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Short Communication

Synthesis of cyclic peptides and peptide libraries on a new disulfide linker

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Abstract: A new cysteine-based disulfide linker for Fmoc solid phase peptide synthesis was developed (Fmoc-Cys(3-mercapto-3-methylbutanoic acid)OPp) that allows the on-resin assembly and side chain deprotection of cyclic peptides. Model peptides and a cyclic peptide library of the structure [a-a-x-x-a-a-c] composed of D-amino acids were assembled and the synthesis and cleavage conditions studied. The best cyclization results were obtained with PyBOP/HOAt/diisopropylethyl amine. Racemization rates of the cysteine in the analyzed model sequences were between 5.2 and 12.3%. Cleavage of the disulfide bond was best carried out with DTT in 50% 2-propanol/100 mM ammonium bicarbonate. The cleaved peptides can be used directly in biological assays.

Keywords: peptide linker; disulfide linker; head-to-tail cyclization; on-resin cyclization

INTRODUCTION

A large number of methods for the chemical synthesis of cyclic peptides have been reported [1–3]. Especially elegant are procedures for the on-resin synthesis and cyclization, where the side chains can be deprotected and the side products washed away while the peptide is still attached to the solid support. If the unprotected peptides can be cleaved under mild conditions, they can be used directly in biological assays. Immobilization of the peptides has not only been carried out most frequently through an amino acid side chain but also through the backbone nitrogen [4–6].

Methods for the simultaneous cyclization and cleavage have also been developed [7–11]. The disadvantage here is the fact that the cleavage yields are quite variable depending on the sequence, and in cases were the cyclization is hampered the amount of liberated peptide can be very small, or even no free peptide is generated at all. This kind of approach then becomes problematic for the use of peptide libraries if uniform concentrations for biological screening are desired.

We were aiming at a method that would allow the synthesis and liberation of cyclic peptides and yield products that contain a side chain sulfhydryl group, which can be utilized for further selective modifications and also for site-specific immobilization purposes. The initial ring size was chosen to be seven amino acids. This size should allow efficient and reliable cyclization to occur with most sequences. With one defined amino acid and six variable positions, it also allows for a sufficient amount of complexity in the generation of peptide libraries. Furthermore, numerous natural and unnatural biologically active peptides are known, which consist of seven amino acids and/or contain rings with this number of amino acids.

Some reports about the solid phase synthesis of disulfide-anchored peptides have appeared previously. Virgilio *et al.* have reported on a method for the on-resin preparation and thioether cyclization of peptides as ß-turn mimetics [12], while Kemp and Galaktos reported a mercaptodibenzofuran moiety that was coupled as a disulfide to a solid support and that was utilized as a template for peptide bond formation by thiol capture [13]. Lack *et al.* described a disulfide linker that was used for the mass-spectrometric analysis of peptides from single beads that were prepared by the Fmoc strategy [14]. Surprisingly, in this study a di-ethyl-substituted disulfide structure ($-CH_2-CH_2-S-S-CH_2-CH_2-$) was employed, which in our hands and those of others was found to be labile under the basic conditions of Fmoc removal. Loss of peptide loading during chain assembly was not reported in this paper and apparently the remaining amounts were sufficient for mass analysis. Mery *et al.* reported on different disulfide linkers that were used for Boc [15] and Fmoc [16] peptide synthesis of linear peptides. The same group also provided information on a disulfide

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linker that contained a secondary hydroxyl function with which the first amino acid was esterified, again for the synthesis of linear peptides [17].

None of the described procedures seemed to be a straightforward solution for our requirements, and therefore we developed an alternative disulfide linker based on a special cysteine derivative. After evaluating and optimizing the synthesis conditions with a number of model peptides, a library of 361 different cyclic heptapeptides was synthesized on the linker in an automated multiple peptide synthesizer.

Since our aim was for a high biological stability of the synthesized peptides, we used D- rather than L-amino acids in all syntheses.

