Chemistry and Physics of Lipids 165 (2012) 731–736

Contents lists available at SciVerse ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Two new nonacosanetriols from *Ginkgo biloba* sarcotesta

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ARTICLE INFO

Article history:
Received 9 June 2012
Received in revised form 29 August 2012
Accepted 31 August 2012
Available online 6 September 2012

Keywords:
Ginkgo biloba
Sarcotesta
Fatty alcohol
Anticoagulative activity
Stereochemistry

ABSTRACT

Two new fatty alcohols named as (7*S*,8*R*,11*S*)-nonacosanetriol (**1**) and (10*R*,12*R*,15*S*)-nonacosanetriol (**2**), along with eight known compounds including ginkgolic acid (**3**), hydroginkgolic acid (**4**), sciadopitysin (**5**), ginkgetin (**6**), isoginkgetin (**7**), ginkgolide A (**8**), ginkgolide B (**9**) and ginkgolide C (**10**) have been isolated from the petroleum ether extract of *Ginkgo biloba* sarcotesta. Their structures were elucidated by means of chemical and extensive spectroscopic analysis. The absolute stereochemistry of compounds **1** and **2** was elucidated on the spectroscopic analysis of the *R*- and *S*-MTPA esters. Compounds **1** and **2** exhibited slight activity of antithrombin and moderate activity of antiplatelet aggregation *in vitro*. This was the first report regarding the anticoagulative activities of biflavonoids in *G. biloba*, and isoginkgetin (**7**) showed significant antithrombin and antiplatelet aggregation activity.

1. Introduction

Ginkgo biloba is a deciduous and dioecious gymnosperm species (Major, 1967) originated in China and is the sole survivor of the ancient family of Ginkgoaceae (Carrier et al., 1998). Although *G. biloba* has been above 200 million years old, its true value has induced a range of attentions all around the world till the recent two decades. It contains a large number of active compounds, the most important of which are flavanol glycosides and terpene lactones. Its amazing vitality has attracted an increasing exploration into potential application in health, foods and supplements (van Beek, 2002; Singh et al., 2008). Male and female flowers are born on different plants and female plants bear a yellowish-green plum-like "fruit" (aril) (Choukchou-Braham et al., 1994) which is indeed the seed of ginkgo in gymnosperms. The outer malodorous fleshy layer is called sarcotesta. It surrounds an ovoid nut, which is called "Baiguo" in China and "Gin-nan" in Japan (Deng et al., 2011).

Ginkgo seeds have been used in traditional Chinese medicine (TCM) and as a foodstuff for centuries throughout Asia (Jin et al., 2008). However, Gbs, the epicarp of mature seeds which is peeled from *G. biloba* seeds, was treated as waste and discarded abundantly in soil and water, thus it polluted soil and poisoned fish in rivers and lakes (Pan et al., 2006). It has become an urgent problem to the local enterprises and governments of the planting area for its poison to the environment (Wu et al., 2011). Modern studies indicated that Gbs consists of ginkgolic acid and polysaccharide, which were reported to have many biological and pharmacological functions, including antitumor, anti-inflammatory, pesticidal and antibacterial properties (Chen et al., 2007). In Xingan (Guangxi province, China) the fruit juice produced during the peeling process was diluted with water and used as an insecticide by local peasants. Satisfactory results were achieved in the control of many kinds of insects, indicating the presence of some insecticidal constituents (Choi et al., 2009). In our preliminary experiment, 95% EtOH extract of Gbs showed anticoagulative activity. And then, in our present study, different solvent extracts of Gbs were tested, and the results showed that the petroleum ether extract had strong anticoagulative activity. Therefore, the petroleum ether extract of Gbs was chemically investigated, which resulted in the isolation of two new fatty alcohols, named as (7*S*,8*R*,11*S*)-nonacosanetriol (**1**) and (10*R*,12*R*,15*S*)-nonacosanetriol (**2**), together with eight known compounds including ginkgolic acid (**3**), hydroginkgolic acid (**4**), sciadopitysin (**5**), ginkgetin (**6**), isoginkgetin (**7**), ginkgolide A (**8**), ginkgolide B (**9**) and ginkgolide C (**10**). Some nonacosanetriols or similar compounds were isolated from nature, such as 7,8,10-nonacosanetriol and 7,9,10-nonacosanetriol from *Typha*

