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Communication

Peptide Cyclization

Fine-Tuning the Balance between Peptide Thioester Cyclization and Racemization

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Abstract: Ring-closure towards seven-membered bislactams containing an α-amino acid and β-alanine is problematic. Such difficult lactamizations are accompanied by side-reactions such as hydrolysis, oligomerization and racemization. By starting from linear peptide 4-methoxythiophenol esters, dilution to 1 mM in combination with phosphate buffer (pH 6.8) intermolecular reactions and racemization could be largely suppressed. Thioesters with the chiral residue at the C-terminus gave the bislactams in yields up to 60 % and enantiomeric excess up to 99 %. Enantiopure bislactams were obtained exclusively from the reversed sequence bearing β-alanine at the C-terminus.

Introduction

Since the discovery of the antibiotic cyclic decapeptide gramicidin S[1] in 1942, pharmacognostic and synthetic research gave rise to numerous cyclic peptides of which some are used nowadays as therapeutics.[2] Striking examples are valinomycin[3] (the most potent agent against SARS), cyclosporin A[4] (immunosuppressant drug in organ transplantation), nisin[5] and calcitonin.[6] Others are used for cancer treatment[7] and some larger cyclic peptides have shown their potential as drugs against Parkinson's disease.[8]

Cyclic peptides provide several advantages over their linear analogs, such as: 1) better stability towards endo- and exoproteases, due to lack of N- and C-termini, 2) better membrane permeability (less or no charge per peptide sequence) and 3) better interactions with target receptors due to the rigid backbone structure.[9,10] This makes cyclic peptides attractive in the field of drug design.[11]

Despite their relevance in pharmaceutical research, some small cyclic peptides (especially 3–5 amino acid residues) are still not readily accessible.[12] Availability from natural sources is rather low and cannot provide access to a variety of peptides differing in ring-size and sequence. The current synthetic methods offer solutions but they have to overcome a few key points, such as for example ring-strain, (cyclo)-oligomerization, and epimerization of the activated C-terminal amino acid prior to cyclization. An amide bond rather adopts *trans* than *cis* geometry. Therefore, cyclization of larger flexible peptides is possible because of their ability to adopt conformations that allow ring closure.[13,14] On the other hand, the N- and C-termini of smaller linear peptides (especially dipeptides containing β-alanine or tetra- and pentapeptides) are quite remote because of the prevalent extended conformation preventing formation of the cyclic monomer. Auxiliary mediated approaches partly overcome cyclization-step problems but still suffer from several disadvantages such as a site of the ring-closure (i.e. peptide sequence) dependency, or harsh conditions required for auxiliary removal.[15] Cyclization can also be facilitated by backbone amide-bond alkylation promoting the amide bond into a *cis* conformation.[16] However, this method suffers from serious limitations regarding alkyl group removal.[15b] Also the very powerful intramolecular Staudinger ligation[17,18] can provide access to medium-sized cyclic peptides. Generally, in difficult cyclizations of peptides bearing a chiral amino acid at the C-terminus epimerization remains a key aspect. Activation of a peptide C-terminal chiral amino acid increases the risk of partial epimerization. As a result, difficult peptide cyclizations require a subtle fine-tuning of activation that just allows lactamization but avoids epimerization. Recently, we have shown that even with the very epimerization-prone phenylglycine at the C-terminus, no epimerization was observed in a native chemical ligation (NCL) strategy in which a thioester intermediate is involved.[19] This prompted us to study the usefulness of C-terminal peptide thioesters in direct epimerization-free lactamization reactions to small cyclic peptides that are reluctant to ring-closure. To the best of our knowledge, except a few 1,4-diazepane-2,5-diones (cyclo-[βAla-Phe] and cyclo-[βAla-Trp]) no other examples of enantiopure homodiketopiperazines containing unsubstituted β-alanine and proteogenic amino acids are known yet.[20] Here, we report the conditions to minimize epimerization during direct peptide thioester cyclization towards seven-membered cyclic dipeptides containing β-alanine and α-amino acids. The straightforward synthesis of enantiopure linear precursors, mild reaction conditions and aqueous medium makes this a suitable method for peptide cyclizations via activation of a chiral C-terminal amino acid as a thioester.

