Acta Chemica Scandinavica, 1995: 49: 696–700
Printed in Belgium – all rights reserved

ACTA
CHEMICA SCANDINAVICA
ISSN 0904–213X

Synthesis of an *N*-Methyldehydroalanine-Containing Fragment of Microcystin by Combination of Solid Phase Peptide Synthesis and β-Elimination in Solution

Maria Zetterström,[a] Lars Trogen,[b] Lars-Gunnar Hammarström,[b] Lars Juhlin,[b] Bo Nilsson,[b] Charlotta Damberg,[c] Tamas Bartfai[a] and Ülo Langel[a]

[a]Department of Neurochemistry and Neurotoxicology, Arrhenius Laboratories, Stockholm University, S-106 91 Stockholm, Sweden; [b]National Defence Research Establishment, Department of NBC Defence, S-901 82 Umeå, Sweden and [c]Swedish NMR Centre, S-104 62 Stockholm, Sweden

Zetterström, M., Trogen, L., Hammarström, L.-G., Juhlin, L., Nilsson, B., Damberg, C., Bartfai, T. and Langel, Ü., 1995. Synthesis of an *N*-Methyldehydroalanine-Containing Fragment of Microcystin by Combination of Solid Phase Peptide Synthesis and β-Elimination in Solution. – Acta Chem. Scand. 49: 696–700 © Acta Chemica Scandinavica 1995.

A new method for the synthesis of dehydroalanine (ΔAla)-containing peptides has been developed by combining solid phase peptide synthesis (*tert*-butyloxycarbonyl/HF–chemistry) with solution synthesis. A sequence from cyanobacterial hepatotoxin microcystin, Ac-D-γ-Glu-[*N*-Me-ΔAla]-D-Ala-Leu amide was chosen as a model peptide. The precursor for the synthesis of the dehydroalanine-containing peptide, Ac-D-γ-Glu-[*N*,*S*-diMeCys]-D-Ala-Leu, was synthesized on a solid phase followed by sulfonium salt formation on the resin. The resulting *S*,*S*-dimethylated peptide was cleaved from the resin with liquid HF. The HPLC-purified *S*,*S*-dimethylated cysteine-containing precursor peptide was subjected to β-elimination in solution catalysed by DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) in methanol. The final product, Ac-D-γ-Glu-[*N*-Me-ΔAla]-D-Ala-Leu amide, was purified by HPLC, and analysed by mass spectrometry and ^{1}H NMR spectroscopy. The stability of the model peptide under acidic, neutral and basic conditions has been studied.

Toxic blue–green algae such as *Microcystis, Oscillatoria* and *Anabena*, pose an increasing environmental hazard in several areas of the world. Two main types of toxin are produced by these cyanobacteria: peptide hepatotoxins and alkaloid neurotoxins.[1] Of increasing interest are hepatotoxins — cyclic heptapeptides named microcystins, which induce severe intrahepatic haemorrhages and liver necrosis. Microcystins have lately been recognised as potent inhibitors of protein phosphatases 1 and 2A,[2] and as potent liver tumour-promoters.[3]

Development of a defence system against the toxicity of microcystins is therefore of great interest. This work is introductory to a project aimed at the production of catalytic antibodies with the ability to degrade microcystins.[4,5]

Microcystins are cyclic heptapeptides with the chemical structure, cyclo(D-Ala-X-[D-*erythro*-β-methylAsp]-Y-Adda-D-γ-Glu-*N*-Me-ΔAla-) where X and Y are variable L-amino acids. The microcystins differ primarily in the two variable L-amino acids and their nomenclature is based on these differences: microcystin-LR contains leucine and arginine in the various positions, microcystin-YR contains tyrosine and arginine, etc. It has been demonstrated that the common structural feature, Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), is very important for the activity of microcystins.[6] Recently, it has as well been reported that the analogue of microcystin-LR containing saturated *N*-methyldehydroalanine, i.e., *N*-methylalanine, is about half as toxic as the parent compound.[7] Hence, it is reasonable to choose at least one of these two unusual amino acids — Adda or ΔAla — as a target for antibody-catalysed degradation of microcystins. Our choice was a ΔAla-containing fragment of microcystin, Ac-D-γ-Glu-[*N*-Me-ΔAla]-D-Ala-Leu amide.

