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Novel diterpenoid diacylglycerols from marine molluscs: potent morphogens and protein kinase C activators

L. De Petrocellis[a], P. Orlando[b], M. Gavagnin[d], M. Ventriglia[c], G. Cimino[d] and V. Di Marzo[d,*]

[a]*Istituto di Cibernetica, C.N.R., Viale Toiano 6, I-80072 Arco Felice, Napoli (Italy)*
[b]*Istituto di Biochimica delle Proteine ed Enzimologia, C.N.R., Viale Toiano 6, I-80072 Arco Felice, Napoli (Italy)*
[c]*Dipartimento di Chimica Farmaceutica e Tossicologica, Facoltà di Farmacia, Università di Napoli, Via D. Montesono, 49, Napoli (Italy)*
[d]*Istituto per la Chimica di Molecole di Interesse Biologico, C.N.R., Viale Toiano 6, I-80072 Arco Felice, Napoli (Italy), Fax +39 81 04 1770*
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Abstract. Five novel 1,2-*sn*-diacylglycerols with diterpenoid acyl moieties in the *sn*-1 position were isolated and characterized, together with the corresponding 1,3-*sn*-diacylglycerols, from three species of dorid nudibranchs molluscs. Their potent activity as morphogens in vivo in the *Hydra* tentacle regeneration assay and their parallel activity as activators of rat brain protein kinase C (PKC) in vitro are reported here. Our findings promote the use of these compounds as useful molecular probes for both in vivo and in vitro studies on the participation of PKC in cell development.
Key words. Protein kinase C; diacylglycerols; *Hydra*; marine natural products.

Among the enzymes leading to the phosphorylation/dephosphorylation cascades essential to intracellular signal amplification and transduction, PKC undoubtedly participates in the widest range of both short- and long-term biological responses (for review see [1]). In particular, all the biochemical events underlying biological processes such as cell differentiation, proliferation and development involve, at one or more stages, the participation of one or more proteins whose functionality can be regulated through PKC-catalysed phosphorylation (for review see [2]), hence the increasingly higher quest for new drugs to be used as pharmacological tools for the study of this enzyme.

Although several PKC inhibitors have been developed so far (see [3] and references cited therein), only a few PKC activators are used in pharmacological investigations, e.g. linear, long-chain 1,2-*sn*-diacylglycerols, such as 1,2-*sn*-dioleoylglycerol, and the phorbol esters. However, the use of the latter compounds, which are much more potent PKC activators than are linear diacylglycerols in vivo, has sometimes been limited by their powerful tumor-promoting activity [4]. Ideally, PKC activators to be used for in vivo studies should be non-tumor promoting, easily partitioned into the plasma membrane, in order to interact more efficiently with their intramembrane target, and not easily degraded by lipases.

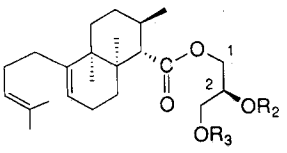
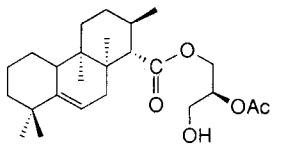
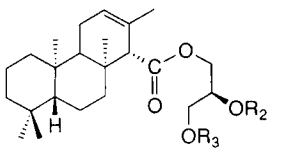
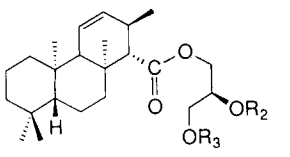
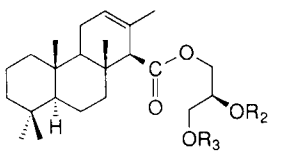
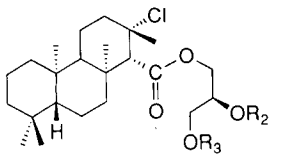
Some years ago, the isolation of diterpenoid 1,2-*sn*-diacylglycerols from dorid nudibranch molluscs, and in particular of verrucosin B (table 1, structure **1**) from the mantle of *Doris verrucosa*, was reported [5, 10]. Following its chemical characterization, the finding of the powerful PKC stimulatory effects of this natural product, which was devoid of tumor-promoting activity (H. Fujiki, pers. commun.), as well as of its PKC-mediated morphogenic properties in vivo in the *Hydra* tentacle regeneration assay [6, 7], prompted the use of verrucosin B in other studies on the participation of PKC in biological responses [7, 8]. These pharmacological properties have recently promoted new investigations aimed, on the one hand, at finding new structurally related 1,2-*sn*-diacylglycerols from marine sources and, on the other, at designing a strategy for their chemical synthesis [9]. Here we report the pharmacological characterization, as well as a preliminary study of the structure activity relationships, of five novel diterpenoid 1,2-*sn*-diacylglycerols and of the corresponding 1,3-*sn*-diacylglycerols (table 1, structures **2**–**11**), recently isolated from *D. verrucosa* and other dorid nudibranch molluscs (Gavagnin, Castelluccio, Ungur and Cimino, unpubl. data).

