agarose (lane B), and the PKC preparation after elution from W7 agarose (lane C). All three lanes contained an 82 kD band characteristic of our PKC preparations [18], and it is apparent that contaminating bands were less abundant in the preparations purified on the W7-agarose and CAPP-agarose columns.

Discussion

In view of the central role of PKC in numerous signal transduction mechanisms, it is of great interest to develop specific PKC inhibitors for mechanistic studies of the enzyme and for potential therapeutic purposes. Several PKC inhibitors that have been identified, including chlorpromazine [2] and W7 [3], appear to inhibit PKC by drug–lipid interactions, but the possibility that there are direct interactions between these drugs and PKC has not been investigated prior to this report. In this paper, we show that PKC interacted directly with W7 and also with a chlorpromazine analog. These interactions did not require the presence of phospholipid, Ca^{2+}, or other PKC cofactors. Our evidence for direct interactions betwen PKC and these inhibitors suggests that the mechanism of inhibition of PKC by cationic amphiphilic drugs does not merely involve drug–lipid interactions.

Certain PKC inhibitors also inhibit calmodulin-dependent enzymes by binding to calmodulin in a Ca^{2+}-dependent manner [3, 10]. In addition, calmodulin binds to W7 Sepharose and to CAPP Sepharose, in a Ca^{2+}-dependent manner [12, 13, 17, 19]. In contrast, we find the PKC binds to W7 agarose and CAPP agarose in a Ca^{2+}-independent manner. A chemically reactive chlorpromazine analog, [^{3}H] norchlorpromazine isothiocyanate, forms a covalent one-to-one complex with calmodulin [20]. It will be of interest to determine whether chemically reactive derivatives of PKC inhibitors bind covalently to the enzyme and to determine the specific sites on the enzyme to which these drugs might bind. Such studies could lead to a rationale for the design of specific PKC inhibitors.

PKC eluted from the W7-agarose column as an asymmetrical peak and from the CAPP-agarose column in two peaks (Figs. 1 and 3). These elution profiles may reflect structural heterogeneity of PKC molecules within the enzyme preparation. Such heterogeneity would be consistent with the evidence for multiple and distinct PKC-encoding cDNAs [18, 21, 22]. Thus, the affinity column procedures described in the present study may also be useful in resolving multiple forms of PKC.

Acknowledgements—We acknowledge the excellent secretarial assistance of Mrs. Nancy Mojica and Ms. Lintonia Sheppard. This work was supported by NCI Grant CA 02656 to I. Bernard Weinstein.

† Author to whom all correspondence should be addressed. Present address: Department of Cell Biology, University of Texas System Cancer Center, 6723 Bertner Ave., Houston, TX 77030.

*Comprehensive Cancer Center/ Institute of Cancer Research, ‡Department of Medicine, and §Department of Genetics and Developmental Biology College of Physicians and Surgeons Columbia University New York, NY 10032, U.S.A.

