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Miniolins A–C, Novel Isomeric Furanones Induced by Epigenetic Manipulation on *Penicillium minioluteum*

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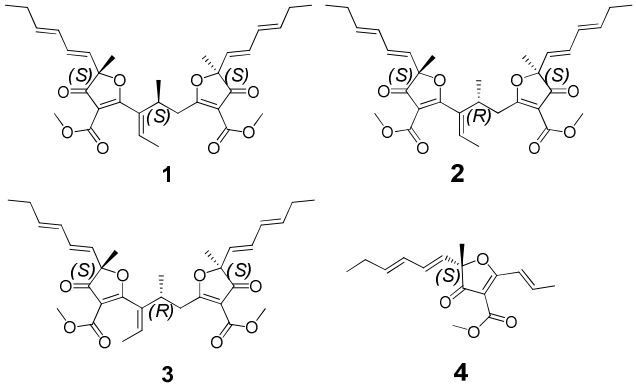
Cultivation of *Penicillium minioluteum* with azacitidine, a DNA methyltransferase inhibitor, led to the isolation of a novel type of aspertetronin dimers, named miniolins A–C (**1**–**3**), along with their precursor aspertetronin A (**4**). The structures of **1**–**3** were elucidated by extensive spectroscopic methods, and the absolute configurations were assigned by chiral HPLC analysis of chemical degradation products and electronic circular dichroism associated with TDDFT computational method (CAM-B3LYP/TZVP). The miniolins showed moderate cytotoxic activity against Hela cell lines.

Introduction

Fungi are talented organisms capable of producing pharmaceutically useful compounds, as exemplified by penicillin, cyclosporine and lovastatin.[1,2] In now post-genomic era, many microorganisms were found harboring significant numbers of biosynthetic pathways to secondary metabolites, such as *Aspergillus oryzae*[3] and *A. fumigatus*[4]. However, many natural-product-encoding gene clusters in microorganisms are silent under common culture conditions. Yet only few of their small molecule products were detected in the laboratory.[5] Thus, in recent years, multiple techniques have been developed to sidestep transcriptional roadblocks and enhance product diversity from the silent biosynthetic pathways. To date, chemical approaches targeting histone and DNA posttranslational processes have shown great potential for rationally directing the activation of silent gene clusters.[6,7]The epigenetic manipulation of fungal gene expression by small molecule, DNAmethyl transferase and/or histone deacetylase (HDAC) inhibitors, influences secondary metabolism in the fungus and is an appropriate method for exploring novel fungal metabolites prepared through cryptic biosynthetic pathways.[8–11]

In our ongoing search for novel bioactives from fungi,[12–15] we found that the addition of azacitidine, a DNA methyltransferase inhibitor, to the culture medium of *Penicillium minioluteum* significantly enhanced the production and diversity of small molecule metabolites (Figure S1), leading to three new polyketidefuranones, named miniolins A, B and C (**1**–**3**), and their precursor (+)-(*E*,*E*)-(*S*)-aspertetronin A (**4**),[16,17], which was originally isolated from various *Aspergillus* sp.[18–20]



Results and discussion

Miniolin A (**1**) was obtained as a colorless oil ($[\alpha]_D^{21}$−85.3 (*c*0.13, $CHCl_3$)). Its molecular formula ($C_{32}H_{40}O_8$) was deduced from HRESIMS at *m/z* 553.2865$[M+H]^+$ (calcd. 553.2796), indicating 13 degrees of unsaturation. The ^{13}C NMR and DEPT spectra (Table1) revealed 32 carbon signals, including eight methyls (including two oxygenated), three methylenes, ten methines (including nine unsaturated) and eleven quaternary carbons, among which include seven double bonds and four carbonyl groups. Apart from those eleven degrees due to the unsaturated bonds, the remaining implied that **1** possesses two rings in the molecule. Inspection of the signals in 1D NMR and MS spectra suggested an aspertetronin A-derived dimer skeleton with a characteristic furan-3(2*H*)-one moiety (δ_C192.4, 107.6, 198.0, 91.3).[16]

Extensive analysis of the 2D NMR spectra (HSQC, ^1H–^1H COSY, and HMBC) further established the connectivities among the substituents and the furan-3(2*H*)-one moieties. Four fragments (**a**, **b**, **c** and **d,** in Figure. 1) were deduced from COSY interaction pairs. Further detailed HMBC

