Polystanins A–D, Four New Protolimonoids from the Fruits of *Aphanamixis polystachya*

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Two new apo-tirucallane triterpenoids with six-membered hemiketal, polystanins A (1) and B (2), and two new tirucallane triterpenoids, polystanins C (3) and D (4) were isolated from the fruits of *Aphanamixis polystachya*. They were elucidated on the basis of detailed spectroscopic analysis including IR, high resolution-electrospray ionization (HR-ESI)-MS, 1 dimensional (1D)- and 2D-NMR. Their absolute configurations were determined by a combination of modified Mosher's method, and electronic circular dichroism (ECD) calculation.

Key words protolimonoid; *Aphanamixis polystachya*; Meliaceae; modified Mosher's method; electronic circular dichroism calculation

Aphanamixis polystachya (Meliaceae) is a timber tree that grows in tropical areas of Asia, such as southern China, India, Malaysia and Indonesia.¹⁾ Previous chemical investigations on the *A. polystachya* have led to the isolation of many structurally diverse limonoids^{2–9)} with a wide range of biological activities, such as anti-feedant, insecticidal, and antioxidant activities,^{10–12)} which inspired a continuing interest of this plant. As a part of our program to seek new biologically active triterpenoids from meliaceous plants, two new rings A, B-*seco* limonoids (aphapolynins A and B)¹³⁾ and eight new ring A-*seco* limonoids (aphanalides A–H),¹⁴⁾ from the ethanol extract of *A. polystachya* have been reported. In continuation of chemical investigation on the fruits of the title plant, two new

apo-tirucallane triterpenoids polystanins A (1) and B (2) with a six-membered hemiketal at the side-chain, and two new tirucallane triterpenoids, polystanins C (3) and D (4) were isolated from the fruits of *A. polystachya*. Herein we described the isolation and structure elucidation of these new compounds.

Results and Discussion

Polystanin A (1) possessed a molecular formula of C₃₃H₅₂O₉, as evidenced by its negative high resolution-electrospray ionization (HR-ESI)-MS at *m/z* 627.3294 [M+Cl][−] (Calcd for C₃₃H₅₂ClO₉, 627.3305, errors in 1.75 ppm), in consistent with ¹H-, ¹³C-NMR, distortionsless enhancement by polarization transfer (DEPT) spectra, indicating 8 degrees of unsaturation.

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Fig. 1. ^1H - ^1H COSY, Key HMBC and ROESY Correlations of **1**

Its IR spectrum indicated the presence of hydroxy (3459 cm^{-1}), carbonyl (1745 cm^{-1}), and olefinic groups (1643 cm^{-1}). The ^1H - and ^{13}C -NMR spectra presented a typical exocyclic double bond [δ_{H} 5.00 (s), 4.90 (s); δ_{C} 147.7 (C-4), 116.3 (C-28)]; a trisubstituted double bond [δ_{H} 5.46 (s, H-15); δ_{C} 161.8 (C-14), 120.9 (C-15)]; a hemiketal carbon signal at δ_{C} 96.6 (C-24); an acetoxy (δ_{H} 2.02; δ_{C} 172.5, 21.2); a methoxy (δ_{H} 3.64, δ_{C} 52.5), and other six tertiary methyls (δ_{H} 1.83, 1.35, 1.25, 1.14, 1.13, and 0.98). Except for one carbon belonging to the methyl ester, and two carbons ascribing to one acetoxy group, the remaining 30 carbons and other spectral features suggested **1** to be of an apo-tirucallol (euphol) skeleton.¹⁵ Furthermore, in addition to the two double bonds and two carbonyl groups, the remaining four degrees of unsaturation indicated **1** to be tetracyclic, suggesting the cleavage of ring A. An ABX coupling system composed of H-1 at δ_{H} 5.48 (d, $J=11.0\text{ Hz}$) and H₂-2 at δ_{H} 2.97 (d, $J=14.0\text{ Hz}$) and 2.45 (dd, $J=14.0$, 11.0 Hz) was revealed by ^1H - ^1H correlation spectroscopy (COSY) spectrum, and in the heteronuclear multiple bond connectivity (HMBC) spectrum of **1** (Fig. 1), the presence of cross-peaks between methoxy protons and a carbonyl carbon at δ_{C} 173.8 indicated the cleavage of seven-membered lactone between C-3 and C-4, forming a methyl ester. The HMBC correlation between H-1 signal and the carbon resonance at δ_{C} 172.5 assigned the only acetoxy to C-1. Furthermore, the HMBC correlations from H₂-28 to C-4 and C-29, implied that the common C-28 methyl was absent, presenting an exocyclic olefinic carbon. Similarly, HMBC correlations from the signal at δ_{H} 3.90 (H-7) to C-5 (δ_{C} 44.3), C-6 (δ_{C} 32.2) and C-9 (δ_{C} 34.5), and from H-5 (δ_{H} 2.69), H₂-6 (δ_{H} 2.25, 1.62), and H₃-30 (δ_{H} 1.14) to C-7 (δ_{C} 73.3) suggested the location of one of the free hydroxy group at C-7. The presence of singlet proton signal H-15 to C-14, C-17 (δ_{C} 59.0), and C-13 (δ_{C} 47.6), and from H₂-16 (δ_{H} 2.25, 2.11), H₃-18 (δ_{H} 1.13) and H₃-30 to C-14 located the trisubstituted double bond at C-14 and C-15, in accordance with typical chemical shifts of apo-tirucall triterpenoids.¹⁶ In the HMBC spectrum, cross-peaks between the oxygenated methylene protons (δ_{H} 3.88, 3.62) and a hemiketal carbon (C-24) necessitated the connection of C-21 to C-24 via an oxygen atom, forming a saturated pyran ring. The terminal two methyl protons H₃-26 (δ_{H} 1.35) and H₃-27 (δ_{H} 1.25) also showed cross-peaks to C-24 in the HMBC spectrum, allowing the position of the 2-hydroxyisopropyl group (C-25, C-26, C-27) at C-24. The other three free hydroxys were located at C-23, C-24 and C-25 according to the chemical shifts of these carbons and the HMBC correlations from both terminal singlet methyls (H₃-26, H₃-27) to C-24 and C-25, and from H-20 (δ_{H} 2.28) and H₂-22 (δ_{H} 1.72) to δ_{C}

