Journal of Peptide Science
J. Peptide Sci. **5**: 203–220 (1999)

REVIEW ARTICLE

Peptide and Glycopeptide Dendrimers. Part II

PAVEL VEPŘEK and JAN JEŽEK*

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Received 4 June 1998
Accepted 12 September 1998

Abstract: Recent progress in peptide and glycopeptide chemistry make the preparation of peptide and glycopeptide dendrimers of acceptable purity, with designed structural and immunochemical properties reliable. New methodologies using unprotected peptide building blocks have been developed to further increase the possibilities of their design and improve their preparation and separation. The sophisticated design of peptide and glycopeptide dendrimers has led to their use as antigens and immunogens, for serodiagnosis and other biochemical uses including drug delivery. Dendrimers bearing peptide with predetermined secondary structures are useful tools in protein *de novo* design. This article covers synthesis and applications of multiple antigen peptides (MAPs), multiple antigen glycopeptides (MAGs), multiple antigen peptides based on sequential oligopeptide carriers (MAP-SOCs), glycodendrimers and template-assembled synthetic proteins (TASPs). In part II the preparation of MAPs, and the utility of glycodendrimers and TASPs are discussed.

Keywords: dendrimers; multiple antigen peptides (MAPs); template assembled synthetic proteins (TASPs); sequential oligopeptide carriers (SOCs); regioselectively addressable functionalized templates (RAFTs); glycodendrimers; peptide synthesis

Contents

Abbreviations: Alloc, allyloxycarbonyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; Dde, 1-(4,4-dimethyl-2,6-dioxohexylidene) ethyl; ELLA, enzyme-linked lectin assay; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate; HIV, human immunodeficiency virus; MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MAP, multiple antigen peptide; Mtt, 4-methyltrityl; NMP, 1-methyl-2-pyrrolidone; Npys, 3-nitro-2-pyridine sulphenyl; PyBOP, benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium-hexafluorophosphate; RAFT, regioselectively addressable functionalized templates; sialosyl-Tn antigen, NeuNAcα2→6GalNAcα1→O-Ser/Thr; SOC, sequential oligopeptide carrier; T antigen, Galβ1→3GalNAcα1→O-Ser/Thr; TASP, template assembled synthetic peptide; Tn antigen, GalNAcα1→O-Ser/Thr.

* Correspondence to: Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 10 Prague 6, Czech Republic. E-mail: jezek@marilyn.uochb.cas.cz

Contract/grant sponsor: Grant Agency of the Czech Republic; Contract/grant number: 203/97/0176

CCC 1075–2617/99/050203-18$17.50

Preparation of MAPs

MAPs as chemical entities can be prepared by either a direct or an indirect approach. As shown in Figure 1, in the direct approach the whole molecule, i.e. the oligolysine core bearing multiple copies of peptide antigen, is prepared by stepwise SPPS. In the

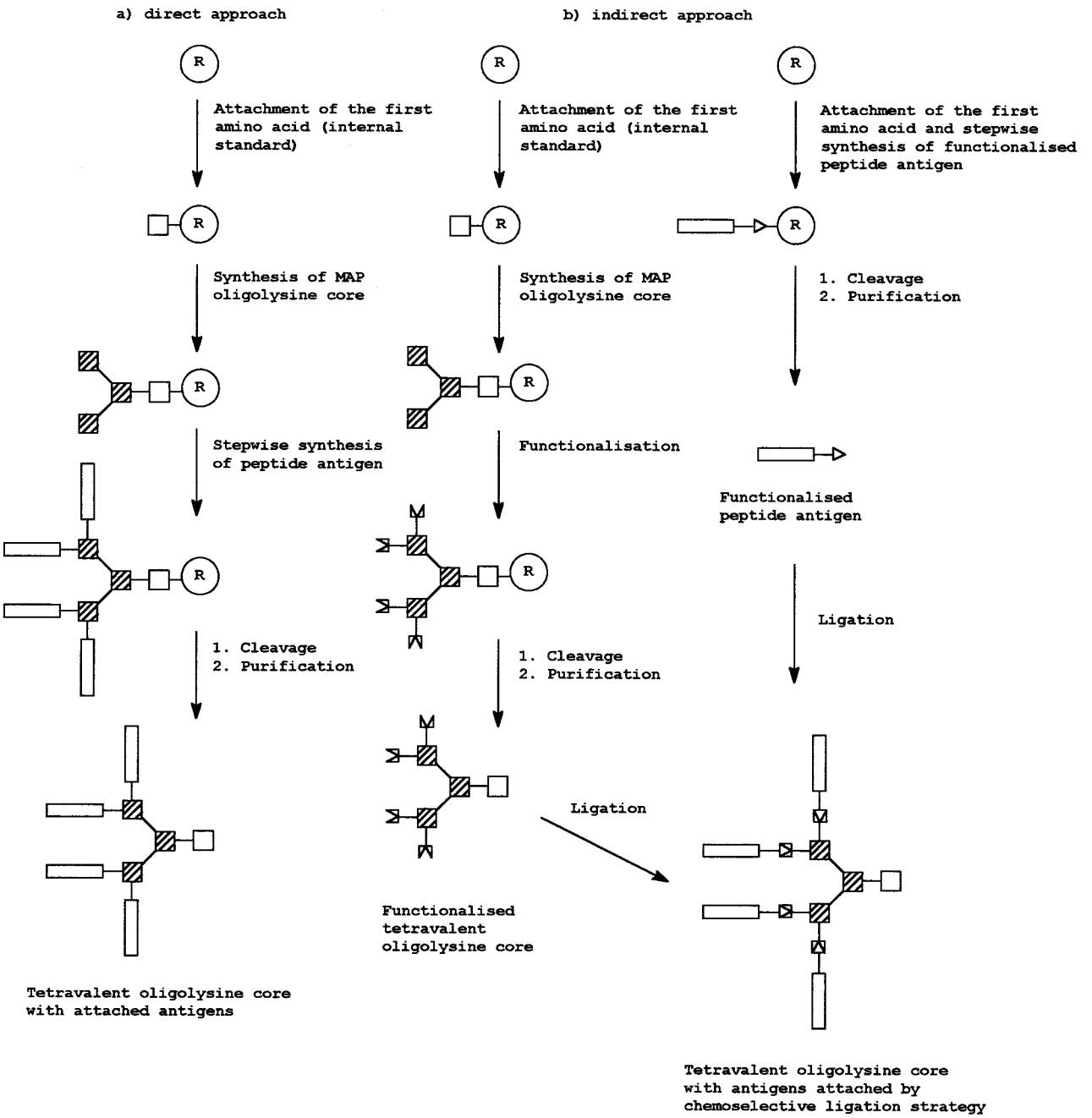


Figure 1 Direct (a) and indirect (b) methods of MAPs preparation.

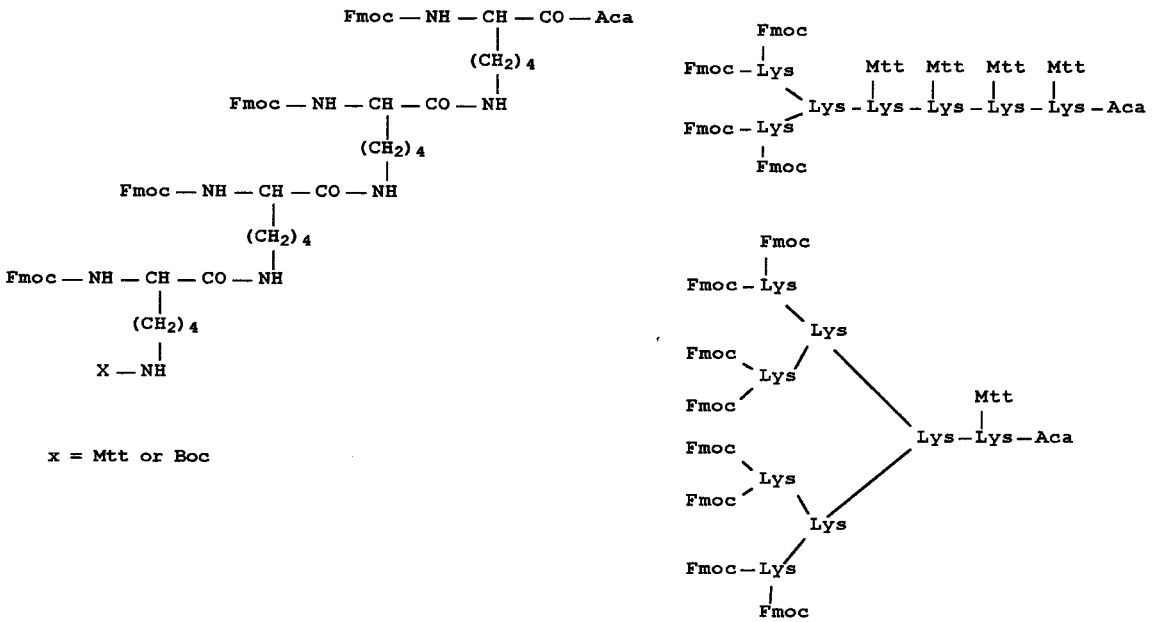


Figure 2 Novel designs of MAP cores.

indirect approach both structural elements are prepared and purified individually and then assembled together according to the conjugation protocols used.

Direct Approach. In the synthesis of MAPs by SPPS [1–3] both Boc and Fmoc chemistry is successfully applied [4,5]. Monoepitopic MAPs and MAPs with chimeric B-T and T-B epitopes [6,7] are synthesized stepwise from the C-terminus of the core matrix to the N-terminus of antigens, employing either Boc-Lys(Boc) or Fmoc-Lys(Fmoc) for the synthesis of the oligolysine core. For the preparation of diepitopic and, generally, multiepitopic MAPs, the orthogonally protected lysine residues are required during the synthesis of the last level of a core [8]. In the MAPs synthesis, six amino-protecting groups have been used so far.