Materials and methods

All compounds under investigation were isolated from *D. verrucosa* as described elsewhere (Gavagnin, Castelluccio, Ungur and Cimino, unpubl. data) with the exception of **5** and **10**, which were extracted from *Archidoris tubercolata* (and, in a previous study, from *A. montereyensis* [10]), and of **3** and **8**, which were found in both *D. verrucosa* and *A. carvi*. Structure elucidation of the new metabolites (**2**, **4**, **6**, **7**, **9**, **11**) was carried out by

* Corresponding author.

Table 1. The effect of 1,2-*sn*- and 1,3-*sn*-diacylglycerols from dorid nudibranch molluscs on PKC and *H. vulgaris* tentacle regeneration.

Substance		Fold potentiation of protein kinase C activity		Effect on *Hydra* ATN			
				EC_{50}	EC_{100}	ATN	RI
		100 nM	6 μM	μM	μM	MAX	
None		1.0	1.0	–	–	5.9	0
	**2** R_2 = Ac, R_3 = H	3.6	5.2	0.16	0.25	9.5	244
	7 R_2 = H, R_3 = Ac	1.2	4.0	0.82	2.5	7.6	12
	**1** verrucosin B	3.6	5.0	0.08	0.25	9.0	210
	**3** R_2 = Ac, R_3 = H	3.2	5.0	0.50	1.0	11.4	93
	8 R_2 = H, R_3 = Ac	1.1	4.2	1.40	2.5	9.3	23
	**4** R_2 = Ac, R_3 = H	3.0	5.4	0.70	1.0	9.3	58
	9 R_2 = H, R_3 = Ac	1.2	3.8	2.90	5.0	11.5	19
	**5** R_2 = Ac, R_3 = H	2.3	4.4	0.80	1.0	9.0	53
	10 R_2 = H, R_3 = Ac	0.9	3.9	3.20	5.0	9.3	12
	**6** R_2 = Ac, R_3 = H	2.0	5.1	0.65	2.6	8.7	18
	11 R_2 = H, R_3 = Ac	1.0	4.4	2.70	5.0	8.3	8

means of spectroscopic (mostly two-dimensional nuclear magnetic resonance [2D NMR]) and mass spectrometric methods. Relative stereochemistry of chiral centers was obtained by nuclear Overhouse effect difference NMR experiments and by analysis of ^{1}H-coupling constant analyses. Absolute stereochemistry was suggested circular dicroism by comparison of profiles with those of known compounds, e.g. verrucosin B, whose configuration had been previously determined by chemical methods [11].

The effect of different concentrations of compounds **1–11** on PKC activity was evaluated by measuring histone H1 phosphorylation in the presence of the enzyme purified from rat brain (Boehringer Mannheim, Germany), γ-[^{32}P]-adenosine triphosphate (ATP), 1,2-*sn*-dioleoylphosphatidylserine (PS, Sigma, 200 μg/ml) and $CaCl_2$ (200 μM), thus exploiting a widely used methodology [12]. Histone H1 was purified from calf thymus as previously described [13]. Rat brain PKC is known to be a mixture of the α, β and γ isoforms of the

enzyme [12]. The assay was conducted in triplicates. The results from at least three separate experiments, expressed as fold potentiation of Ca^{2+}/PS-induced PKC activity, were used to calculate means ± standard deviation (SD) (only means are shown for the sake of clarity; SD was always lower than 5% of the means).