CATHERINE A. O'BRIAN*†
GERARD M. HOUSEY*§
I. BERNARD WEINSTEIN*‡

REFERENCES

1. Y. Nishizuka, *Science* **233**, 305 (1986).
2. T. Mori, Y. Takai, R. Minakuchi, B. Yu and Y. Nishizuka, *J. biol. Chem.* **255**, 8378 (1980).
3. T. Tanaka, T. Ohmura, T. Yamakado and H. Hidaka, *Molec. Pharmac.* **22**, 408 (1982).
4. M. Ito, T. Tanaka, M. Inagaka, K. Nakanishi and H. Hidaka, *Biochemistry* **25**, 4179 (1986).
5. C. A. O'Brian, R. Liskamp, D. H. Solomon and I. B. Weinstein, *Cancer Res.* **45**, 2462 (1985).
6. H-D. Su, G. J. Mazzei, W. R. Vogler and J. F. Kuo, *Biochem. Pharmac.* **34**, 3649 (1985).
7. C. A. O'Brian, R. M. Liskamp, D. H. Solomon and I. B. Weinstein, *J. natn. Cancer Inst.* **76**, 1243 (1986).
8. C. A. O'Brian and I. B. Weinstein, *Biochem. Pharmac.* **36**, 1231 (1987).
9. Y. A. Hannun, C. R. Loomis, A. H. Merrill and R. M. Bell, *J. biol. Chem.* **261**, 12604 (1986).
10. D. R. Marshak, T. J. Lukas and D. M. Watterson, *Biochemistry* **24**, 144 (1985).
11. H-Y. P. Lam, *Biochem. biophys. Res. Commun.* **118**, 27 (1984).
12. D. R. Marshak, D. M. Waterson and L. J. Van Eldyk *Proc. natn. Acad. Sci. U.S.A.* **78**, 6793 (1981).
13. J. F. Harper, *J. Cyclic Nucleotide Protein Phosphorylat. Res.* **9**, 3 (1983).
14. C. A. O'Brian, D. S. Lawrence, E. T. Kaiser and I. B. Weinstein, *Biochem. biophys. Res. Commun.* **124**, 296 (1984).
15. U. K. Laemmli, *Nature, Lond.* **227** 680 (1970).
16. C. R. Merril, R. C. Switzer and M. L. Van Keuren, *Analyt. Biochem.* **105**, 361 (1980).
17. G. A. Jamieson and T. C. Vanaman, *Biochem. biophys. Res. Commun.* **90**, 1048 (1979).
18. G. M. Housey, C. A. O'Brian, M. D. Johnson, P. Kirschmeier and I. B. Weinstein, *Proc. natn. Acad. Sci. U.S.A.*, **84**, 1065 (1987).
19. C. R. Caldwell and A. Haug, *Analyt. Biochem.* **116**, 325 (1981).
20. D. L. Newton, T. R. Burke, K. C. Rice and C. B. Klee, *Biochemistry* **22**, 5472 (1983).
21. L. Coussens, P. J. Parker, L. Rhee, T. L. Yang-Feng, E. Chen, M. D. Waterfield, U. Francke and A. Ullrich, *Science* **233**, 859 (1986).
22. J. L. Knopf, M-H. Lee, L. A. Sultzman, R. W. Kriz, C. R. Loomis, R. M. Hewick and R. M. Bell, *Cell* **46**, 491 (1986).

Biochemical Pharmacology, Vol. 36, No. 23, pp. 4181–4185, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

Cytotoxic activity of cyclic peptides of marine origin and their derivatives: importance of oxazoline functions

(*Received* 6 *May* 1987; *accepted* 10 *June* 1987)

A series of cytotoxic cyclic peptides have been isolated from marine organisms in the last few years [1–6]. All of these peptides contain unusual amino acid moieties involving the thiazole ring as their constituents. Furthermore, except dolastatin 3[5], all of the other peptides have the unique oxazoline ring. Their intriguing structures and cytotoxic activities led us to synthesize some of these peptides: dolastatin 3 (the proposed structure and its 15 isomers) [7, 8], ascidiacyclamide [9, 10], patellamides A[11], B[12, 13], and C[12, 13] (their proposed and revised struc-

tures), and ulithiacyclamide [10, 14]. We then surveyed the cytotoxic activity of these cyclic peptides and their synthetic intermediates. This investigation revealed the importance of the oxazoline ring for cytotoxicity of these peptides.

Materials and methods

Chemicals. Cyclic peptides and their derivatives used for the cytotoxicity test have been prepared according to our previous reports [7–14]. As positive controls, clinically used representive anticancer drugs such as vincristine (VCR), 5-fluorouracil (5FU), adriamycin (ADM), and 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU) were employed. These drugs are gifts from Dr. T. Kato, Aichi Cancer Center Research Institute. Cyclic peptides and anticancer drugs were dissolved in DMSO and saline, respectively, and used for cytotoxicity test.

Growth inhibition of L1210 *cells.* Mouse leukemia L1210 cells were grown in RPMI-1640 medium (Nissui Seiyaku Co. Ltd., Tokyo) supplemented with 10% heat-inactivated fetal calf serum (Gibco, New York), L-glutamine (300 μg/ml), $NaHCO_3$ (2 mg/ml), and kanamycin (100 μg/ml). The cells were incubated at 37° in a humidified incubator with 5% CO_2 in air. For the cytotoxicity assay of the compound, cells (5×10^5) were suspended in 5 ml of the medium in 60-mm plastic dish and incubated for 24 hr. Then 25 μl of DMSO or saline containing appropriate concentration of the test compound was added to the cell culture and the mixture was incubated for another 48 hr. At the end of the incubation, the number of viable cells was counted. Cytotoxicity assay of each compound was done at least twice. The median inhibitory dose, ID_{50}, the concentration of the compound that decreases the growth rate of the cells to 50% of the control cells, was deduced graphically.

