A template for the solid-phase synthesis of conformationally restricted protein loop mimetics

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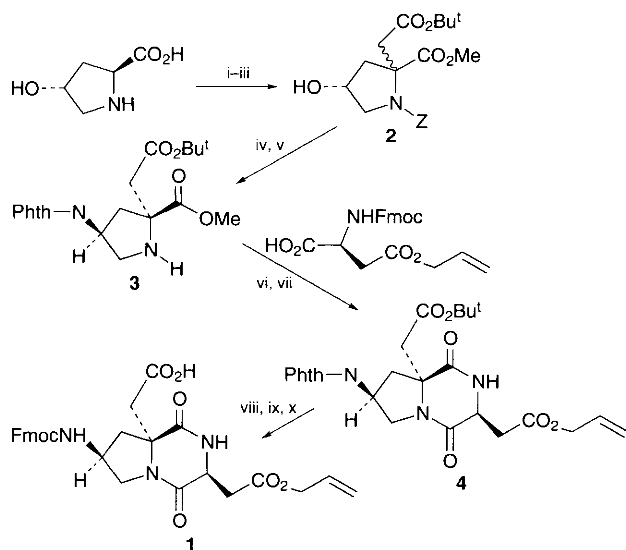
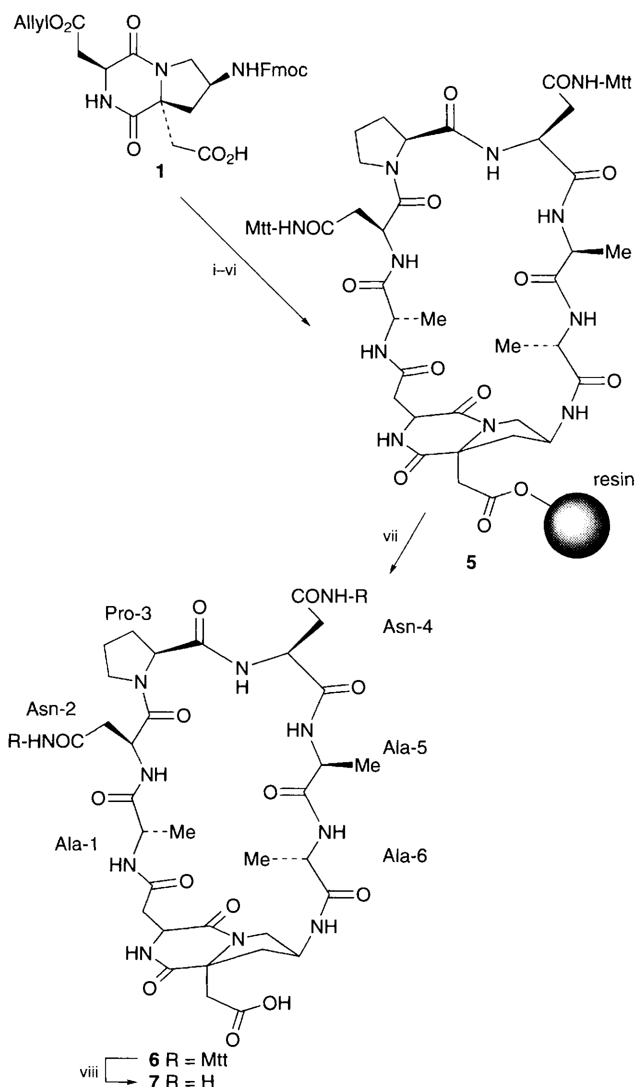
A trifunctional template 1 is synthesized from *trans*-4-hydroxy-L-proline and L-aspartic acid, immobilized on a solid-support, and used for the solid-phase synthesis of a cyclic conformationally restrained hexapeptide loop mimetic containing the motif Asn-Pro-Asn-Ala (NPNA); this is shown by NMR to populate a stable β-turn conformation in aqueous solution.

Surface loops on proteins are of general interest in the design of peptide-based vaccines and inhibitors. Owing to their inherent flexibility, however, linear peptides are often poor mimics of the secondary structural elements found in proteins. A solution to this problem may be found by grafting the peptide onto an organic template whose geometry induces stable, biologically relevant conformations in the sequence of interest. A further advantage might arise, however, if the template could be immobilized, to allow the synthesis of conformationally restrained loop mimetics directly on a solid-phase. We describe here a novel template 1 whose structure and functionality allow the realisation of these goals.

