Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 717–722

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

THE SOLUTION STRUCTURES OF CALYCULIN A AND DEPHOSPHONOCALYCULIN A BY NMR.

Katie E. Volter, Gregory K. Pierens, Ronald J. Quinn*

Queensland Pharmaceutical Research Institute, Griffith University, Nathan, Brisbane, 4111, Australia

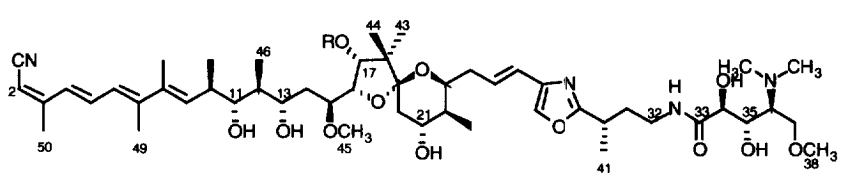
Toshiyuki Wakimoto, Shigeki Matsunaga, Nobuhiro Fusetani

Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agriculture and Agricultural Life Sciences, The University of Tokyo, Tokyo 113, Japan

Received 23 September 1998; accepted 25 January 1999

Abstract: The NMR solution structure of calyculin A (**1**) in chloroform exhibits intramolecular interactions, resembling the original crystal structure[1]. In methanol, calyculin A has the hydrogen bonding moieties solvent exposed. Dephosphonocalyculin A in chloroform resembles calyculin A in chloroform and the crystal structure of calyculin A. Dephosphonocalyculin A in methanol resembles calyculin A in methanol.

Calyculin A (**1**) was first isolated as the major active constituent from the marine sponge *Discodermia calyx* isolated from the Gulf of Sagami.[1] Its unique molecular structure consists of an octamethyl polyhydroxylated 28 carbon fatty acid, with two linked γ-amino acids. It has an Z,E,E,E-tetraene system in the C_{28} moiety, a β-hydroxytetrahydrofuran that is esterified by phosphoric acid and whose hetero oxygen forms a spiro ketal with a γ-hydroxytetrahydrofuran linked to two γ-amino acids.[1] The X-ray crystal structure displays many short intramolecular contacts, one of which is between the C11 hydroxy and the nitrogen of the amide resulting in a constrained ring. Other intramolecular bonds determined from the X-ray crystal structure involve the oxygen atoms of the phosphate group with C13-OH, C36-N(CH_3)$_2$, and with N(oxazole).[1]



(**1**) R = PO_3H_2, (**2**) R=H.

0960-894X/99/$ - see front matter © 1999 Elsevier Science Ltd. All rights reserved.
PII: S0960-894X(99)00066-9

