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Synthesis and analysis of (poly)peptides

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Abstract - (Poly)peptides and proteins are widely distributed over living organisms and are involved in all biological processes. In the laboratory several ways to synthesize these compounds have been investigated; most of the methods involve chemical synthesis either in solution or using the solid phase technique. These and other synthetic methods will briefly be outlined and recent trends in the various areas will be discussed. Strategy and tactics of syntheses will be described, as well as the purification procedures. Finally, methods of analysis of (poly)peptides including recent developments will be mentioned.

INTRODUCTION

The name peptide was coined in 1906 by Emil Fischer who used the nomenclature of saccharides on the one hand and the word peptone, i.e. cleavage products of proteins by enzymes, on the other hand to describe the products formed on linking amino acids via amide bonds (or peptide bonds) (ref. 1). In general the word oligopeptides is used up to a chain length of 10 amino acid residues, polypeptides for a length of 10-100 residues and proteins for even larger molecules. Peptides and proteins exhibit the largest structural and functional variation of all classes of biologically active (macro)molecules. They occur in plants, micro-organisms (peptide antibiotics) and animals, already on a very simple level as in the coelenterate Hydra whose head activator (11 amino acid residues) has been well-studied. Amphibian skin has been found to contain a large variety of peptides, unequalled by any other vertebrate or invertebrate tissue (ref. 2). In man, one can find peptides everywhere, in the central and autonomous nervous system, gastrointestinal tract, atrial tissue, skin, eye etc. Moreover, several peptide hormones have been identified in tumours and mother milk. Peptides and proteins are of prime importance in the regulation and maintenance of all biological processes. Brain peptides, for example, have been found to have effects on homeostatic systems (each system affected by multiple peptides) including the regulation of pain, blood pressure, temperature, thirst, feeding, learning, memory and trophic function (ref. 3). Neuropeptides play a role in several neurological diseases as well as in the immune system. In the last decade or so, many new peptides have been isolated and characterized, largely with immunochemical methods and facilitated by the improvement of analytical techniques like high-performance liquid chromatography (HPLC), amino acid analysis and fast atom bombardment mass spectrometry (FAB-MS). Analysis of nucleotide sequences encoding for proteins has provided further additions to the ever-growing list of new proteins.
In this review I will attempt to describe the state of the art with respect to the synthesis of these interesting compounds. Trends in peptide chemistry, in the purification and in the analysis of peptides will be discussed.

AMINO ACIDS

Of the more than 500 naturally occurring amino acids some 22 have been demonstrated to occur as building blocks of peptides and proteins in living organisms; with the exception of proline which is an imino acid, all of them are α-amino acids and of the L-configuration*) (bacteria excluded). Recently the first exception to this rule has been found: in amphibian skin, a heptapeptide with potent opiate-like activity has been isolated and characterized. The presence of a D-alanine residue in position 2 was unequivocally established (ref. 4). Plants synthesize a large number of non-protein amino acids, some of which are essential for their survival. Micro-organisms represent another rich source of amino acids not present in animal proteins.

Note a: Nomenclature and symbolism for amino acids, peptides and protecting groups is according to the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature, Biochem.J. 219, 345-373 (1984). Other abbreviations are: AMP, adenosine monophosphate; DCC, N,N'-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; MSA, methanesulfonic acid; mRNA, messenger ribonucleic acid; tRNA, transfer RNA; TFMSA, trifluoromethanesulfonic acid.

SYNTHESIS OF (POLY)PEPTIDES

(Poly)peptides can be obtained in different ways: by biosynthesis, chemical synthesis in solution or using solid phase procedures, enzymatic synthesis, semisynthesis or via recombinant DNA technology. I will extensively discuss the chemical procedures and briefly touch upon the other ways of synthesis.

1. *Biosynthesis*

Nearly all polypeptides and proteins are biosynthesized via the ribosomal pathway; this involves several steps including activation of amino acids by formation of a mixed anhydride with AMP and transfer to specific tRNA's, formation of the mRNA-ribosome-initiator-tRNA complex, binding of the aminoacyl-tRNA, peptide bond formation, translocation and termination (ref. 5); it appears significant that the chain is built up from the amino side. After (and even during) the assembly of a linear polypeptide chain, folding into a three-dimensional structure essential for the execution of biological function occurs. It is now known that many small, naturally occurring peptides are obtained by processing of ribosomally synthesized precursor proteins (ref. 3). These precursor proteins may contain several biologically active products (ref. 6).

