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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Phainanoids A–F, A New Class of Potent Immunosuppressive Triterpenoids with an Unprecedented Carbon Skeleton from *Phyllanthus hainanensis*

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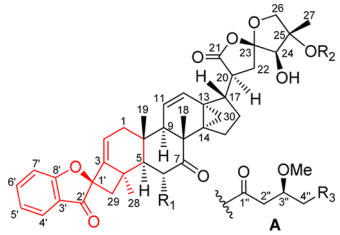
Supporting Information

ABSTRACT: Phainanoids A–F (**1**–**6**), six highly modified triterpenoids with a new carbon skeleton by incorporating two unique motifs of a 4,5- and a 5,5-spirocyclic systems, were isolated from *Phyllanthus hainanensis*. Their structures with absolute configurations were determined by spectroscopic data, chemical methods, and X-ray crystallography. Compounds **1**–**6** exhibited exceptionally potent immunosuppressive activities *in vitro* against the proliferation of T and B lymphocytes. The most potent one, phainanoid F (**6**), showed activities against the proliferation of T cells with IC_{50} value of 2.04 ± 0.01 nM (positive control CsA = 14.21 ± 0.01 nM) and B cells with IC_{50} value of <1.60 ± 0.01 nM (CsA = 352.87 ± 0.01 nM), which is about 7 and 221 times as active as CsA, respectively. The structure–activity relationships of **1**–**6** are discussed.

Phyllanthus is a well-known plant genus which has delivered diverse compound classes of terpenoids, alkaloids, and phenolics with a broad spectrum of bioactivities.[1] Some *Phyllanthus* species have been applied as folk medicines to treat infections, diabetes, and hepatitis B.[2] Immunosuppressants, e.g., cyclosporin A (CsA) and rapamycin, have been successfully used in clinical practice for organ transplant and other immunological associated ailments, but these drugs also cause serious side effects, such as liver and renal toxicity, increased susceptibility to infection, and decreased cancer immunosurveillance.[3] The development of new immunosuppressants with high efficacy and less adverse effects thus remains a high priority. Natural products have been demonstrated as an invaluable source of immunosuppressive agents, which has been attracting broad interests of organic chemists.[4]

In continuing the search for immunosuppressive agents from Chinese medicinal herbs,[5] six potent immunosuppressive compounds, phainanoids A–F (**1**–**6**) (Figure 1), were isolated from *Phyllanthus hainanensis* Merr., which is a shrub only native to the Hainan island of China.[6] Compounds **1**–**6** represent a new carbon skeleton of highly modified triterpenoids by incorporating two unique motifs of 3*H*-spiro[benzofuran-2,1′-cyclobutan]-3-one and 1,6-dioxaspiro[4.4]nonan-2-one. Their structures with absolute configurations were established by spectroscopic and chemical methods, single crystal X-ray crystallography, and CD analysis. In particular, the C-3″ of the C-25 side chain in



1 R_1 = H, R_2 = H
2 R_1 = OH, R_2 = H
3 R_1 = OH, R_2 = Me
4 R_1 = OH, R_2 = $CO(CH_2)_5OH$
5 R_1 = OH, R_2 = **A**, R_3 = OH
6 R_1 = OH, R_2 = **A**, R_3 = OMe

Figure 1. Structures of **1**–**6**.

compounds **5** and **6** was determined as *R*-configured by a combination of chemical degradation, fragment synthesis, and chiral GC/HPLC analysis. Immunosuppressive assays revealed that compounds **1**–**6** exhibited exceptionally potent activities *in vitro* against the ConA-induced proliferation of T lymphocytes with IC_{50} values ranging from 2.04 ± 0.01 to 192.80 ± 0.01 nM (positive control CsA = 14.21 ± 0.01 nM) and against the LPS-induced proliferation of B lymphocytes with IC_{50} values of <1.60 ± 0.01 to 249.49 ± 0.01 nM (CsA = 352.87 ± 0.01 nM). Compounds **3**, **4**, and **6** are more potent than CsA against the ConA-induced proliferation of T lymphocytes, and all the compounds were stronger than CsA against the LPS-induced proliferation of B lymphocytes. The most potent one, phainanoid F (**6**), is about 7 and 221 times as active as CsA against the proliferation of T and B lymphocytes, respectively.