Table 1. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data of **1-3** in $CDCl_3$

	1		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	14.1	1.91, d (7.2)	14.2	1.91, d (7.2)	15.3	1.65, d (6.9)
2	137.8	6.29, q (7.2)	137.3	6.30, q (7.2)	130.6	5.98, m[a]
3	132.6		133.0		134.3	
4	192.4		192.0		191.1	
5	107.6		107.7		109.2	
6	198.0		197.9		198.1	
7	91.3		91.4		91.4	
8	125.5	5.52, d (15.4)	125.3	5.52, d (15.4)	125.3	5.51, d (15.4)
9	131.8	6.32, dd (15.4, 10.4)	132.4	6.26, dd (15.4, 10.4)	132.2	6.27, dd (15.4, 10.4)
10	127.6	5.98, dd (14.8, 10.4)	127.7	5.95, dd (15.7, 10.4)	127.6	5.96, dd (15.1, 10.4)
11	139.5	5.79, dt (14.8, 7.0)	139.7	5.80, m[a]	139.7	5.78, m[a]
12	25.6	2.10, m[a]	25.6	2.10, m[a]	25.7	2.09, m[a]
13	13.2	1.00, t (7.0)	13.2	0.99, t (7.4)	13.3	0.99, t (7.2)
14	162.8		162.9		162.2	
15	22.2	1.53, s	22.4	1.50, s	22.5	1.51, s
16	51.6	3.74, s	51.6	3.76, s	51.7	3.78, s
1′	19.4	1.30, d (6.9)	18.7	1.24, d (6.9)	18.9	1.18, d (6.6)
2′	31.4	3.60, m	31.2	3.56, m	36.7	3.17, m[a]
3′	35.7	3.16, dd (14.2, 7.2) 3.46, dd (14.2, 8.5)	36.5	3.28, dd (13.6, 7.5) 3.36, dd (13.6, 8.2)	37.1	3.17, m[a] 3.29, dd (10.7, 7.2)
4′	196.1		196.5		196.5	
5′	107.3		107.3		107.3	
6′	198.6		198.6		198.4	
7′	90.9		90.8		91.3	
8′	125.9	5.59, d (15.4)	125.7	5.61, d (15.4)	125.4	5.59, d (15.4)
9′	131.8	6.25, dd (15.4, 10.4)	131.9	6.34, d (15.4, 10.4)	132.1	6.32, dd (15.4, 10.4)
10′	127.8	5.97, dd (15.1, 10.4)	127.9	6.01, dd (15.7, 10.4)	127.7	6.00, dd (14.8, 10.4)
11′	139.4	5.85, dt (15.1, 7.0)	139.3	5.83, m[a]	139.6	5.81, m[a]
12′	25.6	2.10, m[a]	25.6	2.10, m[a]	25.7	2.09, m[a]
13′	13.2	1.00, t (7.0)	13.2	0.99, t (7.4)	13.2	0.99, t (7.2)
14′	163.2		163.1		163.1	
15′	22.5	1.64, s	22.7	1.59, s	22.6	1.61, s
16′	51.5	3.79, s	51.5	3.81, s	51.6	3.80, s

δ_H multi, (J in Hz); [a] overlapped

analysisconfirmed the connectivities of fragments **a**~**d** and two furan-3(2*H*)-one moieties. The HMBC correlations of CH_3-15/C-6, 7, 8 indicated the moiety **a**, H_3C-15 and the carbonyl at C-6 were connected by the oxygen-bearing quaternary carbon C-7. The HMBC correlations of H-1/C-3 and H-2/C-4 indicated the connectivity of unit **b** to C-4 through C-3. The HMBC correlation of CH_3-16/C-14 suggests a methyl ester attached to the central core at C-5. Similar HMBC correlations of the other monomer suggested the same fragments (c, d, and a furan-3(2*H*)-one moiety, Figure 1). Furthermore, the HMBC correlations of H-1′/C-3, and H-3′/C-3 revealed that the two monomers were connected between C-2′ and C-3. These data led to the whole planar structure of miniolin A (**1**).