The synthesis of α,β-dehydroamino acids or dehydroamino acid containing peptides has been studied extensively,[8-10] mainly due to the isolation of numerous biologically active α,β-dehydropeptides from natural sources over the last two decades. As in the case of microcystins, the majority of α,β-dehydroamino acids have been found in low-molecular weight cyclic peptides from microbial sources. Dehydroamino acid containing peptides have unique chemical and stereochemical properties which affect both the chemical reactivity and the conformation of the peptides. Therefore the synthesis of new analogues

of α,β-dehydroamino acid containing peptides is of interest.

Numerous methods for the synthesis of α,β-dehydroamino acid containing peptides mainly include their synthesis in solution. The elimination of water from, e.g., serine-, threonine- or phenylserine-containing analogues, eliminations from corresponding tosyl and selenium analogues and base-induced elimination from the corresponding sulfonium salts have been reported (for reviews cf. Refs. 9 and 10). The solid phase synthesis of a dehydroalanine-peptide from asparagine-containing sequences on Merrifield resin has also been described.[11]

However, development of alternative methods for the synthesis of peptides containing dehydroalanine are still needed. Here we report a new method for the preparation of dehydroalanine-containing peptides by combining solid phase peptide synthesis and solution synthesis. A partial sequence of cyanobacterial hepatotoxin microcystin was chosen, and studies of its stability at pH 5.0, 7.4 and 11.0 were made.

Results and discussion

The model peptide-sequence from microcystin-LR was synthesised according to the scheme outlined in Fig. 1. Manual assembly of the tetrapeptide **1** on *p*-methylbenzhydrylamine (MBHA) resin was achieved by coupling of the *tert*-butyloxycarbonyl (*t*-Boc) amino acids with TBTU–HOBt–DIEA and deprotection after each step by treatment with trifluoroacetic acid (TFA)–CH_2Cl_2 (cf., the Experimental). The synthesis was followed by dimethyl sulfonium salt formation of the *N,S*-dimethyl-cysteine residue of the bound peptide yielding the sulfonium analogue **2** of the peptide. This was cleaved from the resin with HF and purified by HPLC. The resulting peptide **3** gave a calculated molecular weight of 518.7 which was confirmed by an $[M+1]^+$ ion of *m/z* of 519.5 in the PDMS analysis (Fig. 2). Its purity was ≥98%. The product **4** was obtained (yield 54% as calculated from the amount of peptide **3** as the starting material) from the subsequent elimination of dimethyl sulfide from the *S,S*-dimethylcysteine residue of the sulfonium salt (cf., the Experimental). The product was purified by HPLC and analysed by mass spectrometry (Fig. 2). A different retention time was obtained for the final product **4** as compared with the *S,S*-dimethylated precursor peptide **3** (Fig. 2) and the mass obtained, $[M+1]^+$ ion of *m/z* of 456.3, corresponded to the calculated value for the *N*-Me-ΔAla analogue (M_W 455.5). The peptide structure was verified by ^1H NMR analysis (cf. Experimental and Table 1), suggesting that the methylene group existed in both s*E*- and s*Z*-forms (Fig. 3).

The stability of the microcystin analogue **4** in water solution at different pH was examined, with results presented in Fig. 4.

The data presented demonstrate that we have been able to synthesise the ΔAla containing fragment of microcystin, Ac-D-γ-Glu-[*N*-Me-ΔAla]-D-Ala-Leu amide, by combining solid phase peptide synthesis (SPPS) with β-elimi-