2. *Chemical synthesis in solution*

a. Protecting groups. Formation of a peptide bond requires the activation of the carboxylic acid function of one amino acid residue (see under 2b.). In order to obtain a defined product, reactive functions other than those involved in the formation of the peptide bond must, in general, be blocked by protecting groups. These reactive functions also include side-chain functions such as the thiol, guanidino, imidazole, indole and hydroxyl functions. For some groups protection is obligatory, for others it is facultative (imidazole, indole, hydroxyl) depending on the reaction conditions. In the course of several decades a large number (> 130) of protecting groups for the NH_2 function have been developed (ref. 7-9). They can be classified either on the basis of their nature, e.g. derived from carboxylic acids, urethane groups (derived from primary, secondary or tertiary alcohols), alkyl protecting groups or derivatives of sulfur or phosphorus, or on the basis of their removal: acidolysis, proton abstraction, reduction/oxidation, nucleophilic displacement and photolysis (ref. 7-9). Since most peptides are fairly stable under acidic conditions, N-protecting groups (not only for NH_2, but also for the imidazole and guanidino nitrogens) with graded sensitivity to cleavage by acids of different strengths have played and still play a dominant role in peptide chemistry. Some of these groups can also be removed by catalytic hydrogenation, and these two deblocking methods are used in over 80% of all syntheses (ref. 8). Representatives of the most widely used urethane-derived amino protecting groups are the benzyloxycarbonyl group, which is removable by catalytic hydrogenation and acidolysis, and the tertiary butyloxycarbonyl (Boc) group which is stable towards hydrogenation and cleavable by mild acid (TFA). Of a more recent date is the 9-fluorenylmethyloxycarbonyl (Fmoc) group which is acid-stable but can be removed by mild basic treatment. With the improvement of chromatographic methods and the increase in sensitivity of analytical techniques it became apparent that small amounts of by-products, like dipeptides and alkylated or racemized side-products can be formed upon the introduction of NH_2 protecting groups via chloroformates (ref. 10). Recent trends include the search for alternative methods of introduction of, in particular, the urethane-derived protecting groups by using compounds such as five-membered heterocycles (2-benzoxazolethiol, 2-benzothiazolethiol and 2-benzimidazolethiol, ref. 11), 2-pyridylcarbonates and thiocarbonates (ref. 12) or chloroalkylcarbonates (ref. 13). Other protecting groups are also being developed, e.g. the 2-(trifluoromethyl)-6-chromonylmethylenecarbonyl (Tcroc, ref. 14), 2-(4-pyridyl)ethoxycarbonyl (4-Pyoc; ref. 15) and the 3,5-dinitro-1-(4-nitrophenyl)-4-pyridone group (Dnpy, ref. 16). Another recent trend is the increased interest in attempts to reduce the formation of alkylated side-products during the acidolytic removal of protecting groups. The addition of a wide variety of scavengers (to bind the formed carbonium ions) has been extensively studied by Merrifield and co-workers (ref. 17) in the case of HF treatment and by several other groups in the case of TFA (ref. 18,19), methanesulfonic acid or trifluoromethanesulfonic acid treatment (ref. 19). Another approach is the development of phosphorus-based protecting groups that are unable to give such side-reactions (ref. 20).
It is possible to perform syntheses without protection of the carboxylic acid function of the amine component. Side-reactions leading to (minor) impurities and solubility problems are usually the reasons for masking them. A similar classification to that for amino protecting groups can be made here based on their nature, i.e. esters of the benzyl, methyl or ethyl, tert.-butyl or aryl type and hydrazides (ref. 9) or based on their removal (the same mechanisms as for amino groups (ref. 21). Recently, several new carboxyl protecting groups have been described which are variants of existing groups (or counterparts from N-protecting groups) and give rise to more selectivity and mild conditions for removal. Examples are the allyl ester (ref. 22), 9-fluorenylmethyl ester (ref. 9), 2-(pyridyl)ethyl ester (ref. 23), the 4-sulfobenzyl ester (ref. 24) and cyclic esters especially for the β-COOH function of aspartic acid (ref. 25).

Side-chain protection. When histidine is the C-terminal amino acid residue, several side-reactions can occur upon activation (formation of cyclic imidazolides, N^{im}-amidino derivatives and racemization; ref. 8,9). Protection of the imidazole function with groups as shown in Table 1 (upper part), leads predominantly to τ-substitution and therefore may not prevent racemization via cyclization. This can be effectively suppressed by specifically blocking the π-nitrogen atom with a phenacyl (ref. 26), benzyloxymethyl or tert.-butyloxymethyl group (ref. 27).

TABLE 1. Imidazole protecting groups

Group	Abbreviation	Stable	Labile
benzyl-	Bzl-	acids	H_2/cat.;Na/NH_3
2,4-dinitrophenyl-	Dnp-	acids	thiolysis;N_2H_4
benzyloxycarbonyl-	Z-	HCl/organic	H_2/cat.;acids
tert.-butyloxycarbonyl-	Boc-		
adamantyloxycarbonyl-	Adoc-		
substituted benzenesulfonyl-	Tos-(4-CH_3)	H_2/cat. (TFA)	HF;Base;Na/NH_3;HOBt
	Mos-(4-OCH_3)		
	Mtr-(2,4,6-CH_3)		
phenacyl-(π)	Pa-	strong acid	Zn/HOAc;photolysis
benzyloxymethyl-(π)	Bom-	mild acid	strong acid;H_2/cat.
tert.-butyloxymethyl-(π)	Bum-	H_2/cat.	mild acid

The basic guanidino group of arginine can be protected by protonation. The low solubility of such derivatives in organic solvents and the observed acylation of the guanidino function often require other types of protection; examples are given in Table 2. The tosyl related groups (ref. 28) find an increasing application both in solution and solid phase synthesis.

TABLE 2. Guanidino protecting groups

Group	Abbreviation	Stable	Labile
nitro-	NO_2-	TFA;alkali	reduction;HF;N_2H_4
benzyloxycarbonyl-	Z-;Z_2-	TFA	H_2/cat.;strong acid
adamantyloxycarbonyl-	$Adoc_2$-	H_2/cat.; alkali	TFA
4-methylbenzenesulfonyl-	Tos-	H_2/cat.	HF
4-methoxybenzenesulfonyl-	Mbs-		increase in lability towards acids ↓
2,4,6-trimethylbenzenesulfonyl-	Mts-		
2,3,4,5,6-pentamethylbenzenesulfonyl-	Pms-		
4-methoxy-2,3,6-trimethylbenzenesulfonyl-	Mtr-		TFA

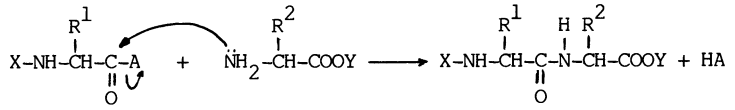
Mercaptans, unlike alcohols, are potent nucleophiles which can compete with amino groups for acylating agents to form thiol esters, hence the mandatory protection of the side-chain of cysteine (Cys). Classification of SH protecting groups is usually made on the basis of their nature (ref. 29,30), the thioethers forming the largest group. For some selected examples see Table 3. Many modified benzyl groups have been investigated as candidates for more acid sensitive protection, but without real success in this respect. A more promising line of research led to the triphenylmethyl (trityl) group which has found wide application because of the diversity of removal conditions (see Table 3; ref. 29). The acetamidomethyl (Acm) group can also be removed with mercury ions at pH 4 and with iodine with concomitant oxidation to the disulfide (the Trt can be selectively oxidized in the presence of the Acm group), but is completely stable towards acids.

