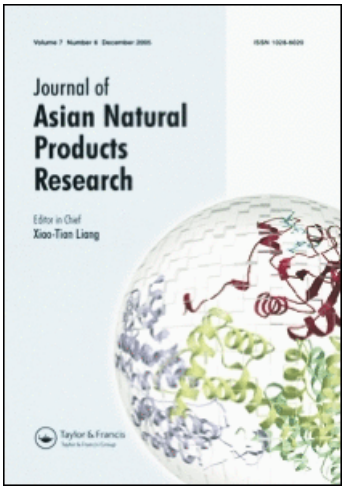

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Four new long-chain aliphatics from the feces of *Trogopterus xanthipes*

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Four new long-chain aliphatics from the feces of *Trogopterus xanthipes*

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Chemical investigation of Trogopterus feces has led to the isolation of four new long-chain aliphatics, including two new fatty alcohols, 8,15-nonacosanediol (**1**) and 6,13-nonacosanediol (**2**), one new fatty acid ester, dihexyl 7,7′-oxydiheptanoate (**3**), and one new ceramide, (2*R*)-2-hydroxy-*N*-[(2*S*,3*S*,4*R*,11*E*)-1,3,4-trihydroxydocos-11-en-2-yl]heneicosanamide (**4**). Their structures were elucidated by means of chemical and extensive spectroscopic analysis. Compound **3** exhibited moderate activity of antithrombin *in vitro*.

Keywords: Trogopterus feces; fatty alcohol; fatty acid ester; ceramide; antithrombin

1. Introduction

Trogopterus feces, called 'Wulingzhi' in Chinese, are the dry stools of *Trogopterus xanthipes* Milne-Edwars. Trogopterus feces have the function of invigorating blood and dissolving stasis, and are often used for the treatment of amenorrhea, menses pain, post-partum abdominal pain, and lochia retention in Chinese medicine. Modern studies have indicated that Trogopterus feces mainly consisted of hyaluronidase inhibitory active 6*H*-dibenzo[*b*,*d*]-pyran-6-ones, cytotoxic triterpenes, and anticoagulative diterpenes and phenolic acids [1–4]. In our present study, different solvent extracts of Trogopterus feces were screened, which showed that the ethyl acetate extract was the important active part. Therefore, the ethyl acetate extract was chemically investigated and four new long-chain aliphatics, including two new fatty alcohols, one new fatty acid ester, and one new ceramide, were isolated. Fatty alcohol, fatty acid ester, and ceramide are important long-chain aliphatics, which were reported to have many pharmacological functions of inhibiting cholesterol synthesis, increasing low-density lipoprotein processing, and anticoagulation [5–8]. In this paper, we deal with the isolation and structural elucidation of two new fatty alcohols, 8,15-nonacosanediol (**1**) and 6,13-nonacosanediol (**2**), one new fatty acid ester, dihexyl 7,7′-oxydiheptanoate (**3**), and one new ceramide, (2*R*)-2-hydroxy-*N*-[(2*S*,3*S*,4*R*,11*E*)-1,3,4-trihydroxydocos-11-en-2-yl]heneicosanamide (**4**) (Figure 1), and report some of their anticoagulative activities. Their structures were elucidated by means of chemical and extensive spectroscopic analysis.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. The molecular for-

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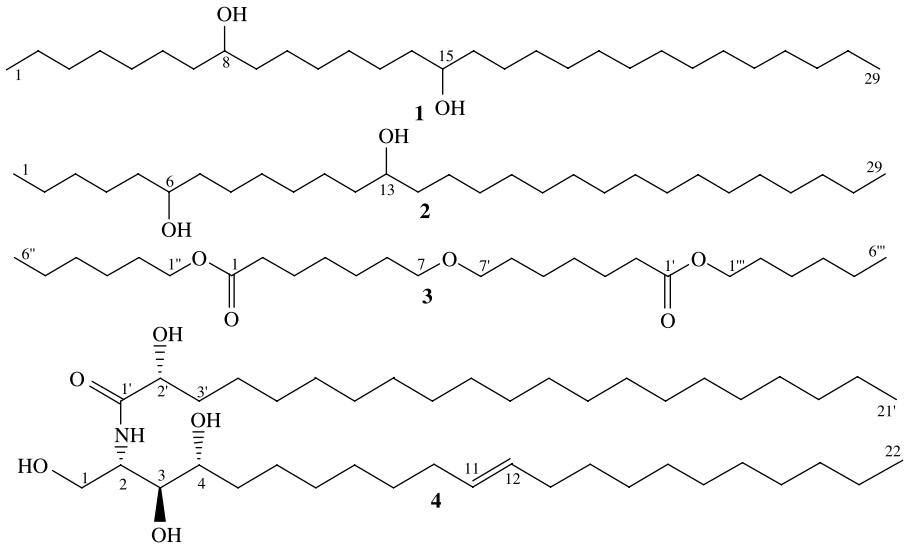