Herein, the isolation, structure characterization, biological evaluation, and a brief structure–activity relationship (SAR) discussion of these highly modified triterpenoids are presented.

Phainanoid A (**1**), colorless crystals, had a molecular formula $C_{38}H_{42}O_8$ with 18 double-bond equivalents (DBEs) as established by HRESI(−)MS at *m*/*z* 671.2863 $[M + HCO_2]^-$ (calcd for $C_{39}H_{43}O_{10}$, 671.2856). The IR spectrum displayed absorptions for hydroxy (3445 cm^{-1}) and carbonyl (1779 and 1714 cm^{-1}) groups. The ^1H NMR data (Table S1) showed typical resonances for four tertiary methyls (δ_H 1.18, 1.23, 1.43, and 1.69, each 3H, s), three olefinic, and four aromatic protons.

Received: November 18, 2014
Published: December 18, 2014

DOI: 10.1021/ja511813g
J. Am. Chem. Soc. 2015, 137, 138–141

The ^{13}C NMR data (Table S2), with the aid of DEPT and HSQC experiments, further revealed the presence of a 1,2-disubstituted benzene, two double bonds, three carbonyls (δ_C 212.8, 198.5, and 177.2), eight sp^3 methylenes (one oxygenated), five sp^3 methines (one oxygenated), and eight sp^3 quaternary carbons (three oxygenated). The diagnostic resonances of two quaternary carbons (δ_C 36.2 and 32.0) and one methylene (δ_H 1.24, 0.82; δ_C 14.1) indicated the presence of a 1,1,2,2-tetrasubstituted cyclopropane. Two proton resonances at δ_H 2.05 and 1.86 showed no correlations with any carbons in the HSQC spectrum and were assigned to the hydroxyls. The aforementioned functionalities accounted for 10 DBEs, and the remaining DBEs thus required the existence of eight additional rings in the molecule.

The planar structure of **1** was established by interpretation of 2D NMR spectra, especially HMBC (Figure S1). Six proton-carrying fragments as drawn in bold bonds were defined by $^1H-^1H$ COSY spectrum and were then assembled through the quaternary carbons and oxygen atoms to delineate the scaffold of **1** by the HMBC correlations. The multiple HMBC correlation networks of H_3-28/C-3, C-4, and C-5; H_3-19/C-1, C-5, C-9, and C-10; H_3-18/C-7, C-8, C-9, and C-14; H_2-30/C-12, C-13, C-14, C-15, and C-17; H-2/C-3; and H_2-6/C-7 established the tetracyclic triterpenoid scaffold bearing a cyclopropyl moiety. The HMBC cross-peaks of H-20/C-21; H_2-22/C-21, C-23, and C-24; H_2-26/C-23; and H_3-27/C-24, C-25, and C-26 revealed the presence of a 5,5-spiroketal motif similar to that of dichapetalin-type triterpenoids,[7] but with two hydroxyls at C-24 (δ_C 83.2) and C-25 (δ_C 77.5) as evidenced by the chemical shifts. The connection between the 1,2-disubstituted benzene and the core backbone via a novel 4,5-spirocyclic fragment was accomplished by the HMBC correlations of H-2/C-3 and C-1′; H_3-28/C-29; H_2-29/C-1′ and C-2′; H-4′/C-2′; and H-7′/C-3′ and C-8′. The planar structure of **1** was thus delineated.

