Molecularly imprinted cavities template the macrocyclization of tetrapeptides†

Dar-Fu Tai* and Yee-Fung Lin

Received (in Cambridge, UK) 4th August 2008, Accepted 26th August 2008
First published as an Advance Article on the web 29th September 2008
DOI: 10.1039/b813439a

Cavities formed using cyclic tetrapeptides (CTPs) or heat-induced conformers act as templates for cyclization; the cavities bind to linear tetrapeptides and enforce turn conformations to enhance cyclization to constrained CTPs.

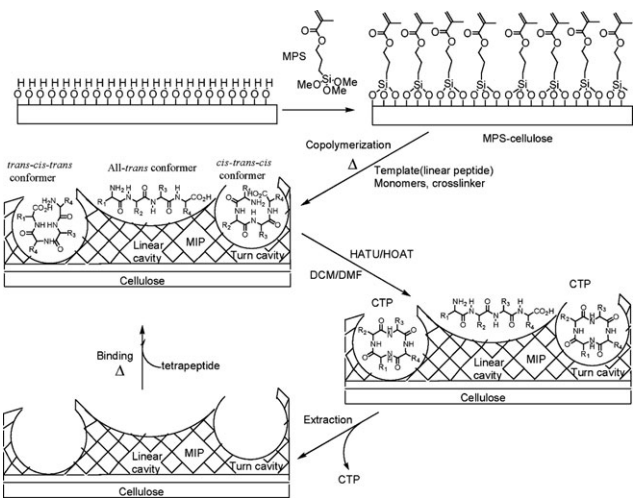
Constrained cyclic peptides incorporating β-turn structures have been designed and studied[1] to provide conformational insight[2] for receptor binding.[3] In a cyclic tetrapeptide ring the distance between C alpha (*i*) and C alpha (*i* + 3) is less than 7 Å[4] which is suitable for metal ion chelation or incorporating small molecules between the respective side chains. Although CTPs have been isolated and structurally characterized, there has been difficulty in synthesizing derivatives without functional side chains.[5] Typical examples are the antiproliferative agent *cyclo*-(Pro-Leu-;Pro-Leu)[6] and tyrosinase inhibitor *cyclo*-(Pro-Val-Pro-Val).[6,7] Aracil and Francisco[6] reported the synthesis of these two peptides having certain biological activities; other groups[6,7] tried to resynthesize these compounds, but obtained compounds without the reported biological activities and with different NMR spectra. The *cyclo*-(Pro-Val-Pro-Val) molecule has a *cis-trans-cis-trans* backbone-ring conformation.[7] It is possible to build an all-*cis* cyclic peptide computationally.[8] However, little effort has been made to construct these diverse and novel structures.[9] Macrocycles, not only those with β-turns, have been prepared using solid-phase methods.[10] Although highly strained CTPs are not readily available, CTP-like molecules (β-turn mimetics) are important substitutes[11] often used for interaction with protein targets. The low hit rates commonly encountered in high-throughput screening of these analogs suggest that improved macrocyclization approaches to CTP's will be welcomed.

CTPs are known to undergo conformational interconversions at high temperature involving a series of *cis-trans* amide isomerizations.[11,12] Recently, a tripeptide fragment, Ac-Ala-Ala-Ala-NH_2, was folded into a β-turn through encapsulation by a porphyrin-assembled synthetic host.[13] Our strategy to improve the yield of cyclization has been to maintain such a turn or partial turn of a linear peptide in a nano sized cavity as a template.

Molecularly imprinted polymers (MIPs)[14] are seldom prepared at high temperatures (> 110 °C). At high temperatures, a linear peptide is prone to adopt a turn conformation owing to its higher propensity to form *cis*-amide conformations. These shapes should facilitate the subsequent cyclization process (Scheme 1). This protocol shows promise as a recognition-based system involving a cavity of nanometric dimensions (the distance represented by a β-turn is approximately 10 Å).

