Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 719–722

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

The C4 Hydroxyl Group of Phorbol Esters is Not Necessary for Protein Kinase C Binding

Minoru Tanaka,[a] Kazuhiro Irie,[a,*] Yu Nakagawa,[a] Yoshimasa Nakamura,[b] Hajime Ohigashi[a] and Paul A. Wender[c]

[a]*Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*
[b]*Laboratory of Food and Biodynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*
[c]*Department of Chemistry, Stanford University, Stanford, CA 94305-5080, USA*

Received 27 November 2000; accepted 13 January 2001

Abstract—To investigate the role of the hydroxyl group at position 4 of the phorbol esters in protein kinase C (PKC) binding and function, 4β-deoxy-phorbol-12,13-dibutyrate (4β-deoxy-PDBu, **5a**) and 4β-deoxy-phorbol-13-acetate (**6a**) were synthesized from phorbol (**1**). The binding affinities of these 4β-deoxy compounds (**5a**, **6a**) to the 13 PKC isozyme C1 domains were quite similar to those of the corresponding 4β-hydroxy compounds (**4a**, **4b**), suggesting that the C4 hydroxyl group of phorbol esters is not necessary for PKC binding. Moreover, functional assays showed that 4β-deoxy-PDBu (**5a**) exhibited biological activities (Epstein-Barr virus induction and superoxide generation) equally potent to those of PDBu (**4a**). © 2001 Elsevier Science Ltd. All rights reserved.

Protein kinase C (PKC) is a family of serine/threonine-specific protein kinases that is activated by endogenous diacylglycerol (DAG) as well as by exogenous agents such as the plant derived, tumor promoting phorbol esters.[1,2] Comprising more than 10 isozymes, PKC plays important roles in cellular signal transduction involving gene expression, cell growth and differentiation. However, the function of each PKC isozyme in these cellular events and in tumor promotion is not fully understood, a situation attributable in part to the lack of isozyme selective regulators.

Phorbol esters bind to PKC at the cysteine-rich C1 domains (C1A and C1B)[3] consisting of about 50 amino acid residues.[4] The structural basis for this binding has been the subject of several recent investigations based on X-ray,[5] NMR,[6–8] and photoaffinity labeling.[9,10] Zhang et al.[5] reported the X-ray crystal structure of a complex of PKCδ-C1B and phorbol-13-acetate (**4b**). While this study used a phorbol ester with a low PKC affinity and did not address the role of the phospholipid in binding, it suggested that the C20 hydroxyl, C3 carbonyl, and C4 hydroxyl oxygens of **4b** in the complex form hydrogen bonds with certain residues of the δ-C1B domain. Hommel et al.[6] and Inagaki et al.[7] determined independently the three-dimensional structure of PKCα-C1B in solution using NMR spectroscopy. Based on the solution structure, Wang et al.[11] analyzed the binding interactions of phorbol-12,13-dibutyrate (PDBu, **4a**) with PKCα-C1B using molecular dynamics simulations. They calculated the hydrogen-bonding energy of each hydrophilic group of **4a** with the binding site of α-C1B. They also analyzed the interaction between phorbol-13-acetate (**4b**) and δ-C1B by the similar method using Zhang's X-ray structure. The C20 hydroxyl and C3 carbonyl oxygens of **4a** interact significantly with the binding sites of both α-C1B and δ-C1B; the percentage of the hydrogen bonding energy in the α-C1B and δ-C1B binding were 39.5% and 44.4% for the C20 oxygen, and 35.7% and 23.6% for the C3 oxygen, respectively. However, the magnitudes of the interactions of the C4 hydroxyl oxygen with α-C1B and δ-C1B were quite different. In the α-C1B binding, the C4 hydroxyl group accounted for only 2.0% of the total hydrogen-bonding energy, while it contributed 32.1% to the δ-C1B binding. These results suggest that 4-deoxy-phorbol esters would bind to α-C1B more strongly than to δ-C1B since the hydroxyl group at position 4 would