Pretreatment of ulithiacyclamide (**10**) *with dithiothreitol.* One mg of **10** and 10 eq. mol of dithiothreitol (2 mg) in 500 μl of DMSO were incubated at 37° for 12 hr. By this treatment, **10** was almost completely changed to another product; probably the disulfide bond cleaved compound which was detected by TLC (silica gel plate, MeOH:$CHCl_3$ = 1:19 v/v, R_f 0.60; cf. R_f of **10**, 0.77) after the DMSO in the reaction mixture was removed by freeze-drying and the residue was solubilized in a small amount of $CHCl_3$. For the cytotoxicity assay, an aliquot of the above DMSO solution was added to the cell suspension. As a control, **10** in DMSO without dithiothreitol was also examined and we found no decrease of the cytotoxic activity compared with that of the non pre-treated **10**.

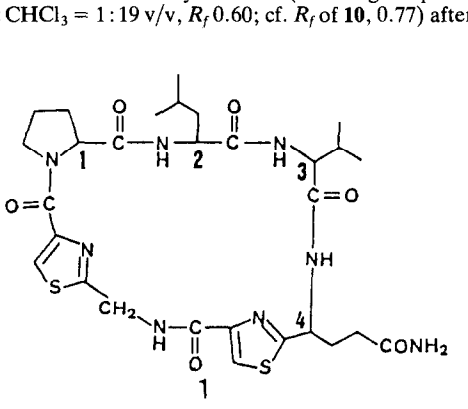
Results and discussion

The compounds were evaluated for the cytotoxic effect against the L1210 cells. Dolastatin 3 (**1a**) containing two thiazole rings but no oxazoline rings has been claimed by Pettit and coworkers [5] to show a high cell growth inhibition ($ED_{50} < 1 \times 10^{-4}$–$1 \times 10^{-7}$ μg/ml) against murine P388 lymphocytic leukemia cell line. On the contrary, synthesized dolastatin 3 having the proposed structure **1a** and its 7 diastereomers (**1b–1h**) shown in Scheme I had no activity against L1210 leukemia cells ($ID_{50} > 250$ μg/ml). The reverse isomer **2a** of **1a**, suggested [5] as an alternative structure, and its 7 diastereomers (**2b–2h**) also had no activity ($ID_{50} > 250$ μg/ml). We have already demonstrated that the melting points, the optical rotations, and the spectral data of these 16 synthesized samples are different from those of natural dolastatin 3, and concluded that the structures **1** and **2** assigned to dolastatin 3 require revision [15, 16].

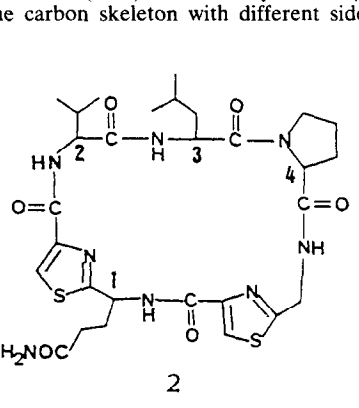
The structures of other compounds tested are shown in Scheme II, and their cytotoxic data are summarized in Table 1.

Of the compounds tested, ulithiacyclamide (**10**) exhibits the most potent cytotoxicity which is comparable with that of presently clinically used useful anticancer drugs. Survey of Table 1 indicates that the oxazoline function is essential to display cytotoxicity since the cytotoxicity depends on whether the oxazoline function is present or not. Even small peptides having the oxazoline function such as **14** and **15** show moderate but distinct cytotoxicity. This also suggests that the cyclic skeleton of peptides might not be essential for cytotoxicity.

