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PAPER

Protolimonoids and norlimonoids from the stem bark of *Toona ciliata* var. *pubescens*†

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Six new tirucallane protolimonoids, toonapubesins A–F (**1–6**), one new rearranged tirucallane protolimonoid, toonapubesin G (**7**), and two new 21,22,23-trinorapotirucallane limonoids, toonapubesic acids A (**8**) and B (**9**), possessing an unprecedented carbon skeleton, along with five known tirucallane protolimonoids (**10–14**) and one known apotirucallane limonoid (**15**), were isolated from the stem bark of *Toona ciliata* var. *pubescens*. Their structures and relative configurations were determined by detailed spectroscopic analysis and by chemical methods. The proposed structures of **8** and **11** were confirmed by X-ray diffraction analysis of their respective derivatives (**8a** and **11a**). The absolute configuration of **8** was determined by a novel solid-state TDDFT ECD approach on its derivative **8a** while the absolute configuration of **10** was determined by the modified Mosher's method. In addition, the structures of dyvariabilin H (**10c**) proposed by Sticher *et al.* and cneorin-NP_{36} (**11b**) by Mondon *et al.* were corrected as **10** and **11**, respectively. Toonapubesin G (**7**) showed promising inhibitory activity against CDC25B with an IC_{50} value of 2.1 μM, while compound **8a** showed significant cell protecting activity against H_2O_2-induced SH-SY5Y cell damage with 11.5% increase in cell viability.

Introduction

Plants of the family Meliaceae are a rich source of limonoids, which are a structurally diverse group of natural products with highly complex polycyclic skeletons.[1] These unusual structural features have attracted great attention as challenging targets for total synthesis,[2] bioactivity evaluation, and biosynthetic studies.[3] The genus *Toona* (family Meliaceae) is composed of only 5 species, but phenotypic plasticity and genetic variation are responsible for much of the taxonomic complexity reflected in the literature.[4] Some *Toona* species, such as *T. ciliata* and *T. sinensis*, are used as folk medicines in China for the treatment of diarrhea, dysentery and ringworm.[5] Previous phytochemical studies of *Toona* species have led to the isolation of a series of limonoids and their biogenetic precursors (protolimonoids), some of which showed various biological activities, such as insect antifeedant, antifungal, and antitumor activities.[6]

Toona ciliata var. *pubescens* is a tall timber tree widely distributed in southern China.[7] In the course of our ongoing search for bioactive metabolites from Chinese medicinal plants,[8] we have chemically investigated the stem bark of the title plant collected from Jiangxi Province, China, resulting in the isolation and identification of five new pregnane steroids,[9] namely toonasterones A and B, (*Z*)-aglawone, (*Z*)-toonasterone C, and (*E*)-toonasterone C. Continuing chemical study on the methanol extract of this plant has led to the characterization of six new tirucallane protolimonoids, named toonapubesins A–F (**1–6**), one new rearranged tirucallane protolimonoid, named toonapubesin G (**7**), and two new 21,22,23-trinorapotirucallane limonoids, named toonapubesic acids A (**8**) and B (**9**), along with six known related analogues (**10–15**). Herein, we report the isolation, structure elucidation, and bioassay results of these new compounds.

Results and discussion

The stem bark of *T. ciliata* var. *pubescens* was extracted exhaustively with MeOH. The MeOH extract was suspended in water and partitioned with $CHCl_3$. The $CHCl_3$-soluble portion was repeatedly chromatographed over silica gel, Sephadex LH-20, and reverse phase HPLC to afford pure compounds **1–15**.

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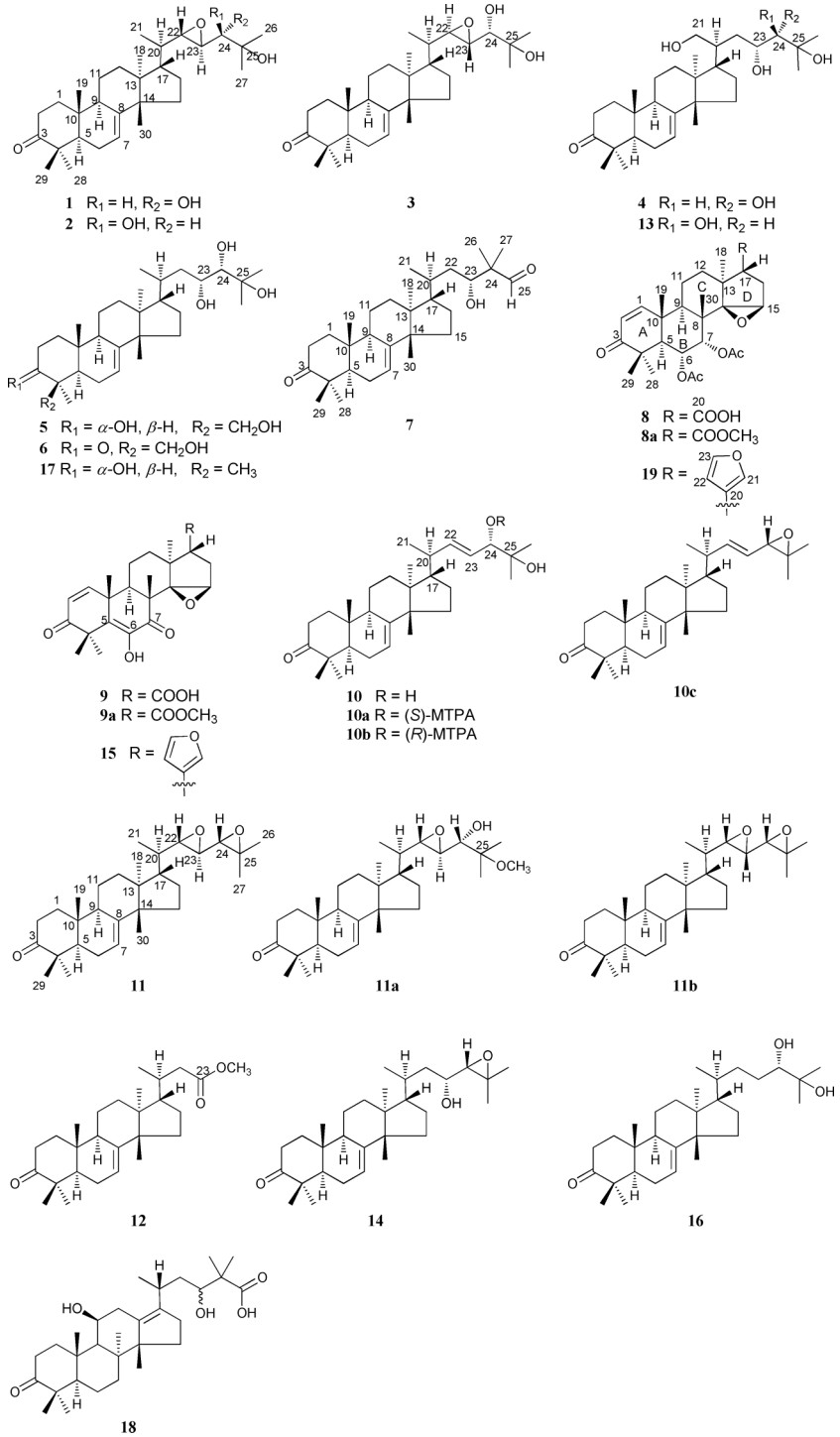


The structures of known compounds were readily identified as dyvariabilin H (**10**),[10] cneorin-NP$_{36}$ (**11**),[11] methyl 24,25,26,27-tetranortirucall-7-en-3-oxo-23-oate (**12**),[12] aglaiodiol (**13**),[13] niloticin (**14**),[14] and cedrelone (**15**),[15] by comparison of their spectroscopic data with those reported in the literature. It may be worth pointing out that, among these known compounds, although **12** was reported as a synthetic oxidation product of bourjotinolone C,[12] this is the first report of its isolation from a natural source. In addition, the ^{13}C NMR data of **12** (Table 2) are also reported for the first time.

Among the 9 new compounds, compounds **1–7** showed NMR data characteristic for tirucallane-type protolimonoids, like that of co-occurring **10** and **11**, while the NMR spectra of **8** and **9** were reminiscent of that of co-occurring **15**, displaying an apotirucallane limonoid nucleus (rings A–D). Moreover, careful analysis of their ^{1}H and ^{13}C NMR data revealed that compounds **1–7** possessed nearly identical tirucallane-type protolimonoid nuclei (rings A–D) and varied only in the side chain at C-17. By contrast, compound **8** differs from **9** only by the different oxidative patterns at ring B. In light of these observations, it

Table 1 ^{1}H NMR spectroscopic data (300 MHz, $CDCl_3$) for compounds **1–7**, **10**, **11a**, and **12**[a]