The relative configuration of **1** was partially assigned by a ROESY spectrum (Figure S1), in which the correlations of H-6*β* with H_3-18, H_3-19, and H-29*β* and H-15*β* with H_3-18, and H-17 revealed that H-6*β*, H-15*β*, H-17, Me-18, Me-19, and CH_2-29 were cofacial and randomly assigned to be *β*-oriented. The large coupling constant (14.6 Hz) of $J_{5,6\beta}$ indicated that H-5 and H-6*β* were diaxially bonded, and H-5 was put in an *α*-orientation. Consequently, the ROESY cross-peaks of H-5/H-9 and H-9/H-30a showed that H-9 and CH_2-30 were *α*-oriented. The assignment for the relative stereochemistry of 4,5- and 5,5-spiro moieties in the two termini of **1** proved challenging due to the absence of available ROESY data and/or the rotary nature of the C-17−C-20 bond. Fortunately, the qualified crystals acquired in an optimized binary solvent system (MeOH/H_2O, 10:1) allowed a successful performance of single crystal X-ray diffraction, in which the anomalous dispersion of Cu K*α* radiation was applied. This not only confirmed its planar structure but also unambiguously determined the absolute configuration of **1** by both the absolute structure parameter [0.02(11)] and the refinement of Hooft parameter [0.01(5)],[8] as 4*R*, 5*R*, 8*R*, 9*R*, 10*S*, 13*S*, 14*R*, 17*S*, 20*S*, 23*R*, 24*R*, 25*S*, 1′*R* (Figure 2).

Phainanoid B (**2**) was assigned a molecular formula $C_{38}H_{42}O_9$ on the basis of HRESI(−)MS, suggestive of an oxygenated derivative of **1**. This deduction was corroborated by the NMR data (Tables S1 and S2), in which the diagnostic signals (δ_H 4.67, δ_C 71.7) for an oxymethine and the alteration of H-5 signal from a double doublet in **1** to a doublet ($J_{5,6}$ = 13.1 Hz) in **2**, indicated the presence of a 6*α*-OH in **2**. The structure of **2** was finally confirmed on the basis of X-ray crystallography study (Figure 3)

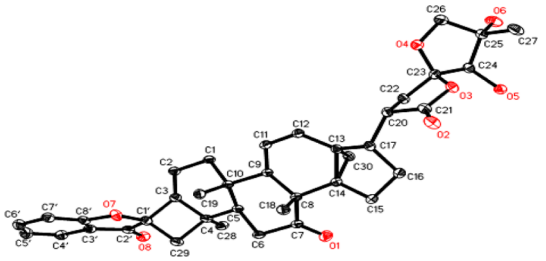


Figure 2. X-ray structure of **1**.

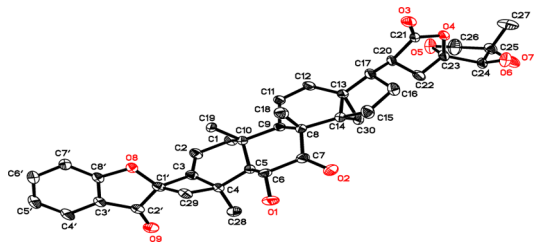


Figure 3. X-ray structure of **2**.

by using the anomalous dispersion of Cu K*α* radiation, which also determined the absolute configuration as depicted on the grounds of the absolute structure parameter [0.0(3)] and the refinement of Hooft parameter [0.1(2)].[8]

Phainanoid C (**3**) was assigned to be a methylated analogue of **2** by HRESIMS ($C_{39}H_{44}O_9$) and NMR data analysis (Tables S1 and S2). The methoxy group (δ_H 3.29, δ_C 51.2) was located to C-25 by the key HMBC correlation of OCH_3/C-25 (δ_C 81.7) (Figure S31). This was supported by the deshielded C-25 resonance ($\Delta\delta_C$ 4.3) due to the etherification effect and the shielded C-24 ($\Delta\delta_C$ −1.8), C-26 ($\Delta\delta_C$ −1.0), and C-27 ($\Delta\delta_C$ −5.3) resonances owing to the *γ*-gauche effects from the methoxy group as compared with those of **2**. The relative configuration of **3** was assigned to be identical with that of **2** based on the ROESY spectrum (Figure S31) and their similar NMR patterns.