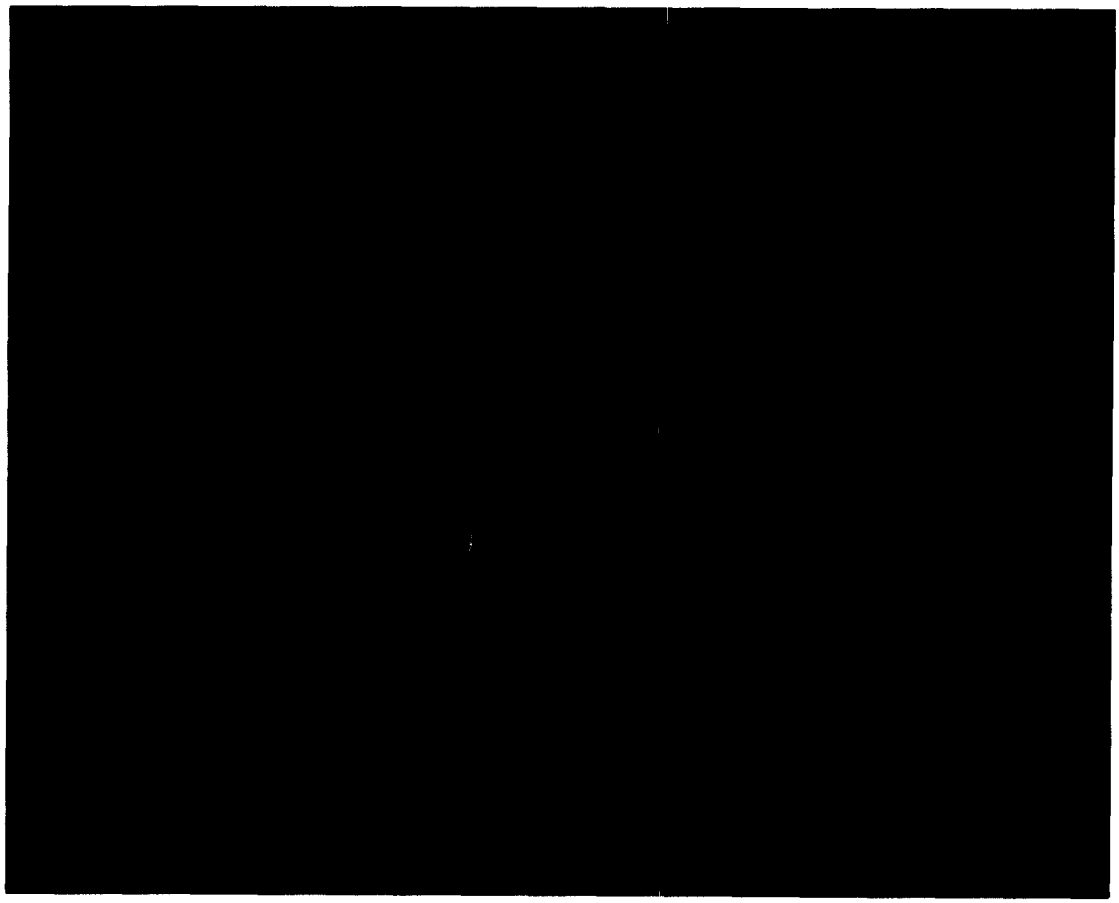


Figure 1: The crystal structure of Calyculin A. Carbon atoms are coloured white, oxygen atoms red, nitrogen atoms blue, and phosphorus atoms orange. The protons have not been displayed. This figure and Figure 2 were pictured using the SYBYL software package (Tripos Associates).

The absolute stereochemistry of calyculin A and other calyculins were later determined.[2] The CD spectrum of a pentanoic acid, obtained by degradation of calyculin A indicated a 2S stereochemistry for the pentanoic acid, which corresponds to 34S of the calyculins.[2] A series of other calyculins, des- N- methylcalyculin and calyculinamides have since been isolated from *D. calyx*[3] and from other marine sponge sources including the New Zealand deep- water marine sponge, *Lamellomorpha strongylata*.[4]

Recently a dephosphorylated form of calyculin A **(2)** has been isolated from *D. calyx* and its structure determined by COSY, HMQC, and HMBC analyses and its stereochemistry unambiguously confirmed to be the same as calyculin A.[5] This was achieved by the conversion of calyculin A into an 11, 13-acetonide

derivative, and dephosphorylation using a mixture of dioxane and pyridine to form the acetonide derivative of dephosphonocalyculin A.[5] Biological testing of the calyculins has shown strong activity in cytotoxicity assays (P388 and L1210 leukemia cells)[1-5] and more specifically, inhibitory activity against protein phosphatases 1 and 2A (PP1, PP2A) with IC_{50} values of 0.5 to 1 nM and 2 nM, respectively.[6] Dephosphonocalyculin A has IC_{50} values of 3.0 and 8.2 nM against PP1 and 2A, respectively. It has an IC_{50} value of 18 ng mL^{-1} in cytotoxicity tests against the P388 cell line.[5]

Modeling of microcystin-LR, motuporin, okadaic acid and calyculin A has allowed for the generation of a pharmacophore model of the proposed binding site of these molecules with PP1. The pharmacophore model proposed consists of a central core containing a conserved acidic site and two potential conserved hydrogen bonding sites, and a non-polar side chain.[7] This study has been used for development of a combinatorial library, based on the results of the modeling study and the compounds studied have been determined to inhibit cells in the G_0 phase.[8] Since the advent of the published X-ray crystal structure of microcystin-LR with PP1,[9] molecular modeling studies showing calyculin A in the binding site have been published.[10] These studies indicate calyculin A interacts within the central binding site. Two of the modeling studies have indicated that calyculin A could occupy the central binding area, extending the hydrophobic nitrile tail into the hydrophobic groove, with the central phosphoric acid moiety positioned over the central metal binding region, and the polyhydroxylated, amine (or "basic") portion of the inhibitor extending into the acidic groove, and possibly interacting with E256.[10b,10c]

We have performed an analysis of the NMR solution structures of calyculin A in order to determine the relationship between the solution structures and the proposed docked structures of calyculin A into PP1. Due to the critical role the phosphate played in the modeling analyses of calyculin A and PP1, and lack of a reported modeled analysis of dephosphonocalyculin A in PP1, an NMR analysis for the solution structures of these compounds have been performed. The assignments of calyculin A in chloroform-d, and methanol-$d_3$were performed by COSY (in $CDCl_3$) and TOCSY ($CDCl_3$ and CD_3OH).[11] Dephosphonocalyculin A in chloroform and methanol-d_3 were assigned by COSY, TOCSY, and HMQC ($CDCl_3$ only) analyses.[11] Solution structure conformations for these two molecules were performed by NOESY and ROESY analyses, at varying mixing times ranging from 50 ms to 250 ms, followed by solution structure generation using MacroModel.[12] Of the twenty lowest energy structures generated for each set of solution structures, only one conformation was noted for each set.