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Results and Discussion

Inspired by the robustness of NCL[21,22] as developed by Dawson and Kent[23] and on our recent work[19] showing that even *C*-terminal phenylglycine may be used in epimerization free NCL, we initiated studies to the synthesis of seven-membered cyclic peptides (homodiketopiperazines) by direct thioester lactamization in an aqueous buffered medium.[24,25] Interestingly, very recently Albericio and co-workers published a complementary study that demonstrated the synthesis of cyclic hexapeptides from thioesters in aprotic solvent, which in some cases required the separation of epimers.[26] As a starting point we chose Boc-βAla-Phe-SPh **1a**, a sequence that is known to be very reluctant to ring-closure. In addition, phenylalanine was chosen for its sensitivity to racemization. To our surprise, despite the previously mentioned difficulties reported by us, we found that, after Boc-removal, thioester **1a** was converted into the desired monocyclic product *cyclo*-[βAla-Phe] **2** in substantial amounts (Scheme 1). Upon standing of Boc-deprotected thioester **1a** in a THF/aqueous buffer solution at pH 6.8 for 4 days the monocyclic product could be isolated in 32 % yield.
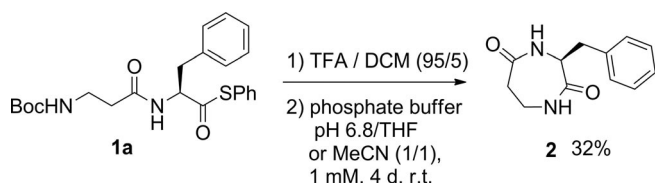
Scheme 1. Direct peptide thioester cyclization.

The difficulties arise because in seven-membered bislactams both amide bonds possess mainly the *cis* conformation implying that ring closure must follow isomerization of the amide bond in the linear dipeptide thioester from predominant *trans* to *cis*.[18] Most probably, dilution to 1 mM in combination with phosphate buffer (pH 6.8) successfully prevented intermolecular reactions since only a small fraction of the peptide *N*-terminus becomes deprotonated, gaining time for amide isomerization.

For our lactamization studies to optimize the yield without sacrificing stereochemical integrity, we aimed to start with enantiopure thioesters. Even though purifications of thioesters **1a** synthesized by HATU, EDC and phosphonium coupling reagents were very straightforward (Table 1, entries 1–4), enantiopure products were obtained exclusively by DCC/HOBt activation, showing the importance of slightly acidic conditions to avoid racemization (entry 5).

Probably the basic reaction conditions of entries 1–3 caused racemization either by an oxazolone or thioester enolate intermediate (vide infra). Because we realized that the success of the lactamization to give seven-membered bislactam **2** relies on the subtle thioester activation also the enantiopure phenyl thioesters **1b**, **c** were made bearing an electron-donating 4-MeO group and an electron-withdrawing 4-Cl atom in yields of 60 % and 30 %, respectively (entries 6, 7). In addition, using the same method the aliphatic benzyl thioester **1d** was made in 82 % yield albeit with an *ee* of 91 % (entry 8).

With the precursors in hand, we optimized the reaction conditions for efficient cyclization and minimizing racemization by

Table 1. Thioester synthesis.

Entry	R	Coupling reagent	Conditions	Thioester yield/*ee* [%]
1	Ph	HATU	DIPEA, THF, r. t.	68/60
2	Ph	PyBrOP	DIPEA, DCM, r.t.	25/91
3	Ph	PyBOP	DIPEA, DCM, r.t.	76/92
4	Ph	EDC/HOBt	EtOAC, −10 °C → r.t.	10/93
5	Ph	DCC/HOBt	EtOAC, −10 °C → r.t.	80/99, **1a**
6	4-MeOC$_6$H$_4$	DCC/HOBt	EtOAC, −10 °C → r.t.	60/>99, **1b**
7	4-ClC$_6$H$_4$	DCC/HOBt	EtOAC, −10 °C → r.t.	30/>99, **1c**
8	Bn	DCC/HOBt	EtOAC, −10 °C → r.t.	82/91, **1d**

using phosphate buffered aqueous media (Table 2). Initially, upon standing of **1a** at pH 6.8 for 5 days, a remarkably high isolated yield of **2** up to 60 % was obtained, although accompanied by significant loss in optical purity to 70 % *ee* (entry 1). Warming the reaction mixture to 50 °C resulted in a lower yield caused by faster thioester hydrolysis accompanied by an even lower *ee* of 40 % only (entry 2). Slightly raising the pH to 7.25 gave bislactam **2** in both lower yields and *ee* of 52 % and 64 %, respectively (entry 3). A further increase of the pH to 7.6 promoted racemization resulting in an *ee* of **2** of 48 % (Table 2, entry 4). At pH 7.25, the electron-donating 4-MeO substituent improved the *ee* to 85 % although the bislactam was isolated in a yield of 40 % at maximum only (entry 5). Starting from the same precursor at pH 6.8, bislactam **2** was obtained in a slightly improved isolated yield of 44 %. More importantly, no loss of *ee* was observed from the chiral HPLC trace (entry 6). The electron-withdrawing 4-Cl substituent that proved to be optimal for peptide thioester cyclizations in previous work by the Albericio group gave in the slow lactamizations towards the seven-mem-

Table 2. Optimization of the reaction conditions for the direct peptide thioester cyclization towards *cyclo*-[βAla-Phe].

