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Spiroconyone A, a new phytosterol with a spiro [5,6] ring system from *Conyza japonica*†

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Spiroconyone A (**1**), the first rearranged phytosterol featuring an unusual spiro [5,6] ring system, and nine known compounds (**2–10**) were isolated from the aerial parts of *Conyza japonica*. The structure of **1** was elucidated through spectroscopic methods, and its absolute configuration was determined by single-crystal X-ray diffraction analysis. Enzyme-based assay revealed that spiroconyone A showed weak TDP1 inhibition and compounds **7** and **10** showed TDP1 inhibition with IC_{50} values of 36 μM and 16 μM, respectively. MTT assay indicated that **7** and **10** showed a strong synergistic effect with the clinical TOP1 inhibitor topotecan in MCF-7 cells. Compound **5** displayed the most potent cytotoxicity against MCF-7 cells with a GI_{50} value of 3.3 μM. Furthermore, a hypothetical biosynthetic pathway for **1** was proposed. This work provides valuable information that the secondary metabolites from *Conyza japonica* could be developed as anticancer agents.

Introduction

Tyrosyl–DNA phosphodiesterase 1 (TDP1), a member of the phospholipase D superfamily, has been found to be a DNA repair enzyme, which can repair DNA topoisomerase 1 (TOP1) mediated DNA damage.[1,2] Studies have demonstrated that overexpression of TDP1 reduces the sensitivity of cancer cells to TOP1 inhibitors.[3,4] Conversely, cells deficient in TDP1 and TDP1 knockout mice show hypersensitivity to camptothecin, a well-known TOP1 inhibitor.[5,6] And TDP1 inhibitors have the ability to sensitize cancer cells to TOP1 inhibitors.[7–9] These studies suggested that TDP1 is a promising target for the development of antitumor agents.[10] Therefore, the discovery of TDP1 inhibitors has attracted much attention. Natural compounds are important sources for the discovery of novel bioactive agents because of their significant chemical diversity and their complementarity to biological targets. In our research for TDP1 inhibitors from natural products, the petroleum ether extract of the aerial parts of *Conyza japonica* (Thunb.) Less. showed TDP1 inhibitory activity and synergistic activity with the TOP1 inhibitor topotecan (TPT) in MCF-7 cells (Fig. S1†), which inspired us to study its secondary metabolites.

C. japonica, a species of the family Compositae, is an annual or biennial herb widely distributed in the southern regions of China.[11] Previous chemical investigations on *C. japonica* showed there are a variety of constituents such as strictic acid,[12] flavonoids and their glycosides,[13] phenylpropanoid glycosides,[14] triterpenoid saponins,[15] diterpenoids and their glycosides,[16] and sesquiterpenoids.[17] Our phytochemical research on the secondary metabolites of the aerial parts of *C. japonica* led to the discovery of a new skeleton phytosterol spiroconyone A (**1**) and nine known compounds (**2–10**). In addition, TDP1 inhibition and the cytotoxicity of the isolates were tested. The synergistic effect of TDP1 inhibitors **7** and **10** with topotecan was also evaluated. Herein, we report the isolation, structural elucidation and hypothetical biogenetic pathway of the new skeleton phytosterol, and the biological evaluation of all isolates.

Results and discussion

The petroleum ether extract from the aerial parts of *C. japonica* was fractionated by silica gel column chromatography (CC) and further purified using various columns to obtain ten compounds, including a new skeleton phytosterol, named spiroconyone A (**1**), and nine known compounds (Fig. 1), which

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†Electronic supplementary information (ESI) available: Potentiation of the cytotoxic action of TPT by a petroleum ether extract of *C. japonica* in MCF-7 cells, TDP1 inhibition curves of **7** and **10**, IR, CD, HRESIMS, 1D and 2D NMR spectra of compound **1**. CCDC 1983137. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/d0ob00666a

