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Nivetetracyclates A and B: Novel Compounds Isolated from *Streptomyces niveus*

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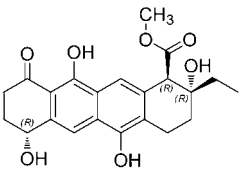
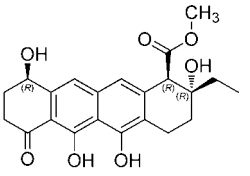
ABSTRACT



Nivetetracyclate A (**1**)



Nivetetracyclate B (**2**)

A high-throughput screening of a microbial natural product library led to the discovery of two novel compounds named nivetetracyclates A and B (1 and 2), which were produced by *Streptomyces niveus* designated as LS2151. The backbone of the compounds contains a hydrotetracyclate not previously reported from a natural source. The structures of the compounds were elucidated by spectroscopic methods. The nivetetracyclates exhibited activity against human HeLa cells.

Bioactive natural compound discovery today is driven by high-throughput screening of large and unique chemical libraries.[1] Natural product libraries combined with different target- and cell-based screening greatly enhance the efficiency of the discovery of bioactive agents.[2] A batch of 129 actinomycete strains isolated from a soil sample collected from Xundian Secondary Forest of Yunnan Province, China, were fermented on a small scale, and the methanol extracts of the fermentation were subjected to anti-*Mycobacterium bovis* BCG, anti-Multidrug Resistant *Staphylococcus aureus* (MRSA), antifungal, and antitumor screening. The *in vitro* MTT assay using human Hela cells was used to detect the antitumor activity of the secondary metabolites, and the primary screening results are listed in the Supporting Information (SI). The crude extract of LS2151 showed an inhibition ratio above 90% at the dose of 100 μg/mL. After HPLC-DAD analysis of the crude extracts, LS2151 was selected for further investigation, owing to the presence of a UV characteristic rare in our database. Large scale fermentation and isolation led to the discovery of two compounds with a unique hydrotetracyclate core structure. We named them nivetetracyclates A and B (**1** and **2**, Figure 1).

The strain LS2151 was identified by analysis of the 16S rRNA gene sequence (GenBank Accession Number: JF740060) and morphology (see the SI). The strain was closely related to the type strain of *Streptomyces niveus* (99.64% 16S rRNA gene similarity), which was first reported

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in the mid-1950s to produce novobiocin (Streptonivicin),[3] an aminocoumarin antibiotic which binds to DNA gyrase and blocks adenosine triphosphatase (ATPase) activity.[4]

Nivetetracyclates A (**1**) and B (**2**) were isolated from the active fractions eluted from an HP-20 resin column with 40% and 60% aqueous acetone. The 40% acetone fraction gave pure nivetetracyclate A (**1**) using semipreparative RP-HPLC equipped with an Eclipse XDB-C_{18} column, and using 55% methanol in water as the mobile phase. From the 60% acetone fraction, the pure nivetetracyclate B (**2**) was isolated using the same methods.

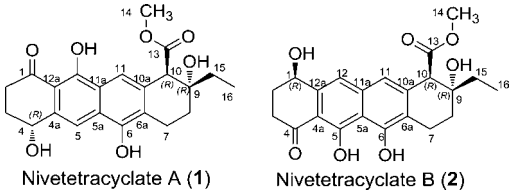

Figure 1. Structures of nivetetracyclates A and B (**1** and **2**).

Nivetetracyclate A (**1**) was obtained as a yellow powder with the molecular formula $C_{22}H_{24}O_7$ (HRESIMS m/z 399.1444, calcd 399.1438 for $[M-H]^-$), thus implying 11 degrees of unsaturation. IR analysis indicated two carbonyl groups (1727 and 1666 cm^{-1}). The UV spectrum of **1** showed maximal absorbance at 225.0, 273.0, 310.0 (sh), and 394.0 nm.[5]

In the ^1H NMR spectrum of **1**, two aromatic protons (δ_H 7.55 and 7.74), five aliphatic methylene units, and one aliphatic methyl group (δ_H 0.98) were found. One phenolic OH group at δ_H 9.21 and one hydrogen-bonded phenolic OH group at δ_H 14.19, two aliphatic OH groups at δ_H 4.53 and 5.59, and one methoxy group at δ_H 3.59 were detected. The other two coupled signals at δ_H 1.43, 1.58 and δ_H 0.98 could be assigned to an ethyl group.

