in glacial acetic acid[16,18,19] (Fig. 2; R = Fmoc, Z, or H). The tritylamide derivatives of Asn and Gln are all crystalline and have much better solubilities in organic solvents than their non-tritylated analogs. The solubilizing property of the Trt group is also effective in peptides containing Asn or Gln protected by this residue. The ω-tritylamides are completely stable to bases and nucleophiles, to catalytic hydrogenation and, unexpectedly, to mineral acid in aqueous-organic solutions. The half-lives of the Trt-cleavage of Fmoc-Asn(Trt)-OH in various TFA solutions, as commonly used for the removal of side-chain protecting groups in the Fmoc-method, are listed in Tab. 1. The values indicate that the rates of cleavage are quite similar to those of tert.-butyl-type protecting groups. Tab. 2 indicates the labilities in TFA-dichloroethane (1:1) of ω-tritylamides of different compounds, and includes also the comparison with the

Mbh and Tmob groups. Trt in the side-chain of Gln is always split off faster than in Asn. Trt-protected Asn with a free amino group in the *N*-terminal position of a peptide is an exception: due to the proximity of the protonated α-amino group, the cleavage of its Trt-group is 20 to 30 times slower than normal.

Table 1 Cleavage of the Trt group of Fmoc-Asn(Trt)-OH

Solvent (v:v)	$t_{1/2}$ [min]
TFA	<1
TFA-H_2O (95:5)	2
TFA-dichloroethane (1:1)	8
TFA-dichloroethane (1:9)	~500
TFA-H_2O-ethanedithiol (76:4:20)	3
TFA-dimethylsulfide-ethanedithiol (77:20:3)	6

Table 2 Cleavage of the amide protection in TFA-dichloroethane 1:1

Compound	$t_{1/2}$ [min]
Fmoc-Gln(Trt)-OH	2
Fmoc-Gln(Mbh)-OH	27
Fmoc-Gln(Tmob)-OH	9
Fmoc-Asn(Trt)-OH	8
Z-Asn(Trt)-OH	7
Ac-Pro-Asn(Trt)-Gly-Phe-Gly-OH	9

The ω-tritylamide protection prevents all reactions of activated α-carboxyl groups with the side-chains of Asn and Gln. For this reason, their active esters are stable in DMF solution containing HOBt and DIEA, in contrast to those of the two unprotected amino acids, which decompose to cyclic imides and side-chain nitriles. Likewise, the N^{α}-Fmoc-*N*-carboxyanhydrides of Asn and Gln can be prepared in stable, crystalline forms when protected as tritylamides[20]. Coupling reactions with ω-tritylamides of Asn and Gln, either as amino- or carboxy-components, do not seem to be noticeably affected by steric hindrance. They can also be esterified easily to hydroxy functions of solid-phase resins, in contrast to the side-chain-unprotected amino acids. Some model peptide syntheses have proven the clear advantage of Trt-protected versus non-protected carboxamide side-chains and, in the case of Trp-containing compounds, also of Trt versus Tmob as protecting group[16].

Arginine. For the protection of the guanidine side-chain function of Arg the Mtr substituent was found to be the most suitable[21]. It was commonly used in Fmoc peptide syntheses, although the conditions for its cleavage by TFA are more vigorous than for the other side-chain protecting groups. Later, Ramage and coworkers[22] provided an improvement by the development of the Pmc group, which is cleaved 20-fold faster than Mtr.

All the sulfonyl-type protecting groups require special care upon cleavage in order to suppress byproduct formation. Again, the unprotected indole moiety of Trp-containing peptides is very exposed to substitution. The most conspicuous byproducts formed upon deprotection in anhydrous TFA of a peptide containing both Trp and Pmc-protected Arg are compounds with Pmc, mainly in position 2, of indole. Other side-reactions occur as well, such as transfer of Pmc to the phenolic hydroxyl of Tyr, formation of sulfate esters with Tyr, Ser, Thr, and even sulfonations of aromatic rings[23,24]. Upon cleavage in 95% or 90% TFA, these latter side-reactions can be avoided, since water does not allow the existence of the aggressive mixed anhydride between TFA and the Pmc-sulfonic acid. However, the suppression of the indole-Pmc products is more difficult and requires the presence of large amounts of cation-scavengers. The optimal cleavage medium is a freshly prepared mixture of TFA, H_2O, and EDT 76:4:20 (v/v), which in comparison with other recommended scavenger cocktails for the deprotection of peptides always gave equal or better results. The half-life for the cleavage of the guanidine Pmc protection in the above-mentioned solution is 20 min, which means that the

complete removal of the Pmc group requires about 2 - 3 hours at room temperature. The formation of small amounts of Pmc-substituted Trp-peptides, despite scavengers, cannot be completely avoided. Since they are more lipophilic than the desired peptides, their removal during purification does not usually pose problems.