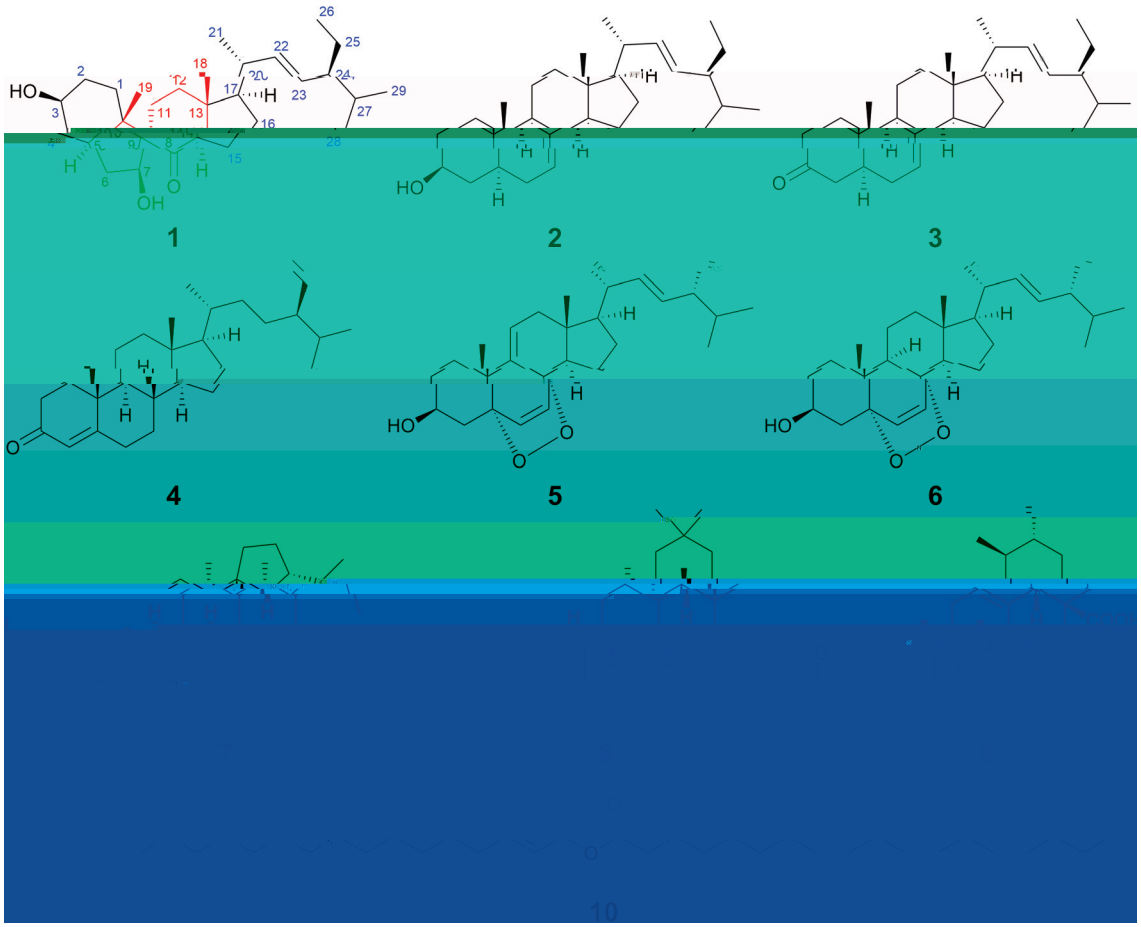

Fig. 1 Structures of compounds **1–10**.

were identified as spinasterol (**2**),[18] α-spinasterone (**3**),[19] β-sitostenone (**4**),[20] 5,8-epidioxy-(3β,5α,8α,22*E*)-ergosta-6,9(11),22-trien-3-ol (**5**),[21] (3β,5α,8α,22*E*)-5,8-diepoxy-ergosta-6,22-dien-3-ol (**6**),[21] simiarenol (**7**),[22] epifriedelanol (**8**),[23] 3β-*O*-acetyl ursolic acid (**9**),[24] phytyl (9*Z*,12*Z*,15*Z*)-linolenate (**10**),[25] by comparing the detailed NMR spectroscopic data with those reported in the literature.

