# Tinospinosides D, E, and Tinospin E, Further Clerodane Diterpenoids from *Tinospora sagittata*

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**Chemical investigation on the roots of *Tinospora sagittata* resulted in the isolation of three novel *cis*-clerodane diterpenoids, tinospinosides D, E, and tinospin E, together with two known compounds, columbin and columbin glucoside, and their structures were determined by extensive spectroscopic analyses, chemical reactions and computer-assisted calculations. The inhibitory activity of the isolated compounds and their chemical derivatives on nitric oxide production in lipopolysaccharide and interferon-$\gamma$ activited J774.1 macrophage-like cells was also evaluated.**

**Key words** *Tinospora sagittata*; Menispermaceae; clerodane diterpenoid; nitric oxide

The genus *Tinospora* (Menispermaceae) has about thirty species, mainly distributing in the tropic area of Asia and Africa, and many of the species have been used in traditional medicines, *e.g.*, *T. codifolia* has been used in Ayurveda (Indian traditional medicines) as a hepatoprotectant[1]; the stem of *T. sinensis* has been used in Traditional Chinese Medicine (TCM) in the treatment of rheumatism and muscle pain; the root of *T. capillipes* and *T. sagittata* has been used in TCM to relieve sore throat, expel superficial infection, and stop diarrhea.[2] The main chemical constituents of *Tinospora* species have been reported to be diterpenoids, which has 12-furano *cis*-clerodane skeleton as their characteristic structure.[3–5] As a part of our continuing phytochemical research on *Tinospora* species, we have previously reported the isolation of three novel clerodane diterpenoids, tinosposinensides A–C, from the stems of *T. sinensis*,[6] and three novel clerodane diterpenoids, tinospinosides A–C, from the roots of *T. sagittata*.[7] In a further investigation on the roots of *T. sagittata*, we herein report the isolation and structural determination of three new clerodane diterpenoids, tinospinoside D (**1**), tinospin E (**2**), and tinospinoside E (**3**) (Fig. 1). The inhibitory activity of these compounds and their chemical derivatives on nitric oxide production in lipopolysaccharide (LPS) and interferon (INF)-$\gamma$ activited J774.1 macrophage-like cells was also evaluated.

## Results and Discussion

The EtOAc fraction from the MeOH extract of roots of *T. sagittata* was fractionated by repeated column chromatography followed by HPLC purification to afford five compounds **1**–**5**. The known compounds were identified to be columbin (**4**) and columbin glucoside (**5**) by comparing the physical and spectroscopic data in literature.[8,9]

Tinospinoside D (**1**) was isolated as a colorless solid, $[\alpha]_D^{22}$ −28.2 ($c$=0.21, MeOH). Its molecular formula was established as $C_{27}H_{36}O_{13}$ by high resolution (HR)-FAB-MS. The $^1$H-NMR spectroscopic data of **1** (Table 1) showed a set of characteristic downfield resonances at $\delta_H$ 6.66 (1H, br d, $J$=1.6 Hz), 7.67 (1H, t, $J$=1.6 Hz) and 7.79 (1H, br d, $J$=1.6 Hz) for C-12 furan ring in *cis*-clerodane type diterpenoids from *Tinospora* sp.[10] The $^1$H- and $^{13}$C-NMR spectroscopic data also indicated the presence of two carbonyl moieties at $\delta_C$ 173.5 and 174.4, as well as a $\beta$-glucopyranosyl moiety with the anomeric resonances at $\delta_H$ 5.64 (1H, d, $J$=7.1 Hz) and $\delta_C$ 100.5, respectively (Tables 1, 2). The $\beta$-glucopyranosyl moiety was determined to be in the D-form by GLC analysis of its trimethylsilylthiazolidine derivative after acid hydrolysis of **1**. The $^1$H- and $^{13}$C-NMR spectroscopic data of **1** showed resemble B ring and C ring resonances, but different A ring resonances in comparison with tinospinoside C.[7] Namely, resonances of an epoxy moiety at $\delta_C$ 54.8 (C-2) and 53.8 (C-3), and an oxyganated quaternary carbon resonance at $\delta_C$ 83.9 (C-4) were observed in **1**, instead of resonances of C-2 oxyganated methine and C-3,4 olefin in tinospinoside C. The epoxy moiety was assigned at C-2 and C-3 by sequential $^1$H–$^1$H correlation spectroscopy (COSY) correlations between resonances of H-10, $H_{\alpha,\beta}$-1, H-2, and H-3, and a heteronuclear multiple bond connectivity (HMBC) correlation from $\delta_H$ 2.69 (H-10) to $\delta_C$ 54.8 (C-2) (Fig. 2). The oxylated quaternary carbon resonance at $\delta_C$ 83.9 was assigned to C-4, by HMBC correlations from $\delta_H$ 2.14 ($H_3$-19) and $\delta_H$