Abbreviations: ADP, adenosine diphosphate; COSY, correlation spectroscopy; DMAP, 4-dimethylaminopyridine; ESI, electrospray ionization; Gbs, *Ginkgo biloba* sarcotesta; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear singular quantum correlation; HR, high resolution; MS, mass spectrometry; NMR, nuclear magnetic resonance; PPP, platelet poor plasma; PRP, platelet rich plasma; *R*-MTPA, *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride; *S*-MTPA, *S*-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride; TMS, tetramethylsilane; Tris, trishydroxymethylaminomethane; TT, thrombin time.

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http://dx.doi.org/10.1016/j.chemphyslip.2012.08.003

angustifolia (Tao et al., 2010), 8,15-nonacosanediol and 6,13-nonacosanediol from *Trogopterus xanthipes* (Yang et al., 2009a), and 9,10,11-trihydroxy-(12*Z*)-12-octadecenoic acid from *Tuber indicum* (Gao et al., 2001). The structures of the two new compounds were elucidated by means of chemical and extensive spectroscopic analysis. Furthermore, their anticoagulative activities were also evaluated.

2. Experimental

2.1. Materials and chemicals

Gbs was collected on November 2010 from Taizhou, Jiangsu Province, China, and identified as the sarcotesta of *Ginkgo biloba* L. by Dr. Hui Yan (Department of Pharmacognosy, Nanjing University of Chinese Medicine, China). A voucher specimen (BG-20101120) was deposited at the Herbarium in Jiangsu Key Laboratory for TCM Formulae Research.

Silica gel for column chromatography (CC) (60–80 μm) and thin-layer chromatography plates (10–40 μm) was purchased from Qingdao Marine Chemical (Qingdao, China). All solvents used were of analytical grade (Nanjing Chemical Plant, Nanjing, China). *R*-MTPA and *S*-MTPA were obtained from Sigma Chemical Co. (St. Louis, MO). Thrombin was purchased from Xisen Sanhe (Leling, China). Tris was the product of Shanghai Jingxi Chemical Industry (Shanghai, China). Sodium citrate was purchased from Shanghai Lingfeng Chemical Reagent (Shanghai, China). Heparin sodium (biotech grade, 150 U mg^{-1}) was purchased from Amresco (Solon, USA). ADP was purchased from Beijing Zhongqin Scientific Instrument Co. Ltd. (Beijing, China). A rabbit (3.8 kg) was supplied by Shanghai Sikelai Experimental Animal (Shanghai, China).

2.2. Apparatus

Melting points were determined with a WRS-lB melting point apparatus (Shanghai Precision and Scientific Instrument, Shanghai, PR China) which was uncorrected. Optical rotation was measured on a Perkin-Elmer 341 polarimeter. NMR spectra were measured on a Bruker AV-500 MHz (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR), using TMS as internal standard; chemical shifts were recorded as δ (ppm) values. ESI-MS and HR-ESI-MS spectra were obtained on a Micromass Q/TOF mass spectrometer. Anticoagulative assay was performed on an LG-PABER-I coagulation-analysis instrument. The blood sample was treated on an Anke TDL-40B centrifuge.