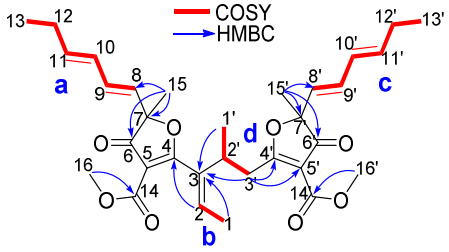


Figure 1. Selected COSY and HMBC correlations of **1**

The geometry of the four double bonds on two diene side chains was determined by NOESY correlations and coupling constants. The large vicinal coupling constants (15.4 Hz of H-8 and H-9 and H-8′ and 9′, 14.8 of H-10 and H-11, and 15.1 Hz of H-10′ and H-11′) and the NOESY correlations of H-9/H-11 and H-9′/H-11′ (Figure 2) confirmed all *trans* geometry of the double bonds on C-8, C-10, C-8′ and C-10′. Furthermore, the NOE signals of H-1/H-2′ suggested an *E*-geometry of the Δ^2 olefin. However, the relative configurations of three chiral centers (C-7, 2′ and 7′) in **1**could not be deduced from NOE correlations because of its high flexibility.

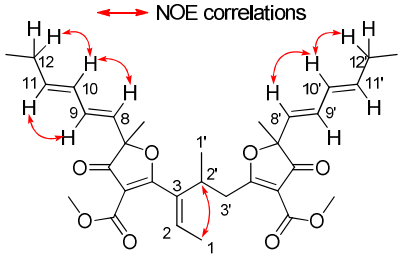


Figure 2. Key NOESY correlations of **1**

HR ESIMS data of miniolins B (**2**) and C (**3**) suggested they possess the same molecular formula as that of miniolin A (**1**).

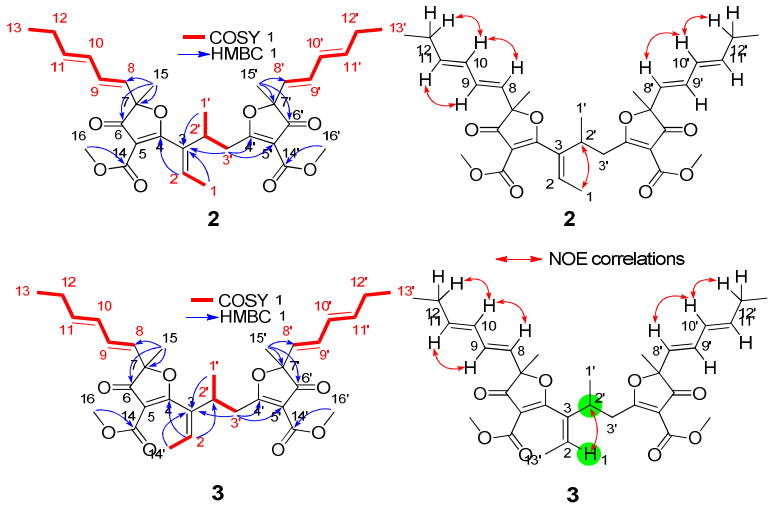
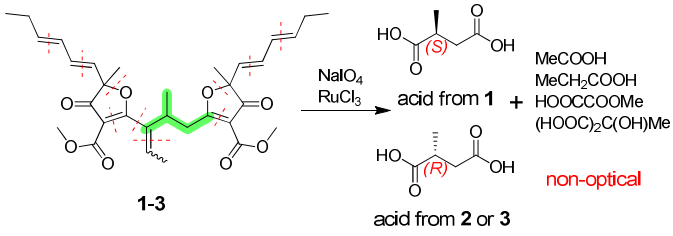


Figure 3. Selected 2D NMR correlations of **2** and **3**

Similar NMR data of all the miniolins indicated they are stereoisomeric. Very similar 1D and 2D NMR data of **2** to those of **1** (Table 1 and Figure 3) indicated both compounds shared the same planar structure with *E*-geometry of all double bonds. Compound**3** also had similar ^{1}H and ^{13}C NMR data (Table 1), COSY and HMBC correlations (Figure 3) to those of **1**, with only one exception of the NOESY correlations of H-2/H-2′ which indicated an *Z*-geometry of the Δ^2 olefin in **3**. Likewise, we were unable to employ NOE data to assign stereochemistry of the chiral centers in **2** and **3**due to their high flexibilities.



