Tetrahedron Vol. 49, No. 47, pp. 10817-10830, 1993
Printed in Great Britain

0040-4020/93 $6.00+.00

Photolabile Derivatives of Indole Alkaloid Tumor Promoter Teleocidins: Synthesis, Biological Activities and Photoaffinity Labeling Studies

Kazuhiro Irie*, Shigenori Okuno, Fumito Koizumi and Koichi Koshimizu

Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

Hoyoku Nishino and Akio Iwashima

Department of Biochemistry, Kyoto Prefectural University of Medicine, Kyoto 602, Japan

(*Received in Japan* 16 *August* 1993)

Key Words: tumor promoter; teleocidin; indolactam; photoaffinity labeling; protein kinase C; Vilsmeier reaction

Abstract: *New photolabile teleocidin derivatives (Az-A-1, Az-C7 and Az-C2) were synthesized and examined by three in vitro bioassays related to tumor-promoting activity. Az-A-1 (15) and two epimers of Az-C7 (11a, 11b) were approximately 10 to 100-fold more active than (-)-indolactam-V (1), the fundamental structure of teleocidins. Synthesis of the ^{3}H-labeled probes with specific activity of more than 40Ci/mmol was achieved by use of commercially available ^{3}H-labeled succinimidyl-4-azidobenzoate. Specific binding of [^{3}H]Az-A-1 and [^{3}H]Az-C7 to the mouse epidermal particulate fraction, the target tissue of teleocidins, was saturable at approximately 20nM. Photoaffinity labeling on the particulate fraction using [^{3}H]Az-A-1 supported the recent hypothesis that the alkyl chain at position 7 of teleocidins is involved in the interaction with the phospholipids close to the receptor site. SDS gel electrophoresis of the photolabeled particulate fraction suggested the existence of two proteins (ca.30 and 50kD) specifically photolabeled by [^{3}H]Az-A-1 and [^{3}H]Az-C7. However, no specific labeling was detected at the 70 to 80kD region, which corresponded to protein kinase C, a well-characterized receptor for tumor promoters.*

INTRODUCTION

Naturally occurring tumor promoters such as phorbol esters, teleocidins and aplysiatoxins, which have similar pleiotropic effects on intact cells, are shown to bind to the same receptor on the membrane fraction in a high affinity manner.[1] The major binding site of these promoters is believed to be protein kinase C (PKC),[2] a key enzyme in the cellular signal transduction. Although several target proteins of PKC have been identified,[3] it is not completely understood how activation of PKC is connected to the proliferation and carcinogenesis of cells by these tumor promoters. Previous studies on the cellular uptake of fluorescent derivatives of phorbol esters indicated that these probes entered into the cells.[4,5] We have also shown that fluorescent teleocidin derivatives localized in the cytoplasm and nuclear membrane rather than the cell membrane of HeLa cells, and that such cellular uptake and the tumor-promoting activity both required pertinent hydrophobicity of the compound.[6] These findings suggest that phorbol esters and teleocidins enter the cell and directly interact with target molecules

other than PKC. In fact, the existence of a tumor promoter-specific binding protein (CN-TPBP) other than PKC in HL-60 and HeLa cells has recently been demonstrated.[7] Direct identification of the tumor promoter binding sites other than PKC is, therefore, indispensable to understand the pleiotropic effects of TPA-type tumor promoters.

Photoaffinity labeling is a promising approach to investigate the binding sites of these tumor promoters. However, several attempts to label the binding proteins with photolabile phorbol esters have resulted in labeling of only phospholipids.[8,9] Since teleocidins are structurally different from phorbol esters, we have tried the photoaffinity labeling using teleocidin derivatives with high specific radioactivity of more than 40Ci/mmol. In our preliminary publication, we have mentioned the synthesis of new photolabile indolactam derivatives having an aryl azide at position 7 of the indole ring (Az-C_7 and Az-C_2).[10] This paper is a full report on the synthesis and biological activities of these probes together with a new additional photolabile teleocidin derivative (Az-A-1). Photoaffinity labeling on the mouse epidermal particulate fraction, the target tissue of these tumor promoters, is also mentioned.

RESULTS

Synthesis of photolabile teleocidin derivatives

Commercially available ^{3}H-labeled succinimidyl-4-azidobenzoate, which has high reactivity to amino groups, enables the simultaneous introduction of an aryl azide and tritium label. In the synthesis of radioactive photolabile teleocidin derivatives, therefore, an amino group must be introduced onto (-)-indolactam-V (**1**). The most suitable position for introducing a photolabile group is position 7 of the indole ring since the structural requirement at this position is quite low.[11] In the previous paper, we have reported the synthesis of (-)-7-(2'-aminoethyl)indolactam-V (**10**) by Friedel-Crafts formylation, nitrovinylation and reduction.[12] However, the Friedel-Crafts formylation with dichloromethyl methyl ether and titanium tetrachloride, gave the desired 7-formyl derivative (**3**) with a low yield of less than 25%. Improvement of this formylation step was necessary to synthesize a variety of photolabile teleocidin derivatives.

After investigation of several reaction conditions, Vilsmeier formylation was proved to be most effective. Formylation of (-)-14-*O*-acetylindolactam-V (**2**) with *N*-methylformanilide (MFA) and phosphorus oxychloride in methylene chloride gave (-)-14-*O*-acetyl-7-formylindolactam-V (**3**) at 76% yield. Formylation using *N,N*-dimethylformamide (DMF) also gave **3** exclusively at 65% yield. In these reactions, regioisomers such as (-)-14-*O*-acetyl-5-formylindolactam V (**4**) were hardly detected. On the other hand, formylvinylation of **2** gave a quite different orientation. Treatment of **2** with 3-(*N*-methylanilino)-2-propenal[13] and phosphorus oxychloride in methylene chloride gave (-)-14-*O*-acetyl-5-(2'-formylvinyl)indolactam-V (**6**) as a major product (35% yield). (-)-14-*O*-Acetyl-7-(2'-formylvinyl)indolactam-V (**5**) was obtained as a minor product (6% yield). Similar orientation was observed in the reaction with 3-(dimethylamino)acrolein; the yield of **5** and **6** was 6% and 27%, respectively. These results are summarized in Table I. The configuration of the formylvinyl groups of **5** and **6** was deduced to be *trans* from coupling constants of vicinal olefin protons (15.9Hz for **5**; 16.2Hz for **6**).

Photolabile indolactam derivatives were synthesized from **3** as shown in Fig. 1. Since hydrophobic substituents at position 7 of **1** enhanced the biological activities related to tumor promotion,[11] **3** was converted to (-)-14-*O*-acetyl-7-(2'-nitro-1'-heptenyl)indolactam-V (**7**) by condensing with 1-nitrohexane in the presence of

ammonium acetate at 25% yield. Ammonium acetate was most effective; triethylamine or sodium bicarbonate instead of ammonium acetate gave very low yield of less than 6%. Compound **7** was reduced by lithium aluminum hydride in the presence of aluminum chloride in tetrahydrofuran. The resultant amines (**9a, 9b**) were partially purified by column chromatography, and used for the following reaction.

