Enzymatic Cyclisation of Peptidomimetics with Incorporated (*E*)-Alkene Dipeptide Isosteres

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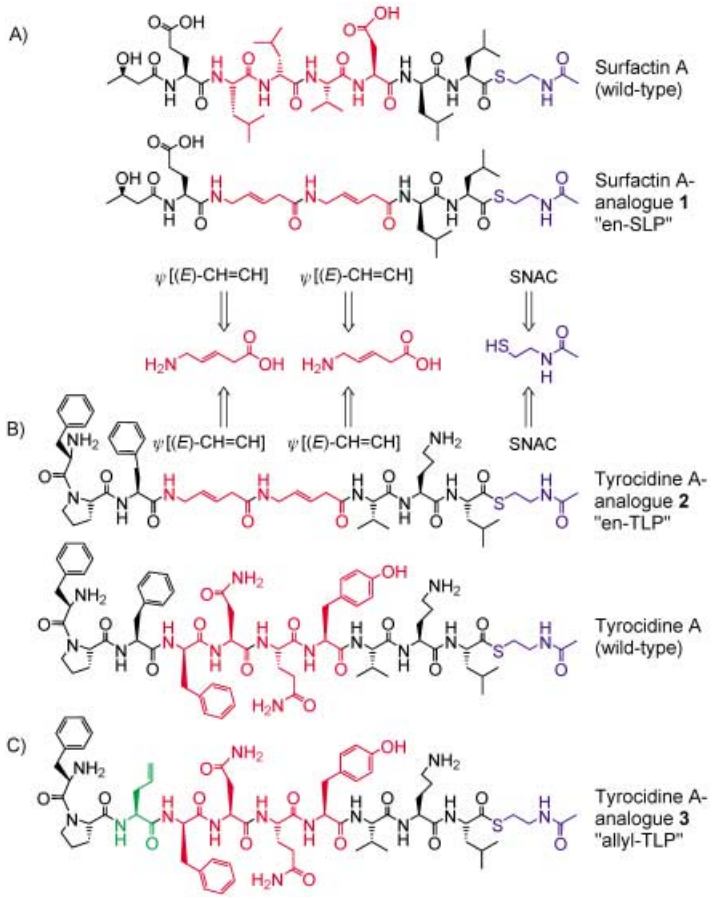

Scheme 1. *Comparison of the SNAC-peptides "en-SLP" (A), "en-TLP" (B) and "allyl-TLP" (C) with the corresponding linear wild-type sequences of the antibiotics surfactin A (A) and tyrocidine A (B/C). Black: recognition sequence for the cyclase, red:* (E)*-alkene dipeptide isostere and the replaced amino acids, blue:* N*-acetylcysteamine-based thiolester (SNAC), green: (2S)-2-amino-4-pentenoic acid.*

Naturally synthesised peptides, either gene-encoded or assembled on multifunctional peptide synthetases, exhibit a wide range of biological activities and find application for example as antibiotics and immunosuppressants.[1,2] There is a great interest in exploring natural peptidic scaffolds by introducing new structural elements in order to obtain novel compounds with desired activity or increased metabolic stability. It has previously been shown, in the case of peptide-based enzyme inhibitors,[3,4] that the substitution of normal peptide bonds by structurally quite similar,[5] noncleavable (*E*)-alkene bonds, which exhibit the same spatial orientation as the native ones, is a promising approach to achieve this goal. Therefore, we decided to explore the incorporation of such a structural element into the linear peptide precursors of the nonribosomal antibiotics surfactin A and tyrocidine A (Scheme 1) to test the possibility of subsequent cyclisation by the corresponding peptide cyclases (TE) Srf-TE[6] and Tyc-TE,[7] respectively. This peptidomimetic approach would not only enhance cyclic peptide stability, but would also allow for postsynthetic modifications of the functionalised C=C double bonds (e.g. glycosylation). The (*E*)-alkene bond belongs to a glycine–glycine dipeptide isostere, which forms a predominant part of the linear peptide precursor's backbone (red in Scheme 1). The N- and C-terminal residues incorporated into the peptides shown in Scheme 1 are necessary for the enzymatic recognition to catalyse the formation of a macrolactone (surfactin A)[8] or a macrolactam (tyrocidine A).[7] In order to achieve cyclisation of novel peptidomimetics by cyclases, acylation at the enzyme's active-site serine[8,9] has to be ensured by a small C-terminal thiolester leaving group (SNAC) (blue in Scheme 1).[7]