69.3 (C-23). Thus, the gross structure of **1** was established as shown.

The relative configuration of **1** was deduced mainly from the analysis of its rotating frame Overhauser effect correlation spectroscopy (ROESY) (Fig. 1B), in which correlations of H-1/H₃-19, H-1/H₃-29, H-7/H-30, H-7/H-15, H-17/H₃-30 revealed that they were co-facial and were arbitrarily assigned to be in β -orientation. Consequently, OAc-1, OH-7, H-5, H-9, and H₃-18 were established as α -oriented, in accordance with those of similar compound, *e.g.* chisopanin A, the structure of which has been determined by the X-ray crystallographic analysis.¹⁶ Therefore, the structure of **1** was elucidated as depicted.

Compound **1** is the first report on the occurrence of apo-tirucallane triterpenes with ring A-*seco* and a six-membered hemiketal ring at the side-chain from *A. polystachya*. Compound **1** exhibited signals either broad or in pair when recorded in CDCl_3 (a pair of mixture with the proportion of 3:1), and such phenomenon was not found when recorded in CD_3OD (with the proportion of 8:1), which might due to the minute acid impurity in solvent CDCl_3 , catalyzing a configurational change at C-24 (Chart 1). To confirm the proposed mechanism, compound **1** was acetylated to afford **1a**, which showed the presence of four additional new acetate groups and a downfield shift of H₂-21 (H-21a, *ca.* $\Delta\delta$ +0.45; H-21b, $\Delta\delta$ +0.34). The typical hemiketal carbon was disappeared, replaced by a carbonyl at δ_{C} 206.6. A downfield shift of C-23 to δ_{C} 74.5 (*ca.* $\Delta\delta$ +5.2) was also observed. These data indicated the cleavage of the side-chain by the acetylation, which also supported the presence of the equilibrium system.

The absolute configuration of **1** was determined by a modified Mosher's method^{17,18} and calculated electronic circular dichroism (ECD). Treatment of **1** with (*R*)-(-)- and (*S*)-(+)- α -methoxy- α -(trifluoromethyl)-phenylacetyl chloride (MTPA-Cl) gave the (*S*)- and (*R*)-MTPA esters **1b** and **1c**, respectively. The ESI-MS exhibited a molecular ion peak at m/z 1042 $[\text{M}+\text{NH}_4]^+$, indicated double MTPA esters added to the compounds **1b** and **1c**, respectively. The ^1H -NMR signals of the two MTPA esters were assigned to C-7 and C-21 on the basis of their ^1H - ^1H COSY spectra, and the $\Delta\delta_{\text{H(S-R)}}$ values were then calculated (Fig. 2). The two bulk MTPA esters were in proximity in space, both affecting the $\Delta\delta_{\text{H(S-R)}}$ values of signals between them in rings C and D, thus unreliable for the determination of the absolute configuration. Protons in *seco*-ring A was far away from the C-21 MTPA ester's interference, and the $\Delta\delta_{\text{H(S-R)}}$ values could be used for the determination of the absolute configuration of C-7 to be *R*. The hydroxy

Chart 1. Equilibrium System of the Side Chain of Compound **1**

Fig. 2. Values of $\Delta\delta_{\text{H}(S-R)}$ (Measured in CD_3OD) for the MTPA Esters of Compound **1**

Fig. 3. Assignment of the Absolute Configuration of **1** by Comparison of the Experimental CD Spectrum with that Calculated Using TDDFT Method

neighboring ketone group was not acylated by MTPA-Cl, possibly due to steric hindrance between OMTPA-21 and the C-23 carbonyl carbon.