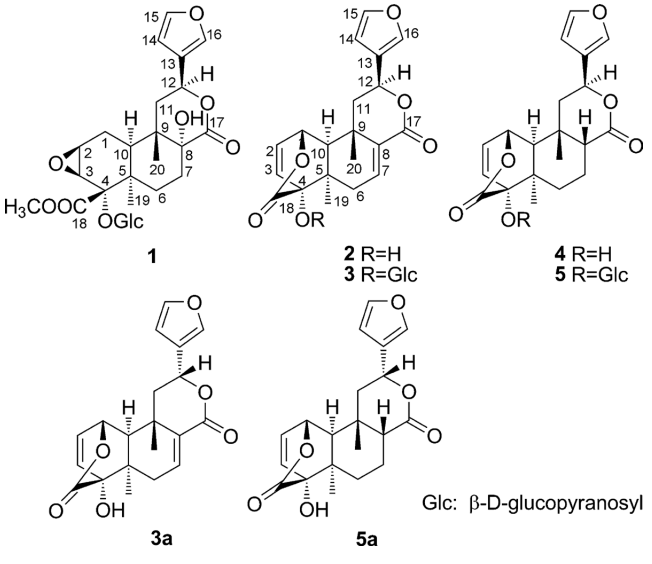


Fig. 1. Chemical Structures of Compounds **1**–**5**, **3a** and **5a**

The authors declare no conflict of interest.

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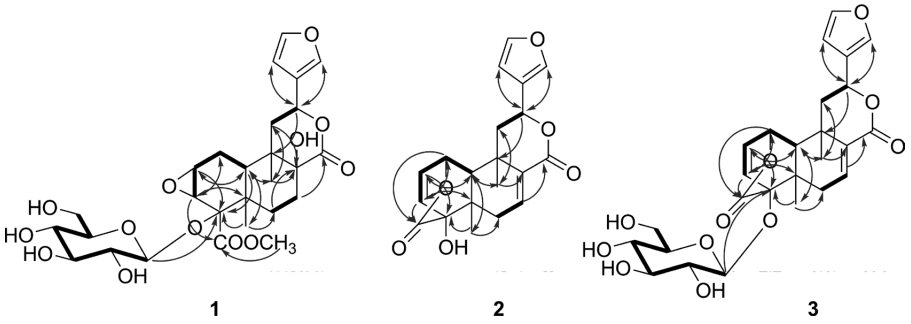


Fig. 2. Key $^1$H–$^1$H COSY and HMBC Correlations for Compounds **1**–**3**

2.69 (H-10) to $\delta_C$ 83.9 (C-4). The *O*-*β*-glucopyranosyl and carbonyl moieties connected to C-4 were determined by the HMBC correlations from $\delta_H$ 5.64 (Glc-H-1) to $\delta_C$ 83.9 (C-4), and $\delta_H$ 4.44 (H-3) to $\delta_C$ 174.4 (C-18), respectively. The C-8 hydroxy moiety was determined by HMBC correlations from $\delta_H$ 2.22, 2.03 ($H_{\alpha,\beta}$-6), 2.18, 1.97 ($H_{\alpha,\beta}$-11) and 1.23 ($H_3$-20) to $\delta_C$ 74.6 (C-8).