MATERIALS AND METHODS

Materials

Fmoc-D-Cys(tBu)-OH was purchased from Bachem AG, Bubendorf, Switzerland. 2-Phenylisopropyltrichloro acetimidate was prepared from 2-phenyl-2-propanol and trichloroacetonitrile according to [18] and used in its unpurified form. 3-Mercapto-3-methylbutanoic acid was prepared according to a literature procedure [19]. Npys-Cl is commercially available, but the quality was not reliable, probably due to the instability of the compound on storage. For that reason the compound was freshly prepared according to Ref. [20] through the disulfide 2,2′-dithiobis(3-nitropyridine) by treatment with sulfuryl chloride.

Peptides were synthesized on TentaGel S NH_2 (Rapp Polymere, Tübingen, Germany), particle size 90 μm, with initial loading of approximately 290 μmol/g.

Fmoc-D-amino acids and coupling reagents were from MultiSynTech, Witten, Germany.

Methods

Silica gel column chromatography was carried out on silica gel 'For Flash Chromatography' (J.T. Baker), particle size 40 μm. For optimal activity the material was heated at 140 °C for 12 h before use.

Analytical HPLC used a Phenomenex Jupiter 4 μ Proteo 90A, 2 × 50 mm column. For the analysis of products for the linker synthesis linear gradients from 5% acetonitrile in water to 100% acetonitrile in 20 min, both containing 0.1% trichloroacetic acid (TFA) were used, at a flow rate 0.7 ml/min and detection at 280 nm. Crude peptides were analyzed with the same solvents and flow rates at 220 nm. Preparative HPLC was carried out with linear gradients of methanol and H_2O (without TFA) at a flow rate of 25 ml/min on a 250 × 40 mm RP18 Nucleosil column, with detection at 280 nm. Details of the elution of the particular compounds are given in the corresponding synthetic procedure.

All solvents and chemicals used were at least of analytical grade.

The peptides were synthesized on a 5 μmol scale with a Syro I multiple peptide synthesizer (MultiSynTech, Witten, Germany), equipped with a reaction block containing 96 individual polypropylene reaction vessels of 1.4 ml volume with a polyethylene frit, arranged in a microtiter plate format.

Racemization analyses of model peptides were carried out by 'C.A.T. Chromatographie und Analysentechnik' (Tübingen, Germany) via GC-MS of deuterium labeled hydrolysis products.

Synthesis of Fmoc-D-Cys(tBu)-OPp (3, Scheme 1)

The phenyl isopropyl protection was introduced according to a general procedure [21]. Fmoc-D-Cys(tBu)-OH (5 g, 12.5 mmol) was dried in a desiccator over phosphorus pentoxide under vacuum for 16 h. The compound was dissolved in dichloromethane (DCM) (50 ml) and 2-phenylisopropyltrichloroacetimidate (5.23 g, 18.75 mmol) was added. Reaction control was carried out by thin-layer chromatography with cyclohexane/ethyl acetate 8 : 2 (R_f 0.6) or by analytical HPLC (elution of the product at 85% acetonitrile, the adduct elutes at 64%). After 1 h, the solid precipitate was filtered off and the solvent removed. The residue was taken up in cyclohexane/ethyl acetate 9 : 1 (5 ml) and purified by column chromatography on silica gel (150 g) with cyclohexane/ethyl acetate 9 : 1 as the eluent. Fractions of approximately 70 ml were collected and the compound was isolated in pure form in fractions 10–14 after evaporation as a light yellow oil. Yield 12 g (23 mmol, 92%). Mol. wt. 517.7. NMR ($CDCl_3$): 7.74–7.79 (2H, 2s, Fmoc), 7.59–7.62 (2H, 2s, Fmoc), 7.24–7.44 (9H, Fmoc & Ph), 5.57–5.63 (1H, d, NH), 4.62–4.70 (1H, ddd, CHα), 4.35–4.42 (2H, d, CH_2-Fmoc), 4.20–4.27 (1H, tr, CH-Fmoc), 3.01–3.15 (2H, dddd, $-CH_2-S$), 1.85–1.90 (3H, s, CH_3-iPr), 1.80–1.85 (3H, s, CH_3-iPr), 1.30–1.39 (9H, s, CH_3-tBu).

