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Structure and absolute configuration assignments of ochracines F–L, chamigrane and cadinane sesquiterpenes from the basidiomycete *Steccherinum ochraceum* HFG119†

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Ochracines F–L (**1–7**), seven previously undescribed chamigrane and cadinane sesquiterpenoids, together with four known chamigranes were isolated from cultures of the wood-decaying fungus *Steccherinum ochraceum* HFG119. Ochracines F–L were structurally characterized by extensive analysis of HRMS and NMR spectroscopic data. The relative configurations were assigned through a combination of NOE correlations and *J*-based configuration analysis (JBCA), while the absolute configurations were determined by X-ray single-crystal diffraction, and calculated methods (ECD, [α], ^{13}C NMR). All the new isolates were evaluated for their cytotoxicity against five human cancer cell lines HL-60, SMMC-7721, A549, MCF-7, and SW-480, and inhibitory activity on NO production in RAW 264.7 macrophages.

Introduction

Mushroom-derived sesquiterpenes are recognized as the most structural among the reported mushroom natural products.[1] Chamigrane sesquiterpenoids featuring a spiro backbone, are rare among higher fungi.[2,3] Most reported chamigranes are generally produced by the red alga of the genus *Laurencia* (Family Rhodomelaceae)[4–8] and endophytic fungi.[9–14]

Our previous chemical study on *Steccherinum ochraceum* resulted in the isolation of four rare endoperoxide type chamigranes, namely steperoxides A–D, of which steperoxides C and D exhibited antimicrobial activity.[2,3] Since natural endoperoxides are of special interest, they have attracted and continue to attract considerable attention for their bioactivities.[15–17] To explore additional peroxy natural products from the cultures of *S. ochraceum*, we scaled up the fermentation, leading to the isolation of five chamigrane-related norsesquiterpene derivatives, ochracines A–E,[18] and 9 chamigrane sesquiterpenes including five new ones, namely ochracines F–J (**1–5**), and two cadinane sesquiterpenes, namely ochracines K (**6**) and L (**7**) (Fig. 1).

As some studies have shown that the production of secondary metabolites is highly dependent on fermentation conditions and modes,[19–21] the fermentation conditions for producing endoperoxide chamigranes might be special.

On the other hand, the assignment of absolute configurations of small organic natural molecules sometimes is challenging due to the lack of chromophores which result in non-appropriate CD spectrum, and the overlapped proton signals in NMR spectrum causes problems of interpreting the NOE

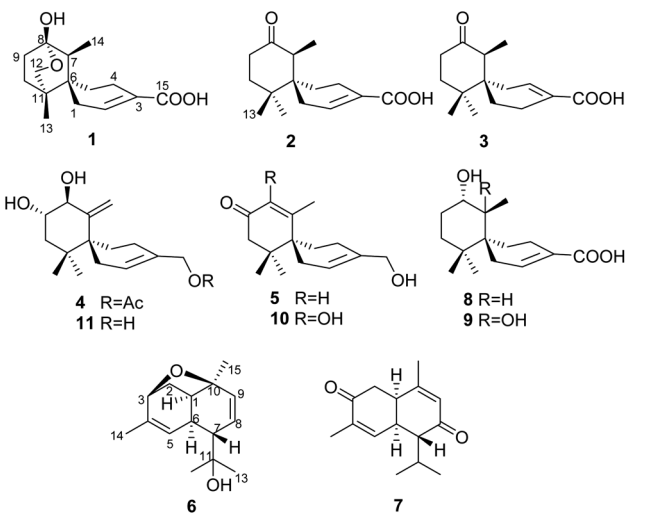

Fig. 1 Structures of compounds **1–11**.

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correlations, *etc.* Here, we described the isolation, structure elucidation, and more importantly, the absolute configuration assignment of the sesquiterpenoids from the cultures of the basidiomycete *S. ochraceum* by using combinatorial methods, including *J*-based configuration analysis (JBCA), calculated ECD, [α] and NMR. Moreover, the fermentation conditions for production endoperoxide type are discussed.

Results and discussion

Ochracine F (**1**), colourless needles (MeOH), afforded the molecular formula $C_{15}H_{22}O_4$ deduced from the HRESIMS ion at *m/z* 289.1408 $[M + Na]^+$, requiring five degrees of unsaturation. Combined analysis of ^1H, ^{13}C NMR data (Tables 1 and 2), and the HSQC spectroscopic correlations disclosed the presence of one singlet methyl, one doublet methyl, six sp^3 methylenes [one

Table 1 ^1H NMR data of compounds **1–5** (600 MHz)

No.	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]
1	2.33, brd (20.0), 2.16, brd (20.0)	2.11, brd (17.9), 2.06, brd (17.9)	2.38, brd (19.3), 2.00, brd (19.3)	2.18, brd (18.5), 2.07, brd (18.5)	2.29, brd (18.5), 2.11, brd (18.5, 2.0)
2	7.07, brs	7.12, brs	7.11, brs	5.70, brs	5.82, brs
4	2.49, brd (18.0), 2.27, brd (18.0)	2.45, overlapped, 2.30, m	2.31, m, 2.25, m	1.95, brd (18.0), 1.71, brd (18.0)	2.19, m, 1.94, overlapped
5	1.87, overlapped, 1.50, overlapped	1.72, overlapped, 1.67, overlapped	1.67, ddd (16.0, 10.0, 6.2), 1.58, ddd (16.0, 4.5, 4.5)	1.90, brd (13.0), 1.54, brd (13.0)	1.90, m, 1.85, m
7	1.74, q (7.2)	2.67, q (7.0)	2.61, q (7.0)		
8				4.08, brd (9.3)	5.88, s
9	1.90, overlapped, 1.69, overlapped	2.42, overlapped, 2.40, overlapped	2.51, ddd (14.7, 6.0, 6.0), 2.38, ddd (14.7, 8.5, 6.5)	3.46, ddd (11.5, 9.3, 5.0)	2.64, d (17.0), 2.08, d (17.0)
10	1.87, overlapped, 1.46, overlapped	1.81, m, 1.71, overlapped	1.81, ddd (13.7, 8.5, 6.0), 1.75, ddd (13.7, 6.5, 6.0)	1.81, dd (13.3, 11.5), 1.59, dd (13.3, 5.0)	
12	4.07, d (9.0), 3.57, d (9.0)	1.16, s	1.10[c], s	0.93, s	0.96[c], s
13	0.70, s	0.98, s	1.11[c], s	0.83, s	1.04[c], s
14	0.91, d (7.2)	1.04, d (7.0)	1.07, d (7.0)	5.39, d (1.7), 4.78, d (1.7)	1.98, s
15				4.40, d (12.0), 4.36, d (12.0)	4.07, d (13.4), 4.03, d (13.4)
15-OAc				2.05, s	

[a] Measured in CD_3OD. [b] Measured in $CDCl_3$. [c] Interchangeable assignments.