The most classical amino-protecting groups are well known Boc and Fmoc groups. Boc-Lys(Fmoc) and Fmoc-Lys(Boc) can be used both in the Boc and Fmoc chemistry [8–10]. Two new groups have recently been developed for the Fmoc chemistry: Dde [1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl [8, 11,12] and Mtt [4-methyltrityl] group [13]. Dde cleavage by 2% hydrazine in DMF (v/v) does not influence the stability of the Fmoc protecting group. One of the limitations of its use concerns the recently described migration of this group during the Fmoc removal. Augustyns *et al.* [14] described Dde migration from an α-NH_2 or an ε-NH_2 group to an unprotected ε-NH_2 during the Fmoc removal from the side-chain by treatment with piperidine. Dde migration can be prevented by the use of 2% DBU [1,8-diazabicyclo[5.4.0]undec-7-ene] instead of piperidine [15]. Srinivasan *et al.* [16] reported intramolecular migration of the Dde group from the β-NH_2 group of diaminopropionic acid to the α-position during the Fmoc removal with piperidine. Mtt is a highly acid-labile amino-protecting group removable by 1% TFA in DCM or by a mixture of HOAc:TFE:DCM (1:2:7) (v/v/v). Under these conditions, the stability of the *t*Bu protecting groups or an ester bond binding the growing polypeptide chain to the resin is not influenced [13]. These groups are used for the protection of both α and ε amino groups of lysine, i.e. Fmoc-Lys(Dde), Dde-Lys(Fmoc), Fmoc-Lys(Mtt), Mtt-Lys(Fmoc). Another amino-protecting group is Npys [3-nitro-2-pyridine sulphenyl] group [17–20]. Npys is stable toward treatment with TFA and HF [21] but can be removed by triphenylphosphines or thiols [22]. It is usually used for the side-chain protection, i.e. Fmoc-Lys-(Npys) or Boc-Lys(Npys). And finally, the Alloc [allyloxycarbonyl] group [23–26]; the Alloc represents catalytically removable protecting group and is typically removed by ($Pd(PPh_3)_4$) in the presence of a nucleophile acting as an allyl group scavenger [27]. This is compatible with the presence of the acid labile *t*Bu, Boc and base labile Fmoc, provided that in the case of Fmoc a nucleophilic scavenger of low basicity is used [28].

These new orthogonally protecting groups enable preparation of novel types of oligolysine cores [13]. Asymmetrically branched cores and cores with an extended C-terminal part by one or more lysines have been designed to further enhance the variability and flexibility in the MAP design, see Figure 2.

The solid-phase synthesis of MAPs has been repeatedly described [10,29]. Here we would like to emphasize several points that we consider to be the determining factors for obtaining products of high homogeneity and yield.

Low-loading resins have to be used in the synthesis of MAPs to minimize inter-chain interactions leading to chain clustering that mimic cross-linking, limit resin swellability, reagent permeability and coupling efficiency [30]. Typically, resins with a loading capacity of 0.1 mmol/g are recommended. For tetravalent MAPs this represents a final concentration of N-reactive ends of 0.4 mmol/g, and for octavalent MAPs of 0.8 mmol/g, respectively. The latter is the loading capacity of the resins generally used for the synthesis of linear peptides. Thus, the use of resins with higher loading, especially for octavalent MAPs, has to be avoided.

Branched character and close proximity of N-reactive ends of a growing molecule require a 4-fold excess of activated amino acid to be used during the coupling steps. The efficiency of coupling has to be thoroughly monitored [31,32]. If necessary, coupling steps should be repeated using different activating agents (DCC/HOBt, BOP, PyBOP, HBTU etc.), solvents (DCM, DMF, NMP and mixtures such as 'magic mixture' [33]), increased temperature [34,35], microwaves [36] etc.

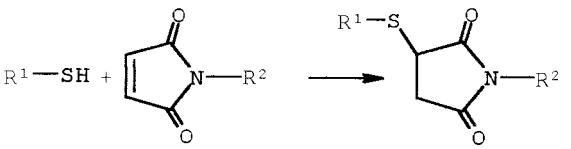
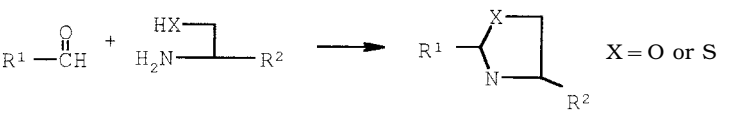
Driving coupling reactions to their completion is even more important than in the synthesis of linear peptides. MAPs are branched structures and accumulation of deletion or multi-dendritic chain products during the synthesis represents a challenging objective for the conventional RP-HPLC characterization and purification to achieve adequate resolution and/or recovery. In most of the immunological studies with MAPs, an extraction or dialysis step has been used with the crude product, followed by further purification by high-performance gel permeation chromatography or ion-exchange chromatography [10,29] to obtain a product of high purity. In this way, only low-molecular by-products or by-products with multiple deletions are removed. Only several studies have been devoted to the full characterization and purification of MAPs [4,37–39]. MAPs are usually purified by HPLC and characterized by HPCE, AA analysis, NMR, ES-MS and MALDI-TOF spectrometry. An alternative approach toward the characterization of peptides is the recently described partial cleavage by trypsin endopeptidase combined with RP-HPLC analysis of cleavage products [40].

In addition, preparation of MAPs by the direct approach is technically straightforward and can be automated. It does not require complicated chemistry, and more importantly does not introduce non-peptide bonds into the construct compared with the indirect approach discussed in the following section.

Indirect Approach. The preparation of high molecular polypeptides, proteins or enzymes is one of the challenging tasks for peptide chemists. Even though peptide chemistry has been undergoing rapid development since the introduction of SPPS, it still encounters various hindrances that make the final goal, i.e. the synthetic preparation of proteins in one procedure, difficult. The most limiting factors are the efficiency of coupling steps and the inter-chain interactions of growing polypeptide chains during the synthesis. To eliminate some of these factors, the idea of preparation of high molecular compounds by fragment condensation has been introduced. In principle, the required structure comprises of several building blocks prepared and purified individually prior to their final assembly. This has a great impact on the purification because the resulting structure differs significantly in its physico-chemical properties from the building blocks and thus all by-products are generally easily separable (for a review see [41]).

Two general strategies could be adopted. One is a classical solution fragment condensation of fully protected peptides in organic solution. This strategy had been widely used in the liquid phase peptide synthesis. Low yield of a product, slow coupling reaction, often poor solubility of fully protected peptide segments, as well as the tendency for racemization are the principal problems of this strategy. On the other hand, the possibility to purify intermediates is an important advantage of this approach [42]. The other strategy is the condensation of unprotected or partially protected peptide fragments, that are usually prepared by SPPS, in aqueous solution under mild conditions. The latter is especially advantageous because it avoids the use of fully protected fragments and utilizes highly selective chemistry. Here we give a brief account of the ligation strategies with a focus on MAP preparation. For further details we refer readers to some excellent reviews [39,43,44].

Table 1 A Survey of Ligation Methods Used in the MAP Preparation (for Details See [39,43,44])

Method	Principle
Thiol chemistry	
Thiol alkylation	R^1–SH + X–CH_2COR^2 → R^1–S–CH_2COR^2, X = Cl or Br R^1–COSH + X–CH_2COR^2 → R^1–CO–S–CH_2COR^2, X = Cl or Br
Thiol addition	
Thiol-disulphide exchange	R^1–SH + Ar–S–S–R^2 → R^1–S–S–R^2
Weak base-aldehyde chemistry	
Hydrazon, oxime formation	$R^1CHO + NH_2$–X–R^2 → R^1–CH=N–X–R^2, X = NH or O
Oxazolidine, thiazolidine ring formation	 X = O or S

In the MAPs synthesis via the indirect approach there are two ligation strategies used, based on either the thiol chemistry or the weak base-carbonyl chemistry (see Table 1).

A reactive pair which consists of a nucleophile at the C-terminus, and an electrophile at the N-terminus is usually chosen and placed on the synthetic peptide monomer and oligolysine core matrix during their solid-phase synthesis. Usually weak bases are used as nucleophiles because their pK_a values are lower than those of the α- and ε-amino groups of lysines and thus they can be selectively addressed during the ligation in aqueous buffers below pH 7.

Utilizable weak bases are alkyl thiol, acyl thiol, 1,2 aminothiol (N-terminal cysteine), 1,2-aminoethanol (N-terminal serine or threonine), hydroxylamine, acylhydrazine and arylhydrazine. As electrophiles haloacetyl, activated asymmetrical disulphide, aldehyde or maleinimide groups are usually used.

Thiol Chemistry. Thioalkylation is very popular in protein chemistry for attaching ligands, oligopeptides, and crosslinking [45,46] and has been applied for the indirect approach of MAP synthesis [47]. The application of thioalkylation on MAPs was first demonstrated by Lu *et al.* in 1991 [48]. Chloroacetyl groups were incorporated on the oligolysine core matrix and coupled with purified synthetic peptide with cysteine at the N-terminus. In the preparation of MAPs by thioldisulphide exchange, the thiolated core matrix reacted with activated S-(Npys)-cysteinyl peptide yielding MAP constructs with an S–S linkage [21,22,49]. The preparation of peptides with thioether linkage by adding a thiol group of cysteine residue to an activated double bond of 4-maleimido group represents another possibility [45].

Weak Base-Aldehyde Chemistry. Ligation methods using carbonyl chemistry are based on the same principle as thiol ligation chemistry, but they use different types of nucleophiles to react with aldehydes to yield other types of non-peptide linkages. The determining criterion for the selection of weak bases has been their ability to react selectively with aldehyde groups under acidic condition. Under these conditions side-chain nucleophiles are protected by protonation so that they cannot attack the aldehyde functionality and thus form undesirable by-products. Generally, there are two groups of nucleophiles used for this purpose. First, conjugated amines with their basicities lowered by neighbouring electron withdrawing groups, such as hydroxylamines and hydrazine derivatives. And compounds with a 1,2-disubstituted motif, such as 1,2-aminoethanol of serine or threonine and 1,2-aminoethanethiol group of cysteine. The aldehyde moiety can be introduced to the molecule in several ways. Firstly, by the $NaIO_4$ oxidation of N-terminal cysteine, serine or threonine under neutral conditions yielding an α-oxoacyl moiety [50]. The second method utilizes coupling of a suitably protected

aldehyde group to the N-terminus or the side-chain of lysine residue: 5,5-dimethoxy-1-oxopentanoic acid [51] and 2,2-dimethoxyacetic acid [39]. Finally, the masked aldehyde group is added to the C-terminus and then ligated to the free amino terminal group enzymatically or chemically with the Ag^+/HOSu method [52].

Even if the principles of the oxime [53], hydrazone [54] and thiazolidine [55] formation have long been known, the real contribution to the peptide preparation can be traced back to the early 1990s. Since then, more articles dealing with chemoselective ligation strategies and their use for the preparation of MAPs have been published.

In the oxime ligation, an aldehyde group reacts with aminooxyacetic functionality and gives a linkage stable toward HF or TFA at room temperature and at pH 2–7. The utility of this group for the MAP preparation as well as other branched peptides preparation can be well documented [39,56].

The hydrazide–aldehyde ligation chemistry, that is usually carried out at pH 4.5–5.0, gives a hydrazone bond which is quite unstable under acidic conditions. In order to stabilize the new linkage in the molecule, the bond is usually reduced by treatment with $NaBH_3CN$ to a hydrazine linkage [57,58]. In dendrimers, the hydrazone linkage has been found to be sufficiently stable at neutral pH in comparison with the linear peptides, probably due to the branched character of the construct [59].

The thiazolidine and oxazolidine ring formation has also been exploited for the preparation of branched peptides [38,52]. During the thiazolidine and oxazolidine ring formation, the thio- and oxo-analogues of proline amino acid are formed. This method further increases selectivity of ligation because a 1,2-disubstitued pattern is required for successful ligation.