Tryptophan. Despite a strong need to protect the indole moiety of Trp against electrophilic attack in the acidolytic deprotection step of Fmoc-syntheses, no suitable means of protection existed until very recently, and this amino acid is still used in many laboratories with an unprotected side-chain. The breakthrough came only in 1991 with a paper by White[25] describing the synthesis of Fmoc-Trp(Boc)-OH[26] (Fig. 1, **5**) and its use for Fmoc-syntheses. This work was based on an earlier investigation of Ragnarsson and coworkers[27] who prepared Boc-Trp(Boc)-OMe and found that the indole Boc-group is cleaved by TFA to the *N*-carboxy derivative, which lowers the reactivity of the aromatic ring system towards electrophiles. Similar to White's results, we found a rapid conversion of the *N*-Boc to the *N*-carboxy compound (t1/2 in TFA-H_2O-EDT 76:4:20 < 1 min) and a very slow decarboxylation of the latter to the free indole (t1/2 ca. 10 h in the same solution). The long half-life of the *N*-carboxy derivative gives a good - though not complete - protection against electrophilic substitutions in the indole moiety. The time required for the complete deprotection of peptides in the said solution is usually less than 3 hours. In 0.1 M aqueous acetic acid the half-life of *N*-carboxy-Trp was found to be 20 min[25]. In the usual RPLC analysis (0.1% TFA in a H_2O-CH_3CN gradient) unsubstituted and N-carboxy-Trp peptides have very small differences of elution time, while in TLC-analysis the latter behave clearly more hydrophilic and are partially decarboxylated on the plate.

We highly recommend the use of the N-Boc protection of the indole ring for Fmoc syntheses of Trp peptides in order to reduce both byproduct formation and irreversible binding of the peptide to the resin in the acidolytic cleavage.

THE HMPB LINKER

The most commonly used polystyrene resin for SPS by the N^{α}-Fmoc method has been developed by Wang[28] and contains the p-benzyloxy-benzylalcohol group, to which the *C*-terminal amino acids are esterified. It does not permit one to prepare protected peptides, since the benzylester bond to the resin needs concentrated TFA for the cleavage. Sheppard and coworkers[29,30] were the first to develop benzylester linkers with higher acid labilities, in order to release peptides in their protected form from the resin and use them for segment condensation syntheses. However, their most acid-labile linker, 4-hydroxymethyl-3-methoxyphenoxy-acetic acid, is somewhat too stable to 1% TFA in DCM, which means that it is not sufficiently selective with respect to the most acid-labile side-chain protecting groups. Mergler and coworkers[31] later developed a polystyrene resin with a higher selectivity for the acidolytic release of protected peptides.

We were trying to find a linker with an optimal balance of selectivity to the side-chain functions and of good stability to weakly acid components of coupling reactions, and which could be bound through its carboxy group to any solid-phase resin containing amino functions. The result was 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid[32] (HMPB, **6** in Fig. 3), which was obtained in 3 steps from 4-hydroxy-3-methoxy-benzaldehyde. It was coupled by TPTU[33] or TBTU[33] to benzhydrylamine-polystyrene resin[34] and esterified with Fmoc-amino acids by the 2,6-dichlorobenzoyl-chloride method[35], resulting in substitution levels of 0.3 to 0.4 mmol/g.

HMPB is analogous to Sheppard's linker[30], but the additional two methylene groups of the butyric acid chain make it about 30-fold more acid-labile. The half-lives of cleavage of various Fmoc-amino acids bound through HMPB to benzhydrylamine-polystyrene resin were determined by direct UV measurement at

300 nm in a solution of 1% TFA in DCM and found to range from 18 sec (Pro) to 33 sec (Ile). This implies that in contact with a large molar excess of 1% TFA in DCM, protected peptides will be cleaved from the HMPB resin in a few minutes. If TFA is neutralized immediately afterwards, the side-chain protecting groups will remain completely stable, except for His(Trt), which in practice often loses a few percent of the Trt-group.