Table 2 ^{13}C NMR data of compounds **1–7** (150 MHz)

No.	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]	**6**[b]	**7**[a]
1	29.4, CH_2	27.4, CH_2	32.7, CH_2	29.6, CH_2	27.9, CH_2	40.0, CH	39.5, CH
2	142.4, CH	142.4, CH	141.8, CH	125.0, CH	123.5, CH	35.6, CH_2	40.9, CH
3	131.7, C	129.3, C	129.4, C	132.1, C	137.7, C	77.3, CH	199.6, C
4	22.2, CH_2	22.7, CH_2	22.1, CH_2	23.8, CH_2	24.0, CH_2	138.4, C	137.4, C
5	31.0, CH_2	28.3, CH_2	23.9, CH_2	26.6, CH_2	30.6, CH_2	126.1, CH	149.2, CH
6	39.0, C	44.5, C	43.6, C	45.4, C	44.0, C	37.1, CH	40.2, CH
7	47.2, CH	49.9, CH	50.5, CH	146.7, C	170.9, C	51.4, CH	59.1, CH
8	99.3, C	214.3, C	214.2, C	74.5, CH	127.3, CH	123.8, CH	202.6, C
9	28.0, CH_2	37.7, CH_2	37.0, CH_2	73.3, CH	49.1, CH_2	136.5, CH	128.1, CH
10	30.6, CH_2	38.2, CH_2	37.1, CH_2	43.1, CH_2	198.8, C	77.4, C	162.1, C
11	35.8, C	37.5, C	37.5, C	37.5, C	40.6, C	72.9, C	28.1, CH
12	73.3, CH_2	23.7, CH_3	25.2, CH_3	24.7, CH_3	25.0, CH_3	28.3, CH_3	21.0, CH_3
13	17.7, CH_3	26.2, CH_3	24.6, CH_3	24.1, CH_3	24.0, CH_3	29.8, CH_3	21.5, CH_3
14	13.8, CH_3	11.8, CH_3	12.5, CH_3	109.8, CH_2	24.5, CH_3	21.5, CH_3	15.7, CH_3
15	170.8, C	170.4, C	170.9, C	68.3, CH_2	67.0, CH_2	28.4, CH_3	21.9, CH_3
15-OAc				171.1, C			
15-OAc				21.2, CH_3			

[a] Measured in CD_3OD. [b] Measured in $CDCl_3$.

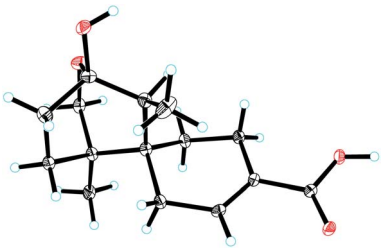

Fig. 2 ORTEP drawing of compound **1**.

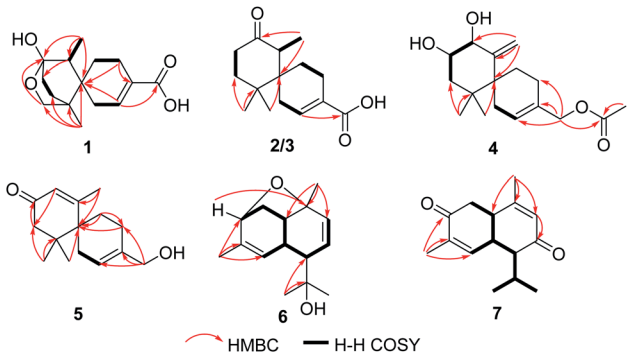

Fig. 3 Selected HMBC and $^{1}H-^{1}H$ COSY correlations of **1**–**7**.

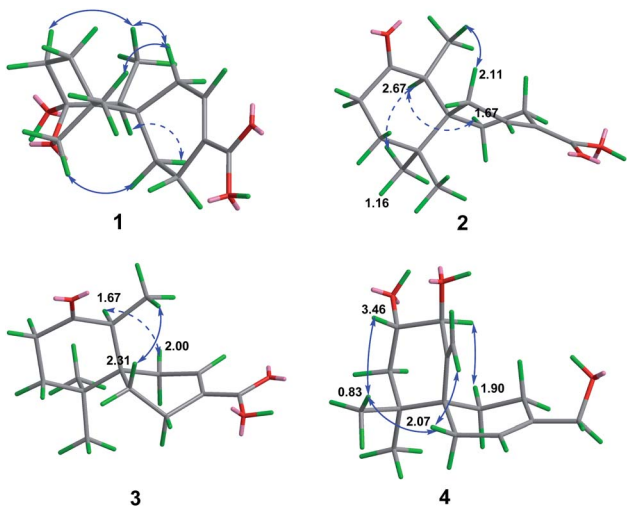

Fig. 4 Selected NOE correlations of **1**–**4**.

oxygenated ($\delta_{C/H}$ 73.3/4.07, 3.57)], one sp^3 methine, one trisubstituted double bond ($\delta_{C/H}$ 142.4/7.07; δ_C 131.7), and four quaternary carbons, including one hemiketal carbon (δ_C 99.3) and a conjugated carboxylic carbon (δ_C 170.8). The above assignments (an α,β-unsaturated carboxyl) account for two of five hydrogen deficiencys, indicating that **1** is a tricyclic compound. Further analysis of the $^{1}H-^{1}H$ COSY spectrum (Fig. 3) established five spin systems of H_2-1/H-2, H_2-4/H_2-5, H_3-14/H-7, and H_2-9/H_2-10. The HMBC spectrum (Fig. 3) showed correlations from H_3-13 to C-6/C-10/C-11/C-12, H_3-14 to C-6/C-7/C-8, and H-7 to C-9, establishing a partial structure of a six-membered ring with a hemiketal (C-8), 12-CH_2OH, 13-CH_3 and 14-CH_3, and correlations from H_2-4 to C-3/C-6, and H-2 to C-15, confirming the presence of a cyclohexene ring with a carboxylic group (15-COOH) substituted at C-3. These two rings share C-6 as the spiro carbon judging from the HMBC correlations of H_3-13/H_3-14/H_2-4/H-2 to C-6. The molecular formula, $C_{15}H_{22}O_4$, and the HMBC correlations from H_2-12 to C-8 suggested that C-8 and C-12 are linked through an ether bond, which rendered compound **1** a novel chamigrane sesquiterpene featuring a 6/6/6 tricyclic system. As shown in Fig. 4, the relative configuration was determined by NOE correlations. Fortunately, single crystal X-ray analysis using CuKα radiation not only confirmed the structure validity of **1**, but also established its absolute configuration (Fig. 2). Moreover, in order to provide integrated data for the structure of this type, experimental CD and calculated ECD spectra of (6*R*,7*S*,8*S*,11*R*)-**1** were carried out (Fig. 5) (ESI†).