The effect of different concentrations of compounds **1–11** on an in vivo response induced by PKC activation, i.e. the enhancement of average tentacle number in excised polyps of the hydrozoan *Hydra vulgaris*, known as the *Hydra* tentacle regeneration assay, was studied as described previously [6, 7] on at least 10 excised polyps for each of the doses tested. After 24 h incubation with drugs, polyps were washed and left to regenerate their head and tentacles. After 10 days the average tentacle number was measured and compared with that of control polyps. Results are reported as EC_{100} and EC_{50} (i.e. the μM concentration causing, respectively, maximal and half-maximal enhancement of ATN), highest average tentacle number observed (ATN MAX) and regeneration index (RI), i.e. the maximal percent increase of ATN divided by the μM concentration at which this maximal increase is observed. ATN values used for evaluation were the means of those observed in the 10 polyps used for the experiment.

Results and discussion

The PKC preparation used in the present study is known to be composed mainly of the Ca^{2+}/PS-dependent α, β and γ isoforms of the enzyme [12]. Therefore, the effect of diacylglycerols on PKC was studied in the presence of Ca^{2+} and PS. As summarized in table 1, both 1,2- and 1,3-*sn*-diacylglycerols significantly enhanced Ca^{2+}/PS-induced PKC activity in a dose-dependent manner, the effect being maximal at a 2–6 μM concentration. At this dose, 1,3-*sn*-diacylglycerols were almost equipotent to 1,2-*sn*-diacylglycerols, while being completely inactive at 100 nM. At this latter concentration 1,2-*sn*-diacylglycerols caused a 2–3.6-fold potentiation of PKC activity. The most active compound was **2**, whose EC_{50} was at least five times lower than that of the widely used PKC activator 1,2-*sn*-dioleoylglycerol (20 nM vs 100 nM, fig. 1), in analogy with data reported here and previously [6, 7] for verrucosin B. Interestingly, compound **2**, unlike verrucosin B and the other compounds under study, possesses an isoprene acyl chain with only two rings and a 2-methyl-pent-2-enyl chain as the substituent of the cycloesenyl ring, thus suggesting that integrity of all three rings is not required for enhanced PKC stimulatory activity. Conversely, the two less active 1,2-*sn*-diacylglycerols (**6**, **5**) possess either of two chemical moieties absent both in verrucosin B (**1**) and in the two most active metabolites (**2**, **3**), i.e. a chlorine substitutent or a completely inverted stereochemistry at all chiral centers of the tricyclic isoprene acyl chain, respectively, thus showing

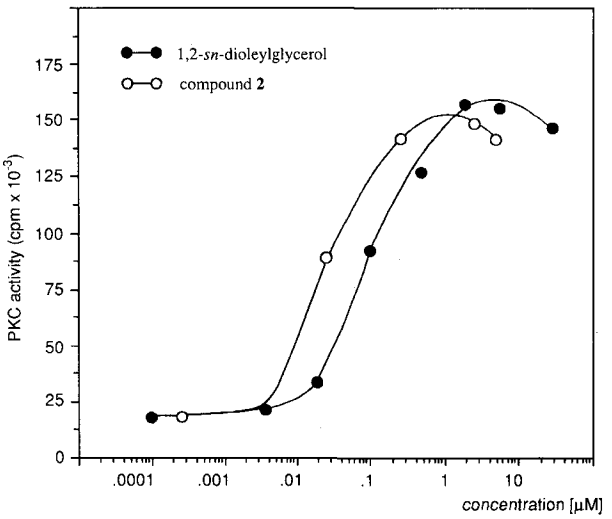


Figure 1. Dose-response curve for the effect of 1,2-*sn*-dioleoylglycerol and compound **2** on Ca^{2+}/PS-induced rat brain PKC activation. The effect was measured as radioactivity (^{32}P) incorporated into histone H1.

that these structural modifications negatively influence PKC stimulatory activity. The effect of 1,3-*sn*-diacylglycerols **7–11** on PKC activity, which was observed only at high concentrations, may be due to their partial conversion to the corresponding 1,2-*sn*-diacylglycerols during the assay [14]. No experiments were carried out with purified PKC isoforms, nor with the Ca^{2+}-independent ε, ξ and δ PKC isoenzymes, since these were not commercially available when the present study was undertaken. Thus, none of the compounds were tested for PKC isoenzyme selectivity.

As shown in table 1, the order of potency of the metabolites in the *Hydra* regeneration assay (measured by using the RI) parallels that observed in the PKC assay, with compound **2** being the most active and compound **6** the less active among the 1,2-*sn*-diacylgly-cerols, and the 1,3-analogs being less active than 1,2-*sn*-diacylglycerols. Also in this case, the effect of 1,3-*sn*-diacylglycerols might be due, in part, to acetyl migration from the *sn*-3 to the *sn*-2 position during the 24 h of the incubation with *Hydra* [14].