Proton	1	2	3	4	5	6	7	10	11a	12
1α	1.47, m	1.47, m	1.46, m	1.46, m	1.41, m	1.57, m	1.48, m	1.46, m	1.46, m	1.46, m
1β	1.99, m	1.99, m	1.98, m	1.99, m	1.52, m	2.04, m	1.98, m	2.02	1.98, m	1.99, m
2α	2.24, dt (14.5, 3.5)	2.24, dt (14.1, 3.7)	2.24, dt (14.3, 3.3)	2.24, dt (14.1, 3.3)	1.90, m	2.39, dt (14.7, 3.5)	2.27, dt (14.5, 3.5)	2.24, dt (14.3, 3.5)	2.24, dt (14.5, 3.5)	2.24, dt (14.3, 3.5)
2β	2.76, td (14.5, 5.7)	2.74, td (14.1, 5.3)	2.77, td (14.2, 5.2)	2.74, td (14.1, 5.4)	1.66, m	2.67, td (14.7, 5.7)	2.75, td (14.4, 5.4)	2.75, td (14.3, 5.4)	2.75, td (14.5, 5.7)	2.75, td (14.3, 5.1)
3β					3.95, brs					
5	1.72, m	1.72, m	1.72, m	1.72, m	1.92, m	1.90, m	1.72, m	1.72, m	1.74, m	1.72, m
6α	2.10, m	2.10, m	2.10, m	2.10, m	2.11, m	2.14, m	2.09, m	2.11, m	2.10, m	2.10, m
6β	2.10, m	2.10, m	2.10, m	2.10, m	1.94, m	1.98, m	2.09, m	2.11, m	2.10, m	2.10, m
7	5.32, m	5.31, m	5.32, m	5.31, m	5.23, m	5.28, m	5.30, br s	5.29, m	5.32, m	5.31, m
9	2.32, m	2.30, m	2.30, m	2.28, m	2.35, m	2.13, m	1.51, m	2.31, m	2.30, m	2.28, m
11	1.58, m	1.59, m	1.60, m	1.58, m	1.60, m	1.60, m	1.59, m	1.63, m	1.58, m	1.56, m
12α	1.64, m	1.66, m	1.64, m	1.60, m	1.63, m	1.65, m	1.52, m	1.66, m	1.64, m	1.65, m
12β	1.83, m	1.85, m	1.83, m	1.78, m	1.82, m	1.84, m	1.82, m	1.86, m	1.83, m	1.82, m
15	1.54, m	1.54, m	1.54, m	1.54, m	1.48, m	1.48, m	1.52, m	1.52, m	1.54, m	1.52, m
16α	1.35, m	1.38, m	1.63, m	1.28, m	1.32, m	1.33, m	1.32, m	1.32, m	1.35, m	1.32, m
16β	1.98, m	2.13, m	2.04, m	2.04, m	2.02, m	2.05, m	2.01, m	2.09, m	1.98, m	1.93, m
17	1.69, m	1.70, m	1.73, m	1.68, m	1.50, m	1.52, m	1.56, m	1.62, m	1.69, m	1.51, m
18	0.80, s	0.79, s	0.80, s	0.85, s	0.82, s	0.83, s	0.81, s	0.82, s	0.80, s	0.85, s
19	1.01, s	1.00, s	1.01, s	0.99, s	0.73, s	0.96, s	1.00, s	1.00, s	1.00, s	1.00, s
20	1.30, m	1.31, s	1.40, m	1.70, m	1.38, m	1.40, m	1.58, m	2.09, m	1.28, m	1.90, m
21	1.03, d (6.2)	1.01, d (6.3)	0.95, d (6.5)	3.31, dd (9.1, 7.1) 3.96, d (9.1)	0.92, d (5.7)	0.92, d (6.1)	1.02, d (6.3)	0.99, d (6.7)	1.04, d (6.4)	0.93, d (6.3)
22	2.76, dd (8.1, 2.1)	2.70, dd (8.1, 2.4)	2.81, dd (7.2, 2.4)	1.66, m	1.88, m	1.88, m	1.12, m	5.55, dd (15.5, 8.2)	2.70, dd (8.1, 2.0)	2.43, dd (13.8, 3.0)
				1.78, m	1.22, m	1.22, m	1.64, m			2.01, m
23	3.11, dd (2.1, 2.0)	2.97, dd (6.0, 2.4)	2.90, dd (4.2, 2.4)	3.97, d (8.9)	4.12, m	4.11, m	3.81, d (7.6)	5.43, dd (15.5, 6.7)	3.16, dd (2.0, 1.8)	
24	3.49, br s	3.24, d (6.0)	3.30, d (4.2)	3.06, br s	3.17, d (8.4)	3.16, d (6.8)		3.84, d (6.7)	3.53, br s	
25							9.51, s			
26	1.29, s	1.30, s	1.28, s	1.29, s	1.30, s	1.30, s	1.08, s	1.11, s	1.19, s	
27	1.28, s	1.29, s	1.28, s	1.27, s	1.32, s	1.32, s	1.03, s	1.20, s	1.25, s	
28	1.04, s	1.03, s	1.03, s	1.04, s	1.07, s	1.17, s	1.04, s	1.04, s	1.04, s	1.05, s
29	1.12, s	1.10, s	1.11, s	1.10, s	3.83, d (10.2)	4.06, d (11.3)	1.11, s	1.14, s	1.11, s	1.11, s
					3.65, d (10.2)	3.62, d (11.3)				
30	1.03, s	1.02, s	1.03, s	1.00, s	0.96, s	1.00, s	1.00, s	1.00, s	1.02, s	1.00, s
OCH_3									3.26, s	3.67, s

[a] The assignments were based on DEPT, ^{1}H-^{1}H COSY, HSQC and HMBC experiments.

appeared that for tirucallane-type protolimonoids (**1–7**) we had only to establish the substitution pattern and configuration of the most abundant compound **10** to give insight into the whole series. Therefore, the structure elucidation of these new metabolites was conducted starting from **10**, followed by the corrected structure of cneorin-NP$_{36}$ (**11**), then the protolimonoids **1–7** and the 21,22,23-trinorapotirucallane limonoids **8** and **9**. Accordingly, the structure elucidation details of these new isolates are described in this order as follows.

Compound **10**, namely dyvariabilin H (**10c**),[10] was obtained as an optically active white amorphous powder. Its molecular formula, $C_{30}H_{48}O_3$, was established by the HRESIMS pseudo-molecular ion peak at *m/z* 479.3500 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_3Na$, 479.3501). The IR absorption bands at 3429 and 1705 cm^{-1} indicated the presence of hydroxyl and carbonyl groups. The ^{1}H NMR data (Table 1) showed characteristic proton signals of seven tertiary methyls [δ_H 1.20, 1.14, 1.11, 1.04, 1.00 (Me × 2), and 0.82 (each 3H, s)], a secondary methyl [δ_H 0.99 (3H, d, *J* = 6.7 Hz)], an oxymethine [δ_H 3.84 (1H, d, *J* = 6.7 Hz)], and three olefinic protons [δ_H 5.55 (1H, dd, *J* = 15.5, 8.2 Hz), 5.43 (1H, dd, *J* = 15.5, 6.7 Hz), and 5.29 (1H, m)], implying that **10** possessed a tirucallane protolimonoid skeleton. The ^{13}C NMR spectrum (Table 2) revealed 30 carbon resonances, which were classified by DEPT and HSQC experiments as one ketone carbonyl (δ_C 217.0), one trisubstituted double bond (δ_C 145.7 and 117.8), one disubstituted double bond (δ_C 140.8 and 125.9), eight methyls, seven sp^3 methylenes, five sp^3 methines, and five sp^3 quaternary carbons. Comparison of its NMR data with those of 24*S*,25-dihydroxytirucall-7-en-3-one (**16**),[16] a tirucallane protolimonoid previously isolated from *Owenia cepiodora*, indicated that compound **10** is an analogue of **16**. Careful comparison of the ^{1}H and ^{13}C NMR data of **10** and **16** revealed that the two compounds shared the same tirucall-7-en-3-one tetracyclic ring system, and the only difference was the presence of a disubstituted double bond (δ_H 5.55; δ_C 140.8 and δ_H 5.43; δ_C 125.9) in the side chain of **10**, in agreement with the 2 mass units difference between the

Table 2 ^{13}C NMR spectroscopic data (100 MHz, $CDCl_3$) for compounds **1–7**, **10**, **11a**, and **12**[a]