The glycine–glycine (*E*)-alkene dipeptide isostere (abbreviated as ψ[(*E*)-CH=CH]) was synthesised by a Schmidt rearrangement of (*E*)-3-hexenedioic acid according to an instruction by Allan and co-workers.[10] The subsequent coupling of a Fmoc protecting group to the amine function under standard condi-

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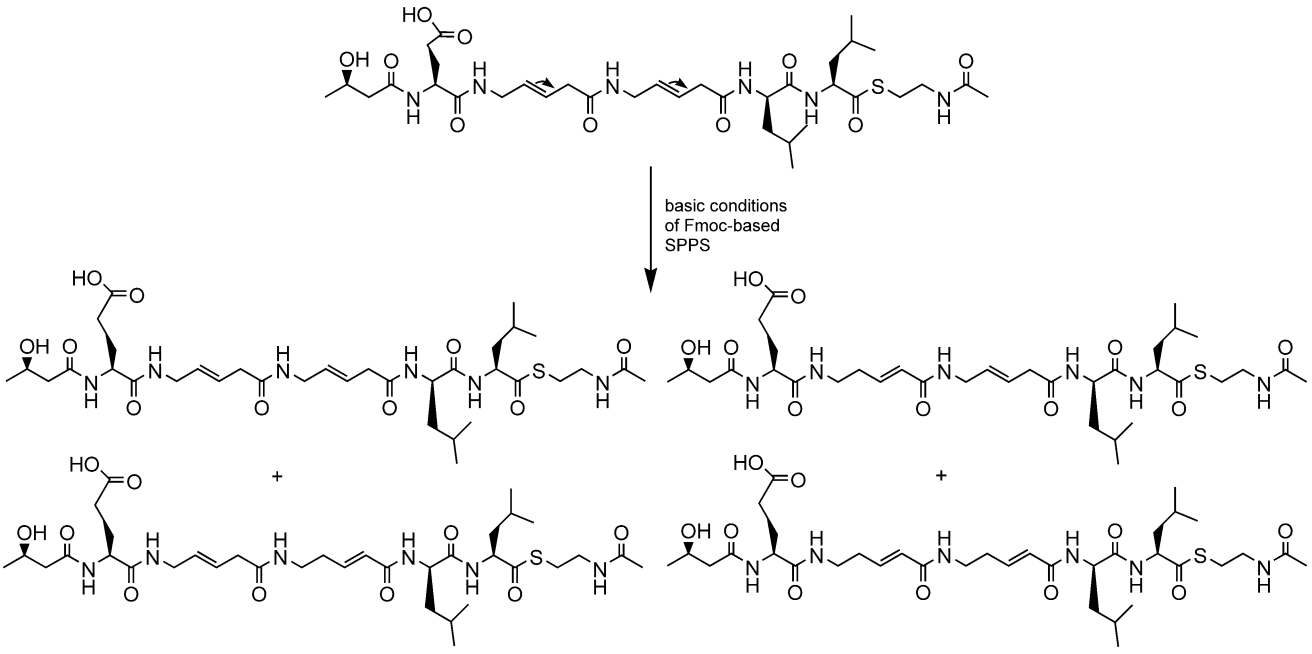
tions led us to a building block (41% yield over two steps), which could be incorporated into the backbone of the linear peptide precursor. For the building of the linear peptide precursor we used Fmoc/*t*Bu-based solid-phase peptide synthesis (SPPS). Here it was important, due to the later modification of the C terminus, that the amine function of the N-terminal amino acid was provided with a Boc protecting group. After cleavage from the employed 2-chlorotrityl chloride resin under mild acidic conditions (yield: 81%), the C-terminal part of the peptide was modified to give the thiolester mentioned above. Finally, all the protecting groups remaining on the peptide precursor were removed with a mixture of trifluoroacetic acid (TFA) and CH_2Cl_2 (1:1). All synthesised linear SNAC-peptide precursors were characterised by mass analysis with MALDI-TOF (see Supporting Information).