Phainanoid D (**4**) had a molecular formula of $C_{44}H_{52}O_{11}$ as determined by the sodiated molecular ion peak at *m*/*z* 779.3391 $[M + Na]^+$ (calcd 779.3407) in HRESI(+)MS. Its NMR data (Tables S1 and S2) were highly similar to those of **2**, but with the absence of the proton signal for OH-25, and in concomitant the presence of the additional proton and carbon resonances for an ester carbonyl (δ_C 175.0) and five methylenes (one oxygenated at δ_C 62.7), suggesting the existence of a new substituent at C-25, which was then identified to be 6-hydroxyhexanoyloxy group by ESIMS, $^1H-^1H$ COSY, and HMBC spectra (Figure S42). Similar to the case of **3**, the carbon resonance changes of the deshielded C-25 and the shielded C-24, C-26, and C-27 further supported this assignment. The relative configuration of **4** was established to be identical to that of **2** by the ROESY spectrum (Figure S42) and the similar ^1H NMR patterns for the common parts of the two cometabolites.

Phainanoid E (**5**) was assigned a molecular formula $C_{43}H_{50}O_{12}$ by the HRESI(+)MS ion at *m*/*z* 759.3394 $[M + H]^+$ (calcd 759.3381). Its ^1H and ^{13}C NMR data (Tables S1 and S2) showed many similarities to those of **4**, with the main differences occurring for the NMR signals of the 25-substituent, which in **5** comprised the resonances of an ester carbonyl (δ_C 172.6), an oxygenated methine (δ_H 3.70, δ_C 77.9), a methylene (δ_H 2.54, 2.62; δ_C 36.9), an oxygenated methylene (δ_H 3.57, 3.75; δ_C 63.1), a methoxyl (δ_H 3.42, δ_C 57.8), and a hydroxyl (δ_H 2.03). Further analysis of the 2D NMR especially $^1H-^1H$ COSY and HMBC

DOI: 10.1021/ja511813g
J. Am. Chem. Soc. 2015, 137, 138−141

spectra (Figure S53) established a 4-hydroxy-3-methoxybutanoyloxy group for the 25-substituent. Based on the ROESY spectrum (Figure S53) and the similar NMR patterns, the relative configurations of all the stereogenic centers except for that of the C-3″ in the C-25 appendage were assigned to be identical with those of **2**.

Phainanoid F (**6**) gave a molecular formula $C_{44}H_{52}O_{12}$ on the basis of HRESI(+)MS ion at *m*/*z* 773.3544 $[M + H]^+$ (calcd 773.3537). The NMR data (Tables S1 and S2) of **6** highly resembled those of **5** except for the presence of additional signals (δ_H 3.36, δ_C 59.4) for one OCH_3 replacing the 4″-OH (δ_H 2.03) in **5**, suggesting that a 3,4-dimethoxybutanoyloxy group was located at C-25 in **6**. This was verified by the HMBC correlation from OCH_3 to C-4″ (δ_C 73.4) and further supported by the deshielded C-4″ ($\Delta\delta_C$ 10.3) as compared with that of **5**. The relative configurations of the stereogenic centers of **6** except for that of the C-3″ in the 25-substituent were established to be the same as those of **2** by the ROESY experiment (Figure S64) and their excellent NMR resemblances.

Considering the co-occurrence with compounds **1** and **2** in the same species, the absolute configurations of **3**−**6**, except for the C-3″ in the C-25 appendages of **5** and **6**, were proposed as depicted on the basis of chemical evidence and biogenetic considerations, which were further corroborated by their highly similar CD curves to those of **1** and **2** in the CD spectra (Figure 4). The assignment of the C-3″ stereochemistry in **5** and **6** was

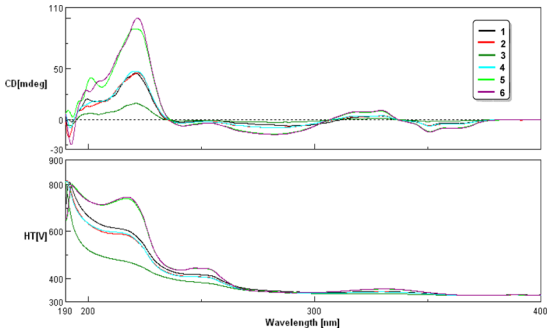


Figure 4. CD spectra of compounds **1**−**6**.