Carbon	1[b]	2[b]	3[b]	4[b]	5[b]	6[b]	7[b]	10[b]	10[c]	11a[b]	12[b]
1	38.5, CH_2	38.5, CH_2	38.5, CH_2	38.5, CH_2	31.0, CH_2	37.3, CH_2	38.5, CH_2	38.5, CH_2	39.0, CH_2	38.5, CH_2	38.5, CH_2
2	34.9, CH_2	34.9, CH_2	34.9, CH_2	34.9, CH_2	25.3, CH_2	35.5, CH_2	35.0, CH_2	34.9, CH_2	35.3, CH_2	34.9, CH_2	34.9, CH_2
3	216.9, C	217.0, C	216.9, C	217.1, C	70.5, CH	216.6, C	217.0, C	217.0, C	215.5, C	216.9, C	216.9, C
4	47.9, C	47.8, C	47.9, C	47.8, C	42.3, C	53.1, C	47.9, C	47.9, C	48.3, C	47.9, C	47.8, C
5	52.3, CH	52.3, CH	52.3, CH	52.2, CH	45.4, CH	53.4, CH	52.3, CH	52.3, CH	53.0, CH	52.3, CH	52.3, CH
6	24.3, CH_2	24.3, CH_2	24.3, CH_2	24.3, CH_2	23.6, CH_2	24.6, CH_2	24.3, CH_2	24.3, CH_2	25.0, CH_2	24.3, CH_2	24.3, CH_2
7	118.2, CH	118.1, CH	118.2, CH	118.1, CH	117.8, CH	118.0, CH	117.9, CH	117.8, CH	118.7, CH	118.1, CH	118.0, CH
8	145.4, C	145.5, C	145.5, C	145.4, C	145.9, C	145.9, C	145.8, C	145.7, C	146.7, C	145.5, C	145.7, C
9	48.4, CH	48.4, CH	48.4, CH	48.3, CH	48.7, CH	48.2, CH	48.4, CH	48.4, CH	49.2, CH	48.4, CH	48.4, CH
10	35.0, C	35.0, C	35.0, C	34.9, C	34.5, C	35.0, C	34.9, C	35.0, C	35.7, C	35.0, C	35.0, C
11	18.1, CH_2	18.2, CH_2	18.2, CH_2	18.2, CH_2	18.2, CH_2	18.6, CH_2	18.3, CH_2	18.2, CH_2	18.9, CH_2	18.1, CH_2	18.2, CH_2
12	33.2, CH_2	33.3, CH_2	33.4, CH_2	33.1, CH_2	33.9, CH_2	33.8, CH_2	33.6, CH_2	34.0, CH_2	34.3, CH_2	33.2, CH_2	33.4, CH_2
13	43.8, C	43.9, C	43.9, C	43.2, C	43.5, C	43.5, C	43.6, C	43.5, C	44.2, C	43.8, C	43.6, C
14	50.7, C	50.7, C	50.7, C	51.3, C	51.2, C	51.2, C	51.2, C	51.2, C	52.0, C	50.7, C	51.2, C
15	34.2, CH_2	34.3, CH_2	34.3, CH_2	33.7, CH_2	33.9, CH_2	34.0, CH_2	34.0, CH_2	33.4, CH_2	34.8, CH_2	34.2, CH_2	34.0, CH_2
16	27.3, CH_2	27.7, CH_2	26.7, CH_2	28.3, CH_2	28.4, CH_2	28.4, CH_2	28.5, CH_2	28.5, CH_2	29.3, CH_2	27.2, CH_2	28.1, CH_2
17	50.4, CH	50.3, CH	52.6, CH	49.4, CH	53.7, CH	53.7, CH	53.7, CH	52.4, CH	53.5, CH	50.4, CH	52.8, CH
18	22.0, CH_3	21.9, CH_3	22.0, CH_3	21.6, CH_3	22.0, CH_3	22.1, CH_3	21.8, CH_3	22.1, CH_3	22.4, CH_3	22.0, CH_3	22.0, CH_3
19	12.8, CH_3	12.7, CH_3	12.8, CH_3	12.7, CH_3	13.7, CH_3	13.5, CH_3	12.8, CH_3	12.7, CH_3	13.0, CH_3	12.8, CH_3	12.8, CH_3
20	38.3, CH	38.9, CH	38.5, CH	43.1, CH	33.7, CH	33.6, CH	36.3, CH	40.3, CH	41.2, CH	38.9, CH	34.1, CH
21	16.4, CH_3	16.3, CH_3	15.6, CH_3	65.0, CH_2	18.9, CH_3	18.9, CH_3	20.1, CH_3	20.0, CH_3	20.7, CH_3	16.5, CH_3	19.2, CH_3
22	60.3, CH	61.4, CH	61.7, CH	38.1, CH_2	40.5, CH_2	40.4, CH_2	38.6, CH_2	140.8, CH	139.1, CH	59.6, CH	41.4, CH_2
23	58.9, CH	58.5, CH	55.7, CH	71.9, CH	69.7, CH	69.7, CH	75.1, CH	125.9, CH	128.3, CH	58.3, CH	174.0, C
24	73.9, CH	77.5, CH	76.4, CH	77.8, CH	74.8, CH	74.8, CH	51.0, C	79.6, CH	79.8, CH	72.8, CH	
25	72.8, C	72.3, C	72.3, C	73.9, C	74.3, C	74.3, C	206.8, CH	72.9, C	72.8, C	77.2, C	
26	25.4, CH_3	24.5, CH_3	24.9, CH_3	26.4, CH_3	26.2, CH_3	26.2, CH_3	16.4, CH_3	24.5, CH_3	24.7, CH_3	19.8, CH_3	
27	25.4, CH_3	26.9, CH_3	26.0, CH_3	27.0, CH_3	27.2, CH_3	27.5, CH_3	18.8, CH_3	26.3, CH_3	26.3, CH_3	21.3, CH_3	
28	24.5, CH_3	24.2, CH_3	24.5, CH_3	24.5, CH_3	21.3, CH_3	20.7, CH_3	24.5, CH_3	23.7, CH_3	25.0, CH_3	24.5, CH_3	24.5, CH_3
29	21.6, CH_3	21.6, CH_3	21.6, CH_3	22.1, CH_3	65.7, CH_2	65.8, CH_2	21.6, CH_3	21.6, CH_3	21.8, CH_3	21.6, CH_3	21.6, CH_3
30	27.5, CH_3	27.5, CH_3	27.4, CH_3	27.3, CH_3	27.5, CH_3	27.5, CH_3	27.4, CH_3	27.4, CH_3	27.8, CH_3	27.5, CH_3	27.4, CH_3
OCH_3										52.3, CH_3	51.4, CH_3

[a] The assignments were based on DEPT, ^1H–^1H COSY, HSQC and HMBC experiments. [b] Recorded in $CDCl_3$. [c] Recorded in acetone-d_6.

molecular weights of **10** and **16**. The location of the olefin at Δ^{22} was supported by both the connections of H-17/H-20(/Me-21)/H-22/H-23/H-24 in the ^1H–^1H COSY spectrum and the significant long-range correlations from Me-21 to C-17, C-20 and C-22 in the HMBC spectrum. The *E*-geometry of Δ^{22} was quickly established by the large coupling constant of H-22 with H-23 (J = 15.5 Hz). In addition, The HMBC correlations of H-24/C-25 and Me-26 (Me-27)/C-24 placed two hydroxyl groups at C-24 (δ_C 79.6, CH) and C-25 (δ_C 72.9, C), respectively.

The ROESY spectrum showed that the relative configuration of the tetracyclic core in **10** was identical to that of **16**. Thus, the ROESY correlations of Me-19/H-1β, Me-19/H-11β, Me-29/H-2β, Me-30/H-12β, Me-30/H-16β, Me-30/H-17, and Me-21/H-17 indicated that they were cofacial and were randomly assigned as β-orientations. Subsequently, the ROESY correlations of Me-18/H-9, Me-18/H-16α, Me-18/H-20, Me-28/H-5, H-5/H-9, and H-5/H-1α suggested that they were all α-oriented.

There is a chiral center at C-24 of **10**. Its absolute configuration was determined by using the modified Mosher's method. Thus, compound **10** was esterified separately with (*R*)- and (*S*)-2-methoxy-2-trifluoromethylphenylacetic (MTPA) chloride in dry CH_2Cl_2 at room temperature to yield the corresponding (*S*)-MTPA ester (**10a**) and (*R*)-MTPA ester (**10b**), respectively. Assignment of the ^1H NMR signals of the esters was achieved by carefully analyzing the 2D NMR spectra. The $\Delta\delta$ ($\delta_{S\text{-ester}} - \delta_{R\text{-ester}}$) values of the protons near the chiral carbon (C-24) are summarized in Fig. 1. Negative $\Delta\delta$ values were recorded for the protons of H-20, Me-21, H-22, H-23, and H-24, and positive $\Delta\delta$ values

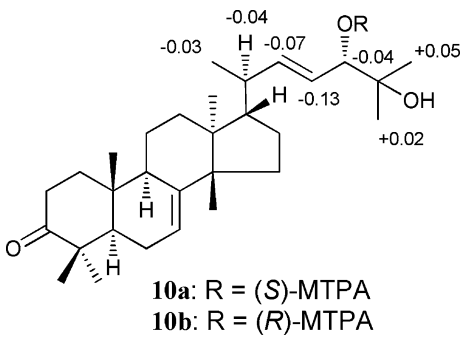


Fig. 1 $\Delta\delta$ ($\delta_S - \delta_R$) values (in ppm) for the MTPA esters of **10**.

were observed for the protons of Me-26 and Me-27. According to Mosher's determination rule, the absolute stereochemistry of the hydroxyl group at C-24 was established as *S*. Based on the above evidence, the structure of compound **10** was unambiguously determined as 24*S*,25-dihydroxytirucall-7,22-dien-3-one, which is a 22,23-dihydro derivative of **16**.

Interestingly, a search of the literature revealed that the spectroscopic characteristics of **10** were identical to those of dyvariabilin H (**10c**) previously isolated from *Dysoxylum variabile* by Sticher *et al.*,[10] except for the molecular weight and the depicted structure therein. To clarify this confusion, the ^1H and ^{13}C NMR data of **10** and **10c** were carefully compared. This comparison immediately revealed that ^{13}C NMR chemical shifts of δ_C 79.6 (C-24) and 72.9 (C-25) in **10c**[10] were erroneously assigned to an epoxy ring, instead

of to a vicinal diol as in **10**. Moreover, the reported HREIMS data (*m/z* 438.3496) for dyvariabilin H was also incorrect and it should be ascribed to the $[M - H_2O]^+$ ion peak of **10**. Based on the above observation, it is clear that the structure of dyvariabilin H (**10c**) should be corrected as **10**.

Compound **11**, gave a pseudomolecular ion peak at *m/z* 477.4 in the positive ESIMS. Its structure determination was not straightforward. In fact, the structure of **11** was firstly identified as a known protolimonoid, cneorin-NP_{36} (**11b**).[11] But careful interpretation of its ^{1}H NMR data indicated that the assigned relative configuration for H-22 and H-23 of **11b**[11] was questionable because the coupling constant observed for H-22 and H-23 (J = 2.1 Hz) did not support the *cis* orientation of both protons.[17] In fact, the structure, in particular, the stereochemistry, of **11b** reported by Mondon *et al.*[11a] was "far from conclusive" as pointed out by Gray *et al.*[11b] In order to confirm the structure of **11**, the crystallization of **11** was tried in $CHCl_3$–MeOH (1 : 9) solution and a suitable crystal (**11a**) for X-ray diffraction analysis was obtained (Fig. 2). Surprisingly, the HRESIMS measurement suggests the molecular formula of **11a** is $C_{31}H_{50}O_4$ {509.3599 $[M + Na]^+$ (calcd for $C_{31}H_{50}O_4Na$, 509.3607)}, which 32 mass units more than that of **11**. Successive X-ray diffraction analysis on the single crystal disclosed the structure of **11a** as depicted in Fig. 2. The suggested relative configuration by X-ray analysis not only supports the correctness of the proposed structure for **11**, but also indicates that **11a** is actually derived by the methanolic addition at the terminal epoxy ring (C-24, C-25) of **11**. It should be noted that the configuration of C-24 would not be changed *via* an S_N1 reaction process (Scheme 1). Thus, the structure of **11** was unambiguously elucidated as 22*S**,23*S**,24*S**,25-diepoxy-tirucalla-7-en-3-one. It is clear that the structure of cneorin-NP_{36} (**11b**) should be corrected as **11**.

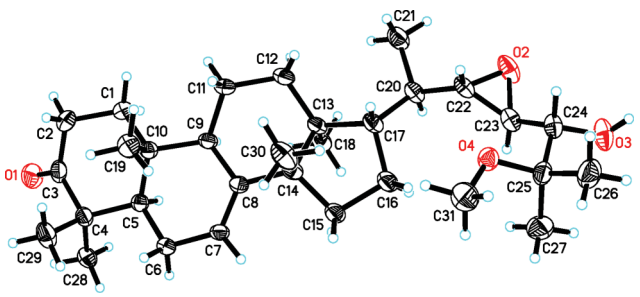


Fig. 2 Single-crystal X-ray structure of **11a**.