The first model we used to see whether such a linear SNAC-peptide can be enzymatically cyclised was based on the scaffold of the nonribosomal macrolactone antibiotic surfactin A (Scheme 1A). After the solid-phase peptide synthesis, we ended up with a mixture of different peptide precursors, since the C=C double bond isomerised under the basic conditions needed for Fmoc-SPPS (Scheme 2). The isomerisation is favoured by the establishment of a conjugated π-electron system of the C=C double bond with the neighbouring amide bond. As the structural differences of all possible isomers were very small, it was impossible to separate them from each other by reverse-phase HPLC. Therefore, we decided to try a cyclisation assay employing the mixture of the SNAC-peptide precursors (250 μM) at 25 °C as a substrate for the excised Srf-TE[6] (2.5 μM), which was dissolved in aqueous HEPES buffer (pH 7.0). After stopping the enzymatic activity by adding aqueous TFA, the assay was subsequently analysed by HPLC-MS. In addition to the hydrolysed substrate, which led to the corresponding acid, cyclised isomers were detected (Figure 1A). Since the peptide substrate was obtained as a mixture, we have not determined the ratio between the hydrolysis and the cyclisation products.

In order to overcome the isomerisation problem of the dipeptide isostere's double bond, we decided to change the conditions during the SPPS of the second model substrate "en-TLP", an analogue of the antibiotic tyrocidine A (Scheme 1B), in two ways. First, the time for the basic cleavage of the Fmoc protecting group with piperidine solution (20% *v*/*v* in *N*,*N*-dimethylformamide) was shortened from 20 to 2.5 minutes. Second, the amino acid coupling conditions were changed. Here a combination of two different approaches was applied: The former coupling reagents 1-hydroxybenzotriazole (HOBt), *N*-((1*H*-benzotriazol-1-yl)(dimethylamino)methylene)-*N*-methanaminium hexafluorophosphate *N*-oxide (HBTU) and diisopropylethylamine (DIPEA) were substituted by the reagents diisopropylcarbodiimide (DIC) and 1-hydroxy-7-azabenzotriazole (HOAt). Moreover, the whole N-terminal tripeptide with the necessary protecting groups (Scheme 1B) was coupled to the C-terminal part of the peptide, which was still bound to the resin. This strategy led us to SNAC-peptide **2**, which contained no isomerised C=C double bond. This was shown by $^{1}H,^{13}C$ NMR correlation spectroscopy and ^{13}C NMR analysis where the carbon atoms of the C=C double bond of the SNAC-peptide as well as of the dipeptide isostere showed the same chemical shifts (Figure 2).

The HPLC-MS analysis after incubation of the SNAC-peptide with the excised tyrocidine cyclase[7] (Tyc-TE) according to the procedure used for surfactin, resulted in a ratio of hydrolysis to cyclisation product of 1:1.6 (Figure 1B). The K_M and k_{cat} values for the hydrolysis reaction were 109 μM and 24 min^{-1} and for the cyclisation reaction 141 μM and 33 min^{-1}, respectively. The



Scheme 2. *Mixture of possible surfactin peptidomimetics after isomerisation of the C=C double bond of the dipeptide isostere(s), due to the basic conditions of the solid-phase peptide synthesis.*