Figure 1. The structures of compounds **1**–**4**.

mula of **1** was determined to be $C_{29}H_{60}O_2$ by high-resolution negative ion ESI-MS at *m/z* 439.4504 $[M-H]^-$, and by positive ion ESI-MS at *m/z* 463.4520 $[M+Na]^+$. The IR spectrum showed the presence of hydroxy (3375 cm^{-1}). The ^1H NMR spectrum of **1** showed the presence of two terminal methyl protons at δ 0.88 (6H, t, $J = 7.1$ Hz), methylene protons at δ 1.23–1.31 (br m) and 1.37–1.48 (br m), and two oxygenated methine protons at δ 3.52 (2H, m). The ^{13}C NMR spectrum of **1** revealed oxygenated methine carbons resonating at δ 73.0. The above data indicated a long-chain hydrocarbon with two hydroxyl functions in **1**. The positive ESI-MS of **1** gave the quasi-molecular ion at *m/z* 441 $[M+H]^+$, and its MS/MS (Figure 2) showed characteristic peaks at *m/z* 441($[M+H]^+$, 10), 341 ($C_{22}H_{45}O_2$, 100), 311 ($C_{21}H_{43}O$, 45), 243 ($C_{15}H_{31}O_2$, 14), 227 ($C_{15}H_{31}O$, 20), 197 ($C_{14}H_{29}$, 18), 128 ($C_8H_{16}O$, 35), and 97 (C_7H_{13}, 13). These characteristic fragment ions were derived from β-fission of the hydroxyl group, which suggested two OH groups present at C-8 and C-15. Compound **1** was subjected to acetylation, and the final reaction product was analyzed by ESI-MS and MS/MS, and its MS/MS (Figure 2) gave characteristic peaks at *m/z* 547 ($[M+Na]^+$, 77), 487 (M+Na-HOAc, 100), 427 (M+Na-2 × HOAc, 14), 425 ($C_{26}H_{49}O_4$, 7), 353 ($C_{23}H_{45}O_2$, 6), 327 ($C_{19}H_{35}O_4$, 8), 269 ($C_{17}H_{33}O_2$, 5), 255 ($C_{16}H_{31}O_2$, 3), 197 ($C_{14}H_{29}$, 9), 171 ($C_{10}H_{19}O_2$, 5), and 99 (C_7H_{15}, 10). These fragment ions were derived from loss of HOAc or β-fission of the acetoxyl group, which revealed two OAc groups present at C-8 and C-15. Thus, compound **1** was elucidated as 8,15-nonacosanediol. $^1H-^1H$ COSY, HSQC, and HMBC experiments were done to further prove the structure of **1**.

Compound **2** was obtained as a white amorphous powder. The molecular formula of **2** was determined to be $C_{29}H_{60}O_2$ by high-resolution negative ion ESI-MS at *m/z* 439.4502 $[M-H]^-$, and by positive ion ESI-MS at *m/z* 463.4512 $[M+Na]^+$. The IR spectrum showed the presence of hydroxy (3371 cm^{-1}). Compound **2** showed close resemblances with **1** in their ^1H and ^{13}C NMR spectra. The positive ESI-MS of **2** gave the quasi-molecular ion at *m/z* 441 $[M+H]^+$, and its MS/MS (Figure 2) showed