Toonapubesins A (**1**) and B (**2**) were both assigned a molecular formula of $C_{30}H_{48}O_4$, as determined from their HRESIMS spectra. In fact, the ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **1** and **2** were very similar to those of compound **11a** except for the lack of typical signals for an oxymethyl (δ_H 3.26; δ_C 52.3), suggesting that **1** and **2** are de-*O*-methyl derivatives of **11a** in agreement with the 14 mass units difference between **1**, **2** and **11a**. Furthermore, careful comparison of ^{13}C NMR chemical shifts of **2** with those of **1** revealed that the C-24 δ value of **2** is apparently different from that of **1** implying that **1** and **2** are epimeric with each other at C-24. Due to the different configuration at C-24, the coupling constants and the splitting pattern of H-24 in **1** and **2** are quite different. The broad singlet of H-24 (δ 3.53) in **1** implied an approximately 90° dihedral angle of H-23/H-24, requiring the bond between C-23 and C-24 to be fixed. This fact suggested that a hydrogen bond should be formed between the 25-OH and the epoxy oxygen atom, as in the similar cases described for protoxylocarpins A–E.[18] A favorable conformation of the hydrogen bond toward the epoxy ring was established (Fig. 3a), according to the structure modeling calculated by MOPAC minimizing energy. As shown in the Fig. 3a, the broad singlet of H-24 was reasonably attributed due to an axial–equatorial coupling in **1**, while the large coupling constant ($J_{23,24}$ = 6.0 Hz) in **2** was attributed due to an axial–axial coupling (Fig. 3b). Accordingly, the configuration of the hydroxyl group at C-24 was assigned as *S* in **1** and *R* in **2**. All the ^{1}H and ^{13}C NMR

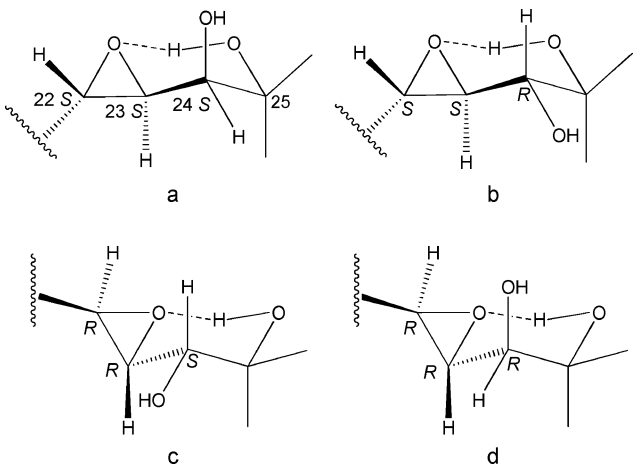
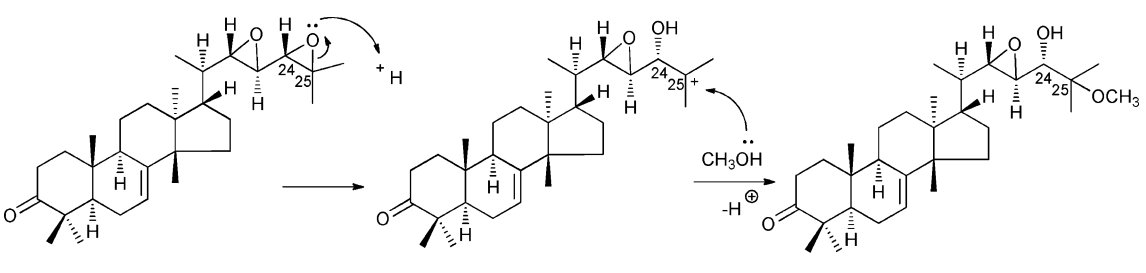


Fig. 3 The hydrogen bond formation between 25-OH and the epoxy group. (a) C (23*S*,24*S*) caused an axial–equatorial coupling of H-23 and H-24 (J = *ca.* 0 Hz). (b) C (23*S*,24*R*) caused an axial–axial coupling of H-23 and H-24 (J = *ca.* 5 Hz). (c) C (23*R*,24*S*) caused an axial–axial coupling of H-23 and H-24 (J = *ca.* 5 Hz). (d) C (23*R*,24*R*) caused an axial–equatorial coupling of H-23 and H-24 (J = *ca.* 0 Hz).



Scheme 1 Plausible mechanism of the formation of **11a** from **11** in weakly acidic solution ($CHCl_3$–MeOH, 1 : 9).

assignments according to structures **1** and **2** were confirmed by ^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY experiments.

The molecular formula of toonapubesin C (**3**), $C_{30}H_{48}O_4$, was established by HRESIMS, ^{13}C NMR, and DEPT spectra, the same as for **1** and **2**. A comparison of the overall ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **3** with those of **1** and **2** suggested that the three compounds had the same planar structure. In fact, **3** also showed a pair of *trans*-coupled epoxide protons [δ_H 2.81 dd, (J = 7.2, 2.0 Hz, H-22) and δ_H 2.90, dd (J = 2.0, H-23)].[17] However, the ^{13}C NMR data of C-21 (δ 15.6), C-22 (δ 61.7), and C-23 (δ 55.7) and the coupling constant ($J_{20,22}$ = 7.2 Hz) were slightly different from those in **11a**, **1**, and **2**, indicating that the configuration of the 22,23-epoxy group in **3** had to be opposite (*R*,*R*) to that of **11a**, **1**, and **2**. In addition, the 4.2 Hz coupling constant between the protons at δ_H 3.30 (H-24) and δ_H 2.90 (H-23) revealed an axial–axial coupling (Fig. 3c), the same as that in **2**, suggesting the hydroxyl group in the side chain also had a 24*S* configuration. Therefore, the structure of toonapubesin C was determined as 22*R*,23*R*-epoxy-24*S*,25-dihydroxy-7-tirucallen-3-one.

The molecular formula of toonapubesin D (**4**) was determined to be $C_{30}H_{50}O_5$ by HRESIMS (pseudomolecular ion peak at m/z 513.3555 [M + Na]$^+$). Its ^{1}H and ^{13}C NMR data (Tables 1 and 2) are almost identical to those of co-occurring aglaiodiol (**13**),[13] suggesting the presence of a tirucall-7-en-3-one nucleus and a saturated side chain at C-17 that contains four hydroxyl groups. In fact, the only difference between **4** and **13** was the NMR data for H-24 and C-24 [δ_H 3.06 (br s), δ_C 77.8 in **4** and δ_H 3.24 (d, J = 7.3 Hz), δ_C 79.3 in **13**]. Thus, the structure of compound **4** was determined as the C-24 epimer of **13**. Because the configuration of C-24 in **13** was assigned as *R**,[13] the relative configuration of C-24 in **4** was consequently determined to be opposite (*S**) to that of **13**.

Toonapubesin E (**5**) gave a sodiated molecular ion peak at m/z 515.3710 [M + Na]$^+$ (calcd for $C_{30}H_{52}O_5Na$, 515.3712), corresponding to the molecular formula $C_{30}H_{52}O_5$. The ^{1}H and ^{13}C NMR spectra of **5** showed close similarities to those of hispidol A (**17**), previously isolated from *Trichilia hispida*.[19] It was immediately apparent from the ^{1}H and ^{13}C NMR data comparison that **5** differs from hispidol A only by the substituents at C-4. The appearance of two typical AB-type downfield signals at δ 3.83 (J = 10.2 Hz) and 3.65 (J = 10.2 Hz) in the ^{1}H NMR spectrum of **5** accompanying the disappearance of Me-29 signal in hispidol A clearly indicated that the Me-29 of hispidol A was oxidized as an alcohol. Finally, the β-orientation of C-29 hydroxymethyl was confirmed by ROESY correlations of H_2-29/Me-19 and H_2-29/H-3 according to the proposed structure of **5**.

Toonapubesin F (**6**) was obtained as an optically active white amorphous powder. The molecular formula of **6** was determined to be $C_{30}H_{50}O_5$ (m/z 513.3564 [M + Na]$^+$), 2 mass units less than that of **5**. The NMR spectra of **6** revealed a close relationship with those of **5**. In fact, the ^{1}H NMR resonances of **6** are almost the same as **5** except for the lack of oxymethine signal at C-3. The replacement of 3-OH in **5** by a ketone group in **6** was easily recognized by the downfield ^{13}C NMR signal at 216.6 accompanied by the disappearance of ^{13}C NMR signal at δ 70.5 in the ^{13}C NMR spectrum of **6**. All the 2D NMR experiments confirmed structure **6**.

The molecular formula of toonapubesin G (**7**), $C_{30}H_{48}O_3$, was deduced by HRESIMS pseudomolecular ion peak at m/z 479.3517 [M + Na]$^+$ (calcd 479.3501). The IR absorption bands at 3435 and 1708 cm^{-1} indicated the presence of hydroxyl and carbonyl groups in the structure of **7**. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **7** were found to be closely related to those of co-occurring **10**, suggesting that they shared the same tirucall-7-en-3-one skeleton. In fact, **7** differs from **10** only at the side chain. In the downfield region of the ^{1}H NMR spectrum in **7**, besides the typical Δ^7 proton signal of the tetracyclic core, an aldehyde [δ_H 9.51, (1H, s, H-25)] and an oxymethine [δ_H 3.81, (1H, d, J = 7.6 Hz, H-23)] proton signals were also observed. The location of the hydroxyl group at C-23 in the side chain was deduced from the ^{1}H–^{1}H COSY experiment (Fig. 4). Thus, starting from H-23, a distinct spin–spin system from H-23 to Me-21 could be recognized (Fig. 4). Furthermore, the HMBC (Fig. 4) correlations observed between H-25 and C-23, C-24, C-26, C-27; H-23 and C-20, C-24, C-25; H-26 (δ_H 1.08, s) and C-23, C-24, C-25; H-27 (δ_H 1.03, s) and C-24, C-25, respectively, permitted the connections of C-23 (oxymethine), C-26 and C-27 (tertiary methyls), and C-25 (aldehyde carbonyl) *via* C-24 (quaternary carbon). Assignments of the proton and carbon signals for the side chain of **7** were secured by comparison with the ^{1}H and ^{13}C NMR data of the side chain of model compound alisol O (**18**) (aldehyde group in **7**; carboxylic acid group in **18**).[20] Based on the above evidence, the structure of compound **7** was established as 3-oxo-23-hydroxy-24,24-dimethyl-26,27-dinortirucall-7-en-25-al.