The ligation techniques discussed so far produce linkages of non-peptide origin which are thought to be potential candidates for new epitopes in molecules. In the peptide chemistry several methods have been introduced to overcome these shortcomings yielding classic peptide bonds at the ligation sites.

Blake [60] coupled a peptide thiocarboxylic acid derivative with a free α-amino group of partially protected peptide upon silver ion activation. The reaction proceeded in DMF/water (1/1, v/v) solution at pH 7 and provided the desired peptide in a comparatively good yield.

Kemp and Fotouhi [61,62] reported peptide synthesis by thiol capture scheme. Generally cysteine is used as a nucleophile at the site of ligation to form a covalent disulphide bond with acyl fragment. This coupling is immediately followed by intramolecular proximity-driven acyl shift to form a peptide bond. This method can be used even if nucleophilic side-chain functionalities such as ε-amino groups of lysine and imidazole functionality of histidine are left unprotected during ligation [62–64].

Zhang and Tam [65] published another scheme in which peptide thiocarboxylic acid reacted with unprotected N-terminal histidyl peptide fragment. In the presence of a suitable thiophilic promotor such as Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic) acid), the acyl segment is captured by N^{im} of histidine residue and immediately undergoes N^{im} to N^{α} intramolecular acyl shift to form a peptide bond. Similarly, the peptide bond can be formed by the intramolecular acyl transfer of acyl segment as thioester, captured by β-bromo amino acid derivative or N-terminal cysteine [66].

Chemoselective ligation strategies, which have been published, can be further exploited in the preparation of conformationally constrained peptide antigens to more accurately mimic the structural arrangements in natural proteins. Multiple cyclic antigen peptides (McAPs) represent a new and interesting approach in antibody preparation. Antigens can be cyclized either prior to their attachment to the oligolysine core [67,68] or after [69] by means of ligation techniques described above. Here, instead of intermolecular reaction between the MAP core and peptide antigen, the intramolecular reaction is employed to give cyclic peptides [70,71]. A novel, convenient method to carry out intramolecular, to obtain cyclic peptides, and intermolecular, to obtain ligated peptides, directly from the solid support has been reported [72].

Cyclic peptides and namely McAPs are believed to have better properties when used as immunogens or antigens compared with the corresponding linear analogues. As immunogens, it is likely to suppose that antibodies induced by the McAPs will be of higher affinity for the native proteins than those induced by MAPs with linear antigens. The use of McAPs as antigens in serodiagnosis should further increase the detection sensitivity and the affinity to specific antibodies [69,71].

Sequential Oligopeptide Carriers in Multiple Antigen Peptide Design

The use of synthetic low molecular branched oligolysine carriers, for attaching multiple copies of

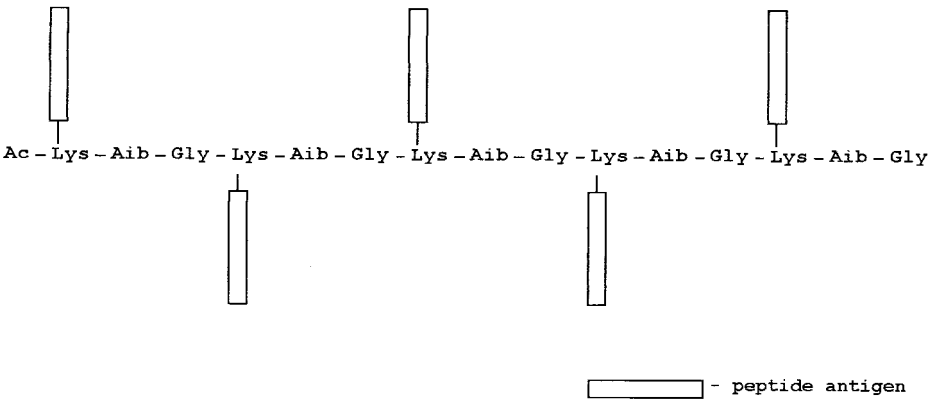


Figure 3 Schematic presentation of Sequential Oligopeptide Carriers with attached multiple copies of antigenic peptide, MAP-SOC$_n$, where $n = 5$ [73].

antigens to form synthetic molecules of immunological value, represents an important step toward development of fully synthetic vaccines. However, these carriers do not adopt any defined spatial conformation and thus one may speculate about the character of interactions in the structure. These interactions are believed to have, to some extent, an unfavourable effect on the presentation of antigens on the immune system. Inter- and intramolecular aggregation of antigens can limit their accessibility as well as alternate the secondary structure of antigens and thus immunogenicity.

A novel class of oligopeptide carriers termed **Sequential Oligopeptide Carriers** (SOCs) has been recently designed by Sakarellos *et al.* [73]. SOCs are linear molecules with a Lys-Aib-Gly tripeptide sequential motif. By varying the number of tripeptide units, carriers of general formula SOC$_n$, (Lys-Aib-Gly)$_n$, where $n = 2–7$ have been prepared [74]. The molecules of carriers adopt a predetermined secondary structure which is given by the presence of Aib, an unnatural amino acid with a known propensity to induce ordered helicoid backbone [75]. This helicoidal structure contributes to the reduction of steric hindrance and conformational restrictions in the molecule, and thus allows peptides to retain their original structure (Figure 3) as confirmed by NMR studies [74]. MAP-SOC$_n$ so far published have been prepared by stepwise SPPS according to the standard synthetic protocols using orthogonally protected lysine, Boc-Lys(Fmoc).

Two antigenic sequences: (1) the [Ala^{76}] derivative of the main immunogenic region (MIR, Trp^{67}-Asn-Pro-Ala-Asp-Tyr-Gly-Gly-Ile-Lys^{76}) of the Torpedo nicotinic acetylcholine receptor (AChR) and (2) the gp63-SRYD (Ile^{250}-Ala-Ser-Arg-Tyr-Asp-Gln-Leu^{257}) fragment of gp63, the major surface glycoprotein of *Leishmania* were selected to assess the utility of MAP-SOC$_n$ as antigens in solid-phase immunoassays and immunogens in eliciting anti-peptide antibodies. MIR-SOC$_n$ showed enhanced binding capacity to the anti-AChR mAbs in comparison with the MIR dodecapeptide in ELISA assays [74,76] and gp63-SRYD-SOC$_n$ were capable of inducing higher titres of Ab capable to cross-react with gp63 of intact parasite [77].

These results indicate that the MAP-SOC$_n$ concept is plausible for the preparation of synthetic antigens and immunogens. The spatial arrangement and regular secondary structure of carriers favour an optimal orientation and accessibility of antigens and thus their recognition by antibodies in immunoassays and by the cells of the immune system in eliciting anti-peptide antibodies. Furthermore, one can assume that the use of a wide spectra of amino-protecting groups and ligation strategies known from the MAP approach will be utilized for the preparation of diepitopic and generally multiepitopic SOCs or lipoSOCs to further improve their immunological properties.

GLYCODENDRIMERS

Carbohydrates derived from various cell surface glycoproteins, proteoglycans, and glycolipids are spearhead molecules of cell surface membranes involved in a variety of biological processes. To study these processes at the molecular level, glycobiology, a new field of research has emerged [78].

Cell surface carbohydrates are involved in many interactions, including cell growth control, regula-

tion and differentiation, inflammatory responses, cellular trafficking, and cancer cell metastasis [79–81]. For example, sialyl-Le^X is a tetrasaccharide that is often found at the terminus of all-surface oligosaccharides of neutrophils and tumour cells [82,83], and has been identified as a ligand of the endothelial leukocyte adhesion molecule [84,85], i.e. a glycoprotein involved in inflammatory response. T, Tn and sialosyl-Tn structures have been recognized as tumour associated antigens [86,87]. Some mono- and oligosaccharides have been determined as receptors in bacterial, toxin, mycoplasma and viral infection. In this connection the reader is referred to the relevant review articles [79,88–90].

A detailed understanding of these interactions will be of particular interest toward the development of new therapeutic inhibitors for the prevention of various phatogenic infections and related diseases.

Recent progress in the synthesis of glycoconjugates [91–96] makes readily available materials suitable for the study of these processes. Unfortunately, individual carbohydrate–protein interactions are weak compared with protein–protein interactions [97]. In order to compensate the low attractive forces of natural oligosaccharides in the design of potent inhibitors, carbohydrate clusters [98], low-valent telomers [99], neoglycoconjugates [100] and glycopolymers [101] have been designed as model compounds with improved inhibitory properties. In some cases these multivalent conjugates were successful in the inhibition of bacterial and viral adhesions *in vitro*.

To better demonstrate the potential of multivalent carbohydrates as potential candidates for therapeutic purposes and to avoid the use of heterogenic and low defined materials, Roy *et al.* [102,103] designed a new class of multivalent dendrimers called glycodendrimers. This new class of low-molecular weight, multiantennary carbohydrate-containing biopolymers provides chemically defined and homogenous materials of high valencies and of high protein avidities which are necessary for the design of potent inhibitors of viral and bacterial cell adhesion processes.

Glycodendrimers, with a variety of shapes, core molecules, carbohydrate residues, and valencies have been synthesized using both convergent and divergent approaches. Cores with (a) 2^n carbohydrate haptens, based on lysine scaffolds [103], 3,3′-iminobis(propylamine) core [104], or methyl-3,5-dihydroxybenzoates coupled with oligoethylene glycol azidotosylate spacers, or (b) 3^n carbohydrate haptens based on methyl-3,4,5-trihydroxybenzoates coupled with oligoethylene glycol azidotosylate spacers [102], and (c) mixed valencies based on e.g. phosphotriester backbones or tris(2-aminoethyl)amine [102,105,106] were developed (see Figure 4).

A key step in the preparation of homogenous glycodendrimers is the coupling of glycosides to the core. The assembly is based on the use of chemoselective ligation strategies, well known from the MAPs. The most abundant is thioalkylation. Briefly, *N*-chloroacetylated dendrimer core reacts selectively in DMSO with thiolated derivatives of saccharide hapten to form glycodendrimer. The utility of this reactive pair is especially advantageous because it makes the monitoring of the completeness of the coupling step by the ^{1}H-NMR facile, a residual chloroacetyl group gives a characteristic signal at δ 4.2 in dimethylsulfoxide-d_6. An alternative strategy is the preparation of the thiourea-bridged glycodendrimers [107].