Patellamides A–C (**3a–c**) and ascidiacyclamide (**3d**) having the same carbon skeleton with different side chains



1

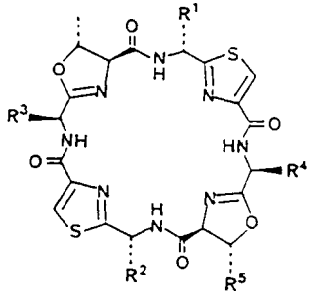


2

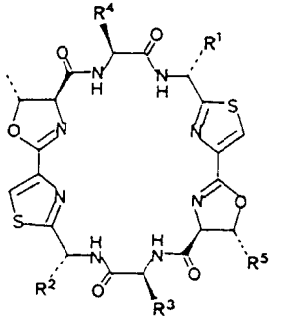
	C-1	C-2	C-3	C-4
1a	L	L	L	L
b	L	L	L	D
c	L	L	D	L
d	L	D	L	L
e	D	L	L	L
f	L	L	D	D
g	L	D	L	D
h	D	L	L	D

	C-1	C-2	C-3	C-4
2a	L	L	L	L
b	D	L	L	L
c	L	D	L	L
d	L	L	D	L
e	L	L	L	D
f	D	D	L	L
g	D	L	D	L
h	D	L	L	D

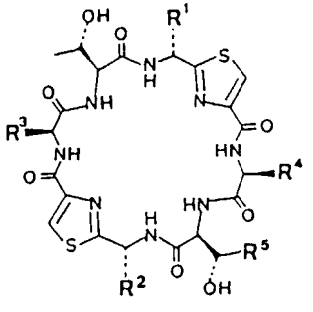
Scheme I



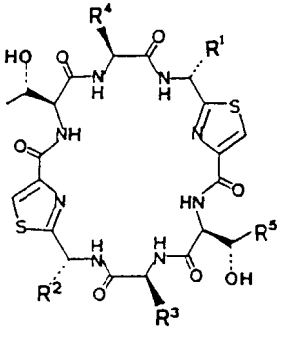
3a–d



4a–c



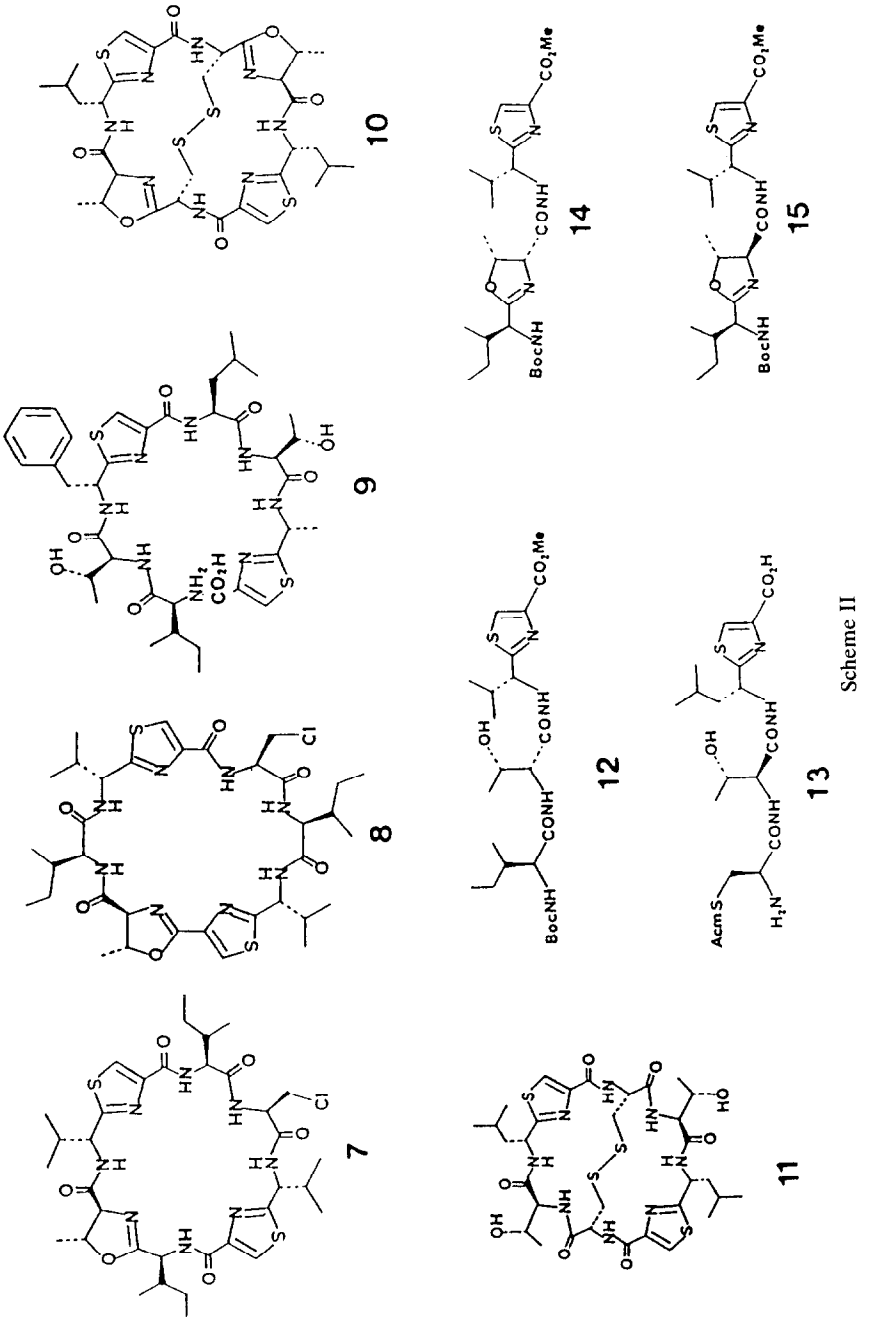
5a–d



6a–c

	R^1	R^2	R^3	R^4	R^5
3a-6a:	$(CH_3)_2CH$	$(CH_3)_2CH$	$CH_3CH_2CH(CH_3)$	$CH_3CH_2CH(CH_3)$	H
3b-6b:	$C_6H_5CH_2$	CH_3	$CH_3CH_2CH(CH_3)$	$(CH_3)_2CHCH_2$	CH_3
3c-6c:	$C_6H_5CH_2$	CH_3	$CH_3CH_2CH(CH_3)$	$(CH_3)_2CH$	CH_3
3d:	$(CH_3)_2CH$	$(CH_3)_2CH$	$CH_3CH_2CH(CH_3)$	$CH_3CH_2CH(CH_3)$	CH_3
5d:	$(CH_3)_2CH$	$(CH_3)_2CH$	$AcmSCH_2$	$AcmSCH_2$	CH_3

Acm = $CH_3CONHCH_2$



Scheme II

Table 1. Effects of cyclic peptides and their derivatives against L1210 leukemia cells

Compd	ID_{50} (μg/ml)	Compd	ID_{50} (μg/ml)
3a	80	**7**	25
3b	15	**8**	100
3c	12	**9**	>250*
3d	22	**10**	0.04
4a	90	**10**†	0.08
4b	6	**11**	>125*
4c	33	**12**	>300*
5a	>125*	**13**	>300*
5b	210	**14**	100
5c	>125*	**15**	33
5d	>125*	VCR	0.013
6a	>125*	5FU	0.21
6b	>250*	ADM	0.14
6c	>125*	ACNU	0.50

* At this concentration, the cell viability was 100% of that of control cells.

† Ulithiacyclamide (**10**) was pre-treated with 10 eq. mol of dithiothreitol as shown in the Experimental Section.

show more or less similar potency except that patellamide A (**3a**) is much weaker. Our results agree, in part, with the reported ones [2], in which **3a** displays cytotoxicity of the same order of magnitude as others (**3b** and **3c**). Ascidiacyclamide is also reported [6] to show an extremely lethal effect on PV_4 culture cells transformed with polyoma virus, the result of which is parallel to our cytotoxic data. We have already revised the proposed structures **4a–c** [2, 3] of patellamides A–C to **3a–c** by synthetic studies [11–13]. Interestingly, however, patellamides with the originally proposed structures **4a–c** also exhibited similar cytotoxicity to those with the revised structures **3a–c**. This again suggests the cyclic skeleton of peptides is not necessarily essential.