As previously reported, synthesis of *cyclo*-(Phe-Phe-Phe-Phe)[15] is a challenging task, as is the synthesis of *cyclo*-(Gly-Gly-Gly-Gly)[16] and of *cyclo*-(Pro-Pro-Pro-Pro).[16] Therefore, these three tetrapeptides and the two disputed peptides (Leu-Pro-Leu-Pro, Pro-Val-Pro-Val)[6,7] were selected as examples to test the new synthesis of CTPs. A three-stage procedure to facilitate CTPs formation is shown in Scheme 2. The yields of cyclization can be used as a report on the conformation in a turn or partial turn of a linear peptide in a nano size cavity.

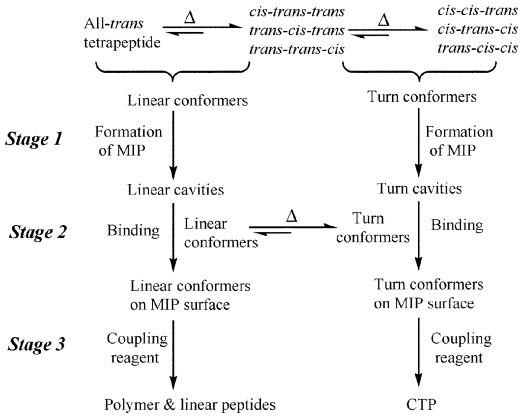
The first stage is the construction of the desired turn-inducing cavities for the respective tetrapeptides. Cellulose fibers were chosen as the platform on which to construct the surface due to its performance and convenience. The highly hydrophilic character of cellulose-based composite materials were modified with 3-methacryloxypropyltrimethoxysilane (MPS) to form a hydrophobic monolayer on the cellulose surface.[17] The grafted cellulose fiber was hydrophobic enough and suitable for fabrication of molecularly imprinted polymers. This nano-cage was constructed by polymerization with acrylamide, *N*-acryltyramine (ATA), *N*,*N*′-ethylene bisacrylamide (EBAA) and 2,2′-azobisisobutyronitrile (AIBN) in the presence of the target tetrapeptide at high temperature. To generate hydrophobic cavities and avoid amino group and carboxylic group interactions on the template the ionic monomer is limited. The linear



Scheme 1 Schematic representation of tetrapeptide induced linear/turn cavities and their potential in cyclization reactions.

Department of Chemistry, National Dong-Hwa University, Hualien, Taiwan. E-mail: dftai@mail.ndhu.edu.tw; Fax: 886 38633570; Tel: 886 38633579

† Electronic supplementary information (ESI) available: Experimental section. See DOI: 10.1039/b813439a



Scheme 2 Possible participation of linear/turn conformers with linear/turn cavities for tetrapeptide cyclization.

peptide contains both favorable (turns) and unfavorable (linear) conformations for cyclization, and the interconversion barriers from linear to turn conformations are different for each peptide. Indeed, the polymerization temperature played a crucial role in the results from the materials synthesized.[18] The MIP can "lock" the conformer during polymerization in a manner similar to memory effects. The higher the curing temperature, the higher the fraction of turn cavities generated.

The solubility was also important. The tetrapeptide was mixed in H_2O and ethylene glycol (1 : 1) to dissolve all the components and allow the formation of MIP-cellulose at high temperature. Some CTPs exhibit one conformation in nonpolar solvents or when a *cis*-amide is present, but CTPs usually exist as multiple conformations in polar solvents.[11] This property offers a unique way to control the system. During polymerization, the equilibrium can be shifted toward the turn conformer using solvent and temperature effects.[18] Generation of more turn cavities would assure the binding of the turn conformer and reduce the binding of the linear form. Peptide bonds, which originally contain less turn character and preferentially adopt a *trans* conformation in solution, could be transformed to a *cis* conformation at high temperature and incorporated into the turn cavities. As shown in Table 1, linear tetrapeptides were used as the template to prepare imprinted polymers with turn cavities. The amount of effective cavities generated was around 60% of the template used. The effective cavities contained both turn cavities and linear cavities.