*cis* Relationship of H-10 and $CH_3$-19 was elucidated by the nuclear Overhauser effect spectroscopy (NOESY) correlation between $\delta_H$ 2.69 (H-10) and 2.14 ($H_3$-19), and *trans* relationship of H-10 and $CH_3$-20 by absence of the NOESY correlation between $\delta_H$ 2.69 (H-10) and 1.23 ($H_3$-20) (Fig. 3). This conclusion was further supported by an overall observation of the NOESY correlations between $H_3$-20/$H_\beta$-6, $H_\beta$-7, and $H_3$-19/$H_\alpha$-6, $H_\alpha$-7. The *β*-orientation of furan ring at C-12 was deduced from NOESY correlations between $\delta_H$ 1.23 ($H_3$-20) and 7.79 (H-16), $\delta_H$ 2.18 ($H_\alpha$-11) and 6.41 (H-12), and $\delta_H$ 1.23 ($H_3$-20) and 1.97 ($H_\beta$-11). Although the orientation of C-8 hydroxy moiety could not have been directly concluded only by NOESY correlations, a good agreement of the C ring NMR spectroscopic data between **1** and tinospinoside C indicated that they have same *β*-oriented C-8 hydroxy moiety. The NOESY correlations between $\delta_H$ 2.14 ($H_3$-19) and 5.64 (Glc-H-1), and $\delta_H$ 5.64 (Glc-H-1) and 4.44 (H-3) indicated their *cis* relationship, namely, *β*-oriented 2,3-epoxyl and 18-carbonyl moieties, and *α*-oriented 4-*O*-glucopyranosyl moiety. A clear 1,4-transannular correlation between $\delta_H$ 2.22 ($H_\beta$-6) and 1.23 ($H_3$-20) was also observed in the NOESY spectrum. Compound **1** adopting a unique all boat conformation for its tricyclic ring system was also indicated by energy calculation of the computer generated conformers using Discovery Studio Ver. 2.1 software, in which, a total energy for the conformer with boat-boat-boat ring conformation was calculated lower than that with chair-chair-boat conformation. This fact can

Table 1. $^1$H-NMR Spectroscopic Data ($\delta$) of Compounds **1**–**3** and **5a** (500 MHz in Pyridine-$d_5$)