*Corresponding author. Tel.: +81-75-753-6282; fax: +81-75-753-6284; e-mail: irie@kais.kyoto-u.ac.jp

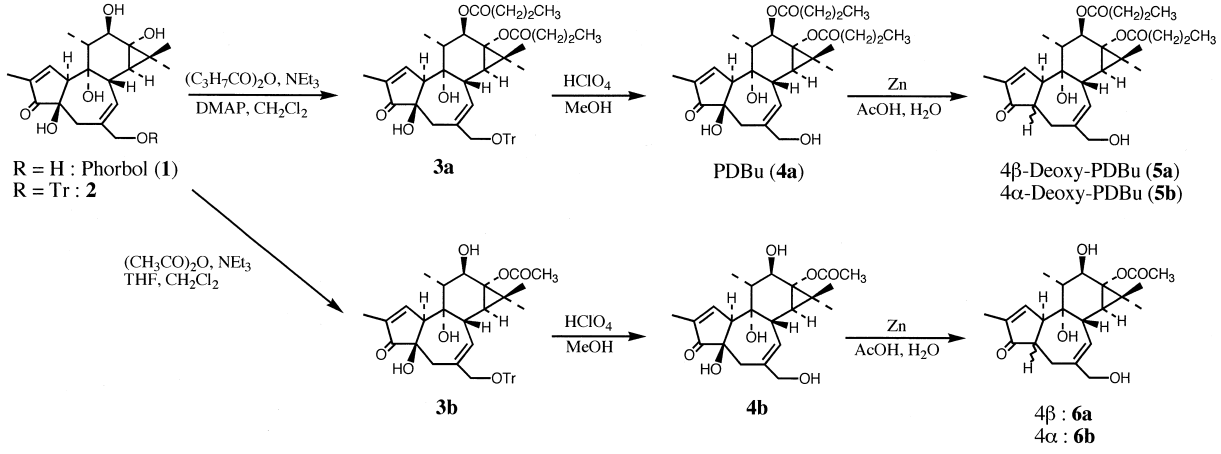
0960-894X/01/$ - see front matter © 2001 Elsevier Science Ltd. All rights reserved.
PII: S0960-894X(01)00045-2

contribute little to the α-C1B binding. This prompted us to synthesize the 4β-deoxy-phorbol esters (**5a** and **6a**) as possible leads for PKC isozyme selective binding and regulation (Scheme 1).

We adopted the acyloin reduction reported by Jacobi et al.[12] for the synthesis of 4β-deoxy-PDBu (**5a**). The hydroxyl group at position 20 of phorbol (**1**) was protected with trityl group (67%). Dibutanoylation of **2** was accomplished with butyric anhydride in the presence of 4-dimethylaminopyridine (DMAP) and triethylamine to yield 20-trityl-PDBu (**3a**, 99%). After deprotection of the trityl group under the acidic condition (99%), acyloin reduction of the C4 hydroxyl group of **4a** gave two 4-deoxy compounds (**5a**, **5b**)[13] in 4% and 46% yield, respectively, along with unreacted **4a** (32%). Since **5a** isomerized in part to **5b** by heating or chloroform treatment, it was purified by HPLC on a reversed-phase column using acetonitrile–water, concentrated in vacuo under 35 °C, and lyophilized. Compound **5a** did not isomerize to **5b** in ethanol and Tris buffer (pH 7.4) at 4 °C. By comparison with the ^{1}H NMR spectrum of 4β-deoxy-phorbol-12,13,20-triacetate reported by Hecker et al.,[14] we determined the stereochemistry at position 4 of **5a** and **5b** as 4β and 4α, respectively (Table 1). NOE experiments supported this assignment; a remarkable NOE enhancement between H-4 (δ 2.79) and H-10 (δ 3.50) characteristic of the 4α-deoxy compound was observed in **5b**, while significant NOEs between H-4 (δ 2.47) and H-8 (δ 2.37)/H-11 (δ 1.54) peculiar to the 4β-deoxy compound were detected in **5a**. Since the yield of the 4β-deoxy compound (**5a**) was low (4%), we attempted the isomerization of the 4α-deoxy compound (**5b**) under the acidic conditions. However, no 4β-deoxy compound (**5a**) was obtained by refluxing **5b** in acetic acid–water. Moreover, acyloin reduction of the C4 hydroxyl group of 4α-phorbol-12,13-dibutyrate (4α-PDBu) prepared from 4α-phorbol gave a result similar to that of 4β-PDBu (**4a**).