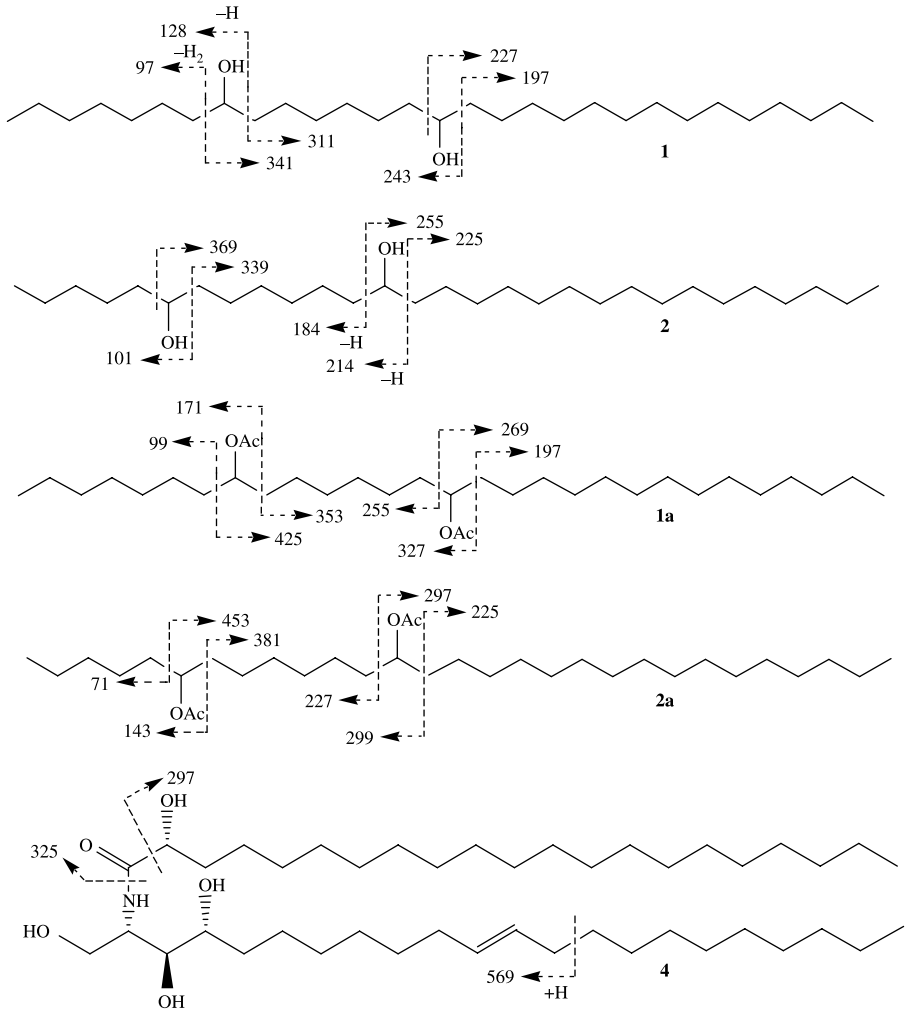

Figure 2. ESI-MS/MS fragmentation of compounds **1**, **2**, **1a**, **2a**, and **4**.

characteristic peaks at *m/z* 441 ($[M+H]^+$, 15), 369 ($C_{24}H_{49}O_2$, 100), 339 ($C_{23}H_{47}O$, 35), 255 ($C_{17}H_{35}O$, 12), 225 ($C_{16}H_{33}$, 10), 214 ($C_{13}H_{26}O_2$, 9), 184 ($C_{12}H_{24}O$, 25), and 101 ($C_6H_{13}O$, 16). These characteristic fragment ions were derived from β-fission of the hydroxyl group, which suggested two OH groups present at C-6 and C-13. Compound **2** was also subjected to acetylation and the final reaction product was analyzed by ESI-MS and MS/MS, and its MS/MS (Figure 2) gave characteristic peaks at *m/z* 547 ($[M+Na]^+$, 69), 487 (M+Na-HOAc, 100), 453 ($C_{28}H_{53}O_4$, 9), 427 (M+Na-2 × HOAc, 24), 381 ($C_{25}H_{49}O_2$, 9), 299 ($C_{17}H_{31}O_4$, 5), 297 ($C_{19}H_{37}O_2$, 12), 227 ($C_{14}H_{27}O_2$, 9), 225 ($C_{16}H_{33}$, 7), 143 ($C_8H_{15}O_2$, 11), and 71 (C_5H_{11}, 5). These fragment ions were derived from loss of HOAc or β-fission of the acetoxyl group, which revealed two OAc groups present at C-6 and C-13. Thus, compound **2** was elucidated as 6,13-nonacosanediol. $^1H-^1H$ COSY, HSQC, and HMBC experiments were done to further prove the structure of **2**.

