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Review

Carbohydrates as templates for control of distance-geometry in de novo-designed proteins

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Abstract. An understanding of very complex natural systems can often only be achieved through detailed studies of systems with a reduced complexity. Thus, de novo design of proteins allows the study of fundamental forces determining protein folding and stability, as well as protein-protein interactions, by analyses of protein models of structural motifs. In addition, de novo design may lead to new biomimetic molecules with novel properties. In a synthetic approach to achieve structural economy, rigid templates, sometimes called topological scaffolds, have been used to connect secondary-structure elements, most notably α-helices. By positioning the helices on the template, the unfavorable entropy of protein folding is reduced. In a novel class of chimeric molecules called carboproteins, carbohydrates are used as templates for de novo design of protein models. Recently, a strategy relying on chemoselective ligation of C-terminal peptide aldehydes to tetra-aminooxy functionalized monosaccharides has provided 7-kDa 4-α-helix bundle carboproteins.

Key words. De novo design; template-assembled synthetic protein (TASP); carboprotein; 4-α-helix bundle; chemical synthesis; carbohydrate template; chemoselective ligation; peptide aldehyde.

Introduction

During the protein-folding process, a linear polypeptide chain goes from a random-coil conformation into a fully folded and unique three-dimensional structure. 'The protein folding problem,' i.e., how the primary structure of a protein determines its tertiary structure, continues to be the major unsolved problem in structural molecular biology [1; ref. 2 pp. 347–371]. Despite very significant advances, successfully predicting the three-dimensional geometry of a protein from its amino acid sequence is still not *generally* possible, although good approximations can be reached for certain motifs. Predictive methods start from the prediction of secondary structure, i.e., which amino acid residues have a tendency to induce α-helix or β-strand formation in peptides. Although the problem of predicting the secondary structure has not been solved *in general*, which would allow predictions for different types of proteins with a high degree of confidence, predictions are rather reliable for transmembrane helices and α-helical coiled coils [ref. 2 pp. 347–371]. Once the secondary structure is known, or has been predicted correctly, knowledge of the rules that govern the packing of the secondary structural elements against each other can be used to predict which globular folds are formed. The rules for packings to form helical bundles are relatively well understood.

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The folding of peptides into an α-helical conformation relies on hydrogen bonding between backbone COs in *i* positions and the NHs in *i*+4 positions. Amphiphilic α-helices are made of 'heptad repeat' elements, also referred to as 4–3 hydrophobic repeats [3], with the general sequence $(abcdefg)_n$. With hydrophobic residues on one face of the helix and hydrophilic residues on the other face, amphiphilic α-helices can form coiled-coil structures or helix bundles, of which the most prominent are the 4-α-helix bundles. Loop regions connect the α-helical segments; four appropriately positioned amphiphilic α helices can undergo a 'hydrophobic collapse,' expelling the water in between their hydrophobic faces, to form a 4-α-helix bundle. This gives a separation of immiscible phases into distinct domains, which is frequently a 'structural imperative' and thus a driving force for the formation of defined structures in biology [3]. The α-helices can be aligned with anti-parallel or, less common, parallel directions. The residues in the heptad repeat can be divided into three categories, core, boundary, or surface, with *a* and *d* residues being in core, *e* and *g* being in boundary, and *b*, *c*, and *f* being in surface positions. Whereas core residues are predominantly hydrophobic (often Leu), boundary residues are frequently charged (often Lys and Glu) and participate in intra- and interhelical stabilization through formation of salt bridges. Residues on the surface are in general hydrophilic, but variations are allowed in these positions. Furthermore, as the side chains of residues in α-helices are arranged in helical rows, the packing of core side chains becomes important when the helices aggregate in a bundle. The fitting motifs have been described in terms of 'ridges in grooves' or 'knobs in holes' patterns. In 4-α-helix bundles, the most common 'ridges in grooves' packing mode results in an interhelical crossover angle of about 20º [4; ref. 2 pp. 40–41].

De novo protein design has emerged as a valuable tool to critically test our understanding of protein folding and structure [5]. Here, the details of the folding and function of a protein are being confronted. Also, the very complex interactions in natural proteins can be studied in greater detail in smaller systems [6]. De novo design may eventually lead to new biomimetic molecules with novel properties. Over the last decade, DeGrado, Pavone and others [5] have explored the properties of de novo-designed zinc-, mercury-, iron/sulfur-, and heme-binding proteins, and Baltzer and coworkers [7, 8] have made significant progress in the design of 4-α-helix bundles with reactive sites for esterase activity. An important step towards practical applications of de novo design was taken when Kim and coworkers very recently reported a strategy to prepare a 5-α-helix bundle as a novel type of inhibitor of HIV cell membrane fusion [9]. This impressive feat was made possible by the progress in de novo design of proteins and the improved understanding of the factors covering protein stability and structure.

Template-assisted protein design

The entropic cost of folding a protein into a single native state increases with the number of unfolded conformations. Thus, if the number of unfolded conformations can be reduced, the stability of the native state is increased [ref. 2 pp. 354–355]. Interestingly, van Gunsteren and coworkers have recently argued that a better understanding of the conformers making up the denatured state of a protein is a key to solving the 'protein-folding problem' [10]. By far the most common way to reduce the number of unfolded conformations in natural proteins by covalent bonds is to introduce suitably located disulfide bridges. However, protein scaffolds, the part that provides the three-dimensional structure, often seem larger than they need to be to position the functional moieties of the molecule. In the last decade, the question of how small a protein can be, while still retaining its function, has been addressed experimentally. This can be viewed as a question of *structural economy*, exemplified by Dutton's molecular 'maquettes' [11, 12] and Pavone's miniaturized hemoproteins or 'mimochromes' [13].