Scheme 1. Oxidative cleavages of **1-3** to methylsuccinic acids

The absolute configurations of the chiral centers (C-7, C-2′, and C-7′) in **1**–**3** were assigned by chiral HPLC analysis of chemical degradation products and ECD spectra. To determine the chirality at C-2′ in **1**–**3**, we performed an oxidative cleavage reaction[21,22] to remove unrelated branches as nonchiral mixed organic acids and to obtain the products with retention of the stereochemistry of C-2′, as shown in Scheme 1. Unexpectedly, oxidation of **1**–**3** with RuO_4[23]produced only 2′-methylsuccinic acid, which was identified by LC/ESI-MS^2 analysis (Figure S2).The absolute stereochemistry of the 2′-methylsuccinic acid wasassigned by chiral HPLC analysis on the basis ofcomparing the t_R with those of standard*S*-or *R*-methylsuccinic acid (Figure 4). Therefore, the absolute configuration of C-2′ in **1** was assigned as *S*, while that in **2** and **3**both was*R*. In addition, the presence of the methylsuccinic acids also supported the linkage between C-2′ and C-3 in **1**–**3**.

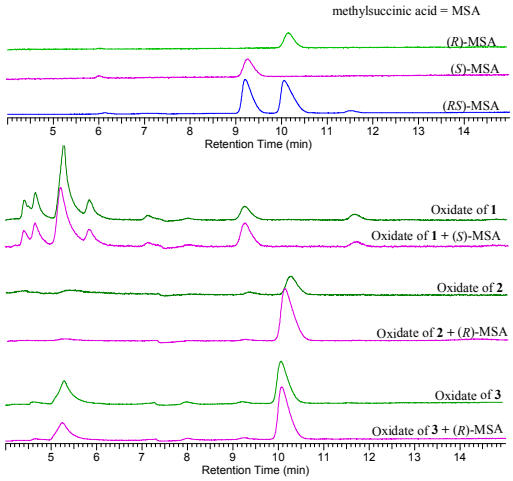


Figure 4. Chiral analysis of the resulting methylsuccinic acids on CHIRALPAK AD-H chiral column

The absolute configurations of the oxygen-bearing quaternary centers C-7 and C-7′ in **1-3** werebiogenetically related to those of **4** (scheme 2). Compound**4** showed the same sign of the optical rotations ($[\alpha]_D^{21}$ +209.8, *c*0.13 in$CHCl_3$) to that reported forboth synthetic($[\alpha]_D^{20}$ + 166.1, *c*0.55 in$CHCl_3$[16]) and natural ($[\alpha]_D$ +133, *c*0.30in$CHCl_3$[19])aspertetronin A,disclosingits 7*S*-configuration. The configurations of C-7 and C-7′ in **1**–**3**were also proposed to be *S*, thus revealing the same chirality.

The stereochemistry of **1** and **4** was further corroborated by their CD spectra. In recent years, TDDFT is becoming a powerful tool to provide high precise CD curves.[24]Comparison of theoretical CD curve with experimental spectrum provides a convenient approach to identify absolute configuration for trace

and non-crystal natural products.[25–27]However, for the flexible molecules, it is still difficult to obtain high accurate results in limited time since the flexibility leads to too numerous conformations to be favorable for calculations. In order to balance accuracy and computational time, we utilized economical molecular force field (MMFF94S) in Conflex 6.7[28] to acquire meaningful conformers, followed by DFT optimizations at B3LYP/6-31G* level for **1** and B3LYP/6-31+G(d,p) for **4** in Gaussian 09[29]. Theoretical CD curves were calculated by TDDFT method at CAM-B3LYP/TZVP level and simulated using SpecDis[30] according to Boltzmann distributions. The resulting calculated CD spectra of (*S*)-**4** well matched its experimental CD spectra (Figure 5), which provided another ceritification to the configuration of C-7 and C-7′ in the monomer and dimers.The similarity in CD spectra of the dimers (**1-3**) indicate that the configurations of the chiral C-2 do not have a great influence on their CD Cotton effects. thus **1** was selected to perform CD calculation (CAM-B3LYP/TZVP) to check the whole absolute configuration assignments. Accordingly, the calculated CD curve showed a positive Cotton effects for the n−π* transition of the furan-3(2*H*)-one moiety and conjugated alkene around the crossover point 265 nm corresponded to the experimental positive Cotton effects observed around 260 nm. Thus, the entire absolute structures of **1**–**3** were establishedas (–)-(7*S*,2′*S*,7′*S*)-**1**, (–)-(7*S*,2′*R*,7′*S*)-**2**, and (–)-(7*S*,2′*R*,7′*S*)-**3**.