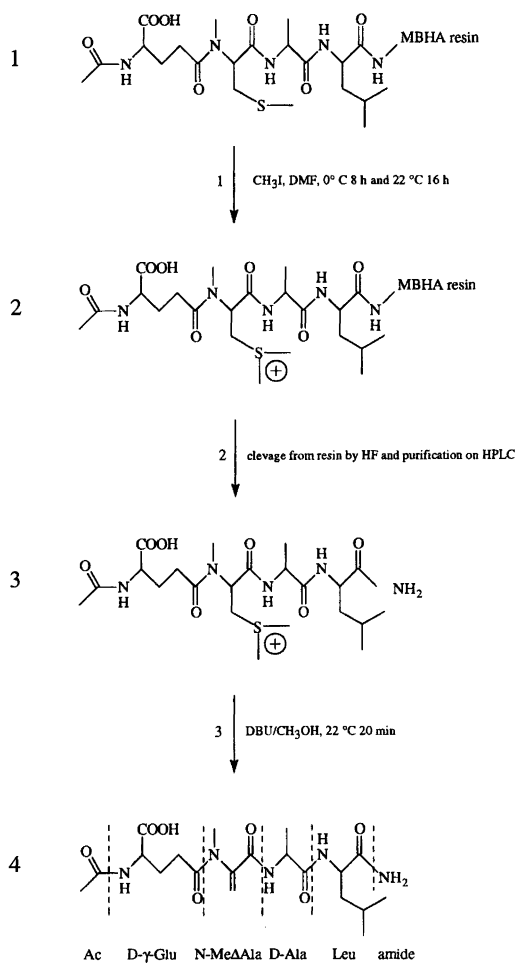

Fig. 1. Sequence of steps involved in the synthesis of the model peptide sequence from microcystin-LR.

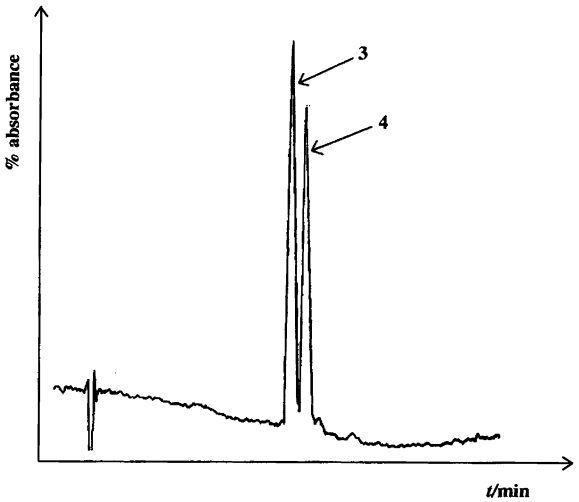

Fig. 2. HPLC elution profile of purified peptides **3** (t_R 12.00 min, *m/z* 519.5) and **4** (t_R 12.80 min, *m/z* 456.3) shown on Fig. 1.

Table 1. Chemical shifts for residues in peptide **4**.

Residue	H_α	H_β	H_γ	H_δ	Other protons
γ-Glu	4.38 (m, 1 H)	2.19 (dt, J=13.5, J=8.1, 1 H) 1.96 (dt, J=13.5, J=8.1, 1 H)	2.67 (m, 1 H) 2.39 (m, 1 H)		
Ala	4.31 (m, 1 H)	1.43 (t, J=7.3, 3 H)			
Leu	4.31 (m, 1 H)	1.64 (m, 1 H)	1.68 (m, 1 H)	0.92 (d, J=5.2, 3 H) 0.86 (d, J 5.2, 3 H)	
ΔAla		6.26/5.87 (s, 1 H)/(s, 1 H) 5.77/5.53 (s, 1 H)/(s, 1 H)			
N-Me					3.30/3.06 (s, 3 H)/(s, 3 H)
Ac					2.04 (s, 3 H) 2.01 (s, 3 H)

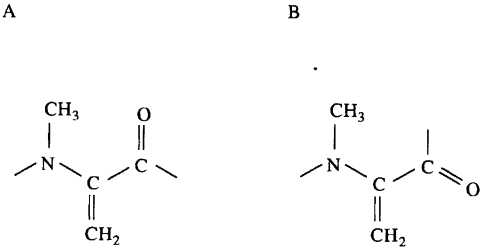

Fig. 3. The two proposed conformations of the peptide **4**: (A) s*E*-form and (B) s*Z*-form.