4β-Deoxy-phorbol-13-acetate (**6a**) was similarly synthesized from phorbol-13-acetate (**4b**). Acyloin reduction of **4b** gave two deoxy compounds (**6a**, **6b**)[15] in 2.4% and 31% yield, respectively, along with unreacted **4b** (8%). The stereochemistry at position 4 of **6a** and **6b** was determined to be 4β and 4α, respectively, by the NMR chemical shifts[15] and the NOESY spectra (data not shown).

The binding affinity to PKC isozymes was evaluated by inhibition of the specific binding of [^{3}H]PDBu to synthetic 50–70-mer C1 peptides under the assay condi-



Scheme 1. Synthesis of 4β-deoxy-phorbol-12,13-dibutyrate (**5a**) and 4β-deoxy-phorbol-13-acetate (**6a**).

Table 1. ^{1}H NMR spectra of PDBu (**4a**), 4α-deoxy-PDBu (**5b**), and 4β-deoxy-PDBu (**5a**) in $CDCl_3$

Position	δ (Multiplicity, *J* in Hz)		
	PDBu (300 MHz)	4α-Deoxy-PDBu (500 MHz)	4β-Deoxy-PDBu (500 MHz)
1	7.60 (s, 1H)	7.04 (s, 1H)	7.55 (br.s, 1H)
4	—	2.79 (m, 1H)	2.47 (m, 1H)
5a	2.48 (d, 1H, *J* = 19.0)	2.49 (dd, 1H, *J* = 15.6, 5.0)	2.15 (dd, 1H, *J* = 18.1, 10.1)
5b	2.55 (d, 1H, *J* = 19.0)	3.45 (dd, 1H, *J* = 15.6, 2.6)	2.84 (dd, 1H, *J* = 18.1, 9.4)
7	5.68 (br.s, 1H)	5.12 (br.s, 1H)	5.53 (d, 1H, *J* = 4.1)
8	3.24 (t, 1H, *J* = 5.7)	1.96 (br.s, 1H)	2.37 (m, 1H)
10	3.25 (s, 1H)	3.50 (m, 1H)	3.25 (m, 1H)
11	2.14 (m, 1H)	1.66 (m, 1H)	1.54 (m, 1H)
12	5.42 (d, 1H, *J* = 10.3)	5.47 (d, 1H, *J* = 10.4)	5.42 (d, 1H, *J* = 9.7)
14	1.06 (d, 1H, *J* = 5.2)	0.78 (d, 1H, *J* = 5.0)	1.05 (d, 1H, *J* = 5.3)
16	1.21 (s, 3H)	1.17 (s, 3H)	1.19 (s, 3H)
17	1.24 (s, 3H)	1.20 (s, 3H)	1.20 (s, 3H)
18	0.89 (d, 3H, *J* = 6.4)	1.09 (d, 3H, *J* = 6.4)	0.92 (d, 3H, *J* = 6.4)
19	1.78 (dd, 3H, *J* = 2.9, 1.3)	1.78 (s, 3H)	1.73 (s, 3H)
20a	4.02 (m, 2H)	3.90 (br.d, 1H, *J* = 12.4)	4.02 (m, 2H)
20b	—	4.01 (br.d, 1H, *J* = 12.4)	—