Synthesis of Fmoc-D-Cys(Npys)-OPp (4, Scheme 1)

To Fmoc-D-Cys(tBu)-OPp (1 g, 1.9 mmol) in DCM (20 ml) two equiv. of Npys-Cl in DCM (20 ml) were added. After stirring the mixture for 10 min at room temperature, methanol/DMF 1 : 1 (100 ml) was added. The solvents were evaporated to leave a viscous slightly yellow residue, which was used as such in the next reaction. For analytical purposes one of the products was purified by preparative HPLC with a linear gradient from 70% methanol/H_2O to 100% methanol in 60 min at a flow rate of 25 ml/min. Elution of the product occurred at 97–98% methanol. Molecular weight: 615.7. NMR ($CDCl_3$): 8.55–8.61 (1H, dd, Npys), 8.42–8.50 (1H, dd, Npys), 7.72–7.79 (2H, 2s, Fmoc), 7.52–7.60 (2H, 2s, Fmoc), 7.19–7.42 (10H, Fmoc, Ph, Npys), 6.87–6.93 (1H, d, NH), 4.56–4.64 (1H, ddd, CHα), 4.43–4.50 (2H, d, CH_2-Fmoc), 4.18–4.24 (1H, tr, CH-Fmoc), 3.50–3.59 (1H, dd, $-CH_2-S$), 3.23–3.32 (1H, dd, $-CH_2-S$), 1.72–1.80 (6H, s, CH_3-iPr).

Synthesis of Fmoc-D-Cys(3-S-3-methyl-butanoic acid)-OPp (5, Scheme 1)

The crude Fmoc-D-Cys(Npys)-OPp (approximately 2 mmol) was dissolved in DMF (20 ml) containing diisopropylethyl amine (2 mmol) and 3-mercapto-3-methylbutanoic acid (402 mg, 2 mmol). After 10 min the red solution was evaporated. The residue was redissolved in dioxane and the product was purified in fractions by preparative HPLC. Elution was at 93–96% methanol. Yield 3.67 g (6.84 mmol, 85%). Molecular

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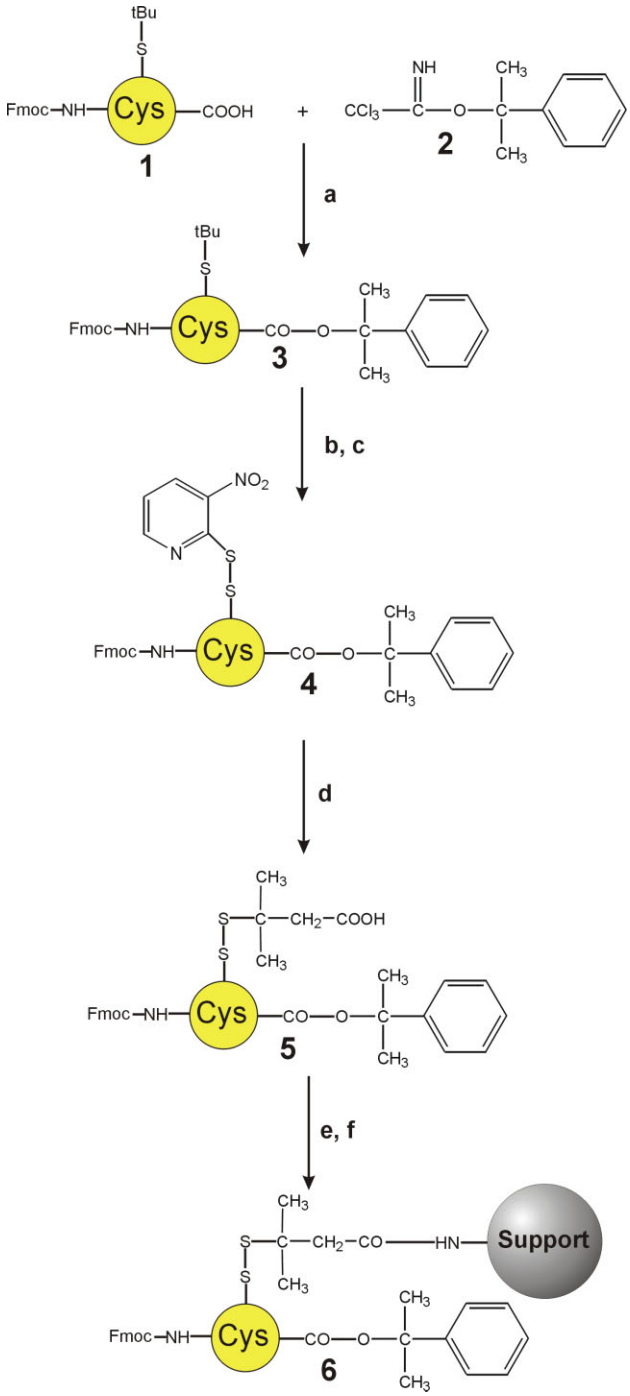