Ochracine G (**2**), $C_{15}H_{22}O_3$, had comparable 1D NMR data (Tables 1 and 2) with those of co-isolate merulinol (**8**),[11] implying that **2** is a chamigrane sesquiterpene. Analysis of the spectroscopic data showed that 8-CHOH in **8** was oxygenated to a ketone (δ_C 214.3) in **2**, which was corroborated by HMBC correlations from H_3-14 to C-8 (Fig. 3) and the HRMS result. The relative configuration of **2** was deduced by NOE correlations of H_3-12 (δ_H 1.16)/H-7 (δ_H 2.67)/H_{eq}-5 (δ_H 1.67), and H_3-14 (δ_H 1.04)/H_{ax}-1 (δ_H 2.11) (Fig. 4), showing that the relative stereochemistries of **2** are 6*R** and 7*S**.

The molecular formula of ochracine H (**3**), $C_{15}H_{22}O_3$, is identical with that of **2**, implying that they are analogues. The quite similar 1D NMR spectra of **2** and **3** (Tables 1 and 2), except for minor differences of chemical shifts of C-1, C-5, and C-6, suggested that they share the same planar structures, but with different configurations, which was further verified by $^{1}H-^{1}H$ COSY and HMBC spectra (Fig. 3). The configuration difference of C-6 between **2** and **3** led to changes of the chemical shifts at C-1, C-5 and C-6, suggesting the chiral carbon C-6 should be *S** in **3** rather than the *R** in **2**. In the ROESY experiment (Fig. 4), cross peaks of H-7 (δ_H 2.61) with H_{eq}-1 (δ_H 2.00), and H_3-14 (δ_H

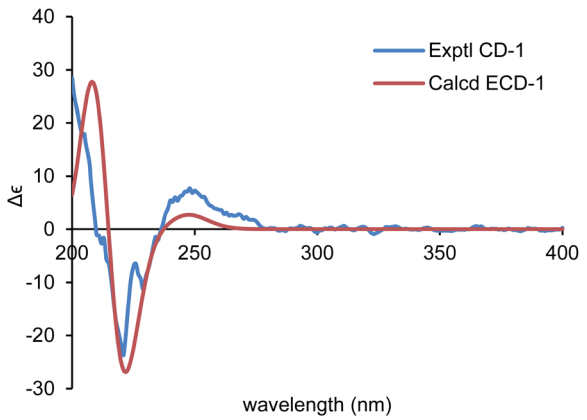

Fig. 5 Comparison of the calculated ECD spectrum for (6*R*,7*S*,8*S*,11*R*)-**1** (σ = 0.22 eV, UV shift 0 nm) with the experimental spectrum of 1 in MeOH.

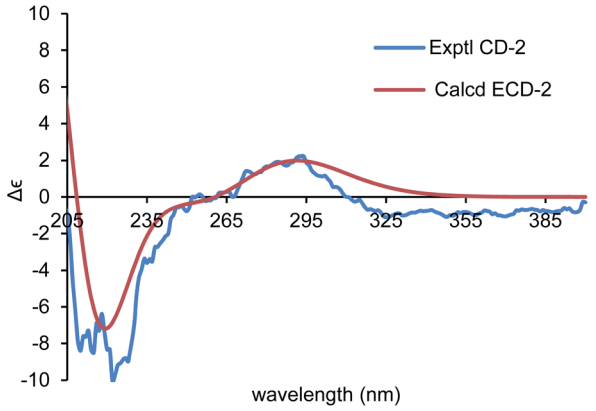

Fig. 6 Comparison of the calculated ECD spectrum for (6*R*,7*S*)-**2** (σ = 0.40 eV, UV shift −1 nm) with the experimental spectrum of **2** in MeOH.

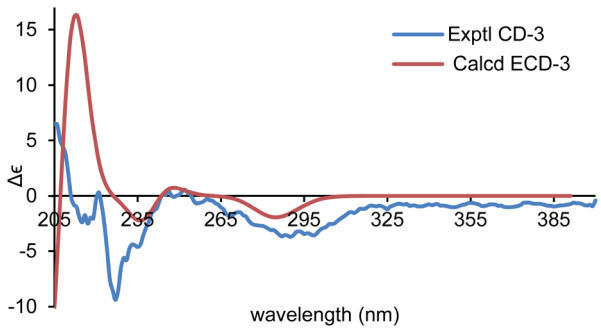

Fig. 7 Comparison of the calculated ECD spectra for (6*S*,7*S*)-**3** (σ = 0.16 eV, UV shift −8 nm) with the experimental spectrum of **3** in MeOH.

1.07) with H_{ax}-5 (δ_H 1.67) observed revealed that relative configurations of **2** are 6*S** and 7*S**.

To establish the absolute configurations of **2** and **3**, calculated electronic circular dichroisms (ECDs) for (6*R*,7*S*)-**2** and (6*S*,7*S*)-**3** were carried out (ESI†), respectively. As shown in Fig. 6 and 7, comparison with the experimental of (6*R*,7*S*)-**2**, and (6*S*,7*S*)-**3** with the calculated ones, the calculated ECD spectra of presented good fitting, which are in accord with octant rule to the Cotton effect near 290 nm observed in the ECD spectra. Thus, the absolute configurations of **2** and **3** were determined with no doubt as suggested above.

Ochracine I (**4**) had a molecular formula $C_{17}H_{26}O_4$ with five degrees of unsaturation. Its ^{1}H and ^{13}C NMR (Tables 1 and 2) data showed a close similarity to those of the co-isolate compound, acaciicolinol F (**11**).[13] Analysis of these data indicated that the hydroxyl at C-15 in **11** was changed into an acetate in **4**, which was supported by HMBC correlations from H_2-15 to carboxyl carbon (Fig. 3). Similar to **11**, a large coupling constant (J = 9.3 Hz) between H-8 and H-9 observed for **4**, together with NOE cross peaks of H-8 (δ_H 4.08)/H_{eq}-5 (δ_H 1.90), and H-9 (δ_H 3.46)/H_3-13 (δ_H 0.83)/H_{eq}-1 (δ_H 2.07)/H-14 (δ_H 4.78) (Fig. 4), suggested that compound **4** is the acetyl derivative of **11**, and they share the same relative configuration at C-6, C-8, and C-9. In addition, the optical rotation of **4** showed positive sign ($[\alpha]_{20\ D}$ + 45.5) similar to of **11** ($[\alpha]_{26\ D}$ + 79.7),[13] leading to the assignment of the absolute stereochemistry of **4** as 6*S*, 8*S*, 9*S* (Fig. 1).

