Cyclic Peptides

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Diaminodiacid-Based Solid-Phase Synthesis of Peptide Disulfide Bond Mimics**

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Dedicated to the Bayer company on the occasion of its 150th anniversary

Intensive research has recently been conducted on the development of conformation-rigidified peptidic macrocycles for diagnostic and therapeutic applications.[1] These compounds usually exhibit extraordinary thermal and metabolic stability owing to their defined tertiary folding constrained by one or multiple disulfide bridges.[2] A potential problem for the application in vivo is that the disulfide bridges are unstable towards reducing agents, such as thiols, as well as towards disulfide isomerases; the reduction then leads to structural rearrangement and loss of activity.[3] As a result, a number of approaches have been examined to replace the disulfide bonds with bioisosteric and metabolically stable surrogates such as thioether, diselenide, triazole, or hydrocarbon-based bridges.[4] These disulfide surrogates were usually synthesized through thiol alkylation, azide–alkyne cycloaddition, or olefin metathesis reactions occurring at the peptide side chains after the peptide skeletons are fully assembled (Figure 1). Limited types of disulfide surrogates can be generated with the above post-chain-assembly cyclization strategy, because many bridge formation reactions are not readily compatible with the peptide backbones. Moreover, when more than one disulfide surrogate need to be installed, the post-chain-assembly cyclization method may sometimes encounter difficulties in controlling the regioselectivity of the cross-linking reactions.[5]

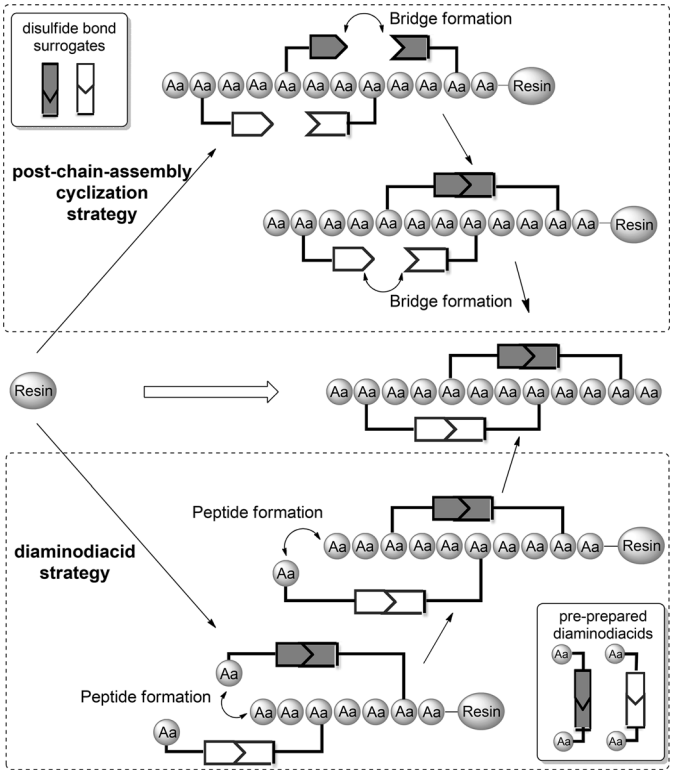

Figure 1. Two strategies for making disulfide-bond surrogates.

In our studies on bioactive peptidic macrocycles[6] we need to generate disulfide surrogates with a higher degree of structural diversity. Thus we became interested in the use of pre-prepared diaminodiacids in the synthesis of peptide disulfide bond mimics (Figure 1). In this strategy all cyclization steps are achieved through peptide coupling reactions, so that the difficulty for making nonpeptidic bridges on peptides can be avoided. Indeed, a few previous studies showed that the diaminodiacid strategy was useful for the synthesis of bioactive disulfide-bond mimics, albeit hitherto containing only one disulfide bridge.[7] To explore this strategy for peptides bearing two or more disulfide surrogates, we now describe the synthesis and bioactivities of thioether and biscarba analogues of tachyplesin I (TPI-1), which is a 17-residue bicyclic peptide with high antimicrobial activity.[8] TPI-1 adopts a β-hairpin conformation stabilized by two cross-strand disulfide bonds. Meldal and Holland-Nell recently showed that TPI-1 analogues with triazole bridges