In a different approach to achieve structural economy, rigid templates or topological scaffolds have been used to connect secondary-structure elements, most notably α-helices, to 'force' the helices into juxtaposition, reducing the number of unfolded conformations, and thus overcoming some of the unfavorable entropy of protein folding. Mutter and coworkers [14, 15] have suggested a 'template-assembled synthetic protein' (TASP) concept which relies on a multifunctional topological carrier molecule, which directs the spatial accommodation of the peptides to form a well-defined tertiary structure (fig. 1). Predominantly peptide templates have been explored by the group of Mutter, including a number of linear oligopeptides with Pro-Gly β turns [14], a decapeptide sequence, cyclized by a disulfide bridge between a C- and an N-terminal Cys residue [16], as well as a number of cyclic decapeptides, referred to as 'regioselectively addressable functionalized templates' (RAFTs) [17]. Similar cyclic decapeptide templates have been used by

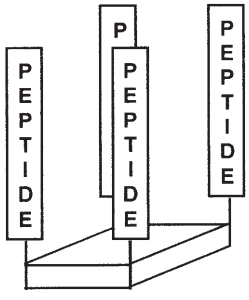


Figure 1. Schematic illustration of the TASP concept. A protein model is assembled by positioning peptides with secondary-structure elements on a rigid template.

Haehnel and coworkers in the synthesis of cytochrome b models [18, 19], as well as in the recent combinatorial synthesis of 4-α-helix bundle hemo- [20] and copper proteins [21]. The copper protein synthesis is an example of true de novo design, as natural soluble monocopper proteins consist largely of β-sheets.

Other research groups have applied the TASP concept to non-peptide templates. Sasaki and Kaiser [22] reported the synthesis of 'Helichrome,' a 4-α-helix bundle TASP based on a coproporphyrin template, while DeGrado and coworkers [23] synthesized a similar 4-α-helix bundle, 'Tetraphilin,' on a tetraphenylporphyrin template. More recently, Fairlie and coworkers applied a number of aromatic scaffolds in the synthesis of 4-α-helix bundle TASPs [24], while Sherman and coworkers [25, 26] have reported the assembly of 4-α-helix bundles on cyclic tetrathiophenol macrocycles. Furthermore, Goodman and coworkers have used Kemp's triacid as a template for the assembly of 3-α-helix collagen models [27], while Ghadiri and coworkers have used complexation with metal ions to form both 3- and 4-α-helix bundles [28–30].

These examples from the literature illustrate the main advantage of the synthetic TASP strategy, a bypassing of the problematic folding step of linear polypeptides, thereby allowing more freedom in the sequence design for the construction of small structures with the desired properties. The constructs have served as models for understanding natural proteins, their folding and stability, and recently also function. Functional TASP structures have focused on ion channeling and redox properties. DeGrado's 'Tetraphilin' served as a proton channel [23], while later studies have included a 4-α-helix bundle melittin model [31], and recently a 5-α-helix bundle ion channel [32]. The function of redox and electron-transporting proteins has been mimicked by the heme-binding TASPs prepared by Haehnel [19], as well as by Ghadiri's metal-coordinated structures [30].

Chemoselective ligation of unprotected peptides

One of the most important advances in chemical peptide and protein synthesis over the past decade has been the development of efficient techniques for chemoselective ligation of unprotected peptide segments. It has significantly extended the reach of synthetic peptide chemistry to allow the reliable synthesis of small proteins. Common to these methods is the chemoselective reaction of two mutually reactive functionalities, one on each segment.

Some methods rely on the formation of a non-natural (i.e., other than amide) bond at the site of ligation, such as the oxime and hydrazone ligations, developed by Rose [33], in which aminooxy or hydrazine nucleophiles react with aldehydes or ketones. Due to the α-effect of the neighboring heteroatom, aminooxy derivatives are weak bases but reactive nucleophiles toward carbonyl groups; this is especially favorable at an acidic pH range of 4–5.5 at which basic side chain nucleophiles are protonated. Oximes are stable under neutral to mildly acidic conditions [34]. Other chemoselective reactions are thioether and thioester ligations, in which a thiol or thioacid nucleophile, respectively, react with a bromoacetyl moiety. A thioether linkage is also the outcome of the selective reaction of a Cys thiol with a maleimidopropionyl (Mp) group. By Michael addition to the pyrrole-2,5-dione ring, the Mp group is converted to a 3-succinimidopropionyl (Sp) group. Finally, a ligation strategy relying on a Pd(0)-catalyzed Sonogashira coupling in water has recently been reported [35]. Peptides functionalized with a terminal 4-iodobenzoyl moiety were ligated to a terminal alkyne compound in aqueous solution in good yields.

Among the methods for establishing an amide bond at the site of ligation, the so-called 'native chemical ligation' is the most popular. First published by Kent and coworkers, the strategy relies on a thiol capture reaction between a thioester and an N-terminal Cys residue [36]. The method was later extended to other N-terminal amino acids [37, 38]. Furthermore, Liu and Tam [39] have demonstrated that an N-terminal Cys can be used for chemoselective ligation to a C-terminal glycolaldehyde moiety, forming a thiazolidine ring at the site of ligation. The 5-membered thiazolidine ring mimics a Pro residue. Finally, the chemoselective reaction between a phosphinothioester and an azido group has very recently been reported to ligate peptide strands, resulting in an amide bond via the Staudinger reaction [40].