very challenging due to the flexible nature and the remote location from the core structure. In order to define the absolute configurations of C-3″, they were subjected to hydrolysis under a basic condition aiming to obtain the derivatives of the C-25 acyl groups. It is quite interesting that **5** yielded the hydrolytic product of a five-membered lactone (**8**) but not the expected free acid (Scheme 1, SI). According to the hydrolytic products of **5** and **6**, two pairs of enantiomers **7**/**8** and **9**/**10** were thus synthesized (Schemes 1 and 2, SI). With the authentic samples in hand, chiral GC/HPLC analysis was then carried out to establish the absolute configurations of C-3″ in **5** and **6**. A major GC peak (t_R = 15.429 min) in the hydrolysis mixture of **5** matched well to that of **7** (*R*) by coinjection, indicating that the C-3″ of **5** is *R*-configured (Figure S2). Similarly, a HPLC peak (t_R = 33.663 min) of the hydrolysis product of **6** matched that of **9** (*R*), revealing that the C-3″ of **6** is also in an *R*-configuration (Figure S3).

It is quite interesting that the chemical shifts and coupling constants of O<u>H</u>-24 showed big alterations between compound groups **1**−**3** (OCH_3-25) and **4**−**6** (RCO_2-25) (Table S1) due to the formation of different intramolecular H-bonds with the OR_2-25 moieties (Figure 5).[9] For **1**−**3**, the O<u>H</u>-24 resonated upfield with large coupling constants (δ_H 1.99−2.26, d, $J_{24,OH}$ = 10.1−

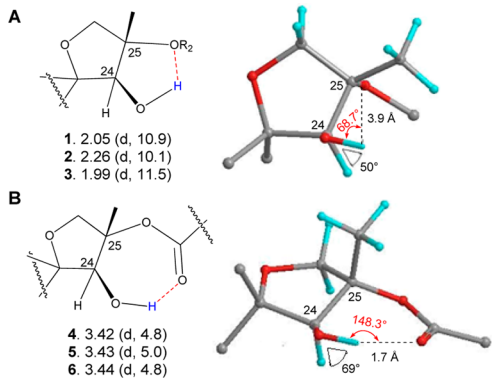


Figure 5. Chemical shifts (δ_H) and coupling constants (Hz) of O<u>H</u>-24; the optimized 3D structures (A: OMe-25 represents **1**−**3**, B: OAc-25 represents **4**−**6**) generated by Hartree−Fock/3-21G showing the dihedral angles of H−C−O−H (black) and H-bond angles (red) and lengths (Å).

11.5 Hz), suggesting that the H-bonds were formed between O<u>H</u>-24 and the oxygen atoms of OR_2-25 in a five-membered ring (Figure 5A), in which the H-bond angle and length simulated for OMe-25 were 68.7° and 3.9 Å, respectively,[10] and the dihedral angle between H-24 and O<u>H</u>-24 was 50° as generated by Hartree−Fock/3-21G. While for **4**−**6**, the stronger H-bonds were formed between O<u>H</u>-24 and the O atoms of the acyl carbonyls furnishing a seven-membered ring (Figure 5B) with more favorable H-bond angles and lengths (148.3° and 1.7 Å, respectively, represented by OAc-25),[10] which resulted in the downfield chemical shifts and small coupling constants for the O<u>H</u>-24 (δ_H 3.42−3.44, $J_{24,OH}$ = 4.8−5.0 Hz) as compared with those of **1**−**3**, owing to the deshielding effects of acyl groups and the increased dihedral angles (69° for OAc-25). The coupling constants of H-24/O<u>H</u>-24 and the dihedral angles in the simulated conformers of **1**−**6** (Figure 5) satisfied the Karplus equation.[11]