Biological Properties of Glycodendrimers

Glycodendrimers have been primarily designed to give deep insight into the carbohydrate–protein interactions. Mammalian lectins, usually surface carbohydrate-binding proteins having a great array of carbohydrate specificities, are involved in the binding of various carbohydrates. To assess binding and inhibitory properties of novel glycodendrimers, plant lectins having similar binding properties have been usually used as model in carbohydrate–protein inhibition studies. The study of coating capacity of various oligolysine based sialoglycodendrimers showed for octa- and hexadecavalent dendrimers comparative results as for homologous polymers. This indicates that synthetic glycodendrimers are promising tools for various immunochemical assays, such as ELISA or ELLA. These sialodendrimers were also found to be good inhibitors of haemagglutination of human erythrocytes by influenza A (strain X-31) viruses [103,108]. Even divalent dendrimer was found to be five times more potent than a monosialoside, and a hexadecavalent one was found to be as potent as homologous polymer [109]. In another inhibition study, when oligolysine based α-D-mannopyranosyldendrimers with two, four, eight, and 16 valencies were used, excellent binding inhibitory properties were detected. Again, the 16-valent glycodendrimer was found to be 578- and 2139-fold more potent than methyl-α-D-mannopyranoside, and 66- and 1383-fold more potent that *p*-nitro-

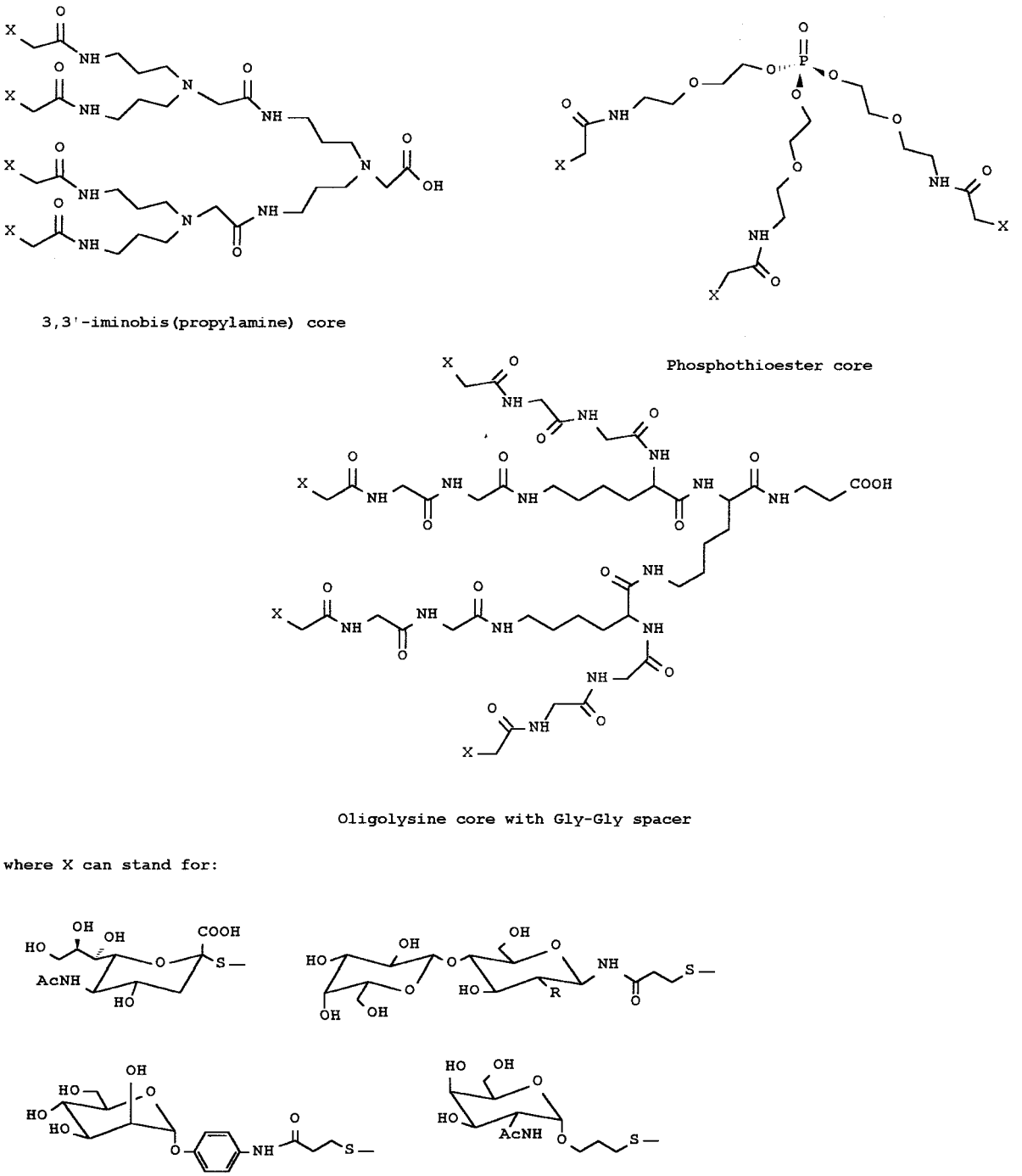


Figure 4 Examples of biologically-active glycodendrimers scaffolded on various cores [102].

phenyl-α-D-mannopyranoside in the inhibition of concavalin A and pea lectins, respectively [110]. The effect of multivalency on the inhibition of binding human α1-acid glycoprotein (orosomucoid) by the α-thiosialosideglycodendrimers scaffolded on the 3,3′-iminobis(propylamine) core further confirmed the usefulness of this approach. As the best inhibitor proved to be the tethered dodecavalent glycodendrimer that was roughly 200 times better than 5-acetamido-5-deoxy-D-glycero-α-D-galacto-2-nonulopyranose [111]. Moreover, the glycodendrimers with Tn (GalNAc-α1→O-Ser/Thr) antigens seem to be interesting molecules in cancer research [112].

TEMPLATE-ASSEMBLED SYNTHETIC PROTEINS

The preparation of new artificial proteins and enzymes with predetermined three-dimensional structure, and tailor-made chemical, biological, and catalytic properties represents an ultimate goal for the peptide and protein chemists [113]. However, the successful design of large and complex systems is limited, as our current understanding of the protein folding mechanism does not allow us to reliably predict whether a given amino acid sequence will fold to a well defined tertiary structure or not [114,115]. The major inherent problem of protein folding is the competition between intermolecular aggregation and intramolecular folding, and the high loss of entropy of chain within the folding process [114–116].

Interesting, but rare, examples of successful protein design, that show that even simple molecules are capable to catalyse difficult reactions, are preparation of a simple enzyme-like 14-residue α-helical system catalysing the decarboxylation of dianionic substrate [117], and the synthesis of a 33-residue synthetic peptide ligase motif, which efficiently catalyses the condensation of two shorter peptide fragments with high sequence- and diastereoselectivity [118].

In principle, the common feature of all the contemporary strategies in the protein design is the assembly of medium-sized peptide blocks that adopt amphiphilic secondary structures to a more complex folding topology. Consequently, a detailed knowledge of processes of secondary structure formation and stability is essential in the successful design of peptide blocks.

The peptide blocks have been selected on the basis of our present knowledge of the critical chain length, solvent and sequence dependence of helices and β-structures, that comes out of numerous studies using homo- and co-oligopeptides [119] or host–guest techniques [120]. In the design of peptide blocks with defined secondary structure the methods of choice are: (1) the selection of amino acid sequences that are known to fold in defined secondary structures; (2) the incorporation of C^{α}-alkylated amino acids to stabilize helical conformation of peptide [121,122]; (3) the incorporation of trifunctional amino acids (i.e. Cys, His at positions i and $i+4$) allowing helix stabilization via complexation of amino acid side-chains with a transition-metal ion [123–125]; and (4) the use of conformationally constrained molecules as templates by geometrical fixing of the first amino acid in the proper orientation for helix or β-sheet initiation [126].

The assembly of secondary structures forming polypeptide blocks into more complex tertiary structure-like topology can be achieved by several routes. First, by the metal ion-assisted self-organizing molecular process [127] in which the peptide blocks undergo metal-ion induced assembly via N-terminal ligands, and usually form structures with four-helix bundle [128], or three-helix bundle [127,129] topology. For example, four-helicoid 15-residue long polypeptides with pyridyl functionality at the N-termini undergo an intramolecular self-assembly process upon the complexation with Ru(II) and form stabile 60-residue of parallel four-helix bundle metalloprotein [130]. Metalloporphyrins instead of metal ions have been used to induce self-assembly processes [131,132]. These complexes are essential intermediates in the synthesis of molecular 'maquettes', a novel class of simplified versions of the metalloproteins involved in redox catalysis and in energy conversion in respiratory and photosynthetic electron transfer. An alternative route is the covalent attachment of peptide blocks to various template molecules, e.g. dibenzofurans [133], coproporphyrins [134], and tetraphenylporphyrins [128].

The template-assembled synthetic protein (TASP) concept as a new general strategy for the construction of artificial proteins with predetermined tertiary structure [135,136] was developed to avoid the well known protein folding problem, the major obstacle encountered in protein *de novo* design [116,137]. Amphiphilic secondary-structure forming oligopeptides (α-helices and β-sheets) are fixed on multifunctional tailor-made molecule which directs the peptide blocks to fold in a protein-like packing arrangement.

For this purpose computer-assisted molecular modelling has been successfully utilized. The template molecule plays an important role. It favours intramolecular interactions of particular peptide blocks and determines the structure of the molecule by the number and spatial arrangement of its anchoring points. By the selection of a proper template, TASPs with $\beta\alpha\beta$-, α-helix bundle, β-barrel-like tertiary structure can be obtained (Figure 5). In this respect, templates should be considered as 'built-in' folding devices and not only as structure-stabilizing cross-links [138].

Template molecules consist of two antiparalell β-sheet oligopeptides containing lysine residues as branching/anchoring points, linked through β-turn-

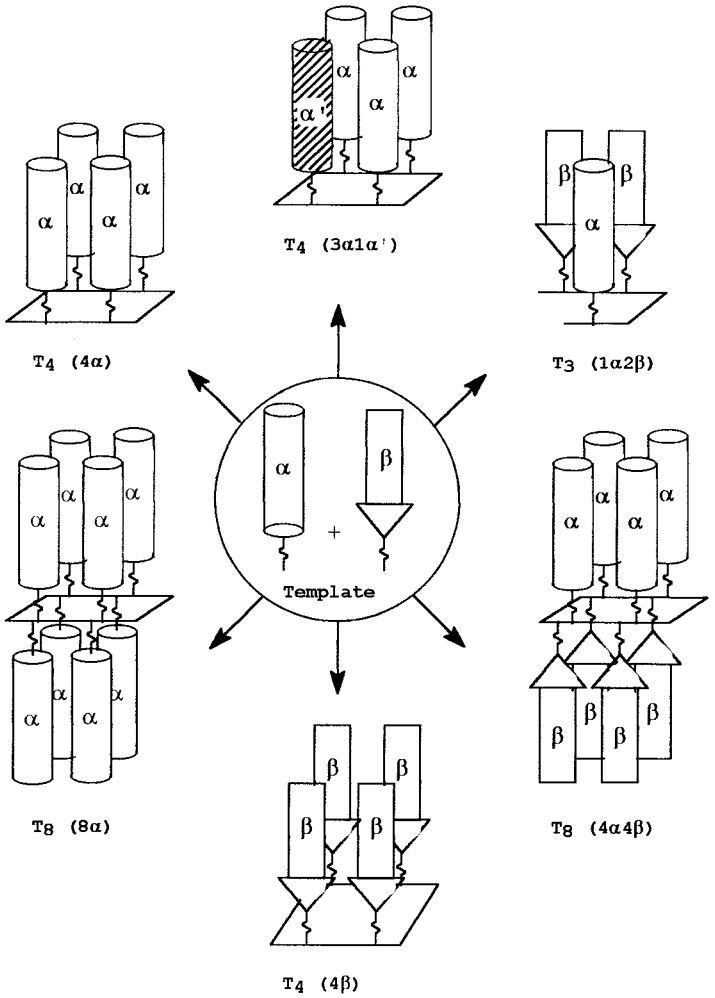


Figure 5 Schematic preparation of TASPs with different packing arrangements. Amphiphilic peptides forming α-helices and β-sheets are grafted onto a template to form a structure with complex tertiary topology (T_x, T – template molecule, x – number of branching sites [116].

inducing mimics. Several designs of templates have been published so far: (1) open-chain oligopeptides with central Pro-Gly dipeptide motif securing the desired U form [116,135,139]; (2) cyclic oligopeptides with or without Pro-Gly motif [140] and oligopeptides cyclized by disulphide bridge formation [141,142]; and (3) cyclic templates with non-peptidic β-turn inducing mimics like (R,S) 8-aminomethyl-5,6,7,8-tetrahydronapth-2-oic acid (Amhn) or 8-aminomethyl-napth-2-oic acid (Amn) [143–145] (Figure 6).

