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Mannolides A–C with an Intact Diterpenoid Skeleton Providing Insights on the Biosynthesis of Antitumor *Cephalotaxus* Troponoids

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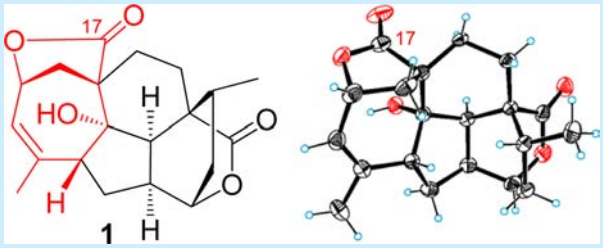
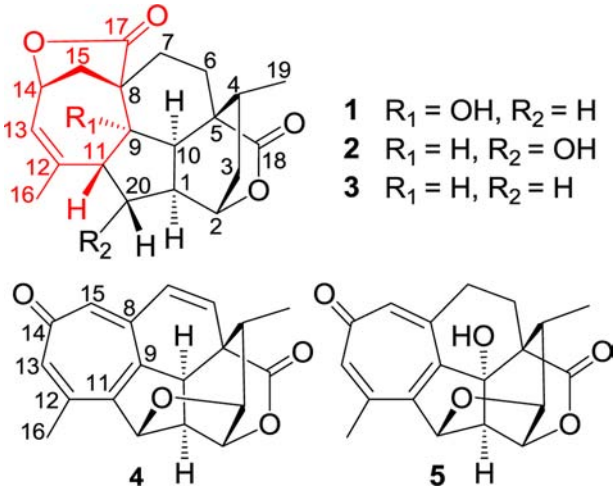
Supporting Information

ABSTRACT: Three new diterpenoids, mannolides A–C (**1**–**3**), and two new *Cephalotaxus* troponoids, **4** and **5**, were isolated from *Cephalotaxus mannii* and structurally characterized by spectroscopic data and X-ray crystallography. The discovery of compounds **1**–**3** featuring a new intact carbon skeleton, proposed as cephalotane, sheds new light on the biogenesis of *Cephalotaxus* troponoids, a rare class of antitumor C_{19} norditerpenoids. Antitumor tests showed that the tropone motif is essential for the activity.



Cephalotaxus is the only genus of the Cephalotaxaceae family and is well-known for its interesting secondary metabolites such as alkaloids (e.g., cephalotaxine and homoerythrina alkaloids) and terpenoids (e.g., abietanes and troponoids) with a variety of biological properties, particularly antitumor activity.[1] For instance, homoharringtonine from *C. fortune* was approved in October 2012 by the Food and Drug Administration for the treatment of chronic or accelerated phase chronic myeloid leukemia with resistance and/or intolerance to two or more tyrosine kinase inhibitors. Among the *Cephalotaxus* metabolites, the troponoids represent a rare class of C_{19} norditerpenoids incorporating a highly rigid tetracyclic carbon skeleton. The first member of the *Cephalotaxus* troponoids, harringtonolide (also known as hainanolide), was isolated and structurally characterized from the seeds of *C. harringtonia* in 1978 by Buta et al.[2] and exhibited various bioactivities, such as plant growth inhibitory[2] and antiviral[3] activities. Of particular note, it was reported by Sun and co-workers to be an antitumor agent against Lewis lung carcinoma, Walker carcinoma, and Sarcoma-180, as well as L-1210, L-615, and P-388 leukemia cells in animal experiments.[4] In a recent report, Nay's group further demonstrated that harringtonolide was also active toward human KB and HT-29 and murine 3T3 EF tumor cell lines, with a selectively potent inhibitory effect on KB cells (IC_{50} = 43 nM).[5] In the past decade, great efforts have been devoted to the total synthesis of this norditerpenoid scaffold due to its unique ring system and promising antitumor acitvities.[6]

Cephalotaxus troponoids are a rare class of antitumor C_{19} norditerpenoids, and there have been only five *Cephalotaxus* troponoids reported to date due to their low natural abundance.[2,5,7] The biogenesis of these intriguing structures remained unknown until recently Abdelkafi and Nay proposed a biosynthetic pathway involving a pimarane precursor.[1] Unfortunately, this plausible pathway lacks supportive evidence such as the discovery of a real compound possessing the carbon skeleton of the proposed key intermediate **iib** (Scheme 1, route B). To further explore the potential medicinal values of *Cephalotaxus* species and discover new natural analogues that might help to understand the biosynthesis of this fascinating compound class, we thus investigated for the first time *Cephalotaxus mannii* Hook f. that grows in Xishuangbanna of China, which led to the isolation of three new diterpenoids, namely, mannolides A–C (**1**–**3**), and two new *Cephalotaxus* troponoids, 6-en-harringtonolide (**4**) and 10-hydroxyharringtonolide (**5**).