The second stage is the binding of the linear peptide to their turn cavities. The orientation and hydrophobicity of the side chain on the tetrapeptide can have stabilizing interactions with the MIP to form MIP–peptide complexes. The MIP cage can accommodate tetrapeptides using solvents such as water, acetonitrile, benzene, toluene or *p*-xylene, but it also shows an unexpected selectivity. Generally, nonpolar solvents performed better at reflux and toluene was found to be the best solvent for adsorption. Binding of appropriate guests occurs best in those solvents that cannot fit well inside the cavities, and refluxing toluene is appropriate to force most tetrapeptides in but not out. In general, the β-turn conformation was not favored in water. Here, it was observed that despite the presence of the linear conformer, the turn conformation dominated because of efficient host–guest interaction at high temperature. After refluxing in toluene for 6 h, 60–70% of linear tetrapeptides were bound to the MIP–cellulose fiber, as determined by HPLC analysis (Table 2). Rinsing with water removed the nonspecifically bound tetrapeptides. Further evidence was obtained using Raman spectroscopy. Captured turn conformer within the cavity is revealed by the Raman band[1] of amide I for β-turn within $\sim$1690 to $\sim$1660 cm^{-1}. In particular, Gly-Gly-Gly-Gly shows two large peaks (1670 and 1679 cm^{-1}) that indicated that the ratio of turn conformers was increased inside the cavity as compared to their free form in the solution. In contrast, no increase was observed upon attaching Pro-Pro-Pro-Pro to its MIPs. Gly-Gly-Pro-Gly, a β-turn peptide,[19] was also examined for comparison (ESI†).

The third stage is the cyclization of the peptide on the surface. Most of the substrates bound to the MIP-cellulose possess a turn

Table 1 MIPs formed at high-temperature[a]

Template (%)	Gly-Gly-Gly-Gly	Phe-Phe-Phe-Phe	Leu-Pro-Leu-Pro	Pro-Val-Pro-Val	Pro-Pro-Pro-Pro
Template in solution[b]	26.9	24.0	26.9	28.5	30.6
Effective cavities[c]	58.1	65.4	62.2	58.1	56.6
Template embedded[d]	15.0	10.6	10.9	13.4	12.8

[a] MIP was prepared by heating at 140 °C for 25 min on MPS-cellulose fiber with 120 μL of H_2O and ethylene glycol (1 : 1), containing template peptide (3.2 μmol)/acrylamide/ATA/EBAA at 8 : 1 : 4 : 15 molar ratio. [b] The amount of template in solution was measured by HPLC, using *N*-carbobenzyloxythreoine methyl ester (1 mM) as the internal standard. [c] The effective cavities were measured using HPLC, based on the amount of tetrapeptide washed out from the MIP. [d] The embedded template was calculated by deducting the amount of template in solution and the amount of tetrapeptide in the cavities from the amount of template used (3.2 μmol).

Table 2 Binding of tetrapeptides at high-temperature[a]

Substrate (%)	Gly-Gly-Gly-Gly	Phe-Phe-Phe-Phe	Leu-Pro-Leu-Pro	Pro-Val-Pro-Val	Pro-Pro-Pro-Pro
Total binding[b]	65.9	71.6	70.0	67.5	67.2
Nonspecific binding[c]	15.9	16.0	24.1	20.9	19.7
Specific binding[d]	50.0	55.6	45.9	46.6	47.5
The yields of CTP[e]	19.6	46.2	24.3	27.8	0
Total binding of NIP[f]	21.3	21.3	15.9	17.4	13.1
The yields of CTP[f]	0	1	0	0	0

[a] Binding was performed using tetrapeptide (3.2 μmol) in toluene (1 mL) at reflux for 6 h. [b] The amount of total binding was measured by HPLC, using *N*-carbobenzyloxythreoine methyl ester (1 mM) as the internal standard. Yields were measured based on the amount of tetrapeptide used (3.2 μmol). [c] The amount of tetrapeptide rinsed out with water from the MIP. [d] The specific binding was calculated by deducting the amount of tetrapeptides nonspecifically bound to the cavities from the amount of total binding. [e] The MIP–peptide complex reacted in DCM–DMF (3 : 1) with HATU–HOAt at room temperature for 6 h. [f] Nonimprinted polymers (NIP) were prepared under the same conditions without the template.