The ^{13}C NMR spectrum of **1** revealed the presence of 22 carbon atoms. The sp^2 carbon region contained two carbonyls (δ_C 205.1 and 172.7), two aromatic methines (δ_C 109.7 and 114.9), and eight aromatic quaternary carbon atoms, two of which (δ_C 149.7 and 161.3) could be assigned to aromatic carbons connected to oxygen atoms. The aliphatic region contained two carbons connected to oxygen atoms (δ_C 66.6 and 69.7), one methoxy (δ_C 51.9), one methine (δ_C 55.4), four methylenes (δ_C 20.6, 27.8, 30.7, and 34.8), and one ethyl group (δ_C 32.5 and 6.9). The structure of **1** was then determined by 2D NMR experiments (HSQC, HMBC, and ROESY). All protonated carbons were assigned by HSQC analysis. Analysis of the $^1H-^1H$ COSY spectrum (Figure 2) led to three proton-bearing fragments, H_2-2/H_2-3/H-4, H_2-7/H_2-8, and H_2-15/H_3-16. The key HMBC correlations shown in Figure 2 were essential for proposing the tetracyclic ring system. The proton at δ_H 9.21 assigned to 6-OH showed cross peaks with C-5a and C-6a. Moreover, the proton at δ_H 14.19 assigned to 12-OH showed correlations to C-11a and C-12a. These signals combined with the mutual ROESY correlations between H-5, H_2-7a/b, and 6-OH as well as between H-11 and 12-OH (Figure 3a) supported the correct location of the phenolic hydroxyls of **1**. According to the HSQC and HMBC correlations, the assignments for protons and carbons of **1** were determined unambiguously as shown in Table 1 and the planar structure of **1** was established.

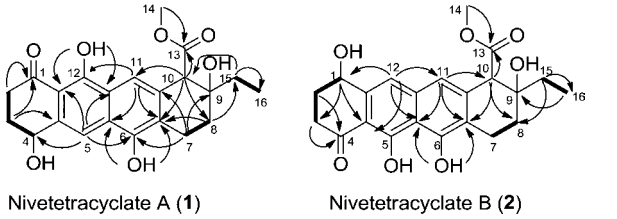

Figure 2. Key HMBC correlations (H→C) and gCOSY (bold line) of nivetetracyclates A and B (**1** and **2**).

Nivetetracyclate B (**2**) was obtained as a yellow powder after lyophilization. The molecular formula of **2** was determined as $C_{22}H_{24}O_7$ (HRESIMS m/z 401.1593, calcd 401.1595 for $[M+H]^+$), thus implying 11 degrees of unsaturation. The IR absorption peaks indicated two carbonyl groups (1716 and 1630 cm^{-1}). The UV spectrum of **2** showed maximal absorbance at 227.0, 273.0, 310.0 (sh), and 411.0 nm.[6]

Analysis of the ^1H and ^{13}C NMR spectroscopic data of **2** revealed similarity with those of **1**. The sp^2 carbon region contained two carbonyls (δ_C 204.5 and 172.2), two aromatic methines (δ_C 118.5 and 115.3), and eight aromatic quaternary carbon atoms, two of which (δ_C 165.1 and 154.1) could be assigned to aromatic carbons connected to oxygen atoms. The aliphatic region contained two carbons connected to oxygen atoms (δ_C 65.9 and 69.8), one methoxy (δ_C 52.0), one methine (δ_C 55.4), four methylenes (δ_C 19.3, 27.9, 31.0, and 33.9), and one ethyl group (δ_C 32.4 and 7.0). The HSQC and $^1H-^1H$ COSY (Figure 2) analysis led to three proton-bearing fragments, H-1/H_2-2/H_2-3, H_2-7/H_2-8, and H_2-15/H_3-16. The HMBC correlations (Figure 2) helped to assign the NMR data (Table 1). The proton at δ_H 10.01 assigned to 6-OH showed cross peaks with C-5a and C-6a.