Spiroconyone A (**1**) was obtained as colorless crystals (MeOH). Its HRESIMS gave an ion peak at *m*/*z* 445.3660 [M + H]$^+$, suggesting a molecular formula of $C_{29}H_{48}O_3$ (calcd for $C_{29}H_{49}O_3$, 445.3676) with six degrees of unsaturation. The IR spectrum showed characteristic absorption for the hydroxy group (3372 cm^{-1}) and the carbonyl group (1702 cm^{-1}). The ^{1}H NMR spectral data (Table 1) displayed signals of six methyl groups [δ_H 0.62 (s), 0.79 (d, J = 6.3 Hz), 0.80 (t, J = 7.5 Hz), 0.84 (d, J = 6.3 Hz), 1.03 (d, J = 6.6 Hz), and 1.29 (s)], two trans-olefinic protons [δ_H 5.04 (1H, dd, J = 15.2, 8.6 Hz) and 5.14 (1H, dd, J = 15.2, 8.5 Hz)], and two oxymethine protons [δ_H 3.54 (m) and 4.45 (m)]. The ^{13}C NMR and DEPT spectroscopic data (Table 1) showed the presence of 29 carbon resonances, including one ketone (δ_C 211.0), one double bond (δ_C 137.5 and 130.5), six methyl groups, nine methylenes, eight sp^3 methines (two oxygenated), and three sp^3 quaternary carbons.

Table 1 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of **1** in $CDCl_3$ (δ in ppm)

No.	δ_H (J in Hz)	δ_C, type	No.	δ_H (J in Hz)	δ_C, type
1α	1.37, m[a]	30.4, CH_2	15α	1.41, m	19.1, CH_2
1β	1.74, m		15β	1.78, m	
2α	1.77, m	30.7, CH_2	16α	1.76, m	29.0, CH_2
2β	1.59, m		16β	1.36, m	
3	3.54, m	71.6, CH	17	1.48, m	57.2, CH
4α	1.82, m	34.2, CH_2	18	0.62, s	12.6, CH_3
4β	1.50, m		19	1.29, s	16.2, CH_3
5	1.53, m	41.0, CH	20	2.03, m	40.4, CH
6α	2.30, dt (13.8, 7.6)	39.6, CH_2	21	1.03, d (6.6)	21.2, CH_3
			22	5.14, dd (15.2, 8.5)	137.5, CH
6β	1.35, m				
7	4.45, m	77.2, CH	23	5.04, dd (15.2, 8.6)	130.5, CH
8		211.0, C			
9		65.9, C	24	1.54, m	51.4, CH
10		44.0, C	25a	1.44, m	25.5, CH_2
11α	1.53, m	30.1, CH_2	25b	1.18, m	
11β	1.88, m		26	0.80, t (7.5)	12.4, CH_3
12α	1.77, m	36.5, CH_2	27	1.53, m	32.0, CH
12β	1.93, m		28	0.84, d (6.3)	21.2, CH_3
13		51.3, C	29	0.79, d (6.3)	19.1, CH_3
14	2.87, dd (11.5, 7.2)	62.5, CH			

[a] "m" means multiplet or overlapped with other signals.

The ketocarbonyl and one double bond unit accounted for two degrees of unsaturation. The remaining four degrees of unsaturation were due to a tetracyclic skeleton.