Entry	R	pH	T [°C]	Yield[a]/*ee* [%]
1	Ph[b]	6.8	18	60/70
2	Ph	6.8	50	53/40
3	Ph	7.25	21	52/64
4	Ph	7.6	21	47/48
5	4-MeOC$_6$H$_4$	7.25	21	40/85
6	4-MeOC$_6$H$_4$	6.8	21	44/99
7	4-ClC$_6$H$_4$	6.8	21	54/70
8[c]	Bn	6.8	21	no reaction
9[c]	Bn	7.25	21	no reaction

[a] Isolated yields. [b] HCl in dioxane was used instead of TFA. [c] Boc-βAla-ᴅPhe-SBn was used as the starting material.

bered bislactam **2** substantial loss in *ee* down to 70 % (entry 7). By starting from the unreactive alkyl-type benzylic thioester both at pH 6.8 and 7.25 no formation of bislactam **2** was observed even upon standing for 11 days (entry 8 and 9).

At this stage, we set out to study where in the sequence of events loss of stereointegrity occurs. To rule out the possibility that racemization took place during TFA-mediated removal of the *N*-terminal Boc-protective group of the linear precursors we synthesized a similar acid-stable *N*-Cbz protected thioester and exposed it to TFA/DCM (95:5) and subsequent evaporation and prolonged heating on a rotavap (not shown). As expected, no change in *ee* was observed. To study the configurational stability under basic conditions, Boc-βAla-ᴅPhe-SPh (97.4 % *ee*) was subjected to DiPEA at 0.004 ᴍ and 0.4 ᴍ concentrations in THF. Upon standing for six days, a drop of *ee* was observed to 95.7 % and 85.5 %, respectively, indicating the sensitivity to base (Figure 1).
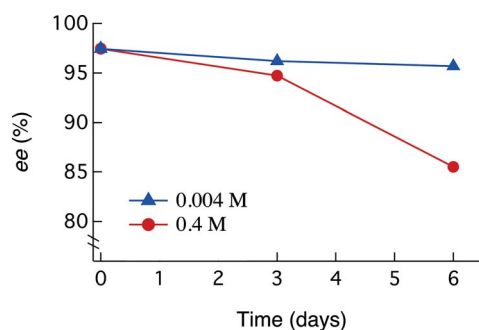

Figure 1. Rate of racemization of peptide thioester Boc-βAla-ᴅPhe-SPh (1 mᴍ) in THF containing 0.004 ᴍ and 0.4 ᴍ DiPEA base in time.

Remarkably, the *ee*s of the lactams derived from H₂N-βAla-ᴅPhe-SPh upon standing in aqueous buffered solutions were significant lower (entries 1–4, Table 2). When Boc-protected thioesters **1a**, **1b** and **1c** were left standing in the buffer/THF (pH 7.25) solution for six days, a significant drop in *ee* was observed only for the 4-Cl-phenyl thioester **1c** (Figure 2).
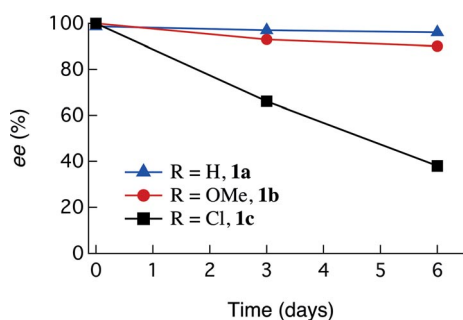

Figure 2. Rate of racemization of terminal *N*-Boc-protected peptide thioesters **1** in THF/0.2 ᴍ buffer pH 7.25, (1:1) in time.

Upon standing of *N*-terminal Boc-protected **1a** for six days in the buffer/THF mixture at pH 7.25 the *ee* was still 96 %. However, comparison with the significantly lower *ee* of 64 % of the cyclic product **2** (Table 2, entry 3), that was derived from **1a**, suggests that the free *N*-terminal amine also takes part in the racemization process as a specific base, probably via an intramolecular deprotonation–oxazolone formation sequence (Scheme 2). Similar findings were observed with the more elec-