TABLE 3. Sulfhydryl protecting groups

Group	Abbreviation	Stable	Labile
benzyl-	Bzl-	acids; iodine	Na/NH_3;HF
4-methoxybenzyl-	MBzl-	TFA; iodine	TFMSA;R-S-Cl
3-nitro-2-pyridinesulfenyl-	Npys-	HF;TFA;$NaHCO_3$	$(n\text{-}Bu)_3P$;1M NaOH/acetone
triphenylmethyl-(trityl)	Trt-	weak acid;base	TFA;I_2;Ag,Hg(II)salts;R-S-Cl
acetamidomethyl-	Acm	acids incl.HF;base	I_2;Hg(II)salts;R-S-Cl
9-fluorenylmethyl-	Fm	TFA;boiling HCl	NH_3/MeOH;piperidine
dimethylphosphinothioyl	Mpt	TFA;HCl	Ag;Hg(II)salts; alkali

Protection of the side-chain hydroxyl groups of serine, threonine and tyrosine is in general not required. However, when applying strong acylating agents and/or large excesses of activating species, protection should take place (e.g. in solid phase syntheses). The use of side-chain unprotected Ser, Thr and Tyr derivatives may sometimes lead to side-reactions that do not occur in the case of "global" protection but on the other hand complete removal of side-chain protecting groups at the end of a synthesis may also pose problems. The current standard method for blocking the OH functions is to convert them into their benzyl or tert.-butyl ethers (for a comprehensive review, see ref. 31).

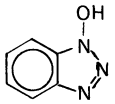
b. Coupling methods. In order to form a peptide bond, one of the groups involved must be activated. No practical solution for the activation of the α-amino group has been found as yet. Activation of the carboxylic acid group of a monovalent amino acid derivative followed by coupling with the NH_2 group of a suitably protected amino acid will give the desired peptide bond.



A wide variety of leaving groups A with electron-withdrawing properties exists, decreasing basicity of A resulting in a higher activation of R-CO-A. The possibility of racemization of peptides which are to be activated is of crucial importance here.
Classification of coupling methods can be made according to the groups: azides, anhydrides, active esters and coupling reagents (ref. 9,32,33). Excellent reviews on the application of azides (ref. 34), active esters (ref. 35), mixed anhydrides (ref. 36) and the carbodiimide coupling method (ref. 37) have appeared. Only some recent activities in these areas will be mentioned here.
Azides. Initially it was assumed that no racemization occurs during this still very popular coupling reaction, but detailed studies later revealed that this is not true. Avoiding even the slightest excess of base is essential in this case. In addition to the classical route via (protected) hydrazides (ref. 34), protected peptides with a free COOH terminal can be treated with diphenyl phosphorazidate to give the desired azide (ref. 38); this procedure has become popular in the synthesis of cyclic peptides (e.g. ref. 39).
Active esters. Of the many active esters described in the literature (ref. 32,35) only a limited number have found extensive use (see Table 4). The very active OPfp ester has become more popular recently when used in combination with the N^{α}-Fmoc protecting group (ref. 40).

TABLE 4. Active esters, X-NH-CHR-CO-A

A=	Abbreviation	
4-nitrophenyloxy-	-ONp	
2,4,5-trichlorophenyloxy-	-OTcp	higher activation,
succinimido-oxy-	-ONSu	increase in
pentafluorophenyloxy-	-OPfp	aminolysis rate



HOBt, is a very efficient catalyst of active ester reactions (ref. 41, 42).

Anhydrides. Symmetrical anhydrides of acylamino acids, which are highly reactive species, have only been used in stepwise synthesis, mainly in solid phase procedures. From an economic point of view (half of the amount is not used) these are not attractive, in contrast to mixed anhydrides. Mixed anhydrides of N-protected amino acids with carbonic acids have been the most popular especially since conditions were described that minimize the risk of racemiza- (ref. 33,36). A thorough, successful study of the reduction of urethane formation and racemization, investigating the effect of base and solvent, has been published recently by Benoiton and co-workers (ref. 43); the use of N-methylpiperidine and dichloromethane is recommended for routine use in mixed anhydride couplings. To overcome undesired urethane formation, derivatives of sulfuric acid but especially of phosphorus have been described (ref. 33). Recently, several studies on the use of acyloxyphosphonium species in amide bond formation have been published (ref. 44-47).
Carbodiimide method. Despite shortcomings the use of this coupling reagent has become very popular (and is still used exclusively in solid phase peptide synthesis). Some of the shortcomings, notably racemization and rearrangements, can be suppressed by the use of additives, of which HOBt (ref. 41) is most often used both in the condensation of fragments (segments) and in stepwise syntheses (ref. 9,37,42): the "DCC-HOBt procedure" is today's most favoured coupling method. An interesting unconventional coupling method in which amide bond formation occurs intramolecularly and is preceded by a covalent capture step ("amine capture" or more recently, "thiol capture") has been proposed by Kemp (ref. 48).

c. Racemization. Racemization at the chiral C^{α} atom may take place as a result of the activation of the carboxyl group. This can be via azlactone formation, direct abstraction of the C^{α} proton or via β-elimination (accelerated by assistance of some side-chains like those of Cys(Bzl), Asp(OtBu), Ser(tBu), Phe, Tyr and His). Relatively safe is the activation of urethane-protected amino acids, e.g. by active esters, the azide method with acylamino acids including peptides (provided no excess of base is present) and the addition of non-acylating acids as a source of protons, like HOBt, in coupling reactions (e.g. ref. 9,49,50).
d. Deprotection. Powerful reagents like HF are able to remove all type of protecting groups but are also the cause of side-reactions (both damage to the peptide chain and side-chains, ref. 51). Deprotection under alkaline conditions causes several problems (ref. 52); therefore acidolytic deprotection has been reinvestigated, both using acids milder than HF (e.g. TFMSA and MSA, ref. 53) and using scavengers under different conditions (ref. 17,19). Interesting in this respect is the finding that the addition of thioanisole in acidic deprotection reactions not only has a suppressing effect on side-reactions but also a promoting effect on the cleavage reaction ("push-pull" mechanism) (ref. 19,53). Final deprotection may still remain problematical because of the "individuality of peptides" (dissimilarity of side-chains and in sequence).

