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Molecular structure of the cyanobacterial tumor-promoting microcystins

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Abstract

The three-dimensional structure of the two hepatotoxic microcystins LR and LY has been determined by two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy and distance geometry calculations. For the microcystin LY a single family of highly convergent structures was obtained. This family is characterized by a relatively compact boat-like ring structure with the large side chain of the Adda residue protruding from the concave side, in close proximity to the Tyr side chain. Conversely, for the microcystin LR the calculations result in three conformational families characterized by an even more compact ring structure. The Adda and Arg side chains protrude from the ring distal from one another caused by the repulsion between the guanido function of Arg and the hydrophobic Adda. The lower toxicity of the LY microcystin could result from the restricted access of the Adda side chain, an essential residue for activity, which results from the close proximity of the aromatic Tyr residue. A significant enthalpic cost would be expected for disturbance of this hydrophobic collapse and correspondingly lower binding affinity to receptor molecules would be predicted. From the structures of the two related microcystins, and homology with other known toxins, we propose a working hypothesis of the Adda side chain interacting with a hydrophobic pore of the phosphatases while the rest of the microcystin acts as a scaffold to help stabilize the interdigitation of the Adda with additional intermolecular interactions.

Key words: Microcystin; Three-dimensional structure; NMR; Distance geometry; Structure–function relationship

1. Introduction

Cyanobacteria, so called water blooms, occur worldwide in mass in eutrophic fresh and brackish waters. Many of these water blooms are toxic, causing the death of fish, birds, wild animals and agricultural livestock and a health risk to human beings, thus leading to a serious problem for water management and public health [1–6]. Two main types of toxins are produced by cyanobacteria: peptide hepatotoxins and alkaloid neurotoxins [7–9]. The hepatotoxins are cyclic heptapeptides, named microcystins, which have been recognized as potent inhibitors of protein phosphatases 1 and 2A [10–12]. As both phosphatases play key roles in cell division, their inactivation should lead to tumor promotion and indeed microcystins were found to promote hepatic tumors initiated by diethylnitrosamine in rats [13,14]. The three-dimensional (3D) structures of microcystins will facilitate the elucidation of the molecular mechanism of phosphatase inhibition.

As shown in Fig. 1, the chemical structure of microcystins corresponds to the cyclic heptapeptide cyclo-(D-Ala[1]-X[2]-D-Me-iso-Asp[3]-Y[4]-Adda[5]-D-iso-Glu[6]-Mdha[7]-) where X and Y are variable L-amino acids, Adda the unusual b-amino acid (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, Mdha the *N*-methyl-dehydroalanine and D-Me-Asp the D-erythro-β-methyl-aspartic acid [15]. Additionally the two acidic amino acids D-Glu and D-Me-Asp are iso-linked. Over 40 variants of the microcystin have been isolated and their chemical composition characterized ([16] and references therein). In addition to the variable L-amino acids, common differences include demethylation of amino acids 3 and/or 7 [17,18]. Among all the variants the most abundant, and correspondingly the best studied, is microcystin LR. Recently we succeeded in the identification and structure determination of an LY variant in which the basic Arg residue is replaced by the aromatic Tyr [19]. This modification adjacent to the Adda residue, the essential component for hepatotoxicity [20,21], significantly lowered, but did not abolish, the bioactivity [22]. Thus, a comparative conformational analysis of these two microcystins may allow insight into the structural requirements for the toxicity of the microcystins.

2. Materials and methods

The microcystins LR and LY were obtained from J.T. Wu, Academia Sinica, Nankang, Taipei, and were fully characterized in terms of homogeneity and chemical structure [19].

NMR spectra were measured on a Bruker AM500 spectrometer at 300 K; the data were processed on a Bruker X32 computer with UXNMR software. The samples containing 1 mg peptide/500 μl DMSO-d_6 were prepared in 5 mm tubes. For each peptide ^{1}H, phase-sensitive TOCSY [23,24], DQF-COSY [25], NOESY [26] and ROESY [27] were recorded using standard pulse sequences and the following parameters. 1D-^{1}H NMR (64 acquisitions, size 32K, sweep width 6250 Hz). TOCSY (microcystin LR: size 2K, mixing time for MLEV17 40 ms, 96 acquisitions, 800 increments, sweep width in F_1 and F_2 5000

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Abbreviations: The two variable L-amino acids present in the microcystin sequences are used to specify the toxins by single capital letter codes [15].