Ulithiacyclamide (**10**), the most potent cytotoxic peptide, also has the same carbon skeleton as those of patellamides A–C (**3a–c**) and ascidiacyclamide (**3d**), but it has a unique disulfide bridge which will assist to fix the conformation of the molecule. Interestingly, decrease of its cytotoxicity was observed by treatment of **10** with dithiothreitol known as a reagent to cleave the disulfide bond. This result suggests that the fixed conformation and/or disulfide bridge may be necessary to improve cytotoxic activity. Although we must wait for further investigation on why these peptides display cytotoxicity, the above data show the necessity of the oxazoline function for the cytotoxicity of cyclic peptides and their derivatives. The study on the mechanism is in progress.

In summary, cytotoxic activity of cyclic peptides of marine origin and their derivatives was investigated using L1210 murine leukemia cells in culture. Ulithiacyclamide (**10**) having the oxazoline function and the disulfide bridge has shown marked cytotoxicity. The importance of the oxazoline function for cytotoxicity has been well documented.

† To whom correspondence should be addressed.

Acknowledgements—We sincerely thank Professor Y. Kawazoe of our Faculty for stimulating discussions and Miss Y. Nakao for her assistance on toxicity assay. We thank Dr T. Kato of Aichi Cancer Center Research Institute for his helpful advice on cytotoxicity assay and for providing L1210 cells and anticancer drugs. Authors at the Department of Synthetic Organic Chemistry are grateful to the Ministry of Education, Science, and Culture, Japan for a partial financial support of this research by a Grant-in-Aid for Special Project Research (No. 61224011).

**Department of Synthetic Organic Chemistry*
Faculty of Pharmaceutical Sciences
Nagoya City University
Nagoya 467, Japan

TAKAYUKI SHIOIRI*†
YASUMASA HAMADA*
SHINJI KATO*
MAKOTO SHIBATA*
YUTAKA KONDO*

‡Department of Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
Nagoya City University
Nagoya 467, Japan

HIROAKI NAKAGAWA‡
KOHFUKU KOHDA†‡

REFERENCES

1. C. Ireland and P. J. Scheuer, *J. Am. chem. Soc.* **102**, 5688 (1980).
2. C. M. Ireland, A. R. Durso, Jr., R. A. Newman and M. P. Hacker, *J. org. Chem.* **47**, 1807 (1982).
3. J. E. Biskupiak and C. M. Ireland, *J. org. Chem.* **48**, 2302 (1983).
4. J. M. Wasylyk, J. E. Biskupiak, C. E. Costello and C. M. Ireland, *J. org. Chem.* **48**, 4445 (1983).
5. G. R. Pettit, Y. Kamano, P. Brown, D. Gust, M. Inoue and C. L. Herald, *J. Am. chem. Soc.* **104**, 905 (1982).
6. Y. Hamamoto, M. Endo, M. Nakagawa, T. Nakanishi and K. Mizukawa, *J. Chem. Soc., Chem. Commun.* 323 (1983).
7. Y. Hamada, M. Kokuryu and T. Shioiri, in *Peptide Chemistry 1983* (Ed. E. Munekata), p. 173. Protein Research Foundation, Osaka (1984).
8. Y. Hamada, K. Kohda and T. Shioiri, *Tetrahedron Lett.* **25**, 5303 (1984).
9. Y. Hamada, S. Kato and T. Shioiri, *Tetrahedron Lett.* **26**, 3223 (1985).
10. S. Kato, Y. Kondo, T. Sugiura, Y. Hamada and T. Shioiri, in *Peptide Chemistry 1985* (Ed. Y. Kiso), p. 67. Protein Research Foundation, Osaka (1986).
11. Y. Hamada, M. Shibata and T. Shioiri, *Tetrahedron Lett.* **26**, 6501 (1985).
12. Y. Hamada, M. Shibata and T. Shioiri, *Tetrahedron Lett.* **26**, 5155 (1985).
13. Y. Hamada, M. Shibata and T. Shioiri, *Tetrahedron Lett.* **26**, 5159 (1985).
14. S. Kato, Y. Hamada and T. Shioiri, *Tetrahedron Lett.* **27**, 2653 (1986).
15. U. Schmidt and R. Utz, *Angew. Chem. Int. Ed. Engl.* **23**, 725 (1984).
16. G. R. Pettit, P. S. Nelson and C. W. Holzapfel, *J. org. Chem.* **50**, 2654 (1985).