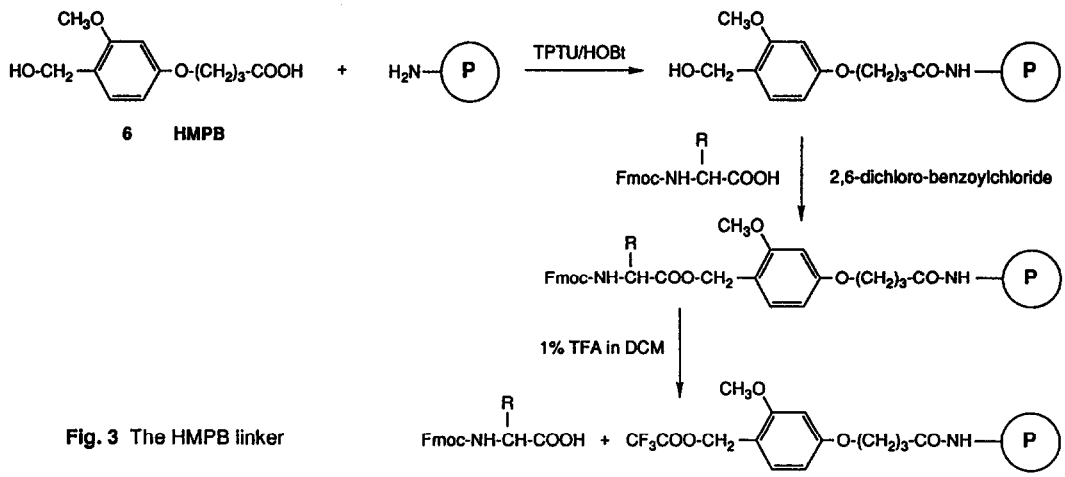

Fig. 3 The HMPB linker

Prior to the cleavage experiments, we tried to obtain some information on the acid strength of TFA and its interaction with peptides in DCM solution. After the addition of 0.002% each of quinaldine red and triphenylmethanol, the acidity is indicated by a color change from red (0% TFA) to yellow (1% TFA). This allows the "titration" of peptides in DCM solution, and we found that all the amide bonds get protonated by TFA in this aprotic medium. This is consistent with an early report by Tam and Klotz[36], who showed by NMR that the addition of 1% TFA leads to protonation of the peptide bonds of polyalanine in chloroform solution. Other sites of protonation are alcohols, esters, ethers, carboxyls, and some other groups. They all function as bases and neutralize, together with the peptide bonds, considerable molar amounts of the added TFA, before its strength is high enough to effect the fast cleavage of protected peptides from the HMPB linker. New types of solid-phase resins have recently been developed, containing polyoxyethylene chains grafted on the polystyrene matrix. These resins require an additional amount of TFA for the protonation of the large number of ether functions. Furthermore, it is essential to use DCM without traces of water or alcohols, which can be obtained by storage in the dark over 4 Å molecular sieves.

An ideal way to cleave protected peptides from resins with the HMPB linker would be a continous-flow procedure, with immediate neutralization of TFA in the column effluent. However, due to shrinking of the resin column, the flow of the TFA solution may become uneven. As an alternative, a batchwise procedure with several short cleavage steps is possible, as described in the experimental part. The solubility of the protected peptides in the 1% TFA/DCM solution has - due to the protonating effect - always been good, even with compounds which are insoluble in neutral DCM.

During the acidolytic cleavage, peptides containing Trp or Cys have a tendency to be bound irreversibly to the resin by reaction of the side-chains with cations generated from the linker. Some examples are given in Tab. 3, which also demonstrates the beneficial effect of the addition of EDT as a scavenger. We

found that the presence of 5% EDT decreases the rate of the cleavage by approximately a factor of 2. Here again, the advantage of Trp protection by Boc is evident.

Table 3 Irreversible binding [%] to the HMPB resin

Esterified Amino acid	Cleavage solution	
	1% TFA in DCM	1% TFA + 5% EDT in DCM
Fmoc-Trp-	84	18
Fmoc-Trp(Boc)-	17	4
Fmoc-Cys(Trt)-	41	7
Fmoc-Cys(Acm)-	20	2

THE LIPOPHILIC SEGMENT COUPLING METHOD

Many efforts have been made to synthesize large peptides from smaller protected building blocks, with the intention to purify them prior to their use for chain elongations. However, purification has often been difficult due to low solubility, as discussed by Atherton and coworkers[37] in the synthesis of the bacteriophage Cro protein containing 66 residues. On the other hand, a consequent protection of all the amino acid side-chains by the groups discussed here generally leads to rather lipophilic peptide segments which can easily be purified. Considering the frequency of the various amino acids in natural peptides and proteins, we find that during peptide syntheses about half of all the side-chains must be protected, and that almost 16% are either Asn, Gln, His, or Arg, which are substituted by the very lipophilic groups Trt or Pmc. Peptide segments with 10 to 20 amino acids, containing several of these groups, are usually soluble in $CHCl_3$, THF, or even in EtOAc.