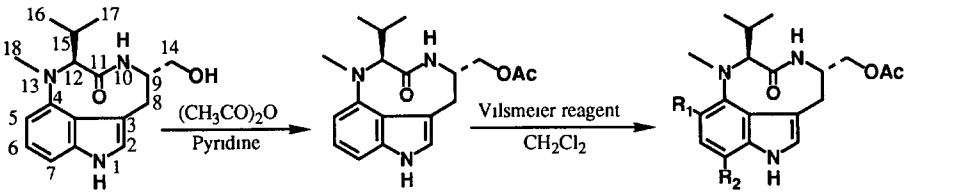

Table I Vilsmeier reaction of (-)-14-*O*-acetylindolactam-V (**2**)[a]

No	Reagent	Yield (%)				Recovery of **2** (%)
		3	**4**	**5**	**6**	
1.	$Ph(CH_3)NCHO$	76	4	—	—	10
2.	$(CH_3)_2NCHO$	65	ND[b]	—	—	16
3.	$Ph(CH_3)NCH{=}CHCHO$	—	—	6	35	10
4.	$(CH_3)_2NCH{=}CHCHO$	—	—	6	27	6

[a]Molar ratio of $POCl_3$, **2** and a formylation reagent (No 1 or 2) was 1.1 3.7, that of $POCl_3$, **2** and a formylvinylation reagent (No 3 or 4) was 1:1:2. These ratio gave the optimal yield. [b]Not detected, less than 1%.

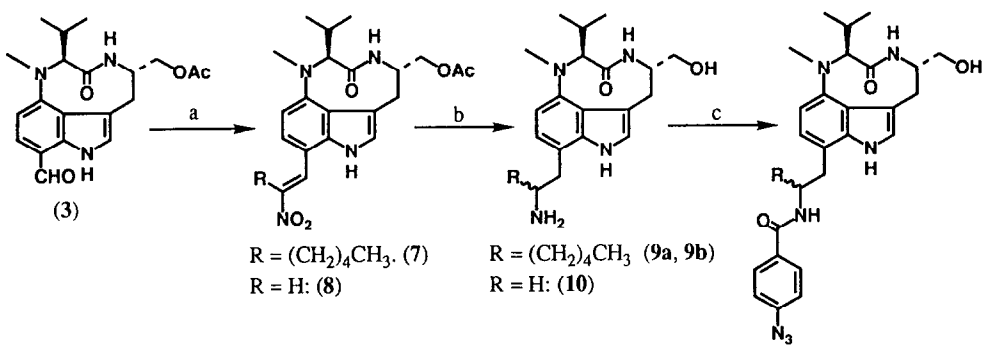

Fig. 1 Synthetic scheme of photolabile indolactam derivatives

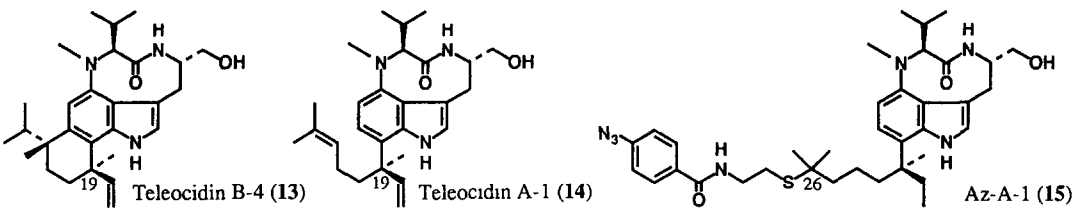

Fig. 2 Structure of teleocidin B-4 (13), A-1 (14) and Az-A-1 (15).

Treatment of the partially purified amines (**9a, 9b**) with succinimidyl-4-azidobenzoate in 2-propanol gave the corresponding two epimers of (-)-[2'-*N*-(4"-azidobenzoyl)aminoheptyl]indolactam-V (Az-C_7, **11a and 11b**), which were separated on silica gel HPLC and respectively designated **11a** and **11b** according to the order of the retention time. The overall yield of **11a** and **11b** from (-)-indolactam-V (**1**) was 3%, respectively. A less hydrophobic photolabile indolactam derivative, (-)-7-[2'-*N*-(4"-azidobenzoyl)aminoethyl]indolactam-V (Az-C_2, **12**) was similarly synthesized from **1** by use of nitromethane instead of 1-nitrohexane with an overall yield of 25%. The yield of the condensation reaction of **3** with nitromethane (76%) was higher than that with 1-nitrohexane (25%). This is probably due to a steric effect of the alkyl chain of 1-nitrohexane.

Since teleocidins (for example, **13** and **14**) are reported to be biologically more active than (-)-indolactam-V (**1**),[14] photolabile compounds derived from teleocidins are expected to have fairly strong biological activities. We have modified teleocidin B-4 (**13**) and A-1 (**14**) to obtain another type of photoaffinity probe. Introduction of a halogen or an amino group onto the vinyl group at position 19 of teleocidin B-4 (**13**) by hydroboration was attempted at first. 14-*O*-Acetylteleocidin B-4 was treated with boron trifluoride in tetrahydrofuran and then with each of the following reagents: bromine, iodine monochloride, ammonium hydroxide and sodium hypochlorite, and azidotrimethylsilane. None of these treatments, disappointingly, gave the desired products. Moreover, treatment of teleocidin B-4 (**13**) with 2-aminoethanethiol hydrochloride in trifluoroacetic acid gave no adduct though this type of addition was successful in teleocidin A-2,[15] a C-19 epimer of teleocidin A-1. After all, an amino function was introduced into teleocidin A-1 (**14**) by the method of Levine *et al.*[15] to give 26-(2'-aminoethylthio)tetrahydroteleocidin A-1, whose condensation with succinimidyl-4-azidobenzoate gave 26-[2'-*N*-(4"-azidobenzoyl)aminoethylthio]tetrahydroteleocidin A-1 (Az-A-1, **15**) with a 42% yield from **14**.

Biological activities of the photolabile teleocidin derivatives (Az-A-1, Az-C_7 and Az-C_2)

Biological activities of the photoaffinity probes were measured by the following three *in vitro* bioassays: binding affinity to the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) receptor in the mouse epidermal particulate fraction, activation of PKC and ability to enhance incorporation of inorganic phosphate into phospholipids of HeLa cells.

Binding affinity of the photolabile teleocidin derivatives to the TPA receptor was evaluated by inhibition of the specific binding of [^{3}H]TPA to the mouse epidermal particulate fraction. This inhibition assay was carried out by the cold acetone filter method.[16,17] Binding affinity was evaluated by the concentration required to cause 50% inhibition, ID_{50}, which was calculated by a computer program (SAS) with a probit procedure.[18] These values are summarized in Table II. Two epimers of Az-C_7 (**11a, 11b**) were found to have a binding affinity 15-

to 18-fold greater than the lead compound (-)-indolactam-V (**1**), while Az-C_2 (**12**) had almost the same binding affinity as **1**. Az-A-1 (**15**) showed approximately a binding affinity 270-fold greater than **1**, which was comparable to TPA and teleocidin B-4 (**13**).

Since PKC is a well-characterized receptor for TPA type tumor promoters, activation of PKC by these photolabile probes was examined. PKC was partially purified from rat brain by the method reported previously.[19] PKC activity was expressed by incorporation of ^{32}P into H1 histone from [γ-^{32}P]ATP. The results in Table II clearly show that Az-A-1 and the two epimers of Az-C_7 are potent activators of PKC comparable to TPA and teleocidin B-4. Less lipophilic Az-C_2 caused weak activation similar to (-)-indolactam-V.

Finally, we measured the stimulation of radioactive inorganic phosphate (^{32}Pi) incorporation into HeLa cell phospholipids because the enhancement of phospholipid metabolism has been reported to play an important role in tumor promotion.[20,21] TPA and teleocidin B-4 caused a maximum increase of about 4-fold over the control in ^{32}Pi incorporation into phospholipids of HeLa cells.[22] As shown in Table II, Az-A-1 and the two epimers of Az-C_7 maximally stimulated at 10^{-6}M, while Az-C_2 hardly stimulated at 10^{-6}M.

As shown above, the three biological activities correlated very well for each derivative. The results strongly suggest that at least Az-A-1 and the two epimers of Az-C_7 have sufficient biological activities to be used as photoaffinity probes.