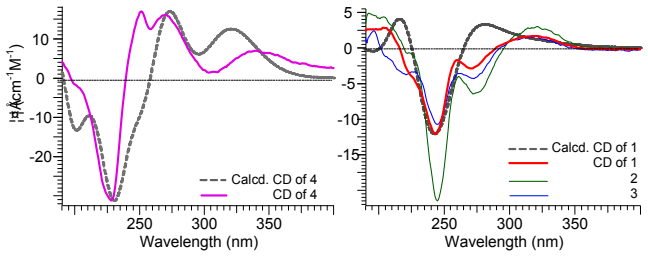


Figure 5. Calculated and experimental CD spectra of **1**–**4**

Members of the furanone structural class of natural oxygenated heterocycles had been reported from fungi of the genera *Cephalosporium* (for example, gregatins and graminin A), *Aspergillus* (aspertetronins, huaspenones A and B),[18–20]*Penicillium* (penicilliol) and *Paraconiothyrium* (gramininB),[31]and they showed a wide range of bioactivities, such as phytotoxic[32]and DNA polymerases inhibitory effects.[33]To the bestof our knowledge, miniolins A–C (**1**–**3**) represent the first members of aspertetronin dimers in nature. Particularly, it should be pointed out that the originally proposed structures of gregatins A-D and aspertetroninsA and B have been revised twice by Burghart-Stoll and Brücknerthrough the totalsynthesis.[16,17]

Biogeneticcally, aspertetronin A (**4**) is the precursor of miniolins A-C (**1**–**3**), which were envisioned to be biosynthesized via polyketide pathways. However, they were not artificial products during the purification procedures but were produced by fermentation (Figure S1). A plausible biogenetic pathway for miniolins A-C (**1**–**3**) is proposed in Scheme 2. The precursor aspertetronin A (**4**) was epoxidized by $NADP^+$ followed by a ring-opening to forme a carbonium (**ii**). **ii** underwent the addition reaction with the other molecule **4** to generate the 2′-*epi*-intermediate (**iii**). Then hydriding of **iii**yiled**iv**,which was followed by elimination of 2-hydroxyl group to afforded the title compounds **1**–**3**.

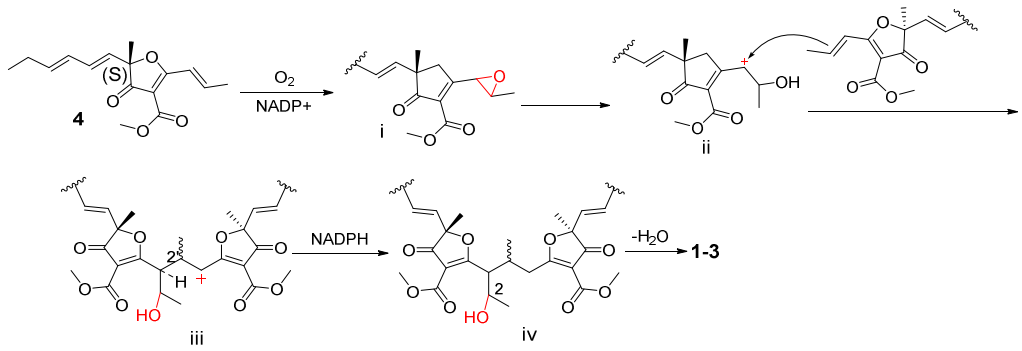
The cytotoxicity of **1**–**4**was evaluated against Hela cell lines by SRB colorimetric assay with etoposide as the positive control. Compounds **1-3**displayed moderate inhibition with IC_{50}values of 33.3, 28.7, and 21.4 μM, respectively, while **4**was non-cytotoxic.