Compound **3** was isolated as a white powder. The molecular formula of **3** was established as $C_{26}H_{50}O_5$ by high-resolution

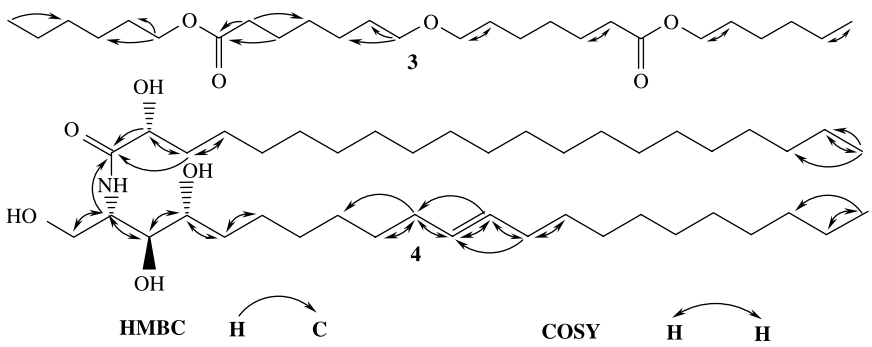

Figure 3. The key HMBC and COSY correlations of compounds **3** and **4**.

ESI-MS at *m/z* 465.3567 $[M+Na]^+$. The positive ESI-MS showed a quasi-molecular ion peak at *m/z* 443 $[M+H]^+$ and 465 $[M+Na]^+$. The ^1H and ^{13}C NMR spectra were typical of a symmetric fatty acid ester. When **3** was methanolyzed with methanolic HCl, a fatty acid methyl ester (FAME) was obtained together with a fatty alcohol. On the basis of ESI-MS analysis, the FAME was characterized as dimethyl 7,7′-oxydiheptanoate from a quasi-molecular ion peak at *m/z* 303 $[M+H]^+$, and the fatty alcohol was identified as hexanol from a quasi-molecular ion peak at *m/z* 103 $[M+H]^+$. The protons at δ 4.06 (4H, t, $J = 6.6$ Hz) and 3.52 (4H, t, $J = 6.7$ Hz) were assigned to be H-1″, 1‴ and H-7, 7′, and the carbons at δ 66.0 and 73.3 were assigned to be C-1″, 1‴ and C-7, 7′, respectively. Assignments of all protons and carbons in **3** were also made by $^1H-^1H$ COSY, HMQC, and HMBC spectra (Figure 3). Thus, the structure of compound **3** was determined as dihexyl 7,7′-oxydiheptanoate.

Compound **4** was isolated as a white powder. The molecular formula of **4** was established as $C_{43}H_{85}NO_5$ by high-resolution ESI-MS at *m/z* 718.6340 $[M+Na]^+$. The positive ESI-MS showed a quasi-molecular ion peak at *m/z* 696 $[M+H]^+$, and its MS/MS showed characteristic peaks at *m/z* 569 (an ion formed by McLafferty rearrangement of the olefinic bond), 297 (an ion derived from β-fission of the OH group), and 325 (an ion derived from α-fission of the NH group; Figure 2). The ^1H and ^{13}C NMR spectra were typical of a ceramide possessing 2-hydroxy fatty acid. Assignments of all protons and carbons in **4** can be made by $^1H-^1H$ COSY, HMQC, and HMBC spectra (Figure 3). In the $^1H-^1H$ COSY spectrum, two methylene protons at δ 3.53 correlated with the methine proton at δ 3.89, the methine proton at δ 3.89 correlated with the methine proton at δ 3.38, the methine proton at δ 3.38 correlated with the methine proton at δ 3.35, which suggested three hydroxyl groups at C-1, C-3, and C-4. An HMBC experiment was run to support these assignments. The fatty acid linked to C-2 of the sphingosine has been confirmed by the correlation between H-2 and the carbonyl carbon at δ 174.4. HMBC correlations of the carbonyl carbon with the H-2′ and the proton HO-2′ confirmed the presence of an α-hydroxy fatty acid side chain. The structure of the α-hydroxy fatty acid side chain in **4** was examined. When **4** was methanolyzed with methanolic hydrochloric acid, the FAME was obtained together with a long-chain base (LCB). On the basis of ESI-MS analysis, the FAME was characterized as methyl 2-hydroxyheneicosanoate from the protonated molecular ion at *m/z* 357. The ^1H NMR spectrum also revealed a pair of olefinic protons at δ 5.36 attributable to the presence of one olefinic bond. The fragment ion at *m/z* 569 due to elimination