3. *Solid phase synthesis*

Since Bruce Merrifield's first publication in 1963, a vast amount of literature on all aspects of solid phase peptide synthesis (spps) have appeared (for an excellent, extensive review see ref. 54). In the beginning spps was based on the Boc-amino acid/benzyl-resin (a copolymer of polystyrene and divinylbenzene) approach, with HBr/TFA or HF as the final deprotection agent. Several improvements have been introduced over the years, such as increasing the differential acid sensitivity of the N^{α} and side-chain protecting groups, improvements in the stability of the benzyl ester type matrix to TFA, the monitoring of the coupling reactions, identifying and eliminating problems like termination, deletion and modification peptides and, last but not least, the suppression of side-reactions introduced by HF in the final deprotection (ref. 54,55). The handling of HF and especially its promotion of side-reactions (ref. 51,54) led at the end of the seventies to an alternative approach: the use of the base-labile Fmoc group for N^{α}-protection. This makes possible the use of mild acid (TFA) for both the cleavage of the peptide from the resin (p-alkoxybenzyl alcohol type resins) and the simultaneous removal of tert.-butyl-derived side-chain protecting groups (ref. 56, 57). The first step here is the acylation of the hydroxymethyl function on the resin by an Fmoc-amino acid derivative using DCC and the basic catalyst N,N'-dimethylaminopyridine (DMAP). This was later found to cause some dipeptide formation and racemization. The problem of these two side-reactions could be solved separately, but we have found a simpler solution to both problems simultaneously, namely the use of HOBt at a lower reaction temperature (ref. 58).
A further application of the base-labile properties of Fmoc/Fm groups (ref. 9) has been proposed by Mutter, who has synthesized the base-labile anchoring group 9-(hydroxymethyl)fluorene-4-carboxylic acid for polymer-supported peptide synthesis; acid-labile N^{α} protecting groups like Boc and Bpoc can then be used (ref. 59).
The solid support used by Sheppard's group (ref. 57) was not a polystyrene based resin, but a polar, poly(dimethylacrylamide) gel which is permeated and solvated by the polar solvent dimethylformamide. A recent development is that this gel is polymerized within the pores of rigid macroporous kieselguhr particles; solid phase synthesis is then carried out under low pressure and continuous flow conditions with continuous spectrometric monitoring (ref. 60). Fully automated instruments are now commercially available for this type of syntheses, in addition to many recent new adaptations of the original apparatus. Two other recent developments are worthwhile mentioning. First, the report of Geysen et al. (ref. 61) on the concurrent synthesis of hundreds of peptides on polyethylene rods that have been γ-irradiated in acrylic acid. Coupling with the N^{ε} group of lysine using Boc-Lys-OMe gives a solid support that enables conventional spps. At the end of the synthesis, side-chain protecting groups are removed with borontris(trifluoroacetate) in TFA and the rods are then used in an enzyme-linked immunosorbent assay. Another method for the simultaneous solid phase synthesis of large numbers of peptides has recently been described by Houghten (ref. 62). Small polypropylene bags are filled with 50-100 mg of Boc-amino acid resin and then sealed and labeled. Standard classical spps procedures including the use of HF are then performed to give 10-20 mg amounts of peptides (ref. 62).
A combination of features from the spps and syntheses in solution, e.g. facilitation of the separation of intermediates and reactions that are carried out in a homogeneous phase, has been proposed by Bayer using polyethyleneglycol (PEG) polymers (ref. 63).

4. *Enzymatic synthesis*

Although the reversal of the catalytic action of a hydrolytic enzyme (papain), i.e. the enzyme-catalyzed peptide synthesis, was already known some 50 years ago, the application of enzymes in peptide synthesis has only clearly increased over the past 10-15 years (ref. 64). This area has gained in interest especially since the enzymatic synthesis of human insulin from porcine insulin. Numerous model compounds have been synthesized using enzymes from different classes, e.g. chymotrypsin, trypsin, thermitase, papain, subtilisin, pepsin, thermolysin and carboxypeptidase Y. The synthesis of biologically active peptides using enzymes in several or all steps has been described, e.g. Leu- and Met-enkephalin, angiotensin II, caerulein, cholecystokinin-octapeptide, dynorphin, and oxytocin; also the

sweetener aspartame (H-Asp-Phe-OMe) has been obtained in this way (using thermolysin). A disadvantage of the enzymatic peptide synthesis is the fact that for each new combination of amino acid derivatives or peptides, suitable (new) reaction conditions have to be found. Once the best conditions have been established, large amounts of that peptide can be obtained (e.g. kg amounts of the luteinizing hormone-releasing hormone by Carlsberg Biotechnology Ltd.). The general belief that enzymes will only couple L-amino acid derivatives or L-amino acid containing peptides has to be abandoned. Petkov et al. (ref. 65), Thorbeck and Widmer (ref. 66) and West and Wong (ref. 67) have recently described practical procedures for the synthesis of D-amino acid-containing peptides. Optimization of this type of unusual catalysis could be obtained by changing the concentrations of substrates, the nature of the reaction medium and the pH.

5. *Semisynthesis*

One can envisage here two approaches: in the first, one makes use of a naturally occurring protein or peptide to obtain fragments (enzymatically or chemically, e.g. using cyanogen bromide) which are then used in a re-synthesis with other, synthetic, fragments. Several proteins and (poly)peptides like trypsin inhibitors, myoglobin, lysozyme, acyl carrier protein, insulin, β- and α-MSH have been studied in this way (ref. 68,69), but the best example is cytochrome c with 104 amino acid residues. Cleavage with CNBr at the two Met residues (using a special protection of the lysine side-chains) gives rise to 2 or 3 fragments (depending on the conditions); reconstitution of the protein using these and completely synthetic fragments has already provided many analogues of cytochrome c for structure-activity relationship (SAR) studies (ref. 69,70).
In the second approach one or more amino acid residues are selectively removed from the N- or C-terminal of the native molecule and the remaining (poly)peptide is then coupled with analogues of those amino acids or peptides, either by chemical means or enzymatically. In this respect many analogues of e.g. insulin (ref. 68) and phospholipase A_2 (ref. 71) have been synthesized.