Synthesis of the tritium labeled probes and their binding affinity to the mouse epidermal particulate fraction

Introduction of tritium label into the photolabile teleocidins was performed by use of commercially available tritium-labeled succinimidyl-4-azidobenzoate (40-60Ci/mmol). Tritium labeled Az-A-1, Az-C_7 and Az-C_2 were obtained by the coupling of [^{3}H]succinimidyl-4-azidobenzoate to each corresponding amine. [^{3}H]Az-C_7 was synthesized as a mixture of the two epimers (**11a** and **11b**) since both epimers showed very similar biological activities. All of these tritium probes were found to have radiochemical purity of more than 95% by silica gel TLC analysis.

Table II Biological activities of photolabile teleocidin derivatives (Az-A-1, Az-C_7 and Az-C_2)

Compound	Inhibition of specific [^{3}H]TPA binding ID_{50} (log1/M)	Activation of PKC[a] cpm at 10^{-6}M	Incorporation of ^{32}Pi into phospholipids of HeLa cells relative cpm/mg of protein at 10^{-6}M
Az-A-1 (**15**)	8.40 (0.20)[b]	16,800	4.36 (0.30)[b]
Az-C_7 (**11a**)	7.22 (0.26)	16,800	3.76 (0.30)
Az-C_7 (**11b**)	7.14 (0.29)	16,500	4.04 (0.50)
Az-C_2 (**12**)	5.90 (0.13)	11,700	1.19 (0.10)
Teleocidin B-4 (**13**)	8.71 (0.23)	16,100	4.27 (0.30)
(-)-Indolactam-V (**1**)	5.97 (0.12)	9,900	3.66 (0.02)
TPA	8.89 (0.12)	16,800	3.67 (0.20)
Control (DMSO)	—	1,000	1.00 (0.06)

[a]This data represents one simultaneous experiment using the same partially purified enzyme. Similar results were obtained in another experiment. [b]Standard deviation.

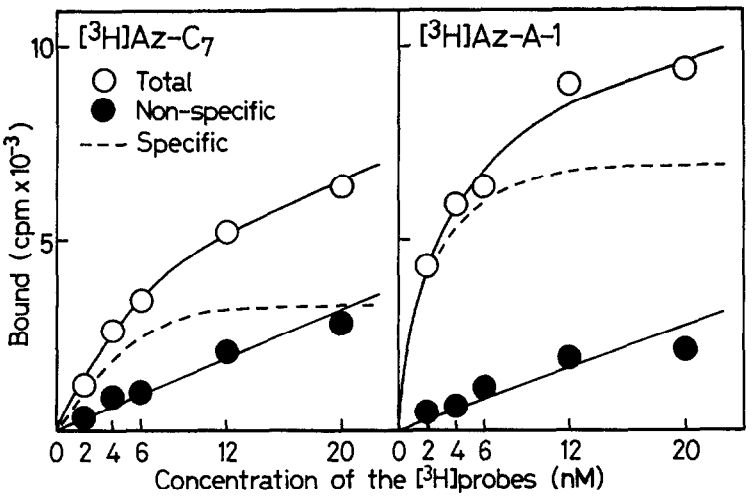

Fig. 3 Binding of [^{3}H]Az-A-1 and [^{3}H]Az-C_7 on the mouse epidermal particulate fraction. One hundred micrograms of protein from the particulate fraction was incubated with each tritiated probe. Total binding (O) and non-specific binding (●) represent the average from duplicate determinations with less than 10% variation. Specific binding (dotted line) is the difference between them. Non-specific binding for each concentration was determined in the presence of a 500-fold excess of a non-radioactive corresponding probe.

Binding affinity of these probes to the mouse epidermal particulate fraction was measured by the cold acetone filter method.[16,17] The results of [^{3}H]Az-A-1 and [^{3}H]Az-C_7 showed saturable and high affinity binding; the saturated concentration was approximately 20nM (Fig. 3). K_D Values of this binding could not be calculated since the free ligand concentration could not be measured by this assay. On the other hand, specific binding of [^{3}H]Az-C_2 was not saturated even at 300nM (data not shown). These results indicate that [^{3}H]Az-A-1 and [^{3}H]Az-C_7 have sufficient binding affinity to the particulate fraction for photoaffinity labeling.

Photoaffinity labeling of the mouse epidermal particulate fraction

The mouse epidermal particulate fraction (500μg protein/1ml Tris-HCl, pH7.4) was incubated with 20nM of [^{3}H]Az-A-1 or [^{3}H]Az-C_7 at 0°C for 2 hours in the dark. The mixture was then irradiated by a UV lamp through a Pyrex filter for 5 minutes under argon. Irradiation of 5 minutes decomposed about 95% of Az-A-1 under the same condition (data not shown). This irradiation did not significantly decrease the specific binding of [^{3}H]TPA to the mouse epidermal particulate fraction (data not shown). The irradiated particulate fraction was centrifuged and washed with acetone to remove the free tritium-labeled photoaffinity probes.

The resultant pellet was extracted with chloroform-methanol (1:2, v/v) containing 2% acetic acid to get total lipids[23] since specific photolabeling in phospholipids was reported in the previous studies using photolabile phorbol esters.[8,9] Radioactivity of the lipid fraction photolabeled with [^{3}H]Az-A-1 or [^{3}H]Az-C_7 is shown in Table III. The radioactivity of lipids photolabeled with [^{3}H]Az-A-1 was significantly reduced in the presence of 1000-fold of non-radioactive Az-A-1, suggesting that the alkyl chain at position 7 of Az-A-1 is involved in the hydrophobic interaction with membrane lipids on the receptor site. The identification of the photolabeled lipids was impossible since silica gel TLC analysis of the photolabeled lipids gave a fairly complex pattern (data not shown). On the other hand, no difference was observed in the case of [^{3}H]Az-C_7 between the radioactivity in the presence and in the absence of 1000-fold of non-radioactive Az-C_7.

Table III Radioactivity of the lipid extracts photolabeled with [^{3}H]Az-A-1 or [^{3}H]Az-C$_7$[a]

Condition	Total dpm	(SD)
[^{3}H]Az-A-1 (20nM) only	73,700	(2,600)
[^{3}H]Az-A-1 (20nM) + 1000-fold non-radioactive Az-A-1	55,900	(2,600)
[^{3}H]Az-C$_7$ (20nM) only	50,300	(3,600)
[^{3}H]Az-C$_7$ (20nM) + 1000-fold non-radioactive Az-C$_7$	48,600	(4,600)

[a]The acetone-washed pellet derived from the mouse epidermal particulate fraction (500μg protein) was extracted with chloroform-methanol (1:2,v/v) containing 2% acetic acid. The radioactivity of the chloroform layer (25μl) was counted. Total dpm represents the total count of the chloroform layer (193μl).

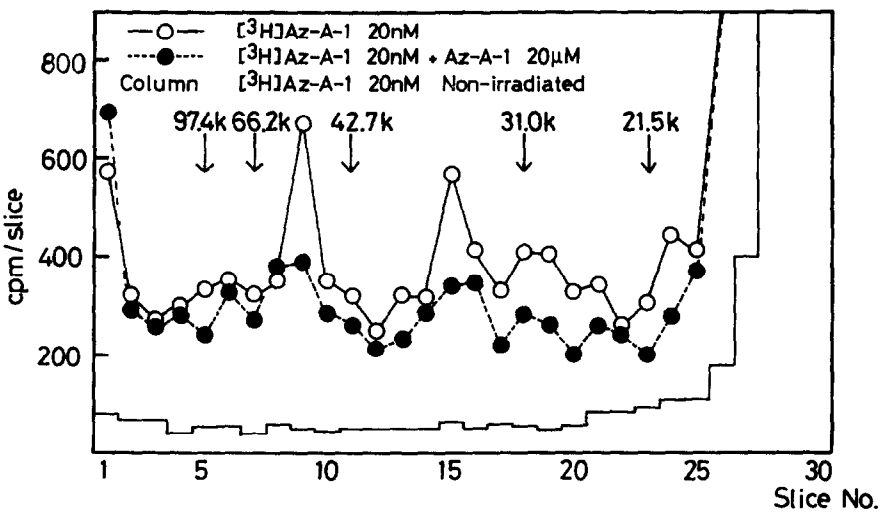

Fig. 4 SDS gel electrophoresis of the mouse epidermal particulate fraction photolabeled with [^{3}H]Az-A-1. Similar result was obtained in another experiment. The particulate fraction (500μg protein) was treated with [^{3}H]Az-A-1 (20nM) in the presence (●) or absence (○) of non-radioactive Az-A-1 (20μM), and irradiated. The resultant proteins were separated by 12.5% SDS-PAGE. The radioactivity in the various regions of the gel was determined by slicing the gel into 2mm fractions. Molecular masses (in kD at top figure) of the marker proteins are indicated by arrows. The proteins in the particlate fraction could hardly be labeled without irradiation (*vid.* columns).