The ECD spectrum of **1** was calculated with Gaussian-09 program package using time-dependent density functional theory (TDDFT) at the B3LYP/TZV(d,p) level,^{19,20} in good agreement with the experimental one (Fig. 3), allowing the assignment of the absolute configuration of **1** as depicted.

The HR-ESI-MS of **2** displayed a molecular ion at m/z 595.3050 $[M+Cl]^-$, consistent with the molecular formula of $C_{32}H_{48}O_8$, requiring 9 degrees of unsaturation. The IR spectrum indicated the presence of hydroxy (3444 cm^{-1}), ester carbonyl (1730 , 1694 cm^{-1}) and double bond (1632 cm^{-1}) functionalities. The ^1H - and ^{13}C -NMR of **2** were very similar to those of **1** in rings B–D and the side-chain, distinguished in signals for ring A and the acetoxy group. Comparison of the 1D-NMR data of **2** with those of gentinin²¹⁾ revealed a similar features in ring A: an α,β -unsaturated ketone [δ_{H} 6.74 (d, $J=12.5\text{ Hz}$, H-1), 5.89 (d, $J=12.5\text{ Hz}$, H-2); δ_{C} 159.8 (C-1), 120.7 (C-2), and 170.3 (C-3)], an oxygenated quaternary carbon at C-4 (δ_{C} 87.1) correlated with two singlet methyls at δ_{H} 1.39 (H₃-28) and 1.48 (H₃-29) in the HMBC spectrum. The sole acetoxy group was located at C-7 by the HMBC cross-peak from δ_{H} 5.19 (H-7) to δ_{C} 172.0. The relative configuration of **2** was determined to be the same as that of **1** by their similar NMR data and the ROESY spectrum.

Our initial attempt to determine the absolute configuration of **2** by use of the circular dichroic (CD) exciton chirality method failed. Since the coupling of two chromophores of the negative Cotton effect at 198 nm²²⁾ (double bond, $\Delta\epsilon$ -6.43, π - π^* transition) and the positive Cotton effect observed at 222 nm²³⁾ (α,β -unsaturated ketone, $\Delta\epsilon$ +7.00, π - π^* transition)

were far away and almost in the same planar, the application of empirical CD rules is problematic. Therefore we turned to computational methods (ECD calculation), the same way as compound **1**. The results showed that the spectrum calculated for the 5*R*, 7*R*, 8*R*, 9*R*, 10*R*, 13*S*, 17*S*, 20*S*, 23*R*, 24*S* was nearly identical with the experimental one of **2** (Fig. 4) over the whole range of wavelengths under investigation. The absolute configuration of compound **2** were thus assigned

Polystanin C (**3**) was obtained as a white amorphous powder. Its molecular formula was established as $C_{33}H_{54}O_6$ on the basis of a pseudo-molecular ion peak at m/z 545.3844 ($[M-H]^-$) in its HR-ESI-MS. Analysis of the ^{13}C -NMR data aided with DEPT technique resolved 33 carbon resonances categorized into nine methyls, eight methylenes, nine methines (one olefinic, and four oxygenated), and seven quaternary carbons (one oxygenated, one olefinic carbon, and one ester carbonyl). These functionalities accounted for two out of the seven degrees of unsaturation, and the remaining five degrees of unsaturation suggested **3** to be pentacyclic. The 1H - and ^{13}C -NMR pattern of **3** were similar to that of the known compound aphanamin,²⁴⁾ coexisting in the same plant. The 1H - 1H COSY, HMBC, and ROESY experiments deduced **3** to be a tetracyclic tirucall-type triterpenoid.²⁵⁾ The acetoxy group was assigned to be at C-3 by the HMBC correlation between δ_H 4.69 (brs, H-3) and δ_C 170.8, showing cross-peak with a downfield methyl at δ_H 2.06. A doublet proton signal at δ_H 4.80 (d, $J=3.5$ Hz, H-21) correlated with a methine carbon signal at δ_C 109.1 in the heteronuclear single quantum coherence (HSQC)

Fig. 4. Assignment of the Absolute Configuration of **2** by Comparison of the Experimental CD Spectrum with that Calculated Using TDDFT Method

spectrum, characteristic of an acetal carbon, which in turn has an HMBC correlation with methoxy protons at δ_{H} 3.35 (3H, s), locating itself at C-21. The remaining linkages were similar to other $\Delta^{7(8)}$ tirucallane-type triterpenoids reported before.^{26–28} The relative configuration of H-3 was determined to be α -oriented by the characteristic broad singlet of H-3 and the chemical shift of C-3.²⁵ Other relative configurations of **3** were elucidated mainly by the ROESY spectrum. The ROESY cross-peaks of H₃-18/H-9, H-9/H-5 and H-5/H₃-28, indicated that H-5, H-9, H₃-18 and H₃-28 were co-facial, arbitrarily assigned to be α -oriented. Other ROESY cross-peaks of H₃-29/H₃-19, and H₃-30/H-17, suggested a β -orientation for H-17, H₃-19, H₃-29 and H₃-30. The relative configurations of the side-chain at C-17 were determined by the ROESY cross-peak between H-17 and H-21, determining the OMe-21 to be α -directed. The configurations of H-23 and OH-24 were determined to be in α -orientation by their carbon shifts [δ_{C} 76.8 (C-23) and 75.6 (C-24)] compared to other analogues with such side-chain.²⁹