A number of 4-α-helix bundle TASPs have been assembled via these chemical ligation strategies. Dawson and Kent [41] functionalized one of Mutter's linear peptide templates with bromoacetyl groups in a thioester ligation to peptides with a C-terminal Gly-$^{\alpha}$COSH residue. Rau and Haehnel applied the thioether chemistry in their synthesis of 4-α-helix bundles, initially by ligation of peptides modified with a bromoacetyl moiety at an N- or C-terminal Lys residue to four Cys residues in the template sequence [18], later by ligation of the template Cys residues to N- or C-terminally maleimidopropionyl functionalized peptides [19–21]. The oxime ligation strategy was applied by Tuchscherer and coworkers [42] to an open-chain anti-parallel β sheet-type template, functionalized with aminooxyacetic acid. An amino acid derivative with a diethyl acetal-protected aldehyde moiety was incorporated at the C terminal of one peptide sequence, and at the N terminal of another, and an anti-parallel 4-α-helix bundle was assembled in solution after acetal deprotection.

Why use carbohydrates as templates? The carboprotein concept

We reasoned that carbohydrates would be promising candidates for templates, as monosaccharides are polyfunctional molecules, pyranose ring forms are relatively rigid, and epimers of sugars are often accessible. Furthermore, a vast literature describes the regiospecific manipulation of their functional groups. Using the primary and secondary hydroxyls of mono- or disaccharides should provide flexible control of the directionality and distances between anchoring points for peptide chains in de novo design of protein models. Our resulting chimeric polypeptide-carbohydrate constructs combine two of nature's ubiquitous constituents in a novel way, using carbohydrates as natural, polyfunctional templates to aid in the construction of proteins. Depending on their size and degree of folding, we proposed the terms *carbopeptides* and *carboproteins* for the members of this class of chimeras. The terms were chosen to differentiate these structures from naturally occurring and synthetic glycopeptides, glycoproteins, proteoglycans, and synthetic neoglycoproteins, as well as Nicolaou's carbopeptoids [43] and Lindhorst's octopus glycosides [44].

The reports by Hirschmann, Nicolaou, Smith, and coworkers on the use of monosaccharides as templates for the design of small non-peptidyl peptidomimetics were an inspiration to the original carboprotein concept [45, 46]. Whereas modified monosaccharides have also been used by Kessler as dipeptide isosteres [47], Leu-enkephalin has been coupled to a cyclodextrin derivative [48], and several groups have used anchoring of peptide chains through the modified O-6 of cyclodextrin scaffolds as a means to improve the pharmacological properties of peptides [49, 50], to aid in drug targeting [51], or to allow molecular sensing [52], the large synthetic potential of carbohydrates had apparently not been exploited for the de novo design of proteins.

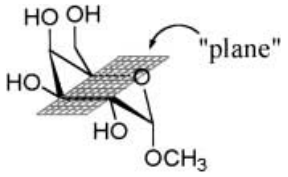


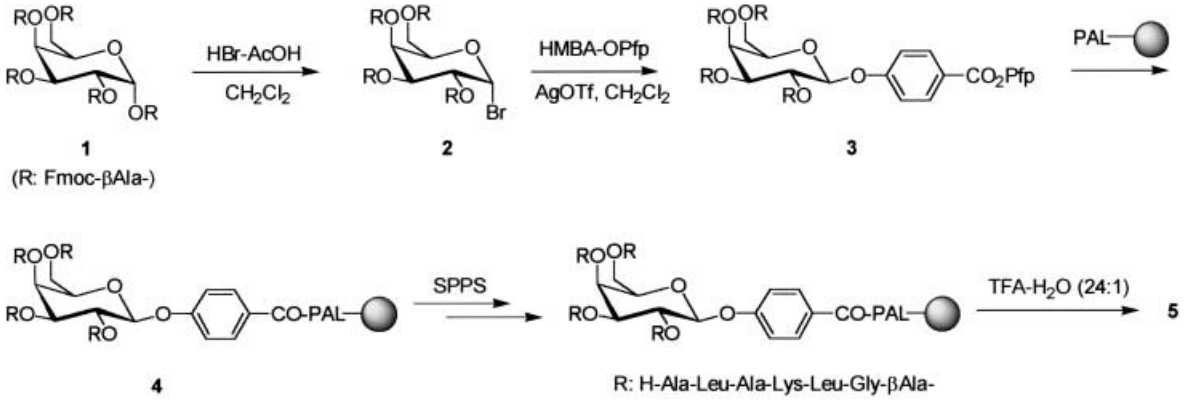
Figure 2. Methyl α-D-Gal*p* shown in $^4C_1$ conformation. All hydroxyl groups are in or above a plane defined by C-2, C-3, C-5, and O-5. The equatorial hydroxyls on C-2 and C-3, and the hydroxymethyl on C-5 are considered to be approximately in the plane, while the axial hydroxyl on C-4 is above the plane.

In our first approaches, we employed a methyl α-D-galactopyranoside (Gal*p*) derivative as template, as all non-anomeric hydroxyls are in or above a plane defined by C-2, C-3, C-5, and O-5 (fig. 2). This design strategy left the anomeric position available for selective functionalization.

The first generation

In the first implementation of this concept, a solid-phase synthetic strategy relying on the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group was employed [53]. In this strategy, the monosaccharide was per-*O*-acylated with four Fmoc-βAla-OH moieties and linked through C-1 to a solid support, following which the peptides were assembled on all four 'arms' (scheme 1). The approach was chosen after initial unsuccessful experiments with per-*O*-acylation of the resin-bound carbohydrate.