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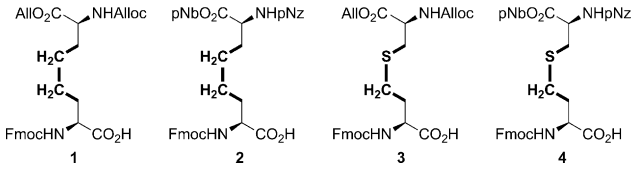
can largely maintain the biological activity.[4i] Here, we found that the folding and activity of TPI-1 analogues were rather subtly sensitive to the structure of the disulfide surrogates, thus underlining the necessity of a flexible synthetic route for generating disulfide bond surrogates with high structural diversity.

To implement the diaminodiacid approach for the synthesis of TPI-1 analogues with double disulfide surrogates, we took inspiration from the recent successful total synthesis of lantibiotics pioneered by the research groups of Vederas[9] and van der Donk.[10] Two sets of orthogonally protected diaminodiacids compatible with Fmoc (9*H*-fluorenyl-methoxycarbonyl) solid-phase peptide synthesis (SPPS) were prepared first (Scheme 1). The allyl/allyloxycarbonyl (Alloc)



Scheme 1. Pre-prepared orthogonally protected diaminodiacids.

and *p*-nitrobenzyl (pNb)/*p*-nitrobenzyloxycarbonyl (pNz) protecting groups were employed, because they can be selectively removed by using $[Pd(PPh_3)_4]/PhSiH_3$ and $SnCl_2$/HCl, respectively. From protected L-glutamic acids we synthesized the biscarba diaminodiacids (**1** and **2**) through Kolbe electrolytic decarboxylative cross-dimerization.[11] From protected L-homoserine and L-cystine we made the thioether-containing diaminodiacids (**3** and **4**).[7]

With the diaminodiacid building blocks in hand, we commenced the synthesis of biscarba TPI-1 analogue TPI-2 (Scheme 2) by using the Rink amide AM resin (loading = 0.3 mmol g^{-1}). A linear octapeptide **5** containing both **1** and **2** was first assembled onto the resin by using HCTU (5-chloro-1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate) as the coupling reagent. Subsequently we removed the Alloc and allyl groups on **5** by using $[Pd(PPh_3)_4]/PhSiH_3$. After the N-terminal Fmoc group was removed with 20% piperidine in DMF, peptide **6** was obtained by lactamization using PyBOP ((benzotriazole-1-yloxy)-tripyrrolidino-phosphonium hexafluorophosphate), HOBt (1-hydroxy-1*H*-benzo-triazole), and NMM (*N*-methylmorpholine). Next, three more amino acids (i.e. Val, Arg, Phe) were assembled onto the peptide leading to **7**. The pNb and pNz groups on **7** were removed by using $SnCl_2$ (6 M)/HCl (1.6 mM). After removal of the N-terminal Fmoc group, peptide **8** was obtained by lactamization using PyBOP/HOBt/NMM. Finally, Trp and Lys were attached to **8** producing the bicyclic peptide **9**. After acidic cleavage from the resin with concomitant removal of all the protecting groups, we obtained crude TPI-2.