Experimental

General Procedures

UV spectra were obtained using a Thermo Scientific Evolution 300 UV-vis spectrophotometer. IR spectra were recorded on a Bruker Tensor 27 spectrophotometer with KBr disks. Optical rotations were measured on a Rudolph Autopol III automatic polarimeter. CD spectra were obtained on a Chirascan CD spectrometer. ESI-MS was performed on a Thermo Fisher LTQ Fleet instrument spectrometer. HR ESIMS was performed on an Agilent 6520 Accurate-Mass Q-TOF LC/MS spectrometer. 1D and 2D NMR spectra were recorded on a Bruker AVANCE III (500 MHz) instrument. Chemical shifts were recorded using solvent residual as the internal standard. High performance liquid chromatography (HPLC) analysis and semi-preparation was performed on a Waters 1525 instrument (Waters Corp.). Chiral separation was performed on a CHIRALPAK AD-H column. Column chromatography was performed on silica gel (90−150 μm; Qingdao Marine Chemical Inc., Qingdao, China), MCI gel (75−150 μm; Mitsubishi Chemical Corp., Tokyo, Japan), Sephadex LH-20 (40−70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden), and Lichroprep RP-18 gel (40−63μm; Merck, Darmstadt, Germany). GF_{254} plates (Qingdao Marine Chemical Inc.) were used for thin-layer chromatography (TLC).

Fungal Material, Extraction, and Isolation.



Scheme 2. Plausible biosynthetic pathway of **1–3**

Penicillium minioluteum was purchased from ChinaGeneralMicrobiologicalCultureCollectionCenter (CGMCC, No. 3.5723). The fungus was incubated in potato dextrose agar (PDA) at 25°C for 4 days. For the cultural media tests, one piece (approximately 7 mm^2) of mycelium was inoculated aseptically to three 500 mL Erlenmeyer flasks containing 150 mL of potato dextrose (PD) liquid mediumwith 500μM 5-azacitidine (AZA) as a chemical epigenetic modifier. The flasks were then cultured at 27±0.5 °C for 10 days with shaking at 150 rpm. As a parallel control, another three flasks of medium excluded the AZA and cultured under the same conditions. Ethyl acetate (EtOAc) extraction of the cultures and the controls were compared by HPLC with an Agilent TC-C18 column (250×4.6 mm). The results (Figure S1) showed that 5-azacitidine enriched the diversity of metabolites. For the scale-up metabolite production, seed mycelium of the fungus was inoculated 150 Erlenmeyer flasks containing 22.5 L of potato dextrose (PD) liquid medium in total and cultured under the tested conditions.

Extraction, isolation and purification.

The cultures of *P. minioluteum* were extracted withpetroleum ether (PE) to yield 2.0 g of residue. The residuewas fractionated by silica flash chromatography eluted with gradient PE-EtOAc to give four fractions (Fr.1-Fr.4). Fr.2 (70mg) was subjected to PTLC with developing solvent of $CHCl_3$-acetone(50:1) to yield compound **1** (9mg) and a mixture(32mg). The latter was then separated on a semi-preparative HPLC C18 column (Thermo BDS Hypersil 250×10 mm) and eluted by 75% MeCN to yield compounds**2**(1.9mg) and**3**(3.2mg). Fr.3 was chromatographed on Sephadex LH-20 gel column then purified by a HPLC C18 semi-preparative column with 75% MeOH as elution to afford **4** (8 mg).

Miniolin A (**1**): colorless oil; $[\alpha]_D^{21}$–85.3 (*c*0.13, $CHCl_3$); UV(MeCN)λ_{max}(logε) 231 nm (4.27); CD(MeCN)λ_{max}($\Delta\varepsilon$) 244 (–21.3), 260 (–3.0), 270 (–5.0), 318 (+3.0) nm; IR(KBr)ν_{max} 3444, 2958, 2922, 2852, 1732, 1716, 1703, 1685, 1651, 1635, 1458, 1116cm^{-1}; ^{1}H NMR and^{13}C NMR data, see Table 1; ESIMS*m/z*553.00$[M+H]^+$; HRESIMS *m/z* 553.2865$[M+H]^+$ (calcd. for $C_{32}H_{40}O_8H$ $[M+H]^+$553.2796).

Miniolin B (**2**): colorless oil; $[\alpha]_D^{21}$—103.7 (*c*0.1, $CHCl_3$); UV (MeCN) λ_{max}(logε) 233 nm (3.48); CD (MeCN) λ_{max} ($\Delta\varepsilon$) 244 (–21.3), 260 (– 3.0), 270 (– 5.0), 318 (+ 3.0) nm; IR (KBr)ν_{max}, 3446, 2956, 2922, 2850, 1716, 1651, 1579, 1560, 1440, 1384 cm^{-1}; ^{1}H NMR and^{13}C NMR data, see Table 1; ESIMS*m/z*552.97 $[M+H]^+$; HRESIMS *m/z* 553.2826 $[M+H]^+$(calcd. for $C_{32}H_{40}O_8H$ $[M+H]^+$553.2796).