6. *Synthesis by recombinant DNA technology*

This relatively new field has seen a rapidly growing development. Well over 100 papers describing the expression of foreign genes in micro-organisms (mainly E. coli, but also yeast and B. subtilis) have appeared, interferones, growth hormone, hepatitis B core antigen and (pre)proinsulin being among the proteins studied (ref. 72). Problems that can arise are the difficulties in completely removing impurities (proteins from host cells) and assuming the "heat-kill" of remainders of the organism. A disadvantage of the method up till now is the fact that post-translational modifications like disulfide bond formation, N^{α}-terminal acetylation, C-terminal amidation, and the removal of the N-terminal Met residues (i.e. the start of protein biosynthesis) are either not (yet) possible in the laboratory or have given rise to conflicting data (ref. 72). On the other hand one now has the possibility of producing (large) amounts of proteins, previously not accessible by chemical means or by isolation of naturally occurring material (lack of sufficient sources as with the interferones). Another advantage of this route is the absence of infectious impurities (hepatitis or AIDS virus in blood products) which can be present. Recent literature in this field clearly points to an increase in the use of these methods for the synthesis of protein/polypeptide analogues (site-directed mutagenesis); Camble recently reported on SAR studies with interferon α_2 (ref. 73).

AIM, STRATEGY AND TACTICS

The aims of the synthesis of peptides can be of a widely different nature:
1. The (urgent) need of a certain peptide.
2. In addition to a desired peptide, as many fragments as possible should be obtained, in the free form, for biological/pharmacological testing.
3. To investigate the possibilities of scaling up a synthesis: safety and economic aspects are becoming very important.
4. Synthesis on a microgram scale, e.g. with radioactive material.

Depending on the aim, the strategy and tactics of the synthesis have to be formulated. The strategy describes the main lines of the route and takes into account structural features like disulfide bridges. It defines whether fragment condensations or stepwise elongation should be employed, when to use a certain type of protecting group and how to prevent the occurrence of insolubility and of racemization. Tactics include performance of the synthesis using minimally or completely protected intermediates, and which protecting groups and methods of activation and coupling are the most appropriate. The final question, which must be envisaged straight from the beginning is: how can the end-product be deprotected?
Some examples are given below:

1. Target molecule

One of the best examples to illustrate strategy and tactics principles is still the total synthesis of human insulin by the Ciba-Geigy group in 1974 (ref. 74). Insulin consists of two chains of 21 and 30 amino acid residues, respectively, connected by 2 disulfide bridges (and one intra-chain S-S bridge in the A-chain). Directed formation of these three disulfide bridges at different stages of the fragment condensation approach was carried out using two

SH protecting groups, Trt and Acm, differing in sensitivity towards iodine which is used to deprotect and unify two thiol functions. A new, very mild method was developed for the selective removal of the temporary amino protecting groups occurring simultaneously at the two peptide chains (A and B) in one molecule. Following the completion of the synthesis of the protected molecule (Boc- and OtBu functions), final deprotection was achieved in one step by acidolysis. In spite of the complexity of the molecule, many analogues have been obtained using this elegant synthesis (ref. 75).
Since the successful synthesis of the adrenocorticotropic hormone (ACTH, 39 residues) in 1963 (ref. 76), several other large (poly)peptides have been synthesized over the past decades using the fragment condensation approach. These include calcitonin (32 amino acid residues), calcitonin-gene related peptide (37), growth hormone-releasing hormone (44), thymopoietin II (49), urogastrone (human epidermal growth factor, 53) and a fragment of ribonuclease T_1 of 81 residues. A milestone has been reached recently by the solution synthesis of crystalline, fully active ribonuclease A, consisting of 124 amino acid residues (ref. 77). Thirty fragments were synthesized using the azide and active ester methods, while employing established protecting groups for the several side-chains. After the final(azide) coupling, removal of the protecting groups was carried out by TFMSA/TFA in the presence of scavengers. Several purification steps after formation of the 4 disulfide bridges by air oxidation provided pure material that could be crystallized.
Numerous papers have been published describing the solid phase synthesis of a wide variety of peptides; many long peptides have been synthesized by fully automated procedures. Although correct sequences have been obtained in general, one should be very careful in analyzing the compounds, not only the long peptides (ref. 78) but also shorter ones (ref. 79), in order to check for deletion peptides and other impurities. Recently, the synthesis of interleukin-3 (IL-3), a protein of 140 amino acids, was carried out using a new type of automated peptide synthesizer, and was shown to have the biological activities attributed to native IL-3 (ref. 80). When the overall efficiency of the chain assembly was assessed by quantitative sequence analysis of the protected chain-bound peptide it was found that 41% of the molecules had the target sequence, the remaining 59% being a mixture of closely related molecules consisting primarily of peptides with a single internal amino acid missing (ref. 80).

2. Target molecule and fragments as biological probes

When we at Organon International started the synthesis of the 31 amino acids containing molecule human β-endorphin some nine years ago it was known on the one hand that in addition to its opiate-like properties this peptide possessed many other activities, and that on the other hand enzymatic processing generates naturally occurring fragments with diminished opiate activity. Our strategy consisted of the synthesis of fragments that were later coupled to obtain longer peptides and finally β_h-endorphin itself (a so-called "pyramidal" approach: several people working simultaneously on different fragments in the beginning, only one at the end (ref. 81,82). The choice of segmentation was guided by two kinds of arguments. We wanted to obtain as many of the naturally occurring fragments as possible (e.g. fragments 1-5, 1-9, 1-16 i.e. α-endorphin and 1-17 i.e. γ-endorphin), but then there were also synthetic considerations; for example, coupling of peptides with a C-terminal prolyl or glycyl residue is to be preferred (racemization-safe).