2.3. Extraction and isolation

Air-dried and powdered Gbs (25 kg) were extracted two times with 95% EtOH (2 × 250 L) under reflux for 2 h at 70–80 °C each time. The extracts were concentrated under reduced pressure, and then they were suspended in H_2O and extracted successively with petroleum ether, ethyl acetate and *n*-butanol to give the respective extracts after solvent removal. The combined petroleum ether extracts were evaporated under reduced pressure to leave a residue (250 g) which was chromatographed on silica gel (2.5 kg) eluted with a petroleum ether–EtOAc stepwise gradient (100:0 → 1:1) and 5 fractions were collected. Fr. 4 (30 g) was separated by silica gel (petroleum ether–EtOAc, 5:1) to obtain compounds **1** (50 mg) and **2** (30 mg); Fr. 1 (60 g) was separated by silica gel (petroleum ether–EtOAc, 20:1) to obtain compounds **3** (100 mg) and **4** (60 mg); Fr. 3 (40 g) was separated by silica gel (petroleum ether–EtOAc, 8:1) to obtain compounds **5** (50 mg), **6** (10 mg) and **7** (10 mg), and Fr. 5 (40 g) was separated by silica gel (petroleum ether–EtOAc 3:1) to obtain compounds **8** (20 mg), **9** (30 mg) and **10** (10 mg).

(7*S*,8*R*,11*S*)-nonacosanetriol (**1**) white powder, $[\alpha]_D^{20}$ + 3.0° (*c* = 0.045, MeOH), m.p. 160–162 °C, ^{1}H NMR (CD_3OD, 500 MHz) δ 3.80 (1H, m, H-11), 3.58 (1H, m, H-8), 3.40 (1H, m, H-7), 1.76 (1H, t, *J* = 3.5 Hz, H-10a), 1.73 (1H, t, *J* = 3.5 Hz, H-9a), 1.53 (1H, m, H-9b), 1.51 (1H, m, H-10b), 1.45–1.50 (4H, br. m, H-6, 12), 1.24–1.39 (40H, br. m, H-2–5, 13–28), 0.89 (6H, t, *J* = 7.0 Hz, H-1, 29); ^{13}C NMR (CD_3OD, 125 MHz) δ 76.4 (C-7), 76.0 (C-8), 72.4 (C-11), 40.4 (C-9, 10), 39.1 (C-6, 12), 34.1 (C-3, 27), 30.9–31.3 (C-4, 14–26), 27.4 (C-5), 26.9 (C-13), 24.1 (C-2, 28), 14.8 (C-1, 29). ESI-MS: *m*/*z* 455 $[M-H]^-$, 457 $[M+H]^+$, 479 $[M+Na]^+$; HR-ESI-MS: *m*/*z* 479.7761 $[M+Na]^+$ ($C_{29}H_{60}O_3Na$, calc. 479.7747).

C(7),C(8),C(11)-tris-(*S*)-MTPA ester of compound **1** [(*S*)-MTPA-**1**] white powder, m.p. 175–180 °C, ^{1}H NMR (CD_3OD, 500 MHz) δ 7.58 (6H, m, MTPA-ArH-3, 5), 7.41 (9H, m, MTPA-ArH-2, 4, 6), 3.83 (1H, m, H-11), 3.60 (1H, m, H-8), 3.56 (9H, s, MTPA-OCH_3), 3.42 (1H, m, H-7), 1.75 (1H, m, H-9a), 1.70 (1H, m, H-10a), 1.56 (1H, m, H-9b), 1.52 (1H, m, H-10b), 1.38–1.43 (4H, m, H-6, 12), 1.27–1.38 (40H, m, H-2–5 and H-13–28), 0.90 (6H, t, *J* = 7.0 Hz, H-1, 29). ESI-MS: *m*/*z* 1105 $[M+H]^+$.