Miniolin C (**3**): colorless oil; $[\alpha]_D^{21}$—106.2 (*c*0.05, $CHCl_3$); UV (MeCN) λ_{max}(logε) 195 nm (5.58); CD (MeCN) λ_{max} ($\Delta\varepsilon$) 244 (– 21.3), 260 (– 3.0), 270 (– 5.0), 318 (+ 3.0) nm; IR(KBr)ν_{max} 3446, 2954, 2920, 2850, 1733, 1706, 1703, 1651, 1577, 1458, 1377 cm^{-1}; ^{1}H NMR and^{13}C NMR data, see Table 1; ESIMS*m/z*552.64 $[M+H]^+$; HRESIMS *m/z* 553.2810 $[M+H]^+$ (calcd. for $C_{32}H_{40}O_8H$ $[M+H]^+$553.2796).

Aspertetronin A (**4**): colorless oil; $[\alpha]_D^{21}$ +209.8 (*c* 0.13, $CHCl_3$); UV(MeCN)λ_{max}(logε) 231 (4.09) nm; CD(MeCN)λ_{max}($\Delta\varepsilon$) 228 (– 18.6), 251 (+ 7.1), 270 (+ 7.4), nm; IR(KBr)ν_{max} 3423，2962，1743，1706，1645，1438，1132，991，788cm^{-1};^{1}H NMR (400 MHz, $CDCl_3$)δ(multi, *J* in Hz)2.06(H_3-1, dd,6.8, 1.4 Hz), 7.20(H-2, dq, 15.7,6.8 Hz), 7.33(H-3, dq, 15.7,1.4 Hz), 5.57(H-8, d, 15.4 Hz), 6.27(H-9, dd, 15.4, 10.3 Hz), 5.97(H-10, dd, 15.1, 10.3 Hz), 5.80(H-11, dt, 15.1,6.5 Hz), 2.10(H_2-12, dq, 7.4, 6.5 Hz), 0.99(H_3-13, t, 7.4 Hz), 1.54(H_3-15, s), 3.84(H_3-16, s); ^{13}C NMR (100 MHz, $CDCl_3$)δ(in ppm) 19.3(C-1), 144.6(C-2), 120.8(C-3), 185.2(C-4), 103.7(C-5), 198.3(C-6), 90.4(C-7), 126.1(C-8), 131.5(C-9), 127.8(C-10), 139.2 (C-11), 25.6(C-12), 13.2(C-13), 163.5(C-14), 22.5(C-15), 51.5(C-16); ESIMS*m/z*277.00$[M+H]^+$.

Oxidative cleavage reactions and chiral HPLC analysis.

Compounds **1** (2.1 mg), **2** (1.9 mg) and **3** (1.5 mg) were, respectively, treated with 50 eq. $NaIO_4$ and 2.2%mol $RuCl_3.xH_2O$ in solvent system H_2O/CH_3CN/AcOEt (3:2:2). The mixture was stirred for 4h at room temperature. After addition of 0.5 mL 1N HCl for suppression of methylsuccinic acid ionization, the mixture was extracted with water saturated n-butyl alcohol(3×1.0 mL). The combined organic extracts were dried over $MgSO_4$. The resulting products and reference

methylsuccinic acid were carried out for comparative analysis on a thermo LCQ Fleet LC/MS with an Agilent TC-18 column (250 × 4.6 mm) and CHIRALPAK AD-H column (250 × 4.6 mm). t_R and MS/MS data were collected.

Computational method

A preliminary conformational search was performed in Conflex 6.7 using MMFF94S force field. Conformers with 3 kcal/mol were saved and further optimized in Gaussian 09 software package at B3LYP/6-31+G (d,p) level for compound **4** and B3LYP/6-31G* level for **1**. The stable conformers with distributions greater than 1% and without imaginary frequencies were submitted to ECD calculation by the TDDFT (CAM-B3LYP/TZVP) method associated with CPCM solvent model in MeCN. The excitation energies (E), oscillator strength (f), rotatory strength of the lowest 30 excited states were calculated. ECD spectra of different conformers were simulated using SpecDis with a half bandwidth of 0.5 eV. The final ECD spectra were generated according to the Boltzmann-calculated distribution of each conformer.

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Notes and references

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TOC (Graphic Abstract):