| Position | **1** | **2** | **3** | **5a** |
|---|---|---|---|---|
| 1 | 2.29 (ddd, 15.5, 7.5, 3.2) | 5.41 (d, 5.0) | 5.37 (d, 5.0) | 5.24 (d, 5.1) |
| | 2.20[a)] | | | |
| 2 | 3.64 (dt, 4.6, 3.2) | 6.43 (dd, 7.8, 5.0) | 6.50 (dd, 8.2, 5.0) | 6.36 (dd, 8.0, 5.1) |
| 3 | 4.44 (d, 4.6) | 6.53 (dd, 7.8, 1.4) | 7.37 (dd, 8.2, 1.1) | 6.49 (dd, 8.0, 1.8) |
| 6 | 2.22[a)] (*β*) | 2.74 (dd, 15.8, 8.1) | 2.97 (dd, 16.1, 8.1) | 2.08 (brdd, 13.4, 7.1) |
| | 2.03 (dt, 12.8, 2.7) | 2.35 (dd, 15.8, 3.5, *β*) | 2.41 (dd, 15.2, 3.0, *β*) | 1.78 (ddd, 13.4, 6.6, 5.8, *β*) |
| 7 | 2.76 (m, *β*) | 7.27 (dd, 8.1, 3.5) | 7.19 (dd, 8.1, 3.0) | 2.84 (m) |
| | 2.22[a)] | | | 1.86 (m, *β*) |
| 8 | | | | 2.79 (dd, 10.5, 3.4) |
| 10 | 2.69 (m) | 2.08 (s) | 2.07 (s) | 1.58 (s) |
| 11 | 2.18 (dd, 13.3, 8.5) | 2.41 (d, 14.7) | 2.41 (d, 14.6) | 2.27 (dd, 14.2, 2.6, *β*) |
| | 1.97 (dd, 13.3, 8.5, *β*) | 2.13 (dd, 14.7, 11.5, *β*) | 2.11 (dd, 14.6, 11.5 *β*) | 2.18 (dd, 14.2, 11.7) |
| 12 | 6.41 (t, 8.5) | 5.35 (d, 11.5) | 5.33 (d, 11.5) | 5.67 (dd, 11.7, 2.6) |
| 14 | 6.66 (brd, 1.6) | 6.72 (t, 0.9) | 6.68 (t, 0.9) | 6.72 (dd, 1.9, 0.7) |
| 15 | 7.67 (t, 1.6) | 7.65 (t, 1.6) | 7.63 (d, 1.6) | 7.67 (t, 1.6) |
| 16 | 7.79 (brd, 1.6) | 7.78 (dd, 1.3, 0.7) | 7.72 (t, 0.7) | 7.84 (t, 0.7) |
| 19 | 2.14 (s) | 1.23 (s) | 1.39 (s) | 1.36 (s) |
| 20 | 1.23 (s) | 1.24 (s) | 1.23 (s) | 1.42 (s) |
| $OCH_3$ | 3.77 (s) | | | |
| Glc-1 | 5.64 (d, 7.1) | | 5.40 (d, 7.3) | |
| Glc-2 | 4.26[a)] | | 4.24 (t, 7.3) | |
| Glc-3 | 4.25[a)] | | 4.27[a)] | |
| Glc-4 | 4.15 (t, 8.5) | | 4.27 (t, 7.1) | |
| Glc-5 | 3.93 (ddd, 8.5, 6.5, 2.5) | | 4.02 (m) | |
| Glc-6 | 4.48 (dd, 11.5, 2.5) | | 4.54 (dd, 12.0, 1.3) | |
| | 4.22 (dd, 11.5, 6.5) | | 4.34 (dd, 12.0, 5.7) | |

*a*) Overlapped resonances.

be interpreted as that C5–C4 bond with large substitutions of $\beta$-D-glucopyranosyl moiety and carbonyl methyl ester at C-4, preferentially taking a more stable bowsprit orientation toward B ring, consequently resulting A ring in boat form. Although the absolute configurations of the asymmetric centers in **1** were not determined, it was assumed to have the same absolute configurations in the diterpene backbone with those structure and biosynthesis related *cis*-clerodane diterpenoids isolated in this study and in a previous study.[7)]

Tinospin E (**2**) and tinospinoside E (**3**) were both isolated as colorless solid. The molecular formulas were established as $C_{20}H_{20}O_6$ and $C_{26}H_{30}O_{11}$, respectively by the HR-FAB-MS data. Compounds **2** and **3** were also *cis*-clerodane type diterpenoids, which were indicated by the typical C-12 furan ring downfield resonances in the $^1$H-NMR spectrum (Table 1). The superimposable $^1$H- and $^{13}$C-NMR spectroscopic data of **3** and **2**, except for resonances of an additional $\beta$-glucopyranosyl moiety in **3** suggested that **3** was a $\beta$-glucopyranoside of **2** (Tables 1, 2). Thus, structural elucidation of **2** was carried out first.