C(7),C(8),C(11)-tris-(*R*)-MTPA ester of compound **1** [(*R*)-MTPA-**1**] white powder, m.p. 169–173 °C, ^{1}H NMR (CD_3OD, 500 MHz) δ 7.44 (6H, m, MTPA-ArH-3, 5), 7.36 (9H, m, MTPA-ArH-2, 4, 6), 3.81 (1H, m, H-11), 3.64 (1H, m, H-8), 3.55 (9H, s, MTPA-OCH_3), 3.40 (1H, m, H-7), 1.76 (1H, m, H-9a), 1.73 (1H, m, H-10a), 1.57 (1H, m, H-9b), 1.55 (1H, m, H-10b), 1.35–1.40 (4H, br. m, H-6, 12), 1.30–1.35 (40H, br. m, H-2–5, 13–28), 0.89 (6H, t, *J* = 7.0 Hz, H-1, 29). ESI-MS: *m*/*z* 1105 $[M+H]^+$.

(10*R*,12*R*,15*S*)-nonacosanetriol (**2**) white powder, $[\alpha]_D^{20}$ − 8.0° (*c* = 0.015, MeOH), m.p. 185–187 °C, ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.88 (3H, m, H-10, 12, 15), 1.61 (1H, m, H-11a), 1.58 (1H, m, H-11b), 1.42–1.48 (8H, br. m, H-9, 13, 14, 16), 1.26–1.29 (38 H, br. m, H-2–8, 17–28), 0.88 (6H, t, *J* = 6.6 Hz, H-1, 29); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 75.4 (C-12), 74.5 (C-15), 72.8 (C-10), 38.6 (C-11), 38.0 (C-13, 14), 36.8 (C-9, 16), 32.0 (C-3, 27), 29.4–29.7 (C-4–7, 18–26), 26.0 (C-8), 25.4 (C-17), 22.6 (C-2, 28), 14.0 (C-1, 29). ESI-MS: *m*/*z* 457 $[M+H]^+$, 479 $[M+Na]^+$; HR-ESI-MS: *m*/*z* 479.7706 $[M+Na]^+$ ($C_{29}H_{60}O_3Na$, calc. 479.7747).

C(10),C(12),C(15)-tris-(*S*)-MTPA ester of compound **2** [(*S*)-MTPA-**2**] white powder, m.p. 209–213 °C, ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.58 (6H, m, MTPA-ArH-3, 5), 7.42 (9H, m, MTPA-ArH-2, 4, 6), δ 3.90 (3H, m, H-10, 12, 15), 3.56 (9H, s, MTPA-OCH_3), 1.73 (1H, m, H-11a), 1.70 (1H, m, H-11b), 1.52 (4H, m, H-13, 14), 1.45 (4H, m, H-9, 16), 1.25–1.31 (38 H, m, H-2–8 and H-17–28), 0.88 (6H, t, *J* = 6.6 Hz, H-1, 29). ESI-MS: *m*/*z* 1105 $[M+H]^+$.

C(10),C(12),C(15)-tris-(*R*)-MTPA ester of compound **2** [(*R*)-MTPA-**2**] white powder, m.p. 193–196 °C, ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.44 (6H, m, MTPA-ArH-3, 5), 7.36 (9H, m, MTPA-ArH-2, 4, 6), 3.89 (3H, m, H-10, 12, 15), 3.55 (9H, s, MTPA-OCH_3), 1.68 (1H, m, H-11a), 1.65 (1H, m, H-11b), 1.55 (4H, m, H-13, 14), 1.45 (4H, m, H-9, 16), 1.23–1.30 (38 H, m, H-2–8 and H-17–28), 0.88 (6H, t, *J* = 6.6 Hz, H-1, 29). ESI-MS: *m*/*z* 1105 $[M+H]^+$.

2.4. Acetylation of compounds *1* and *2*

Compounds **1** (2 mg) and **2** (2 mg) were dissolved in 3 mL of Ac_2O–pyridine (1:1) and kept at room temperature for 48 h to yield crude products, respectively. 3 mL of water was added into the reaction products, and the solution was extracted three times with EtOAc (3 × 3 mL), respectively. The resulting extractions were concentrated under reduced pressure to afford their corresponding diacetate derivatives (**1a** and **2a**), which were analyzed by ESI-MS–MS.