of nonene from the molecular ion indicated that the olefinic bond in the LCB of **4** was located at C-11. Furthermore, the C-atom signals at δ 33.1 and 32.8 confirmed the *E* geometry of the olefinic bond at C-11 in the LCB [9]. Considering the biogenesis and steric hindrance of sphingolipids, the chemical shifts of the carbon signals of C-2–C-4, C-1′, and C-2′ of sphingolipids generally were acknowledged to determine the absolute stereochemistry of the phytosphingosine moiety. The carbon signals at δ 52.4 (C-2), 75.6 (C-3), 72.0 (C-4), 174.4 (C-1′), and 72.2 (C-2′) in **4** were nearly identical to those of the (2*S*,3*S*,4*R*,2′*R*)-sphingolipids reported in the literatures [10–14]. Thus, the structure of compound **4** was established as (2*R*)-2-hydroxy-*N*-[(2*S*,3*S*,4*R*,11*E*)-1,3,4-trihydroxydocos-11-en-2-yl]heneicosanamide.

In vitro anticoagulative activities of the different solvent extracts and compounds **1**–**4** were tested using the thrombin time (TT) method assay with heparin sodium as a positive control in this study. The results were summarized in Table 1, which suggested that compound **3** could moderately prolong TT with a good dose-effect manner. Trogopterus feces are the dry stools of *T. xanthipes,* which feed on leaf twigs in *Biota orientalis*, and compounds **1**–**4** might be metabolic products of *T. xanthipes*. The coagulative assay results suggested that the isolated fatty acid ester **3** was one of the important anticoagulative constituents of Trogopterus feces.

Table 1. Antithrombin activities of compounds **1**–**4** ($\bar{x} \pm s$, $n = 6$–8).

Sample	Dosage ($\mu g\,ml^{-1}$)	TT prolongation rate (%)
Compound **1**	100	2.75 ± 0.29
Compound **2**	100	5.34 ± 0.69
Compound **3**	100	54.49 ± 2.98
	50	20.22 ± 1.61
	25	9.30 ± 0.91
	10	3.13 ± 0.44
Compound **4**	100	2.37 ± 0.27
Heparin sodium	50	73.25 ± 3.14

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter. IR spectra were taken on a Nicolet IR-100 FT-IR spectrometer in KBr disks. NMR spectra were measured on a Bruker AV-500 MHz (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) using tetramethylsilane as the internal standard, and chemical shifts were recorded as δ values. ESI-MS and HR-ESI-MS spectra were obtained on a Micromass Q/TOF Mass Spectrometer. Anticoagulative assay was performed on a coagulation analysis instrument LG-PABER-I. The blood sample was treated on an Anke TDL-40B Centrifugal Machine. Silica gel for column chromatography (CC, 200–300 mesh) and thin layer chromatography plates (10–40 μm) were the products of Qingdao Marine Chemical Co., Ltd (Qingdao, China). All solvents used were of analytical grade (Nanjing Chemical Plant, Nanjing, China). Thrombin was purchased from Xisen Sanhe Co., Ltd (Leling, China). Tris-hydroxymethylaminomethane (Tris) was the product of Shanghai Jingxi Chemical Industry Co., Ltd (Shanghai, China). Sodium citrate was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd (Shanghai, China). Heparin sodium (biotech grade, $150\,U\,mg^{-1}$) was purchased from Amresco (Solon, OH, USA). Rabbit (3.8 kg) was supplied by Shanghai Sikelai Experimental Animal Co., Ltd (Shanghai, China).

3.2 Materials

Trogopterus feces were collected in June 2008 from Hebei Province of China, and identified as the dry stools of *T. xanthipes* by Associate Professor Nianyun Yang of Nanjing University of Traditional Chinese

Medicine. A voucher specimen (GS-20080610) is kept in the Herbarium of Nanjing University of Traditional Chinese Medicine.