tions of Sharkey and Blumberg[16] with slight modifications.[17] We have recently determined the dissociation constants (K_d) of PDBu and all PKC C1 peptides except for θ-C1A, enabling the comparative measurement of the binding constants (K_i) of other PKC activators by this [^{3}H]PDBu inhibition assay. Some of the K_d values of [^{3}H]PDBu in this assay have been revised when better performance or stability were obtained at 4 °C incubation or by elongation of the 50-mer peptides.[18,19] Table 2 summarizes the K_i values of 4β-deoxy-PDBu (**5a**) together with PDBu (**4a**) for all PKC C1 peptides. 4α-Deoxy-PDBu (**5b**) showed 100–1000-fold weaker binding affinity (data not shown) as expected from previous reports.[20] 4β-Deoxy-PDBu (**5a**) showed potent binding affinities to all PKC C1 peptides, comparable to PDBu (**4a**). The binding affinity of **5a** to the novel PKC-C1B peptides (δ, ε, η, θ) was even higher than PDBu (**4a**). These results indicate that the C4 hydroxyl group of phorbol esters is not necessary for PKC binding. In addition, deletion of the C4 hydroxyl group did not significantly change the PKC isozyme selectivity between α-C1B and δ-C1B. These results are unexpected from the X-ray structure[4] in which the C4 hydroxyl group is implicated in δ-C1B binding.

Table 2. K_i values (nM) for inhibition of the specific [^{3}H]PDBu binding to all PKC C1 peptides by 4β-deoxy-PDBu (**5a**) and PDBu (**4a**)

PKC C1 peptides	4β-Deoxy-PDBu (**5a**)	PDBu (**4a**)
α-C1A-long[a]	4.05 (0.33)[b]	1.55 (0.37)
α-C1B-long[a]	13.9 (2.5)	17.2 (0.81)
β-C1A-long[a]	2.78 (0.64)	1.80 (0.06)
β-C1B	2.23 (0.28)	1.97 (0.04)
γ-C1A	5.23 (0.91)	2.91 (0.52)
γ-C1B	1.84 (0.09)	1.58 (0.08)
δ-C1A	70.2 (10.4)	107 (26)
δ-C1B	0.55 (0.11)	1.03 (0.06)
ε-C1A	4.49 (0.65)	14.9 (1.6)
ε-C1B	0.58 (0.09)	1.35 (0.11)
η-C1A	14.9 (0.71)	10.2 (2.1)
η-C1B	0.35 (0.02)	0.95 (0.12)
θ-C1A	NT[c]	NT[c]
θ-C1B	0.73 (0.10)	1.54 (0.08)

[a]The 72-mer peptide in which 10 residues of both N- and C-termini of the original 50-mer C1 peptide are elongated to get good solubility and/or high folding efficiency.[19]
[b]Standard deviation of at least two separate experiments.
[c]Not tested.

In order to investigate the role of the butanoyl groups at positions 12 and 13 of **5a** in the unexpected binding results, the K_i values of 4β-deoxy-phorbol-13-acetate (**6a**) and phorbol-13-acetate (**4b**) for δ-C1B and α-C1B were measured. The binding constants of **6a** for δ-C1B and α-C1B (5.26±0.58 μM and 77.1±12.6 μM) were similar to those of **4b** (12.4±0.51 μM and 87.4±13.5 μM), respectively, suggesting that the hydrophobic side chains do not significantly alter isozyme selectivity. These binding data correspond well with the binding model calculated from the solution structure of α-C1B[6] rather than that from the crystal structure of δ-C1B.[5] Photoaffinity labeling experiments using native PKC[10] clearly showed that the ester carbonyl group at position 13 of phorbol esters is close to the binding site of PKC, supporting the binding model derived from the solution structure of α-C1B. Collectively, these results indicate that the solid-state structure[5] might not adequately or fully reflect the true interaction in solution between tumor promoters and PKC.