The absolute configuration of **3** was also determined by the modified Mosher's method. Treatment of **3** with (*R*)- and (*S*)-MTPA-Cl gave the (*S*)- and (*R*)-MTPA esters **3a** and **3b**, respectively. The ¹H-NMR signals of the two MTPA esters were assigned on the basis of their ¹H–¹H COSY spectra, and the $\Delta\delta_{\text{H}(S-R)}$ values were then calculated (Fig. 5). The results indicated that the absolute configuration of C-24 was *S*. Thus, the absolute configuration of **3** was elucidated as 3*R*, 5*R*, 9*R*, 10*R*, 13*S*, 14*S*, 17*S*, 20*S*, 21*R*, 23*R*, and 24*S*.

Compound **4** gave a molecular formula of C₃₃H₅₄O₆, the same as that of **3**, as determined by the observed pseudo-molecular ion at *m/z* 545.3847 [M–H][–] (Calcd for C₃₃H₅₃O₆, 545.3848) in the HR-ESI-MS. Its NMR data showed great similarity to those of **3** with same functionalities, however, some chemical shift discrepancies were observed for signals in the side-chain at C-17. The large chemical shift difference (*ca.* $\Delta\delta$ –4.1) at C-21 suggested it to be an epimer of **3** at this position, which was confirmed by the ROESY correlation between H-21 and H-20 instead of the observed cross-peak of H-21 and H-17 in **3**. Lots of C-21 methoxylated epimers have been reported in family Meliaceae, which could be distinguished

Fig. 5. Values of $\Delta\delta_{\text{H}(S-R)}$ (Measured in CDCl₃) for the MTPA Esters of Compound **3**

according to the chemical shift of C-21: OMe-21*α* (δ_{C} 109.0 ± 0.5) and OMe-21*β* (δ_{C} 105.0 ± 0.5),²⁹ which also supported our inference, δ_{C} 109.1 for OMe-21*α* in **3** and δ_{C} 105.0 for OMe-21*β* in **4**.

Experimental

Optical rotations were measured with a Jasco P-1020 polarimeter. CD spectra were obtained on a Jasco 810 spectrometer. IR (KBr-disc) spectra were recorded by Bruker Tensor 27 spectrometer. NMR spectra were recorded on Bruker Avance-III NMR instrument (¹H: 500 MHz, ¹³C: 125 MHz) with TMS as internal standard. Chemical shifts were given in values of ppm and coupling constants in Hertz. Mass spectra were obtained on an MS Agilent 1100 Series LC/MSD ion-trap mass spectrometer (ESI-MS) and an Agilent 6520B Q-TOF spectrometer (HR-ESI-MS), respectively. Semi-preparative HPLC was carried out on a Shimadzu LC-8A instrument (flow rate set at 10 mL/min) with a SPD-20A detector using a Shim-pack PRC-ODS column (5 μ m, 250 × 20 mm). Silica gel (100–200, 200–300 mesh, Qingdao Haiyang Chemical Co., Ltd., China), Sephadex LH-20 (Pharmacia, U.S.A.), and RP-C₁₈ (40–63 μ m, YMC, Japan) were used for column chromatography. The pre-coated silica gel GF₂₅₄ plates (Qingdao Marine Chemical Plant, Qingdao, China) were used for TLC. Spots were visualized by heating silica gel plates immersed in vanillin–H₂SO₄ in ethanol. All solvents used were of analytical grade (Jiangsu Hanbang Science and Technology Co., Ltd., China).

Plant Material The fruits of *A. polystachya* (WALL.) R. N. PARKER were collected from Xishuangbanna, Yunnan Province, China, in March 2010, and authenticated by Prof. Jingyun Cui of Xishuangbanna Tropical Garden, Chinese Academy of Sciences. A voucher specimen (No. AP-Fruits-2010-03-ZY) has been deposited in the Department of Natural Medicinal Chemistry, China Pharmaceutical University.

Extraction and Isolation Air-dried fruits of the plant material (5 kg) were extracted by refluxing with 95% EtOH three times (10 L × 3, 3 h, 3 h, 2 h) to give 875 g of crude extract, which was suspended in 1.5 L water and then extracted with petroleum ether (PE) to remove the fatty components completely. The defatted crude extracts were further partitioned with CHCl₃ to give a CHCl₃-soluble fraction (90 g). The CHCl₃ fraction was subjected to silica gel column chromatography (CC) eluted successively with PE–ethyl acetate (EtOAc) gradient (20:0–0:1, v/v) to obtain six fractions (A–F). Fraction C (15 g) was applied to a silica gel column eluted with CHCl₃–MeOH (50:1–1:1, v/v) as eluant to give three fractions. Fraction CA (2.1 g) was separated over silica gel column again (CH₂Cl₂–Me₂CO, 50:1–5:1, v/v) to afford five major fractions, CAB (40 mg) was purified by semi-preparative

HPLC to afford compounds **3** (15 mg, t_R =17.6 min) and **4** (6 mg, t_R =18.1 min) using 95% methanol in water. Fraction CAD (1.3 g) was separated over a reversed-phase silica gel column (MeOH–H₂O, 65:35–100:0, v/v) to afford two major fractions, CAD2 (620 mg) was purified by semi-preparative HPLC to afford compounds **1** (125 mg, t_R =25.4 min) and **2** (3 mg, t_R =28.3 min) using 75% isocratic methanol in water.