SDS gel electrophoresis of the mouse epidermal particulate fraction photolabeled with [^{3}H]Az-A-1 is shown in Fig. 4. Comparison between the open circles and the columns clearly indicated that many proteins in the particulate fraction were labeled by [^{3}H]Az-A-1 upon photolysis, and comparison between the open circles and the closed circles suggested that *ca.* 30 and 50kD peaks were specifically photolabeled. These two peaks were also detected when photolabeled with [^{3}H]Az-C$_7$ (data not shown). However, no specific labeling was detected at the 70-80kD region, which corresponded to PKC.

DISCUSSION

New photolabile indolactam derivatives (Az-A-1, Az-C_7 and Az-C_2) were synthesized to analyze the binding sites in the mouse epidermal particulate fraction, the target tissue of TPA-type tumor promoters. Formylation at position 7 of (-)-indolactam-V (**1**) was a key step for the synthesis of Az-C_7 (**11a, 11b**) and Az-C_2 (**12**). The Vilsmeier reaction using MFA or DMF was found to be far more effective than the previous Friedel-Crafts formylation[12] to get desired (-)-14-*O*-acetyl-7-formylindolactam-V (**3**). As is the case of Friedel-Crafts acylation of **1**,[12] the 5-substituted derivative was hardly obtained. In addition, another Vilsmeier reaction using 3-(*N*-methylanilino)-2-propenal or 3-(dimethylamino)acrolein gave a different orientation; the 5-substituted derivative (**6**) was the major product. Although the reason for this drastic change in orientation is not clear, this reaction enables the synthesis of a variety of 5-substituted indolactam derivatives, which are especially useful for examining the conformation-activity relationship since introduction of a substituent onto position 5 of **1** hinders the resonance by a steric interaction between the substituent and the *N*-methyl group to fix the molecule in the sofa conformer[12]; teleocidins and (-)-indolactam-V (**1**) exist as two stable conformers in solution at room temperature, the sofa and the twist type.[24]

The biological activities of these photolabile derivatives (Az-A-1, Az-C_7 and Az-C_2) were measured by three *in vitro* bioassays related to tumor-promoting activity: inhibition of specific [^{3}H]TPA binding, activation of PKC and incorporation of ^{32}Pi into phospholipids of HeLa cells (Table II). Of these derivatives, Az-A-1 and Az-C_7 were approximately 10 to 100-fold more active than (-)-indolactam-V (**1**), the fundamental structure of teleocidins. Az-C_2 showed almost the same activity as **1**, suggesting that hydrophobic alkyl chain at position 7 is indispensable to a high level of activity such as in Az-A-1 and Az-C_7, and that the *p*-azidobenzoylaminoethyl moiety at position 7 of **1** did not increase the level of activity at all. These results strongly indicate that Az-A-1 and Az-C_7 have sufficient activity to be used as photoaffinity probes. This was confirmed by using the corresponding tritium labeled probes; [^{3}H]Az-A-1 and [^{3}H]Az-C_7 showed saturable binding on the mouse epidermal particulate fraction at 20nM (Fig. 3).

Photoaffinity labeling of the mouse epidermal particulate fraction by [^{3}H]Az-A-1 resulted in the specific photolabeling on lipids (Table III), indicating that the alkyl chain at position 7 of Az-A-1 or teleocidins (for example, **13** or **14**) was involved in the hydrophobic interaction with membrane lipids on the receptor site. This also supports the conclusion of our previous quantitative structure-activity studies with a series of 7-substituted indolactam derivatives.[11] Similar results are reported using photolabile phorbol esters with most of the specific irreversible binding being to phospholipids rather than to proteins.[8,9] On the basis of these results, one of the major receptors of teleocidins in the mouse epidermal particulate fraction seems to be a lipid-protein complex, of which a most plausible candidate is PKC. On the other hand, no specific photolabeling on lipids was observed using [^{3}H]Az-C_7 (Table III). This is presumably due to the position where the photolabile group is attached; the azido moiety of Az-A-1 is located at the end of the long alkyl side chain, and that of Az-C_7 is close to the indole moiety, which has several polar functional groups indispensable to the receptor binding. The azido group of Az-C_7 might be a little bit far from lipids on the receptor site.

SDS gel electrophoresis of the mouse epidermal particulate fraction photolabeled with [^{3}H]Az-A-1 or [^{3}H]Az-C_7 suggested the existence of two proteins (*ca.* 30 and 50kD) photolabeled specifically. Although we have no idea about these proteins at present, this is the first report on the specific photolabeling on proteins in the mouse epidermal particulate fraction. Further investigations must be done to identify these proteins. Contrary to

our expectation, no specific labeling was detected at the 70 to 80kD region, which corresponded to PKC. Two explanations are possible about this observation; the azido groups of the photoaffinity probes (Az-A-1 and Az-C_7) interact only with phospholipids when bound to PKC, or the content of PKC in the particulate fraction is too low to be detected by this photoaffinity labeling. On the basis of recent structure-activity studies[11,14] and computer graphic studies,[25] at least the azido group of Az-A-1 is deduced to interact only with phospholipids in the PKC-ligand complex. However, since no specific photolabeling on lipids was detected using [^{3}H]Az-C_7 (Table III), failure of PKC photolabeling using this probe supports the latter possibility. Photoaffinity labeling of a pure PKC using these probes is necessary for better understanding of these results. Recently, one of the authors (K.I.) has synthesized new photolabile phorbol esters for PKC photoaffinity labeling in collaboration with Wender *et al.*[26] Photoaffinity labeling of a pure PKC is in progress using Az-A-1 and Az-C_7 as well as these new phorbol esters.

Acknowledgements

The authors thank Drs H. Hayashi and M. Arai of the Department of Agricultural Chemistry at the University of Osaka Prefecture for cultivating the micro-organisms; Mr. R. Imamura of the Faculty of Science at Kyoto University and Ms. K. Omine of the Institute for Chemical Research at Kyoto University for the ^{1}H NMR measurements; Dr. N. Takeda of the Faculty of Pharmacy at Meijo University for the HR-FABMS and HR-in-beam-EIMS measurements. The authors also thank Dr. Y. Hatanaka of the Faculty of Pharmaceutical Sciences at Toyama Medical & Pharmaceutical University for valuable discussion on photoaffinity labeling. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (K.I. and S.O.), and the Kato Memorial Bioscience Foundation (K.I.).

Experimental

General remarks

Melting points are not corrected. The following spectroscopic and analytical instruments were used: UV, Shimadzu UV-200; ORD, Jasco Model J-5; ^{1}H NMR, JEOL GX400 (400MHz, ref. TMS, 27°C); HPLC, Waters Model 600E with Model 484 UV detector; MS, JEOL JMS-DX300 (70eV, 300μA).