Construction of the 2D structure for **1** was accomplished by interpretation of 2D NMR spectral data. The ^{1}H–^{1}H COSY correlations between H-2/H-3/H-4, H-6/H-7, H-14/H-15, (H-17) H-21/H-20/H-22/H-23/H-24/H-25/H-26, and H-28/H-27/H-29 as drawn in red bold bonds (Fig. 2) built up five spin–spin coupling systems. The HMBC correlations from H-19 to C-1 (δ_C 30.4), C-5 (δ_C 41.0), and C-10 (δ_C 44.0), H-3 to C-1 (δ_C 30.4), H-2 to C-10 (δ_C 44.0), and H-4 to C-5 (δ_C 41.0) generated a six-membered carbon ring A. The HMBC correlations from H-19 to C-5 (δ_C 41.0), C-9 (δ_C 65.9), and C-10 (δ_C 44.0), H-5 to C-7 (δ_C 77.2), and H-6 to C-9 (δ_C 65.9) and C-10 (δ_C 44.0) generated a five-membered carbon ring B, which was fused to ring A with C-5 and C-10. The HMBC correlations from H-18 to C-13 (δ_C 51.3), C-14 (δ_C 62.5), and C-17 (δ_C 57.2), H-15 to C-17 (δ_C 57.2), and H-16 to C-14 (δ_C 62.5) generated a five-membered carbon ring D. The HMBC correlation from H-15 to C-8 (δ_C 211.0) and the chemical shift of H-14 (δ_H 2.87) indicated that C-8 connected with C-14. The HMBC correlations from H-18 to C-13 (δ_C 51.3), C-14 (δ_C 62.5), and C-12 (δ_C 36.5), H-11 to C-8 (δ_C 211.0) and C-13 (δ_C 51.3), and H-12 to C-9 (δ_C 65.9) and C-14 (δ_C 62.5) generated a six-membered carbon ring C, which was fused to ring D with C-13 and C-14. The HMBC correlations from H-28 and H-29 to C-24 (δ_C 51.4) confirmed the connection of C-27 with C-24, implying that C-20 of the side chain was connected to ring D at C-17, which was consistent with the ^{1}H–^{1}H COSY result. Based on the above-mentioned observations, it can be inferred that rings B and C should be connected at C-9, forming a unique spiro [5,6] ring system, which was also confirmed by the HMBC cross peak between H-7 and C-11 and the chemical shift of C-9 (δ_C 65.9). Therefore, a planar structure of **1** was elucidated as shown in Fig. 1.

Following the establishment of the planar structure, the relative configuration of compound **1** was then assigned by analysis of NOESY data (Fig. 2). The NOESY correlations of H-7/H-5/H-3/H-4α (δ_H 1.82) and H-19/H-4β (δ_H 1.50) indicated that OH-3, OH-7, H-4β, and Me-19 were on the same side of rings A and B, assigned the β-orientation, while H-3, H-4α, H-5, and H-7 were on the opposite side. The NOESY correlation of H-7 and H-14 suggested that OH-7 and the ketocarbonyl were on the same side of the spiral carbon C-9. The typical NOESY correlation of H-14 and H-17 indicated that they were on the same side of rings C and D, assigned the α-orientation. The NOESY correlation of H-18 and H-20 indicated that Me-18 and the side chain were on the same side, assigned the β-orientation. The configuration of the remaining chiral center C-24 could not be assigned by the NOESY experiment because the ethyl and isopropyl groups could rotate in solution. Fortunately, the qualified crystals of **1** were obtained in MeOH, which allowed the successful performance of the single-crystal X-ray diffraction (XRD) analysis, using the Cu Kα radiation [flack parameter = −0.02(4)] (Fig. 3). This not only confirmed the planar structure of compound **1**, but also unambiguously determined its absolute configuration as 3*S*,5*R*,7*S*,9*R*,10*S*,13*R*,14*R*,17*R*,20*R*,24*S*. The trivial name spiroconyone A was given for compound **1**.

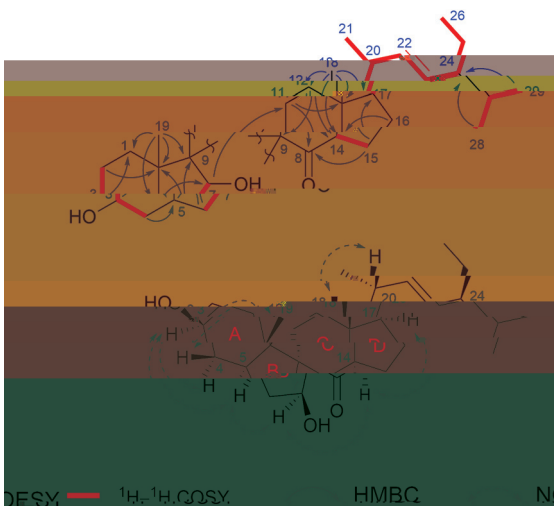

Fig. 2 The key 2D NMR correlations of **1**.

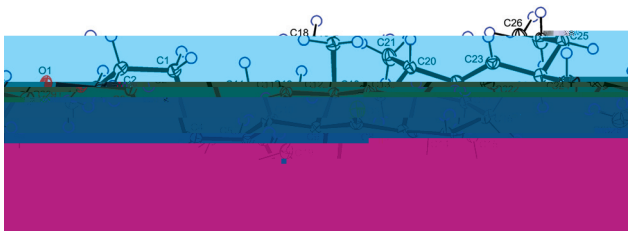

Fig. 3 ORTEP diagram of **1**.