In most segment coupling syntheses described in the literature, the various protected segments were added stepwise to the resin-bound *C*-terminal part. In many cases, such coupling reactions proceeded slowly or incompletely, even with a large excess of the activated carboxyl component. Quite often, difficult reactions are caused by intermolecular aggregations, however this phenomenon is rather concealed than prevented by the binding to a solid-phase resin. Our own experience with lipophilic protecting groups led us to prefer a combination of solid-phase and solution methods. We apply the SPS-method to the stage of peptide segments, which are further processed to the end-products in solution. Fig. 4 illustrates the build-up of a peptide from three protected segments, synthesized separately by SPS on a resin with the HMPB linker and detached by 1% TFA in DCM. The length of segments may vary from a few up to about 20 amino acids, and preferentially they contain Gly or Pro at the carboxyl end.

Before the *C*-terminal peptide can be used for coupling to its neighbour segment, it must be changed to a tert.-butylester with a free α-amino group. Armstrong and coworkers[38] described the acid-catalyzed alkylation of alcohols and carboxylic acids by tert.-butyl trichloroacetimidate, (TBTA, Fig. 5). Due to the presence of acid-labile groups, the conditions had to be modified, and with protected peptide segments it is usually performed in $CHCl_3$-TFE-TBTA 7:2:1 (v/v) for 1 h at room temperature[39,40]. For the production of

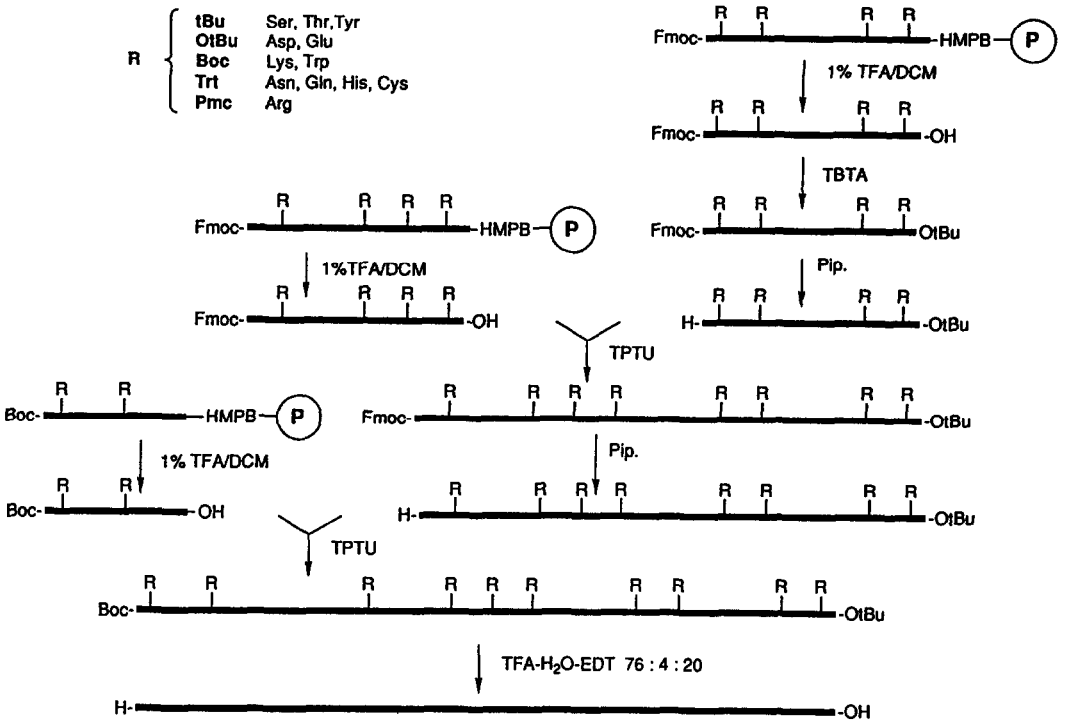

Fig. 4 The lipophilic segment coupling method

TBTA

Fig. 5 Formation of tert.-butyl esters from C-terminal carboxy groups

peptide amides, the *C*-terminal segments can be amidated instead of esterified. However, peptide amidations seem to be extremely prone to racemization, since we have not found conditions for coupling with ammonia or ammonium salts with complete retention of the L-form[41].

Most peptides with lipophilic side-chain protections are freely soluble in NMP, and coupling reactions in this solvent with tetramethyluronium reagents (e.g. TPTU + HOBt) are often complete in a few min at room temperature. In contrast to the amidations, we did not so far observe partial racemizations (≤ 1%) of residues in *C*-terminal positions. The common byproducts of Fmoc cleavage or coupling reactions, such as piperidine, dibenzofulvene, tetramethylurea, HOBt, etc. can often be removed by extractions or precipitations. Protected peptides can be purified by flash or medium pressure chromatography on silicagel, with mixtures or gradients of $CHCl_3$, MeOH, AcOH, and H_2O for elution. The purity is analyzed by TLC and RPLC. C_{18}-columns can be used if the peptides contain a relatively low proportion of lipophilic protecting groups. For most protected segments, however, Vydac "Diphenyl" columns are a better choice, since even very lipophilic peptides can be eluted from this type of adsorbent.