The molecular formula of ochracine J (**5**), is $C_{15}H_{22}O_3$ deduced by HREIMS ion at *m*/*z* 250.1567 $[M]^+$. The 1D NMR (Tables 1 and 2) and HSQC spectra displayed signals for three methyl singlets, five methylenes (one oxygenated), an α,β-unsaturated ketone (δ_C 170.9; $\delta_{C/H}$ 127.3/5.88, s; 198.8), one pair of trisubstituted double bond carbons, and two sp^3 quaternary carbons. These data shared close similarity to those of acaciicolinol L (**10**)[13] which was also isolated from in this study, the only difference between them being the absence of hydroxyl at C-8 in **5**. This difference was confirmed by the presence of a singlet olefinic proton (δ_H 5.88) and a long-range correlation from H_3-14 to C-8 in the HMBC experiment (Fig. 3). Since there is only one chiral carbon, the absolute stereochemistry of C-6 was defined *S* by comparison of the experimental value of optical rotation ($[\alpha]_{20\ D}$ + 39.4) with both the reported data of **10** ($[\alpha]_{27\ D}$ + 159.6) and the calculated data ($[\alpha]_D$ + 22.3) (ESI†). Hence, the structure of **5** was identified as shown in Fig. 1.

Ochracine K (**6**), presented a molecular formula $C_{15}H_{22}O_2$ based on the HRESIMS (observed *m*/*z* 257.1512 $[M + Na]^+$), requiring for five indices of double bond equivalents. The ^{13}C NMR and DEPT spectra of **6** (Table 2) showed 15 carbon signals corresponding to four methyls (δ_C 21.5, 28.3, 28.4, and 29.8), one sp^3 methylene (δ_C 35.6), three sp^2 methines (δ_C 123.8, 126.1, and 136.5), four sp^3 methines (δ_C 37.1, 40.0, 51.4, and 77.3), and three quaternary carbons (one sp^2 δ_C 138.4, and two oxygenated δ_C 72.9, 77.4). The ^{1}H NMR spectrum of **6** (Table 3) displayed signals of four singlet methyl groups at δ_H 1.21 (H_3-15), 1.26 (H_3-13), 1.31 (H_3-12), and 1.67 (H_3-14), one oxymethine at δ_H 4.04 (d, J = 5.0 Hz, H-3), three olefinic protons at 4.86 (brs, H-5), 5.66 (dd, J = 10.3, 6.2 Hz, H-8), 5.99 (d, J = 10.3 Hz, H-9). These described NMR characteristic signals and MS data suggested **6** should be a sesquiterpene. Starting from obvious HMBC correlations of H_3-12/13 to C-7/11, H_3-14 to C-3/4/5, H_3-15 to C-1/

Table 3 ^{1}H NMR data of compounds **6** and **7** (150 MHz)

No.	**6**[b]	**7**[a]
1	2.60, dd (4.8, 4.8)	3.24, m
2	2.21, ddd (11.0, 5.0, 4.8), H_{eq}, 1.87, d (11.0), H_{ax}	2.86, dd (16.3, 5.3), 2.78, dd (16.3, 5.6)
3	4.04, d (5.0)	
5	4.86, brs	6.55, brs
6	2.93, brs	3.32, overlapped
7	1.99, d (6.2)	2.17, dd (8.5, 4.3)
8	5.66, dd (10.3, 6.2)	
9	5.99, d (10.3)	5.81, brs
11		2.02, m
12	1.31, s	1.14, d (7.0)
13	1.26, s	0.92, d (7.0)
14	1.67, s	1.72, s
15	1.21, s	1.92, s

[a] Measured in CD_3OD. [b] Measured in $CDCl_3$.

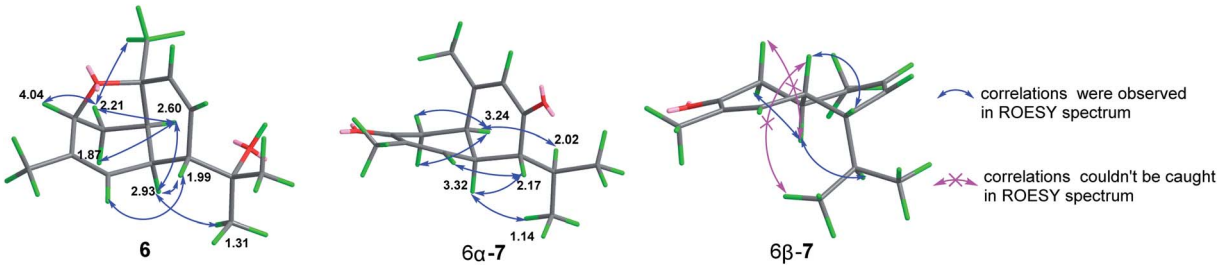

Fig. 8 Selected NOE correlations of **6** and **7**.

9/10, coupled with the successive ^{1}H–^{1}H COSY correlations of H-3/H_2-2/H-1/H-6/H-7/H-8/H-9 and H-5/H-6 (Fig. 3) suggested that **6** is a cadinane with two pair of double bonds at C-4(5) and C-8(9), and three oxygen baring carbons (C-3, C-10, and C-11). However, the MS result and the above assignments indicated that **6** is tricyclic. Further analysis of HMBC, a key correlation from H-3 to C-10 implied that the third ring is formed between 3-OH and 10-OH *via* a dehydration reaction. The relative stereochemistry of **6** (1*S**, 3*R**, 6*R**, 7*R**, 10*R**) was determined by a combination of JBCA and ROESY spectrum (Fig. 8). Firstly, dihedral angles of H-3–C-3–C-2–H_{ax}-2 and H_{ax}-2–C-2–C-1–H-1 close to 90° deduced by the JBCA (H-3, δ_H 4.04, d, J = 5.0 Hz; H_{ax}-2, δ_H 1.87, d, J = 11 Hz), together with the correlations of H-3/H_2-2/H-1 (δ_H 2.60), H_3-15/H_{eq}-2 (δ_H 2.21) in the ROESY spectrum (Fig. 8), suggested that the orientations of H-1, H-3, and H_3-15 are β. The broad singlet signal for H-6 indicative of 90° dihedral angles between H-6 and other protons at adjacent carbons, coupled NOE cross peaks of H-1/H-6 (δ_H 2.93)/H_3-12 (δ_H 1.31), and H-7 (δ_H 1.99)/H-5 (δ_H 4.86), illustrated that the orientation of H-6 is β, and the orientation of H-7 is α.