(3) (a) Hoeksema, H.; Bergy, M. E.; Jackson, W. G.; Shell, J. W.; Hinman, J. W.; Fonken, A. E.; Boyack, G. A.; Caron, E. L.; Ford, J. H.; de Vries, W. H.; Crum, G. F. *Antibiot. Chemother.* **1956**, *6*, 143–148. (b) Smith, C. G.; Dietz, A.; Sokolski, W. T.; Savage, G. M. *Antibiot. Chemother.* **1956**, *6*, 135–142.

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(5) Nivetetracyclate A (**1**): (4*R*,9*R*,10*R*)-9-ethyl-4,6,9,12-tetrahydroxy-1-oxo-1,2,3,4,7,8,9,10-octahydrotetracene-10-methyl carboxylate; yellow powder; $[\alpha]_D^{20}$ +174.3 (*c* 0.002, MeOH); UV (MeOH) λ_{max} nm (log ε) 225.0 (4.98), 273.0 (5.12), 310.0 (sh) (4.38), 394.0 (4.35); IR ν_{max} 3315, 2948, 1727, 1666, 1621, 1493, 1436, 1395, 1358, 1318, 1245, 1198, 1026, 1008, 946 cm^{-1}; HRESIMS m/z 399.1444 $[M-H]^-$ (calcd for $C_{22}H_{23}O_7$ 399.1438); ^1H and ^{13}C NMR data can be found in Table 1.

(6) Nivetetracyclate B (**2**): (1*R*,9*R*,10*R*)-9-ethyl-1,5,6,9-tetrahydroxy-4-oxo-1,2,3,4,7,8,9,10-ocathydrotetracene-10-methyl carboxylate; yellow powder; $[\alpha]_D^{20}$ +182.3 (*c* 0.002, MeOH); UV (MeOH) λ_{max} nm (log ε) 227.0 (4.98), 273.0 (5.06), 310.0 (sh) (4.46), 411.0 (4.40); IR ν_{max} 3393, 2930, 1716, 1630, 1512, 1433, 1396, 1289, 1253, 1203, 1148, 1030, 1016, 948 cm^{-1}; HRESIMS m/z 401.1593 $[M+H]+$ (calcd for $C_{22}H_{25}O_7$ 401.1595); ^1H and ^{13}C NMR data can be found in Table 1.

Table 1. ^{1}H and ^{13}C NMR (in DMSO-d_6) Assignments and HMBC Correlations of **1** and **2**