Finally, two in vitro bioassays relevant to in vivo tumor promotion were carried out to investigate the agonist activities (functional) of 4β-deoxy-PDBu (**5a**). The Epstein-Barr virus early antigen (EBV-EA)-inducing abilities[21,22] and the superoxide (O_2^-) generation-inducing abilities in differentiated HL-60 cells (Table 3) of the agents were determined.[23,24] EBVs are under the strict control of the host human lymphoblastoid Raji cells. They are activated by tumor promoters to produce the early antigen (EA). The EBV-EA-inducing activity is expressed as the percentage of EA-positive cells. Under our experimental conditions, PDBu exhibited maximum induction between 30% and 40% at 10^{-7} M. Superoxide (O_2^-) generation is triggered by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in epithelial cells and leukocytes through the xanthine oxidase and NADPH oxidase systems, respectively. The ability is expressed as the level of O_2^- production. Recent investigation has revealed that PKCβ plays an important role in signaling for the O_2^- production.[25] Under our experimental conditions, TPA produced about 2–3 nmol/mL min of O_2^- at 10^{-7} M. Both 4β-deoxy-PDBu (**5a**) and PDBu (**4a**) showed maximum EA-induction at 10^{-7} M, and potent superoxide generation induction at 10^{-7} M. In a control experiment, 4α-deoxy-PDBu (**5b**) showed weak EA-induction and only at much higher concentration (10^{-5} M). In the O_2^- generation test, **5b** was inactive even at 10^{-5} M (data not shown). These results clearly indicate that **5a** exhibits functional potency similar to **4a**.

Table 3. EBV-EA-inducing ability and superoxide (O_2^-) generation-inducing ability of PDBu (**4a**) and 4β-deoxy PDBu (**5a**) and 4α-deoxy PDBu (**5b**)

	% of EA-positive cells at the indicated concentrations (M)					nmol/10^{-6} cell/min of superoxide generation at the indicated concentrations (M)			
Compound	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-9}	10^{-8}	10^{-7}	10^{-6}
4a	3.6 (0.4)[a]	10.8 (1.3)	33.1 (0.5)	29.9 (1.1)	NT[b]	0.00 (0.03)	0.11 (0.09)	3.11 (0.01)	3.32 (0.45)
5a	1.2 (0.3)	7.0 (1.0)	31.1 (1.8)	25.6 (0.3)	NT	0.11 (0.03)	0.08 (0.10)	3.16 (0.48)	3.14 (0.07)
5b	NT	NT	NT	2.7 (0.4)	17.9 (0.6)	NT	NT	NT	0.05 (0.02)

[a]Standard deviation of two separate experiments.
[b]Not tested.

It is well-known that 4-deoxy-phorbol esters are active tumor promoters.[20] However, their precise binding to PKC C1 domains has not yet been reported. The present study unambiguously shows that the hydroxyl group at position 4 of phorbol esters is not necessary for PKC binding and functional activity (related to tumor promotion). These binding data differ in regard to C4 from the binding model derived from the crystal structure complex of PKCδ-C1B and phorbol-13-acetate (**4b**).[5] Further investigation is needed to determine whether the solid-phase structure is relevant to the solution-phase structure of the PKC isozymes.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (C) (2) (No. 11660109) and on Priority Areas (A) (2) (No. 12045241) from Ministry of Education, Science, Culture and Sports of Japan (for K.I.) and from Japan Society for the Promotion of Science for Young Scientists (Y.N.), and by grants (CA31841, CA31845) from the National Institutes of Health (P.A.W.).