The findings presented in this paper clearly demonstrate that diterpenoid 1,2-*sn*-diacylglycerols isolated from dorid nudibranch molluscs may be used as pharmacological tools for PKC activation in some in vivo studies since a good correlation exists between their activation of PKC in vitro and their effect on a typical PKC-mediated biological response such as the enhancement of ATN in *H. vulgaris*. Moreover, diterpenoid 1,2-*sn*-diacylglycerols are not likely to be tumor promoters or good substrates for enzymatic hydrolysis since they are structurally very similar to verrucosin B, which is not significantly hydrolysed by *Hydra* homogenates (De Petrocellis and Di Marzo, unpubl. data) and was found not to induce tumors in mouse skin (H. Fujiki pers. commun.). This would

make these natural products ideal pharmacological tools for in vivo experiments on PKC-mediated short- and long-term responses such as those concerned with cell differentiation, proliferation and motility. As new strategies for their chemical synthesis become available (like the one described in a separate paper [9]), an ever-increasing use in pharmacological studies, as molecular probes for PKC activation, can be predicted for diterpenoid 1,2-*sn*-diacylglycerols.

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1 Nishizuka Y. (1986) Studies and perspectives of protein kinase C. Science **233:** 305–312
2 Tilly B. C. and Moolenar W. H. (1989) Inositol lipids in growth factor action. In: Inositol Lipids in Cell Signalling, pp. 485–519, Michell R. H., Drumond A. H. and Downess C. P. (eds), Academic Press, San Diego
3 Hannun Y. A., Merrill A. H. and Bell R. M. (1991) Use of sphingosine as inhibitor of protein kinase C. Methods Enzymol. **201:** 316–328
4 Diamond L., O'Brien T. G. and Baird W. M. (1980) Tumor promoters and the mechanism of tumor promotion. Advances Cancer Res. **32:** 1–74
5 Cimino G., Gavagnin M., Sodano G., Puliti R., Mattia C. A. and Mazzarella L. (1988) Verrucosin-A and -B, ichthyotoxic diterpenoic acid glycerides with a new carbon skeleton from the Dorid nudibranch *Doris verrucosa*. Tetrahedron **44:** 2301–2310
6 De Petrocellis L., Di Marzo V., Arcà B., Gavagnin M., Minei R. and Cimino G. (1991) The effect of diterpenoidic diacylglycerols on tentacle regeneration in *Hydra vulgaris*. Comp. Biochem. Physiol. **100C:** 603–607
7 De Petrocellis L., Di Marzo V. and Cimino G. (1993) The possible involvement of protein kinase C and phospholipase A_2 activation in *Hydra* tentacle regeneration. Experientia **49:** 57–64
8 Di Marzo V., Vial D., Sokoloff P., Schwartz J.-C. and Piomelli D. (1993) Selection of alternative Gi-mediated signaling pathways at the dopamine D_2-receptor by protein kinase C. J. Neurosci. **13:** 4846–4853
9 Ungur N., Gavagnin M. and Cimino G. (1996) Synthesis of diastereoisomeric *ent*-isocopalic acid glycerides. Tetrahedron Letts. **37:** 3549–3552
10 Gustafson K. and Andersen R. J. (1985) Chemical studies of British Columbia nudibranchs. Tetrahedron **41:** 1101–1108
11 Gavagnin M., Spinella A., Cimino G. and Sodano G. (1990) Stereochemistry of ichthyotoxic diacylglycerols from opisthobranch molluscs. Tetrahedron Lett. **31:** 6093–6094
12 Kitano T., Go M., Kikkawa U. and Nishizuka Y. (1986) Assay and purification of protein kinase C. Methods Enzymol. **124:** 349–352
13 De Petrocellis L., Quagliarotti G., Tomei L. and Geraci G. (1986) Structuring of H1 histone: evidence of high-affinity binding sites for phosphate ions. Eur. J. Biochem. **156:** 143–148
14 Ganong B. R. and Bell R. M. (1987) Synthesis of cell-permeant diacylglycerol analogs for structure-function analysis of protein kinase C and other enzymes. Methods Enzymol. **141:** 313–320