By using of the same quantum chemistry mechanic methods as **1–3**, ECD of (1*S*,3*R*,6*R*,7*R*,10*R*)-**6** (**6a**) and ^{13}C NMR of (1*S**,3*R**,6*R**,7*R**,10*R**)-**6** were calculated (ESI†) to establish its absolute configurations and prove its structure validity, which shared an opposite curve and a highly comparable linear regression (R^2 = 0.9993) compared with its experimental CD/NMR spectra (Fig. 9 and 10), respectively, confirming the absolute configurations of **6** are 1*R*,3*S*,6*S*,7*S*,10*S*. And the structure of **6** is (1*R*,3*S*,6*S*,7*S*,10*S*)-amorpha-5α,10α-epoxy-4(5),8(9)-dien-11-ol.

Ochracine L (**7**), was obtained as a colourless oil, and its molecular formula, $C_{15}H_{20}O_2$, was determined from the HREIMS (observed *m*/*z* 232.1466 $[M]^+$), requiring six degrees of unsaturation. The UV spectrum of **7** presented maximal absorption at 230.5, and 248.0 nm implying a conjugated group. Combined analyses of ^{1}H, ^{13}C, and HSQC data of **7** (Tables 2 and 3) revealed the presence of two down-shifted methyl singlets and two secondary methyls, one sp^3 methylene, two sp^2 and four sp^3 methines, five quaternary carbons (two conjugated ketones, two olefinic carbons). Since the two ketones and two double bonds takes up 4 degrees of hydrogen deficiency, **7** should be bicyclic. The existence of a propyl unit supported by

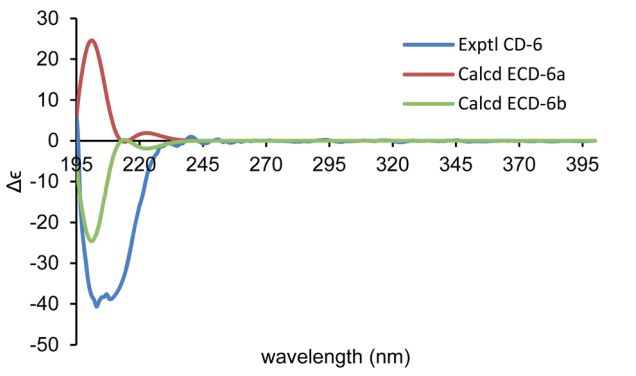

Fig. 9 Comparison of the calculated ECD spectra for (1*S*,3*R*,6*R*,7*R*,10*R*)-**6** (**6a**) (σ = 0.22 eV, UV shift 0 nm) and (1*R*,3*S*,6*S*,7*S*,10*S*)-**6** (**6b**) with the experimental spectrum of **6** in MeOH.

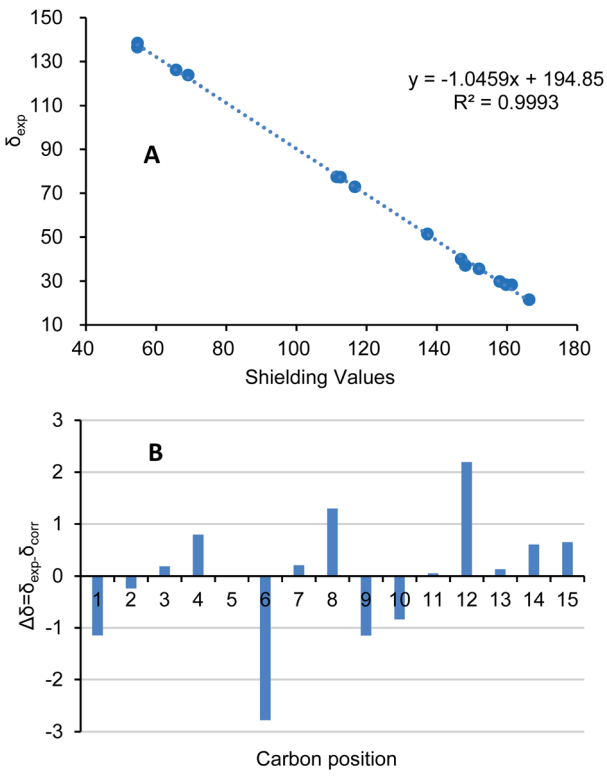

Fig. 10 (A) Regression analysis of experimental *versus* calculated ^{13}C NMR chemical shifts of **6** at GIAO B3LYP/6-31G(d) level; linear fitting was shown as a line. (B) Relative chemical shift errors between scaled ^{13}C NMR and experimental ^{13}C NMR for **6**.