The template 1 was synthesized from readily available *trans*-4-hydroxy-L-proline and L-aspartic acid, following the route shown in Scheme 1. α-Alkylation of Me_3Si-protected N^{α}-Z-4-hydroxyproline methyl ester, and removal of the temporary $OSiMe_3$ protecting group with aqueous citric acid, gave 2 as a mixture of diastereoisomers (*ca.* 2 : 1, with an excess of the desired isomer). After a Mitsunobu reaction[1] and removal of the Z-group, the diastereoisomers were then separated by flash chromatography on silica (CH_2Cl_2–Et_2O–hexane, 1 : 1 : 1) to afford 3. The relative configuration of 3 was confirmed by ^1H NOE-difference experiments. The coupling of 3 with Fmoc-Asp(Oallyl)-OH proceeded optimally using HATU† and HOAt for activation,[2] and the resulting dipeptide was cyclized directly after extraction, without further purification, to give 4. Removal of the phthalimide protecting group in 4 was problematic using hydrazine, since the allyl ester was also attacked by this reagent. However, selective deprotection could be achieved in good



Scheme 1 *Reagents and conditions*: i, MeOH, $SOCl_2$ (93%); ii, $PhCH_2OCOCl$ (90%); iii, Me_3SiCl, Et_3N, then LDA (1.2 equiv.), then $BrCH_2COOBu^t$, then H_3O^+ (64%); iv, Ph_3P, DEAD, PhthNH, −23 °C, then room temp. (75%); v, Pd–C, H_2 and separation of diastereoisomers (42%); vi, HATU, HOAt, CH_2Cl_2; vii, DBU, MeCN (65%, 2 steps); viii, $MeNHNH_2$, DMF; ix, Fmoc-Succin. (65%, 2 steps); x, TFA (90%).



Scheme 2 *Reagents and conditions*: i, 1 (2 equiv.), Tentagel-SAC, CIP (6 equiv.), Pyr–CH_2Cl_2; ii, piperidine, DMF; iii, peptide assembly using HBTU (3 equiv.), HOBt (3 equiv.) in DMF, DIEA, and Fmoc-protected amino acid (3 equiv.) in each coupling step, piperidine in DMF for Fmoc-removal; iv, $Pd(PPh_3)_4$ (3 equiv.), NMM, AcOH, DMF; v, piperidine, DMF; vi, BOP (1.5 equiv.), DIEA (3.0 equiv.), NMP, Me_2SO; vii, 1% TFA–CH_2Cl_2 (*ca.* 30% yield over last 4 steps); viii, 95% TFA–5% $Pr^i{}_3SiH$

yield using methylhydrazine, and reprotection was then performed directly, without purification of the free amine, using Fmoc-*N*-hydroxysuccinimide.

To establish methods for assembling peptide loops on the template, whilst bound to a solid-support, the target **7** was chosen (Scheme 2). This contains the Asn-Pro-Asn-Ala (NPNA) motif, which in a tandemly repeated form comprises the immunodominant epitope on the circumsporozoite surface protein of the malaria parasite *Plasmodium falciparum*.[3] Earlier NMR studies[4] have shown that synthetic peptides of the type $(NPNA)_n$ (n = 1,2,3) display a tendency to adopt β-turn conformations in aqueous solution. Moreover, the β-turns can be stabilized by replacing Pro with α-methylproline (P^{Me}), without compromising the ability of the dodecamer peptide $(NP^{Me}NA)_3$ to elicit antibodies that cross-react with *P. falciparum* sporozoites.