References and Notes

1. Nishizuka, Y. *Nature* **1984**, *308*, 693.
2. Nishizuka, Y. *FASEB J.* **1995**, *9*, 484.
3. Hurley, J. H.; Newton, A. C.; Parker, P. J.; Blumberg, P. M.; Nishizuka, Y. *Protein Sci.* **1997**, *6*, 477.
4. Ono, Y.; Fujii, T.; Igarashi, K.; Kuno, T.; Tanaka, C.; Kikkawa, U.; Nishizuka, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 4868.
5. Zhang, G.; Kazanietz, M. G.; Blumberg, P. M.; Hurley, J. H. *Cell* **1995**, *81*, 917.
6. Hommel, U.; Zurini, M.; Luyten, M. *Nat. Struct. Biol.* **1994**, *1*, 383.
7. Ichikawa, S.; Hatanaka, H.; Takeuchi, Y.; Ohno, S.; Inagaki, F. *J. Biochem.* **1995**, *117*, 566.
8. Xu, R. X.; Pawelczyk, T.; Xia, T.-H.; Brown, S. C. *Biochemistry* **1997**, *36*, 10709.
9. Irie, K.; Ishii, T.; Ohigashi, H.; Wender, P. A.; Miller, B. L.; Takeda, N. *J. Org. Chem.* **1996**, *61*, 2164.
10. Uotsu, K.; Sodeoka, M.; Shibasaki, M. *Bioorg. Med. Chem.* **1998**, *6*, 1117.
11. Wang, S.; Kazanietz, M. G.; Blumberg, P. M.; Marquez, V. E.; Milne, G. W. A. *J. Med. Chem.* **1996**, *39*, 2541.
12. Jacobi, P.; Härle, E.; Schairer, H. U.; Hecker, E. *Liebigs Ann. Chem.* **1970**, *741*, 13.
13. Compound **5a** (4β-deoxy-PDBu): $[\alpha]_D$ +63.1° (*c* = 0.154, MeOH, 27.6 °C); UV λ_{max} (MeOH) nm (ε): 227 (7100); ^{13}C NMR (500 MHz, CD_3OD, 16 mM, 27 °C) δ ppm: 10.17, 13.88, 13.99, 15.48, 17.25, 19.20, 19.65, 24.13, 27.44, 30.25, 36.63, 37.15, 37.23, 43.21, 43.37, 45.37, 55.49, 66.83, 67.66, 78.34, 79.38, 127.49, 137.67, 143.94, 161.27, 175.27, 177.65, 212.31; HR-FABMS *m*/*z*: 489.2870 (MH^+ calcd for $C_{28}H_{41}O_7$, 489.2852); compound **5b** (4α-deoxy-PDBu): $[\alpha]_D$ −48.0° (*c* = 0.492, MeOH, 30.3 °C); UV λ_{max} (MeOH) nm (ε): 232 (8100); ^{13}C NMR (500 MHz, CD_3OD, 31 mM, 27 °C) δ ppm: 10.26, 12.21, 13.87, 13.98, 16.71, 19.15, 19.73, 24.49, 26.71, 27.26, 37.14, 37.27, 38.28, 42.28, 44.12, 48.53, 50.36, 66.66, 69.13, 76.99, 79.66, 124.84, 137.87, 144.10, 159.00, 175.25, 177.52, 214.32; HR-FABMS *m*/*z*: 489.2858 (MH^+ calcd for $C_{28}H_{41}O_7$, 489.2852).
14. Fürstenberger, G.; Hecker, E. *Tetrahedron Lett.* **1977**, *11*, 925.