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Hz; microcystin LY: size 2K, mixing time for MLEV17 60 ms, 96 acquisitions, 1024 increments, sweep width in F_1 and F_2 6250 Hz). NOESY (microcystin LR: size 2K, mixing time 200 ms, sweep width in F_1 and F_2 5000 Hz, 64 acquisitions, 600 increments; microcystin LY: size 2K, mixing time 200 ms, sweep width in F_1 and F_2 6250 Hz, 64 acquisitions, 600 increments). DQF-COSY (microcystin LY in DMSO-d_6: size 2K, sweep width in F_1 and F_2 6250 Hz, 128 acquisitions, 1024 increments; microcystin LY in DMSO-d_6/ND_3/D_2O: size 2K, sweep width in F_1 and F_2 6250 Hz, 64 acquisitions, 600 increments); ROESY (microcystin LR: size 2K, mixing time 100 ms, 4 kHz spin lock field, 32 acquisitions, 800 increments, sweep width in F1 and F2 6250 Hz; microcystin LY: size 2K, mixing time 100 ms, 4 kHz spin lock field, 64 acquisitions, 800 increments, sweep width in F1 and F2 6250 Hz). Prior to transformation of the TOCSY, NOESY and ROESY spectra gaussian window functions in F_2 and shifted sine-bell functions in F_1 were used. Shifted sine-bell function in F_1 and no window function in F_2 were applied prior to transformation of both DQF-COSY spectra. The NOESY spectra with 200 ms mixing time were used for integration of NOE crosspeaks which were translated into interproton distances after calibration by a r^{-6} correlation (two spin approximation ±5%). The distances of pseudoatoms were corrected by adding 0.9 Å for methylen, 1.0 Å for methyl groups and 2.0 Å for aromatic protons to the lower distance value. The ROESY spectra were applied for distinguishing ROE and chemical exchange.

From high quality 2D-TOCSY and DQF-COSY spectra all of the amino acids of the microcystin LR and LY were unambiguously assigned (for the chemical shifts see [19]). The sequential assignment of both cyclic peptides was derived from the connectivities in the NH-CαH region of NOESY spectra, and the iso-linkage of D-Me-Asp and D-Glu confirmed by NOESY spectra and by DQF-COSY experiments as a function of pD [19]. Moreover, the (4*E*,6*E*) configuration of the double bond in the Adda residue was proven by the 2D-NOESY spectra, as stereogenic variations at this center are known to produce non-toxic agents [20,21].

The 3D structures of the cyclic peptides were determined by distance geometry calculations using a homewritten FORTRAN program which utilizes the random metrization method described by Havel [28]. An all-atom representation was used, except for the CβH2 and CγH2 methylenes of D-iso-Glu and Arg (for microcystin LR) which could not be diastereotopically assigned. The NOE intensities were converted to distances using a reference distance of 1.78 Å for the iso-Glu β/β cross peaks. For the computations 64 (30 intraresidue, 21 sequential and 13 long range) and 70 (46 intraresidue, 15 sequential and 9 long range) NOE-derived distance constraints were used for the microcystin LY and LR, respectively. One hundred structures were generated from the distance matrices containing the holonomeric and experimental distances. The structures were first generated in four dimensions, optimized using the distance driven dynamics method [29], reduced to three-dimensions using the EMBED algorithm [30] and then optimized again. The optimization of the structures, in both four and three dimensions were carried out in two steps of (i) 100 ps at a temperature of 1000 K with tight coupling to a temperature bath [31] and (ii) 50 ps with a weak coupling to a temperature bath of 1 K, to allow for slow removal of the kinetic energy.