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   1              5              9
H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-
    17                  22                  27              31
Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
```

Three main protected fragments 1-9, 10-17 and 18-31 were planned for the synthesis of β- and γ-endorphin (ref. 82). The sequence -Lys-Asn-Ala- is present twice and we used this in the planning of the synthesis of four fragments spanning the C-terminal 14-peptide. Tert-butyl derived side-chain protecting groups for amino and carboxyl functions were employed and the hydrogenolytically-labile Z group for α-amino protection; see Fig.1.
The fragments were coupled using DCC and HOBt and the final deprotection step was carried out in TFA (ref. 82).
In addition, all (partially) protected fragments were obtained in their free form, purified and analyzed (ref. 81-85). Pharmacological evaluation of these fragments has resulted in interesting structure-activity relationships (ref. 86,87).

3. Scaling up

It may be clear that a successful, satisfactory synthesis of peptides on a laboratory scale is not necessarily applicable to a (repetitive) commercial production scale. A comprehensive review on the complexity of developing peptide production facilities has been given by Feurer (ref. 88). The choice of protecting groups, their way of introduction and removal, the activation procedures as well as the availability of suitable reagents have to be considered beforehand (e.g. when the azide reaction is used, a limited amount can be reacted in view of the liberated hydrazoic acid). Purification of the end-product on a scale of >100 kg-year, for example would not be practicable with currently used methods (see below) both from a technical and financial point of view; crystallization would be the appropriate method. Nine years ago all but one of the large scale syntheses were carried out in solution

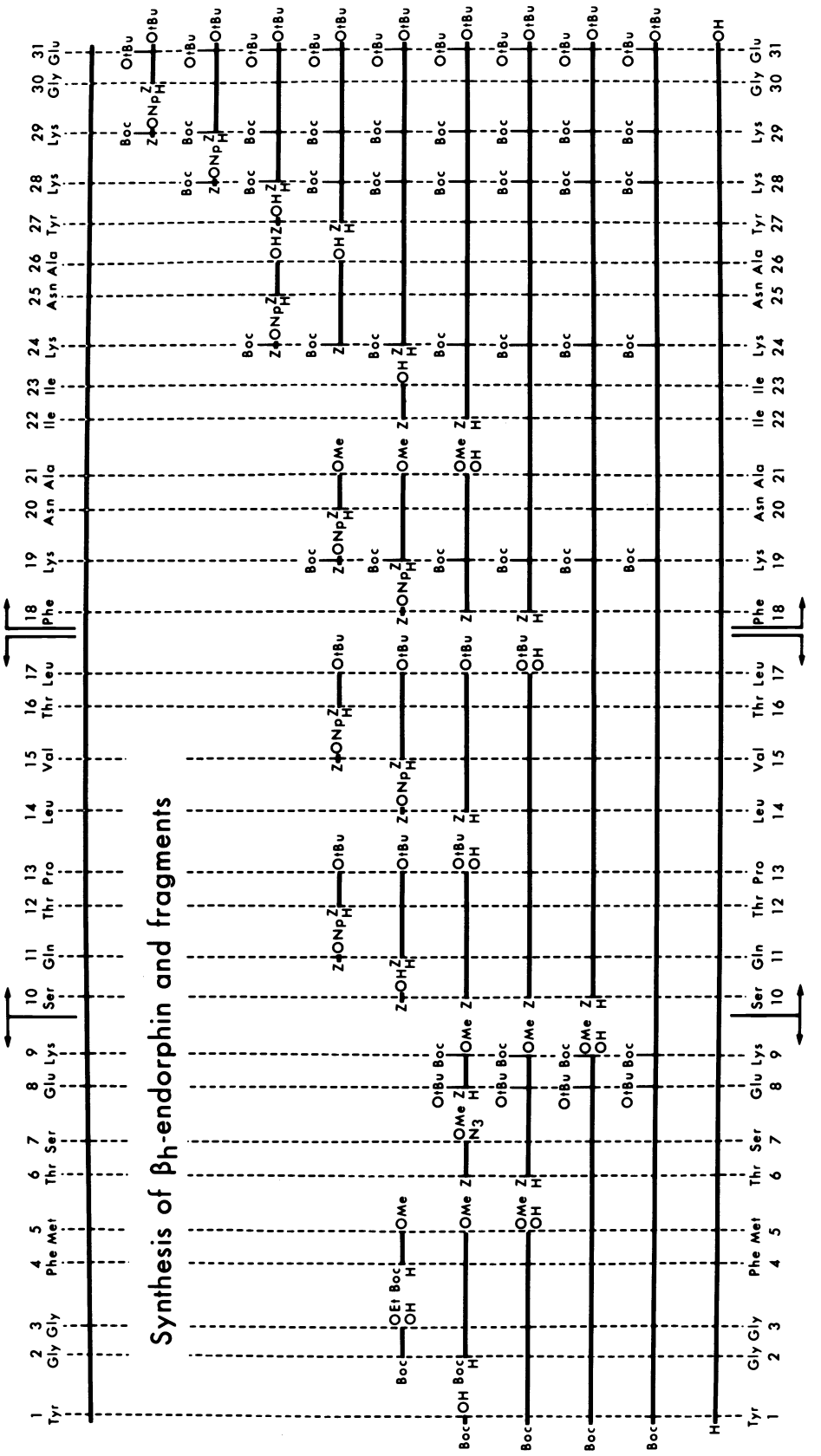

Fig.1 Synthesis of β_h-endorphin and several fragments using the fragment condensation approach.

(ref. 88). Although several peptides are now being synthesized by the solid phase method on a scale ranging from 50 g to 1 kg, the majority (including those peptides produced on a multi-kg scale) is still obtained by solution synthesis. A recent review by Meienhofer compares the opportunities and constraints of large scale peptide synthesis in solution and solid phase synthesis (ref. 89).

4. Synthesis on a microgram scale

Different requirements also play a role in strategy and tactics when working on a microgram scale, as is for instance the case with radioactive material, although safety and economical aspects are also important here. As an illustration the following example is given. In the past we have worked out a synthesis of fragments and analogues of arginine vasopressin (AVP) and oxytocin containing a cystine residue in position 6 (ref. 90). We used the triphenylmethyl (Trt) function for SH protection during the synthesis of the protected peptides and at the end converted the -Cys (Trt)- into the corresponding -Cys (Scm)- peptides (Scm stands for methoxycarbonylsulfenyl). Reaction with free cysteine provided the asymmetrical disulfide; deprotection with acid was followed by purification (ref. 90). In the synthesis of a ^{35}S labelled AVP fragment we changed our strategy in such a way that the reaction with [^{35}S]cysteine was the final reaction step (ref. 91).
Different reaction conditions, e.g. excess reactant, solvent and time were developed partly due to the restrictions imposed on us by the commercially available radioactive material (ref. 91).

PURIFICATION

For the purification of (poly)peptides and proteins one can make use of the following possibilities: crystallization, extraction and chromatography. As (poly)peptides, in contrast to proteins, are difficult to crystallize one usually goes straight to the other techniques. In extraction, use is made of the fact that, in general, the main component and by-products of a synthesis have different distribution coefficients in two non-miscible (mixtures of) solvents. Automation of multiple extraction steps has resulted in the counter current distribution machine in which the upper and lower layer move in opposite directions. Much more applied in practice is the so-called Craig machine, where in the multiplicative distribution, the lower phase retains its position while the upper phase is mobile (ref. 92). A large number of combinations of solvents of widely varying nature is possible in order to find optimal separation of the peptide components (ref. 32). Craig machines are available with small (5 ml phase volume) and large (1 L phase) separation tubes. Purification of peptides by liquid-liquid chromatography is also possible in variants of the Craig multiplicative distribution like for example counter-current chromatography (a continuous, non-equilibrium process) as described by Ito and Bowman (ref. 93). Application in the purification of peptides, however, is still limited (ref. 94). In a variation of counter current chromatography (CC), named droplet CC, the solute partitions between droplets of moving phase passing through columns of stationary liquid phase (ref. 95). Although a commercially available apparatus (Büchi) has been available for some 8 years, this method has not found widespread use in peptide purification.