Scheme 1 Synthesis of the disulfide linker and coupling to an amino-support (all reactions performed at ambient temperature). **(a)** DCM, 1 h; **(b)** 3-nitro-2-pyridinesulfenyl chloride, DCM, 10 min; **(c)** excess methanol/dimethylformamide (DMF) 1 : 1; **(d)** 3-mercapto-3-methylbutanoic acid, 1 equiv. diisopropylethyl amine, DMF, 10 min; **(e)** TentaGel S NH_2 resin (max. 1 equiv. amino functions), TBTU, diisopropylethyl amine, DMF, 3 h; **(f)** 10% acetic acid anhydride/5% diisopropylethyl amine in DMF, 1 h. This scheme is available in colour online at www.interscience.wiley.com/journal/jpepsci.

weight 593.8. NMR: NMR ($CDCl_3$): 7.70–7.78 (2H, 2s, Fmoc), 7.53–7.61 (2H, 2s, Fmoc), 7.20–7.42 (9H, Fmoc, Ph), 5.60–5.69 (1H, d, NH), 4.61–4.71 (1H, ddd, CHα), 4.28–4.47 (2H, m, CH_2-Fmoc), 4.17–4.27 (1H, tr, CH-Fmoc), 3.28–3.39 (1H, dd, $-CH_2-$S), 3.14–3.26 (1H, dd, $-CH_2-$S), 2.61–2.69 (2H, s, CH_2-COOH), 1.80–1.87 (3H, s, CH_3-iPr), 1.74–1.80 (3H, s, CH_3-iPr), 1.40–1.49 (6H, s, S–C(CH_3)$_2$).

Coupling of Fmoc-D-Cys(3-S-3-methyl-butanoic acid)-OPp to TentaGel S NH_2 Resin

Fmoc-D-Cys(3-S-3-methyl-butanoic acid)-OPp (3.97 g, 6.8m mol), TBTU (2.2 g, 6.8 mmol) and diisopropylethyl amine (4.8 ml, 13.6 mmol) dissolved in a few milliliters of DMF were added to TentaGel S NH_2 resin (23.52 g, corresponding to 6.8 mmol amino functions). Enough additional DMF was added to ensure covering of the resin with the solvent. The mixture was agitated slowly over 3 h, after which the solution was filtered off and the resin was washed three times with DMF (100 ml each). Ten percent acetic acid anhydride in DMF containing 5% diisopropylethyl amine was added and the resin was slowly agitated for additional 1 h. The resin was washed successively with DMF and methanol and left to dry in air for 16 h. The Fmoc group was cleaved by a treatment of the resin with 20% piperidine in DMF for 10 min. The loading of the resin with the disulfide linker was typically 180–200 µmol/g.

Peptide Assembly on the Resin

The model peptides cyclo-[a-a-w-w-a-a-c], cyclo-[a-a-r-w-a-a-c] and cyclo-[a-a-s-w-a-a-c] were assembled manually on the resin by using TBTU/diisopropylethyl amine activation in a fivefold excess for 1 h. The Fmoc group was cleaved with 20% piperidine in DMF for 5 min.

For the synthesis of the peptide libraries, first two Fmoc-D-alanine entities were coupled to the entire linker-coupled resin in a filtration funnel. After cleavage of the Fmoc group and coupling of the second alanine, the resin was washed several times with DMF and DCM. The dried resin was distributed into the reaction vessels (5 µmol loading each) in the 96mer assembly block according to the balanced density method as a suspension in a mixture of DMF and tetrachloroethylene. The optimal solvent composition was determined with a few milligrams of the resin in a 1 ml glass cuvette in 500 µl DMF by the stepwise addition of 50 µl portions of tetrachloroethylene. The distribution of the resin into the reaction vessels was carried out with a stepper pipette equipped with a 12.5 ml pipetting piston of which a few millimeters of the tip was cut off in order to increase the internal diameter. This prevents the clogging of the device by the resin particles. The peptides were assembled on the instrument with 0.5 M amino acid stock solutions, with TBTU and diisopropylethyl amine activation. Double couplings of 1 h each were carried out. The Fmoc groups were cleaved with 20% piperidine in DMF within 5 min. After the final coupling and Fmoc deprotection, the resin was washed repeatedly with DMF followed by DCM.