The acidolytic deprotection of the completed peptide is usually performed in TFA-H_2O-EDT 76:4:20. The time required should be determined individually for each compound. It does not only depend on the types of protecting groups, but also on their position and relative arrangement in the peptide chain.

The problems of "difficult sequences" in SPS and insolubility of peptides in polar solvents are both caused by intermolecular aggregation of peptide chains in the form of hydrogen-bonded ß-sheet structures. In SPS, this process manifests itself into shrinking of the resin bed and slow or incomplete reactions. With organic solvents, high viscosity or gelatinous precipitations as well as unusual behavior in TLC and RPLC may occur. For SPS, Stewart and Klis[42] advocate the addition of chaotropic salts like KSCN, LiBr, or $NaClO_4$ in order to break secondary structures and to improve the coupling efficiency. Seebach and coworkers[43] studied the influence of various lithium salts on resin swelling, coupling rates, byproduct formation, and racemization. Depending on the solvents and coupling methods used, these authors found positive as well as negative effects (increased racemization) of lithium salts. Narita et al.[44] investigated the influence of various solvent combinations on ß-sheet formation and found that HFIP and TFE have a very strong potential to disrupt these secondary structures. The two fluorinated alcohols, also in mixtures with other solvents, have recently been found to dissolve otherwise insoluble protected peptides and to improve coupling reactions in solution[45,46]. Earlier applications for SPS have not been very convincing, however it seems important to obtain more information on the scope of TFE and HFIP in peptide syntheses, both on solid-phase and in solution. The formation of ß-sheet secondary structures is very pronounced with small lipophilic side chains like Leu, Ile, Val, Ala. There is also evidence that an accumulation of Ser, Thr, Lys, Asp, and Glu with their tert.-butyl-type protecting groups may have a similar effect. On the other hand, Trt and Pmc groups tend to moderate aggregations. (Among the 8 protected peptides of Fig. 6, only the decapeptide **13** was insoluble in NMP.)

We have synthesized a variety of different peptides by the segment coupling method, involving the isolation and purification of protected intermediates of small or moderate length. Our experiences have in general been good, especially concerning the yield and purity of the end-products. The method should be of a general utility for the synthesis of large peptides, and also in large quantities.

APPLICATIONS OF SEGMENT COUPLING

h.Calcitonin-(1-33).[47] The assemblage of the complete chain from the three intermediates **7**, **8**, and **9** (Fig. 6, a) seemed obvious due to the presence of Pro(23) and Gly(10) in *C*-terminal positions of segment coupling reactions. The precursor of **7**, obtained in very high purity by cleavage from the HMPB linker using 1% TFA in DCM, was converted to **7** by esterification with TBTA, followed by cleavage of N^{α}-Fmoc with piperidine in THF-DMA solution. The middle segment **8** was obtained in 90% purity and further purified by flash chromatography. Intermediate **9**, with two Trt-protected Cys residues, was cleaved from the resin by DCM-TFA-EDT 94:1:5. It was obtained only in 75% yield, since some of it was bound irreversibly to the HMPB linker. **9** was oxidized to the cyclic disulfide by iodine in $CHCl_3$-TFE solution[48], and then also purified by flash chromatography. The segment coupling of **7** with **8**, followed by cleavage of Fmoc from Thr(11) and coupling of the resulting product with the disulfide obtained from **9**, were performed in DMF by TBTU in the presence of HOBt and DIEA. Both coupling reactions were very fast and quantitative. The protected endproduct was again purified by flash chromatography and then deprotected by TFA-H_2O-EDT 90:5:5 for 45 min at 30° C. The free peptide was precipitated by diisopropyl-ether, converted from the trifluoroacetate to the acetate form, and finally purified by countercurrent distribution.