Table 3 Summary of the results of difficult CTPs syntheses[a]

Substrate yield/conv. (%)	Gly-Gly-Gly-Gly	Phe-Phe-Phe-Phe	Leu-Pro-Leu-Pro	Pro-Val-Pro-Val	Pro-Pro-Pro-Pro
By MIPs formed at high-temperature	14.1[b] (25.6)[c]	40.5[b] (62.9)[c]	—	—	0[b] (0[c]
By MIPs formed and binding at high-temperature	19.6[b] (39.4)[c]	46.2[b] (83.1)[c]	24.3[b] (53.1)[c]	27.8[b] (59.7)[c]	0[b] (0)[c]
By MIPs formed by UV irradiation	0[b] (0)[c]	37.1[b] (70.2)[c]	—	—	0 (0)[c]
By CTP-templated MIP[e]	27.7[b] (87.8)[c]	49.3[b] (93.9)[c]	—	—	—
By CTP templated MIP[e] with binding at high-temperature	42.8[b] (84.5)[c]	54.4[b] (90.2)[c]	—	—	—
Lit. yield (%)	4.4[17]	1[16]	5[6]	10[7]	0[17]

[a] The MIP-peptide complex reacted in DCM–DMF (3 : 1) with HATU–HOAt at room temperature for 6 h. Yields were measured using HPLC. [b] Yields were measured based on the amount of CTP, divided by the amount of tetrapeptide used for binding (3.2 μmol). [c] Conversions were measured based on the amount of CTP, divided by the amount of tetrapeptide specifically attached to the MIPs (Table 2). [d] The MIPs were prepared by irradiation of MPS-cellulose fiber (28.8 mg) at 350 nm with 100 μL of the mixed solvent (CH_3CN–H_2O = 1 : 1) containing template peptide–acrylamide–ATA–EBAA at 8 : 1 : 4 : 15 molar ratio. [e] The MIPs were prepared by the above method using CTP as template.

conformation reducing the number of undesired conformers. The poor reversibility and high hydrophobicity of the turn cavities exposes the tetrapeptide amino and carboxylic groups to the coupling reagents, which can significantly improve the macrocyclizations. The linear tetrapeptides that are in contact with the turn cavities are also shielded from each other and this protection enhances intramolecular reactions with increased rates and shorter reaction times. The activated ester could be held in complexes hidden from hydrolysis and side reactions, so as to enhance the yield of cyclization. The coupling reagent adopted for cyclization was *O*-(7-azabenzotriazol-1-yl)-l,l,3,3-tetramethyluronium hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt).[20] The solvent system was modified by increasing the ratio of dichloromethane (DCM) to *N*-dimethylformamide (DMF). As shown in Table 3, DCM–DMF used at a 3 : 1 ratio resulted in higher yields of CTPs. The MIPs effectively bring the termini of tetrapeptides together for cyclization, overcoming strain related resistance. In comparison with UV irradiation, the CTP synthesis of Gly-Gly-Gly-Gly was improved from none to near 20%. However, it was still not possible to achieve cyclization for Pro-Pro-Pro-Pro to *cyclo*-(Pro-Pro-Pro-Pro). The energy difference between the turn conformer and linear conformer must be too large. This is consistent with Raman spectra. Fortunately, no heterochiral CTP was generated, indicating no racemization occurred during the third-stage process. For those linear peptides with a minimal amount of turn conformation, the yield for the intramolecular peptide bond formation was improved using this procedure. With *cyclo*-(Pro-Leu-Pro-Leu), the ^{13}C NMR spectrum has nine peaks in the alkyl region; while the broad ^{1}H NMR spectrum more closely resembled that of Haddadi and Cavelier.[6] As for *cyclo*-(Pro-Val-Pro-Val), only one set of Pro-Val signals was observed and it did not resemble the spectrum reported by Aracil and Francisco.[6]

In some cases where the ratio of turn conformers was still low at 140 °C, the fabrication method was modified by using CTPs as the templates. As CTPs were available, using them as the templates during UV irradiation ensured the formation of the β-turn cavities on MIPs. The yield of cyclization was improved to 27.7% for the less hydrophobic tetrapeptide: Gly-Gly-Gly-Gly, and even higher (42.8%) using capture at high temperature. The yield of *cyclo*-(Phe-Phe-Phe-Phe) was also improved to 54.4%. It appears that 85–90% of the tetrapeptides attached to the MIPs were converted into CTPs.