According to HPLC analysis, the crude product contained a single major component (Figure 2), which can be easily purified to the desired product (TPI-2) with an overall yield of isolated product of 7%. Thus, the diaminoacid strategy can readily afford the desired disulfide mimics with fairly good efficiency. By using the same approach we also prepared the thioether-containing TPI-3 with an overall yield of 5% after isolation (Table 1). Note that in the recent preparation of a triazole-bridged TPI-1 analogue through Cu^I-mediated

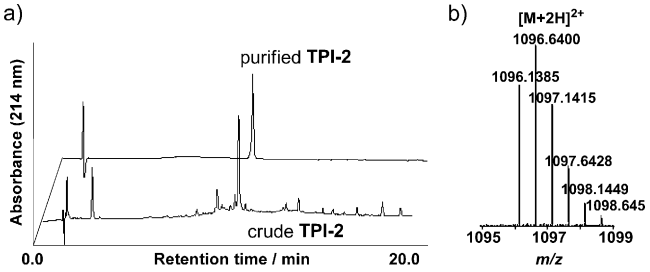
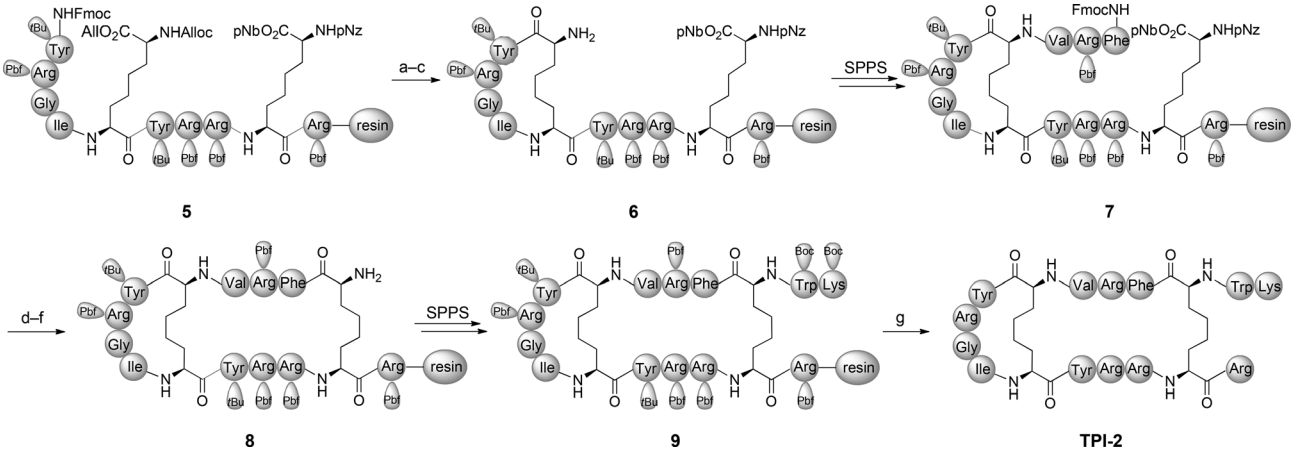
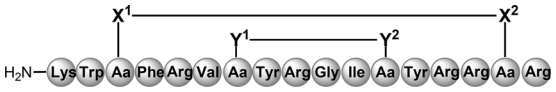


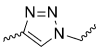
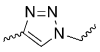
Figure 2. a) HPLC traces of crude and purified TPI-2. b) ESI-QTOF-MS spectrum of TPI-2 (expected mass: 2190.2551).



Scheme 2. Solid-phase synthetic route for the preparation of TPI-2 through the diaminodiacid strategy. a) $[Pd(PPh_3)_4]/PhSiH_3$, b) 20% piperidine/DMF, c) PyBOP/HOBt/NMM, d) $SnCl_2$ (6 M)/HCl (1.6 mM), e) 20% piperidine/DMF, f) PyBOP/HOBt/NMM, g) trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5; *v*/*v*/*v*). The following protecting groups for amino acid side chains were used: *tert*-butyl (for Tyr), 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf; for Arg), and *tert*-butyloxycarbonyl (Boc; for Trp and Lys).