tron-rich thioester Boc-βAla-ᴅPhe-4-MeO-SPh; in a buffer/THF mixture the *ee* value dropped from 98.5 % to 96 % over six days while *cyclo*-[βAla-ᴅPhe] was found in 85 % *ee* only (Table 2, entry 5). Involvement of the basic *N*-terminus can be envisioned in effecting oxazolone formation (Scheme 2, route **A**) or enolization (Scheme 2, route **B**) both leading to an achiral intermediate. It may be concluded that even using mild activation as a thioester and neutral conditions, during difficult/slow cyclizations of peptides bearing a chiral *C*-terminal residue partial epimerization may be inevitable.
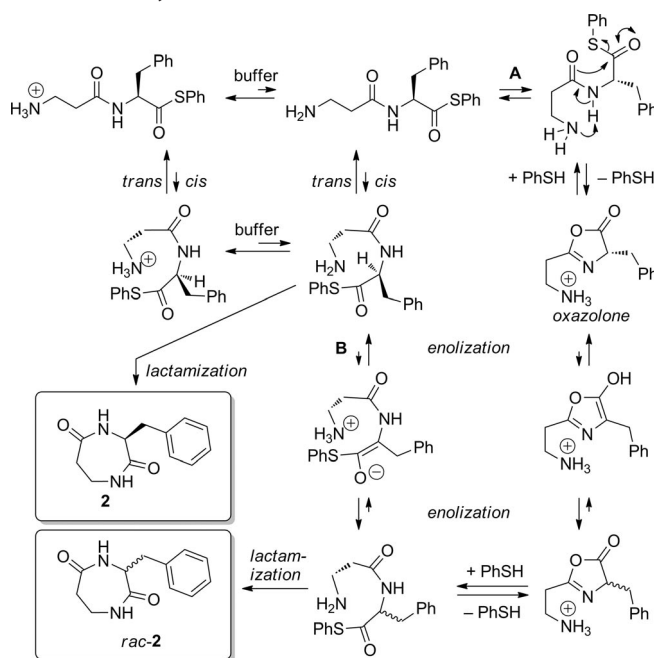
Scheme 2. Proposed cyclization and racemization mechanistic pathways from dipeptide thioester H₂N-βAla-Phe-SPh.

We also investigated the cyclization efficiency of the reverse peptide sequence bearing achiral β-alanine at the *C*-terminus thus avoiding racemization. A series of *C*-terminal β-alanine dipeptide thioesters were synthesized by solution phase peptide synthesis using HATU couplings avoiding DCU formation (see Supporting Information).

Both PhSH- and BnSH-derived peptide thioesters underwent cyclization in buffered solutions (pH 6.8) and afforded the enantiopure bislactams (Table 3). The obtained yield of 51 % for *cyclo*-[Phe-βAla] starting from H₂N-Phe-βAla-SPh (entry 1) was similar as for the reverse sequence (52 %, Table 2, entry 3). At a slightly elevated temperature also the benzyl thioester gave *cyclo*-[Phe-βAla] in about the same yield (entry 2). By starting from H₂N-Val-βAla-SBn a significant drop in yield of the bislactam was observed (entry 3). Even when starting from the more reactive SPh ester, for obtaining *cyclo*-[Leu-βAla] an elevated temperature was required (entries 4 and 5). The lower lactamization yields for these sequences incorporating Val and Leu may be explained by a combination of the more hindered *N*-terminus and faster hydrolysis of the less hindered thioesters. Indeed, less *N*-terminal steric hindrance in the linear lactamization precursors gave *cyclo*-[Trp-βAla], *cyclo*-[Lys(*Z*)-βAla] and *cyclo*-[Ser(Bn)-βAla], all in acceptable yields even at room temperature (entries 6–8).

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Table 3. Synthesis of enantiopure homodiketopiperazines.

Entry	R	T [°C]	Product	Yield [%][a]
1	Ph	r.t.	cyclo-[Phe-βAla]	51
2	Bn	50	cyclo-[Phe-βAla]	49
3	Bn	50	cyclo-[Val-βAla]	23
4	Ph	r.t.	cyclo-[Leu-βAla]	—[b]
5	Ph	50	cyclo-[Leu-βAla]	42
6	Bn[c]	r.t.	cyclo-[Trp-βAla]	63
7	Bn	r.t.	cyclo-[Lys(Z)-βAla]	45
8	Ph[c]	r.t.	cyclo-[Ser(Bn)-βAla]	52

[a] Isolated yields after flash chromatography. [b] Mainly cyclic dimer formed. [c] MeCN was used instead of THF.

It is important to note that the presence of buffer proved to be crucial for successful lactamization, because dimers and oligomers were barely detected.

Conclusions

Efficient synthetic methodology providing seven-membered bislactams containing an α-amino acid and β-alanine was developed. These lactams are difficult to access because the linear precursors are reluctant to ring-closure and therefore a good model for difficult peptide cyclizations. The synthesis of enantiopure racemization-prone peptide thioester lactamization precursors was achieved using DCC in respectable yields and very high ee values. Conditions were found that drive the delicate balance of the kinetics of lactamization, hydrolysis, racemization, and oligomerization to lactamization. During difficult lactamizations of peptides with a chiral C-terminal residue racemization can be avoided by choosing 4-methoxythiophenol ester activation at pH 6.8. Linear peptide thioesters seem to be a good choice as precursors obviating the need for additional auxiliaries or backbone-amide alkylation to facilitate ring closure. Studies are ongoing to further broaden the scope by introducing substituted β-alanines and moving towards larger cyclic peptides that are reluctant to ring-closure.

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