Polystanin A (**1**): White, amorphous powder; $[\alpha]_D^{25}$ –76.8 (c =0.23, MeOH); CD (acetonitrile) $\lambda(\Delta\epsilon)$ =190 (+3.06), 207 (–7.64) nm; UV (acetonitrile) $\lambda_{\max}$ (log ϵ) 197 (4.08) nm; IR $\nu_{\max}$ (film) cm^{-1} : 3459, 2954, 2930, 1745, 1643, 1440, 1388, 1243, 1172, 1098, 1028, 976, 896, 837, 675, 630, 517, 471, 419; for ¹H- and ¹³C-NMR data, see Table 1; ESI-MS m/z 593.2 [M+H]⁺, 610.3 [M+NH₄]⁺, and 627.6 [M+Cl][–]; HR-ESI-MS [M+Cl][–] m/z 627.3294 (Calcd for C₃₃H₅₂ClO₉ 627.3305).

Preparation Acetylated Ester Derivatives of Compound 1

1 Compound **1** (10 mg) was acetylated with Ac₂O–CH₂Cl₂ using *N,N*-dimethyl-4-aminopyridine (4-DMAP, 20 mg) as catalyst at room temperature for 2 h, and the reactant was partitioned between H₂O and CH₂Cl₂. The solution was washed successively with 1 M HCl (10 mL), NaHCO₃-saturated H₂O (10 mL), and saturated brine (5 mL). The organic layer was dried over Na₂SO₄ and then evaporated under reduced pressure. The residue was purified by silica gel CC with PE–EtOAc (4:1) as eluant to give **1a** (8 mg, yield 80%). ¹H-NMR (500 MHz, CD₃OD) δ_H 5.51 (1H, dd, J =11.0, 1.5 Hz, H-1), 2.93 (1H, dd, J =14.5, 1.5 Hz, H-2a), 2.50 (1H, dd, J =14.5, 11.0 Hz, H-2b), 2.55 (1H, dd, J =13.0, 3.0 Hz, H-5), 2.29 (1H, signal overlapped, H-6a), 1.62 (1H, signal overlapped, H-6b), 5.15 (1H, brt, J =1.0 Hz, H-7), 2.28 (1H, signal overlapped, H-9), 1.97 (1H, m, H-11a), 1.72 (1H, signal overlapped, H-11b), 1.89 (1H, dt, J =13.5, 2.5 Hz, H-12a), 1.55 (1H, m, H-12b), 5.33 (1H, d, J =2.5 Hz, H-15), 2.28 (1H, signal overlapped, H-16a), 2.12 (1H, signal overlapped, H-16b), 1.80 (1H, dd, J =5.5, 3.5 Hz, H-17), 1.11 (3H, s, H-18), 1.01 (3H, s, H-19), 2.03 (1H, signal overlapped, H-20), 4.33 (1H, dd, J =11.5, 3.0 Hz, H-21a), 3.96 (1H, dd, J =11.5, 6.0 Hz, H-21b), 2.11 (1H, signal overlapped, H-22a), 1.72 (1H, signal overlapped, H-22b), 5.64 (1H, dd, J =8.0, 4.0 Hz, H-23), 1.52 (3H, s, H-26), 1.62 (3H, s, H-27), 5.02 (1H, s, H-28a), 4.92 (1H, s, H-28b), 1.83 (3H, s, H-29), 1.22 (3H, s, H-30), 3.65 (3H, s, OMe), 2.01, 2.03, 2.07, 2.08 and 2.10 (each 3H, s, 5×OAc); ¹³C-NMR (125 MHz, CD₃OD) δ_C 78.4 (C-1), 36.4 (C-2), 173.6 (C-3), 147.1 (C-4), 45.6 (C-5), 30.6 (C-6), 76.4 (C-7), 43.8 (C-8), 35.9 (C-9), 45.5 (C-10), 19.8 (C-11), 35.7 (C-12), 47.6 (C-13), 161.0 (C-14), 120.2 (C-15), 35.9 (C-16), 57.3 (C-17), 20.2 (C-18), 15.6 (C-19), 37.2 (C-20), 66.8 (C-21), 32.9 (C-22), 74.5 (C-23), 206.6 (C-24), 84.7 (C-25), 25.3 (C-26), 24.1 (C-27), 116.8 (C-28), 23.4 (C-29), 27.3 (C-30), 52.6 (OMe), 21.4, 172.4 (OAc-1), 20.9, 172.1 (OAc-7), 21.1, 173.2 (OAc-21), 21.0, 171.8 (OAc-23) and 21.4, 172.2 (OAc-25); ESI-MS m/z 778.5 [M+NH₄]⁺, 760.8 [M+H]⁺, 701.3 [M+H–CH₃COOH]⁺, 641.3 [M+H–2×CH₃COOH]⁺, 581.4 [M+H–3×CH₃COOH]⁺, 521.4 [M+H–4×CH₃COOH]⁺, and 461.3 [M+H–5×CH₃COOH]⁺.