The starting monosaccharide D-galactose was converted to (Fmoc-βAla)$_5$-α-D-Gal*p* (**1**) by per-*O*-acylation with (Fmoc-βAla)$_2$O in CH_2Cl_2-pyridine (1:1) in the presence of 4-dimethylaminopyridine (DMAP). Treatment of **1** with 30% HBr-HOAc in CH_2Cl_2 provided α-galactosyl bromide (**2**), which upon reaction with 4-hydroxymethylbenzoic acid pentafluorophenyl ester (HMBA-OPfp) in



Scheme 1. Synthesis of the first-generation carboprotein **5** by simultaneous assembly of four peptide strands on a resin-bound Gal*p* template.

CH_2Cl_2 in the presence of silver triflate gave (Fmoc-βAla)$_4$-β-D-Gal*p*-(1-*O*)-MBA-OPfp (**3**), in 45% purified yield. Due to its scarcity, the glycosyl bromide was used as the limiting reagent in these glycosylations. Template **3** showed a three-bond scalar coupling constant $^3J_{1,2}$ of 8.1 Hz indicative of a *diaxial* configuration of the coupling protons in β-D-Gal*p* in the $^4C_1$ conformation. Thus, we concluded that the hydroxyls were properly positioned for the envisaged application. The carbohydrate moieties of carbopeptides and -proteins could potentially serve as structural markers for conformational changes caused by aggregating peptide chains in a helix bundle, i.e., distortion of the pyranoside $^4C_1$ conformation could reflect conformational changes in the peptide chains.

Next, template **3** was coupled smoothly to an acidolyzable PAL-PEG-PS [54, 55] support to give tetra-Fmoc-protected template resin **4**. Simultaneous sequential peptide chain elongation on all four 'arms' of the support-bound template by consecutive Fmoc removal and coupling of Fmoc-protected amino acids (Fmoc-AA-OH), followed by a final cleavage with TFA-H_2O (24:1) gave the peptidyl-template (H-Ala-Leu-Ala-Lys-Leu-Gly-βAla)$_4$-β-D-Gal*p*-(1-*O*)-MBA-NH_2 (**5**), the *first* carbopeptide. The crude product was evaluated by analytical C18 reversed-phase high-performance liquid chronatography (RP-HPLC) and shown to be >70% pure. Direct infusion electrospray ionization mass spectroscopy (ESI MS) of crude **5** gave the expected mass.

The second generation

The carbopeptide concept was extended in a second-generation approach with the goal to prepare protein-sized structures, starting with the relatively well-understood 4-α-helix bundles [56, 57]. Inspired by the progress in chemoselective ligation chemistry, a convergent modular synthesis strategy was adopted to increase the freedom of the design. Peptides and template were accordingly prepared and purified prior to a final coupling step. Not only should this facilitate preparation of the large structures in good purity, the modular approach would, ultimately, also allow attachment of non-identical peptides and non-parallel helices to the template. Oxime ligation [33] was chosen as the key reaction to combine the building blocks. In our experience, this reaction is robust, fast, and high yielding. Reaction conditions are very mild and without complications, unlike the disulfide formation often seen in thiol-based ligation chemistries. Furthermore, one of us recently developed a new method for the synthesis of C-terminal peptide aldehydes using a backbone amide linker (BAL) strategy [58, 59]. From long peptide aldehydes, a second-generation carboprotein could be assembled *via* ligation to an aminooxy-functionalized carbohydrate template (fig. 3).

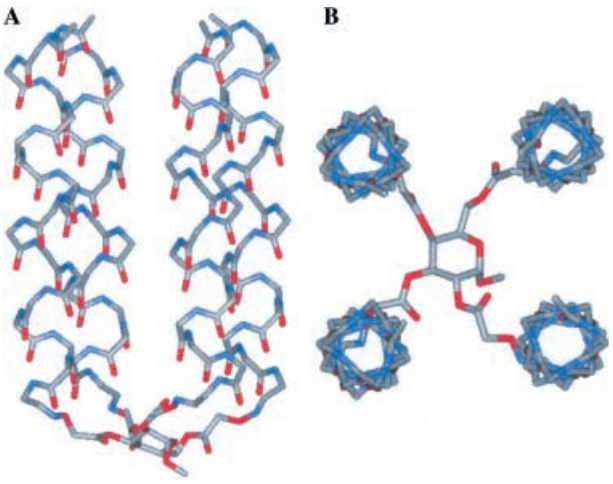


Figure 3. Structure of a 4-α-helix bundle carboprotein, side (*A*) and top (*B*) view. The template is aminooxyacetyl-functionalized methyl α-D-Gal*p*. Peptide strands each with 16 residues (of which only the backbone is shown) are attached with *E*-oxime linkages. This is a simplified illustration, as the peptide stands are aligned in parallel (i.e., no interhelical crossover) and locked in α-helical conformation, with only the template structure being energy minimized (Tripos force field). Generated with Sybyl 6.