	compound **1**			compound **2**		
no.	δ_C,[a] *mult*	δ_H,[b] *mult* (*J* in Hz)	HMBC (H→C#)	δ_C,[a] *mult*	δ_H,[b] *mult* (*J* in Hz)	HMBC (H→C#)
1	205.1, C			65.9, CH	4.76, dd (8.4, 3.6)	3, 4a, 12, 12a
2	34.8, CH_2	2.85, dt (17.5, 5.0)	1, 3, 4, 12a	31.0, CH_2	2.16, ddd (16.8, 9.0, 4.8)	
		2.76, m, overlap	1, 3, 4		2.02, m	3, 4, 12a
3	30.7, CH_2	2.22, m	1, 2, 4, 4a	33.9, CH_2	2.87, ddd (18.0, 7.2, 4.8)	
		2.05, m	1, 2, 4, 4a		2.81, m, overlap	1, 2, 4
4	66.6, CH	4.84, br dd (7.5, 3.0)	2, 3, 4a, 5	204.5, C		
4a	139.4, C			108.3, C		
5	109.7, CH	7,74, s		165.1, C		
5a	127.0,C			110.6, C		
6	149.7, C			154.1, C		
6a	124.5, C			119.7, C		
7	20.6, CH_2	2.96, dd (18.0, 6.5)	6, 6a, 8, 9, 10a	19.3, CH_2	2.82, m, overlap	
		2.79, m, overlap	6, 6a, 8		2.69, ddd (18.0, 11.6, 7.0)	
8	27.8, CH_2	2.12, dd (13.5, 6.5)	6a, 7, 9,10	27.9, CH_2	2.06, ddd (13.3, 11.7, 7.1)	
		1.82, dd (13.5, 6.5)	6a, 7, 9, 10, 15		1.81, dd (13.2, 7.2)	
9	69.7, C			69.8, C		
10	55.4, CH	3.92, s	6a, 8, 10a, 11, 13, 15	55.4, CH	3.87, s	8, 6a, 13
10a	133.0, C			130.2, C		
11	114.9, CH	7.55, s	5a, 6a, 10, 12	118.5, CH	7.07, s	5a, 6a, 10, 12
11a	122.2, C			136.2, C		
12	161.3, C			115.3, CH	7,21, s	1, 4a, 5a a, 11
12a	109.2, C			139.9, C		
13	172.7, C			172.2, C		
14	51.9, CH_3	3.59, s	13	52.0, CH_3	3.59, s	13
15	32.5, CH_2	1.58, dq (14.0, 7.0)	8, 9, 10, 16	32.4, CH_2	1.57, dq (14.4, 7.2)	16
		1.43, dq (14.0, 7.0)	8, 9, 10, 16		1.42, dq (14.4, 7.2)	8, 16
16	6.9, CH_3	0.98, t (7.0)	9, 15	7.0, CH_3	0.98, t (7.2)	9, 15
1-OH					5.62, br, s	
4-OH		5.59, br, s				
6-OH		9.21, s	5a, 6, 6a		10.01, s	6, 5a, 6a
9-OH		4.53, s			4.57, br, s	
12-OH		14.19, s	11a, 12, 12a			

[a] Recorded at 125 MHz. [b] Recorded at 600 MHz.

The hydroxy (5-OH) coupled carbon (δ_C 165.1) was assigned after all the other carbons were correctly located. Thus the planar structure of **2** was determined.

In nivetetracyclate A (**1**), the mutual ROESY correlations between H-10 and 9-OH as well as between H-10, 9-OH, and H-8b revealed that H-10 and 9-OH were oriented to be *syn* with regard to H-8b. In a similar manner the mutual ROESY correlations between 14-CH_3 and the ethyl group confirmed their *syn* orientation in the axial position.

In nivetetracyclate B (**2**), the observed ROESY correlations between H-10 and H_2-15, H_3-16 alone cannot confirm their *syn* orientation in particular with the absence of correlation between H-8a/b and the ethyl group. Also, the vicinal relationship of H-10 and the ethyl group makes a ROESY correlation between them possible despite the position of the ethyl group in either the axial or equatorial position. But the nearly same ^{1}H and ^{13}C NMR data for C-8, C-9, C-10, C-13, C-14, C-15, and C-16, and H_2-8, H-10, H_3-14, H_2-15, and H_3-16 revealed that the relative configuration for C-9 and C-10 of nivetetracyclate B should be consistent with those of nivetetracyclate A.

The mutual key ROESY correlations observed in **1** and **2** did not allow the configurational analysis to be completed. Therefore, in order to determine the absolute configuration of **1** and **2**, the quantum-chemical calculation of electronic circular dichroism (ECD) of all eight possible stereoisomers of each compound (shown in the SI) was performed utilizing time-dependent density functional theory (TDDFT). First, a systematic conformational analysis of

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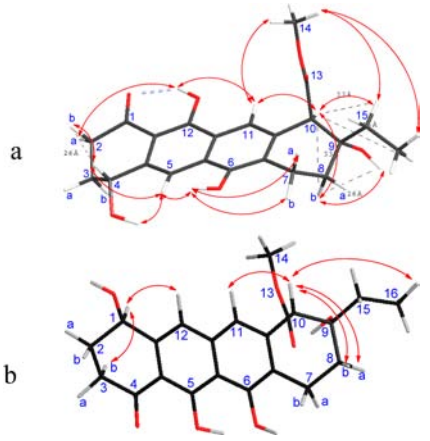

Figure 3. ROESY correlations of **1** (a) and **2** (b).