Calyculin A in chloroform, as seen in Figure 2A, strongly resembled the crystal structure, with a number of strong intramolecular ROE's observed between the basic portion of calyculin A (the C34-C38 region) and the spiroacetal region, specifically between C32-C16, C38-C13 and between C9-amide. Restrained molecular dynamics and subsequent the minimisation procedure were performed in a "virtual" chloroform solvent. Because of the nature of this solvent, the potential hydrogen bonding moieties, the amide, the

tertiary amine, and the poly-hydroxy moieties are internal to the molecule, shielded from the solvent by the carbon backbone of the molecule. ROE constraint ranges were violated by no greater than 1 Å. Strong intramolecular hydrogen bonds observed include C11-OH and C34-OH; C11-OH and amide NH; C11-OH and C37-OCH_3, and a weaker hydrogen bonding interaction between C21-OH and O16. The twenty lowest energy structures had an average RMSD of 0.8Å ± 0.52Å and had final energies of 40- 90 kJ mol^{-1}.

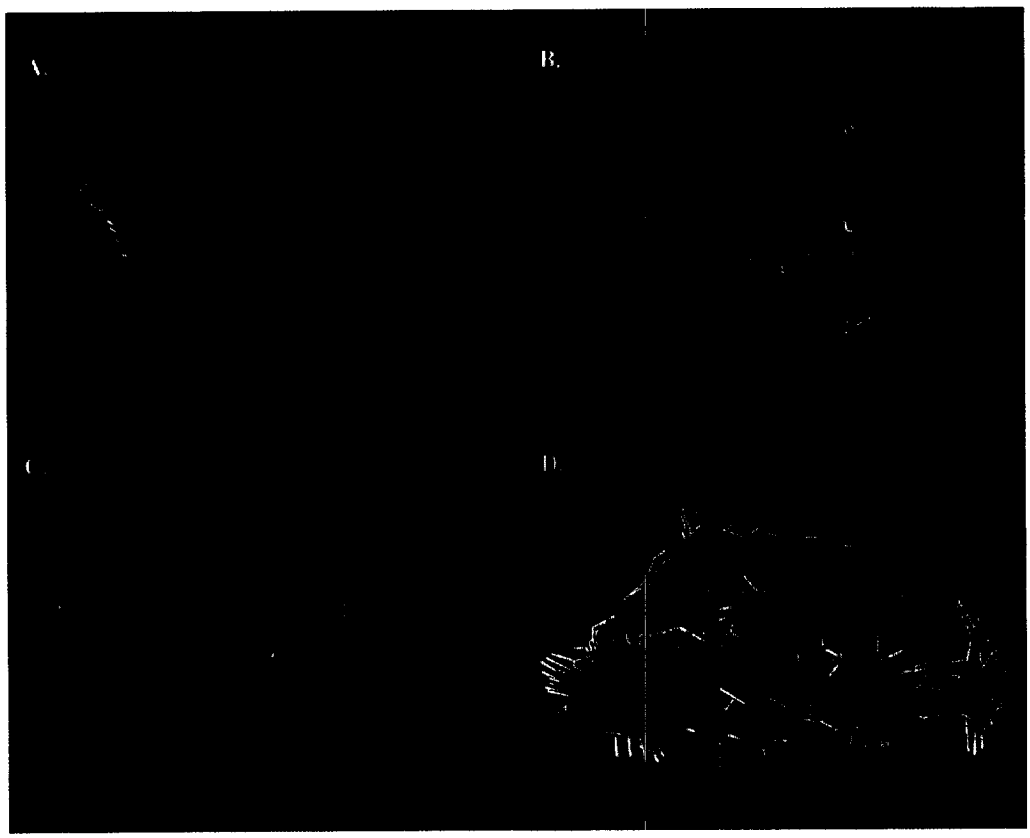


Figure 2: (A) The solution structure of Calyculin A in chloroform. (B) Calyculin A in methanol. (C) Dephosphonocalyculin A in chloroform, and (D) Dephosphonocalyculin A in methanol.