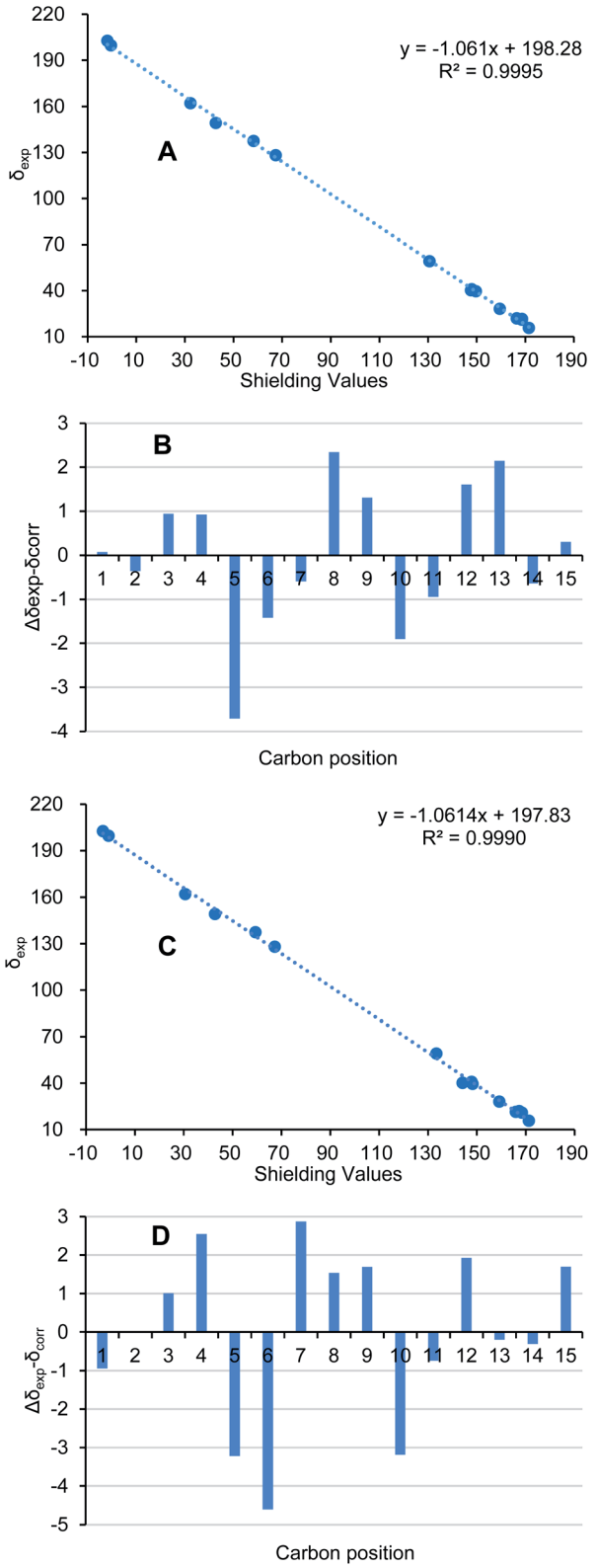

Fig. 11 (A/C) Regression analysis of experimental *versus* calculated ^{13}C NMR chemical shifts of 6α-**7**/6β-**7** at GIAO B3LYP/6-31G(d) level. (B/D) Relative chemical shift errors between scaled ^{13}C NMR and experimental ^{13}C NMR for 6α-**7**/6β-**7**.

^{1}H–^{1}H COSY (Fig. 3) correlations between H_3-12/H-11/H_3-13 indicated that this bicyclic molecule is a cadinane-type sesquiterpene, and shared close similarity to α-amorphene-3-one,[22] except the presence of an additional conjugated ketone (δ_C 202.6) at C-8 in **7** rather than 8-CH_2 in α-amorphene-3-one. The relative configuration of **7** was mainly assigned by ROESY spectrum. Correlations of H-1 (δ_H 3.24)/H-11 (δ_H 2.02) suggested that H-1 and H-7 are in opposite side. It was difficult to deduce the relative configuration of H-6 by NOE correlation and JBCA analysis due to the close chemical shifts of H-1 and H-6, and the partially overlapped signal of H-6. Therefore, NOE correlations of the two possible stereoisomers, 6α-/6β-**7**, were compared. As shown in Fig. 8, only the 6α-**7** can satisfy these NOE correlations of H_2-2/H-1, and H_3-12/H-6/H-7, so the relative configurations of **7** are 1*R**,6*S**,7*R** (6α-**7**). To further ensure this deduction that the structure of **7** is 6α-**7** other than 6β-**7**, their ^{13}C NMR were calculated as shown in Fig. 11 (ESI†). Obviously, 6β-**7** could be excluded by the R^2 value (6α-**7**, $R^2 = 0.9995$; 6β-**7**, $R^2 = 0.9990$).

Considering the biosynthetic pathway and the calculated optical rotation ($[\alpha]_D$ + 83.2) of 6α-**7** (ESI†), the absolute configurations of **7** were assigned as 1*R*,6*S*,7*R*. Thus, the structure of **7** is (1*R*,6*S*,7*R*)-amorpha-4,9(10)-diene-3,8-dione.

The structures of merulinol (**8**), acaciicolinols C (**9**), L (**10**), and F (**11**) were established by comparing the NMR data with the published values.[11,13]

All the new isolates (**1**–**7**) were tested for their cytotoxicity against five human cancer cell lines: HL-60, SMMC-7721, A549, MCF-7, and SW-480, and against nitric oxide (NO) production. Unfortunately, they were devoid of any cytotoxicity and NO-production inhibitory activity.

Conclusions

In summary, eleven sesquiterpenes, including nine chamigrane-type ones and two cadinane-type ones, were isolated from the fermentation broth of the basidiomycete *S. ochraceum*. Their planar structures and absolute stereostructures were elucidated by spectroscopic analysis and calculated methods. Compounds **1** and **6**, each featured by a six or five-membered ether ring with the absolute configurations established *via* a single X-ray diffraction experiment and/or computational methods, are quite novel tricyclic sesquiterpene in the chamigrane or cadinane families. All the new isolates were evaluated for their cytotoxicity against five cancer cell lines and inhibition against NO production. One case should be noted is that the reason fails to obtain endoperoxide chamigranes may be attributed to the culture medium used for *S. ochraceum*. It was suggested that potato dextrose broth (PDB)[2,3,13,14] is more suitable for production of the endoperoxide chamigranes than sabouraud dextrose broth (SDB)[11] or glucose peptone broth (GPB) used in this research.

Experimental section

General experimental procedures

The crystal data were collected on an APEX II DUO spectrophotometer (Bruker AXS GmbH, Karlsruhe, Germany) using

graphite-monochromated Cu Kα radiation (λ = 1.54178 Å) as the X-ray source. Melting point was measured on an X-4 microscopic melting point meter (Yuhua Instrument Co., Ltd, Gongyi, China). Optical rotations were obtained on a JASCOP-1020 digital polarimeter (Horiba, Kyoto, Japan). UV spectra were recorded on a Shimadzu UV-2401PC UV-visible recording spectrophotometer (Shimadzu, Kyoto, Japan). A Chiral scan circular dichroism spectrometer (Applied Photophysics Limited, Leatherhead, Surrey, UK) was used to record the CD spectra. 1D and 2D NMR spectra were obtained on a Bruker Avance III 600 MHz spectrometer (Bruker Corporation, Karlsruhe, Germany). An Agilent 6200 Q-TOF MS system (Agilent Technologies, Santa Clara, CA) was used to acquire the HRESIMS data. HREIMS were recorded on a Waters AutoSpec Premier P776 MS system. Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) and silica gel (Qingdao Haiyang Chemical Co., Ltd) were used for column chromatography (CC). Medium pressure liquid chromatography (MPLC) was performed on a Büchi Sepacore System equipped with pump manager C-615, pump modules C-605 and fraction collector C-660 (Büchi Labortechnik AG, Flawil, Switzerland), and columns packed with Chromatorex C-18 (40–75 μm, Fuji Silysia Chemical Ltd., Kasugai, Japan). Preparative high performance liquid chromatography (prep-HPLC) was performed on an Agilent 1260 liquid chromatography system equipped with Zorbax SB-C18 columns (particle size 5 μm, dimensions 9.4 mm × 150 mm, flow rate 8 mL min^{-1}) and a DAD detector (Agilent Technologies, Santa Clara, CA, USA).