Application and Prospectives of TASPs

The complex character of TASPs gives them structural features of both synthetic peptides (branched character) and globular proteins (amphiphilic peptide blocks, hydrophilic surface and hydrophobic core). The value of the TASPS concept in the design of artificial proteins with complex tertiary structures was demonstrated by successful designs and preparations of molecules with four-helix bundle [143,145], β-meander [146] or β-barrel [147] topologies. The incorporation of functional properties into the molecules is the next step in the protein design. The preparation of biologically-active peptides, peptides with suitable immunological properties, catalysts, enzyme-like models valuable for the structure-function studies, ion-channel or receptor mimics etc. are the prospectives of the protein design using the TASP concept.

Recently, the utility of TASPs as immunogens inducing specific antibodies was described [140,148]. The antibody response of both MHC-TASP and (LDH-C4)-TASP used in the studies was

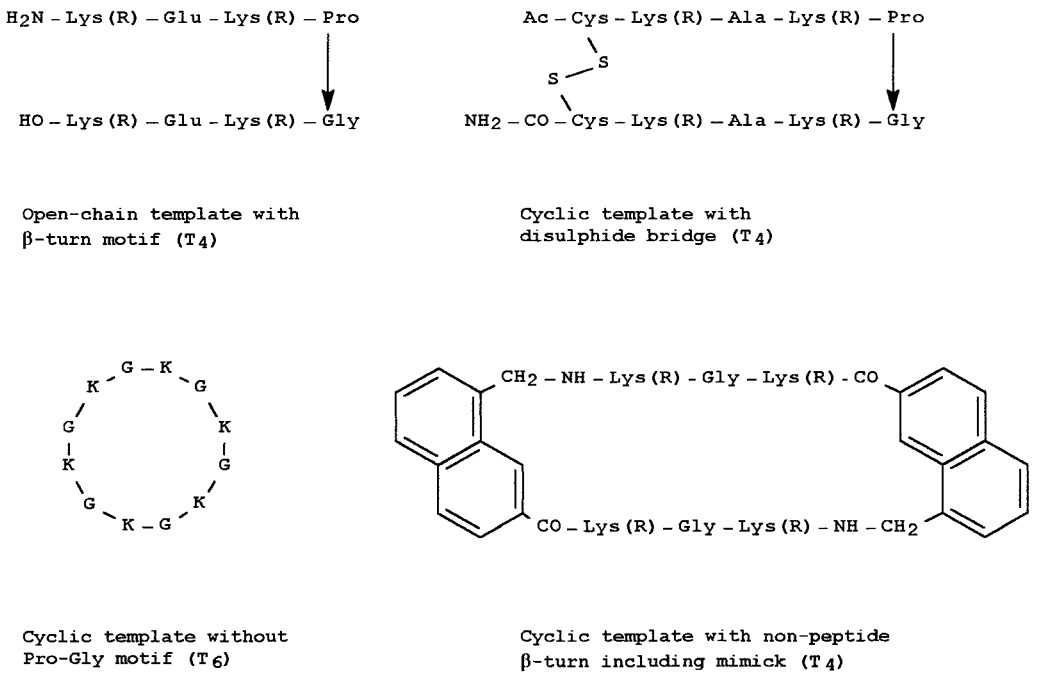


Figure 6 Templates used in TASP concept.

obtained without coupling the peptide to a carrier protein. These preliminary findings indicate the great potential of TASPs in synthetic vaccine development. Similarly to the MAP approach, here also the use of heterogeneous, high molecular protein carriers can be avoided by substituting them with low molecular oligopeptide synthetic carriers. As stressed above, in the MAP section, both the peptide sequence and the preserving of the native structure in the immunogen are essential conditions for eliciting high-affinity antibodies toward the native molecules against which the antibodies are directed. The TASP concept makes the preparation of more specific vaccines possible. As mentioned above, the template molecule can induce and hold the complex packing arrangement of the attached peptide blocks. Grafting antigenic regions to the TASP molecule upon the preserving of the spatial arrangement in the native molecule might represent an important breakthrough in this field. Another approach towards surface mimics is the preparation of TASP with attached peptide loops mimicking, for example, the binding site of an antibody, or the ligand binding site of a receptor [142]. This new approach exploits the recent advances in the chemoselective ligation strategies [39,44] and the new class of regioselectively addressable functionalized templates (RAFTs) [149] with specifically addressable lysine side-chain amino groups (see Figure 7). Pentavalent TASPs with tripeptide KPR, RPK or pseudopeptide [KΨ(CH_2NH)PR] with reduced peptide bond have been found to be potent and specific inhibitors of human immunodeficiency virus (HIV) infection by preventing viral entry into permissive cells, syncytium formation and triggering cell death by apoptosis [150,151].

Preparation and Properties of TASPs

In the initial reports TASPs were synthesized exclusively on the solid-phase support by stepwise SPPS following standard synthetic protocols of peptide chemistry [135,140]. By using orthogonally protected lysines, TASPs with several different peptide blocks in the molecule can be prepared by selective deprotection of each protecting group followed by the assembly of a particular peptide sequence. Due to the macromolecular and branched character of the TASPs the purification step is considerably more laborious and a combination of two orthogonal preparative purification techniques, e.g. RP-

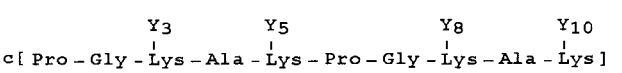


Figure 7 Tetravalent cyclic RAFT molecule with selectively addressable side-chains: Y_3 = Boc, Y_5 = Alloc, Y_8 = Dde, and Y_{10} = Fmoc [152].

HPLC and CZE or IEC, is usually necessary to obtain a product of a high purity [141].

The development of novel protection groups and chemoselective ligation techniques [43] is also reflected in the synthetic approaches used in the TASP concept. New methodology known from the MAP approach as an indirect approach, has been adapted for the use in the TASPs preparation [139,142,143,145]. In short, structural units of TASPs are synthesized and purified individually and then linked together by means of chemoselective ligation strategies. It is evident that the purification step in an indirect approach is significantly less laborious and provides products of higher purity even if only one purification technique was used.

Conformational characterization of TASPs by means of CD and NMR spectroscopy is in agreement with postulated secondary and tertiary structures. A strong secondary-structure inducing effect of templates to the molecule has been demonstrated on TASPs with four-helix bundle topology [141]. TASPs molecules showed higher helicoidal content than linear helicoid-structure forming peptide blocks, clearly suggesting the important role of the template in secondary as well as tertiary structure formation. RAFT templates in which symmetry is removed by differential protection permit their full characterization by NMR spectroscopy. 600 Mhz COSY, TOCSY, NOESY experiments, CD spectra and molecular dynamic simulations demonstrated that RAFT adopt stable, well defined conformation. The averaged (Φ, Ψ) values obtained for the Pro and Gly residues suggest that the RAFT molecule adopts a slightly distorted type II β-turn [153,154].

Acknowledgements

This work was supported by grant No. 203/97/0176 of the Grant Agency of the Czech Republic to Jan Ježek. The authors would like to thank J. Velek PhD and M. Ledvina PhD for their critical reading of the manuscript and fruitful discussion.

REFERENCES

1. R.B. Merrifield (1963). Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc. 85*, 2149–2154.
2. R.B. Merrifield. Solid-phase peptide synthesis, in: *Peptides: Synthesis, Structures, and Applications*, B. Gutte, Ed., p. 93–169, Academic Press, San Diego, 1995.
3. G.B. Fields and R.L. Noble (1990). Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Peptide Protein Res. 35*, 161–214.
4. A. Basak, A. Boudreault, A. Chen, M. Chretien, N.G. Seidah and C. Lazure (1995). Application of the multiple antigenic peptides (MAP) strategy to the production of prohormone convertases antibodies: synthesis, characterization and use of 8-branched immunogenic peptides. *J. Peptide Sci. 1*, 385–395.
5. B. Nardelli, Y.-A. Lu, R.D. Shiu, C. Delpiere-Defoort, A.T. Profy and J.P. Tam (1992). A chemically defined synthetic vaccine model for HIV-1. *J. Immunol. 148*, 914–920.
6. J.P. Tam, P. Clavijo, Y.-A. Lu, V. Nussenzweig, R. Nussenzweig and F. Zavala (1990). Incorporation of T and B epitopes of the circumsporozoite protein in a chemically defined synthetic vaccine against malaria. *J. Exp. Med. 171*, 299–306.
7. S.K. Chai, P. Clavijo, J.P. Tam and F. Zavala (1992). Immunogenic properties of multiple antigen peptide systems containing defined T and B epitopes. *J. Immunol. 149*, 2385–2390.
8. N. Ahlborg (1995). Synthesis of a diepitope multiple antigen peptide containing sequences from two malaria antigens using Fmoc chemistry. *J. Immunol. Methods 179*, 269–275.
9. J.P. Tam and Y.-A. Lu (1989). Vaccine engineering: enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T- and B-cell epitopes. *Proc. Natl. Acad. Sci. USA 86*, 9084–9088.
10. J.P. Tam (1989). High-density multiple antigen-peptide system for preparation of antipeptide antibodies. *Methods Enzymol. 168*, 7–15.
11. B.W. Bycroft, W.C. Chan., S.R. Chhabra and N.D. Hove (1993). A novel lysine-protecting procedure for continuous flow solid phase synthesis of branched peptides. *J. Chem. Soc., Chem. Commun.*, 778–779.
12. B. Kellam, B.W. Bycroft and S.R. Chhabra (1997). Solid-phase applications of Dde and the analogue Nde: synthesis of trypanothione disulphide. *Tetrahedron Lett. 38*, 4849–4852.
13. A. Aletras, K. Barlos, D. Gatos, S. Koutsogianni and P. Mamos (1995). Preparation of the very acid-sensitive Fmoc-Lys(Mtt)-OH. Application in the synthesis of side-chain to side-chain cyclic peptides and oligolysine cores suitable for the solid-phase assembly of MAPs and TASPs. *Int. J. Peptide Protein Res. 45*, 488–496.
14. K. Augustyns, W. Kraas and G. Jung. A side reaction of the Dde protecting group: migration to an unprotected lysine, in: *Book of Abstracts, 24th Symposium of the EPS*, Abstract No. 399, Edinburgh, Scotland, 1996.
15. K. Augustyns, W. Kraas and G. Jung (1998). Investigation of the stability of the Dde protecting group used in peptide synthesis: migration to an unprotected lysine. *J. Peptide Res. 51*, 127–133.