In the $^1$H-NMR spectrum of **2**, in addition to the 12-oxymethine proton resonance at $\delta_H$ 5.35, another oxymethine proton at $\delta_H$ 5.41 was also observed, which was assigned to be H-1 by the $^1$H–$^1$H COSY correlation between $\delta_H$ 5.41 (H-1) and 2.08 (H-10). In the $^1$H–$^1$H COSY spectrum, H-1 was showed to be correlated with a pair of coupled olefin proton resonances at $\delta_H$ 6.43 (H-2) and $\delta_H$ 6.53 (H-3), suggesting presence of an olefin moiety at C-2 and C-3. Another downfield olefin proton resonance at $\delta_H$ 7.27 was assigned to be H-7 by the HMBC correlation from $\delta_H$ 7.27 (H-7) to C-17 lactone carbonyl carbon at $\delta_C$ 166.8 (Fig. 2). The HMBC correlation from $\delta_H$ 5.41 (H-1) to carbonyl carbon at $\delta_C$ 175.3, suggested the lactone between C-1 and C-4. The hydroxylated quaternary carbon C-4 was assigned by HMBC correlations from $\delta_H$ 1.23 ($H_3$-19) and 6.43 (H-2) to its carbon resonance at $\delta_C$ 81.3. By these analyses, the gross structure of **2** was elucidated.

In comparison to the $^{13}$C-NMR spectroscopic data of **2** (Table 2), a glycosylation shift (+5.2 ppm) was observed at C-4 in **3**. The $\beta$-D-glucopyranosyl moiety at C-4 was further determined by the HMBC correlation from $\delta_H$ 5.40 (Glc-H-1) to $\delta_C$ 86.5 (C-4).

On acid hydrolysis of **3**, it afforded a pair of compounds. One was **2**, and the other was identified to be fibleucin (**3a**), a known compound which was firstly isolated from *Fibraurea chloroleuca* MIERS,[11)] and its structure was well established by X-ray analysis.[12)] Compound **2** have very similar but different $^1$H- and $^{13}$C-NMR spectroscopic data in comparison with **3a**. Compound **2** has same relative configurations for A ring and B ring as **3a**, since same NOESY correlations between H-3/$H_3$-19, H-10/$H_3$-19, $H_\beta$-6/$H_3$-20, and $H_\alpha$-6/$H_3$-19 were observed (Fig. 3). The opposite H-12 orientations of **2** and **3a** were deduced from their different NOESY correlations between H-12/H-10 for **2**, and H-12/$H_3$-20 for **3a**. Thus, the structure of **2** was determined to be with a $\beta$-orientated 12-furan ring.

It is necessary to note the production of a pair of 12-epimeric compounds, columbin (**4**) and a new compound **5a** after acid hydrolysis of columbin glucoside (**5**). The

Table 2. $^{13}$C-NMR Spectroscopic Data ($\delta$) of Compounds **1**–**3** and **5a** (125 MHz in Pyridine-$d_5$)[a)]

| Position | **1** | **2** | **3** | **5a** |
|---|---|---|---|---|
| 1 | 22.1 | 74.6 | 73.8 | 74.6 |
| 2 | 54.8 | 130.7 | 131.2 | 129.4 |
| 3 | 53.8 | 138.6 | 134.0 | 138.5 |
| 4 | 83.9 | 81.3 | 86.5 | 81.7 |
| 5 | 42.6 | 43.2 | 44.5 | 38.8 |
| 6 | 29.1 | 31.7 | 31.7 | 26.5 |
| 7 | 27.0 | 141.3 | 141.2 | 16.8 |
| 8 | 74.6 | 137.9 | 137.8 | 42.9 |
| 9 | 40.6 | 37.2 | 37.2 | 36.4 |
| 10 | 45.5 | 54.9 | 54.3 | 55.2 |
| 11 | 43.8 | 44.4 | 44.2 | 48.8 |
| 12 | 71.1 | 70.2 | 70.2 | 70.1 |
| 13 | 127.8 | 124.6 | 124.5 | 125.3 |
| 14 | 109.6 | 109.5 | 109.4 | 109.5 |
| 15 | 144.4 | 144.1 | 144.0 | 144.3 |
| 16 | 140.3 | 140.8 | 140.8 | 140.7 |
| 17 | 173.5 | 166.8 | 166.7 | 175.2 |
| 18 | 174.4 | 175.3 | 171.8 | 175.6 |
| 19 | 29.0 | 26.5 | 26.5 | 24.1 |
| 20 | 22.4 | 25.5 | 25.6 | 28.8 |
| $OCH_3$ | 52.1 | | | |
| Glc-1 | 100.5 | | 101.8 | |
| Glc-2 | 75.8 | | 75.3 | |
| Glc-3 | 78.8 | | 78.7 | |
| Glc-4 | 72.1 | | 71.5 | |
| Glc-5 | 78.5 | | 78.7 | |
| Glc-6 | 63.0 | | 62.4 | |

