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Phorbasones A and B, Sesterterpenoids Isolated from the Marine Sponge *Phorbas* sp. and Induction of Osteoblast Differentiation

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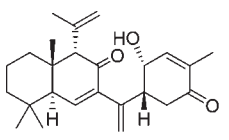
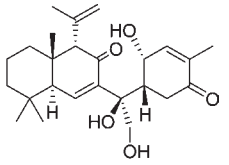
ABSTRACT



Phorbasone A



Phorbasone B

Two new sesterterpenoids, phorbasones A (1) and B (2), were isolated from the Korean marine sponge *Phorbas* sp. Their complete structures were elucidated by spectral data and chemical reactions. Phorbasone A exhibited a positive effect on the calcium deposition activity in C3H10T1/2 cells. The biogenic origin of the core structure is believed to be through a novel rearrangement from the ansellone carbon structure.

Marine sponges have produced many structurally novel secondary metabolites with diverse biological activities.[1] *Phorbas* sp., a marine sponge, is a good example of the structural diversity in secondary metabolites as it produces macrolides,[2] diterpenes,[3] and sesterterpenes.[4] These structurally diverse natural products also showed a wide range of biological activities such as antifungal activity, cytostatic activity, cytotoxicity,[2] inhibition of isocitrate lyase,[3f] and activation of the cAMP pathway.[4b]

As part of an ongoing search for compounds inducing the differentation of the osteoblast responsible for bone formation,[5] an extract of the genus *Phorbas* was screened with a calcium deposition assay in C3H10T1/2 cells. On the

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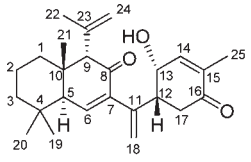
basis of activity guided fractionation, we isolated two new compounds, phorbasones A (**1**) and B (**2**), along with the reported phorbaketals. Phorbasone A showed a striking effect on calcium deposition in the cells.

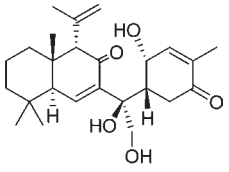
The core structure of phorbasone A provides another interesting point as the structure is related to that of ansellone A isolated from the Nudibranch *Cadlina luteromarginata* and the sponge *Phorbas* species.[4b] Apparently, three classes of sesterterpenes—phorbaketal, ansellone, and phorbasone—obtained from *Phorbas* species appeared to be biogenically associated with one another.

In this paper we present the structure determination of two new phorbasones and their osteogenic properties, as well as a plausible biogenic pathway from geranylfarnesyl pyrophosphate to phorbasone via ansellone and phorbaketal structures.

Following the evaluation of biological activity of the seven fractions from the extract of *Phorbas* sp. collected in 2008, fraction 5 (90% MeOH in water, 1.4 g) showed a calcium deposition effect and contained a compound showing the ^{1}H NMR signal patterns different from those of phorbaketals. Phorbasone A (**1**, 22 mg) was isolated as a minor component in the course of isolating a large quantity of phorbaketal A (1.0 g). Subsequently, phorbasone B (**2**, 5 mg) with a similar structure was further separated from fraction 4 (80% MeOH in water, 150 mg).



1



2

Phorbasone A (**1**) was isolated as a yellowish oil and determined to have a molecular formula of $C_{25}H_{34}O_3$ on the basis of high resolution FABMS ($[M+Na]^+$ peak at m/z = 405.2410, Δ = 0.4 ppm), consistent with nine degrees of unsaturation. The IR spectrum revealed the strong absorption bands at 3434 cm^{-1} (hydroxyl) and 1672 cm^{-1} (carbonyl). The ^{13}C NMR spectrum of **1** displayed eight olefinic resonances (δ_c 114.3, 116.9, 137.4, 142.3, 143.0, 144.9, 147.9, and 151.5) and two ketone carbonyl cabons (δ_c 201.9 and 202.0) (Table 1). Among them, two carbons at δ_c 114.3 and 116.9 were assigned as isolated olefinic carbons based on both the HSQC spectrum and their corresponding carbon chemical shifts. Furthermore, the presence of α, β-unsaturated ketone groups was also proposed by the UV band at 225 nm (log $\varepsilon \approx 4.1$) and the related carbon chemical shifts. Additionally, aliphatic carbons in the upfield region were classified as five methyls, two vinyl methyls, four methylenes, and five methines from analysis of the ^{1}H and HSQC spectra. Collectively, these observations accounted for six of nine double-bond equivalents in the molecule, deducing **1** to be tricyclic.