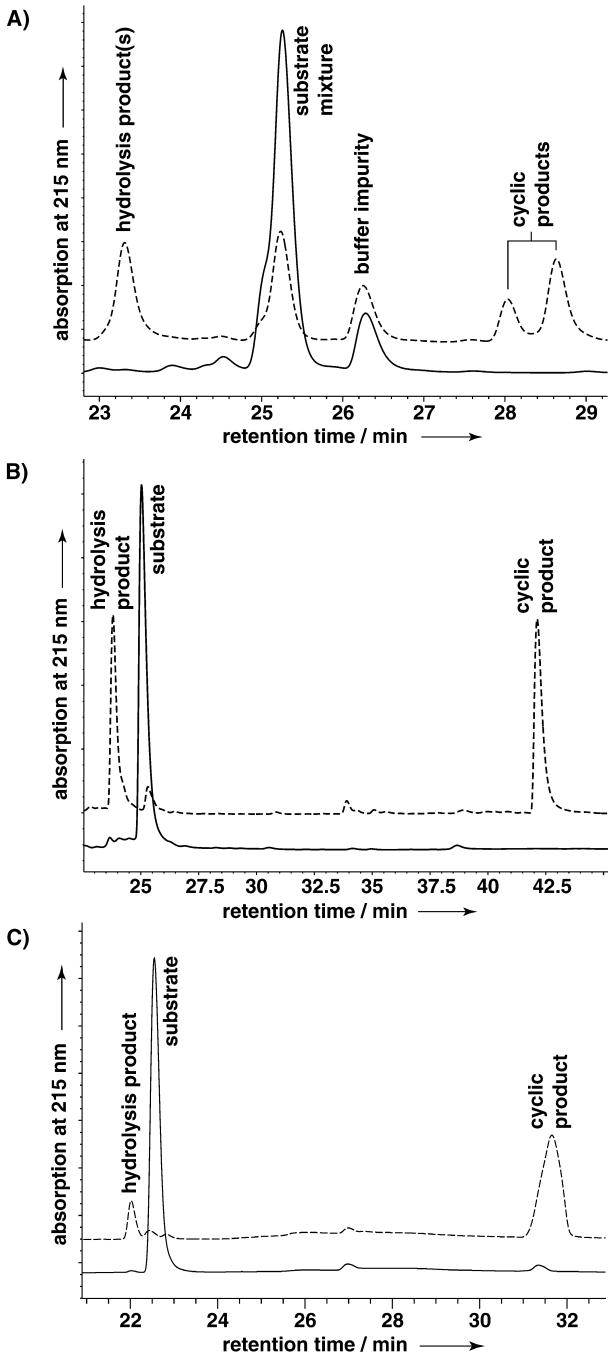


Figure 1. *HPLC traces of the cyclisation assays. A) en-SLP (time of the assay: 45 min). B) en-TLP (time of the assay: 20 min). C) allyl-TLP (time of the assay: 60 min). Solid line: assay without enzyme; dashed line: assay with cyclase. The products were identified by MS-analysis (see Supporting Information).*