*a*) Assignment were based on DQF-COSY, TOCSY, DEPT, NOESY, and HMBC experiments.

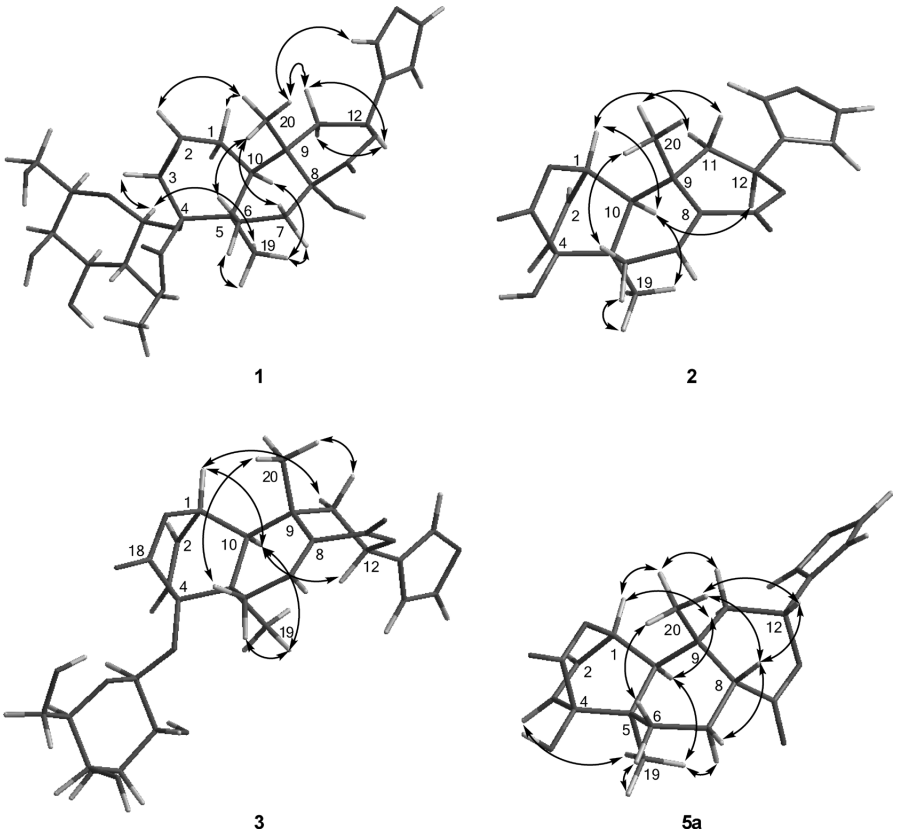


Fig. 3. Key NOESY Correlations for Compounds **1**–**3** and **5a**

structure of **5a** was determined to be 12-epimer of **4** by the characteristic NOESY correlation between H-12/$H_3$-20 (Fig. 3), and therefore was given the trivial name 12-*epi*-columbin.

Compounds **1**, **2**, **3**, **4**, **5** and **5a** were evaluated for their inhibitory activities on nitric oxide production in LPS and INF$\gamma$-activited J774.1 macrophage-like cells. Only compound **5a** showed nitric oxide inhibitory activity with the $IC_{50}$ value of 84 $\mu$M, while the $IC_{50}$ value of the positive control $N^G$-monomethyl-L-arginine (L-NMMA) was 43.1 $\mu$M in this assay. The highest concentration (100 $\mu$M) of other compounds did not reach the half maximal inhibitory concentration.