Detailed analysis of the HSQC, COSY, and TOCSY spectra revealed a linear connectivity of three diastereotopic methylenes in the range of 1.00−2.00 ppm and also a coupling between the proton doublet at δ 2.61 (H-5) and a proton doublet at δ 7.05 (H-6) in the trisubstituted double bond as shown in the Figure 1. An HMBC spectrum showed the long-range correlations of the methine singlet at H-9 and the olefinic methine at H-6 with the ketone carbon at δ 202.0 (C-8). Moreover, each methyl singlet at δ 1.00 (H_3-19), 1.08 (H_3-20), and 1.11 (H_3-21) was correlated with four neighboring carbons, constructing a partial structure of 4,4,10-trimethyltrihydronaphthalen-8-one. The correlation with C-8, one of two very close ketone carbonyl carbon signals, was clearly confirmed by a selective HMBC experiment. In addition, it was observed that the methine singlet at H-9 on the subunit was correlated with an olefinic methyl, a terminal methylene, and a quaternary olefinic carbon in the HBMC spectrum, indicating the attachment of an isopropene moiety on C-9.

In a similar way, the structure of one remaining ring was also established by both consecutive COSY cross peaks from the downfield shifted doublet at δ 6.78 (H-14) to diastereotopic methylene protons at δ 2.44 and 2.80 (H-17) via an oxymethine at δ 4.14 (H-13) and a methine at δ 3.07 (H-12) and the HMBC correlations of H-14 and H-17 with another ketone carbonyl carbon at δ_C 201.9, assigned as a cyclohexenone. This was confirmed by the HMBC correlations between the olefinic methyl at δ 1.76 (H_3-25) and three sp^2 carbons in the ring. Finally, the HMBC

Table 1. ^{1}H and ^{13}C NMR Data of Phorbasone A (**1**)[a]

no.	δ_C, mult[b]	δ_H, mult (J Hz)
1a	37.4, CH_2	1.36, br d (13.2)
1b		1.44, ddd (13.2, 13.2, 3.4)
2a	19.3, CH_2	1.53, br d (13.2)
2b		1.78, br d (13.2)
3a	42.3, CH_2	1.27, m
3b		1.52, br d (13.2)
4	33.7, C	
5	49.1, CH	2.61, d (2.2)
6	151.5, CH	7.05, d (2.2)
7	143.0, C	
8	202.0, C	
9	70.1, CH	2.58, br s
10	43.3, C	
11	147.9, C	
12	44.5, CH	3.07, ddd (12.8, 3.7, 3.2)
13	64.9, CH	4.14, dd (5.8, 3.2)
14	144.9, CH	6.78, dq (5.8, 1.3)
15	137.4, C	
16	201.9, C	
17a	38.0, CH_2	2.44, ddd (16.3, 3.7, 0.7)
17b		2.80, dd (16.3, 12.8)
18a	116.9, CH_2	5.07, dd (1.3, 1.3)
18b		5.12, dd (0.9, 0.9)
19	33.1, CH_3	1.08, s
20	23.2, CH_3	1.00, s
21	22.9, CH_3	1.11, s
22	27.7, CH_3	1.85, dd (1.3, 0.9)
23	142.3, C	
24a	114.3, CH_2	4.79, br s
24b		5.08, dd (1.3, 1.3)
25	15.6, CH_3	1.76, dd (1.3, 0.7)

[a] Measured at 500 MHz (^{1}H) and 125 MHz (^{13}C) in CD_3OD.

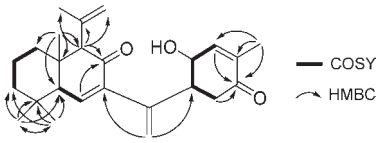


Figure 1. Key COSY and HMBC correlations (H→C) of **1**.

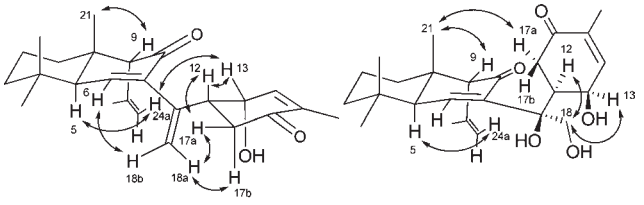


Figure 2. Key NOE correlations of **1** and **2**.

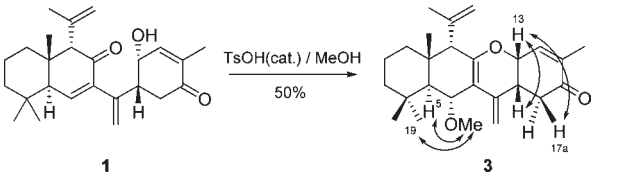
correlations from the unassigned terminal methylene protons to both the quaternay carbon at C-7 in the first bicyclic unit and the methine carbon at C-12 in the cyclohexenone ring assembled two partial moieties and completed the gross structure of **1**, an unprecedented sesterterpene skeleton.