We initially envisioned accessing the template by per-*O*-acylation of methyl α-D-Gal*p* with commercially available tert-butyloxycarbonyl (Boc)-protected aminooxyacetic acid (Boc-Aoa-OH), followed by acidic removal of the Boc groups. However, the reaction, in which the acid was activated with *N,N′*-diisopropylcarbodiimide (DIPCDI) in the presence of catalytic DMAP, yielded a mixture of compounds. The major components of the mixture were identified as methyl (Boc-Aoa)$_5$-α-D-Gal*p* and methyl (Boc-Aoa)$_6$-α-D-Gal*p* arising from N-acylation of the desired tetra-*O*-acyl template. The problem was obviously insufficient protection of the α-nucleophilic nitrogen in Boc-Aoa-OH, a problem previously noticed by Mutter and coworkers [60]. While they circumvented the problem using trityl-protected aminooxyacetic acid (Trt-Aoa-OH), we explored the possibility of attaching a second Boc group to the nitrogen in Boc-Aoa-OH. Boc groups are advantageous, as normally no purification step is required after deprotection and precedents existed for *N,N*-di-Boc protection of amino acids [61]. Accordingly, Boc_2-Aoa-OH was synthesized in high yield from Boc-Aoa-OH in three facile steps with transient protection of the carboxylic acid as the benzyl ester and incorporation of the second Boc group in the presence of DMAP [56].

With Boc_2-Aoa-OH in hand, the protected template was readily prepared in good yield by DIPCDI/DMAP-mediated coupling in pyridine-CH_2Cl_2 (1:1); gratifyingly, final removal of all eight Boc groups with TFA-CH_2Cl_2 (1:1) proved to be fast and quantitative to provide the desired compound, methyl 2,3,4,6-tetra-*O*-Aoa-α-D-Gal*p* (**6**) (fig. 4) [56]. This tetra-functionalized template should

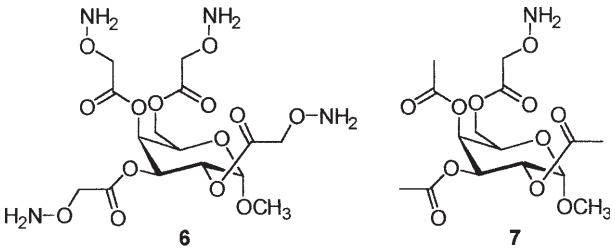
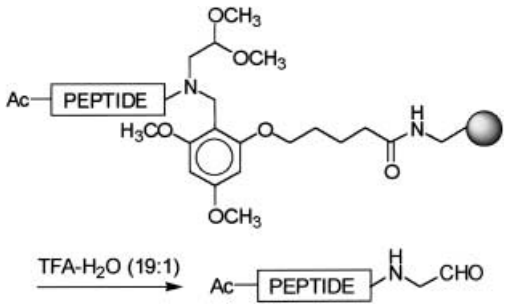


Figure 4. The templates methyl 2,3,4,6-tetra-*O*-Aoa-α-D-Gal*p* (**6**) and methyl 6-*O*-Aoa-2,3,4-tri-*O*-acetyl-α-D-Gal*p* (**7**).



Scheme 2. Synthesis of a C-terminal peptide aldehyde by acidolytic cleavage from the o-BAL handle.

give access to four-stranded carbopeptides and ultimately 4-α-helix bundle carboproteins. However, to have a single-stranded reference to the four-stranded structures, a mono-functionalized template was designed. The synthesis of methyl 6-*O*-Aoa-2,3,4-tri-*O*-acetyl-α-D-Gal*p* (**7**) (fig. 4) followed methods developed for the synthesis of the tetra-functionalized template in combination with well-described carbohydrate chemistry [57]. Conveniently, the templates are water soluble and when kept as a freeze-dried powder at –18 °C, the deprotected structures have proved to be stable for more than 1 year. In solution, nuclear magnetic resonance (NMR) experiments indicated a slow loss of structural integrity of template **6**, presumably due to an intramolecular O-N acyl transfer. However, this degradation did not pose a problem in the use of the template, as the time scale of most oxime ligations was much shorter than for the observed template rearrangement (see below).

A number of C-terminal peptide aldehydes have been synthesized for second-generation carboproteins, starting with the shorter sequences Fmoc-Ser-Gly-Gly-H (**8**) and H-Ala-Leu-Ala-Lys-Leu-Gly-Gly-H (**9**) [56], and later extended to the dodeca- and hexadecapeptide aldehydes Ac-Lys-Ala-Leu-Lys-Glu-Ala-Leu-Ala-Lys-Leu-Gly-Gly-H (**10**) and Ac-Glu-Ala-Leu-Glu-Lys-Ala-Leu-Lys-Glu-Ala-Leu-Ala-Lys-Leu-Gly-Gly-H (**11**) [57]. Briefly, the syntheses followed a standard BAL strategy [58, 59, 62], commencing with coupling of a PALdehyde handle to amino-functionalized resin, followed by anchoring of the glycinal terminal by reductive amination with aminoacetaldehyde dimethyl acetal. Next, acylation of the secondary nitrogen was achieved with $(Fmoc\text{-}Gly)_2O$, whereas subsequent chain elongation followed standard Fmoc methodology for solid-phase peptide synthesis (SPPS). Cleavage with trifluoroacetic acid (TFA) released the peptide with concomitant removal of ^{t}Bu/Boc protecting groups and demasking of the aldehyde (scheme 2). All peptide aldehydes were synthesized from the ortho-isomer of PALdehyde, 5-(2-formyl-3,5-dimethoxyphenoxy)-pentanoic acid, of which we have recently developed a facile and economical synthesis [Boas U., Brask J., Christensen J. B. and Jensen K. J. The ortho Backbone Amide Linker (BAL) is an easily prepared and highly acid-labile handle for solid-phase synthesis. J. Comb. Chem., in press]. The longer sequences were based on a peptide used by Mutter and coworkers in the synthesis of the 4-α-helix bundle TASP, T_4-(4α_{15}-Ac) [16]. Hence, this amphiphilic peptide had already been shown to be capable of forming a stable 4-α-helix bundle on a cyclic peptide template, which offered a comparison with our carbohydrate-based template.