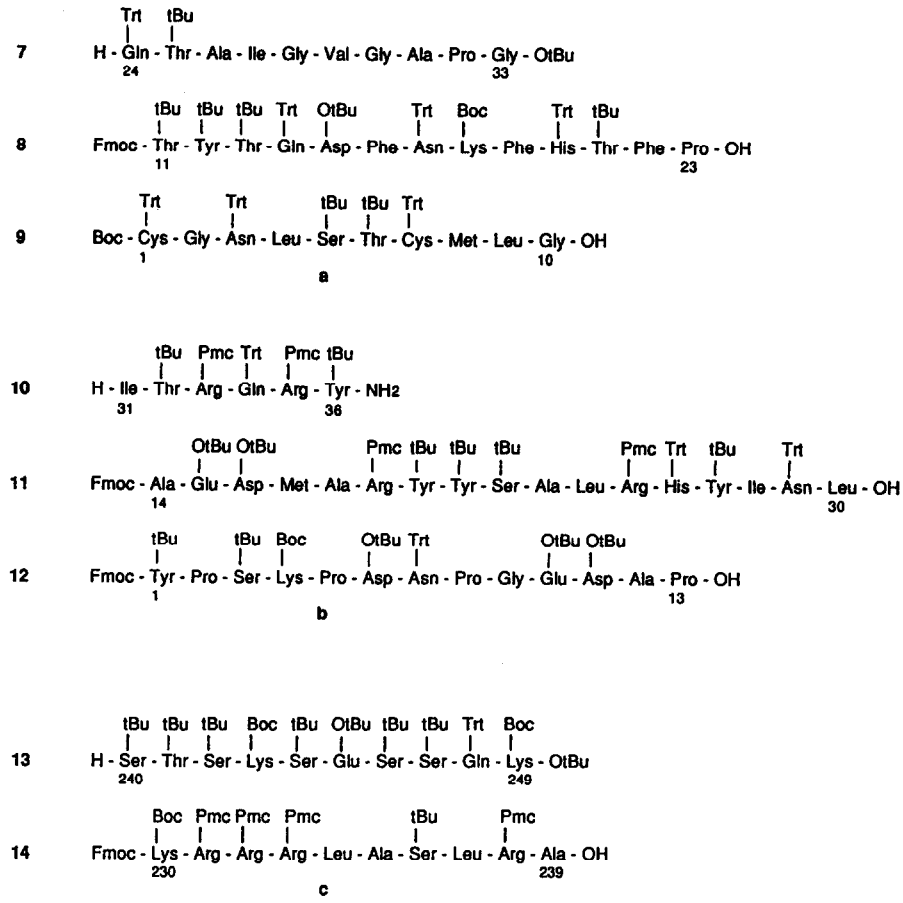

Fig. 6 Lipophilic segments for the syntheses of h.calcitonin-(1-33), **a**; h.neuropeptide Y, **b**; and [Ala^{235}]-70K S6 kinase-(230-249), **c**.

h.Neuropeptide Y.[49] This 36-peptide was composed of the segments **10 - 12** (Fig. 6, b), produced by SPS on HMPB-polystyrene resin and cleavage by 1% TFA in DCM. The primary *C*-terminal 6-peptide, with N^{α}-Fmoc and a free Tyr(36)-α-carboxyl group, was converted to the amide by reaction in NMP with NH_4Br and TPTU. Unexpectedly, 20% of Tyr(36) was epimerized in this process. The diastereomeric peptides were separated on TLC plates and on RPLC. By changing the coupling reagents and conditions, it was possible to reduce, but not eliminate, the partial epimerization. The D-Tyr(36)-amide could finally be removed by repeated flash chromatography of the N^{α}-Fmoc-form of **10**.

The crude, protected segment **11** surprisingly was contaminated with a large proportion (ca. 40%) of a more hydrophilic byproduct, as detected by TLC and RPLC. It was identified as the Asp(16)-Met(17)-succinimide derivative, and again it could be separated by silicagel chromatography. This example demonstrates the importance of purifying the intermediate segments in order to prevent the later occurrence of

byproducts (e.g. ß-peptides from succinimides), which would be hard to remove. Nicolas et al.[50] have pointed to the fact that the Asp(OtBu)-Gly sequence in SPS may give rise to traces (0.3% in 10 min) of the succinimide derivative in each Fmoc-cleavage step by piperidine. If the residue following Asp(OtBu) is not Gly, succinimides are formed even slower, and they do not normally occur in detectable amounts during SPS. It is unknown why in the case of segment **11** the succinimide was formed to such a large extent.

All the protected segments and larger intermediates of this synthesis were soluble in $CHCl_3$ and were purified by flash chromatography or MPLC on silicagel. The two segment coupling reactions - by TPTU + HOBt in NMP - were complete in 20 min, and there was no detectable (≤ 1%) epimerization of Leu(30). N^{α}-Fmoc of the fully protected 36-peptide was removed by piperidine, followed by complete deprotection in TFA-H_2O-EDT 76:4:20 for 2 h at 25° C. The endproduct was purified by preparative RPLC in a Vydac "Diphenyl" column.