The configuration of the bicyclic ring was assigned as a frame of *trans* decalin from the NOE peaks of H_3-21 with adjacent protons, especially between H_3-21 and H-9 (Figure 2). On the basis of this frame, the orientation of the isopropene moiety was opposite to that of H_3-21 from the NOEs of H-5/H-24a and H-9/H_3-23. On the other hand, the NOE signals of H-12/H-13 and H-12/H-17a indicated that three protons are positioned on the same side of the cyclohexenone ring plane. The H-17b was configured in the axial position based on a large coupling constant of 12.8 Hz with H-12. Further analysis of the NOESY spectrum enabled us to determine the relative configuration between decalin and cyclohexenone moieties from the NOEs of H-18a/H17a, H-18a/H-17b, H-18b/H-6, and H-13/H-24a, suggesting a 12*R** configuration at C-13. Notably, the last critical correlation was unequivocally observed by the NOESY 1D spectrum.

The relative stereochemistry of **1** was also confirmed by the NOE experiment of the tetracyclic product obtained from **1** through an acid catalyzed cyclization reaction (Scheme 1). When compound **1** was treated in methanol with an acid catalyst, tetracyclic compound **3** was obtained in 48% yield. The structural identity of **3** was unambiguously confirmed by NOSEY, HSQC, and HMBC. The NOE peaks of **3** clearly showed all the relative stereochemistry; H_3-OCH_3/H_3-19, H_3-OCH_3/H-5, H-13/H-12, and H-13/H-17a (Supporting Information).

The absolute stereochemistry of compound **1** was determined by the Mosher ester method. The shielding effect of the phenyl group in the MTPA ester of **1** gave different

Scheme 1. Acid Catalized Cyclization Reaction of Phorbasone A and Key NOE Correlations of **3**



$\Delta\delta^{SR}$ signs on each side centered on C-13, with positive values for H-15 and H_3-25 and negative values for H-12, H-17, and H-18 (Supporting Information). Accordingly, the configuration at C-13 was assigned as *R*.

Phorbasone B (**2**) was determined as $C_{25}H_{36}O_5$ on the basis of a combination of HRFABMS (*m*/*z* 439.2460 measured for $[M + Na]^+$) and the ^{13}C spectrum. Compared with **1**, the obvious difference is that one terminal methylene group was oxidized, and from this, a new oxymethylene and one additional hydroxy unit were present. From the detailed analysis of 2D NMR data, the partial structures of bicyclic and cyclohexenone moieties were deduced to be identical to those of compound **1**. The well-resolved AB splitting methylene protons at δ 3.96 and 4.00 showed the HMBC correlations with two quaternary carbons at δ 139.8 (C-7), 79.6 (C-11) and the methine carbon at δ 42.2 (C-12). This was indicative of the linkage of two ring structures to the each side of the hydroxy carbon at C-11. Accordingly, the structure of **2** was derived from the hydroxylation at positions C-11 and C-18 of **1**. Each configuration in two partial structures was identical to that of compound **1** from the NOESY spectrum, but their relative orientation was somewhat different from that of **1**. First of all, the H-17a at δ 2.06 (1H, dd, J = 13.7, 2.7 Hz) positioned in equatorial with H-12 showed a strong NOE correlation with H_3-21 on the decalin skeleton. This showed that the cyclohexenone ring was configured perpendicular to the decalin moiety. Based on this configuration, the distinct NOE observation of the oxymethylene protons at H-18 with two protons at H-12 and -13 led to the determination of the stereochemistry at C-11 in **2**, indicating 11*R*.

To the best of our knowledge, the tricyclic skeleton of phorbasone A and B is an unprecedented structure and is presumed to be derived from the carvone skeleton of phorbaketal via an ansellane structure through skeletal rearrangement as shown in Figure 3.

All three sesterterpene structures from *Phorbas* species, phorbaketal, ansellone and phorbasone, are believed to come from geranylfarnesyl pyrophosphate through cationic cyclizations and rearrangements. Initial cyclization of geranylfarnesyl pyrophosphate produces the basic carbon skeleton (**i**) of phorbaketal after γ-hydroxylation. The farnesyl part of the phorbaketal carbon skeleton undergoes cationic cyclization to form the ansellone carbon skeleton (**ii**). The ansellone structure can further rearrange into a phorbasone skeleton through cationic cyclization to form a [2.2.2] bicyclic intermediate (**iii**) and successive