Table 1: Structures of different TPI analogues and their minimal inhibitory concentrations (MIC) against several bacterial strains.[a]



Compound	X^1	X^2	Y^1	Y^2	*B. subtilis*	*S. epidermidis*	*S. enterica*	*P. aeruginosa*	*E. coli*
TPI-1	S	S	S	S	4 (>200[15a])	4 (2.3[15b])	8 (16.2[15a])	16 (8.2[15a])	8 (11.5[15a])
TPI-2	CH_2	CH_2	CH_2	CH_2	8	32	64	256	16
TPI-3	S	CH_2	S	CH_2	16	16	64	128	16
TPI-4	CH_2	CH_2	S	S	8	16	32	64	16
TPI-5	S	S	CH_2	CH_2	8	16	64	64	16
TPI-6	S	CH_2	S	S	8	8	64	64	64
TPI-7	S	S	S	CH_2	8	4	32	16	8
TPI-8	S	S	CH_2	S	16	16	32	64	32
TPI-9	CH_2	S	S	S	4	4	16	16	8
TPI-triazole[4i]					5.5[4i]	8.0[4i]	–	–	10.0[4i]
TPI-triazole′[4i]					4.5[4i]	10.5[4i]	–	–	7.0[4i]

[a] MIC values were obtained by using the standard two-fold dilution protocol and are reported in $\mu g\,mL^{-1}$.

cycloaddition, two cyclic products were obtained with a regioselectivity of 1:1.5 (correct/incorrect).[4i] This problem may cause complications in both the isolation and structure determination of the desired product, thus manifesting the advantage of the diaminodiacid strategy. In contrast, a high degree of selectivity for the intramolecular cyclization versus intermolecular amidation in the diaminodiacid strategy may result from the pseudo-high dilution of the resin-bound peptides.

The circular dichroism (CD) spectrum of TPI-1 in water showed a positive peak at 198 nm, a negative minimum at 208 nm, and a strong positive band at 228–230 nm (Figure 3a). These observations were consistent with the well-defined β-pleat sheet structure reported for TPI-1.[12] In contrast, for TPI-2 we only observed a small negative minimum at 212 nm indicating a less-ordered β-turn conformation. More strikingly, for TPI-3 there was no negative minimum in the CD spectrum, thus suggesting a significant deviation from the β-turn structure. To corroborate the above conclusions, we conducted solution NMR spectroscopy (including DQF-COSY, TOCSY, and NOESY) for TPI-2 and TPI-3 (The NMR structure for TPI-1 was reported[13]). The determined solution structures revealed that while TPI-1 adopted an ordered β-pleat sheet structure (Figure 3b), almost all the carbonyl oxygen atoms of TPI-2 and TPI-3 pointed outward, disfavoring the formation of any interstrand hydrogen bonds (Figure 3c,d). Thus both CD and NMR spectroscopy studies indicated that TPI-2 and TPI-3 did not adopt the authentic β-hairpin structure.

The biological activity of the TPI-1 analogues was studied with antimicrobial assays. Several bacterial strains were grown in the presence of increasing concentrations of the TPI-1 analogues. These strains included two Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus epidermidis*) and three Gram-negative bacteria (*Salmonella enterica*, *Pseudomonas aeruginosa*, and *Escherichia coli*). The minimal inhibitory concentration (MIC) was determined by using a standard two-fold dilution protocol (Table 1).[14] It was found that TPI-1 exhibited comparable bactericidal activities against both Gram-positive and Gram-negative bacteria, an observation consistent with the previous studies.[15] Although the structures of TPI-2 and TPI-3 deviated from the ordered β-pleat sheet structure, they were still effective inhibitors of bacterial growth with MIC values about 2- to 16-fold lower than that of TPI-1. Note that some slightly better MIC values were reported by Meldal and Holland-Nell for the triazole-containing TPI-1 analogues (TPI-triazole and TPI-triazole′, Table 1).[4i] They also reported that neither the linear