Sequence [Ala^{235}]-(230-249)-70K S6 Kinase.[51] Although the synthesis of this peptide (Fig. 6, c) was expected to be a simple routine preparation, it posed more problems than the previous examples. The first attempt was a straightforward SPS of the complete chain. The first eight coupling reactions were fast and quantitative, but Ser(240) was already slower, and in the next cycle, the shrinking of the resin volume to 65% was observed. All further coupling reactions were very slow and incomplete, in spite of several measures taken (including addition of 0.4 M LiCl) to counteract this clear state of aggregation. The endproduct of the synthesis was a complex mixture, not worth being separated.

The 20-peptide was then prepared by coupling in solution of the decapeptides **13** and **14**. The precursor of **13** was synthesized on the HMPB-resin and detached by 1% TFA in DCM. It was purified by MLPC on silicagel in the presence of 5% TFE in order to avoid aggregation. The conversion to the α-tert.-butylester was done as usual, but due to insolubility of the fully protected peptide in THF-DMA, the Fmoc-cleavage was performed in $CHCl_3$-TFE-piperidine 80:4:16 (v/v) for 2 h. The *N*-terminal segment **14** , upon detachment from the HMPB linker, was very pure and easily soluble. Due to the insolubility of **13** in NMP, the coupling with **14** was performed in $CHCl_3$-TFE 9:1. After a 1 h preactivation period of **14** with HOOBt, DIEA, and TPTU in equimolar ratio, a 50% excess of this solution was added to a solution of **13** + 1 equiv. DIEA. The coupling reaction was clearly slower and somewhat less quantitative than the usual segment couplings in NMP solution. However, the optimization of the coupling reaction in the $CHCl_3$-TFE solvent mixture has not been attempted so far. After 15 h the product was precipitated and purified by MPLC, again in the presence of 5% TFE. Due to aggregation, it had to be dissolved in $CHCL_3$-HFIP 7:3 for TLC-analysis and could not be eluted out of the RPLC column. After deprotection by 95% TFA for 2 h at 30° C and precipitation by diisopropylether, the water soluble product was analyzed by RPLC. The N^{α}-Fmoc group was cleaved in 10% aqueous piperidine for 20 min, followed by acidification and purification of the free peptide by preparative RPLC.

The C-terminal Ala(239) residue was not epimerized (≤ 1%) during the segment coupling between **13** and **14** by TPTU in $CHCl_3$-TFE 9:1. However, when the reaction was performed in NMP + 0.4 M LiCl (**13** was soluble upon addition of LiCl) we found 27% of D-Ala(239). In two further experiments, **14** was coupled in twofold excess by TPTU to the *resin-bound* **13.** In $CHCL_3$-TFE 9:1, the 20-peptide was obtained in 22% yield, uncontaminated by the epimer. The second experiment was in NMP + 0.4 M LiCl and gave even less peptide (15%), containing 20% of D-Ala(239).

EXPERIMENTAL

Merck precoated plates Silica Gel 60 F-254 were used for TLC. The progress of all reactions in solution was monitored by TLC. For analytical RPLC of protected (lipophilic) peptides, a Vydac Diphenyl column (4.6 · 150 mm, 5μ, 300Å) was used with linear gradients between 0.1% TFA in H_2O and 0.09% TFA in CH_3CN. With less lipophilic or free peptides, the Nucleosil 300-5C18 ChromCart, 4 · 250 mm, of Macherey-Nagel was used, with the same solvent gradients. CZE was performed with a Beckman PACE 2000 instrument in a CElect P 175 capillary from Supelco. D-Amino acids were determined as N^{α}-trifluoroacetyl-isopropyl-esters by gas chromatography in a capillary coated with Chirasil-Val (Alltech, Deerfield, IL), after hydrolysis (24h 105°C in 6N HCl) of the peptide[52]. Mass spectroscopy was performed in a ZAB HF instrument from Fisons Instruments and in a BIOION 20 instrument from Applied Biosystems, using FAB and 252 Cf PD techniques, respectively. The matrix for the FAB mode was thioglycerol, and in the PD mode, the peptides were adsorbed on nitrocellulose.

Merck Silicagel 60, 40-63μm, was used for flash chromatography of the protected peptides. Solvent systems for elution were composed of $CHCl_3$, MeOH, H_2O, and AcOH in variable ratios. For MPLC, columns filled with Merck Lichroprep Si 60, 15-25 μm, and solvent gradients of DCM and MeOH, often in combination with some H_2O and AcOH, were used. The following is an example: Column filled and peptide dissolved in A + 1% B. Gradient from A + 1% B to A + 10% B within 1 h. A = DCM; B = MeOH-H_2O-AcOH 100:10:5.