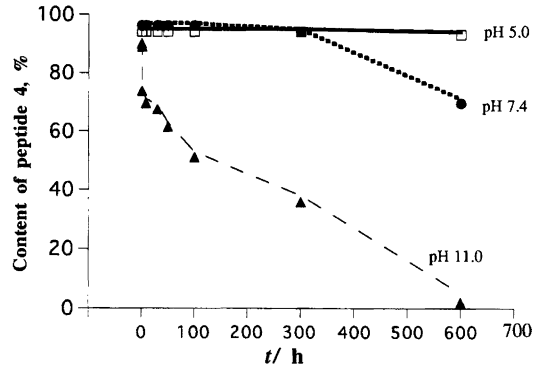

Fig. 4. The stability of peptide **4** at different pH values in 50 mM Na-phosphate buffer (pH 5.0 and 7.4) and 0.1 M CAPS (pH 11.0).

nation in solution, using *N,S*-diMe-Cys as a building block for *N*-Me-ΔAla.

Application of cysteine residues modified on sulfur for β-elimination reactions in solution has often been reported (for a review, see Ref. 10) and the *S,S*-dimethylsulfonium group as a leaving group has been used by Sokolovsky *et al.*[12] However, introduction of the methods compatible with different SPPS methods is of interest.

Synthesis of peptides containing cysteine by SPPS demands the protection of the sulfhydryl group in order to avoid the side reactions or even degradation. In contrast, the *S*-methyl group was compatible with SPPS using *t*-Boc–TFA–HF chemistry as expected from the analogy with Met. Analysis of reaction products after HF-cleavage was carried out at three different stages of the synthesis. The *N,S*-diMe-Cys containing analogue of the peptide **4** (i.e., the cleaved peptide **1**) and peptide **3** were both cleaved from the resin yielding the peptide with the correct molecular mass. However, the attempt to carry out the β-elimination reaction with peptide **2** followed by HF cleavage, was not successful according to the mass spectrometry data (data not shown).

It is noteworthy that the dimethyl sulfonium salt formation carried out by adding CH_3I in DMF (Fig. 1) was successful and the resulting peptide **3** was stable in the cleavage by HF. The method described can be useful for relatively simple synthesis of $-S^+(CH_3)_2-$ containing peptides. It has also been demonstrated that the β-elimi-

nation reaction can be carried out on *N*-methylated residues derived from cysteine.

An unprotected *N*-terminal amino group of the peptide would very likely be methylated during dimethylsulfonium salt formation in solution and therefore only *N*-terminally protected peptides can be used for the synthesis of dehydroalanine-containing peptides with *S*-methylcysteine as a building block. We chose the acetylated *N*-terminal amino group in our model peptide; further evaluation of applicability of other protective groups in peptide chemistry (*t*-Boc, Fmoc, Cbz) is needed. The possibility that the functional groups of amino acids Met, His and Trp are *S*- or *N*-methylated in their side chains is likely, therefore the use of the method described here for peptides including these amino acids is probably connected with formation of numerous side products.

The stability of peptide **4** was studied at pH 5.0, 7.4 and 11.0 (Fig. 4). The model peptide is stable at acidic pH, whereas at pH 11 it is degraded with a half life of approximately 135 h. Even under neutral conditions slight degradation was noted during incubation for 600 h. Transition state analogues for raising catalytic antibodies enabling the degradation of microcystin should hence be designed keeping this in mind.

We have demonstrated the possibility of using Boc-*N,S*-diMe-Cys, a new building block, for the synthesis of a dehydroalanine containing analogue of microcystin-LR, by combining solid phase peptide synthesis with the elimination reaction in solution. Compared with the methods described so far[11,13] for solid phase peptide synthesis of dehydroalanine containing peptides, this method is compatible with Boc–HF chemistry up to the elimination step which should be carried out in solution.

Experimental

General methods. Molecular masses of the peptides were determined using a Plasma Desorption Mass Spectrometer Model BioIon 20, Applied Biosystems. Ten µl of sample were applied to a nitrocellulose sample target, and spectra were recorded both before and after the sample target had been washed with deionized water containing 0.1% TFA (trifluoroacetic acid); the values of molecular masses of the peptides according to their $[M+1]^+$ ions were obtained in each case within the error limits ($\pm 0.1\%$).