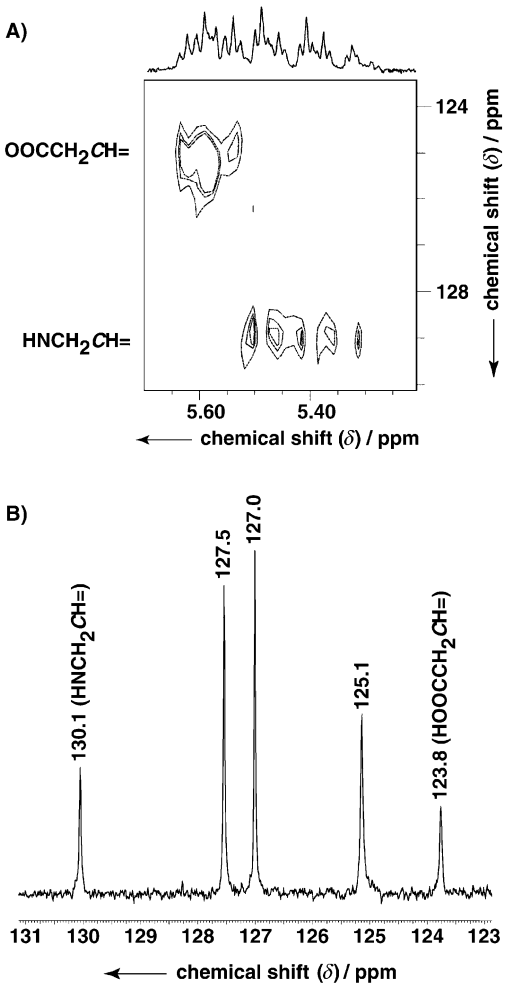


Figure 2. *Comparison of the chemical shift of the olefinic carbon atoms of the glycine–glycine dipeptide isostere incorporated into the backbone of en-TLP (A) and of the Fmoc-protected dipeptide isostere alone (B). A) $^{1}H,^{13}C$ NMR correlation spectrum, 125 MHz, [D_6]DMSO. B) ^{13}C NMR spectrum, 75 MHz, [D_6]DMSO.*

increased K_M and the decreased k_{cat} value of the peptidomimetic **2** in comparison with the native tyrocidine A[6] suggested a reduced substrate preorganisation by intramolecular hydrogen bonds, which was reported to be important for tyrocidine cyclisation.[11] The regioselectivity of the built macrolactam during the enzymatic cyclisation reaction was confirmed by MS/MS-fragmentation analysis (see Supporting Information).

In addition, an alternative strategy for postsynthetic modification of peptide antibiotics was tested. Bulky substituents such as large sugar moieties, which would affect substrate recognition and enzymatic cyclisation, could be attached to the functionalised double bond of the final cyclic product. Therefore, a second novel analogue of tyrocidine A called "allyl-TLP" (Scheme 1C) was tested. In this analogue, the amino acid at position 3 is replaced by (2*S*)-2-amino-4-pentenoic acid, which has a terminal C=C double bond (green in Scheme 1). The SNAC-peptide substrate **3** was synthesised by SPPS under the same conditions as used for the surfactin analogue **1** (see Supporting Information). The incubation of allyl-TLP with Tyc-TE revealed a hydrolysis-to-cyclisation ratio of 1:7.2 (Figure 1C); this demonstrated the preparative utility of this method.

In summary, we have introduced a new methodology for peptidomimetic synthesis by utilising SNAC-peptides, with a large part of the backbone substituted by dipeptide isosteres. These novel substrate analogues are specifically recognised by the corresponding excised peptide cyclases, and an effective cyclisation was observed. A procedure was developed to build up SNAC-peptide substrates by Fmoc/*t*Bu-based SPPS with incorporated (*E*)-alkene dipeptide isosteres, which do not suffer

from isomerisation of the C=C double bond. To increase the antibiotic activity of these novel cyclic peptidomimetics, further modification of the internal or terminal C=C double bonds can be envisioned. For this purpose several preparative, organic reactions can be utilised, for example, olefin cross metathesis or epoxidation and other addition reactions at an olefinic double bond. In contrast to previous attempts, where diversity was generated in the linear peptide sequence,[12] this approach allows libraries of cyclic compounds with scaffolds also arising from postsynthetic modifications to be synthesised and screened.

Experimental Section

Cyclisation assay: The linear SNAC-peptide substrate (250 μM) was incubated at 25 °C with the corresponding excised cyclase (2.5 μM) in HEPES-buffer (25 mM, pH 7.0, NaCl (50 mM); final volume: 50 μL). After different reaction times (en-SLP: 45 min, en-TLP: 20 min, allyl-TLP: 60 min), the enzymatic activity was quenched by the addition of aqueous TFA (35 μL, 4% *v*/*v*). All assays were analysed by HPLC-MS (Nucleodur 120-3 C18 column, 0.3 mL min^{-1}, 40 °C, 20–55% solvent B over 40 min, then 55–95% solvent B over 10 min; solvent A: bidestilled water with 0.1% (*v*/*v*) TFA, solvent B: acetonitrile with 0.1% (*v*/*v*) TFA).

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