With templates and peptide aldehydes in hand, the four-stranded structures **12**–**15** were constructed [56, 57] from template **6** and peptide aldehydes **8**–**11** (table 1, entries 1–4). The single-stranded carbopeptide **16** was obtained [57] by ligation of peptide aldehyde **11** to mono-functionalized template **7** (table 1, entry 5). These reactions were performed by simply dissolving template and peptide aldehyde (in 50% excess) in aqueous acetate buffer, pH

Table 1. Summary of compound numbers. Each entry corresponds to one ligation reaction, forming one carbopeptide/carboprotein.

Entry	Template	Peptide aldehyde	Carboprotein
1	**6** (Gal*p*, 4-ONH_2)	**8** (Fmoc-SGG-H)	**12** (2.1 kDa)
2	**6**	**9** (H-ALAKLGG-H)	**13** (2.9 kDa)
3	**6**	**10** (Ac-KALKEALAKLGG-H)	**14** (5.3 kDa)
4	**6**	**11** (Ac-EALEKALKEALAKLGG-H)	**15** (7.1 kDa)
5	**7** (Gal*p*, 1-ONH_2)	**11**	**16** (2.0 kDa)
6	**17** (Alt*p*, 4-ONH_2)	**19** (Ac-YEELLKKLEELLKKAG-H)	**20** (8.1 kDa)
7	**18** (Glc*p*, 4-ONH_2)	**19**	**21** (8.1 kDa)
8	**6**	**19**	**22** (8.1 kDa)
9	**23** (Gal*p*, 4 -ONH_2)	**11**	**24** (7.2 kDa)

4.76. Due to limited solubility of the short, Fmoc-protected peptide aldehyde **8**, addition of an organic cosolvent as well as prolonged reaction time were required for this particular reaction to go to completion. For unprotected peptide aldehydes, where water solubility was not a problem, the ligations proceeded cleanly to completion within minutes, as monitored by analytical HPLC.

To prove the identity of the large structures, characterization proceeded with ESI MS, ^{1}H-NMR, and analytical HPLC. From the MS analyses, a deconvolution of the series of peaks resulting from multiple protonated species in all cases returned the expected mass of the carbopeptide or carboprotein. The NMR analyses showed an approximate 1:1 ratio of *Z* and *E* oxime isomers, indicating the presence of up to 16 (i.e., 2^4) isomers of each four-stranded structure and two isomers of the single-stranded structure **16**. Broadening of HPLC peaks was pronounced for large carboprotein structures when analyzing them on a C18 RP-HPLC column; substituting a C18 for a C4 RP column significantly sharpened the peaks. We attribute this phenomenon to the presence of equilibria between folded and unfolded bundle structures and their different affinity for the column material [63, 64]. It seems unlikely that it is due to the presence of *E*/*Z* isomers.

Biophysical characterization

According to the design, the most likely candidate to form a stable 4-α-helix bundle was the 4 × 16 AA carboprotein **15** (fig. 5). The folding of this carboprotein was studied by circular dichroism (CD) and NMR spectroscopy, and the results compared with those of the 4 × 12 AA carbopeptide **14** and the 1 × 16 AA single-stranded carbopeptide **16** (fig. 5). The first CD spectra revealed that all three templated structures were α-helical in aqueous buffer, but to varying degrees. The content of α-helix was calculated [65] from the mean residue ellipticity (MRE, θ) at 222 nm to be 29%, 45%, and 67% for **14**, **16**, and **15**, respectively. The low α-helicity for **14**, indicating insufficient length of the truncated peptides to stabilize a bundle, was expected from the random coil-like CD spectrum recorded by Mutter and coworkers for TASP T_4-(4α_{11}), a structure with similar peptide sequence [16].

Next, the potential 4-α-helix bundle **15** and the single-stranded reference **16** were compared in denaturation experiments with guanidinium chloride (GuHCl). A pronounced effect of interhelical stabilization was observed, as **15** in contrast to **16** displayed a concerted denaturation curve with a midpoint at 5.1 M GuHCl. For comparison, Mutter's T_4-(4α_{15}) was reported to have a denaturation midpoint of 3 M GuHCl [16]. As the major difference between **15** and T_4-(4α_{15}) is the template (and the linkage between template and peptides), the observed difference in denaturation resistance could indicate improved bundle stabilization by the D-Gal*p* template compared to the cyclic peptide template in T_4-(4α_{15}). From the denaturation curve of **15**, the free energy of folding in water was calculated to be $\Delta G_{H2O} = -6.9$ kcal/mol, while the slope of the extrapolation, reflecting the cooperativity of fold-