In chromatography, separation of (poly)peptide mixtures takes place on the basis of molecular size, charge and hydrophobic character (ref. 32). Many types of stationary phases, based on silica, sepharose and macroreticular polymers are available in different varieties; in principle an unlimited number of mobile phases are possible although limitations exist as far as pH and volatility of the solvents are concerned. High-performance liquid chromatography (HPLC) has found a tremendous increase in its use for the purification of peptides (e.g. ref. 96) on a small scale using analytical columns or on multi-mg scale using semi-preparative columns. Recently, large commercial HPLC instruments (Whatman, Jobin Yvon) have been developed and these will certainly find their way to producers of large amounts of (poly)peptides and proteins.

METHODS OF ANALYSIS

After purification the peptide has to be analyzed. The purity criteria depend on the aim of the synthesis. For a reference compound criteria can be applied that differ from those necessary for clinical release. In such a first, limited, analysis, thin layer chromatography (in several solvent systems and using different detection methods), and often also electrophoresis is carried out followed by reversed phase HPLC (RP-HPLC), which has become a more or less standard analytical technique in most laboratories. Amino acid analysis has to be performed in order to check for the correct composition; simultaneously the peptide content is obtained, the remainder being water and, in general, acid. That such an amino acid analysis is not superfluou , especially not in solid phase peptide synthesis, can be illustrated by several reports (e.g. ref. 78-80).
Our own research group also routinely makes isotachopherograms of our peptide preparations. Analytical capillary isotachophoresis has been shown to be a rapid qualitative and quantitative method for the simultaneous determination of anions (or cations) in peptide preparations (ref. 97-99). In the synthesis and purification of peptides many different organic and inorganic acids may be used: e.g. TFA, MSA, TFMSA, formic acid, acetic acid and the hydrogen halides. It is known that peptides in general tend to retain variable amounts of

water and acids, as counterions and as residual acids after the final isolation or purification step. In addition peptides are tested and stored preferably as their corresponding acetates or hydrochlorides; in order to convert the end-product into the acetate form an ion-exchange step is necessary. We have developed a system in which 8 frequently occurring anions can be measured simultaneously as is shown in Fig. 2 (formate, citrate, methanesulfonate, trifluoroacetate, monochloroacetate, acetate, p-toluenesulfonate and HOBt, ref. 97,99; a separate system for the halides has been developed, ref. 99).

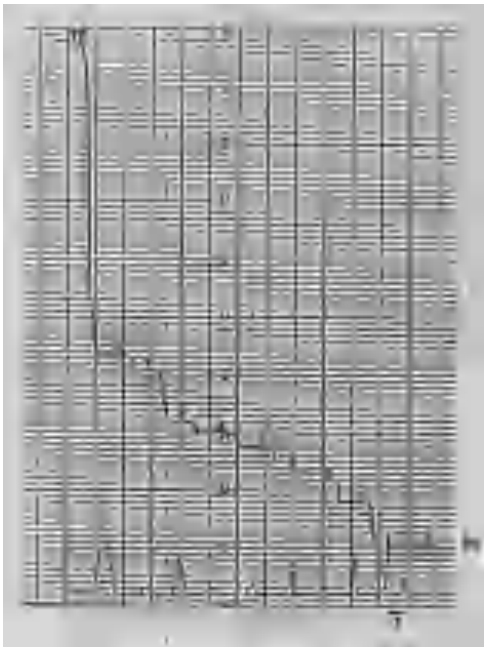

Fig.2 Isotachopherogram of a reference mixture of eight anions. (Reproduced from ref. 99 with permission of Elsevier Science Publ. B.V., Amsterdam).

In addition to the content of the acetic acid in the peptide preparation we simultaneously obtain information on the completeness of the ion-exchange step and on the presence of strange anions originating from the ion-exchange resin (ref. 97) or solvent bottles. Checks by isotachophoresis on desalting of peptide preparations using HPLC can also provide useful information (ref. 100). Analogously, a system that can separate 11 cations (hydrazine, sodium, trimethylamine, tetramethylammonium, pyridine, piperidine, 4-dimethylaminopyridine, N-ethylmorpholine, N,N-diisopropylethylamine, triethylamine and dicyclohexylamine) in one single run has been developed (ref. 98,99).
Analytical isotachophoresis can also be used to give information on the purity of the peptide itself (e.g. 97,101), but in our hands (RP-)HPLC is a more suitable technique to establish the purity of peptides.