3.3 Extraction and isolation

The air-dried and powdered Trogopterus feces (3 kg) were extracted with 60% C_2H_5OH (2× 50 liters) for 2 h under reflux, and the combined extracts were concentrated under reduced pressure. The residue (370 g) was then suspended in H_2O and extracted successively with ethyl acetate and *n*-butanol to give the respective extracts after the removal of the solvent. The combined ethyl acetate extracts were evaporated under reduced pressure to give the residue (277 g), which was chromatographed on silica gel (2 kg) eluting with $CHCl_3$–CH_3OH, stepwise gradient (100:0 → 5:1), and five fractions were collected. Fraction 1 (10 g) was separated by silica gel [CH_2Cl_2–CH_3OH (30:1)] to yield compounds **1** (90 mg), **2** (150 mg), and **3** (255 mg). Fraction 2 (15 g) was separated by silica gel [CH_2Cl_2–CH_3OH (20:1)] to obtain compound **4** (150 mg).

Compound **1** was obtained as a white amorphous powder; $[\alpha]_D^{20}$ + 3 (c = 0.025, MeOH); IR (KBr) ν_{max}: 3375, 2921, 1537, 1474 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 0.88 (6H, t, J = 7.1 Hz, H-1, 29), 1.23–1.31 (38H, br m), 1.37–1.48 (12H, br m), 3.52 (2H, m, H-8, 15); ^{13}C NMR (CD_3OD, 125 MHz): δ 14.7 (C-1, 29), 24.1 (C-2, 28), 27.3 (C-6, 10, 13, 17), 30.8–31.3 (C-4–5, 11–12, 18–26), 33.5 (C-3, 27), 38.8 (C-7, 9, 14, 16), and 73.0 (C-8, 15); ESI-MS: *m/z* 441 $[M+H]^+$; ESI-MS/MS (relative intensity %): *m/z* 441 (10), 341 (100), 311 (45), 243 (14), 227 (20), 197 (18), 128 (35), 97 (13); HR-ESI-MS: *m/z* 439.4504 $[M-H]^-$ (calcd for $C_{29}H_{59}O_2$, 439.4515), *m/z* 463.4520 $[M+Na]^+$ (calcd for $C_{29}H_{60}O_2Na$, 463.4491).

Compound **2** was obtained as a white amorphous powder; $[\alpha]_D^{20}$ − 89 (c = 0.015, MeOH); IR (KBr) ν_{max}: 3371, 2920, 1541, 1472 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 0.88 (6H, t, J = 7.1 Hz, H-1, 29), 1.23–1.31 (38H, br m), 1.37–1.48 (12H, br m), 3.53 (2H, m, H-6, 13); ^{13}C NMR (CD_3OD, 125 MHz): δ 14.7 (C-1, 29), 24.2 (C-2, 28), 27.3 (C-4, 8, 11, 15), 30.8–31.3 (C-9–10, 16–26), 33.5 (C-3, 27), 38.8 (C-5, 7, 12, 14), 73.0 (C-6, 13); ESI-MS: *m/z* 441 $[M+H]^+$; ESI-MS/MS (relative intensity %): *m/z* 441 (15), 369 (100), 339 (35), 255 (12), 225 (10), 214 (9), 184 (25), 101 (16); HR-ESI-MS: *m/z* 439.4502 $[M-H]^-$ (calcd for $C_{29}H_{59}O_2$, 439.4515), *m/z* 463.4512 $[M+Na]^+$ (calcd for $C_{29}H_{60}O_2Na$, 463.4491).

Compound **3**, a white powder; IR (KBr) ν_{max}: 3209, 2909, 1625, 1534, 1470 1274, 1061, 1014, 722 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 4.06 (4H, t, J = 6.6 Hz, H-1″, 1‴), 3.52 (2H, t, J = 6.7 Hz, H-7, 7′), 2.27 (4H, t, J = 7.4 Hz, H-2, 2′), 1.61 (8H, m, H-2″, 2‴, 3, 3′), 1.42 (4H, m, H-6, 6′), 1.28–1.30 (24H, br s, H-3″–5″, 3‴–5‴, 4–6, 4′–6′), 0.88 (6H, t, J = 7.2 Hz, H-6″, 6‴); ^{13}C NMR (CD_3OD, 125 MHz): δ 177.2 (C-1, 1′), 73.3 (C-7, 7′), 66.0 (C-1″, 1‴), 35.7 (C-2, 2′), 33.5 (C-4″, 4‴, 6, 6′), 31.0 (C-2″, 2‴), 30.8 (C-4, 4′), 27.2 (C-3″, 3‴, 5, 5′), 26.8 (C-3, 3′), 24.1 (C-5″, 5‴), 14.8 (C-6″, 6‴); ESI-MS: *m/z* 443 $[M+H]^+$, 465 $[M+Na]^+$; HR-ESI-MS: *m/z* 465.3567 $[M+Na]^+$ (calcd for $C_{26}H_{50}O_5Na$, 465.3556).