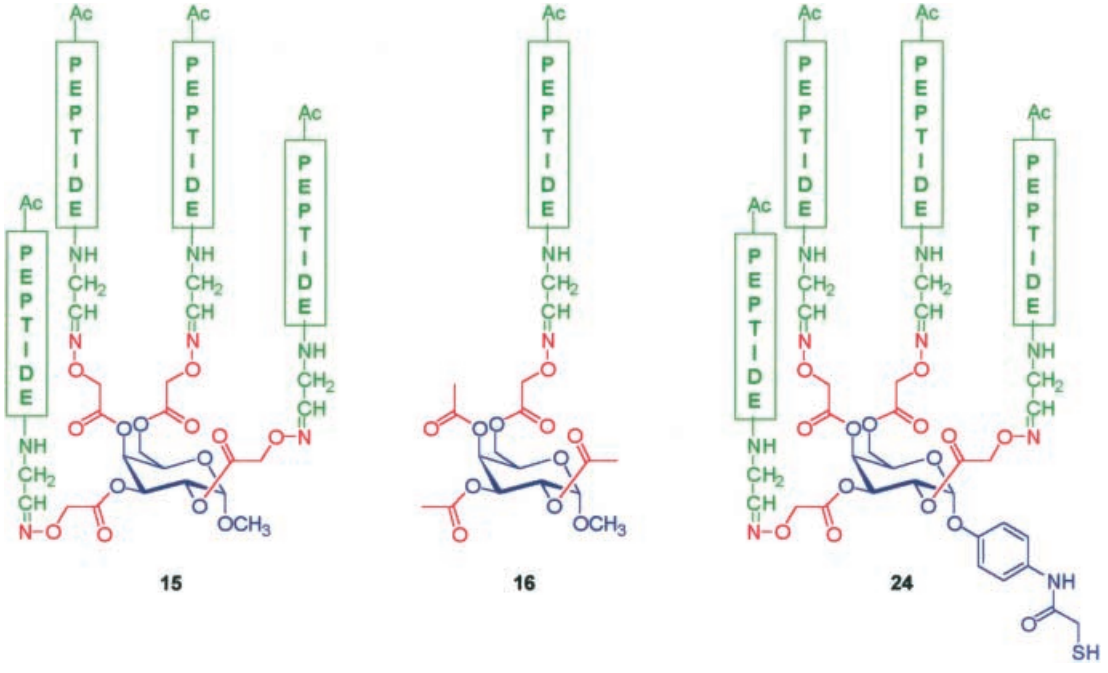


Figure 5. Structures of 4 × 16 AA carboprotein **15**, 1 × 16 AA carbopeptide **16**, and 4 × 16 AA thiol-functionalized carboprotein **24**. All three structures were prepared by ligation to peptide aldehyde Ac-EALEKALKEALAKLGG-H (**11**).

ing, was m = 1.4 kcal/mol/M [66]. These values are comparable with those reported for other templated 4-α-helix bundles [29, 67], though not as high as the values recently reported by Mezo and Sherman [26] for a 4-α-helix bundle on a thiophenol cavitand template (ΔG_{H2O} = –22.9 kcal/mol; m = 3.1 kcal/mol/M).

To conclude the biophysical studies, the solvent exposure of amide protons in **15** and **16** were compared in NMR H-D exchange experiments. As expected, all amides in the single-stranded carbopeptide **16** exchanged within 30 min. In contrast, 42 h was required to exchange all amides in carboprotein **15** with the same peptide sequence, revealing a shielding from the solvent of some amide protons in the structure. We find that the results from the CD and NMR H-D exchange experiments together strongly indicate that the 4 × 16 AA second-generation carboprotein **15** folds to form a 4-α-helix bundle structure.

Effect of the carbohydrate template on carboprotein stability

Given that the Gal*p* template induces 4-α-helix bundle formation in the attached amphiphilic peptides, can proper choice of the carbohydrate template be used to (de)stabilize the carboprotein? Recently, Fairlie and coworkers investigated the influence of template geometry on the formation of 4-α-helix bundles in TASP structures [24]. Based on four different aromatic templates, they concluded that with a sufficiently long linker between the peptide strands and the template, the template geometry was of less importance. However, although Fairlie's templates had different sizes, the peptide-anchoring points were all in the same plane. We speculated that the comparison of a series of carboproteins assembled on different carbohydrate epimers could reveal new answers to this question. Hence, in an ongoing project, new second-generation carboproteins have been prepared on tetra-aminooxyacetyl-functionalized carbohydrate templates [68]. The templates were synthesized, using previously described chemistry, from methyl α-D-altropyranoside (Alt*p*) and methyl α-D-glucopyranoside (Glc*p*) starting materials, to give Alt*p*-template **17** and Glc*p*-template **18** (fig. 6). The trans diaxial arrangement of the O-2 and O-3 hydroxyls in **17** was of special interest, as it was expected to influence the stability of the resulting four-stranded carboprotein compared to the same structure on the Gal*p*-template **6**, with all hydroxyls in or above the same plane (fig. 2). The hydroxyls are the ultimate points of attachment of the peptides and the trans 1,2-diaxial arrangement was initially expected to lead to a *decreased* stability of a 4-α-helix bundle structure.

The two new templates and the Gal*p*-template **6** were ligated to the same peptide aldehyde, Ac-Tyr-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Ala-Gly-H (**19**) [68]. The sequence of this peptide aldehyde was based on the heptad repeat in the very stable 4-α-helix bundles prepared by Sherman [25, 26]. Incorporation of Ala near the C terminal was rationalized from its large helix propensity, whereas N-terminal Tyr allowed concentration determination from the UV absorption. As before, the C-terminal glycinal enabled oxime ligations with an eliminated risk of racemization. The resulting three carboproteins, **20** (Alt*p* based), **21** (Glc*p* based), and **22** (Gal*p* based) (table 1, entries 6–8) all showed a high α helicity in preliminary CD measurements and are all potential 4-α-helix bundles [68]. Interestingly, **20** was found to be *more* α-helical than **21** and **22**. Though unexpected, the observed higher helicity can be explained from the subtle effects on side chain packing when one peptide strand is offset relative to the others. However, further investigations are required to study other possible effects, such as aggregation.