Preparation of the (R)- and (S)-MTPA Ester Derivatives of Compound 1 To a stirred solution of **1** (10 mg) in dehydrated CH₂Cl₂ (200 μ L) was added 4-DMAP (10 mg) and (R)-(-)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride (MTPA-Cl, 10 μ L). The mixture was stirred at room temperature for 12 h, monitored by TLC. Saturated NH₄Cl solution (5 mL) was added to quench the reaction and then

extracted with CH₂Cl₂ (3×5 mL). The combined organic layers were washed with H₂O and brine, dried over Na₂SO₄, and then passed through a silica gel CC and eluted with PE–EtOAc (2:1) to give (S)-Mosher ester **1b** (6.0 mg, yield 60%): ¹H-NMR (CD₃OD, 500 MHz): δ_H 5.443 (1H, dd, J =10.0, 1.0 Hz, H-1), 2.930 (1H, dd, J =13.5, 1.0 Hz, H-2a), 2.411 (1H, dd, J =13.5, 10.0 Hz, H-2b), 2.649 (1H, dd, J =13.5, 3.0 Hz, H-5), 5.885 (1H, dd, J =10.0, 2.0 Hz, H-7), 5.404 (1H, d, J =2.5 Hz, H-15), 2.336 (1H, ddd, J =8.5, 7.0, 3.5 Hz, H-16a), 2.117 (1H, m, H-16b), 1.573 (1H, m, H-17), 0.998 (3H, s, H-18), 0.946 (3H, s, H-19), 1.853 (1H, m, H-20), 4.413 (1H, dd, J =11.5, 3.0 Hz, H-21a), 4.220 (1H, dd, J =11.5, 4.0 Hz, H-21b), 3.859 (1H, br t, J =1.0 Hz, H-23), 1.290 (3H, s, H-26), 1.399 (3H, s, H-27), 4.972 (1H, s, H-28a), 4.869 (1H, s, H-28b), 1.806 (3H, s, H-29), 1.061 (3H, s, H-30); ESI-MS m/z 1042.4 [M+NH₄]⁺, 1059.5 [M+Cl][–]. Treatment of **1** (10.0 mg) with (S)-(+)-MTPA-Cl (10 μ L) with the same procedure as above yielded the corresponding (R)-Mosher ester **1c** (4.8 mg, yield 48%): ¹H-NMR (CD₃OD, 500 MHz) δ_H 5.422 (1H, dd, J =10.0, 1.0 Hz, H-1), 2.923 (1H, dd, J =13.5, 1.0 Hz, H-2a), 2.370 (1H, dd, J =13.5, 10.0 Hz, H-2b), 2.620 (1H, dd, J =13.5, 3.0 Hz, H-5), 5.815 (1H, dd, J =10.0, 2.0 Hz, H-7), 5.338 (1H, d, J =2.5 Hz, H-15), 2.250 (1H, ddd, J =8.5, 7.0, 3.5 Hz, H-16a), 2.054 (1H, m, H-16b), 1.540 (1H, m, H-17), 0.919 (3H, s, H-18), 0.847 (3H, s, H-19), 1.840 (1H, m, H-20), 3.968 (2H, dd, J =11.5, 3.0 Hz, H-21), 3.814 (1H, br t, J =1.0 Hz, H-23), 1.326 (3H, s, H-26), 1.410 (3H, s, H-27), 4.966 (1H, s, H-28a), 4.859 (1H, s, H-28b), 1.799 (3H, s, H-29), 0.935 (3H, s, H-30); ESI-MS m/z 1042.6 [M+NH₄]⁺, and 1060.0 [M+Cl][–].

Polystanin B (**2**): White, amorphous powder; $[\alpha]_D^{25}$ 0.0 (c =0.08, MeOH); CD (acetonitrile) $\lambda(\Delta\epsilon)$ =198 (–6.43), 222 (+7.00); UV (acetonitrile) $\lambda_{\max}$ (log ϵ) 207 (4.08), 199 (4.07) nm; IR $\nu_{\max}$ (film) cm^{-1} : 3444, 2932, 1730, 1694, 1632, 1454, 1383, 1246, 1166, 1129, 1095, 1031, 950, 892, 832, 617, 516; for ¹H- and ¹³C-NMR data, see Table 1; ESI-MS m/z 583.4 [M+Na]⁺, 595.5 [M+Cl][–]; HR-ESI-MS [M+Cl][–] m/z 595.3050 (Calcd for C₃₂H₄₈ClO₈ 595.3043).