HPLC was carried out on YMC packed AM-301 (ODS, 4.6mm i.d. x 100mm), AM-322 (ODS, 10mm i.d. x 150mm), A-023 (silica gel, 10mm i.d. x 250mm) column (Yamamura Chemical Laboratory), μ-Bondasphere C_{18} (19mm i.d. x 150mm) column (Waters Associates), and Asahipak ODP-50 (ODP, 6mm i.d. x 150mm) column (Asahi Chemical Industry). Wako C-100 and C-200 gel (silica gel, Wako Pure Chemical Industries) and YMC A60-350/250 gel (ODS, Yamamura Chemical Laboratory) were used for column chromatography.

(-)-Indolactam-V (**1**), teleocidin B-4 (**13**) and teleocidin A-1 (**14**) were obtained from the culture broth of *Streptoverticillium blastmyceticum* NA34-17 by the method reported previously.[27] Radioisotopes were purchased from NEN Research Products.

Vilsmeier formylation

MFA (267μl) and $POCl_3$ (54μl) were mixed at 0°C, and stood at room temperature for 20 minutes to give an orange color solution. This mixture was added to dry CH_2Cl_2 solution of (-)-14-*O*-acetylindolactam-V (**2**)[27] (200mg/2ml), and the reaction mixture was stirred at 0°C for 70 minutes. After addition of saturated CH_3COONa aqueous solution, the mixture was extracted with EtOAc. The EtOAc extracts were purified by column chromatography on the Wako C-100 gel (acetone-toluene stepwise), followed on the YMC A60-350/250 gel with 30% CH_3CN to give (-)-14-*O*-acetyl-7-formylindolactam-V (**3**)[12] as pale yellow powder (165mg, 76% yield). (-)-14-*O*-Acetyl-5-formylindolactam-V (**4**) was obtained as a minor product (8mg, 4% yield). Compound **4**: amorphous powder, $[\alpha]_D$ -14.3° (*c*=0.49, MeOH, 25°C). UV λ_{max} (EtOH) nm (ε): 295 (4,500), 255 (14,500). ^1H NMR δ ($CDCl_3$, 0.009M, almost exclusively as sofa conformer) ppm: 0.95 (3H, d, *J*=6.7Hz), 1.26 (3H, d, *J*=6.7Hz), 2.00 (3H, s), 2.70 (1H, m), 2.81 (1H, dd, *J*=14.6, 1.5Hz), 2.87 (3H, s), 3.03 (1H, d, *J*=10.7Hz), 3.28 (1H, dd, *J*=14.6, 4.9Hz), 3.82 (2H, m), 4.63 (1H, m), 4.81 (1H, br.d, *J*=10.7Hz, NH-10), 7.08 (1H, d, *J*=2.4Hz), 7.37 (1H, dd, *J*=8.5, 0.9Hz), 7.75 (1H, d, *J*=8.5Hz), 8.56 (1H, br.s, NH-1), 10.80 (1H, s). HR-EIMS *m/z*: 371.1835 (M^+, calcd. for $C_{20}H_{25}N_3O_4$, 371.1845). Similar Vilsmeier reaction using **2** (250mg), DMF (210μl) and $POCl_3$ (70μl) in CH_2Cl_2 (2ml) gave **3** (176mg, 65% yield).

3-(*N*-Methylanilino)-2-propenal[13] (176mg) and $POCl_3$ (51μl) were dissolved in THF (0.5ml) at room temperature. This THF solution was added to dry CH_2Cl_2 solution of **2** (188mg/2ml), and the reaction mixture was stirred at 0°C for 70 minutes. After addition of saturated CH_3COONa aqueous solution, the mixture was extracted with EtOAc. The EtOAc extracts were purified by column chromatography on the Wako C-100 gel (acetone-toluene stepwise), followed by HPLC on μ-Bondasphere C_{18} using 65% MeOH to give (-)-14-*O*-acetyl-5-(2'-formylvinyl)indolactam-V (**6**, 76mg, 35% yield) and (-)-14-*O*-acetyl-7-(2'-formylvinyl)indolactam-V (**5**, 13mg, 6% yield). Compound **6**: yellow rods from MeOH, mp. 240-242°C, $[\alpha]_D$ +190.9° (*c*=0.12, MeOH, 26°C). UV λ_{max} (EtOH) nm (ε): 334 (19,600), 288 (21,100), 266 (16,000). ^1H NMR δ ($CDCl_3$, 0.06M, sofa conformer only) ppm: 0.95(3H, d, *J*=6.7Hz), 1.38 (3H, d, *J*=6.7Hz), 1.99 (3H, s), 2.73 (1H, m), 2.79 (1H, dd, *J*=14.7, 1.5Hz), 2.83 (3H, s), 2.95 (1H, d, *J*=10.7Hz), 3.28 (1H, dd, *J*=14.7, 4.6Hz), 3.79 (2H, m), 4.63 (1H, m), 4.85 (1H, d, *J*=10.7Hz, NH-10), 6.60 (1H, dd, *J*=16.2, 7.2Hz), 7.04 (1H, d, *J*=2.4Hz), 7.35 (1H, d, *J*=8.5Hz), 7.50 (1H, d, *J*=8.5Hz), 8.62 (1H, d, *J*=16.2Hz), 8.80 (1H, br.s, NH-1), 9.76 (1H, d, *J*=7.2Hz). EIMS *m/z*: 397 (M^+). Anal. Calcd. for $C_{22}H_{27}N_3O_4$: C, 66.48; H, 6.85; N, 10.57. Found: C, 66.21; H, 6.87; N, 10.71. Compound **5**: red amorphous powder, $[\alpha]_D$ -724.2° (*c*=0.87, EtOH, 20°C). UV λ_{max} (EtOH) nm (ε): 425 (17,400), 278 (13,100). ^1H NMR δ ($CDCl_3$, 0.07M, twist conformer only) ppm: 0.60 (3H, d, *J*=6.7Hz), 0.95 (3H, d, *J*=6.1Hz), 2.10 (3H, s), 2.62 (1H, m), 2.98 (3H, s), 3.17 (1H, dd, *J*=17.7, 3.7Hz), 3.25 (1H, br.d, *J*=17.7Hz), 4.02 (1H, dd, *J*=11.6, 8.5Hz), 4.23 (1H, dd, *J*=11.6, 3.7Hz), 4.34 (1H, m), 4.47 (1H, d, *J*=10.4Hz), 6.24 (1H, br.s, NH-10), 6.58 (1H, d, *J*=8.2Hz), 6.70 (1H, dd, *J*=15.9, 7.6Hz), 7.04 (1H, s), 7.34 (1H, d, *J*=8.2Hz), 7.68 (1H, d, *J*=15.9Hz), 9.10 (1H, br.s, NH-1), 9.67 (1H, d, *J*=7.6Hz). HR-EIMS *m/z*: 397.2011 (M^+, calcd. for $C_{22}H_{27}N_3O_4$, 397.2002). A similar Vilsmeier reaction using **2** (287mg), 3-(dimethylamino)acrolein (176μl) and $POCl_3$ (78μl) in CH_2Cl_2 (3ml) gave **6** (89mg, 27% yield) and **5** (21mg, 6% yield). 3-(Dimethylamino)acrolein and $POCl_3$ should be carefully mixed to prevent explosion; we added 3-(dimethylamino)acrolein (176μl) to dry CH_2Cl_2 solution (1ml) of $POCl_3$ (78μl) maintained at 0°C. The resultant orange suspension was added to dry CH_2Cl_2 solution of **2** (287mg/2ml).