16. A. Srinivasan, R.R. Wilhelm and M.A. Schmidt. Intramolecular migration of the β-Dde protecting group of diaminopropionyl to the α-position during Fmoc solid phase synthesis, in: *Book of Abstracts*, *15th APS*, Abstract No. 111, Nashville, Tennessee, 1997.
17. R. Matsueda and R. Walter (1980). 3-Nitro-2-pyridinesulfenyl (Npys) group. A novel selective protecting group which can be activated for peptide bond formation. *Int. J. Peptide Protein Res. 16*, 392–401.
18. O. Rosen, S. Rubinraut and M. Fridkin (1990). Thiolysis of the 3-nitro-2-pyridinesulfenyl (Npys) protecting group. *Int. J. Peptide Protein Res. 35*, 545–549.
19. K.W. Hahn, W.A. Klis and J.M. Stewart (1990). Design and synthesis of a peptide having chymotrypsin-like esterase activity. *Science 248*, 1544–1547.
20. K.C. Pugh, L. Cera and J.M. Stewart (1993). Synthesis and stability of 3-nitro-2-pyridinesulfenyl chloride (NpysCl). *Int. J. Peptide Protein Res. 42*, 159–164.
21. S. Rajagopalan, T.J. Heck, T. Iwamoto and J.M. Tomich (1995). Use of the 3-nitro-2-pyridine sulfenyl protecting group to introduce N^{ε}-branching at lysine during solid-phase peptide synthesis. I. Application to the synthesis of a peptide template containing two addressable sites. *Int. J. Peptide Protein Res. 45*, 173–179.
22. F. Baleux and P. Dubois (1992). Novel version of multiple antigenic peptide allowing incorporation on a cysteine functionalized lysine tree. *Int. J. Peptide Protein Res. 40*, 7–12.
23. F. Guibé, O. Dangles, G. Balavoineand and A. Loffet (1989). Use of an allylic anchor group and of its palladium catalyzed hydrostannolytic cleavage in the solid phase synthesis of protected peptide fragments. *Tetrahedron Lett. 30*, 2641–2644.
24. G. Shapiro and D. Buechler (1994). Mild and rapid azide-mediated, palladium catalyzed cleavage of allylester based protecting groups. *Tetrahedron Lett. 35*, 5421–5424.
25. A. Loffet and H.X. Zhang (1993). Allyl-based groups for side-chain protection of amino-acids. *Int. J. Peptide Protein Res. 42*, 346–351.
26. M.H. Lyttle and D. Hudson. Allyl based side-chain protection for SPPS, in: *Peptides. Chemistry and Biology*, J.A. Smith and J.E. Rivier, Eds, p. 583–584, ESCOM, Leiden, 1992.
27. M. Dessolin, M.-G. Guillerez, N. Thieriet, F. Guibé and A. Loffet (1995). New allyl group for palladium catalyzed removal of allylic protections and transacylation of allyl carbamates. *Tetrahedron Lett. 36*, 5741–5744.
28. P. Lloyd-Williams, A. Merzouk, F. Guibé, F. Albericio and E. Giralt (1994). *Tetrahedron Lett. 35*, 4437–4440.
29. D.N. Posnett and J.P. Tam (1989). Multiple antigenic peptide method for producing antipeptide site-specific antibodies. *Methods Enzymol. 178*, 739–746.
30. J.P. Tam and Y.A. Lu (1995). Coupling difficulty associated with interchain clustering and phase transition in solid phase peptide synthesis. *J. Am. Chem. Soc. 117*, 12058–12063.
31. V.K. Sarin, S.B.H. Kent, J.P. Tam and R.B. Merrifield (1981). Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem. 117*, 147–157.
32. W.S. Hancock and J.E. Battersby (1976). A new microtest for the detection of incomplete coupling reactions in solid-phase peptide synthesis using 2,4,6,-trinitrobenzenesulphonic acid. *Anal. Biochem. 71*, 260–264.
33. L. Zhang, C. Goldammer, B. Henkel., F. Zühl, G. Panhaus, G. Jung and E. Bayer. 'Magic Mixture', a powerful solvent system for solid-phase synthesis of 'difficult sequences', in: *Innovation and Perspectives in Solid Phase Synthesis. Peptides, Proteins and Nucleic Acids*, R. Epton, Ed., p. 711–716, Mayflower Worldwide Ltd., Birmingham, 1994.
34. A.K. Rabinovich and J.E. Rivier (1994). Accelerated protocols for solid-phase peptide synthesis at elevated temperature. *Am. Biotechnol. Lab. 12*, 48–51.
35. L.M. Varanda and M.T. Miranda (1997). Solid-phase peptide synthesis at elevated temperatures: a search for and optimized synthesis condition of unsulfated cholecystokinin-12. *J. Peptide Res. 50*, 102–108.
36. S.-T. Chen, P.-H. Tseng, H.-M. Yu, C.-Y. Wu, K.-F. Hsiao, S.-H. Wu and K.-T. Wang (1997). The studies of microwave effects on the chemical reactions. *J. Chin. Chem. Soc. 44*, 169–182.
37. S. Bay, R. Lo-man, E. Osinaga, H. Nakada, C. Leclere and D. Cantacuzéne (1997). Preparation of a multiple antigen glycopeptide (MAG) carrying the Tn antigen. *J. Peptide Res. 49*, 620–625.
38. J.C. Spetzler, J.P. Tam (1995). Unprotected peptides as building blocks for branched peptides and peptide dendrimers. *Int. J. Peptide Protein Res. 45*, 78–85.
39. J. Shao and J.P. Tam (1995) Unprotected peptides as building blocks for the synthesis of peptide dendrimers with oxime, hydrazone, and thiazolidine linkages. *J. Am. Chem. Soc. 117*, 3893–3899.
40. H.H. Keah, E. Kecorius and M.T.W. Hearn (1998). Direct synthesis and characterization of multi-dendritic peptides for use as immunogens. *J. Peptide Res. 51*, 2–8.
41. H. Benz (1994). The role of solid-phase fragment condensation (SPFC) in peptide synthesis. *Synthesis*, 337–358.
42. E.T. Kaiser, H. Nihara, G.A. Laforet, J.W. Kelly, L. Walters, M.A. Findeis and T. Sasaki (1989). Peptide and protein synthesis by segment synthesis-condensation. *Science 243*, 187–192.
43. J.P. Tam. Synthesis and applications of branched peptides in immunological methods and vaccines, in: *Peptides: Synthesis, Structures, and Applications*, B. Gutte, Ed., p. 455–500, Academic Press, San Diego, 1995.
44. J.P. Tam and J.C. Spetzler (1995). Chemoselective approaches to the preparation of peptide dendrimers

and branched artificial proteins using unprotected peptides as building blocks. *Biomed. Peptide Protein Nucl. Acids 1*, 123–132.
45. G.E. Means and R.E. Feney (1990). Chemical modifications of proteins: history and applications. *Bioconjug. Chem. 2*, 2–12.
46. D.S. Wilbur (1992). Radiohalogenation of proteins: an overview of radionuclides, labeling methods, and reagents for conjugate labeling. *Bioconjug. Chem. 3*, 433–470.
47. S.D. Mahale, J. Pereira, U. Natraj and K.S. Iyer (1996). Comparison of antibodies raised against the peptide 10–24 of chicken riboflavin carrier protein (cRCP) by classical and multiple antigen peptide (MAP) approaches. *J. Immunol. Methods 190*, 215–219.
48. Y.A. Lu, P. Clavijo, M. Galantino, Z.Y. Shen, W. Liu, J.P. Tam (1991). Chemically unambiguous peptide immunogen: preparation, orientation and antigenicity of purified peptide conjugated to the multiple antigen peptide system. *Mol. Immunol 28*, 623–630.
49. J.W. Drijfhout and W. Bloemhoff (1991). A new synthetic functionalized antigen carrier. *Int. J. Peptide Protein Res. 37*, 27–32.
50. J.R. Clam and L. Hough (1965). The periodate oxidation of amino acids with reference to studies on glycoproteins. *Biochem. J. 94*, 17–24.
51. L.-C. Chiang, E. Cabezas, J.C. Calvo and A.C. Satterthwait. The synthesis of peptide secondary structure mimetics with covalent hydrogen bond mimics on the solid support, in: *Peptides. Chemistry, Structure and Biology*, R.S. Hodges and J.A. Smith, Eds., p. 278–280, ESCOM, Leiden, 1994.
52. C.F. Liu and J.P. Tam (1994). Peptide segment ligation strategy without use of protecting groups. *Proc. Natl. Acad. Sci. USA 91*, 6584–6588.
53. B.F. Erlanger, F. Borek, S.M. Beiser and S. Lieberman (1957). Steroid–protein conjugates. I. Preparation and characterization of conjugates of bovine serum albumin with testosterone and with cortisone. *J. Biol. Chem. 228*, 713–727.
54. T.P. King, Y. Li and L. Kochoumian (1978). Preparation of protein conjugates via intermolecular disulfide bond formation. *Biochemistry 17*, 1499–1506.
55. M.P. Schubert (1936). Compounds of thiol acids with aldehydes. *J. Biol. Chem. 114*, 341–350.
56. K. Rose (1994). Facile synthesis of homogenous artificial proteins. *J. Am. Chem. Soc. 116*, 30–33.
57. H.F. Gaertner, K. Rose, R. Cotton, D. Timms, R. Camble and R.E. Offord (1992). Construction of protein analogues by site-specific condensation of unprotected fragments. *Bioconjug. Chem. 3*, 262–268.
58. T.P King, S.W. Zhao and T. Lam (1986). Preparation of protein conjugates via intermolecular hydrazone linkage. *Biochemistry 25*, 5774–5779.
59. K.F. Geoghegan and J.G. Stroh (1992). Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine. *Bioconjug. Chem. 3*, 138–146.
60. J. Blake (1981). Peptide segment coupling in aqueous medium: silver ion activation of the thiolcarboxyl group. *Int. J. Peptide Protein Res. 17*, 273–274.
61. D.S. Kemp (1981). The amine capture strategy for peptide bond formation-an outline of progress. *Biopolymers 20*, 1793–1804.
62. N. Fotouhi, N.G. Galakatos and D.S. Kemp (1989). Peptide synthesis by prior thiol capture. 6. Rates of the disulfide bond forming capture reaction and demonstration of the overall strategy by synthesis of the C-terminal 29-peptide sequence of BPTI. *J. Org. Chem. 54*, 2803–2817.
63. D.S. Kemp, N.G. Galakatos, D. Dranginis, C. Ashton, N. Fotouhi and T.P. Curran (1986). Peptide synthesis by prior thiol capture. 4. Amide bond formation: the effect of a side-chain substituent on the rates of intramolecular *O*,*N*-acyl transfer. *J. Org. Chem. 51*, 3320–3324.
64. D.S. Kemp and R.I. Carey (1991). Resolution of the histidine problem for thiol capture-synthesis of a 39-peptide. *Tetrahedron Lett. 32*, 2845–2848.
65. L. Zhang and J.P. Tam (1997). Orthogonal coupling of unprotected peptide segments through histidyl amino terminus. *Tetrahedron Lett. 38*, 3–6.
66. J.P. Tam, Y.A. Lu, C.F. Liu and J. Shao (1995). Peptide synthesis using unprotected peptides through orthogonal coupling methods. *Proc. Natl. Acad. Sci. USA 92*, 12485–12489.
67. T.D. Pallin and J.P. Tam (1996). Assembly of cyclic peptide dendrimers from unprotected linear building blocks in aqueous solution. *J. Chem. Soc., Chem. Commun.*, 1345–1346.
68. L. Zhang and J.P. Tam (1997). Synthesis and application of unprotected cyclic peptides as building blocks for peptide dendrimers. *J. Am. Chem. Soc. 119*, 2363–2370.
69. J.C. Spetzler and J.P. Tam (1996). Self-assembly of cyclic peptides on a dendrimer: multiple cyclic antigen peptides. *Peptide Res. 9*, 290–296.
70. T.D. Pallin and J.P. Tam. Vaccine design: the orthogonal incorporation of cyclic peptides as multiple antigens attached to dendrimeric cores, in: *Peptides: Chemistry, Structure and Biology*, P.T.P. Kaumaya and R.S. Hodges, Eds, p. 853–854, Mayflower Scientific Ltd., Kingswinford, 1996.
71. T.D. Pallin and J.P. Tam (1995). Cyclisation of totally unprotected peptides in aqueous solution by oxime formation. *J. Chem. Soc., Chem. Commun.*, 2021–2022.
72. J.A. Camarero, G.J. Cotton, A. Adeva and T.W. Muir (1998). Chemical ligation of unprotected peptides directly from a solid support. *J. Peptide Res. 51*, 303–316.
73. V. Tsikaris, C. Sakarellos, M.T. Cung, M. Marraud and M. Sakarellos-Daitsiotis (1996). Concept and de-