A comparison of the pairwise rmsd values for the structures of microcystin LR with the smallest distance restraint violations clearly indicate three distinct conformational families. The three families, shown in Fig. 2, have an average distance restraint violation of 0.16 Å. The structures within each family have rmsd of 0.87 Å and 0.30 Å (family A), 0.95 Å and 0.57 Å (family B) and 0.89 Å and 0.23 Å (family C), for all heavy atoms and backbone heavy atoms, respectively. For microcystin-LY, 10 structures with the smallest distance violations were chosen for further analysis and are shown in Fig. 3. The average distance restraint violation for this final set of structures is 0.2 Å. The pairwise root-mean-square deviations (rmsd) for all heavy atoms and backbone heavy atoms are 0.9 and 0.2 Å, respectively.

3. Results and discussion

The three families of structures of microcystin LR exhibit a common general feature consisting of a compact planar array of the peptide backbone of Adda^5-D-iso-Glu^6-Mdha^7 ring portion (Fig. 4). In family A, D-Ala^1 lies in this plane with Leu^2-D-Me-Asp^3 protruding from the plane and with Arg^4 located at the edge of the plane. In family B Arg^4 lies in the plane and Ala^1 at the edge, while in family C Arg^4 becomes part of the protrusion and Leu^2 moves to the edge at the opposite side. Thus, the three families differ mainly in small movements of the residues which protrude from the plane formed by residues 5–7. In this context it is noteworthy that nodularin, a hepatotoxic cyclic pentapeptide, isolated from the blue green algae *Nodularia spumigena* of brackish water, corresponds to residues 3–7 of microcystin LR with replacement of Mdha by a-(methylamino)dehydrobutyric acid [32,33], thus lacking the D-Ala^1-Leu^2 of the microcystins. Since microcystin LR and nodularin have identical hepatotoxicity and binding affinity to protein phosphatases 1 and 2A [10–12], we propose that the dipeptide sequential intron in the microcystins (D-Ala-Leu) must adopt a conformational exon to maintain the correct topological array of the rest of the ring structure, responsible for the selective recognition by the receptor molecules. The backbone structures of microcystin LR obtained in the present study are in contrast to those proposed from modeling studies which predicted a planar ring [34,35].

In all three families of structures the Adda side chain, which is essential for hepatotoxicity [20,21], is protruding above the main plane, distant from the sequentially adjacent Arg side chain (see Fig. 4). The Adda side chain, despite restricted rotational freedom because of double bonds, retains sufficient conformational space for relatively large movements (i.e. in family A the side chain is bent over the ring structure and in family B it is directed away from the ring, an effect even more pronounced in family C). Conversely, in the 3D structure of microcystin LY, as shown in Fig. 5, a surprisingly restricted conformational space is observed for the Adda side chain, most probably as a result of a hydrophobic collapse-type interaction with the adjacent Tyr side chain. This strong interaction leads to movements in the peptide backbone ring structure which adopts a boat-type array significantly different from the half-chair-like array in the LR variant. The aromatic interaction between the side chains of Tyr and Adda may explain the lower toxicity of this microcystin compared to that of the LR variant. The disruption of this hydrophobic core would be accompanied by a large enthalpic cost, and therefore by a lower affinity for the protein phosphatases. This would counteract the enhancement of hepatotoxicity that would be predicted based on the higher hydrophobicity of the microcystin LY [16].

Okadaic acid, a fatty acid polyketal from marine sponges, is also known to inhibit protein phosphatases 1 and 2A with high selectivity and potency (see [36] for review). The identical selectivity as microcystins strongly suggests identical binding sites. However, adaptation of