Furthermore, having achieved a 4-α-helix bundle structure, the question still remains whether the structure is native-like or should more accurately be described as an ensemble of molten globule-like structures [69]. Hence, a number of experiments are required to characterize the three new carboproteins, in addition to those already applied. Near-UV CD spectroscopy can provide information on fluctuations of aromatic chromophores, and after incorporation of, e.g., a Trp residue in the sequence, detection of strong near-UV CD bands would indicate a native-like structure, in contrast to the weaker or absent signals of molten globules [70, 71]. Furthermore, addition of the hydrophobic dye 8-anilino-1-naphthalenesulfonic acid (ANS) is a convenient and common test, as it has

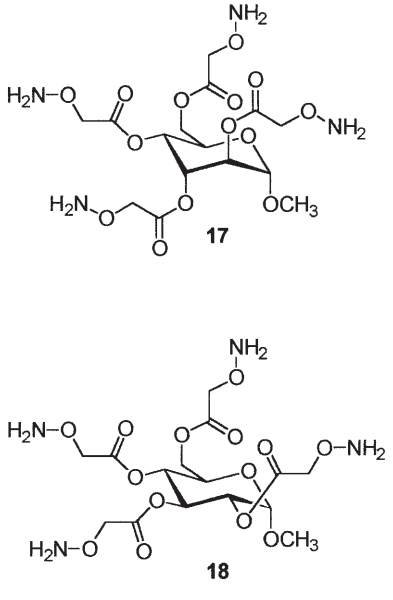


Figure 6. The templates methyl 2,3,4,6-tetra-*O*-Aoa-α-D-Alt*p* (**17**) and methyl 2,3,4,6-tetra-*O*-Aoa-α-D-Glc*p* (**18**).

been found to bind to molten globules but not to native-like structures [70, 72]. Finally, the degree of chemical shift dispersion in the amide region of ^{1}H-NMR spectra recorded in H_2O-D_2O (9:1) can be used to differentiate native-like from molten globule structures, with the former giving the better dispersion [71, 73].

Monolayers of carboproteins: in situ scanning tunneling microscopy

The above CD analyses of the α-helicity measure an average property of carboproteins in solution. In contrast, analysis of monolayers of carboproteins after adsorption to a surface by in situ scanning tunneling microscopy (in situ STM) should allow study of the self-organization of 4-α-helix bundles, and maybe provide insights into interactions between hydrophilic surfaces (exterior) on neighboring carboproteins [74]. For this purpose, a new template with a thiol moiety was prepared to enable adsorption to gold surfaces. By incorporating the thiol in the carbohydrate aglycon, adsorption was expected to occur without interfering with carboprotein folding. The template, 4-(mercaptoacetamido)phenyl 2,3,4,6-tetra-*O*-Aoa-α-D-Gal*p* (**23**), was synthesized in four steps starting from commercially available 4-nitrophenyl α-D-Gal*p*, which was reduced to the amine. Coupling of the aglycon to *S*-trityl-mercaptoacetic acid, followed by Boc_2-Aoa-OH acylation of the carbohydrate hydroxyls provided the protected template. Deprotection of Trt and all Boc groups was performed with TFA-CH_2Cl_2-Et_3SiH (1:1:0.1). Next, the template was ligated to the hexadecapeptide aldehyde **11** as this sequence previously formed a 4-α-helix bundle on a Gal*p*-template. The expected 7.2-kDa thiol-functionalized 4-α-helix bundle carboprotein **24** (table 1, entry 9; fig. 5) was identified by ESI MS. Approximately 10–20% had formed the disulfide, but as both the thiol and the disulfide are expected to undergo adsorption, this was not critical.

In a very recent publication, initial results on the adsorption of carboprotein **24** on single-crystal Au(111) surfaces have been reported and compared with data from the partial structures 4-(mercaptoacetamido)phenyl α-D-Gal*p* (**25**), and *N*-phenyl-mercaptoacetamide disulfide (**26**) [74]. Voltammetry of the three molecules on Au(111) showed reductive thiolate-Au desorption peaks. With the two partial structures **25** and **26**, strong signals were obtained in 10 mM NaOH solution with close to monolayer coverage. With carboprotein **24**, observed in phosphate buffer at pH 6.9, peak potentials and coverages reflected the significantly larger size of the full 4-α-helix bundle. Furthermore, observation of regular domains of fragment **26** was reported as a preliminary result of ongoing STM investigations. Similar images of ordered structures of carboprotein **24** have now been recorded. These results demonstrate that the incorporated thiol moiety enables formation of well-defined monolayers on Au(111) and hold promise for future investigations of functionalized 4-α-helix bundle carboproteins.

Conclusions

The carboprotein concept, the use of carbohydrates as templates for de novo design of protein models, has been implemented for the synthesis of artificial 4-α-helix bundles. The first carbopeptide was synthesized by stepwise assembly of the four 'arms' on a D-Gal*p* template linked to a solid support. The next carbopeptides and, eventually, carboproteins were synthesized by a convergent route in which C-terminal peptide aldehydes were chemoselectively ligated with tetra-aminooxy-functionalized monosaccharides. This allowed reliable preparation of 10–20 mg of purified carboprotein on Gal*p*, Glc*p*, and Alt*p* templates. Biophysical studies with CD and H-D exchange NMR spectroscopy clearly indicated formation of the desired 4-α-helix bundle. Surprisingly, the Alt*p* template provided a carboprotein with a higher content of α-helix compared to similar carboproteins prepared on Gal*p* and Glc*p*. This indicates that the template has a controlling effect on the 4-α-helix bundle structure. Finally, the use of in situ STM to study monolayers of carboproteins offers the prospect to study the self-organization of 4-α-helix bundles at a molecular level.

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