*Synthesis of Az-C_7 (**11a** and **11b**)*

Compound **3** (90mg) was dissolved in 1-nitrohexane (2ml). After addition of ammonium acetate (120mg), the solution was heated to 190°C on an oil bath for 30 minutes. The reaction mixture was partitioned 3 times between EtOAc and water. The EtOAc layer was dried over Na_2SO_4, and evaporated to dryness. The dark red oil was chromatographed on the Wako C-100 gel (acetone-toluene stepwise), followed by HPLC on μ-Bondasphere C_{18} using 85% MeOH to give (-)-14-*O*-acetyl-7-(2'-nitro-1'-heptenyl)indolactam-V (**7**, 30mg, 25% yield). Compound **7**: red amorphous powder, $[\alpha]_D$ -347.6° (*c*=0.96, EtOH, 27°C). UV λ_{max} (EtOH) nm (ε):

457 (10,000), 279 (7,700), 234 (18,200). ^{1}H NMR δ ($CDCl_3$, 0.06M, almost exclusively twist conformer) ppm: 0.62 (3H, d, *J*=6.7Hz), 0.94 (6H, m), 1.42 (4H, m), 1.70 (2H, m), 2.11 (3H, s), 2.63 (1H, m), 2.93 (2H, m), 2.99 (3H, s), 3.15 (1H, dd, *J*=17.5, 3.8Hz), 3.26 (1H, br.d, *J*=17.5Hz), 4.01 (1H, dd, *J*=11.6, 8.5Hz), 4.23 (1H, dd, *J*=11.6, 3.7Hz), 4.36 (1H, m), 4.47 (1H, d, *J*=10.1Hz), 6.18 (1H, br.s, NH-10), 6.59 (1H, d, *J*=8.2Hz), 7.03 (1H, s), 7.25 (1H, d, *J*=8.2Hz), 8.42 (1H, s), 9.18 (1H, br.s, NH-1). HR-EIMS *m/z*: 484.2660 (M^+, calcd. for $C_{26}H_{36}N_4O_5$, 484.2686).

Compound **7** (27mg) was dissolved in dry THF (2ml). $AlCl_3$ (30mg) and $LiAlH_4$ (34mg) in dry THF (3ml) was added to the solution, and the reaction mixture was refluxed for 6 minutes. After addition of EtOAc, the mixture was extracted with 1N HCl, followed by partitioning between EtOAc and NH_4OH (pH 12). The EtOAc extracts were chromatographed on the Wako C-100 gel (MeOH-$CHCl_3$) to give the amines [**9a** and **9b**, EIMS *m/z*: 414 (M^+)] which gave a characteristic coloration with ninhydrin and was used in the next reaction without further purification. Compounds **9a** and **9b** (16mg) were treated with succinimidyl-4-azidobenzoate (145mg) in 2-PrOH (3ml) for 30 hours at room temperature under dark. After partitioning between EtOAc and water 3 times, the EtOAc extracts were chromatographed on the Wako C-100 gel (acetone-toluene stepwise), followed by HPLC on μ-Bondasphere C_{18} using 90% MeOH, and on YMC A-023 using n-hexane, $CHCl_3$ and 2-PrOH (80:16:4) to give(-)-7-[2'-*N*-(4"-azidobenzoyl)aminoheptyl]indolactam-V as two epimers, which were designated **11a** and **11b** according to the order of the retention time in the HPLC on YMC A-023 (flow rate, 3.0ml/min: **11a**, t_R=33.6min; **11b**, t_R=40.8min). Compound **11a**: amorphous powder, 7mg, 22% yield from **7**, $[\alpha]_D$ -245.2° (*c*=0.46, EtOH, 20°C). UV λ_{max} (EtOH) nm (ε): 270 (25,800), 227 (30,300). ^{1}H NMR δ ($CDCl_3$, 0.026M, sofa:twist = 1:2.5) ppm: twist conformer; 0.63 (3H, d, *J*=7.0Hz), 0.83 (3H, t, *J*=6.6Hz), 0.93 (3H, d, *J*=6.2Hz), *ca.*1.20 (5H, m), 1.35-1.55 (2H, m), 1.84 (1H, m), *ca.*2.60 (2H, m), 2.92 (3H, s), 3.06 (1H, dd, *J*=17.6, 3.7Hz), 3.20 (1H, br. d, *J*=17.6Hz), 3.41 (1H, d, *J*=13.6Hz), 3.59 (1H, m), 3.76 (1H, m), 3.98 (1H, m), 4.37 (1H, m), 4.41 (1H, d, *J*=10.3Hz), 6.26 (1H, d, *J*=7.0Hz, NH-26), 6.39 (1H, d, *J*=7.7Hz), 6.78 (1H, d, *J*=7.7Hz), 7.06 (1H, s), 7.12 (2H, m), 7.19 (1H, br.s, NH-10), 7.83 (2H, m), 10.83 (1H, br.s, NH-1): sofa conformer; 2.39 (1H, m), *ca.*2.65 (1H, m), 2 77 (3H, s), 2.87 (1H, d, *J*=14.3Hz), 2.97 (1H, d, *J*=11.0Hz), 3.13 (1H, dd, *J*=14.3, 4.4Hz), 3.49 (1H, d, *J*=13.2Hz), 4.11 (1H, m), 4.80 (1H, br.d, *J*=11.4Hz, NH-10), 6.31 (1H, d, *J*=7.0Hz, NH-26), 6.88 (1H, d, *J*=7.7Hz), 6.94 (1H, d, *J*=7.7Hz), 7.21 (1H, d, *J*=2.6Hz), 11.22 (1H, br.s, NH-1). Other peaks of the sofa conformer had weak intensities and/or overlapped the peaks of the twist conformer. HR-FABMS (matrix, NBA) *m/z*: 559.3276 (M^+, calcd. for $C_{31}H_{41}N_7O_3$, 559.3271). Compound **11b**: amorphous powder, 7mg, 22% yield from **7**, $[\alpha]_D$ -18.7° (*c*=0.49, EtOH, 20°C). UV λ_{max} (EtOH) nm (ε): 270 (26,400), 227 (30,800). ^{1}H NMR δ ($CDCl_3$, 0.03M, sofa:twist = 1:2) ppm: twist conformer; 0.67 (3H, d, *J*=6.6Hz), 0.81 (3H, m), 0.95 (3H, d, *J*=6.2Hz), *ca.*1.20 (4H, m), *ca* 1.25 (1H, m), 1.35-1.60 (2H, m), 1.81 (1H, m), *ca.*2.60 (2H, m), 2.83 (1H, m, 14-OH), 2.91 (3H, s), 3.06 (1H, dd, *J*=17.2, 3.7Hz), 3 21 (1H, br.d, *J*=17.2Hz), 3 37 (1H, d, *J*=13.6Hz), 3.57 (1H, m), 3.75 (1H, m), 4.16 (1H, m), 4.32 (1H, m), 4.42 (1H, d, *J*=10.3Hz), 6.25 (1H, d, *J*=7.0Hz, NH-26), 6.38 (1H, d, *J*=7.7Hz), 6.77 (1H, d, *J*=7.7Hz), 7.06 (1H, s), 7 12 (2H, m), 7.16 (1H, br.s, NH-10), 7.83 (2H, m), 10.72 (1H, br s, NH-1): sofa conformer; 0.95 (3H, d, *J*=6.2Hz), 1.25 (3H, d, *J*=6.6Hz), 2.40 (1H, m), 2.73 (3H, s), 3.02 (1H, d, *J*=11.0Hz), 3.11 (1H, dd, *J*=14.3, 4.4Hz), 3.48 (3H, m), 4 06 (1H, m), 4.47 (1H, m), 4.92 (1H, br.d, *J*=11 0Hz, NH-10), 6 32 (1H, d, *J*=7.0Hz), 6 87 (1H, d, *J*=7.3Hz), 6.94 (1H, d, *J*=7.3Hz), 11.15 (1H, br s, NH-1). Other peaks of the sofa conformer had weak intensities and/or overlapped the peaks of the twist conformer. HR-FABMS (matrix, NBA) *m/z*: 559.3250 (M^+, calcd. for $C_{31}H_{41}N_7O_3$, 559.3271).