Fungal material

The basidiomycete *Steccherinum ochraceum* was collected at Ailao Mountain of Yunnan Province, China, in July 2003, which was identified by Prof. Mu Zang (Kunming Institute of Botany). The voucher specimen was deposited in the Herbarium of Kunming Institute of Botany, CAS. The strain (HFG119) was isolated from the fresh fruiting bodies of *S. ochraceum* and preserved on potato dextrose agar (PDA) medium at 4 Celsius degree.

Fermentation, extraction and isolation

The fungus *S. ochraceum* was cultured on PDA medium for one week in the early stage. The agar was then inoculated into 500 mL of glucose peptone broth (GPB).[23] After incubation for 1 week at 25 °C, the seed liquid was then inoculated into 500 mL Erlenmeyer flasks (150 flasks in all), each containing 400 mL of GPB. After incubation for 21 days, fungal culture was filtered to separate mycelia and broth (total volume of 60 L). The culture broth of *S. ochraceum* (60 L) was filtered and evaporated under reduced pressure, then partitioned between ethyl acetate (EtOAc) and water four times to yield a broth extract. The mycelia were extracted by EtOH (95%) three times. The extract was evaporated and then partitioned between EtOAc and water four times to give an EtOAc layer. Totally, the weight of the crude EtOAc layer was 34 g.

The crude EtOAc layer was first separated by MPLC eluted with a gradient solvent system of MeOH : H_2O (V/V 20 : 80, 40 : 60, 60 : 40, 80 : 20, 100 : 0), which gives twenty-one fractions (A–U). All compounds were purified by preparative HPLC (a flow rate of 8 mL min^{-1}) with Zorbax SB-C18 column using a linear elution of MeCN : H_2O under different volume ratio.

Fraction K (210 mg) was fractionated by Sephadex LH-20 (MeOH) to produce six subfractions (K1–K6). Fractions K3 (73.5 mg), K4 (58.3 mg), and K5 (42.9 mg) were separately subjected to silica gel CC (a gradient solvent system of petroleum ether: acetone (V/V) 5 : 1 to 2 : 1) to yield thirteen fractions K3a–K3m, ten fractions K4a–K4j, and ten fractions K5a–K5j. Compounds **1** (3.5 mg, t_R = 6.0 min), and **10** (5.0 mg, t_R = 10.8 min) were purified by semi-preparative HPLC (MeCN : H_2O (20 : 80 → 40 : 60) in 25 min) from fraction K4b. Similarly, subfractions K3g (MeCN : H_2O (17 : 83 → 32 : 68) in 20 min), K3b (MeCN : H_2O (17 : 83 → 32 : 68) in 20 min) and K3l (MeCN : H_2O (17 : 83 → 28 : 72) in 20 min) were dealt on semi-preparative HPLC affording compounds **5** (1.4 mg, t_R = 15.0 min), **7** (2.1 mg, t_R = 18.9 min) and **11** (6.1 mg, t_R = 14.7 min), respectively. Compound **9** (3.6 mg, t_R = 7.8 min) was isolated from fraction K5b (MeCN : H_2O (20 : 80 → 40 : 60) in 25 min) by preparative HPLC (a flow rate of 8 mL min^{-1}) with Zorbax SB-C18 column.

Subsequently, fraction L (190 mg) was further purified on Sephadex LH-20 (CH_3OH) affording three subfractions (L1–L3). Fraction L2 (80.9 mg) was subjected to Sephadex LH-20 (acetone) to give seventeen subfractions (L2a–L2q). By using preparative HPLC (a flow rate of 8 mL min^{-1}) with Zorbax SB-C18 column, compound **6** (1.0 mg, t_R = 11.3 min, MeCN : H_2O (18 : 82 → 30 : 70) in 20 min) was purified from L2d, and compound **8** (5.4 mg, t_R = 17.0 min) from L2n (MeCN : H_2O (24 : 76 → 39 : 61) in 20 min).

Fractions M (160 mg) and O (113 mg) were further purified by Sephadex LH-20 twice in succession (CH_3OH and then acetone). Fractions M2 and O1 gave 7 (M2a– M2g) and 8 minor fractions (O1a–O1h), among them subfractions M2f (MeCN : H_2O (20 : 80 → 33 : 67) in 20 min) yielding compounds **2** (1.0 mg, t_R = 17.0 min) and **3** (1.1 mg, t_R = 17.5 min), and subfractions M2f (MeCN : H_2O (20 : 80 → 35 : 65) in 20 min) yielding compound **4** (5.0 mg, t_R = 17.0 min) by preparative HPLC (a flow rate of 8 mL min^{-1}) with Zorbax SB-C18 column.

Ochracine F (1). Colourless needles; $[\alpha]_{20\ D}$ + 47.9 (*c* 0.35 MeOH); m.p. 146.6–153.6 °C; UV (MeOH) λ_{max} nm (log ε): 214.2 (3.90); ^{1}H NMR (600 MHz CD_3OD) data (Table 1); ^{13}C NMR (150 MHz CD_3OD) data (Table 2); HRESIMS *m*/*z* 289.1408 $[M + Na]^+$ (calcd for $C_{15}H_{22}O_4Na$, 289.1410).

X-ray crystallographic analysis. Colourless crystal of **1** were obtained by crystallization from MeOH. Crystal data for Cu_**1**_0 m: $2(C_{15}H_{22}O_4)\cdot 3(H_2O)$ M = 586.70 a = 6.1285(5) Å b = 32.973(3) Å c = 7.7602(6) Å α = 90° β = 108.863(3)° γ = 90° V = 1483.9(2) Å^3 T = 100(2) K space group $P21$ $Z = 2\mu(CuK\alpha) = 0.819$ mm^{-1} 13 461 reflections measured 4805 independent reflections (R_{int} = 0.0459). The final R_1 values were 0.0579 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.1595 ($I > 2\sigma(I)$). The final R_1 values were 0.0615 (all data). The final $wR(F^2)$ values were 0.1664 (all data). The goodness of fit on F^2 was 1.049. Flack parameter = 0.14(8).

Ochracine G (2). White powder; $[\alpha]_{19\ D}$ − 52.8 (*c* 0.06 MeOH); ^{1}H NMR (600 MHz $CDCl_3$) data (Table 1); ^{13}C NMR (0.150 MHz $CDCl_3$) data (Table 2); HRESIMS *m*/*z* 273.1467 $[M + Na]^+$ (calcd for $C_{15}H_{22}O_3Na$ 273.1461).