The solution structure of calyculin A in methanol can be seen in Figure 2B. The structure in this solvent forms a conformation with the phosphate moiety and the hydrophilic moieties exposed to the solvent. The dynamics and minimisation analyses were performed in a virtual water solvent. Constraints generated from ROE interactions involved the strong interactions within the hydrophobic region. Other weaker ROE interactions were observed between the basic region (the polyhydroxy region extending from carbon 34 to carbon C38) and the spiroacetal region (from carbon16 to carbon 23), for example between amide NH and

C43; and shorter intramolecular regions, for example between C14 and H11. After dynamics and minimisation analyses, the constraints resulting from ROE's were violated by no greater than 0.9 Å. Significant intramolecular hydrogen bonding interactions for calyculin A in methanol were observed between the amide NH and C35-OH, with less significant interactions observed between C1 nitrile and C21-OH; between C35-OH and C11-OH and between C34-OH and C11-OH. The twenty lowest energy structures observed had an average RMSD of 0.64Å ± 0.24Å, and had final energies ranging from 100 - 123 kJ mol^{-1}.

Calyculin A in chloroform exhibits many intramolecular hydrogen bonds removed from solvent exposure whereas in methanol, these groups are solvent exposed. The solution structure of calyculin A in methanol does appear to show similarity to two of the docked structures previously reported,[10b, 10c] whilst the solution structure of calyculin A in chloroform is very similar to the reported crystal structure.[1]

The solution structures of dephosphonocalyculin A in chloroform are shown in Figure 2C. As seen with calyculin A, ROE interactions were observed primarily between the basic region and the spiroacetal region of the molecule, and between the dihydroxy region (carbons 11 and 13) and the spiroacetal region. Constraints from ROE analysis was violated by no greater than 0.81 Å. Significant observed hydrogen bonding interactions include the interaction between amide C=O and C35-OH, and between C13-OH and C11-OH. The less significant intramolecular interactions occur between amide NH and C15 -OCH_3, and between amide NH and C13 -OH. The twenty lowest energy structures observed had an RMSD of 0.48Å ± 0.21Å, and had final energies ranging from 223- 275 kJ mol^{-1}.

Dephosphonocalyculin A in methanol exhibited primarily short-range ROE interactions, and the resulting solution structure is seen in Figure 2D. Only two longer-range ROE interactions are evident between the basic region and the 11, C13 dihydroxy region of the molecule. After restrained molecular dynamics and minimisation studies performed in water, the analysed constraints arising from the ROE interactions were violated by no greater than 0.9Å. Hydrogen bonding interactions were observed between C13–OH and C11–OH, amide NH and C35–OH, C35–OH and C11–OH, and to a lesser extent, C13–OH and C15–OCH_3, and C34–OH and C1 nitrile. The hydrogen bonding moieties on the molecule are solvent exposed, with the nitrile moiety curled around. The twenty lowest energy structures had a final RMSD of 1.65Å ± 0.90Å and a final energy of 170- 243 kJ mol^{-1}.

The solution structure of dephosphonocalyculin A in chloroform is quite similar to the solution structure of calyculin A in the same solvent, in that the basic region (C34- C37) and the C11, C13 dihydroxy region exhibit weak intramolecular hydrogen bonding, which brings these two regions close. Similarly to calyculin A, dephosphonocalyculin A in methanol exhibits a structure with most hydrogen bonding groups solvent exposed. These results mirror the apparent alteration in conformation of the solution structures of cyclosporin A in solvents of differing polarity.[13] The implication of conformational studies to the interaction of dephosphonocalyculin A with PP1 are currently being investigated by docking experiments.

Acknowledgments: We thank the Australian Research Council for support of this research. The bequest of the late Thomas Patrick Hinch is also gratefully appreciated.

References and Notes:

1. Kato, Y.; Fusetani, N.; Matsunaga, S.; Hashimoto, K.; Fujita, S.; Furuya, T. *J. Am. Chem. Soc.* **1986**, *108*, 2780.
2. Matsunaga, S.; Fusetani, N. *Tetrahedron Letts.* **1991**, *32*, 5605.
3. (a) Kato, Y.; Fusetani, N.; Matsunaga, S.; Hashimoto, K.; Koseki, K. *J. Org. Chem.* **1988**, *53*, 3930. (b) Matsunaga, S.; Wakimoto, T.; Fusetani, N. *J. Org. Chem.* **1997**, *62*, 2640.
4. Dumdei, E.; Blunt, J.; Munro, M.; Pannell, L. *J. Org. Chem.* **1997**, *62*, 2636.
5. Matsunaga S.; Wakimoto, T.; Fusetani, N.; Suganuma, M. *Tetrahedron. Lett.* **1997**, *38*, 3763.
6. Ishihara, H.; Martin, B.; Brautigan, D.; Karaki, H.; Ozaki, H.; Kato, Y.; Fusetani, N.; Watabe, S.; Hashimoto, K.; Uemura, D.; Hartshorne, D. *Biochem. Biophys. Res. Comm.* **1989**, *159*, 871.
7. (a) Quinn, R.; Taylor, C.; Suganuma, M.; Fujiki, H. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1029. (b) Quinn, R.; Taylor, C.; Embrey, K.; Poulsen, S-A. In *Toxic Microcystis*; Watanabe, M. Harada, K-I. Carmichael, W. Fujiki, H., Eds.; CRC; 1995; pp235-255.
8. Wipf, P.; Cunningham, A.; Rice, R.; Lazo, J. *Bioorg Med. Chem.* **1997**, *5*, 165.
9. Goldberg, J.; Huang, H.; Kwon, Y.; Greengard, P.; Nairn, A.; Kuriyan, J. *Nature* **1995**, *376*, 745.
10. (a) Bagu, J.; Sykes, B.; Craig, M.; Holmes, C. *J. Biol. Chem.* **1997**, *272*, 5087. (b) Lindvall, M.K.; Pihko, P.M.; Koskinen, A.M.P. *J. Biol. Chem.* **1997**, *272*, 23312. (c) Gauss, C-M.; Sheppeck, J.E.; Nairn, A.C.; Chamberlin, R. *Bioorg. Med. Chem.* **1997**, *5*, 1751.
11. **Materials and Methods:** Calyculin A (9 mg) and dephosphonocalyculin A (5 mg) were run on a Varian Unity Plus 600 MHz spectrometer using using a 5 mm Triple Resonance Proton Observe Probe and were processed using Sun processing stations. The simulation of the solution structures by Dynamics simulations and minimisations were performed on a Silicon Graphics O2 computer using MacroModel®, Version 6.0 software. Chloroform (D, 99.8%) and methanol-d_3 were obtained from the Cambridge Isotope Laboratories (Andover, Mn, USA). All ROESY spectra were run with mixing times of 50, 100, 150, 200 and 250 ms (2048 data points in both F1 and F2). Constraints were generated from the ROE's obtained and these were used in the simulated annealing and subsequent minimisation on the structures in MacroModel. ROE's were devided by cross- peak volume measurements into strong (1.8-2.5 Å (2.15 ± 0.35 Å)); medium, (1.8-3.5 Å (2.65 ± 0.85 Å)); and weak correlations (1.8-5.0 Å (3.4 ± 1.6 Å)). Simulated annealing involved the simulation of heating the molecule at 1000 K for 0.2 ρs, in the presence of the virtual solvent (using H_2O for CD_3OH and chloroform for $CDCl_3$), and then cooling the structure to 200 K to generate 100 structures. From there minimisation using the TNCG (Truncated Newton Conjugate Gradient) method, MM2* force field, and using the appropriate solvent, was performed on the generated conformations for a maximum of 100 iterations to provide a population of structures for the solution structure analysis. From this, the twenty lowest energy conformers were selected, superimposed and analysed.
12. MacroModel ® Interactive Molecular Modeling System. Version 6.0. Department of Chemistry, Columbia University, New York, New York, 10027, USA.
13. (a) Kessler, H.; Loosli, H-R.; Oschkinat, H. *Helv. Chim. Acta.* **1985**, *68*, 661. (b) Loosli, H-R.; Kessler, H.; Oschkinat, H.; Weber, H-P.; Petcher, T.J.; Widmer, A. *Helv. Chim. Acta.* **1985**, *68*, 682. (c) Kessler, H.; Köck, M.; Wein, T., Gehrke, M.. *Helv. Chim. Acta.* **1990**, *73*, 1818. (d) Verheyden, P.; Jaspers, H.; De Wolf, E.; Van Binst, G. *Int. J. Pept. Prot. Res.* **1994**, *44*, 364. (e) Ko, S.Y.; Dalvitt, C. *Int. J. Pept. Prot. Res.* **1992**, *40*, 380. (f) Lautz, J.; van Gunsteren, W.F.; Weber, H-P.; Wenger, R.M. *Biopolymers*, **1990**, *29*, 1669.