Synthesis of Az-C_2 (12)

(-)-7-(2-Aminoethyl)indolactam-V (**10**)[12] (5.7mg) was treated with succinimidyl-4-azidobenzoate (21.6mg) in 2-PrOH (1.5ml) for 44 hours at room temperature under dark. After partitioning between EtOAc and water 3 times, the EtOAc extracts were chromatographed on the Wako C-100 gel (acetone-toluene stepwise), followed by HPLC on YMC AM-322 using 70% MeOH to give (-)-7-[2'-*N*-(4"-azidobenzoyl)aminoethyl]-indolactam-V (**12**). Compound **12**: amorphous powder, 5.5mg, 68% yield. UV λ_{max} (EtOH) nm (ε): 272 (24,300). 229 (28,300) IR ν_{max} (KBr) cm^{-1}· 2150 (-N_3). ^{1}H NMR δ ($CDCl_3$, 0.02M, sofa:twist = 1:2) ppm:

twist conformer; 0.65 (3H, d, *J*=6.7Hz), 0.94 (3H, d, *J*=6.4Hz), 2.61 (1H, m), 2.92 (3H, s), 3.01-3.23 (4H, m), 3.45-3.77 (4H, m), 4.32 (1H, m), 4.40 (1H, d, *J*=10.1Hz), 6.41 (1H, d, *J*=7.6Hz), 6.63 (1H, br.t, *J*=6.8Hz, NH-21), 6.67 (1H, br.s, NH-10), 6.83 (1H, d, *J*=7.6Hz), 7.04 (1H, s), 7.10 (2H, d, *J*=8.6Hz), 7.80 (2H, d, *J*=8.6Hz), 10.27 (1H, br.s, NH-1): sofa conformer; 0.94 (3H, d, *J*=6.4Hz), 1.24 (3H, d, *J*=7.0Hz), 2.40 (1H, m), 2.75 (3H, s), 2.86 (1H, d, *J*=14.4Hz), 2.98 (1H, d, *J*=11.0Hz), 4.45 (1H, m), 4.81 (1H, br.d, *J*=11.0Hz, NH-10), 6.93 (1H, d, *J*=7.3Hz), 6.96 (1H, d, *J*=7.3Hz), 7.18 (1H, d, *J*=2.5Hz), 10.73 (1H, br.s, NH-1). Other peaks of the sofa conformer had weak intensities and/or overlapped the peaks of the twist conformer. HR-in-beam-EIMS *m/z*: 489.2512 (M^+, calcd. for $C_{26}H_{31}N_7O_3$, 489.2488).

Synthesis of Az-A-1 (15)

26-(2'-Aminoethylthio)tetrahydroteleocidin A-1 was synthesized from teleocidin A-1 (**14**) by the method of Levine *et al.*[15] This amine (20.5mg) was treated with succinimidyl-4-azidobenzoate (103mg) in 2-PrOH (1ml) for 54 hours at room temperature under dark. The reaction mixture was processed as above, and purified by HPLC on YMC AM-322 using 85% MeOH to give Az-A-1 (**15**). Compound **15**: amorphous powder, 19.5mg, 74.3% yield from **14**, $[\alpha]_D$ -99.9° (*c*=0.49, EtOH, 25°C). UV λ_{max} (EtOH) nm (ε): 270 (26,400), 228 (31,200). ^{1}H NMR δ ($CDCl_3$, 0.015M, sofa:twist = 1:5) ppm: 0.53 (3H, d, *J*=6.8Hz), 0.59 (3H, t, *J*=7.3Hz), 0.89 (3H, d, *J*=6.4Hz), 1.06-1.16 (5H, m), 1.17 (3H, s), 1.31-1.43 (5H, m), 1.69-1.82 (2H, m), 1.93-2.01 (2H, m), 2.25-2.38 (2H, m), 2.55 (4H, m), 2.90 (1H, dd, *J*=17.6, 3.4Hz), 3.15 (1H, br.d, *J*=17.6Hz), 3.35 (1H, m), 3.44 (1H, m), 3.71-3.76 (2H, m), 4.16 (1H, m), 4.29 (1H, d, *J*=10.3Hz), 6.13 (1H, m, NH-32), 6.32 (1H, d, *J*=8.3Hz), 6.38 (1H, br.s, NH-10), 6.87 (2H, m), 7.06 (2H, d, *J*=8.8Hz), 7.65 (2H, d, *J*=8.8Hz), 8.22 (1H, br.s, NH-1): sofa conformer; 2.73 (3H, s), 8.69 (1H, br.s, NH-1). Other peaks of the sofa conformer had weak intensities and/or overlapped the peaks of the twist conformer. HR-FABMS (matrix, NBA) *m/z*: 661.3741 (M^+, calcd. for $C_{36}H_{51}N_7O_3S$, 661.3774).

Inhibition of specific [^{3}H]TPA binding to the mouse epidermal particulate fraction

Inhibition of specific [^{3}H]TPA binding was assayed by the cold-acetone filter method[16,17] with slight modifications.[6] An epidemal particulate fraction was prepared from dorsal epidermis of female ICR mice and subjected to a binding assay as reported previously.[6] Binding affinity was evaluated by the concentration required to cause 50% inhibition, ID_{50}, which was calculated by a computer program (SAS) with a probit procedure.[18] Since there existed subtle variations in ID_{50} values, depending on the particulate fraction prepared, all compounds were tested simultaneously using the same particulate fraction.

Activation of PKC

PKC was partially purified from rat brain by DE-52 column chromatography.[19] PKC activity was determined by measuring incorporation of ^{32}P into H1 histone from [γ-^{32}P]ATP as reported previously.[6]

Stimulation of ^{32}Pi incorporation into HeLa cell phospholipids

Incorporation of ^{32}Pi into HeLa cell phospholipids was measured by the method reported previously[22] with slight modifications.[28]

Tritium labeling of Az-C_7 (11a, 11b) and Az-A-1 (15)

The partially purified amines (**9a, 9b**) mentioned above were further purified by HPLC on Asahipak ODP-50 column using 70% MeOH containing 0.5% conc. NH_4OH. The purified amines (250nmol) were treated with [^{3}H]succinimidyl-4-azidobenzoate (2.5nmol, 49.1Ci/mmol) in 2-PrOH (0.125ml). The solution was stood at 30°C for 24 hours. The reaction mixture was transferred to a sample tube (9mm i.d. x 6cm). After addition of toluene (150μl) and 1M AcOH (150μl), the tube was vigorously shaken and centrifuged. The upper layer was removed, and put into an another sample tube. Water (150μl) was added to the tube, which was again vigorously shaken and centrifuged. The upper layer (toluene-2-PrOH solution, 110μl) was used as [^{3}H]Az-C_7 in the following experiments.

[^{3}H]Az-A-1 was similarly synthesized from the corresponding amine, 26-(2'-aminoethylthio)-tetrahydroteleocidin A-1. The amine (500nmol) was treated with [^{3}H]succinimidyl-4-azidobenzoate (2.5nmol, 49.1Ci/mmol) in 2-PrOH (0.125ml). The reaction mixture was processed as above, and the resultant toluene-2-PrOH solution (110μl) was used as [^{3}H]Az-A-1 in the following experiments. The radiochemical purity of these tritiated probes determined by TLC on silica gel using hexane-2-PrOH (85:15) was found to be >95%. The yield of these reactions was >90%.