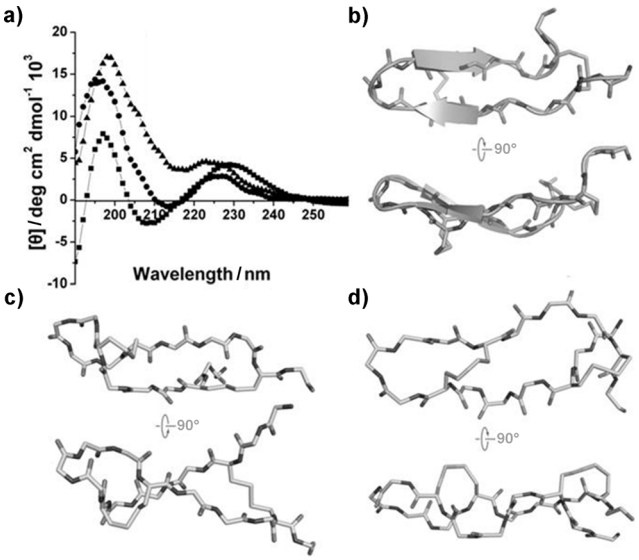


Figure 3. a) CD spectra of TPI-1 (■), TPI-2 (●), and TPI-3 (▲) in water and b–d) structures for TPI-1 (b), TPI-2 (c), and TPI-3 (d) determined by NMR spectroscopy in aqueous solution.

nor the incorrectly cyclized analogues of TPI-1 showed any significant antimicrobial activity.[4i] Collectively, these results indicated that the overall hairpin-like shape, rather than the details of the β-sheet hydrogen bonding patterns, played a key role in determining the bioactivity of TPI-1 analogues. It is important to note that the installation of biscarba or thioether surrogates in TPI-1 improved its stability. Indeed, TPI-1 was completely reduced in one hour in aqueous dithiothreitol (DTT, 1 mM) solution, while TPI-2 and TPI-3 showed no degradation in DTT solution after 48 h. Furthermore, the serum stability experiments revealed that 69% of native TPI-1 was left after 24 h, whereas during the same time 85% of biscarba analogue TPI-2 remained intact.

To scrutinize why the change of disulfide bonds to the thioether and biscarba surrogates caused significant deviations from the β-hairpin structure, we measured the dihedral angles of the cross-strand bridges in the NMR structures. For TPI-1, the dihedral angles between the C–S–S–C atoms were 94.2° (inner bridge) and 97.2° (outer bridge) degrees, respectively. This observation was consistent with the notion that the dihedral angle of the disulfide bond in proteins is usually close to 90°.[16] The dihedral angles of the two bridges in TPI-2 were 59.0° (inner) and 174.7° (outer), whereas for TPI-3 the values were 49.4° (inner) and 143.1° (outer). Such large changes of dihedral angles may indicate that the different patterns of torsional strain among the S–S, C–C, and S–C bonds led to the variation of the global conformation.

One possible solution to alleviate the above problem was to use only one disulfide surrogate in the TPI-1 analogues. It is also interesting to learn which of the two disulfide bonds in TPI-1 was more important for maintaining the β-hairpin structure. Thus we designed and synthesized TPI-1 analogues with only the inner or outer disulfide bond replaced by one biscarba or thioether bridge (TPI-4 to TPI-7, Table 1). To test whether or not the orientation of the thioether bond exerted any significant effect, we also prepared TPI-8 and TPI-9 (Table 1). Our measurements revealed that the MIC values of TPI-4 to TPI-9 were fairly close to each other. These values were more resembling to those of TPI-1, thereby suggesting that TPI-4 to TPI-9 were more active than TPI-2 or TPI-3. In particular, the activity of the thioether-bridged TPI-9 was identical to that of TPI-1 for four strains of bacteria (i.e. *Bacillus subtilis*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Escherichia coli*). Thus the installation of only one disulfide surrogate indeed caused less activity change than the replacement of two disulfide bonds. Unfortunately, our data did not give a clear hint concerning the relative importance of the inner versus outer disulfide bridges.