The intended application, e.g. use in the clinic,requires more detailed analytical work. Several analyses have to be performed: a check on racemization either using enzymes or by gas chromatography (on a chiral column) or HPLC (on chiral columns or with chiral eluents) on hydrolyzed samples, a water content determination, a perchloric acid titration (i.e. a non-destructive method to determine the equivalent weight) and spectroscopic methods like UV, NMR, and mass spectrometry. For proteins several criteria have been described in the literature, partly replacing and partly adding to the above list, e.g. gel electrophoresis, isoelectric focussing, CD spectrophotometry and protease mapping (ref. 102). As far as HPLC for proteins is concerned, the use of (widepore) reversed phase columns often resulted in purification, but lead to denaturation of the protein (although still suitable for sequencing). Later, separation on the basis of hydrophobic character and size exclusion chromatography were used as well as high performance ion-exchange chromatography. The RP-HPLC columns mostly used for oligo- and polypeptides have improved in quality over the years (treatment of the silica matrix, capping, narrower size distribution, smaller particles) and several manufacturers now offer a wide range of columns. A vast number of papers have appeared describing the application of different types of HPLC columns and mobile phases for different purposes in the peptide field (ref. 96). Detection methods other than UV measurement have been applied, e.g. fluorescence and electrochemical detection. Chemiluminescence and laser-induced fluorescence detection lower the detection limit even further into the femto-atogram range; a recent paper by Miyaguchi describes chemiluminescence detection of the N-terminal amino acid of bradykinin after HPLC separation on a microbore column (detection limit around 1 fmol; ref. 103). This type of column (smaller diameter than usual) has several advantages over the "traditional" HPLC columns; its wide acceptance is hampered by some problems related

to the present instrumentation (ref. 104). Sequence analysis of peptides and proteins can also be necessary to perform sometimes in order to verify the structure of the synthesized peptide (ref. 78-80). Recent trends in this area, also employing HPLC, have been reviewed recently (ref. 105). Considerable attention has been devoted in recent years to the application of the HPLC methodology for amino acid analysis. Pre-column derivatization, followed by HPLC separation instead of the post-column reaction with ninhydrin as in the classical Moore and Stein procedure, results in a considerable operational advantage. Reaction of amino acids with o-phthalaldehyde or dansyl chloride gave derivatives that were measured with a fluorimeter; several research groups have reported HPLC separations of these derivatives (for a recent review see ref. 96;106). The drawbacks of the use of these reagents (no reaction takes place with proline and hydroxyproline using o-phthalaldehyde, limited stability, formation of multiple derivatives and interfering peaks, the use of a fluorimeter) are not present with the pre-column derivatization procedure using phenylisothiocyanate (PITC) to give phenylthiocarbamyl (PTC) amino acids which can be separated by RP-HPLC and UV detected. Based on this principle a dedicated apparatus was introduced (Pico.Tag Workstation of Waters) in which gas-phase acid hydrolysis of the peptide takes place followed by PTC-amino acid formation (ref. 107).

We have recently performed a systematic study of the chromatographic parameters stationary and mobile phase, gradient profile, pH, flow rate and column temperature which has resulted in an HPLC system in which 23 PTC-amino acids are distinctly separated (see Fig. 3) (ref. 108).

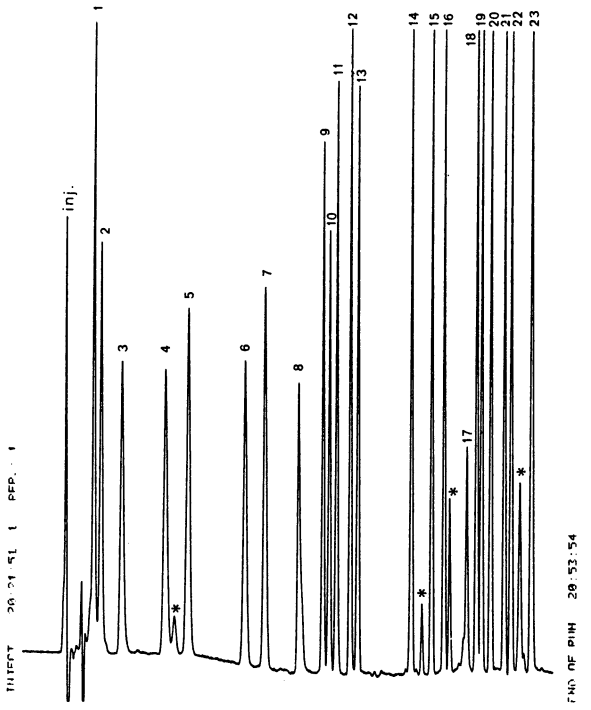

Fig. 3 HPLC analysis of 23 PTC-amino acids; * = reagent-derived peaks.

Applying this system the preceding steps in HPLC amino acid analysis, i.e. gas-phase hydrolysis of the sample and the pre-column derivatization were evaluated and optimized; the sensitivity is in the low pmol range (ref. 108). We have successfully applied this method to the amino acid analysis of several peptides of different nature (ref. 108).

Application of mass spectrometry (MS) in the peptide field has increased when combined with gas chromatography; derivatization, however, is necessary for enhancing sample volatility (for recent reviews see ref. 109,110). Coupling of liquid chromatography and MS has recently become possible but published accounts of its application in sequence analysis to real problems are few (ref. 109,110). The introduction of the ionization method called fast atom bombardment (FAB) some 5 years ago, has offered the possibility of routine analysis of peptides without the need of derivatization. Informative mass spectra of many peptides have already been obtained (e.g. ref. 109), including larger molecules like insulin, and the technique has been proposed as a "must" in the confirmation of the sequence of synthesized peptides (ref. 79). Sequencing of unknown peptides based on FAB-MS still seems to be difficult, resulting normally only in a partial sequence (ref. 111). Nevertheless, this new technique certainly is a welcome addition to the arsenal of analytical methods used to establish the purity of synthetic peptides.

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