## Experimental

**General** Optical rotations were measured with a JASCO P-2200 polarimeter in a 0.5-dm cell. IR spectra were measured on a JASCO FT/IR-4100 fourier transform infrared spectrometer by the KBr disk method. Circular dichroism (CD) spectra were recorded on a JASCO J-720W spectropolarimeter. NMR spectra were measured on a JEOL ECP-500 spectrometer with TMS as the internal reference, and the chemical shifts are expressed in $\delta$ (ppm). Electrospray ionization (ESI)-MS was recorded on an LCQ mass analyzer and HR-FAB-MS on a JEOL JMS-700 MStation spectrometer. Preparative HPLC was performed on a JASCO PU-2086 HPLC system, equipped with a JASCO RI-2301 Differential Refractometer detector and an YMC-Pack RP-$C_{18}$ column (150×20 mm i.d.). Silica gel (Silica Gel 60N, Kanto Chemical Co., Inc., Tokyo, Japan) was used for column chromatography. TLC was conducted using Silica gel 60 $F_{254}$ plates (E. Merck). GLC was carried out on a Perkin-Elmer Clarus 500 GC-MS instrument. Absorbance for bioassay was measured using ImmunoMini NJ-2300 microplate reader.

**Plant Material** Plant collection and identification of the roots of *T. sagittata* were same as previously described.[7]

**Extraction and Isolation** The MeOH extract and EtOAc fraction were obtained by the same procedure as previously reported.[7] The EtOAc fraction (37.8 g) was subjected to silica gel column chromatography with a gradient of $CHCl_3$–MeOH–$H_2O$ to give five fractions (1–5). Fraction 4 (10.4 g) was then subject to ODS column chromatography eluting with MeOH–$H_2O$ (2 : 3). Combination of the eluate by TLC afforded seven sub-fractions S1–S7. Final purification was carried out by preparative HPLC with an ODS column (YMC-Pack Pro C18, 150×20 mm). The flow rate for HPLC was 5.0 mL/min. In detail, from sub-fraction S3 (1.6 g), with 25% $CH_3CN$, compounds **3** (20 mg, $t_R$ 32 min) and **5** (36 mg, $t_R$ 36 min) were isolated; from sub-fraction A4 (0.8 g), with 30% $CH_3CN$, compound **1** (24 mg, $t_R$ 22 min) was isolated; from sub-fraction A6, with 30% $CH_3CN$, compounds **2** (6 mg, $t_R$ 58 min) and **4** (17 mg, $t_R$ 62 min) were isolated.

Tinospinoside D (**1**): Colorless solid. $^1$H- and $^{13}$C-NMR spectroscopic data, see Tables 1 and 2. IR (KBr) cm$^{-1}$: 3423, 2929, 2853, 1735, 1720, 1637, 1628, 1458, 1384, 1255, 1024. HR-FAB-MS *m/z*: 591.2064 $[M+Na]^+$ (Calcd for $C_{27}H_{36}O_{13}Na$: 591.2053). ESI-MS *m/z*: 591.3 $[M+Na]^+$. $[\alpha]_D^{22}$ −28.2 ($c$=0.21, MeOH). CD (MeOH): $[\theta]^{22}$ (nm) −1672 (209), 4762 (235).

Tinospin E (**2**): Colorless solid. $^1$H- and $^{13}$C-NMR spectroscopic data, see Tables 1 and 2. IR (KBr) cm$^{-1}$: 3435, 2928, 1707, 1638, 1542, 1458, 1383, 1272, 1152, 1024. HR-FAB-MS *m/z*: 357.1325 $[M+H]^+$ (Calcd for $C_{20}H_{21}O_6$: 357.1338). ESI-MS *m/z*: 379.1 $[M+Na]^+$. $[\alpha]_D^{22}$ +32.8 ($c$=0.22, MeOH). CD (MeOH): $[\theta]^{22}$ (nm) −8508 (207), 47608 (223), −23207 (247).