sign of a new class of sequential oligopeptide carriers (SOC) for covalent attachment of multiple antigenic peptides. *Biopolymers 38*, 291–293.

74. V. Tsikaris, C. Sakarellos, M. Sakarellos-Daitsiotis, P. Orlewski, M. Marraud, M.T. Cung, E. Vatzaki and S. Tzartos (1996). Construction and application of a new class of sequential oligopeptide carriers (SOCn) for multiple anchoring of antigenic peptides — application to the acetylcholine receptor (AChR) main immunogenic region. *Int. J. Biol. Macromol. 19*, 195–205.
75. V.A. Bindra and A. Kuki (1994). Conformational preferences of oligopeptides rich in alpha-aminoisobutyric acid. III. Design, synthesis and hydrogen bonding in 3(10)-helices. *Int. J. Peptide Protein Res. 44*, 539–548.
76. C. Sakarellos, V. Tsikaris, S. Kosma, M. Sakarellos-Daitsiotis, E. Vatzaki, S.J. Tzaros, M.T. Cung and M. Marraud. A conformationally based rational design of multiple antigenic peptide carriers: the potential for disease treatment, in: *Peptides: Chemistry, Structure and Biology*, P.T.P. Kaumaya and R.S. Hodges, Eds, p. 816–817, Mayflower Scientific Ltd., Kingswinford, 1996.
77. V. Tsikaris, C. Sakarellos, M. Sakarellos-Daitsiotis, M.T. Cung, M. Marraud, G. Konidou, A. Tzinia and K.P. Soteriadou (1996). Use of sequential oligopeptide carriers (SOCn) in the design of potent *Leishmania* gp63 immunogenic peptides. *Peptide Res. 9*, 240–247.
78. R.A. Dwek (1996). Glycobiology: toward understanding the function of sugars. *Chem. Rev. 96*, 683–720.
79. A. Varki (1993). Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology 3*, 97–130.
80. K. Drickamer and M.E. Taylor (1993). Biology of animal lectins. *Annu. Rev. Cell. Biol. 9*, 237–264.
81. T. Feizi (1985). Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. *Nature 314*, 53–57.
82. S. Hakomori (1989). Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. *Adv. Cancer Res. 52*, 257–331.
83. M. Fukuda (1985). Cell surface glycoconjugates as onco-differentiation markers in hematopoietic cells. *Biochim. Biophys. Acta 780*, 119–150.
84. G. Walz, A. Aruffo, W. Kolanus, M. Bevilacqua and B. Seed (1990). Recognition by ELAM-1 of the Sialyl-Le^x determinant on myeloid and tumour cells. *Science 250*, 1132–1135.
85. M.P. Bevilacqua, J.S. Pober, D.L. Mendrick, R.S. Cotran and M.A. Gimbrone (1987). Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc. Natl. Acad. Sci. USA 84*, 9238–9242.
86. G.F. Springer (1984). T and Tn, general carcinoma autoantigens. *Science 224*, 1198–1206.
87. S.I. Hakomori (1991). Possible functions of tumor-associated carbohydrate antigens. *Curr. Opin. Immunol. 3*, 646–653.
88. J. Montreuil (1980). Primary structure of glycoprotein glycans: basis for the molecular biology of glycoproteins. *Adv. Carbohydr. Chem. Biochem. 37*, 157–223.
89. M.J. Horowitz and W. Pigman. *The Glycoconjugates*, vol. I–IV, Academic Press, New York, 1997.
90. H. Lis and N. Sharon (1993). Protein glycosylation. Structural and functional aspects. *Eur. J. Biochem. 218*, 1–27.
91. H. Paulsen (1990). Syntheses, conformations and X-ray structure analyses of the saccharide chains from the core regions of glycoproteins. *Angew. Chem. Int. Ed. Engl. 29*, 823–839.
92. Y. Ichikawa, G.C. Look and C.H. Wong (1992). Enzyme-catalyzed oligosaccharide synthesis. *Anal. Biochem. 202*, 215–238.
93. H. Kunz (1987). Synthesis of glycopeptides, partial structures of biological recognition components. *Angew. Chem. Int. Ed. Engl. 26*, 294–308.
94. D. Qiu and R.R. Koganty (1997). Mucin type glycopeptides: synthesis of Core 2, Core 6, and F1-α building blocks and some unexpected reactions. *Tetrahedron Lett. 38*, 45–48.
95. I. Matsuo, H. Fujimoto, M. Isomura and K. Ajisaka (1997). Chemoenzymatic synthesis of Galα1-3Gal, Galα1-3Galβ1-4GlcNAc, and their PEG-conjugates. *Bioorg. Med. Chem. Lett. 7*, 255–258.
96. R. Roy (1998). The chemistry of neoglycoconjugates. *Carbohydr. Chem.*, 243–321.
97. D.R. Bundle and N.M. Young (1992). Carbohydrate–protein interactions in antibodies and lectins. *Curr. Opin. Struct. Biol. 2*, 666–673.
98. K. Ozaki, R.T. Lee, Y.C. Lee and T. Kawasaki (1995). The differences in structural specificity for recognition and binding between asialoglycoprotein receptors of liver and macrophages. *Glycoconj. J. 12*, 268–274.
99. S. Aravind, W.K.C. Park, S. Brochu and R.Roy (1994). Single step syntheses of lactosylated clusters by telomerizations. *Tetrahedron Lett. 35*, 7739–7742.
100. R.T. Lee and Y.C. Lee. Enhanced biochemical affinities of multivalent neoglycoconjugates, in: *Neoglycoconjugates: Preparation and Applications*, Y.C. Lee and R.T. Lee, Eds, p. 23–57, Academic Press, San Diego, 1994.
101. R. Roy. Design and Syntheses of Glycoconjugates, in: *Modern Methods in Carbohydrate Synthesis*, S.-H. Khan and R.O. Neil, Eds, p. 378–415, Harwood Academic, Amsterdam, Netherlands, 1995.
102. R. Roy (1996). Glycodendrimers: a new class of biopolymers. *Polymer News 21*, 226–232.
103. R. Roy, D. Zanini, S.J. Meunier and A. Romanowska (1993). Solid-phase synthesis of dendritic sialoside inhibitors of influenza A virus haemagglutinin. *J. Chem. Soc., Chem. Commun.*, 1869–1872.
104. D. Zanini and R. Roy (1996). Novel dendritic α-sialosides: synthesis of glycodendrimers based on a 3,3′-iminobis(propylamine) core. *J. Org. Chem. 61*, 7348–7354.