Cleavage of the Pp Protection Group

The Pp group was cleaved with 2% TFA in DCM (250 µl) by treating the peptide-resins on the instrument three consecutive times for 5 min, 2 h and 3 h. Afterwards the cleavage mixture was washed three times with DCM followed

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Table 1 Racemization analyses of the D-Cysteine in model peptides

Sequence	% L-Cysteine
cyclo-[a-a-a-e-a-a-c]	5.17
cyclo-[a-a-h-w-a-a-c]	11.3
cyclo-[a-a-k-q-a-a-c]	10.3
cyclo-[a-a-n-i-a-a-c]	12.3

by two washes with 5% diisopropylethyl amine in DMF and five washes with DMF.

Cyclization of the Peptides on the Resin

Cyclization was carried out by two successive 24 h treatments with 200 µl DMF containing 5 equiv. of PyBOP and HOAt plus 10 equiv. of diisopropylethyl amine. Between the two treatments, the resin was washed twice with 300 µl DMF. After the cyclization, the resin was washed three times with DMF and three times with DCM and left in air to dry.

Side Chain Deprotection of the Immobilized Peptides

Deprotection of the amino acid side chains was carried out by three consecutive treatments with 250 µl TFA containing 5% phenol and 5% water for 5 min, 55 min and 5 h, respectively. Afterwards, the resin was washed three times with acetic acid, followed by three washes with 2-propanol. Drying was carried out for 16 h at 40 °C.

Cleavage of the Peptides from the Support

Peptides were cleaved from the support directly into the reaction vessels by three successive 24 h treatments with two equiv. of DTT (1.54 mg) in 2-propanol/water 1 : 1 (800 µl) containing 100 mM ammonium bicarbonate. After each incubation, the filtrate was collected and the resin was washed with 2-propanol/water 1 : 1 (500 µl). The peptides were analyzed by MALDI-MS directly from the cleavage solution. All filtrates were combined and the solvent was evaporated to yield the crude peptides together with oxidized and reduced DTT. After redissolving the material in 2-propanol/water 1 : 1 (1 ml) and evaporating the solvent again, peptide stock solutions were prepared by redissolving the material in DMSO (500 µl). The stock solutions had concentrations of approximately 3.5 mM, which was determined by quantitative amino acid analysis of several samples. The quality of some of the crude peptides was additionally investigated by analytical HPLC. The stock solutions were stored in deep-well microtiter plates at −70 °C. Racemization analysis of the cysteine in four sequences was carried out and is shown in Table 1.

RESULTS AND DISCUSSION

Linker Synthesis

The first requirement of the linker synthesis was the reliable introduction of a *C*-terminal protection group into a cysteine derivative that is stable under the conditions of standard Fmoc peptide assembly and can be removed selectively. The 2-phenylisopropyl group appeared well suited for this purpose. The ester was reported to be cleaved within 2 h by 2% TFA in DCM without affecting OtBu and Boc groups [21].

The cysteine derivative **3** (Scheme 1) was prepared by treating commercially available Fmoc-D-Cys(tBu)-OH **1** with 2-phenylisopropyltrichloroacetamidate **2** [18] following a published procedure [21], yielding over 90% after column chromatography on silica gel.

Next, the transformation of the tBu-protected sulfur group into the activated Npys derivative was carried out according to a literature procedure [22]. Initially, the formation of the product was unsatisfactory. It was linked to the solubility of the reagent Npys-Cl in DCM, which was limited with certain commercial batches. Npys-Cl is quite unstable on storage [20,23], and much better results were obtained when we prepared fresh material from 2-chloro-3-nitropyridine via the disulfide 2,2′-dithiobis(3-nitropyridine) [20]. We also restricted the reaction time to a few minutes at room temperature and quenched the reaction by the addition of a mixture of methanol and DMF before workup. If these precautions are taken, the product can be obtained in reasonable yield.