Ochracine H (3). White powder; $[\alpha]_{19\ D}$ − 6.1 (*c* 0.11 MeOH); UV (MeOH) λ_{max} nm (log ε): 215.6 (3.97); ^{1}H NMR (600 MHz $CDCl_3$) data (Table 1); ^{13}C NMR (150 MHz $CDCl_3$) data (Table 2); HREIMS *m*/*z* 250.1564 $[M]^+$ (calcd for $C_{15}H_{22}O_3$ 250.1569).

Ochracine I (4). Colourless oil; $[\alpha]_{20\ D}$ + 45.5 (*c* 0.50 MeOH); ^{1}H NMR (600 MHz $CDCl_3$) data (Table 1); ^{13}C NMR (150 MHz $CDCl_3$) data (Table 2); HRESIMS *m*/*z* 317.1728 $[M + Na]^+$ (calcd for $C_{17}H_{26}O_4Na$ 317.1723).

Ochracine J (5). Colourless oil; $[\alpha]_{20\ D}$ + 39.4 (*c* 0.14 MeOH); UV (MeOH) λ_{max} nm (log ε): 243.8 (3.95); ^{1}H NMR (600 MHz $CDCl_3$) data (Table 1); ^{13}C NMR (150 MHz $CDCl_3$) data (Table 2); HREIMS *m*/*z* 250.1567 $[M]^+$ (calcd for $C_{15}H_{22}O_3$ 250.1569).

Ochracine K (6). Colourless oil; $[\alpha]_{20\ D}$ + 76.5 (*c* 0.05 MeOH); UV (MeOH) λ_{max} nm (log ε): 203.0 (3.62); ^{1}H NMR (600 MHz $CDCl_3$) data (Table 3); ^{13}C NMR (150 MHz $CDCl_3$) data (Table 2); HRESIMS *m*/*z* 257.1512 $[M + Na]^+$ (calcd for $C_{15}H_{22}O_2Na$ 257.1512).

Ochracine L (7). Colourless oil; $[\alpha]_{20\ D}$ + 35.8 (*c* 0.21 MeOH); UV (MeOH) λ_{max} nm (log ε): 230.5 (4.16) 248.0 (3.56); ^{1}H NMR (600 MHz CD_3OD) data (Table 3); ^{13}C NMR (150 MHz CD_3OD) data (Table 2); HREIMS *m*/*z* 232.1466 $[M]^+$ (calcd for $C_{15}H_{20}O_2$ 232.1463).

Bioassays

Nitric oxide production in RAW 264.7 macrophages. The RPMI 1640 medium (Hyclone, Logan, UT) containing 10% FBS was used to culture the murine monocytic RAW 264.7 macrophages. The compounds were dissolved in DMSO and further diluted in medium to produce different concentrations. The culture medium and cell mixture were dispensed into 96-well plates (2×10^5 cells per well) and maintained at 37 °C under 5% CO_2 in a humidified atmosphere. After preincubation for 24 h, serial dilutions of the test compounds were added into the cells, up to the maximum concentration of 25 μM, then LPS was added to a concentration 1 μg mL^{-1} and incubation continued for 18 h. After addition of 100 μL of Griess reagent (reagent A and reagent B, Sigma, St. Louis, Mo) to 100 μL of each supernatant from the LPS-treated or LPS- and compound-treated cells in triplicate and incubation for 5 min, NO production of each cell was assessed by sample absorbance at 570 nm by a 2104 Envision Multilabel plate reader. L-N^G-Monomethyl arginine (L-NMMA) was used as a positive control.

Cytotoxicity against five human cancer cell lines. The following five human cancer cell lines were used: the HL-60 (ATCC CCL-240) human myeloid leukemia; SMMC-7721 human hepatocellular carcinoma; A-549 (ATCC CCL-185) lung cancer; MCF-7 (ATCC HTB-22) breast cancer; SW-480 (ATCC CCL-228) human colon cancer. The cell line SMMC-7721 was bought from China Infrastructure of Cell Line Resources (Beijing, China), and others were bought from American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Hyclone) and maintained at 37 °C under 5% CO_2 in a humidified atmosphere. Colorimetric measurements of the amount of insoluble formazan which was produced in living cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) was used to assess cell viability. In brief, each well of a 96-well cell culture plate was seeded with 100 μL of adherent cells and kept for 12 h for adherence, and then added with test compounds, however, suspended cells were seeded before added with test compounds with both the same density of 1×10^5 cells per mL every 100 μL of culture medium. After different concentrations of test compounds addition, each cancer cell line was incubated for 48 h in triplicate. Cisplatin was used as a positive control. After the incubation, each well was treated with MTT (100 μg) and incubation continued for 4 h at 37 °C. After the removal of the 100 μL culture medium, the cells were lysed with 20% SDS-50% DMF (100 μL). The remaining lysates were subjected to a measure of optical density at 595 nm with a 96-well microtiter plate reader.[24]

Quantum chemistry calculation details

Conformation searches on the candidate structures were performed by MMFF94s force field, giving compounds (6*R*,7*S*,8*S*,11*R*)-**1**, (6*R*,7*S*)-**2**, (6*S*,7*S*)-**3** four possible conformers each, (6*S*)-**5** seven ones, (1*S*,3*R*,6*R*,7*R*,10*R*)-**6** (**6a**) three ones, 6α-**7** two ones, 6β-**7** three ones with distribution higher than 1%. These conformers were further optimized on B3LYP/6-31g(d) level of theory in Gaussian 16 program,[25] respectively. The conformers within 3 kcal mol^{-1} of global minimum energy were selected for further calculations. The ECD spectra and optical rotations for the selected conformers were accomplished by B3LYP/6-31g(d) with CPCM model in methanol. The ECD calculation results were processed on SpecDis v1.71 software,[26] by which the ECD spectrum of the antipode, such as (1*R*,3*S*,6*S*,7*S*,10*S*)-**6** (**6b**), was generated directly by the function "enantiomeric ECD". The calculated specific optical rotation data of these conformers were averaged according to the Boltzmann distribution theory and their relative Gibbs free energy. The NMR calculations were carried out by B3LYP/6-31g(d) level of GIAO theory. The NMR calculation were processed by Microsoft Office Excel program (ESI†).

Author contributions

Z. Z. Zhao performed the experiments, data analysis, and experimental planning. H. P. Chen contributed to the calculation work. Q. L. Zhao screened the biological activities. The project was conceived, planned, and supervised by H. P. Chen. and J. K. Liu. The manuscript was written by Z. Z. Zhao, H. P. Chen, and J. K. Liu. All authors reviewed the manuscript.

Conflicts of interest

There are no conflicts to declare.

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