each isomer was carried out using the MMFF94 molecular mechanics force field via the MOE software package.[7] The MMFF94 conformers obtained within an energetic range of 2 kcal mol^{-1} were further optimized using the Becke 3-Lee−Yang−Parr (B3LYP) exchange-correlation functional at the 6-31G(d) basis set level using the Gaussian09 program package.[8] Frequency calculations were then carried out to verify the stability of these conformers and also gave a relevant percentage of the population for subsequent ECD investigations. The 30 lowest electronic transitions were then calculated and the rotational strengths of each electronic excitation were converted to ECD spectra using a Gaussian function with a half-bandwidth of 0.3 eV. The overall ECD spectra were then generated according to the Boltzmann weighting of each conformer. An excellent agreement was found between the theoretical ECD curve for 4*R*,9*R*,10*R* and the experimental CD curve of **1** (Figure 4a). And the best fit was obtained between the experimental spectrum of **2** and the ECD spectrum simulated for the 1*R*,9*R*,10*R* (Figure 4b). So, the absolute configuration of **1** and **2** was assigned to be 4*R*,9*R*,10*R* and 1*R*,9*R*,10*R*, respectively.

The genome of LS2151 was sequenced, and the putative biosynthesis cluster (type II PKS gene cluster) was identified by bioinformatics (accession number KF563088). A biosynthesis pathway for **1** and **2** can be proposed to begin with propionyl CoA rather than actetate, which is the starter unit for most other aromatic polyketides, via type II PKS (Figure 5). Some special gene may be implicated in specifying the unique propionate-starter unit.

Nivetetracyclates A and B (**1** and **2**) were tested on the viability of human HeLa cells, and they showed activity against HeLa cells with IC_{50} values of 11.22 ± 0.13 and 7.57 ± 0.72 μM, respectively. **1** and **2** showed weak antibacterial activities with MIC values of 64 μM each against *methicillin-resistant Staphylococcus aureus* (MRSA) and *Mycobacterium bovis* BCG and a MIC value of more than 32 μM against *Candida albicans* SC5314.

These nivetetracyclates represent a new class of natural products with a previously unreported skeleton. They

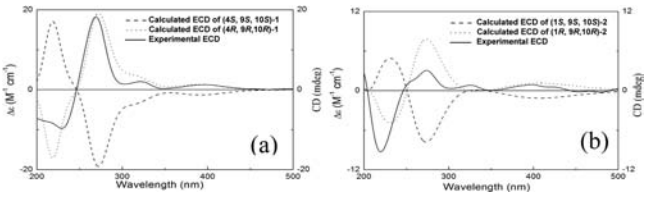

Figure 4. Attribution of the absolute configuration of **1** (a) and **2** (b) by comparison of the calculated CD spectra with the experimental spectra.

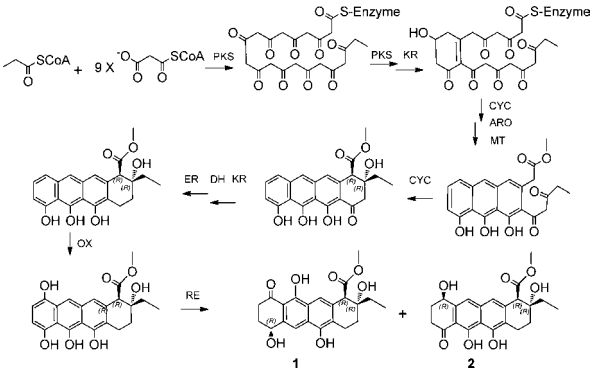

Figure 5. Suggested biosynthetic route of **1** and **2**.

differ significantly from the known tetracyclics, such as anthracycline/anthracyclinone and tetracycline, by having two octahydrogenated side rings fused to a naphthalene. The structures would offer unique binding possibilities for the active sites of drug targets in comparison with other known tetracyclics. The preliminary bioactivity evaluation indicated that this novel nivetetracyclate family deserves more bioactivity and cytotoxicity work as well as structure−activity relationship studies to provide new perspectives.

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Note Added after ASAP Publication. Figure 5 contained errors in the version published ASAP on October 25, 2013; the correct version reposted on October 29, 2013.

Supporting Information Available. Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.