The introduction of a carboxylic group into the side chain was carried out by treating the crude or purified Npys derivative **4** with 3-mercapto-3-methylbutanoic acid [19] in DMF. Initial attempts to use a mercapto acid building block with a primary carbon next to the sulfur showed that the resulting disulfide is not stable under the basic conditions of Fmoc removal, as has also been reported by others [16]. Diisopropylethyl amine catalyses the reaction and converts the product **5** into the more stable corresponding salt. The coupling of **5** to a resin containing amino groups (Rapp S NH_2) resulted in good yield.

Peptide synthesis (Scheme 2). Before the automated synthesis of the peptide library, several model peptides consisting entirely of D-amino acids were assembled manually on the resin by employing standard peptide synthesis protocols. Cleavage of the Fmoc groups by 2% DBU rather than 20% piperidine in DMF was also investigated, but led to a complete loss of the peptides from the support, probably due to a cleavage of the disulfide linkage.

Although it had been reported that in solution a 2 h treatment with 2% TFA in DCM is sufficient for a complete removal of the phenyl isopropyl group [21], on the solid support we found it necessary to extend the incubation time to 5 h. Several conditions for the efficient cyclization of the peptides on the support were investigated: diisopropylcarbodiimide/HOBt and TBTU, HATU or PyBOP/diisopropylethyl amine/HOAt. Also the solvents DMF and NMP were compared,

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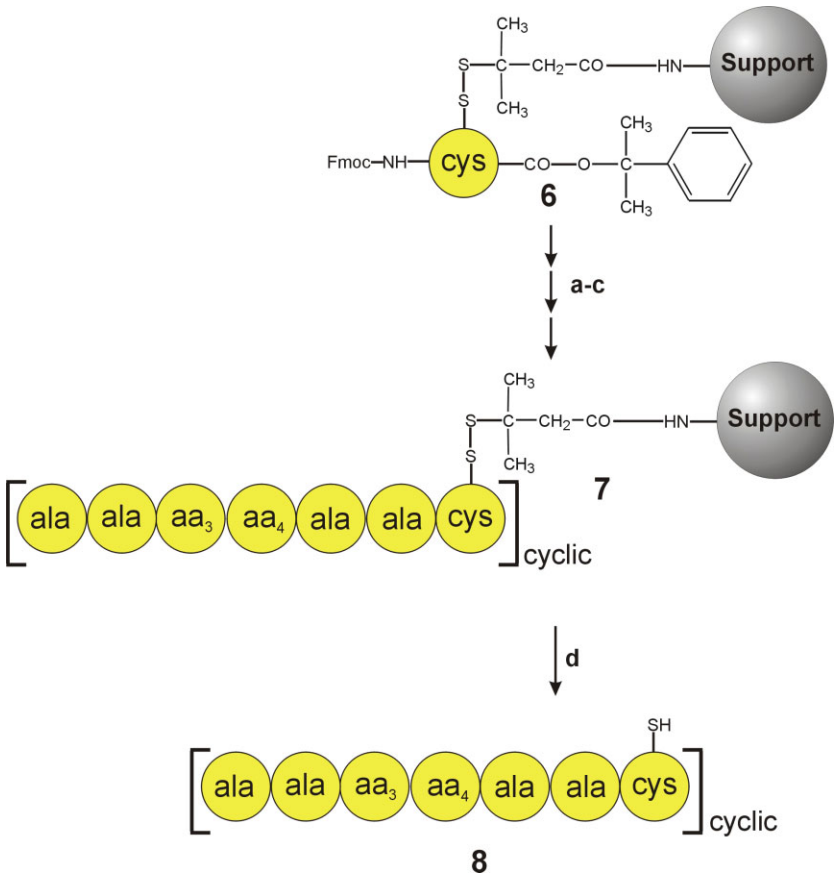

Scheme 2 Synthesis of cyclic peptides on the disulfide linker and cleavage from the support. **(a)** standard Fmoc peptide synthesis protocol with TBTU/diisopropylethyl amine activation and Fmoc-cleavage by 20% piperidine/DMF; **(b)** 2% trifluoro acetic acid in DCM, 5 h; **(c)** 5 equiv. PyBop/HOAt, 10 equiv. diisopropylethyl amine, 2 × 24 h; **(d)** 2 equiv. DTT in 2-propanol/H_20 1: 1, 100 mM ammonium bicarbonate, 3 × 18 h. This scheme is available in colour online at www.interscience.wiley.com/journal/jpepsci.