Polystanin C (**3**): White, amorphous powder; $[\alpha]_D^{25}$ –49.5 (c =0.09, MeOH); UV (acetonitrile) $\lambda_{\max}$ (log ϵ) 194 (3.88) nm; IR $\nu_{\max}$ (film) cm^{-1} : 3451, 2948, 1731, 1637, 1450, 1444, 1376, 1246, 1188, 1158, 1098, 1030, 954, 920, 827, 781, 650, 607, 429; for ¹H- and ¹³C-NMR data, see Table 1; ESI-MS m/z 581.5 [M+Cl][–]; HR-ESI-MS [M–H][–] m/z 545.3844 (Calcd for C₃₃H₅₃O₆ 545.3848).

Preparation of the (R)- and (S)-MTPA Ester Derivatives of Compound 3

Treatment of **3** (5.0 mg) with (R)-(-)-MTPA-Cl (10 μ L) with the stated procedure yielded the corresponding (S)-Mosher ester **3a** (4.2 mg, yield 84%): ¹H-NMR (CDCl₃, 500 MHz) δ_H 2.081 (1H, signal overlapped, H-20), 4.660 (1H, d, J =3.5 Hz, H-21), 3.280 (3H, s, OMe), 1.922 (1H, m, H-22a), 1.256 (1H, m, H-22b), 4.313 (1H, dt, J =11.0, 3.0 Hz, H-23), 5.032 (1H, d, J =3.5 Hz, H-24), 1.335 (3H, s, H-26), 1.224 (3H, s, H-27); ESI-MS m/z 780.2 [M+NH₄]⁺. Treatment of **3** (5.0 mg) with (S)-(+)-MTPA-Cl (10 μ L) with the same procedure yielded the corresponding (R)-Mosher ester **3b** (3.8 mg, yield 76%): ¹H-NMR (CDCl₃, 500 MHz) δ_H 2.162 (1H, m, H-20), 4.759 (1H, d, J =4.0 Hz, H-21), 3.335 (3H, s, OMe), 1.997 (1H, m, H-22a), 1.264 (1H, m, H-22b), 4.328 (1H, dt, J =11.0, 4.0 Hz, H-23), 5.028 (1H, d, J =4.0 Hz, H-24), 1.271 (3H, s, H-26), 1.180 (3H, s, H-27); ESI-MS m/z 780.5 [M+NH₄]⁺.