The structure and the purity of the peptide **4**, Ac-D-γ-Glu-[*N*-Me-ΔAla]-D-Ala-Leu amide, were checked by ^{1}H NMR spectroscopy; the spectra were recorded on a Varian Unity 300 Spectrometer. Chemical shifts (Table 1) are reported in parts per million (ppm), using TSP (sodium 3-trimethylsilyltetradeuteriopropionate) as an internal standard (0 ppm). Splitting pattern notations are: s, singlet; d, doublet; t, triplet; dt, doublet of triplets and m, multiplet. Coupling constants (J) are given in hertz (Hz). The sample was dissolved in D_2O and the spectrum run at room temperature.

*Synthesis of aNH-Boc-*N,S*-dimethylcysteine.* We adopted the methodology for synthesis of the corresponding *S*-benzyl derivative as described by Yamashiro *et al.*[14] and subsequently used by Öhler and coworkers[15] for the preparation of *N,S*-dimethylcysteine with a reported yield of 54%. For solubility reasons we had to resort to a more elaborate work-up procedure involving evaporation to dryness and trituration of the residue with ethanol. This will be described in detail elsewhere (Trogen *et al.*, in preparation). Thus we were able to obtain the desired amino acid in about 70% yield. The α-amino group of the *N,S*-dimethylcysteine was protected with a Boc group as described.[16]

Peptide synthesis on a solid phase. The model peptide, Ac-D-γ-Glu-[*N,S*-diMeCys]-D-Ala-Leu amide, was assembled manually in a stepwise manner on a solid support with TBTU [2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate]–HOBt (1-hydroxy benzotriazole) activation strategy on a 0.2 mmol scale. Amino acids, Boc-Leu, Boc-D-Ala (both from Bachem Feinchemikalien AG), Boc-*N,S*-dimethylcysteine and Boc-D-Glu(α-OBzl) (Sigma), were coupled to MBHA (Bachem Feinchemikalien AG) resin. An individual cycle for each amino acid included deprotection of the Boc-group with 50% TFA in CH_2Cl_2 for 20 min, and acylation with 1.2-fold excess (as compared with the amount of free amino groups on the resin) of the protected amino acids in dimethylformamide (DMF) for 10–30 min. Between each operation several extensive washings were performed with CH_2Cl_2, DIEA (*N,N*-diisopropylethylamine) and DMF. After final coupling and deprotection, the acetylation was carried out using 10% acetic anhydride and 5% DIEA in CH_2Cl_2 for 10 min.

Dimethylsulfonium salt formation on a solid phase. Following acetylation, the peptide on the resin (Ac-D-γ-Glu-[*N,S*-diMeCys]-D-Ala-Leu-resin) was *S*-methylated by addition of a 20-fold excess of methyl iodide in DMF and incubation of the reaction mixture for 8 h at 0 °C and for 16 h at room temperature.

Cleavage and purification of the peptide. The *S*-methylated peptide was cleaved from the resin by making use of dry liquid HF as described earlier[17] to obtain the 'crude peptides'.

Purification of all the cleaved peptides was carried out on an LKB HPLC (high performance liquid chromatography) apparatus (SYSTEM PREP 50) using a Polygosil 60-7 C_{18} reversed-phase column (1.0 × 25.0 cm). The crude peptides were separated at a flow rate of 2.0 ml min^{-1}, using a linear gradient from 0–50% (v/v) water–acetonitrile containing 0.1% TFA for 30 min. The purity of the individual peptides was checked on an analytical Nucleosil 120-3 C_{18} reversed-phase HPLC column (0.4 × 10.0 cm) and determined to be ≥98%.

Synthesis of the ΔAla-containing analogue of microcystin in solution. The *N*-Me-*S,S*-trimethylcysteine residue of the purified peptide has been subjected to elimination by addition of an eightfold excess of DBU in dry methanol and stirring the reaction mixture for 20 min. Water was added and the dehydroalanine-containing peptide was immediately purified by reversed-phase HPLC and its mass spectra analysed as described above.