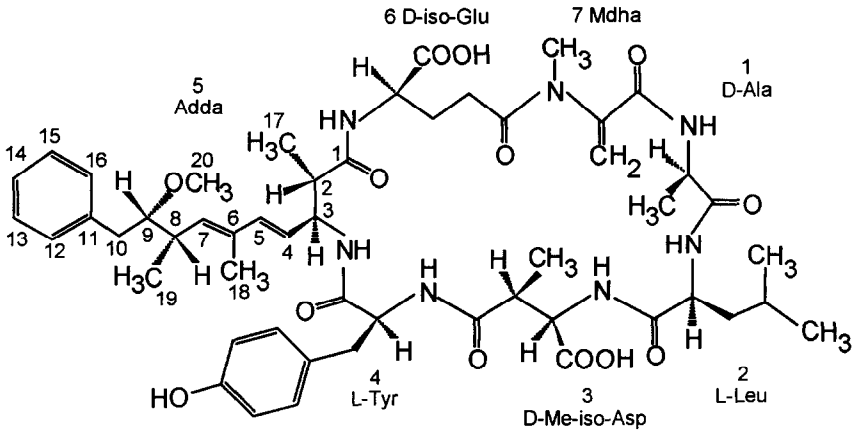

Fig. 1. Structure of microcystin LY; replacement of L-Tyr by L-Arg leads to the structure of microcystin LR.

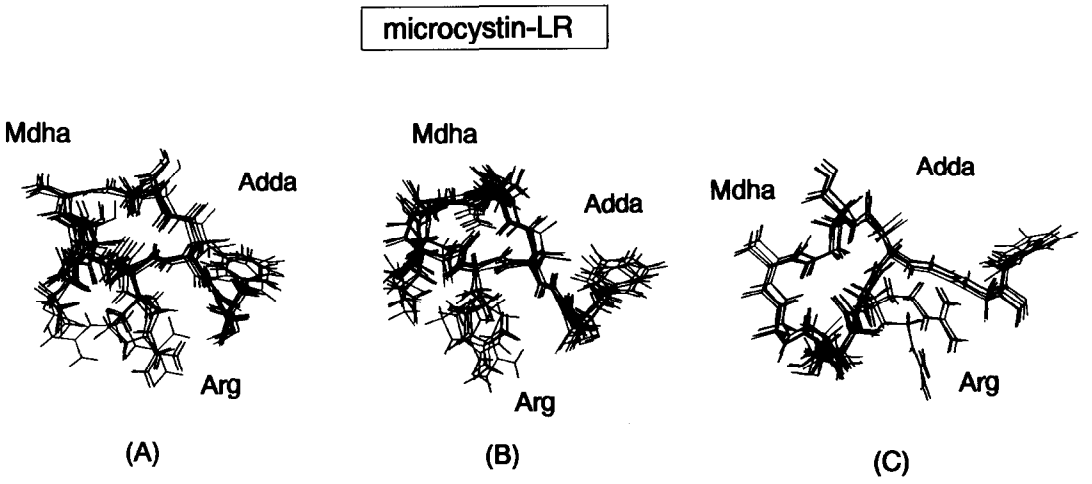

Fig. 2. Superposition of the most convergent microcystin LR structures subdivided in the three conformational families A, B and C.

this large polyketal to the hypothetical binding portion of microcystins is difficult. The carboxylic moiety of okadaic acid could mimic the α-carboxyl function of the invariant D-*iso*-Glu and wrapping around the small planar structure of microcystin LR could introduce the opposite end into the hydrophobic cavity.

Cyclosporin A is known to prevent the hepatotoxicity of microcystins [37,38], but pharmacodynamic studies indicate that the observed effect is most probably associated with the hepatocellular uptake mediated by the multispecific transport system for bile acids, since microcystin uptake can be inhibited by antamanide and rifampicin. Although cyclosporin A is a cyclic undecapeptide containing mostly N-methylated amino acids and the unnatural, unsaturated, hydrophobic amino acid (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine, looking for structure similarities to account for the observed sharing of some membrane transport and cytosol effector mechanism is certainly speculative.