Compound **4**, a white powder; $[\alpha]_D^{20}$ + 11 (c = 0.01, MeOH); IR (KBr) ν_{max}: 3211, 2914, 1627, 1530, 1470 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 7.35 (1H, d, J = 8.9 Hz, NH), 5.36 (2H, br m, H-11, 12), 3.89 (1H, m, H-2), 3.84 (1H, m, H-2′), 3.53 (2H, br d, J = 15.3 Hz, H-1), 3.38 (1H, m, H-3), 3.35 (1H, m, H-4), 1.98 (4H, m, H-10, 13), 1.59 (1H, m, H-3′a), 1.49 (3H, m, H-5, 3′b), 1.26–1.34 (58H, br m, H-6–9, 14–21, 4′–20′), 0.85 (6H, t, J = 6.8 Hz, H-22, 21′); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 174.4 (C-1′), 131.2 (C-11), 130.7 (C-12), 75.6 (C-3), 72.2 (C-2′), 72.0 (C-4), 61.5 (C-1), 52.4 (C-2),

35.4 (C-5, 3′), 33.1 (C-13), 32.8 (C-10), 32.2 (C-20, 19′), 30.2–29.5 (C-7–9, 14–19, 5′–18′), 26.4 (C-6), 25.4 (C-4′), 22.9 (C-21, 20′), 14.8 (C-22, 21′); ESI-MS: *m/z* 696 $[M+H]^+$; ESI-MS/MS: *m/z* 696(8), 569(48), 297(50), 325(37); HR-ESI-MS: *m/z* 718.6340 $[M+Na]^+$ (calcd for $C_{43}H_{85}NO_5Na$, 718.6325).

3.4 Acetylation of compounds 1 and 2

Compound **1** (2.0 mg) in Ac_2O–pyridine (1:1) was kept at room temperature for 48 h to yield a crude product, to which 3 ml of water was added and then extracted with EtOAc (3 ml). The resulting extract was concentrated under reduced pressure to afford its diacetate derivative **1a**. ESI-MS: *m/z* 525 $[M+H]^+$, 547 $[M+Na]^+$; ESI-MS/MS: *m/z* 547 (77), 487 (100), 427 (14), 425 (7), 353 (6), 327 (8), 269 (5), 255 (3), 197 (9), 171 (5), 99 (10). Compound **2** was also acetylated as above to afford its diacetate derivative **2a**. ESI-MS: *m/z* 525 $[M+H]^+$, 547 $[M+Na]^+$; ESI-MS/MS: *m/z* 547 (69), 487 (100), 453 (9), 427 (24), 381 (9), 299 (5), 297 (12), 227 (9), 225 (7), 143 (11), 71 (5).

3.5 Methanolysis of compounds 3 and 4

Compound **3** (1 mg) was heated with 10% HCl in MeOH (1 ml) at 80°C for 14 h. The reaction mixture was then extracted with *n*-hexane, and the upper and lower liquid was individually analyzed by ESI-MS, which showed quasi-molecular ions at *m/z* 303 $[M+H]^+$ and 103 $[M+H]^+$, respectively. The same experiments were done for compound **4**, and gave quasi-molecular ions at *m/z* 357 $[M+H]^+$ and 372 $[M+H]^+$, respectively.

3.6 Anticoagulative evaluation of compounds 1–4

Evaluation of anticoagulative activities of the different solvent extracts and pure compounds **1**–**4** was performed using a TT method. Rabbit common carotid artery was cut off to take a sample of blood, which was mixed with anticoagulant (3.8% sodium citrate) in the proportion of 9:1, and the mixture was centrifuged at 2500 rpm for 15 min to collect the plasma. The plasma (50 μl) was put in a plastic cup for 3 min at 37°C, and 100 μl thrombin solution of 15 U ml^{-1} diluted by 0.1 mol ml^{-1}, pH 7.4, Tris–HCl buffer was also put in the plastic cup along with 10 μl sample solution; meanwhile, the coagulation analysis instrument was started up so that the thrombin clotting time was recorded. The same experiment was done for the positive control drug heparin sodium and the blank solvent ethanol. Each analyte was tested several times, and an average value was applied. TT prolongation rate was calculated to assess the anticoagulative activities of the samples.

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