As shown in Fig. 1, spiroconyone A possesses an unusual spiro [5,6] ring system. With reference to the rearrangement of triterpenoid,[26] and based on the co-isolated known analogue spinasterol (**2**), the plausible biosynthetic route for **1** was proposed as shown in Scheme 1. Briefly, **2** served as the precursor, which underwent hydroxylation at C-9 to afford hydroxylated intermediate **i**, which further underwent epoxidation, and subsequent hydrolysis to afford intermediate **ii**. Finally, a pinacol rearrangement occurred starting from 8,9-diol under acidic conditions to afford **1**. To the best of our knowledge, several abnormal rearranged steroids have been reported, and the rearranged products are characterized with a seven-membered or spiro [4.4] ring system.[27–30] Meanwhile, there are a few rearranged products containing the spiro [5,6] ring system reported, and all of them are rearranged from triterpenes.[26,31–36] However, spiroconyone A is the first example of a rearranged phytosterol with a spiro [5,6] ring system.

The TDP1 inhibitory activity of the isolates was firstly screened through a fluorescence assay at one dose concentration (100 μM) using our reported TDP1 inhibitor, oxynitidine derivative **41a**, as the positive control.[9] As shown in Table 2, six compounds **1**, **3**, **4**, **7**, **9**, and **10** showed TDP1 inhi-

bition with the percentage inhibitions ranging from 15% to 95% at 100 μM. The novel spiroconyone A showed weak TDP1 inhibition (29% at 100 μM). Because their percentage inhibitions were more than 50%, compounds **7** and **10** were further tested for IC_{50} values, defined as the compound concentration that results in 50% enzyme activity inhibition. Linolenate (**10**) showed good TDP1 inhibition with an IC_{50} value of 16 μM (the inhibitory curves in Fig. S2†). The IC_{50} value of simiarenol (**7**) was 36 μM.

The cytotoxicity of the isolates was evaluated through MTT assay against two human cancer cell lines, namely breast cancer MCF-7 and lung cancer A549 cell lines. The GI_{50} values, defined as the compound concentration resulting in 50% cell growth inhibition, are summarized in Table 2. Compound **5** showed the most potent cytotoxicity against MCF-7 cells with a GI_{50} value of 3.3 μM. Compound **9** showed high cytotoxicity against MCF-7 cells (GI_{50} = 6.5 μM). The two active compounds **7** and **10** against TDP1 enzyme showed weak cytotoxicity against these two cancer cell lines.

To evaluate the synergistic activity of compounds **7** and **10** with TOP1 inhibitors, the cytotoxicity of the combination of **7** or **10** with TPT was evaluated in MCF-7 cells by MTT assay. After being incubated for 72 h at 37 °C, the cytotoxicity of TPT against MCF-7 cells significantly increased in the presence of **7** or **10** (Fig. 4, left). The combination analyses of the dose–response data were presented in the form of Combination Index (CI) *vs.* Fraction Affected (Fa) plots (Fig. 4, right), demonstrating that **7** possessed a strong synergistic effect with TPT (CI values < 0.3) and **10** also showed a synergistic effect (CI values < 1) in MCF-7 cells.

Experimental

G n ral xp rim ntal proc dur s

C(ed)6na1DP9442.4((CI)18.4(xor)-x.4((Fig.)]TJ/F131247.061nam2165(vs.)Tj/

diffractometer. A SEP LC-52 equipped with UV-200 and a Dr Maisch C_{18} column (250 × 10 mm, S-5 μm) was used for semi-preparative HPLC separation. RP-C_{18} silica gel (YMC, 50 μm), MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan), silica gel (100–200 and 200–300 Mesh, Qingdao Marine Chemical, Inc., China), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden) were used for column chromatography. TLC was carried out on silica gel GF254 plates (Qingdao Marine Chemical, Inc., China). All solvents were analytical grade (Guangzhou Chemical Reagents Company, Ltd). The TDP1 protein was expressed and purified from *Escherichia coil* BL21 (DE3) cells in our lab. A549 and MCF-7 cell lines were purchased from the China Center for Type Culture Collection.