To conclude, from the 3D structures of the two microcystins in solution it is compelling to propose a tight

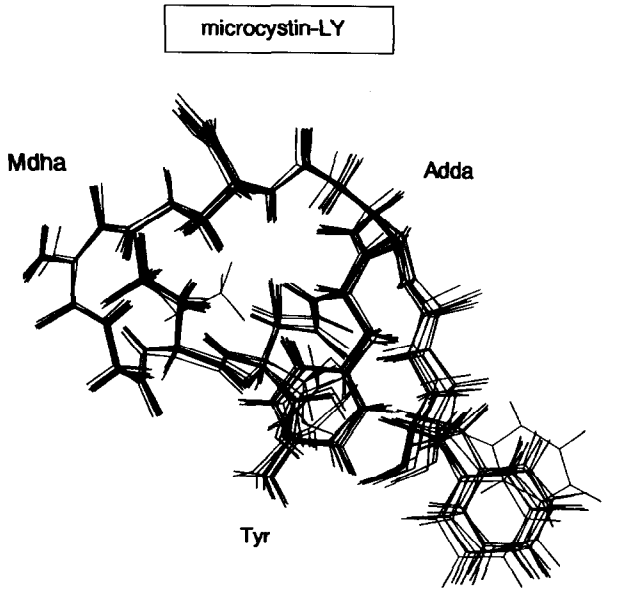

Fig. 3. Superposition of the 10 microcystin LY structures with the smallest distance violations.

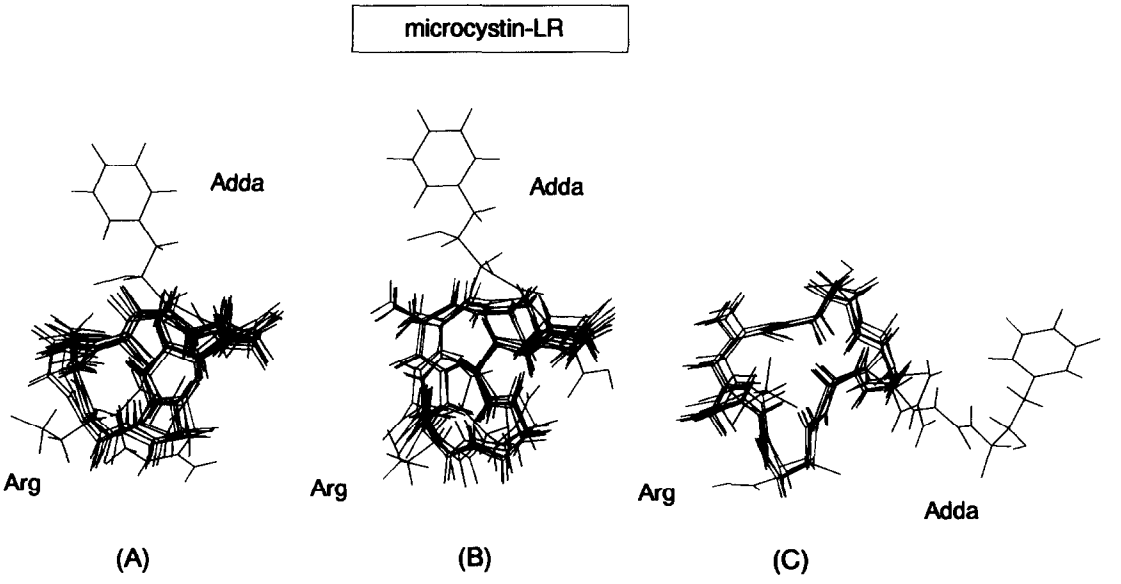

Fig. 4. Superposition of the backbone atoms and the Mdha residue of 6 (family A), 6 (family B) and 5 (family C) structures of microcystin LR including the side chains of one conformer, respectively.

interdigitation of the Adda side chain with a hydrophobic cavity of the protein phosphatases 1 and 2A. However, since this amino residue alone [39] or linear microcystin-related precursor peptides are not toxic [40], it is reasonable to assume that the remainder of the peptide (the planar ring portion) plays an important role in recognition or maintaining the proper orientation for the Adda residue. This inhibition mechanism will certainly remain only a working hypothesis until the first 3D structure of the protein phosphatase/microcystin complex becomes available. Preliminary crystallization data have recently been reported for the phosphatase PP1/microcystin LR complex [41].