Preparation of the HMPB resin. BHA resin from Calbiochem-Novabiochem (Polystyrene-1% DVB, 100-200 mesh, with a content of 0.7 mmol benzhydrylamine per g resin) was shaken in NMP for 1h with 1.2 equiv. each of HMPB (**6**)[32] and TBTU and 3.6 equiv. of DIEA. The resin was then filtered off, washed with DMA, DMA + 20% piperidine, isopropanol, methanol, and finally dried. It contained 0.64 mmol HMPB/g[34]. The C-terminal Fmoc-amino acids of peptides to be synthesized were esterified to the HMPB linker of the resin by the 2,6-dichlorobenzoylchloride method[35].

SPS with the HMPB resin. A semi-automatic shaking-vessel machine for manual addition of the activated Fmoc-amino acids was used. Normally, single coupling reactions were performed in NMP with a twofold excess of preactivated derivatives (1 Fmoc-AA-OH, 1.1 DIEA, 1 TPTU[33], 3 min r.t. in NMP) and monitored by taking 2 μl samples of the resin suspension for ninhydrin tests. These were usually negative after 4 min, and the coupling was terminated after 40 min to 1 h by a 5 min capping period with DMA-pyridine-Ac_2O 8:1:1. The Fmoc-cleavage by 20% piperidine in DMA was continously monitored in a flow-through cell and at the end quantitated from the absorption at 300 nm of the combined cleavage portions.

Detachment of protected peptides from the resin. 1 g of the dried peptide-resin was pre-swollen in DCM in a cylindrical vessel with a sintered glass bottom, shaken for 2 min with 10 ml of 1% TFA in DCM and filtered by N_2 pressure into 2 ml MeOH-pyridine 9:1. This was repeated 3 to 5 times, until TLC tests proved the completeness of cleavage. For peptides containing Trp or Cys, 5 vol-% EDT was added to the TFA solution. The combined cleavage portions were concentrated to 20%, followed by precipitation of the peptide with methyl-tert.-butyl ether, or by addition of $CHCl_3$ and washing with aqueous solutions.

Tert.-butylation of peptide-α-COOH. 5-10% solutions of the protected peptides in $CHCl_3$-TFE-TBTA 7:2:1 were left at r.t. until TLC-probes indicated the completion of the esterification, usually 1 h.[40] The products were then precipitated by the addition of diisopropyl ether or methyl-tert.-butyl ether, if necessary in a 1:1 mixture with hexane.

Cleavage of N^{α}-Fmoc. A similar procedure as above was used, but in THF-DMA-piperidine 80:16:4. For the complete removal of residual piperidine, $CHCl_3$ solutions of protected peptides were extracted with pH 2 buffer, 5% $NaHCO_3$, and H_2O.

Coupling of protected peptide segments. A 20-30 mM solution of the carboxyl-component was preactivated for 3-5 min with 1.25 equiv. each of TPTU, HOBt, and DIEA. 1 equiv. each of the amino-component and DIEA were then added, and after 1-2 h the product was precipitated.

Deprotection of protected peptides. Prior to the actual cleavage, the time necessary for complete deprotection was determined: 1 mg samples were dissolved in 1 ml test tubes in 20 µl TFA-H_2O-EDT 76:4:20 and left for 10, 30, 90, and 270 min at r.t., precipitated by 400 µl methyl-tert.-butyl ether, centrifuged, dried, dissolved in H_2O + TFE and analyzed by RPLC. With the optimal time thus found, the cleavage of the total peptide sample was done accordingly. If Trp(Boc) and Arg(Pmc) did not occur simultaneously, the cleavage was done in 95% TFA, which is faster than with 20% EDT. The free peptides were purified by different methods and usually isolated in the forms of acetate salts by lyophilization from 90% AcOH. The correct structures were verified by FAB-MS and amino acid analysis, and purities were analyzed by RPLC and CZE.

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1. Abbreviations used for amino acids follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in J. Biol. Chem. **1972**, *247*, 977-983. Further abbreviations are as follows: Boc, tert.-butyloxycarbonyl; Bum, tert.-butyloxymethyl; CZE, capillary zone electrophoresis; DCM, dichloromethane; DIEA, diisopropylethylamine; DMA, dimethylacetamide; EDT, ethanedithiol; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; Fmoc, 9-fluorenylmethoxycarbonyl; HMPB, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid; HOBt, 1-hydroxybenzotriazole; HOOBt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; Mbh, 4,4'-dimethoxybenzhydryl; MPLC, medium pressure liquid chromatography; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl: NMP, *N*-methylpyrrolidone; Pmc, 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl; TBTA, tert.-butyl trichloroacetimidate; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Tmob, 2,4,6-trimethoxybenzyl; TPTU, 2-(2-oxo-1(2H)-pyridyl)-1,1,3,3-tetramethyluronium tetrafluoroborate; Trt, triphenylmethyl (trityl); Z, benzyloxycarbonyl.
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