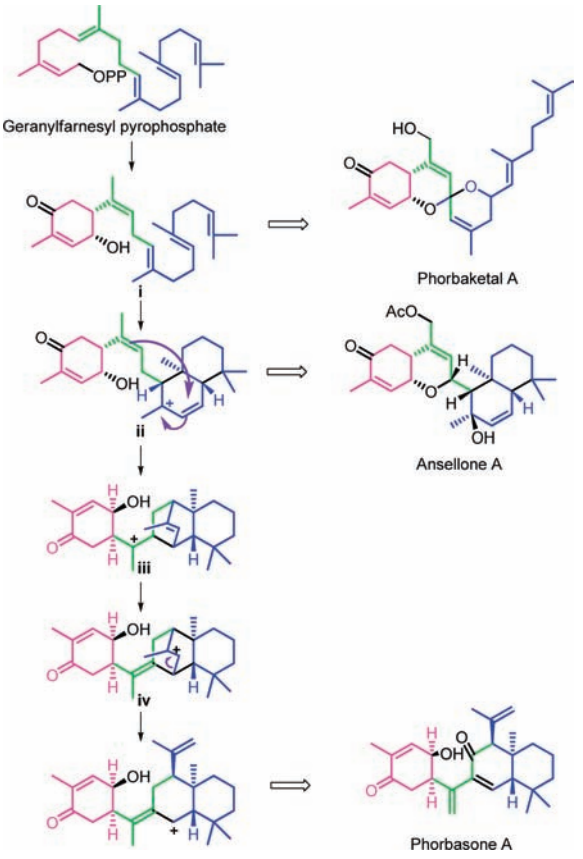


Figure 3. Biosynthetic pathway to ansellone and phorbasone skeleta.

proton migration into (**iv**) followed by ring cleavage. This rearrangement of ansellone to phorbasone also supports the relative and absolute stereochemistry of phorbasone A. Determination of the absolute stereochemistry of these natural products has been obscured by the unusual CD behavior of these compounds, along with the diterpene phorbasin C isolated from *Phorbas* sp. These natural products showed the positive sign of the Cotton effect for the (*S*)-carvone stereochemistry.[6] However, the absolute stereochemistry of phorbasin C was reassigned through total synthesis to have the (*R*)-carvone stereochemistry.[7]The absolute stereochemistry of ansellone A was also confirmed to possess the (*R*)-carvone stereochemistry through X-ray crystallography[4b] which confirmed the absolute stereochemistry of phorbaketal and alotaketal.

We examined the effect of phorbasones A and B on calcium deposition in mensenchymal C3H10T1/2 cells. The

(6) CD spectra of phorbaketal A and phorbasone A showed the positive sign of the Cotton effect.

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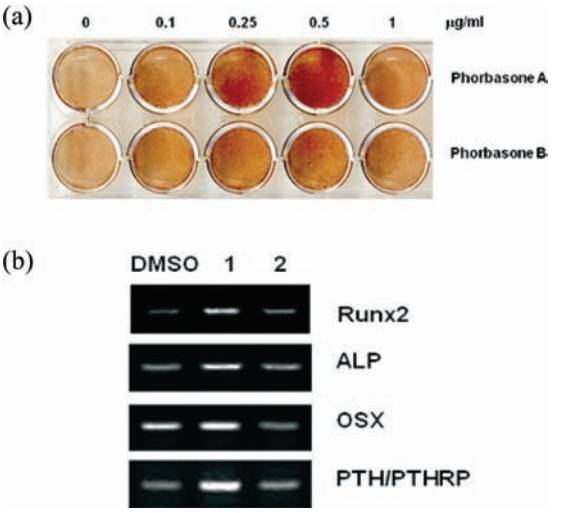

Figure 4. (a) Effect of compounds **1** and **2** on calcium deposition and (b) expression of markers of osteoblast differentiation by RT-PCR in C3H10T1/2 cells .

cells were dose-dependently cultured in differentiation media with phorbasones A and B for 6 days. As demonstrated by Alizarin red staining in Figure 4, phorbasone A showed a distinct calcium deposition effect compared to B. Maximal staining of phorbasone A was observed at a concentration of 0.5 μg/mL.

Furthermore, the gene expression of markers of osteoblast differentiation was observed by reverse transcription (RT)-PCR, where phorbasone A exhibited the increased Runx2 (a Runt protein), ALP (alkaline phosphatase), OSX (osterix), PTH (parathyroid hormone), and PTHrP (PTH-related peptide) mRNA.[8]

In summary, we isolated a new class of sesterterpenoids, phorbasones A and B. Phorbasone A showed an induction of osteoblast differentiation. Currently, we are working on the identification of the molecular target of phorbasone A for osteoblast differentiation.

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Supporting Information Available. Experimental procedures and spectral data of **1**, **2**, and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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