Binding affinity of [^{3}H]Az-C$_7$ and [^{3}H]Az-A-1 to the mouse epidermal particulate fraction

Specific [^{3}H]TPA binding of [^{3}H]Az-C$_7$ and [^{3}H]Az-A-1 was assayed by the cold-acetone filter method.[16,17] An epidermal particulate fraction was prepared from the dorsal epidermis of female ICR mice by the method reported previously.[6] One hundred micrograms of protein from the particulate fraction was incubated at 0°C for 2 hours with various concentrations (2-20nM) of each tritiated probe in 1ml of 20mM Tris-HCl buffer (pH 7.4). 2-Mercaptoethanol was not added to the solution because an aryl azide group might react with this reagent. Non-specific binding was determined by measuring the binding of each tritiated probe in the presence of a 500-fold excess of an non-radioactive corresponding probe. These compounds were added as a DMSO solution. The final concentration of DMSO was below 0.5%.

Photoaffinity labeling of the mouse epidermal particulate fraction

1) Extraction of lipids photolabeled with [^{3}H]Az-A-1 and [^{3}H]Az-C$_7$

The mouse epidermal particulate fraction (500μg protein) was incubated at 0°C for 2 hours with the tritiated Az-A-1 or Az-C$_7$ (20nM) in the presence or absence of a 1000-fold (20μM) corresponding non-radioactive probe in 1ml of 20mM Tris-HCl buffer (pH 7.4). The final concentration of DMSO was also below 0.5% as mentioned above. After 2 hours, each solution was poured on a 3.2cm diameter petri dish. This solution was stirred and irradiated by a UV lamp (8W, 250-400nm, PUV-1, TOPCON) for 5 minutes through a Pyrex filter under argon. The distance between the petri dish and the UV lamp was 2cm. The mixture was then transferred to an Eppendorf tube (1.5ml), and was centrifuged at 12,000rpm for 20 minutes in an Eppendorf microcentrifuge 5412. The supernatant was discarded, and the resultant pellet was washed with acetone (400μl). The acetone suspension was centrifuged at 12,000rpm for 20 minutes. After removal of acetone, the pellet was subjected to total lipid extraction. All lipids were extracted by the method of Bligh and Dyer[23] with a slight modification. In brief, 300μl of chloroform/methanol (1:2, v/v) containing 2% acetic acid was added to the pellet. After vigorous stirring, 100μl of chloroform was added to the pellet suspension. The mixture was centrifuged for 2 minutes at 12,000rpm. The radioactivity in 25μl of the chloroform phase was counted in a liquid scintillation spectrometer.

2) SDS gel electrophoresis of the mouse epidermal particulate fraction photolabeled with [^{3}H]Az-A-1 and [^{3}H]Az-C$_7$

The mouse epidermal particulate fraction (500μg protein) was similarly treated with [^{3}H]Az-A-1 or [^{3}H]Az-C$_7$ in the presence or absence of the 1000-fold corresponding non-radioactive probe described above. In this case, an additional control without irradiation was prepared. After irradiation, the mixture was transferred to an Eppendorf tube (1.5ml), and was centrifuged at 12,000rpm for 20 minutes. After careful removal of the supernatant using Kimwipe, the pellet was subjected to 12.5% SDS-PAGE. The pellet was dissolved in a sample solution (250μl), which consisted of 62.5mM Tris-HCl, 2% SDS, 10% glycerol, 0.01% BPB, 2mM EDTA, 51mM 2-mercaptoethanol. Fifty microliters of the sample solution was analyzed on a 2mm thick 12.5% polyacrylamide slab gel. Quantitation of the radioactivity bound to the proteins in the mouse epidermal particulate fraction was done by slicing the gels. The 2mm gel slices were digested by overnight incubation in 1ml of 15% H_2O_2 at 55-60°C, and the radioactivities were counted in 10ml of scintillation fluid.

REFERENCES

1. Fujiki, H.; Sugimura, T. *Adv. Cancer Res.* **1987**, *49,* 223-264.
2. Nishizuka, Y. *Nature* **1984**, *308,* 693-698.
3. For review see: Aderem, A. *Cell* **1992**, *71,* 713-716.
4. Tran, P. L.; Deugnier, M. A. *Carcinogenesis* **1985**, *6,* 433-439.
5. Liskamp, R. M. J.; Brothman, A. R.; Arcoleo, J. P.; Miller, O. J.; Weinstein, I. B. *Biochem. Biophys. Res. Commun.* **1985**, *131,* 920-927.
6. Irie, K.; Okuno, S.; Koshimizu, K.; Tokuda, H.; Nishino, H.; Iwashima, A. *Int. J. Cancer* **1989**, *43,* 513-519.
7. Hashimoto, Y.; Shudo, K. *Jpn. J. Cancer Res.* **1991**, *82,* 665-675.
8. Delclos, K. B.; Yeh, E.; Blumberg, P. M. *Proc. Natl. Acad. Sci. USA* **1983**, *80,* 3054-3058.
9. Schmidt, R.; Heck, K.; Sorg, B.; Hecker, E. *Cancer Letters* **1985**, *26,* 97-111.
10. Okuno, S.; Irie, K.; Nishino, H.; Iwashima, A.; Koshimizu, K. *Agric. Biol. Chem.* **1990**, *54,* 1885-1887.
11. Irie, K.; Hagiwara, N.; Tokuda, H.; Koshimizu, K. *Carcinogenesis* **1987**, *8,* 547-552.
12. Irie, K.; Hagiwara, N.; Koshimizu, K. *Tetrahedron* **1987**, *43,* 5251-5260.
13. Jutz, C. *Chem. Ber.* **1958**, *91,* 850-861.
14. Fujiki, H.; Suganuma, M.; Ninomiya, M.; Yoshizawa, S.; Yamashita, K.; Takayama, S.; Hitotsuyanagi, Y.; Sakai, S.; Shudo, K.; Sugimura, T. *Cancer Res.* **1988**, *48,* 4211-4214.
15. Levine, L.; Fujiki, H.; Sakai, S.; Gjika, H. B.; Vunakis, H. V. *Carcinogenesis* **1988**, *9,* 1629.
16. Ashendel, C. L.; Boutwell, R. K. *Biochem. Biophys. Res. Commun.* **1981**, *99,* 543-549.
17. Hergenhahn, M.; Hecker, E. *Carcinogenesis* **1981**, *2,* 1277-1281.
18. James, H. G.: Probit procedure. In *SAS User's Guide* **1979**; Jane, T. H.; Kathryn, A. C. Eds.; SAS Institute, Cary, NC; pp.357-360.
19. Kikkawa, U.; Go, M.; Koumoto, J.; Nishizuka, Y. *Biochem. Biophys. Res. Commun.* **1986**, *135,* 636-643.
20. Rohrschneider, L. R.; O'Brien, D. H.; Boutwell, R. K. *Biochim. Biophys. Acta* **1972**, *280,* 57-70.
21. Rohrschneider, L. R.; Boutwell, R. K. *Cancer Res.* **1973**, *33,* 1945-1953.
22. Nishino, H.; Fujiki, H.; Terada, M.; Sato, S. *Carcinogenesis* **1983**, *4,* 107-110.
23. Bligh, E. G.; Dyer, W. J. *Can. J. Biochem. Physiol.* **1959**, *37,* 911-917.
24. Endo, Y.; Shudo, K.; Itai, A.; Hasegawa, M.; Sakai, S. *Tetrahedron* **1986**, *42,* 5905-5924.
25. For review see: Wender, P. A.; Cribbs, C. M. *Adv. Med. Chem.* **1991**, *1,* 1-53.
26. Wender, P. A.; Irie, K.; Miller, B. L. *J. Org. Chem.*, **1993**, *58,* 4179-4181.
27. Irie, K.; Hirota, M.; Hagiwara, N.; Koshimizu, K.; Hayashi, H.; Murao, S.; Tokuda, H.; Ito, Y. *Agric. Biol. Chem.* **1984**, *48,* 1269-1274.
28. Irie, K.; Okuno, S.; Kajiyama, S.; Koshimizu, K.; Nishino, H.; Iwashima, A. *Carcinogenesis* **1991**, *12,* 1883-1886.