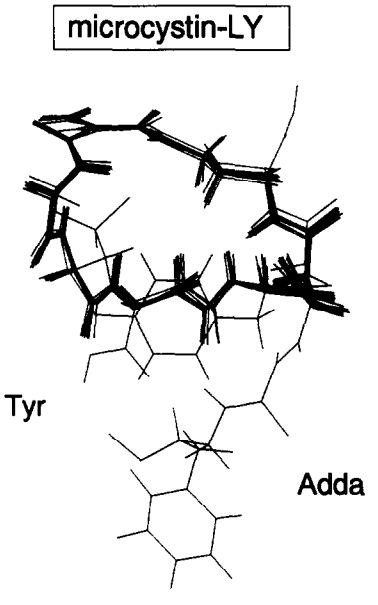

Fig. 5. Superposition of the backbone atoms and the Mdha residue of 10 microcystin LY structures including the side chains of one conformer.

References

[1] Skulberg, O.M., Codd, G.A. and Carmichael, W.W. (1984) Ambio 13, 244–247.
[2] Berg, K.O., Skulberg, O.M., Skulberg, R., Underdal, B. and Willén, T. (1986) Acta Vet. Scand. 27, 440–452.
[3] Beasley, V.R., Dahlem, A.M., Cook, W.O., Valentine, W.M., Lowell, R.A., Hooser, S.B., Harada, K.I., Suzuki, M. and Carmichael, W.W. (1989) J. Vet. Diagn. Invest. 1, 359–365.
[4] Pearson, M.J. (1990) in: Toxic Blue-Green Algae (S.L. Hunt, ed.) Report of the National Rivers Authority; Water Quality Series, no. 2, Peterborough.
[5] Repavich, W.M., Sonzogni, W.C., Standridge, S.H., Wedepohl, R.E. and Meisner, C.F. (1990) Water Res. 24, 225–231.
[6] Sivonen, K., Niemelä, S.I., Niemi, R.M., Lepistü, L., Luoma, T.H. and Räsänen, L.H. (1990) Hydrobiologica 190, 267–275.
[7] Codd, G.A. and Poon, G.K. (1988) Proc. Phytochem. Soc. Eur. 28, 283–296.
[8] Carmichael, W.W. (1989) in: Natural Toxins: Characterization, Pharmacology and Therapeutics (Ownby, C.L. and Odell, G.V. Eds.) pp. 3–16, Pergamon Press, Oxford.
[9] Carmichael, W.W. (1992) J. Appl. Bacteriol. 72, 445–459.
[10] MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P. and Codd, G.A. (1990) FEBS Lett. 264, 187–192.
[11] Matsushima, R., Yoshizawa, S., Watanabe, M.F., Harada, K.-I., Furusawa, M., Carmichael, W.W. and Fujiki, H. (1990) Biochem. Biophys. Res. Commun. 171, 867–874.
[12] Yoshizawa, S., Matsushima, R., Watanabe, M.F., Harada, K.-I., Ichihara, A., Carmichael, W.W. and Fujiki, H. (1990) J. Cancer Res. Clin. Oncol. 116, 609–614.
[13] Falcomer, I.R. and Buckley, T.H. (1989) Med. J. Aust. 150, 351.
[14] Nishiwaki-Matsushima, R., Ohta, T., Nishiwaki, S., Suganuma,