Plant mat rial

The aerial parts of *C. japonica* were collected in Quanzhou, Fujian Province, People's Republic of China in May 2018. The plant was identified by Dr Gui-Hua Tang from the School of Pharmaceutical Sciences, Sun Yat-sen University. A voucher specimen (No. 2018051101) was deposited at the School of Pharmaceutical Sciences, Sun Yat-sen University.

Extraction and isolation

The air-dried and powdered aerial parts of *C. japonica* (20 kg) were extracted with 95% EtOH (40 L × 3) at room temperature to obtain a crude extract (971 g), which was suspended in 10 L H_2O and then partitioned continuously with petroleum ether (10 L × 3) and EtOAc (10 L × 3) to afford the petroleum ether extract (325 g) and EtOAc extract (85 g). The petroleum ether extract was subjected to column chromatography over silica gel with a petroleum ether–EtOAc mixture (gradient from 1 : 0 to 0 : 1, v/v) and $CHCl_3$–MeOH (gradient from 1 : 0 to 0 : 1, v/v) to give twelve fractions (Fr.1–Fr.12). Fr.3 was separated using a silica gel column eluting with petroleum ether–EtOAc to afford Fr.3.1–3.3, which were analyzed by using TLC. Fr.3.1 was fractionated using RP-C_{18} silica gel and silica gel columns (petroleum ether–acetone, 50 : 1, v/v) to afford Fr.3.1.1–Fr.3.1.2. Fr. 3.1.1 was separated using a silica gel column (CH_2Cl_2) to yield **3** (11.2 mg). Fr.3.1.2 was separated using a silica gel column (petroleum ether–acetone, 50 : 1, v/v) to afford **4** (9.3 mg). Fr.3.2 was separated using a Sephadex LH-20 column (CH_2Cl_2–MeOH, 1 : 1, v/v) to afford **8** (13.0 mg). Fr.3.3 was separated using a Sephadex LH-20 column (CH_2Cl_2–MeOH, 1 : 1, v/v) followed by a silica gel column (petroleum ether–EtOAc, 1 : 1, v/v) to afford **7** (6.3 mg). Fr.5 was separated by using a silica gel column eluting with petroleum ether–EtOAc and further purified by using an RP-C_{18} silica gel column to yield **10** (96.5 mg). Fr.7 was fractionated using a silica gel column eluting with a CH_2Cl_2–MeOH mixture (gradient from 1 : 0 to 10 : 1, v/v) to give Fr.7.1–7.2. Fr.7.1 was separated by using a silica gel column, and then recrystallized from methanol to yield **2** (257.7 mg). Fr.7.2 was separated using an MCI gel column (MeOH/H_2O, 50% → 100%, v/v) to afford Fr.7.2.1–7.2.2. Fr.7.2.1 was separated using a silica gel column and further purified by semi-preparative HPLC (92% MeOH in H_2O, 3.0 mL min^{-1}) to yield **1** (9.0 mg, t_R = 18.1 min). Fr.7.2.2 was separated using a silica gel column (CH_2Cl_2–MeOH, 100 : 1, v/v) and further purified by semi-preparative HPLC (95% MeOH in H_2O, 3.0 mL min^{-1}) to yield **5** (3.6 mg, t_R = 20.2 min) and **6** (24.6 mg, t_R = 24.8 min). Fr.8 was separated using an MCI gel column (MeOH/H_2O, 30% → 100%, v/v), followed by a Sephadex LH-20 column (MeOH) and a silica gel column (CH_2Cl_2–MeOH, 200 : 1, v/v) to yield **9** (10.3 mg).

Spiroconyon A (1). Colorless crystals; mp: 147.6–149.1 °C; $[\alpha]_D^{20}$ −3.7 (*c* 0.30, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.50) nm, 223 (3.00) nm; IR (KBr) ν_{max} 3372, 2957, 2919, 1702, 1461, 1384, 1178 cm^{-1}; For ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) data, see Table 1; HRESIMS *m*/*z* 445.3660 $[M + H]^+$ (calcd for $C_{29}H_{49}O_3$, 445.3676).