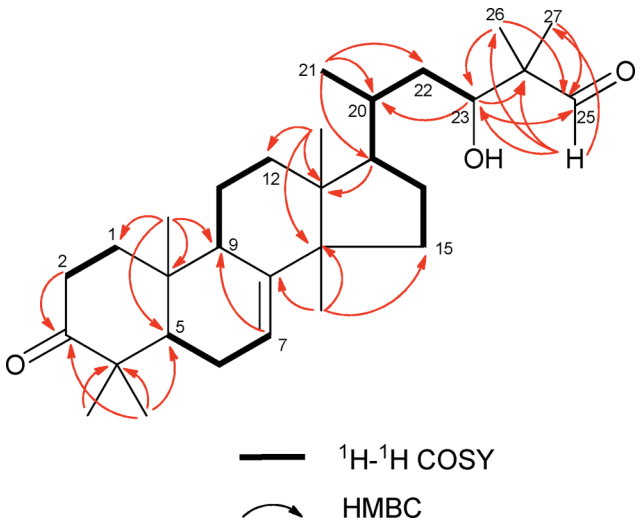
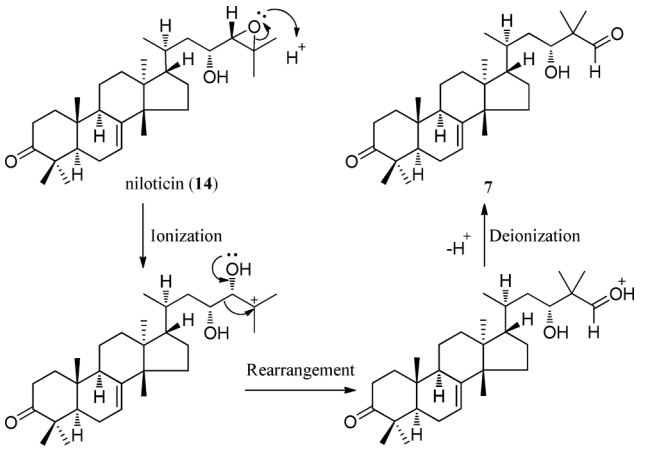


Fig. 4 Selected 2D NMR correlations for compound **7**.

To determine the absolute stereochemistry at C-23, the advanced Mosher's method was applied. Unfortunately, the efforts for obtaining the corresponding Mosher esters failed, probably due to the steric hindrance near C-23 and instability caused by the aldehyde group. The configuration of C-23 remains undefined. Compound **7** represents a new rearranged skeleton of tirucallane protolimonoid, presumably biosynthesized from co-occurring niloticin (**14**).[14] By analogy to Zhao's hypothesis for the biogenetic pathway of alisol O (**18**),[20] a plausible biogenetic pathway for **7** is proposed as shown in Scheme 2.[21] Based on the biogenetic considerations, the C-23 hydroxyl group was assumed adopting the same *R* configuration as that of **14**.[14]

Among all isolates, compounds **8** and **9** are the most polar ones since they both contain a carboxylic acid group, as evidenced by IR absorption bands at 3400 and 1730 cm^{-1}. The high polarity of



Scheme 2 Plausible biogenetic pathway for **7**.

these molecules caused difficulty to obtain reasonable amounts of pure **8** and **9** for structure determination, and the scant amounts of **8** (0.8 mg) and **9** (0.5 mg) prevented us from confidently recording well resolved ^{13}C and 2D NMR spectra. In order to confirm the presence of the carboxylic acid functional group and to obtain large quantities of **8** and **9** for structure elucidation and bioactivity assay, more limonoid-containing fraction was treated with CH_2N_2 yielding an expected methylation mixture. This fraction was successively separated by silica gel CC to afford the expected methyl ester derivatives of **8a** (11.6 mg) and **9a** (10.3 mg), respectively, which showed, in their ^1H NMR spectra, the expected singlet at δ 3.67 integrating for three protons.

Compound **8a** was obtained as colorless crystals (from acetone). Its molecular formula, $C_{28}H_{38}O_8$, was determined by a HRESIMS pseudomolecular ion peak at m/z 525.2466 $[M + Na]^+$ (calcd 525.2464). The IR absorptions revealed the presence of carbonyl (1736, 1672 cm^{-1}) groups. The ^{13}C NMR (Table 3) spectrum displayed 28 carbon resonances, which were classified by DEPT and HSQC experiments as 8 methyls, 3 methylenes, 8 methines (three oxygenated and two olefinic), and 9 quaternary carbons (one ketone and three ester carbonyls). In addition, five tertiary methyls (δ_H 1.25, 1.19, 1.17, 1.16 and 1.14), one methoxy (δ_H 3.67), and two acetyls (δ_H 2.11, 2.01) were distinguished by analysis of its ^1H NMR data (Table 3). The protons at δ_H 7.15 (d, J = 10.2 Hz, H-1) and 5.94 (d, J = 10.2 Hz, H-2) and carbon signals at δ_C 157.4 (C-1), 126.2 (C-2) and 204.6 (C-3) indicated the presence of a conjugated enone system in the molecule. The ^1H and ^{13}C NMR data, in combination with the HMBC correlation analysis (Fig. 5), furnished the planar structure of **8a**. A comparison of overall ^1H and ^{13}C NMR data (Table 3) revealed similarities between model compound 6α-acetoxy-14β,15β-epoxyazadirone (**19**), previously isolated from *T. ciliata*,[6b] and compound **8a**, possessing the same apotirucallane-type triterpenoid nucleus (rings A–D). In fact, **8a** differs from **19** only by the substituent at C-17 (carbomethoxy group for **8a**, furan ring for **19**). The presence of a carbomethoxy group at C-17 of **8a** was evident by the peaks at δ 174.1 and 51.6 in its ^{13}C NMR spectrum. Careful analysis of the 2D NMR (^1H–^1H COSY, HSQC, HMBC) (Fig. 5) allowed unambiguous location of the carbomethoxy group at C-17. Finally, the proposed structure including the relative configuration of **8a**, consequently of **8**, was unambiguously confirmed by single-crystal X-ray diffraction analysis (Fig. 6).

Table 3 ^1H and ^{13}C NMR spectroscopic data of compounds **8a** and **9a** in $CDCl_3$

	8a		**9a**	
no.	δ_H (mult, J in Hz)[a]	δ_C[b]	δ_H (mult, J in Hz)[a]	δ_C[b]
1	7.15 (d, 10.2)	157.4, CH	6.90 (d, 10.2)	152.2, CH
2	5.94 (d, 10.2)	126.2, CH	6.11 (d, 10.2)	127.3, CH
3		204.6, C		203.6, C
4		40.5, C		48.5, C
5	2.49 (d, 12.6)	48.5, CH		134.0, C
6	5.34 (dd, 12.6, 2.7)	70.0, CH		141.1, C
7	5.01 (d, 2.7)	73.5, CH		197.6, C
8		42.7, C		46.6, C
9	2.56 (dd, 12.6, 3.6)	38.9, CH	2.61 (dd, 10.8, 3.0)	43.1, CH
10		45.1, C		40.2, C
11α	1.80 (m)	16.2, CH_2	1.82 (m)	19.2, CH_2
11β	1.93 (m)		1.82 (m)	
12α	2.14 (m)	30.2, CH_2	2.13 (m)	35.3, CH_2
12β	1.85 (m)		1.81 (m)	
13		42.4, C		42.4, C
14		71.8, C		69.0, C
15	3.38 (br s)	56.8, CH	3.74 (br s)	54.9, CH
16α	1.84 (dd, 13.8, 10.5)	29.2, CH_2	2.18 (dd, 13.2, 10.5)	30.6, CH_2
16β	2.05 (dd, 13.8, 6.6)		2.27 (dd, 10.5, 6.6)	
17	2.39 (dd, 10.5, 6.6)	49.0, CH	2.51 (dd, 10.5, 6.6)	50.9, CH
18	1.16 (s)	20.1, CH_3	0.93 (s)	22.9, CH_3
19	1.17 (s)	21.5, CH_3	1.28 (s)	23.9, CH_3
20		174.1, C		173.6, C
28	1.25 (s)	31.6, CH_3	1.57 (s)	26.7, CH_3
29	1.14 (s)	22.4, CH_3	1.49 (s)	21.2, CH_3
30	1.19 (s)	18.8, CH_3	1.08 (s)	20.2, CH_3
OMe	3.67 (s)	51.6, CH_3	3.67 (s)	51.5, CH_3
6-OAc	2.01 (s)	21.2, CH_3		
		169.9, C		
7-OAc	2.11 (s)	21.1, CH_3		
		169.9, C		
6-OH			6.43 (s)	

[a] Recorded at 300 MHz. [b] Recorded at 100 MHz.

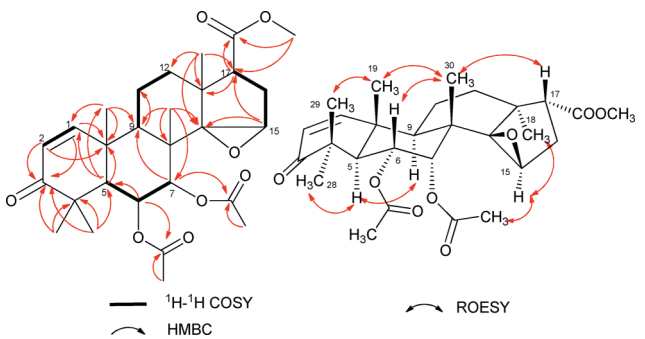


Fig. 5 Selected 2D NMR correlations for compound **8a**.