105. C. Kieburg and T.K. Lindhorst (1997). Glycodendrimer synthesis without using protecting groups. *Tetrahedron Lett. 38*, 3885–3888.
106. P.R. Ashton, S.E. Boyd, C.L. Brown, N. Jayaraman and J.F. Stoddart (1997). A convergent synthesis of a carbohydrate containing dendrimer. *Angew. Chem. Int. Ed. Engl. 36*, 732–735.
107. T.K. Lindhorst and C. Kieburg (1996). Glycocoating of oligovalent amines: synthesis of thiourea-bridged cluster glycosides from glycosyl isothiocyanates. *Angew. Chem. Int. Ed. Engl. 35*, 1953–1956.
108. D. Zanini, W.K.C. Park and R. Roy (1995). Synthesis of novel dendritic glycosides. *Tetrahedron Lett. 36*, 7383–7386.
109. R. Roy, F.O. Andersson, G. Harms, S. Klem and R. Schauer (1992). Synthesis of esterase-resistant 9-*O*-acetylated polysialoside as inhibitor of influenza C virus hemagglutinin. *Angew. Chem. Int. Ed. Eng. 31*, 1478–1481.
110. D. Pagé, D. Zanini and R. Roy (1996). Macromolecular recognition: effect of multivalency in the inhibition of binding of yeast mannan to concanavalin A and pea lectins by mannosylated dendrimers. *Bioorg. Med. Chem. 4*, 1949–1961.
111. D. Zanini and R. Roy (1997). Synthesis of new α-thiosialodendrimers and their binding properties to the sialic acid specific lectin from *Limax flavus*. *J. Am. Chem. Soc. 119*, 2088–2095.
112. S. Hanessian, D. Qiu, H. Prabhanjan, G.V. Reddy and B. Lou (1996). Synthesis of clustered D-GalNAc (Tn) and D-Glaβ(1→3)GalNAc (T) antigenic motifs using a pentaerythritol scaffold. *Can. J. Chem. 74*, 1738–1747.
113. E. Perez-Paya, R.A. Houghten and S.E. Blondelle (1994). Synthetic peptides as binding-step based catalytic mimics. *Peptide Res. 7*, 286–288.
114. T.E. Creihton (1988). Toward a better understanding of protein folding pathways. *Proc. Natl. Acad. Aci. USA 85*, 5082–5086.
115. R.L. Baldwin (1989). How does protein folding get started? *Trends Biochem. Sci. 14*, 291–294.
116. M. Mutter and S. Vuilleumier (1989). A chemical approach to protein design — template-assembled synthetic proteins (TASP). *Angew. Chem. Int. Ed. Engl. 28*, 535–554.
117. K. Johnsson, R.K. Allemann, H. Widmer and S.A. Benner (1993). Synthesis, structure and activity of artificial, rationally designed catalytic polypeptides. *Nature 365*, 530–532.
118. K. Severin, D.H. Lee, A.J. Kennan and M.R. Ghadiri (1997). A synthetic peptide ligase. *Nature 389*, 706–709.
119. S. Abd El Rahman, H. Anzinger and M. Mutter (1980). Relationship between conformation and physicochemical properties of polypeptides. I. Synthesis of homo- and co-oligopeptides by the liquid phase method. *Biopolymers 19*, 173–187.
120. C. Toniolo, G.M. Bonora M. Mutter and V.N.R. Pialli (1981). Linear oligopeptides. 78. Effect of the insertion of a proline residue on the conformation of host peptides. *Makromol. Chem. 182*, 2007–2014.
121. K-.H. Altmann, E. Altmann and M. Mutter (1992). 93. Conformational studies on peptides containing enantiomeric α-methyl α-amino acids. Part I. Differential conformational properties of (R) and (S)-2-methyl aspartic acid. *Helv. Chim. Acta 75*, 1198–1210.
122. V.A. Bindra and A. Kuki (1994). Conformational preferences of oligopeptides rich in α-aminoisobutyric acid. III. Design, synthesis and hydrogen bonding in 3_{10}-helices. *Int. J. Peptide Protein Res. 44*, 539–548.
123. M.R. Ghadiri and C. Choi (1990). Secondary structure nucleation in peptides. Transition metal ion stabilized α-helices. *J. Am. Chem. Soc. 112*, 1630–1632.
124. M.A. Holmes and B.W. Matthews (1980). Structure of thermolysin refined at 1.6 Å resolution. *J. Mol. Biol 160*, 623–639.
125. W.P. Gaykema, A. Volbeda and W.G. Hol (1986). Structure determination of *Panulirus interruptus* haemocyanin at 3.2 Å resolution. Successful phase extension by six-fold density averaging. *J. Mol. Biol 187*, 255–275.
126. D.S. Kemp (1990). Peptidomimetics and the template approach to nucleation of β-sheets and α-helices in peptides. *Trends Biotechnol. 8*, 249–255.
127. M.R. Ghadiri, C. Soares and C. Choi (1992). A convergent approach to protein design. Metal ion-assisted spontaneous self-assembly of a polypeptide into a triple-helix bundle protein. *J. Am. Chem. Soc. 114*, 825–831.
128. K.S. Ackerfeldt, R.M. Kim, D. Camac, J.T. Groves, J.D. Lear and W.F. DeGrado (1992). Tetraphilin: a four-helix proton channel built on a tetraphenylporphyrin framework. *J. Am. Chem. Soc. 114*, 9656–9657.
129. M. Lieberman and T. Sasaki (1991). Iron(II) organizes a synthetic peptide into three-helix bundles. *J. Am. Chem. Soc. 113*, 1470–1471.
130. M.R. Ghadiri, C. Soares and C. Choi (1992). Design of an artificial four-helix bundle metalloprotein via a novel ruthenium(II)-assisted self-assembly process. *J. Am. Chem. Soc. 114*, 4000–4002.
131. C.T. Choma, J.D. Lear, M.J. Nelson, P.L. Dutton, D.E. Robertson and W.F. DeGrado (1994). Design of a heme-binding four-helix bundle. *J. Am. Chem. Soc. 116*, 856–865.
132. D.E. Robertson, R.S. Farid, C.C. Moser, J.L. Urbauer, S.E. Mulholland, R. Pidikiti, J.D. Lear, A.J. Wand, W.F. DeGrado and P.D. Dutton (1994). Design and synthesis of multi-haem proteins. *Nature 368*, 425–432.
133. H. Diaz, J.R. Espina and J.W. Kelly (1992). A dibenzofuran-based amino acid designed to nuleate antiparallel β-sheet structure: evidence for intramolecular hydrogen-bond formation. *J. Am. Chem. Soc. 114*, 8316–8318.

134. T. Sasaki and E.T Kaiser (1989). Helichrome: synthesis and enzymatic activity of a designed hemeprotein. *J. Am. Chem. Soc. 111*, 380–381.
135. M. Mutter, E. Altmann, K.-H. Altmann, R. Hersperger, P. Koziej, K. Nebel, G. Tuchscherer and S. Vuilleumier (1988). 94. The construction of new proteins. Part III. Artificial folding units by assembly of amphiphilic secondary structures on a template. *Helv. Chim. Acta 71*, 835–847.
136. M. Mutter. Synthetic protein with a new three-dimensional architecture, in: *Peptides. Chemistry and Biology*, G.R. Marshall, Ed., p. 349–353, ESCOM, Leiden, 1988.
137. J.S. Richardson and D.C. Richardson (1989). The *de novo* design of protein structures. *Trends Biochem. Sci. 14*, 304–309.
138. R. Floegel and M. Mutter (1992). Molecular dynamics conformational search of six cyclic peptides used in the template assembled synthetic protein approach for protein *de novo* design. *Biopolymers 32*, 1283–1310.
139. P.E. Dawson and S.B.H. Kent (1993). Convenient total synthesis of a 4-helix TASP molecule by the chemoselective ligation. *J.* Am. *Chem. Soc. 115*, 7263–7266.
140. G. Tuchscherer, B. Dorner, U. Sila, B. Kamber and M. Mutter (1993). The TASP concept: mimetics of peptide ligands, protein surfaces and folding units. *Tetrahedron 49*, 3559–3575.
141. M. Mutter, G. Tuchscherer, C. Miller, K.-H. Altmann, R.I. Carey, D.F. Wyss, A.M. Labhardt and J.E. Rivier (1992). Template-assembled synthetic proteins with four-helix-bundle topology. Total chemical synthesis and conformational studies. *J. Am. Chem. Soc. 114*, 1463–1470.
142. M. Mutter, P. Dumy, P. Garrouste, C. Lehmann, M. Mathieu, C. Peggion, S. Peluso, A. Razaname and G. Tuchscherer (1996). Template assembled synthetic proteins (TASP) as functional mimetics of proteins. *Angew. Chem. Int. Ed. Engl. 35*, 1482–1485.
143. I. Ernest, S. Vuilleumier, H. Fritz and M. Mutter (1990). Synthesis of a 4-helix bundle-like template-assembled synthetic protein (TASP) by condensation of a protected peptide on a conformationally constrained cyclic carrier. *Tetrahedron Lett. 31*, 4015–4018.
144. I. Ernest, J. Kalvoda, C. Sigel, G. Rihs, H. Fritz, M.J.J. Blommers, F. Raschdorf, E. Francotte and M. Mutter (1993). Template-assembled synthetic proteins (TASP). Cyclic templates with incorporated turn-inducing mimics. *Helv. Chim. Acta 76*, 1539–1563.
145. G. Tuchscherer (1993). Template assembled synthetic proteins: condensation of a multifunctional peptide to a topological template via chemoselective ligation. *Tetrahedron Lett. 34*, 8419–8422.
146. M. Mutter and S. Vuilleumier (1989). A chemical method for new proteins. Template associated synthetic proteins (TASP). *Angew. Chem. 101*, 551–592.
147. M. Mutter, R. Hersperger, K. Gubernator and K. Muller (1989). The construction of new proteins: V. A template-assembled synthetic protein (TASP) containing both a 4-helix bundle and beta-barrel-like structure. *Proteins 5*, 13–21.
148. P.T. Kaumaya, A.M. VanBuskirk, E. Goldberg and S.K. Pierce (1992). Design and immunological properties of topographic immunogenic determinants of a protein antigen (LDH-C4) as vaccines. *J. Biol. Chem. 267*, 6338–6346.
149. P. Dumy, I.M. Eggleston, S. Cervigni, U. Sila, X. Sun and M. Mutter (1995). A convenient synthesis of cyclic peptides as regioselectively addressable functionalized templates (RAFT). *Tetrahedron Lett. 36*, 1255–1258.
150. C. Callebaut, E. Jacotot, G. Guichard, B. Krust, M. Rey-Cuille, D. Cointe, N. Benkirane, J. Blanco, S. Muller, J. Briand and A.G. Hovanessian (1996). Inhibition of HIV infection by pseudopeptides blocking viral envelope glycoprotein-mediated membrane fusion and cell death. *Virology 218*, 181–192.
151. C. Callebaut, E. Jacotot, B. Krust, G. Guichard, J. Blanco, A. Valenzuela, J. Svab, S. Muller, J.P. Briand and A.G. Hovanessian (1997). Pseudopeptide TASP inhibitors of HIV entry bind specifically to a 95-kDa cell surface protein. *J. Biol. Chem. 272*, 7159–7166.
152. S.E. Cervigni, P. Dumy. P.T.P. Kaumaya, M. Mathieu, O. Nyanguile, C. Peggion, A. Razaname, G. Tuchscherer and M. Mutter. Template-assisted protein design: chimeric TASP by chemoselective ligation, in: *Peptides, Chemistry, Structure and Biology*, P.T.P. Kaumaya and R.S. Hodges, Eds, p. 555–557, Mayflower Scientific Ltd., Kingswinford, 1996.
153. P. Dumy, I.M. Eggleston, G. Esposito, S. Nicula and M. Mutter. Conformational studies of RAFT molecules for protein design, in: *Peptides: Chemistry, Structure and Biology*, P.T.P. Kaumaya and R.S. Hodges, Eds, p. 605–606, Mayflower Scientific Ltd., Kingswinford, 1996.
154. P. Dumy, I.M. Eggleston, G. Esposito, S. Nicula and M. Mutter (1996). Solution structure of regioselectively addressable functionalized templates: an NMR and restrained molecular dynamics investigation. *Biopolymers 39*, 297–308.