Table 1. ¹H- and ¹³C-NMR Spectral Data of Compounds 1–4

No.	1 ^{a)}		2 ^{a)}		3 ^{b)}		4 ^{b)}	
	δ_{H} , mult. (<i>J</i> in Hz)	δ_{C}	δ_{H} , mult. (<i>J</i> in Hz)	δ_{C}	δ_{H} , mult. (<i>J</i> in Hz)	δ_{C}	δ_{H} , mult. (<i>J</i> in Hz)	δ_{C}
1a	5.48, d (11.0)	78.9, CH	6.74, d (12.5)	159.8, CH	1.39 ^{c)}	32.0, CH ₂	1.39 ^{c)}	32.0, CH ₂
1b					1.39 ^{c)}		1.39 ^{c)}	
2a	2.97, d (14.0)	36.4, CH ₂	5.89, d (12.5)	120.7, CH	2.05, m	23.8, CH ₂	2.05, m	23.9, CH ₂
2b	2.45, dd (14.0, 11.0)				1.92, m		1.92, m	
3		173.8 C		170.3, C	4.69, br s	78.4, CH	4.69, t-like (2.5)	78.4, CH
4		147.7 C		87.1, C		36.6, C		36.7, C
5	2.69, dd (13.0, 2.5)	44.3, CH	2.50, dd (13.5, 3.0)	51.0, CH	1.77 ^{c)}	45.6, CH	1.77, dd (11.0, 5.5)	45.7, CH
6a	2.25 ^{c)}	32.2, CH ₂	2.15, td (13.5, 2.0)	28.6, CH ₂	1.88, m	23.0, CH ₂	1.89 ^{c)}	23.0, CH ₂
6b	1.62, dt (14.0, 2.5)		1.82 ^{c)}		1.69, m		1.85 ^{c)}	
7	3.90 ^{c)}	73.3, CH	5.19, brt (1.5)	76.3, CH	5.27, q (3.5)	118.2, CH	5.27, q (3.5)	118.2, CH
8		45.3, C		43.5, C		145.7, C		145.8, C
9	2.25 ^{c)}	34.5, CH	2.28 ^{c)}	42.5, CH	2.36, m	48.6, CH	2.34, m	48.6, CH
10		45.8, C		45.3, C		34.8, C		34.9, C
11a	1.94, m	19.8, CH ₂	1.88 ^{c)}	19.5, CH ₂	1.56 ^{c)}	17.5, CH ₂	1.55 ^{c)}	17.6, CH ₂
11b	1.72 ^{c)}		1.83 ^{c)}		1.56 ^{c)}		1.55 ^{c)}	
12a	1.85, m	36.1, CH ₂	1.88 ^{c)}	36.6, CH ₂	1.75 ^{c)}	31.7, CH ₂	1.86 ^{c)}	31.3, CH ₂
12b	1.48, m		1.60, m		1.57 ^{c)}		1.36 ^{c)}	
13		47.6, C		47.7, C		43.7, C		43.6, C
14		161.8, C		160.3, C		51.1, C		50.9, C
15	5.46, s	120.9, CH	5.33, d (2.0)	120.9, CH	1.58 ^{c)}	33.8, CH ₂	1.56 ^{c)} 1.52, m	34.2, CH ₂
					1.49, m		1.52, m	
16a	2.25 ^{c)}	35.0, C H ₂	2.22, ddd (15.0, 7.5, 3.5)	35.1, CH ₂	1.93, m	27.4, CH ₂	1.89 ^{c)}	27.4, CH ₂
16b	2.11, dd (14.5, 6.0)		1.96, dd (15.0, 2.0)		1.30, m		1.31, m	
17	1.41, m	59.0, CH	1.44, m	59.0, CH	1.79 ^{c)}	50.3, CH	2.05, m	45.0, CH
18	1.13, s	19.8, CH ₃	1.13, s	21.2, CH ₃	0.91, s	22.6, CH ₃	0.88, s	23.3, CH ₃
19	0.98, s	15.6, CH ₃	1.32, s	16.2, CH ₃	0.79, s	13.0, CH ₃	0.79, s	13.0, CH ₃
20	2.28 ^{c)}	31.2, CH	2.25 ^{c)}	31.2, CH	2.18, m	47.8, CH	2.01, m	46.5, CH
21a	3.88, dd (17.5, 4.5)	66.5, CH ₂	3.84, ddd (11.0, 5.0, 1.5)	66.4, CH ₂	4.80, d (3.5)	109.1, CH	4.70, d (3.0)	105.0, CH
21b	3.62, d (17.5)		3.62, t (11.0)					
22b	1.72 ^{c)}	34.5, CH ₂	1.70, m	34.4, CH ₂	1.95, m	34.5, CH ₂	1.91, m	31.7, CH ₂
22b			1.70, m		1.77 ^{c)}		1.39 ^{c)}	
23	3.90 ^{c)}	69.3, CH	3.90, t (3.0)	69.2, CH	4.23, ddd (6.0, 4.5, 1.5)	76.8, CH	4.43, ddd (9.0, 7.5, 2.0)	78.9, CH
24		96.6, C		96.7, C	3.26, s	75.6, CH	3.18, d (2.0)	76.8, CH
25		77.6, C		77.6, C		73.2, C		73.1, C
26	1.35, s	25.4, CH ₃	1.34, s	25.4, CH ₃	1.30, s	26.4, CH ₃	1.27, s	26.4, CH ₃
27	1.25, s	23.7, CH ₃	1.24, s	23.7, CH ₃	1.27, s	26.4, CH ₃	1.27, s	26.3, CH ₃
28	5.00, s	116.3, CH ₂	1.39, s	32.5, CH ₃	0.84, s	27.4, CH ₃	0.84, s	27.4, CH ₃
	4.90, s							
29	1.83, s	23.6, CH ₃	1.48, s	26.9, CH ₃	0.97, s	21.4, CH ₃	0.97, s	21.4, CH ₃
30	1.14, s	27.8, CH ₃	1.24, s	27.3, CH ₃	0.99, s	27.1, CH ₃	1.00, s	27.3, CH ₃
OAc		172.5, C		172.0, C		170.8, C		170.8, C
	2.02, s	21.2, CH ₃	1.99, s	21.3, CH ₃	2.06, s	21.3, CH ₃	2.07, s	21.3, CH ₃
OMe	3.64, s	52.5, CH ₃			3.35, s	55.6, CH ₃	3.36, s	55.3, CH ₃

a) Data measured in CD₃OD at 500 MHz (¹H) and 125 MHz (¹³C). b) Data measured in CDCl₃ at 500 MHz (¹H) and 125 MHz (¹³C). c) Signal pattern unclear due to overlapped.

Polystanin D (4): White, amorphous powder; $[\alpha]_{\text{D}}^{25}$ −2.8 (c=0.10, MeOH); UV (acetonitrile) λ_{max} (log ϵ) 194 (3.88) nm; IR ν_{max} (film) cm^{−1}: 3453, 2970, 1731, 1642, 1463, 1385, 1247, 1051, 617; for ¹H- and ¹³C-NMR data, see Table 1; ESI-MS *m/z* 569.4 [M+Na]⁺ 581.6 [M+Cl][−]; HR-ESI-MS [M−H][−] *m/z* 545.3847 (Calcd for C₃₃H₅₃O₆ 545.3848).

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