Although the X-ray geometry (relative configuration only) and the negative n–π* Cotton Effect (CE) [341 nm, $\Delta\varepsilon$ = –2.92] of the enone chromophore[22] clearly suggested the usual absolute configuration of the triterpenoid skeleton, we intended to test the applicability of the solid-state TDDFT ECD method[23] for the configurational assignment of a complex norlimonoid such as **8a**. This method has been recently developed for the configurational assignment of natural products and it has been found especially useful for large flexible natural products, since the conformational analysis step can be skipped.[23] Thus, ECD spectra of **8a** were recorded in both methanol solution and in the solid state as a KCl disk, which showed similar profiles indicating that the

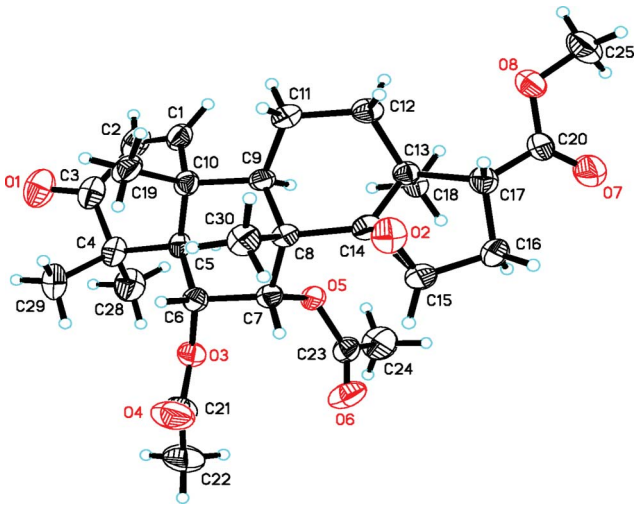


Fig. 6 Single-crystal X-ray structure of **8a**.

same conformation is prevalent in solution and solid state. Then TDDFT ECD spectra were calculated for the optimized geometry of the X-ray structure with three different functionals (B3LYP, BH&HLYP, PBE0) and the TZVP basis set. All the three ECD spectra calculated for the (5*R*, 6*R*, 7*S*, 8*S*, 9*R*, 10*R*, 13*S*, 14*R*, 15*R*, 17*R*) absolute configuration reproduced the experimental solid state ECD spectrum well, confirming its absolute configuration and demonstrating that the solid state TDDFT ECD protocol works well for this class of complex natural products (Fig. 7). To the best of our knowledge, this is the first report on the determination of the absolute configuration of a limonoid by the solid state TDDFT ECD approach.

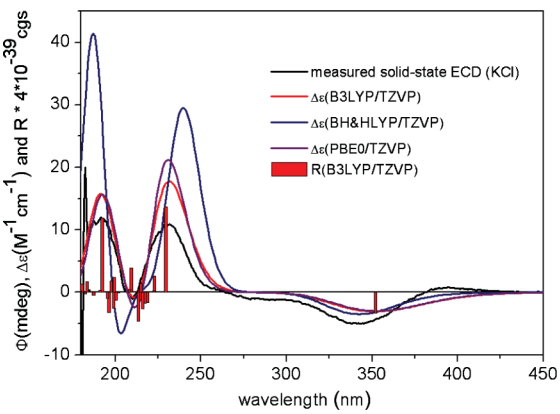
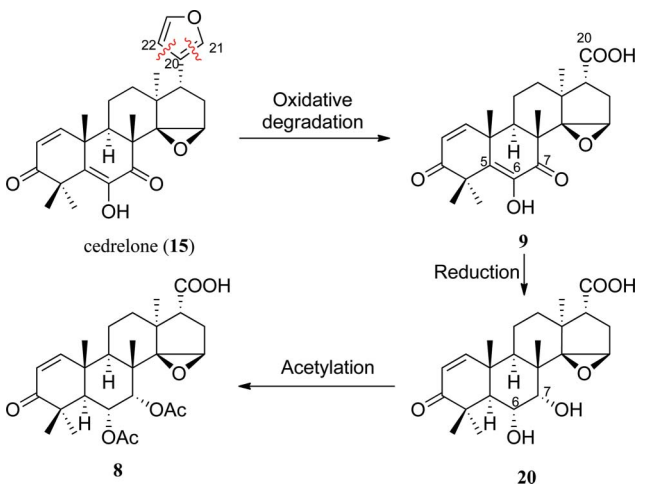


Fig. 7 Experimental solid state ECD spectrum of **8a** recorded as KCl disk (black curve), TDDFT calculated ECD spectra (B3LYP/TZVP, BH&HLYP/TZVP, PBE0/TZVP) for (5*R*, 6*R*, 7*S*, 8*S*, 9*R*, 10*R*, 13*S*, 14*R*, 15*R*, 17*R*) **8a** using X-ray geometry as input; vertical bars represent rotational strengths.

Compound **9a** had a molecular formula of $C_{24}H_{30}O_6$ as determined by HRESIMS [*m*/*z* 437.1954 $[M + Na]^+$, corresponding to $C_{24}H_{30}O_6Na$ (calcd 437.1940)]. Its ^1H and ^{13}C NMR data (Table 3) are similar to those of **8a**, suggesting the presence of a conjugated enone system in the A ring, an epoxy ring between C-14 and C-15, and also a carbomethoxy group at C-17. In fact, the main differences between **9a** and **8a** appeared at ring B, where the two acetoxy groups of ring B in **8a** were absent and the three methines [δ_C 48.5 (C-5), 70.0 (C-6), and 73.5 (C-7)] in **8a** were replaced by the conjugated enone system [δ_C 134.0 (C-5), 141.1 (C-6), and 197.6 (C-7)] in **9a**. In addition, a downfield ^1H NMR signal at δ 6.43 was assignable to the proton of 6-OH, which showed cross-peaks with C-5, C-6, and C-7 in the HMBC experiment supporting the presence of a conjugated enone in the ring B. Based on the above evidence, the planar structure of **9a** was established. The assigned structure for **9a** was confirmed by analysis of its ^1H–^1H COSY, HSQC, and HMBC spectra and by comparison with the corresponding part of co-occurring cedrelone (**15**). The relative configurations of **9a**, and consequently of **9**, at C-5, C-8, C-9, C-10, C-13, C-14, C-15, and C-17 were deduced to be the same as those of **8a** by analysis of its ROESY spectrum. The absolute configuration of all the chiral centers of **9a** was proposed to be the same as **8a** on the basis of biogenetic considerations.

It should be noted that, to the best of our knowledge, this is the first report of C_{23} norlimonoids with an unprecedented 21,22,23-trinorapotirucallane skeleton bearing a carboxylic acid group at C-17. A plausible biogenetic pathway for toonapubesic acids A (**8**) and B (**9**) is proposed as outlined in Scheme 3. Both compounds **8** and **9** could have the common precursor cedrelone (**15**), which, after oxidative degradation of the furan ring accompanying the cleavage of the C-20–C-22 single bond and the C-20–C-21 double bond, would generate the skeleton of toonapubesic acid B (**9**). Further, reduction of the Δ^5 olefin and 7-carbonyl group of **9** and acetylation of the diols at C-6 and C-7 of the intermediate **20**, would yield toonapubesic acid A (**8**).



Scheme 3 Plausible biosynthetic pathway for toonapubesic acids A (**8**) and B (**9**).

Considering the wide range of biological activities as well as the pharmacological properties exhibited by protolimonoids and limonoids, we performed *in vitro* experiments on a panel of cancer and non cancer cell lines in order to investigate the bioactivity of compounds **1–15**. The results showed that all tested compounds were inactive or had only weak cytotoxicity towards the tested tumor cell lines. However, in the bioassay of inhibitory activity against CDC25B dual specificity phosphatase, which is a key enzyme for cell cycle progression and was observed in a variety of cancers with a striking association with tumor aggressiveness and poor prognosis,[24] compound **7** exhibited significant inhibitory activity with an IC_{50} value of 2.1 μM. While in the bioassay of

protecting activity against H_2O_2-caused damage on SHSY5Y cells, a widely used neuroblastoma cell line for study of neurodegenerative disease,[25] compound **8a** showed significant protection (11.5% increase in cell viability).

Conclusions

In summary, six new tirucallane protolimonoids, toonapubesins A–F (**1–6**), one new rearranged tirucallane protolimonoid, toonapubesin G (**7**), and two new 21,22,23-trinorapotirucallane limonoids, toonapubesic acids A (**8**) and B (**9**), possessing an unprecedented carbon skeleton, along with five known related compounds (**10–15**), were isolated from the stem bark of *Toona ciliata* var. *pubescens*. The structures of dyvariabilin H (**10c**) proposed by Sticher *et al.* and cneorin-NP_{36} (**11b**) by Mondon *et al.* were corrected as **10** and **11**, respectively. Toonapubesin G (**7**) showed promising inhibitory activity against CDC25B with an IC_{50} value of 2.1 μM, while compound **8a** showed significant cell protecting activity against H_2O_2-induced SH-SY5Y cell damage with 11.5% increase in cell viability.

Experimental section

General experimental procedures

Melting points were measured on an SGW X-4 melting point instrument and were uncorrected. Optical rotations were measured on a Perkin-Elmer polarimeter 341. UV spectra were recorded on a 756 CRT spectrophotometer. CD spectra were obtained on a JASCO 810 spectrometer. IR spectra were recorded on a Nicolet-Magna FT-IR 750 spectrometer. The NMR spectra were measured on Bruker DRX 400, Varian Mercury 300 and Varian Inova 600 spectrometers. Chemical shifts were expressed in δ (ppm) and coupling constants (J) in Hz. ^{1}H and ^{13}C NMR assignments were supported by ^{1}H–^{1}H COSY, HSQC, HMBC and ROESY experiments. ESIMS and HRESIMS spectra were recorded on a Q-TOF Micro LC-MS-MS mass spectrometer. Reversed-phase HPLC analysis was performed on an Agilent 1100 series liquid chromatography using a VWD G1314A detector at 210 nm and a semi-preparative ZORBAX ODS column (250 mm × 9.4 mm i.d., 5 μm particle size. Commercial silica gel (Qing Dao Hai Yang Chemical Group Co., 200–300 mesh) was used for column chromatography (CC), and precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co., G60 F-254) were used for analytical TLC.

Plant material

The stem bark of the *Toona ciliata* var. *pubescens* was identified by Prof. Ren-Lin Liu, Department of Chemistry and Biological Science, Gan Nan Normal College, and collected in Xinfeng, Jiangxi Province, China, in August 2008. A voucher sample (08-*P*-46) is available for inspection at the Herbarium of Shanghai Institute of Materia Medica, CAS.