15. Compound **6a** (4β-deoxy-phorbol-13-acetate): $[\alpha]_D$ +91.7° (*c* = 0.333, MeOH, 26.9 °C); UV λ_{max} (MeOH) nm (ε): 229 (5800); ^{1}H NMR (500 MHz, CD_3OD, 14.9 mM, 27 °C) δ ppm: 1.06 (3H, d, *J* = 6.4 Hz), 1.07 (1H, d, *J* = 5.5 Hz), 1.20 (3H, s), 1.22 (3H, s), 1.48 (1H, m), 1.71 (3H, s), 2.11 (3H, s), 2.15 (1H, dd, *J* = 18.0, 8.2 Hz), 2.38 (1H, t, *J* = 5.6), 2.63 (1H, m), 2.77 (1H, dd, *J* = 18.0, 9.3 Hz), 3.19 (1H, m), 3.93 (3H, m) 5.48 (1H, d, *J* = 3.7 Hz), 7.62 (1H, s); ^{13}C NMR (500 MHz, CD_3OD, 14.9 mM, 27 °C) δ ppm: 10.18, 16.16, 17.26, 21.07, 24.10, 27.25, 30.37, 36.02, 43.28, 45.12, 45.52, 55.81, 67.76, 69.15, 77.89, 79.17, 128.05, 137.51, 143.62, 161.73, 176.03, 212.46; HR-FABMS *m*/*z*: 391.2137 (MH^+ calcd for $C_{22}H_{31}O_6$, 391.2120); compound **6b** (4α-deoxy-phorbol-13-acetate); $[\alpha]_D$ −82.7° (*c* = 0.396, MeOH, 28.4 °C); UV λ_{max} (MeOH) nm (ε): 233 (7100); ^{1}H NMR (500 MHz, CD_3OD, 43.1 mM, 27 °C) δ ppm: 0.80 (1H, d, *J* = 5.3 Hz), 1.21 (3H, s), 1.22 (3H, s), 1.22 (3H, d, *J* = 5.8 Hz), 1.65 (1H, m), 1.72 (3H, s), 1.93 (1H, br. s), 2.07 (3H, s), 2.24 (1H, dd, *J* = 15.3, 4.5 Hz), 2.71 (1H, m), 3.29 (1H, m), 3.49 (1H, m), 3.85 (2H, m), 3.95 (1H, d, *J* = 10.0 Hz), 5.12 (1H, br. s), 7.30 (1H, s); ^{13}C NMR (500 MHz, CD_3OD, 25.6 mM, 27 °C) δ ppm: 10.26, 12.60, 16.75, 21.14, 24.56, 26.38, 27.26, 37.41, 42.44, 46.23, 48.63, 50.40, 68.96, 69.22, 75.67, 79.42, 125.41, 137.33, 143.94, 159.41, 175.56, 214.50; HR-FABMS *m*/*z*: 391.2112 (MH^+ calcd for $C_{22}H_{31}O_6$, 391.2120).
16. Sharkey, N. A.; Blumberg, P. M. *Cancer Res.* **1985**, *45*, 19.
17. Irie, K.; Oie, K.; Nakahara, A.; Yanai, Y.; Ohigashi, H.; Wender, P. A.; Fukuda, H.; Konishi, H.; Kikkawa, U. *J. Am. Chem. Soc.* **1998**, *120*, 9159.
18. Irie, K.; Nakahara, A.; Ohigashi, H.; Fukuda, H.; Wender, P. A.; Konishi, H.; Kikkawa, U. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2487.
19. Irie, K.; Nakahara, A.; Ikawa, Y.; Tanaka, M.; Nakagawa, Y.; Nakamura, Y.; Ohigashi, H.; Wender, P. A. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 2429.
20. Fürstenberger, G.; Hecker, E. *Z. Naturforsch.* **1985**, *40*, 631.
21. Ito, Y.; Yanase, S.; Fujita, J.; Harayama, T.; Takashima, M.; Imanaka, H. *Cancer Lett.* **1981**, *13*, 29.
22. zur Hausen, H.; Bornkamm, G. W.; Schmidt, R.; Hecker, E. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 782.
23. Market, M.; Andrews, P. C.; Babior, B. M. *Methods Enzymol.* **1984**, *105*, 358.
24. Nakamura, Y.; Murakami, A.; Ohto, Y.; Torikai, K.; Tanaka, T.; Ohigashi, H. *Cancer Res.* **1998**, *58*, 4832.
25. Korchak, H. M.; Rossi, M. W.; Kilpatrick, L. E. *J. Biol. Chem.* **1998**, *273*, 27292.