Compounds **1**−**6** exhibited exceptionally potent immunosuppressive activities and the results were summarized in Table 1. Compounds **3**, **4**, and **6** are more active than CsA against ConA-induced proliferation of T lymphocytes, and all the compounds are more potent than CsA in inhibiting LPS-induced proliferation of B lymphocytes. The most potent one, phainanoid F (**6**), showed activities against the proliferation of T cells with IC_{50} value of 2.04 ± 0.01 nM (positive control CsA = 14.21 ± 0.01 nM) and B cells with IC_{50} value of <1.60 ± 0.01 nM (CsA = 352.87 ± 0.01 nM), which is about 7 and 221 times as active as CsA, respectively. A gross SAR for this compound class can be delineated as follows: (1) The 4,5-spirocyclic ether and/or the 5,5-spiro-lactone moieties are crucial for the immunosuppressive activities since there has been no such strong activities reported for 13,30-*cyclo*-dammarane triterpenoids hitherto,[7] which is presumed to be associated with the length and/or the conformation of this highly modified dispiro-containing 13,30-*cyclo*-dammarane skeleton. (2) As compared with **1**, the presence of a hydroxy group at C-6 in **2** will not obviously change the immunosuppressive activities but will slightly improve the selectivity index (SI). (3) Compounds **3**−**6** with the OR_2-25 (R_2 = methyl or acyl groups) in place of the OH-25 of **2** significantly improved the inhibitory activities, indicating that methylation or acylation of OH-25 will remarkably enhance the immunosuppressive activities, and a fully hydrophobic and/or a higher oxygenated acyl chain is more favorable to the activities, e.g., compound **6** is more potent than **4** and **5**.

DOI: 10.1021/ja511813g
J. Am. Chem. Soc. 2015, 137, 138−141

Table 1. Immunosuppressive Effects of 1−6 on Murine Lymphocyte Proliferation Induced by ConA (5 μg/mL) or LPS (10 μg/mL)[a]

		ConA-induced T-cell proliferation		LPS-induced B-cell proliferation	
cmpd	CC_{50}(nM) ± SD	IC_{50}(nM) ± SD	SI[a]	IC_{50}(nM) ± SD	SI[a]
1	541.40 ± 0.03	184.90 ± 0.03	2.93	122.68 ± 0.03	4.41
2	1112.97 ± 0.01	192.80 ± 0.01	5.77	249.49 ± 0.01	4.46
3	7.62 ± 0.02	6.24 ± 0.02	1.22	2.35 ± 0.02	3.24
4	34.05 ± 0.05	8.68 ± 0.05	3.92	17.04 ± 0.05	2.00
5	97.60 ± 0.01	43.26 ± 0.01	2.26	4.38 ± 0.01	22.28
6	7.79 ± 0.01	2.04 ± 0.01	3.82	<1.60 ± 0.01	>4.87
CsA	>1000 ± 0.01	14.21 ± 0.01	>70.37	352.87 ± 0.01	>2.83

[a]SI is determined as the ratio of the concentration of the compound that reduced cell viability to 50% (CC_{50}) to the concentration of the compound needed to inhibit the proliferation by 50% relative to the control value (IC_{50}).

In summary, phainanoids A−F (**1**−**6**), six highly modified triterpenoid derivatives possessing a new carbon skeleton, were isolated from *P. hainanensis*. Compounds **1**−**6** exhibited exceptionally potent immunosuppressive activities. These compounds are more powerful than CsA in the tested assays, and the most potent one, phainanoid F (**6**), is about 7 and 221 times as active as CsA against the proliferation of T and B lymphocytes, respectively. Compared with CsA, this compound class is equally sensitive to both T and B lymphocytes. This finding has provided a new structural class for the exploration of immunosuppressive agents.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support from the National Natural Science Foundation (no. U1302222; 81273398; 81321092) and the state key project "973" (no. 2012CB721105) from the MOST of the P. R. China are gratefully acknowledged. We thank Prof. S.-M. Huang of Hainan University, for the identification of the plant materials.

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DOI: 10.1021/ja511813g
J. Am. Chem. Soc. 2015, 137, 138−141