M., Kobayama, K., Carmichael, W.W. and Fujiki, R. (1992) J. Cancer Res. Clin. Oncol. 118, 420–424.
[15] Carmichael, W.W., Beasley, V.R., Bunner, D.L., Eloff, J.N., Falcomer, I., Gorham, P., Harada, K.-I., Krishnamurthy, T., Yu, M.-J., Moore, R.E., Rinehart, K.L., Runnegar, M., Skulberg, O.M. and Watanabe, M. (1988) Toxicon 26, 971–973.
[16] Craig, M., McCready, T.L., Luu, H.A., Smillie, M.A., Dubord, P. and Holmes, C.F.B. (1993) Toxicon 31, 1541–1558.
[17] Sivonen, K., Namikoshi, M., Evans, W.R., Carmichael, W.W., Sun, F., Rouhiainen, L., Luukkainen, R. and Rinehart, K.L. (1992) Appl. Environ. Microbiol. 58, 2495–2500.
[18] Luukkainen, R., Sivonen, K., Namikoshi, M., Färdig, M., Rinehart, K.L. and Niemelä, S.I. (1993) Appl. Environ. Microbiol. 59, 2204–2209.
[19] Rudolph-Böhner, S., Wu, J.T. and Moroder, L. (1993) Biol. Chem. Hoppe-Seyler 374, 635–640.
[20] Harada, K.-I., Matsuura, R., Susuki, M., Watanabe, M.F., Oishi, S., Dahlem, A.M., Beasley, V.R. and Carmichael, W.W. (1990) Toxicon 28, 55–64.
[21] Harada, K.-I., Ogawa, K., Matsuura, K.K., Murata, H., Suzuki, M., Watanabe, F.M., Itezono, Y. and Nagayama, N. (1990) Chem. Res. Toxicol. 3, 473–481.
[22] Stoner, R.D., Adams, W.H., Slatkin, D.N. and Siegelman, H.W. (1989) Toxicon 7, 825–828.
[23] Braunschweiler, L. and Ernst, R.R. (1983) J. Magn. Reson. 53, 521–528.
[24] Bax, A. and Davis, D.G. (1985) J. Magn. Reson. 65, 355–360.
[25] Piantini, U., Sorensen, O.W. and Ernst, R.R. (1982) J. Am. Chem. Soc. 104, 6800–6801.
[26] Jeener, J., Meier, B.H., Bachmann, P. and Ernst, R.R. (1979) J. Chem. Phys. 71, 4546–4553.
[27] Bax, A. and Davies, D.G. (1985) J. Magn. Reson. 63, 207–213.
[28] Havel, T.F. (1991) Prog. Biophys. Molec. Biol. 56, 43–78.
[29] Kaptein, R., Boelens, R., Scheek, R.M. and van Gunsteren, W.F. (1988) Biochemistry 27, 5389–5395.
[30] Crippen, G.M. and Havel, T.F. (1988) Distance Geometry and Molecular Conformation, Research Studies Press, Somerset, England; John Wiley, New York.
[31] Berendsen, H.J.C., Postma, J.P.M., van Gunsteren, W.F., DiNola, A. and Haak, J.R. (1984) J. Chem. Phys. 81, 3684–3896.
[32] Rinehart, K.L., Harada, K.-I., Namikoshi, M., Chen, C., Harvis, C.A., Munro, M.H.G., Blunt, J.W., Mulligan, P.E., Beasley, V.R., Dahlem, A.M. and Carmichael, W.W. (1988) J. Am. Chem. Soc. 110, 8557–8558.
[33] Sandström, A., Glemarec, C., Meriluoto, J.A.O., Eriksson, J.E. and Chattopadhyaya, J. (1990) Toxicon 28, 535–540.
[34] Lanaras, T., Cook, C.M., Eriksson, J.E., Meriluoto, J.A.O. and Hotokka, M. (1991) Toxicon 29, 901–906.
[35] Taylor, C., Quinn, R.J., McCulloch, R., Nishiwaki-Matsushima, R. and Fujiki, H. (1992) Bioorg. Med. Chem. Lett. 4, 299–302.
[36] Cohen, P., Holmes, C.F.B. and Tsukitani, Y. (1990) Trends Biochem. Sci. 15, 98–102.
[37] Eriksson, J.E., Grönberg, L., Nygård, S., Slotte, J.P. and Meriluoto, J.A.O. (1990) Biochim. Biophys. Acta 1025, 60–66.
[38] Stoner, R.D., Adams, W.H., Slatkin, D.N. and Siegelman, H.W. (1990) Toxicon 28, 569–573.
[39] Namikoshi, N., Rinehart, K.L., Dahlem, A.M., Beasley, V.R. and Carmichael, W.W. (1989) Tetrahedron Lett. 30, 4349–4352.
[40] Choi, B.W., Namikoshi, M., Sun, F., Rinehart, K.L., Carmichael, W.W., Kaup, A.M., Evans, W.R. and Beasley, V.R. (1993) Tetrahedron Lett. 34, 7881–7884.
[41] Barford, D. and Keller, J.C. (1994) J. Mol. Biol. 235, 763–766.