in pure form and as mixtures with DCM, and the use of collidin as the base. Cyclization reactions generally were found to work best with an excess of PyBOP, HOAt and diisopropylethyl amine in DMF for 2 days. We observed a considerable amount of *N*-terminal guanidine formation with carbodiimide and uronium salts. The formation of guanidine as a side reaction with uronium salts is well known in peptide synthesis [24], but with carbodiimides it has not been reported earlier, to our knowledge. The utilization of the phosphonium salt PyBOP together with HOAt seems the best choice for long reaction times. The activated *C*-terminal cysteine undergoes a certain amount of racemization under those conditions, as was determined for four model peptides (Table 1). For the intended utilization of the cyclic peptides in biological screening campaigns, the racemization extent was considered tolerable, although it needs to be taken into account when structure–activity investigations of positive results ('Hits') are carried out.

Automated Synthesis of the Peptide Library

For the automated synthesis of the peptide library, the structure cyclo-[a-a-x-x-a-a-c] was chosen (with x representing all proteinogenic amino acids in their D-conformation except cysteine, which was omitted). Three hundred and sixty one sequences were thus synthesized. The two *C*-terminal alanines were coupled to the resin in one batch. Afterwards, the resin was distributed into the reaction vessels (1.4 ml volume) that are used in the multiple peptide synthesizer (Syro from MultiSynTech). The balanced density approach was employed for distributing the resin in an amount of 5 µmole each into the reaction vessels.

Cleavage of the Peptides from the Support

Several procedures and conditions for the cleavage of the disulfide linkage were investigated, including mercaptoethanol, mercaptopropanol, trimethyl phosphine and DTT. DTT gave the best results in terms of the quality of the resulting peptides, with only a minor aromatic nuisance. The cleavage progress with DTT was investigated by quantitative amino acid analyses for some model peptides, as shown in Figure 1. In addition, amino acid analyses of the resulting support material after the third cleavage revealed that over 95% of the peptide had been liberated by this procedure (data not shown).

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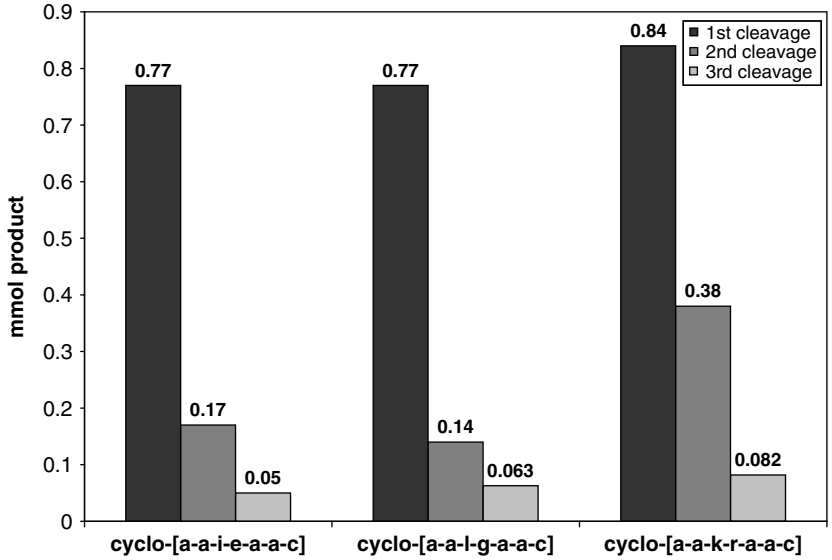

Figure 1 Cleavage of synthesized model peptides from the support by three consecutive 24 h incubations with two equiv. of DTT in 2-propanol/water 1 : 1 containing 100 mM ammonium bicarbonate. The amounts of cleaved peptides were determined by quantitative amino acid analyses.

Initial experiments with the peptides show that the remaining oxidized and reduced DTT is tolerable in bacterial growth assays (to be reported elsewhere).

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