To examine the structural distortion owing to the installation of disulfide surrogates, we measured the backbone Hα and amide H(N) chemical shifts of TPI-1 to TPI-9. Because the proton chemical shift value is sensitive to the chemical environments around the proton, the change of these values from the parent compound to its analogues may reflect the extent of structural variation. In our NMR experiments, proton assignments were achieved for seven peptides (TPI-1, 2, 3, 4, 5, 7, and 9). The root-mean-square deviations of the backbone Hα chemical shifts of 16 residues for TPI-2, 3, 4, 5, 7, and 9 (Figure 4a; note: Lys1 was exposed to the solvent molecules and subject to fast proton exchange) were found to be 0.58, 0.49, 0.40, 0.51, 0.15, 0.37 ppm. Moreover, the root-mean-square deviations of the backbone amide H(N) chemical shifts of 16 residues for TPI-2, 3, 4, 5, 7, and 9 (Figure 4b) were 0.56, 0.56, 0.32, 0.52, 0.13, 0.31 ppm, respectively. Thus, the structures of TPI-4, 7, and 9 were more similar to the structure of TPI-1 than to TPI-2 and 3. This observation confirmed our expectation that the installation of only one disulfide surrogate caused less structural changes than the replacement of two disulfide bonds.

To summarize, we examined for the first time the use of a diaminodiacid-based strategy for the solid-phase synthesis

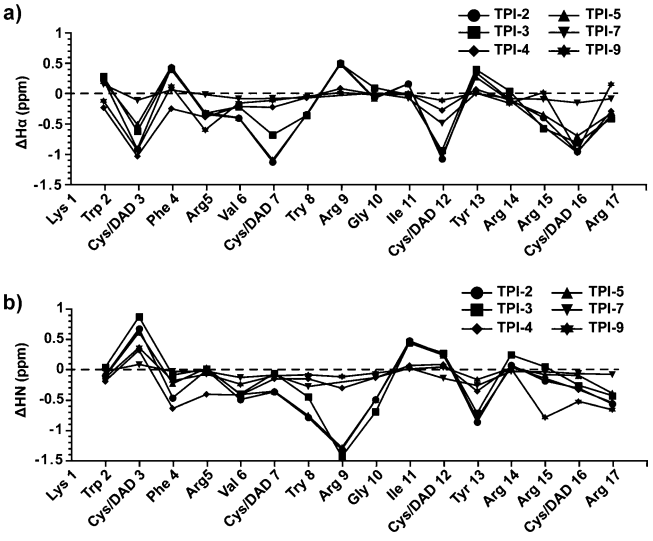


Figure 4. The chemical shift differences of backbone Hα (a) and amide H(N) (b) between the wild-type peptide (TPI-1) and modified peptides (TPI-2, 3, 4, 5, 7, and 9). DAD = diaminodiacid.

of peptide disulfide bond mimics with two cross-linking bridges. With the β-hairpin antimicrobial peptide tachyplesin I as a model, we showed that the diaminodiacid strategy can readily generate the desired disulfide surrogates with good yields and reliable structural control. Both the CD and NMR spectroscopy studies indicated that the biscarba and thioether-containing TPI-1 analogues did not adopt the authentic β-hairpin structure owing to the disruption of interstrand hydrogen bonding. Nonetheless, these analogues were still effective antimicrobial peptides, thus indicating that the overall hairpin-like shape, rather than the details of the β-sheet conformation, dominated the bioactivity of these compounds. More detailed analysis showed that the dihedral angles of the disulfide, biscarba, or thioether bridges in TPI-1 analogues varied dramatically, thereby indicating that the different torsional strains of the S–S, C–C, and S–C bonds may cause the structural distortions. Thus, to pinpoint the optimal peptide disulfide bond mimics, it is important to make and screen an array of disulfide bond surrogates ideally with high structural diversity. Although the present study has only examined the biscarba- and thioether-based bridges, we expect that the preparation and use of diverse structures of diaminodiacids would be practical.

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