In conclusion, this study indicates the possibility of modulating the MIP systems by raising the temperature regime and through induced fit molecular recognition for CTP synthesis.

The present work is partially supported by a Grant from the National Science Council of Taiwan.

Notes and references

1 G. D. Rose, L. M. Gierasch and J. A. Smith, *Adv. Protein Chem.*, 1985, **37**, 1.
2 E. Vass, M. Hollosi, F. Besson and R. Buchet, *Chem. Rev.*, 2003, **103**, 1917.
3 R. M. Freidinger, *J. Med. Chem.*, 2003, **46**, 5553.
4 W. Kabsch and C. Sander, *Biopolymers*, 1983, **22**, 2577; C. M. Wilmot and J. M. Thornton, *J. Mol. Biol.*, 1988, **203**, 221.
5 J. S. Davies, *J. Pept. Sci.*, 2003, **9**, 471.
6 J. M. Aracil, A. Badre, M. Fadli, G. Jeanty, B. Banaigs, C. Francisco, F. Lafargue, A. Heitz and A. Aumelas, *Tetrahedron Lett.*, 1991, **32**, 2609; U. Schmidt and J. Langner, *J. Pept. Res.*, 1997, **49**, 67; M. El Haddadi, F. Cavelier, E. Vives, A. Azmani, J. Verducci and J. Martinez, *J. Pept. Sci.*, 2000, **6**, 560.
7 T. Ueda, *Acta Crystallogr.*, 1984, **C40**, 111; T. Ueda, I. Sada, T. Kato and N. Izumiya, *Int. J. Pept. Protein Res.*, 1985, **25**, 475.
8 R. Poteau and G. Trinquier, *J. Am. Chem. Soc.*, 2005, **127**, 13875.
9 K. Ueno and T. Shimizu, *Biopolymers*, 1983, **22**, 633; W. D. Meutermans, G. T. Bourne, S. W. Golding, D. A. Horton, M. R. Campitelli, D. Craik, M. Scanlon and M. L. Smythe, *Org. Lett.*, 2003, **5**, 2711.
10 K. Burgess, *Acc. Chem. Res.*, 2001, **34**, 826.
11 M. MacDonald and J. Aubé, *Curr. Org. Chem.*, 2001, **5**, 417; M. P. Glenn, M. J. Kelso, J. D. A. Tyndall and D. P. Fairlie, *J. Am. Chem. Soc.*, 2003, **125**, 640; Y. Che and G. R. Marshall, *J. Med. Chem.*, 2006, **49**, 111.
12 U. Link, W. Mastle and M. Rothe, *Int. J. Pept. Protein Res.*, 1993, **42**, 475.
13 S. Tashiro, M. Kobayashi and M. Fujita, *J. Am. Chem. Soc.*, 2006, **128**, 9280.
14 K. Haupt and K. Mosbach, *Chem. Rev.*, 2000, **100**, 2495; M. J. Whitcombe, C. Alexander and E. N. Vulfson, *Synlett*, 2000, **5**, 911; G. Wulff, *Chem. Rev.*, 2002, **102**, 1.
15 F. Cavelier-Frontin, S. Achmad, J. Verducci, R. Jacquier and G. Pépe, *THEOCHEM*, 1993, **286**, 125.
16 K. Titlestad, *Acta Chem. Scand., Ser. B*, 1977, **31**, 641.
17 M. Abdelmouleh, S. Boufi, A. ben Salah, M. N. Belgacem and A. Gandini, *Langmuir*, 2002, **18**, 3203.
18 S. A. Piletsky, E. V. Piletska, K. Karim, K. W. Freebairn, C. H. Legge and A. P. F. Turner, *Macromolecules*, 2002, **35**, 7499.
19 P. Lagant, G. Vergoten, G. Fleury and M. H. Loucheux-Lefebvre, *Eur. J. Biochem.*, 1984, **139**, 137.
20 A. Ehrlich, H. U. Heyne, R. Winter, M. Beyermann, H. Haber, L. A. Carpino and M. Bienert, *J. Org. Chem.*, 1996, **61**, 8831.