Tinospinoside E (**3**): Colorless solid. $^{1}$H- and $^{13}$C-NMR spectroscopic data, see Tables 1 and 2. IR (KBr) cm$^{-1}$: 3423, 1706, 1638, 1542, 1458, 1383, 1271, 1058, 1024. HR-FAB-MS *m/z*: 541.16782 $[M+Na]^+$ (Calcd for $C_{26}H_{30}O_{11}Na$: 541.16858). ESI-MS *m/z*: 541.2 $[M+Na]^+$. $[\alpha]_D^{22}$ +28.7 ($c$=0.22, MeOH). CD (MeOH): $[\theta]^{22}$ (nm) −20993 (209), 33493 (227), −25623 (247).

**Acid Hydrolysis** A solution of **3** (5 mg) in 1 M HCl (dioxane/$H_2O$, 1 : 1, 1 mL) was heated at 80°C for 3 h, Then treated with a Diaion HP-20 column, eluted with $H_2O$ and MeOH to afford subfractions E1 and E2. Subfraction E2 was further purified by reverse-phase HPLC with $CH_3CN$–$H_2O$ (35 : 65) to give the tinospin E (**2**) (1.2 mg) and fibleucin (1 mg). Acid hydrolysis of **5** were carried out using the same conditions, to give columbin (**4**) (0.5 mg) and **5a** (1.2 mg).

**5a**: Colorless solid. $^{1}$H- and $^{13}$C-NMR spectroscopic data, see Tables 1 and 2. $[\alpha]_D^{17}$ +20.3 ($c$=0.12, MeOH). CD (MeOH): $[\theta]^{22}$ (nm) −32629 (205), 18344 (232).

**Determination of Sugar Absolute Configuration** Glucosyl moieties in compounds **1** and **3** were determined to be D-form by the same method described in our previous study.[6)]

**Computer Assistant Chemical Structure Calculation** The planar structure was constructed by Chem Draw Ultra 10.0 software (Chemical Structure Drawing Standard; Cambridge Soft Corporation, U.S.A.). A standard dynamics cascade protocol was used for calculating the three-dimensional structure, by five steps: Minimization, Minimization 2, Heating, Equilibration and Production. The protocol provides the total energy values to standardize validation of any conformations. The conformation generation algorithms are Windows OS compatible; in the current study three-dimensional conformations were generated on a Intel® Core™ i7-920 processor with 3GB RAM and NVIDIA® Quadro® FX1700 512MB, running Windows XP Pro SP3.

**Nitric Oxide Inhibitory Assay** The J774.1 cell line was purchased from Riken Cell Bank (Tsukuba, Japan) and cultured in RPMI 1640 medium supplemented with penicillin G (100 units/mL), streptomycin (100 $\mu$g/mL) and 10% FBS. The cells were seeded in 96-well plastic plates with a density of $1\times10^6$ cells/well and allowed to adhere for 2 h at 37°C in a humidified atmosphere containing 5% $CO_2$. Then the medium was replaced with fresh medium containing LPS (10 ng/mL) and INF$\gamma$ (40 U/mL) together with test compounds at various concentrations, and the cells were further incubated for another 48 h. Nitric oxide production was determined by measuring the accumulation of nitrite in the culture supernatant using Griess reagent. Briefly, 100 $\mu$L of the supernatant from incubates were mixed with equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylene–diamide dihydrochloride in 2.5% $H_3PO_4$) and were allowed to stand for 10 min at 37°C in a humidified atmosphere containing 5% $CO_2$. Cell viability was determined using the mitochondrial respiration-dependent MTT reduction method. Absorbance at 550 nm was measured using a microplate reader.

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