Using CIP for activation,[6] the template **1** was coupled without problem to the acid-sensitive resin Tentagel-SAC (Rapp Polymere, Tübingen) (0.23 mequiv. g^{-1}), to a substitution level of 0.18 mmol g^{-1}. After removal of the Fmoc-group, the required hexapeptide was assembled using HBTU and HOBt for activation[7] (Scheme 2). The allyl ester of the template was then cleaved, and cyclization was induced with BOP and DIEA in NMP–Me_2SO. Analysis of the cyclisation products was carried out by reverse-phase HPLC, following cleavage from the resin and deprotection with TFA. Two major products were detected in approx. 2 : 1 ratio; the required macrocycle **7** {electrospray MS: 806.5 [$(M + H)^+$]} and a dimeric species (ES-MS: 1612 [$(M^+ +H)$], respectively. The latter was not further investigated. Quantitative Fmoc-determination gave the amount of linear peptide on the resin, from which the yield of **7** after purification by HPLC was estimated to be *ca.* 30%. Alternatively, cleavage from the resin with 1% TFA–CH_2Cl_2 afforded **6** with the side-chain protecting groups intact. This form may be valuable for conjugation of the loop mimetic with carrier molecules, such as the polylysine core of a multiple antigen peptide[8] (MAP).

With the fully deprotected cyclic peptide **7** available, its conformation was studied by ^1H NMR spectroscopy in aqueous solution (90% H_2O, 10% D_2O, pH 5). In particular, evidence was found for a stable type-I β-turn within the NPNA motif, analogous to that found earlier in linear peptides containing $(NP^{Me}NA)_n$ (n = 1,2,3) motifs.[5] Thus, the low temperature coefficient (-1.2 ppb K^{-1}) of the Ala-5 NH resonance (Table 1) indicates substantial shielding of this amide proton from solvent, and its likely involvement in intramolecular hydrogen bonding (see Fig. 1). In analogy to earlier studies,[9] a series of ROESY spectra with mixing times (spin lock periods) in the range 75–300 ms revealed the medium range ($i, i + 2$ and $i, i + 3$) NOE connectivities depicted in Fig. 1, as well as a strong Asn[4]-Ala[5] d_{NN} NOE connectivity. These NOE data and temperature coefficients are consistent with formation of a stable type-I β-turn within the NPNA motif in **7**, which is significantly populated on the NMR timescale. Comparable ($i, i + 3$) NOE connectivities and low temperature coefficients are not discernible[4] in linear synthetic peptides of the type $(NPNA)_n$ (n = 1,2,3), thus indicating a stabilization of β-turn secondary structure within the template-bound peptide loop.

In summary, these studies demonstrate that template **1** can be used to construct conformationally restrained loop mimetics on a solid phase, and that at least in the case of **7**, β-turn secondary structure is stabilized in the loop backbone. This template may be useful in the design of other protein loop mimetics, for example, as components of conformationally defined synthetic peptide vaccines, or as inhibitors of ligand–receptor interactions.

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Table 1 Temperature coefficients (ppb K^{-1}) of the peptide amide NH resonances in **7** measured in the range 5–30° in aqueous solution (90% H_2O, 10% D_2O), pH 5.0. Asp-8 and Apro-7 refer to the aspartate and modified aminoproline moieties of the template in **7** (Scheme 2)

Residue	Temperature coefficient
Asp-8	−8.4
Ala-1	−7.6
Asn-2	−6.8
Asn-4	−4.5
Ala-5	−1.2
Ala-6	−6.5
Apro-7	−7.4

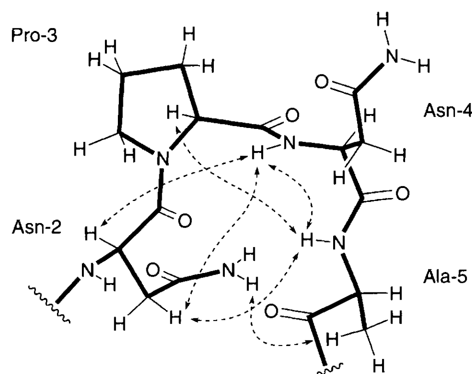

Fig. 1 The NPNA motif in **7** is depicted in a type-I β-turn conformation. The medium range NOE connectivities, and a strong d_{NN} (i, i+1) NOE, detected in ROESY spectra (in 90% H_2O, 10% D_2O, pH 5.0, 300 K), are depicted by dotted arrows. The Ala-5 NH proton may hydrogen bond to the Asn-2 backbone CO, consistent with the low temperature coefficient observed for this amide proton (Table 1).

Footnotes

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‡ Abbreviations: BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; CIP, 2-chloro-1,3-dimethylimidazolidinium hexafluorophosphate; DIEA, *N,N*-diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; Mtt, 4-methyltrityl; NMM, *N*-methylmorpholine; NMP, 1-methyl-2-pyrrolidone.

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