Extraction and isolation

The air-dried powder of the stem bark of *T. ciliata* var. *pubescens* (4 kg) was extracted exhaustively with MeOH at r.t. (40 L × 3, each for 7 days). Removal of MeOH under vacuum gave a yellow extract (500 g), which was subsequently dissolved in water to form a suspension (1 L) and extracted with $CHCl_3$. The $CHCl_3$-soluble fraction (211 g) was separated by silica gel column chromatography (CC) eluted with a gradient of petroleum ether/EtOAc (90 : 10 to 0 : 100) to give 10 fractions. Fraction 1 (2.5 g) was chromatographed on silica gel with petroleum ether/EtOAc (95 : 5 and 90 : 10) to yield **11** (13.2 mg), **14** (15.0 mg), and **15** (4.2 mg). Fraction 8 (11.2 g) was separated on a column of silica gel (petroleum ether/acetone, 90 : 10) to afford fractions 8a and 8b. Fractions 8a and 8b were respectively chromatographed over Sephadex LH-20 eluting with $CHCl_3$/MeOH (50 : 50), and each of them was then purified by reversed phase HPLC eluted with MeOH/H_2O (92 : 8) to yield **7** (8.1 mg). Fraction 9 (8.8 g) was subjected to column chromatography on silica gel eluted with a gradient of $CHCl_3$/MeOH (20 : 1 to 95 : 5) to give four major fractions 9a–9d. Fraction 9b was purified on a column of Sephadex LH-20 eluting with $CHCl_3$/MeOH (50 : 50) to yield **12** (8.2 mg). Fraction 9c subjected to a column of Sephadex LH-20 eluted with $CHCl_3$/MeOH (50 : 50) to give fractions 9c1–9c2. 9c1 was separated by reversed phase HPLC (MeOH/H_2O, 90 : 10) to yield **1** (10.7 mg), **2** (5.2 mg), **3** (5.8 mg) and **10** (25.3 mg). Fraction 10 (13 g) was separated on a column of MCI gel (MeOH/H_2O, 50 : 50 to 90 : 10) to afford subfractions 10a (4.2 g), 10b (2.3 g), 10c (2.5 g), and 10d (2.1 g). Fraction 10b (2.3 g) was a limonoid-containing fraction, which was deduced from its ^{1}H NMR spectrum. 1.1 g of this fraction was chromatographed on silica gel CC with petroleum ether/EtOAc (80 : 20 and 60 : 40) to afford fractions 10b1–10b3. Fractions 10b1–10b2 were chromatographed over Sephadex LH-20 eluting with $CHCl_3$/MeOH (50 : 50), and each of them was then purified by reversed phase HPLC eluted with MeOH/H_2O (85 : 15) to yield **8** (0.8 mg) and **9** (0.5 mg), respectively. More fraction 10b (1.2 g) was treated with CH_2N_2 giving a methylated fraction. This fraction was successively separated by silica gel CC with petroleum ether/EtOAc (80 : 20) to afford methyl ester derivatives of **8a** (11.6 mg) and **9a** (10.3 mg), respectively. Fraction 10c was subjected to a column of silica gel eluted with a gradient of $CHCl_3$/EtOAc (80 : 20 to 60 : 40) to give six subfractions10c1–10c6, and each of 10c1 and 10c2 was then purified by reversed phase HPLC eluted with MeOH/H_2O (70 : 30) to yield **5** (7.2 mg) and **6** (10.4 mg). Fraction 10d was chromatographed on reversed phase HPLC, eluted with MeOH/H_2O (40 : 60 to 80 : 20), to give **4** (14.2 mg) and **13** (12.4 mg).

Toonapubesin A (1). White amorphous powder; $[\alpha]_D^{16}$ −50.7 (*c* 0.08, MeOH); IR (KBr) ν_{max} 3374, 2926, 1715, 1452, 1425, 1385, 1246, 1153, 998 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; positive mode ESIMS *m/z* 495.4 $[M + Na]^+$, 967.8 $[2M + Na]^+$; negative mode ESIMS *m/z* 517.6 $[M + HCOO]^-$; HRESIMS *m/z* 495.3455 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_4Na$, 495.3450).

Toonapubesin B (2). White amorphous powder; $[\alpha]_D^{16}$ −52.6 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3374, 2925, 1718, 1452, 1425, 1385, 1248, 1155, 998 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; positive mode ESIMS *m/z* 495.4 $[M + Na]^+$, 967.7 $[2M + Na]^+$; negative mode ESIMS *m/z* 517.6 $[M + HCOO]^-$; HRESIMS *m/z* 495.3459 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_4Na$, 495.3450).

Toonapubesin C (3). White amorphous powder; $[\alpha]_D^{16}$ −55.0 (*c* 0.04, MeOH); IR (KBr) ν_{max} 3374, 2925, 1719, 1452, 1425, 1388, 1246, 1153, 998 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Tables 1

and 2; positive mode ESIMS *m/z* 495.4 $[M + Na]^+$, 967.8 $[2M + Na]^+$; HRESIMS *m/z* 495.3451 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_4Na$, 495.3450).

Toonapubesin D (4). White amorphous powder; $[\alpha]_D^{20}$ −31.1 (*c* 0.18, MeOH); ECD (MeOH), λ_{max} [nm] ($\Delta\varepsilon$), $c = 1.07 \times 10^{-3}$): 192 (+4.49), 209 (–2.11), 300 (–0.55) nm; IR (KBr) ν_{max} 3417, 2933, 1709, 1466, 1465, 1385, 1286, 1161, 1041 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; positive mode ESIMS *m/z* 513.4 $[M + Na]^+$, 1003.8 $[2M + Na]^+$; negative mode ESIMS *m/z* 535.6 $[M + HCOO]^-$; HRESIMS *m/z* 513.3555 $[M + Na]^+$ (calcd for $C_{30}H_{50}O_5Na$, 513.3556).

Toonapubesin E (5). White amorphous powder; $[\alpha]_D^{20}$ −44.8 (*c* 0.15, MeOH); ECD (MeOH), λ_{max} [nm] ($\Delta\varepsilon$), $c = 1.75 \times 10^{-3}$): 192 (+7.74), 215 (–0.56) nm; IR (KBr) ν_{max} 3415, 2947, 1640, 1458, 1383, 1161, 1031, 957 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; positive mode ESIMS *m/z* 515.4 $[M + Na]^+$, 1007.7 $[2M + Na]^+$; negative mode ESIMS *m/z* 537.6 $[M + HCOO]^-$; HRESIMS *m/z* 515.3710 $[M + Na]^+$ (calcd for $C_{30}H_{52}O_5Na$, 515.3712).

Toonapubesin F (6). White amorphous powder; $[\alpha]_D^{20}$ −70.5 (*c* 0.09, MeOH); ECD (MeOH), λ_{max} [nm] ($\Delta\varepsilon$), $c = 1.46 \times 10^{-3}$): 190 (+6.00), 213 (–1.86) nm; IR (KBr) ν_{max} 3508, 3419, 2949, 2877, 1711, 1637, 1446, 1375, 1163, 1049, 802 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; positive mode ESIMS *m/z* 491.4 $[M + H]^+$, 513.4 $[M + Na]^+$, 1003.6 $[2M + Na]^+$; negative mode ESIMS *m/z* 489.6 $[M - H]^-$; HRESIMS *m/z* 513.3564 $[M + Na]^+$ (calcd for $C_{30}H_{50}O_5Na$, 513.3556).

Toonapubesin G (7). White amorphous powder; $[\alpha]_D^{20}$ −36.0 (*c* 0.13, MeOH); ECD (MeOH), λ_{max} [nm] ($\Delta\varepsilon$), $c = 1.46 \times 10^{-3}$): 192 (+5.39), 209 (–2.46) nm; IR (KBr) ν_{max} 3435, 2926, 1708, 1468, 1385, 1065 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; positive mode ESIMS *m/z* 479.5 $[M + Na]^+$, 935.7 $[2M + Na]^+$; negative mode ESIMS *m/z* 501.4 $[M + HCOO]^-$; HRESIMS *m/z* 479.3517 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_3Na$, 479.3501).

Toonapubesic acid A (8). White amorphous powder; $[\alpha]_D^{25}$ +111.4 (*c* 0.03, MeOH); ECD (MeOH), λ_{max} [nm] ($\Delta\varepsilon$), $c = 1.43 \times 10^{-4}$): 337 (–3.84), 291sh (–0.81), 232 (+16.63), 192 (+16.54); IR (KBr) ν_{max} 3446, 2922, 2850, 1736, 1674, 1466, 1437, 1379, 1250, 1223, 1030 cm^{-1}; ^1H NMR data ($CDCl_3$, 300 MHz) δ 7.16 (d, J = 9.9 Hz, H-1), 5.95 (d, J = 9.9 Hz, H-2), 2.49 (d, J = 12.6 Hz, H-5), 5.34 (dd, J = 12.6, 2.7 Hz, H-6), 5.01 (d, J = 2.7 Hz, H-7), 2.56 (dd, J = 12.6, 3.6 Hz, H-9), 3.38 (br s, H-15), 2.39 (dd, J = 10.5, 6.6 Hz H-17), 1.18 (s, Me-18), 1.20 (s, Me-19), 1.25 (s, Me-28), 1.16 (s, Me-29), 1.22 (s, Me-30), 2.01 (s, OAc-6), 2.11 (s, OAc-7); positive ESIMS *m/z* (rel int) 511.3 $[M + Na]^+$ (100); negative ESIMS *m/z* (rel int) 487.2 $[M - H]^-$ (100).

Compound 8a. Colorless crystals; mp 232–234 °C; $[\alpha]_D^{25}$ +72.0 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 225 (3.57) nm; ECD (MeCN, λ_{max} [nm] ($\Delta\varepsilon$), $c = 1.85 \times 10^{-4}$): 341 (–2.92), 291sh (–0.38), 229 (+12.13), 193 (+12.81); ECD (KCl) λ_{max} (mdeg), 134 μg of **8a** in 250 mg KCl: 343 (–5.00), 278sh (–1.04), 231 (+10.91), 191 (+12.01); IR (KBr) ν_{max} 3448, 2923, 2852, 1736, 1672, 1435, 1379, 1250, 1030, 935 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table 3; HRESIMS *m/z* 525.2466 $[M + Na]^+$ (calcd for $C_{28}H_{38}O_8Na$, 525.2464).