X-ray crystallographic data for spiroconyon A. Spiroconyone A was crystallized from methanol to give colorless crystals. $C_{29}H_{48}O_3$·MeOH (M = 476.71 g mol^{-1}): orthorhombic, space group $P2_12_12_1$ (no. 19), a = 6.61847(5) Å, b = 9.54000(6) Å, c = 44.1844(3) Å, V = 2789.81(3) $Å^3$, Z = 4, T = 100.00 (10) K, μ (Cu Kα) = 0.566 mm^{-1}, D_{calc} = 1.135 g cm^{-3}, 28 419 reflections measured (8.004° ≤ 2Θ ≤ 153.768°), 5762 unique (R_{int} = 0.0280, R_{sigma} = 0.0186) which were used in all calculations. The final R_1 was 0.0279 [$I > 2\sigma(I)$] and wR_2 was 0.0720 (all data). The flack parameter is −0.02(4). Crystallographic data for spiroconyone A have been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 1983137†).

TDP1 Inhibition assay

The TDP1 fluorescence assay was conducted according to our reported method.[9] Briefly, the reaction mixture (50 μL) containing 20 μL of TDP1 solution (0.02 μL of purified TDP1 (100 nM) in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, and 1 mM DTT) was dispensed into a white 384-well plate. The tested compound solution in DMSO (5 μL) was pinned into assay plates and incubated at 37 °C for 30 min. Then the plate was read using a Molecular Devices at Ex_{485}/Em_{510} nm to identify false-positive compounds that had autofluorescence. The linear oligonucleotide substrate (5′-*FAM*-AGGATCTAAAAGACTT-*BHQ*-3′, 25 μL, 35 nM) was dispensed into the wells to start the reaction. The plate was immediately read three times with Ex_{485}/Em_{510} nm. The percentage of TDP1 inhibition was calculated by comparing the rate of enhancement of the fluorescence intensity for the compound-treated wells to that of DMSO control wells.

C ll cultur and cytotoxicity assays

The cells were cultured on DMEM medium at 37 °C under a humidified atmosphere with 5% CO_2. The compounds were evaluated for their cytotoxicity through MTT assay against two human cancer cell lines, namely MCF-7 and A549 cell lines. The cells were treated with the compounds (pre-dissolved in DMSO) for 72 h in a five-dose assay ranging from 0.01 to 100 μM. After treatments, MTT solution (20 μL, 2.5 mg mL^{-1}) in PBS was added. After 4 h of incubation, the formazan crystal formed in the well was dissolved in 100 μL DMSO for

optical density reading at 570 nm. The GI_{50} value was calculated by nonlinear regression analysis (GraphPad Prism). All experiments were conducted for three independent replicates.

Synergistic effect

For drug combination experiments, MCF-7 cells were incubated with the tested compounds and TPT for 72 h at 37 °C, and then measured by MTT assay. The combination index (CI) value was calculated by the Chou–Talalay method (CalcuSyn software). CI values below 1 are indicative of synergism, and CI values below 0.3 are indicative of strong synergism.

Conclusions

In summary, spiroconyone A (**1**), the first example of a rearranged phytosterol containing an unusual spiro [5,6] ring system, and nine known compounds were isolated from the aerial parts of *C. japonica*. It was hypothesized that spinasterol (**2**), as the precursor, underwent successful hydroxylation, epoxidation and pinacol rearrangement to give **1**. The enzyme-based screening of all isolates indicated that compounds **7** and **10** exhibited TDP1 inhibition with IC_{50} values of 36 μM and 16 μM, respectively. And they could significantly increase the cytotoxicity of TPT against MCF-7 cells. In particular, **7** exhibited a strong synergistic effect with TPT (CI values < 0.3). Besides, compounds **5** and **9** showed high cytotoxicity against MCF-7 cells with GI_{50} values of 3.3 μM and 6.5 μM, respectively. The novel spiroconyone A showed weak TDP1 inhibitory activity (29% inhibition at 100 μM concentration) and no cytotoxicity (GI_{50} > 100 μM). These findings might offer the evidence for the discovery of antitumor agents from this plant.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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