The ^{1}H NMR spectra of peptide **4** indicates the possible presence of s*E*- and s*Z* forms of the ΔAla residue in approximately a 1:1 ratio which will be subjected to further investigation (Table 1). Signals at δ 6.26 (s, 1 H)/ 5.87 (s, 1 H) and at 5.77 (s, 1 H)/5.53 (s, 1 H) are assigned to methylene protons of this residue. Signals at δ 3.30 (s, 3 H) and 3.06 (s, 3 H) are assigned to *N*-methyl groups and the acetyl group on D-γ-Glu gives rise to singlets at δ 2.04 (s, 3 H) and 2.01 (s, 3 H).

*Stability of the microcystin analogue, Ac-D-γ-Glu-[*N*-Me-ΔAla]-D-Ala-Leu amide.* The stability of the microcystin analogue was investigated by incubating small amounts of the dehydroalanine peptide at three different pH values (pH 5.0, 7.5 in 0.05 M Na-phosphate buffer and 11.0 in 0.1 M 3-cyclohexylamino-1-propanesulfonic acid; CAPS) from 0 to 500 h. Samples were regularly taken from the solution throughout the entire time, frozen at − 70 °C and examined by analytical HPLC in order to visualise the eventual degradation.

Acknowledgements. This work was supported by grants from the Swedish Medical Research Council, the Swedish Board of Technical Development (NUTEK), the National Institute of Mental Health, the Ivar Bendixsons Foundation and the Pharmacia Research Foundation. We are grateful to Dr. Anders Undén, Kalle Kaljuste and Claudio Rivera of Stockholm University and to Professor Zelig Eshhar, Professor Bernard Green and Dan Tawfik of the Weizmann Institute, Rehovot, for the stimulating discussions.

References

1. Rudolph-Böhner, S., Mierke, D. F. and Moroder, L. *FEBS Lett. 349* (1994) 319.
2. MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P. and Codd, G. A. *FEBS Lett. 264* (1990) 187.
3. Fujiki, H. and Suganuma, M. *Adv. Cancer Res. 61* (1993) 143.
4. Lerner, R.A., Benkovic, S.J. and Schultz, P.G. *Science 252* (1991) 659.
5. Green, B.S. and Tawfik, D.S. *Trends Biotechnol. 7* (1989) 304.
6. Harada, K.-L., Matsuura, K., Suzuki, M., Watanabe, M. F., Oishi, S., Dahlem, A. M., Beasley, V. R. and Carmichael, W. W. *Toxicol. 28* (1990) 55.
7. Namikoshi, M., Choi, B. W., Sun, F. and Rinehart, K. L. *Chem. Res. Toxicol, 6* (1993) 151.
8. Rich, D. H. and Tam, J. P. *J. Org. Chem. 42* (1977) 3815.
9. Schmidt, U., Lieberknecht, A. and Wild, J. *Synthesis* (1988) 159.
10. Noda, K., Shimohigashi, Y. and Izumiya, N. *The Peptides,* Academic Press, New York 1983, Vol. 5, p. 285.
11. Ranganathan, D., Shah, K. and Vaish, N. *J. Chem. Soc., Chem. Commun.* (1992) 1145.
12. Sokolovsky, M., Sadeh, T. and Patchornik, A. *J. Am. Chem. Soc. 86* (1964) 1212.
13. Blettner, C. and Bradley, M. *Tetrahedron Lett. 35* (1994) 467.
14. Yamashiro, D., Aanning, H.L., Branda, L.A., Cash, W.D., Murty, V.V.S. and du Vigneaud, V. *J. Am. Chem. Soc. 90* (1986) 4141.
15. Öhler, E., Prantz, E. and Schmidt, U. *Chem. Ber. 111* (1978) 1058.
16. Stewart, J. M. and Young, J. D. *Solid Phase Peptide Synthesis*, Wiley, New York 1984, p. 61.
17. Land, T., Langel, Ü., Löw, M., Berthold, M., Undén, A. and Bartfai, T. *Int. J. Peptide Protein Res. 38* (1991) 267.

Received February 7, 1995.