Toonapubesic acid B (9). White amorphous powder; $[\alpha]_D^{25}$ −43.6 (*c* 0.02, MeOH); ECD (MeOH), λ_{max} [nm] ($\Delta\varepsilon$), $c = 1.50 \times 10^{-4}$): 345sh (–1.98), 319 (–4.62), 278 (+5.68), 235sh (+1.83), 195 (+6.99); IR (KBr) ν_{max} 3363, 2924, 2852, 1728, 1695, 1682, 1620, 1460, 1342, 1207, 1170, 1033 cm^{-1}; ^1H NMR data ($CDCl_3$, 300 MHz) δ 6.92 (d, J = 9.9 Hz, H-1), 6.11 (d, J = 9.9 Hz, H-2), 2.62 (d, J = 10.2 Hz, H-9), 3.74 (s, H-15), 2.51 (dd, J = 10.5, 6.6 Hz H-17), 1.00 (s, Me-18), 1.28 (s, Me-19), 1.57 (s, Me-28), 1.49 (s, Me-29), 1.09 (s, Me-30); positive ESIMS *m/z* (rel int) 401.3 $[M + H]^+$ (100).

Compound 9a. White amorphous powder; $[\alpha]_D^{25}$ −81.3 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 222 (3.87), 278 (3.84) nm; ECD (MeOH), λ_{max} [nm] ($\Delta\varepsilon$), $c = 1.93 \times 10^{-4}$): 346sh (–3.90), 318 (–9.89) 279 (+12.07), 235sh (+3.65), 192 (+15.46); IR (KBr) ν_{max} 3363, 2922, 2850, 1728, 1695, 1682, 1620, 1460, 1342, 1170, 1034, 785 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table 3; HRESIMS *m/z* 437.1954 $[M + Na]^+$ (calcd for $C_{24}H_{30}O_6Na$, 437.1940).

Dyvariabilin H (10). White amorphous powder; $[\alpha]_D^{20}$ −14.3 (*c* 0.04, MeOH); IR (Film) ν_{max} 3429, 2970, 1705, 1458, 1385, 1167, 974, 756 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; positive mode ESIMS *m/z* 479.5 $[M + Na]^+$, 935.9 $[2M + Na]^+$; HRESIMS *m/z* 479.3500 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_3Na$, 479.3501).

Compound 11a. Colorless crystals; mp 164–166 °C; $[\alpha]_D^{16}$ −64.0 (*c* 0.10, $CHCl_3$); ^1H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; positive mode ESIMS *m/z* 509.4 $[M + Na]^+$, 995.8 $[2M + Na]^+$; negative mode ESIMS *m/z* 531.7 $[M + HCOO]^-$; HRESIMS *m/z* 509.3599 $[M + Na]^+$ (calcd for $C_{31}H_{50}O_4Na$, 509.3607).

Methyl-24,25,26,27-tetranortirucall-7-en-3-oxo-23-oate (12). white amorphous powder; $[\alpha]_D^{20}$ −50.3 (*c* 0.11, MeOH); ^1H and ^{13}C NMR ($CDCl_3$), see Tables 1 and 2; positive mode ESIMS *m/z* 437.3 $[M + Na]^+$, 851.6 $[2M + Na]^+$.

X-ray crystallographic analysis of compounds 8a and 11a. Colorless crystals of **8a** and **11a** were obtained in acetone and $CHCl_3$/MeOH (1 : 9), respectively. Crystal data were obtained on a Bruker Smart Apex CCD diffractometer with monochromated Mo-Kα radiation (λ = 0.71073 Å). The structure was solved by direct methods (SHELXS-97) and refined using full-matrix least squares difference Fourier techniques. All non-hydrogen atoms were refined anisotropically, and all hydrogen atoms were placed in idealized positions and refined as riding atoms with the relative isotropic parameters.†

Crystal data of 8a. Orthorhombic system, $C_{28}H_{38}O_8$, space group $P2_1$ with a = 7.1487(8) Å, b = 13.2022(14) Å, c = 28.693(3), V = 2702.9(5) Å^3; Z = 4; D_c = 1.235 Mg m^{-3}, and $F(000)$ = 1080. Crystal size: 0.280 × 0.265 × 0.137 mm^3. Independent reflections 2887 [R(int) = 0.0774]. The final indices were R_1 = 0.0415, wR_2 = 0.0847 [$I > 2\sigma(I)$].

Crystal data of 11a. Orthorhombic system, $C_{31}H_{50}O_4$, space group $P2_1$ with a = 10.2080(8) Å, b = 15.3825(12) Å, c = 17.7371(12), V = 2785.2(4) Å^3; Z = 4; D_c = 1.161 Mg m^{-3}, and $F(000)$ = 1072. Crystal size: 0.429 × 0.336 × 0.231 mm^3. Independent reflections 2936 [R(int) = 0.0826]. The final indices were R_1 = 0.0574, wR_2 = 0.1384 [$I > 2\sigma(I)$].

Preparation of MTPA esters. (*R*)- and (*S*)-MTPA-Cl (10 µL) and a catalytic amount of DMAP were separately added to two

different aliquots of alcohol **10** (1.0 mg each) in dry CH_2Cl_2 (0.5 mL), and the resulting mixtures were allowed to stand at room temperature for 12 h. After the evaporation of the solution, the mixtures were purified on preparative TLC (SiO_2, light petroleum ether/Et_2O, 7 : 3), affording pure (*S*)- and (*R*)-MTPA esters of **10**, respectively.

(*S*)-MTPA ester of 10 (10a). Selected ^{1}H NMR values ($CDCl_3$, 300 MHz) δ 5.71 (1H, dd, J = 15.5, 8.4 Hz, H-22), 5.32 (1H, dd, J = 15.5, 9.0 Hz, H-23), 5.31 (1H, m, H-7), 5.19 (1H, d, J = 9.0 Hz, H-24), 2.75 (1H, td, J = 14.1, 3.5 Hz, H-2β), 2.24 (1H, dt, J = 14.1, 5.4 Hz, H-2α), 2.11 (2H, m, H-6), 2.08 (1H, m, H-20), 1.21 (3H, s, Me-27), 1.18 (3H, s, Me-26), 1.12 (3H, s, Me-29), 1.05 (3H, s, Me-28), 1.00 (6H, s, Me-19, 30), 0.81 (3H, s, Me-18), 0.96 (3H, d, J = 6.4 Hz, Me-21); ESIMS m/z 695.4 $[M + Na]^+$.

(*R*)-MTPA ester of 10 (10b). Selected ^{1}H NMR values ($CDCl_3$, 300 MHz) δ 5.78 (1H, dd, J = 15.5, 8.2 Hz, H-22), 5.45 (1H, dd, J = 15.5, 9.1 Hz, H-23), 5.30 (1H, m, H-7), 5.23 (1H, d, J = 9.1 Hz, H-24), 2.75 (1H, td, J = 14.1, 3.3 Hz, H-2β), 2.24 (1H, dt, J = 14.1, 5.4 Hz, H-2α), 2.12 (1H, m, H-20), 2.11 (2H, m, H-6), 1.16 (6H, s, Me-26, 27), 1.12 (3H, s, Me-29), 1.05 (3H, s, Me-28), 1.00 (6H, s, Me-19, 30), 0.83 (3H, s, Me-18), 0.99 (3H, d, J = 6.7 Hz, Me-21); ESIMS m/z 695.6 $[M + Na]^+$.

Computational details

Geometry optimization [B3LYP/6-31G(d) level of theory] and TDDFT calculations were performed with Gaussian 03[26] using various functionals (B3LYP, BH&HLYP, PBE0) and TZVP basis set. CD spectra were generated as the sum of Gaussians[27] with 3000 cm^{-1} half-height width (*ca.* 16 nm at 230 nm), using dipole-velocity computed rotational strengths.

CDC25B inhibition assay

CDC25B phosphatase catalytic domain was expressed with the Glutathionine *S*-transferase (GST) and purified by the GSTrap affinity chromatograph. GST-CDC25B active enzyme was stored in 50 mM Tris–HCl Ph 8.0, 50 mM NaCl, 10 mM Glutathionine, 2 mM DTT and 2 mM EDTA at –80 °C. The typical inhibition assay was carried out in a 100 µL system containing 50 mM Tris–HCl PH 8.0, 50 mM NaCl, 2 mM DTT, 2 mM EDTA, 1% glycerol, 10µM OMEP, 2% DMSO and 70–100 nM GST-CDC25B. The reaction was monitored by Victor (Perkin–Elmer; excitation filter 485 nm and emission filter 530 nm) at room temperature. The IC_{50} was calculated with Prism 4 software (Graphpad, San Diego, CA, USA) from the non-linear curve fitting of the percentage of inhibition (% inhibition) *versus* the inhibitor concentration [I] by using the following equation: % inhibition = $100/(1+[IC_{50}/[I]]^k)$, where k is the Hill coefficient.

Neuroprotective activity assay

SH-SY5Y cell survival was evaluated according to the reported protocol with modification.[28] Cells were high passages from the ATCC (American Type Culture Collection) maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded into 96-well plates at a density of 1 × 105 cells mL^{-1} in MEM/F12 medium supplemented with 10% (v/v) fetal bovine serum. Experiments were carried out 24 h after cells were seeded. Test compounds and positive control R-tocopherol (vitamine E) were made to 10^{-2} M stock solutions with DMSO and then diluted to corresponding concentrations with cell culture medium. Cell survival was evaluated by methylthiazolyltetrazolium bromide (MTT) reduction[29] in order to analyze the cytoprotection of the test compounds. In brief, cells were incubated with test compounds (1 or 10 µM) or *R*-tocopherol (10 µM) 2 h prior to treatment with 100 µM H_2O_2 for another 24 h without changing the culture medium. Then 10 µL of MTT (5 mg mL^{-1}) was added to each well and incubated at 37 °C for 4 h. The cells were finally lysed with 100 µL of DMSO, and the amount of MTT formazan was measured at 490 nm spectral wavelength using a microplate reader. The positive control *R*-tocopherol (vitamin E) caused 7.52% increase in cell viability over the H_2O_2 group at 10 µM concentration.

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