Compounds **1**–**3** feature a new intact C_{20} diterpenoid skeleton for which we proposed a trivial name of cephalotane. The discovery of cephalotane-type diterpenoids (**1**–**3**) is particularly noteworthy, as this new skeleton could serve as the key biosynthetic intermediate for the coexistent antitumor troponoids (e.g., **4**, **5**, and harringtonolide). This finding naturally inspired us to propose a more reasonable biosynthetic pathway (Scheme 1, route A) for the antitumor troponoids with

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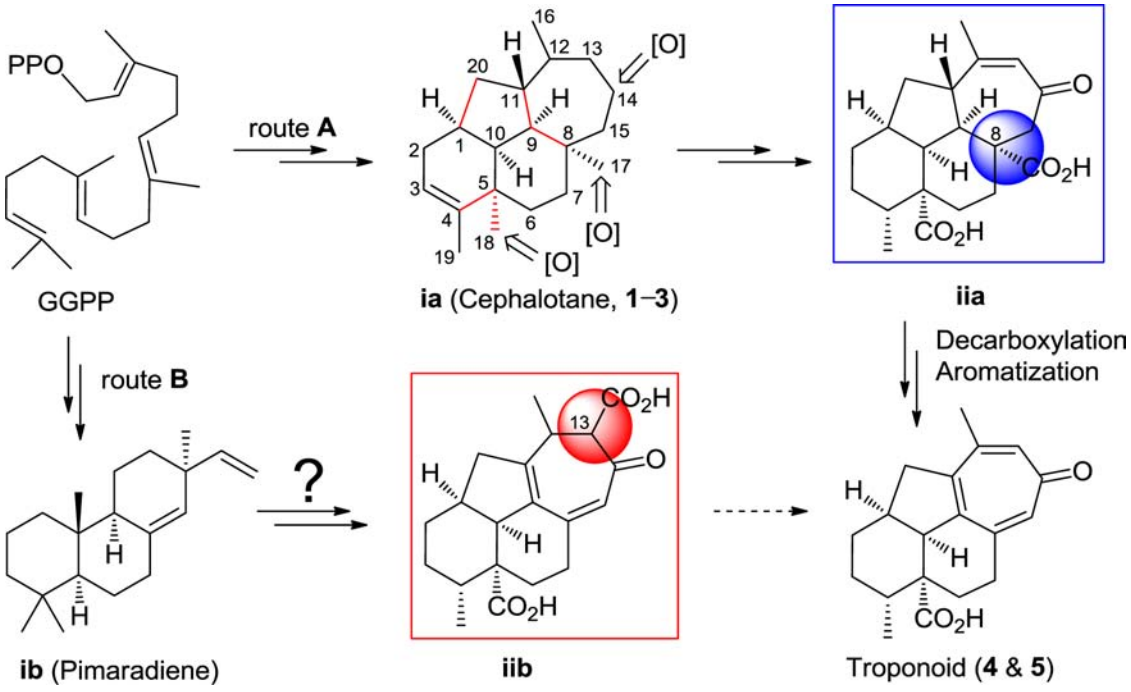
Scheme 1. Proposed Biogenesis for the Diterpenoid Skeletons from *Cephalotaxus* Plants



cephalotane diterpenoids represented by **1**–**3** as the key precursors. Herein, the isolation, structural elucidation, in vitro antitumor activity of these compounds from *C. mannii* Hook f., and, in particular, the biogenetic discussion of this compound class are detailed below.

Mannolide A (**1**) was obtained as a white solid. Its molecular formula was determined to be $C_{20}H_{24}O_5$ by HRESIMS at *m*/*z* 711.3162 $[2M + Na]^+$ (calcd for $C_{40}H_{48}O_{10}Na$, 711.3145) and implied nine double bond equivalents (DBEs). The IR spectrum of **1** revealed the presence of hydroxy (ν_{max} = 3485 cm^{-1}) and lactone carbonyl (ν_{max} = 1755 and 1738 cm^{-1}) groups. The ^1H NMR data [Table S1, Supporting Information (SI)] showed signals for two methyl resonances [δ_H 0.97 (H-19, d, *J* = 6.4 Hz), 1.85 (H-16, s)] and one olefinic signal at δ_H 5.76 (1H, d, *J* = 6.0 Hz) assignable to the proton of a trisubstituted double bond. The ^{13}C and DEPT NMR data (Table S3 and Figure S7, SI) displayed 20 carbon resonances, including two methyl, five methylene, seven methine (two oxygenated and one olefinic), and six quaternary (one oxygenated, one olefinic, and two ester carbonyls) carbons. The aforementioned functionalities accounted for three DBEs, and the remaining six DBEs required **1** to possess a hexacyclic skeleton.

Analysis of $^1H-^1H$ COSY data (Figures S1 and S8, SI) for **1** revealed three structural fragments that were connected through oxygen and quaternary carbon atoms by interpretation of HMBC data (Figures S1 and S10, SI), which established the planar structure of **1** with a new diterpenoid scaffold. The relative configuration of **1** was then deduced from ROESY data (for details, see the Detailed Structural Characterization of **1** in SI). Fortunately, compound **1** afforded fine crystals when kept in MeOH at room temperature (rt). To further corroborate the structure and assign the absolute stereochemistry of **1**, a single-crystal X-ray experiment with Cu Kα (λ = 1.54178 Å) was successfully performed, which not only confirmed the structure assigned by spectroscopic data but also established the absolute configuration of **1**, as shown (Figure 1) [Flack parameter = 0.03(10)].[8]

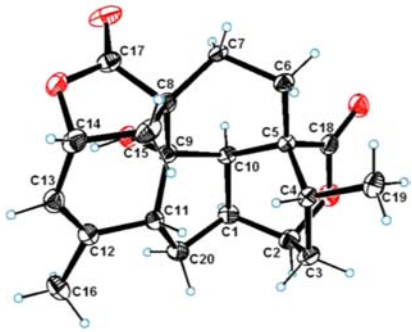


Figure 1. ORTEP drawing of compound **1**.

Mannolide B (**2**) shared the same molecular formula, $C_{20}H_{24}O_5$, as **1** based on the HRESI(+)MS ion at *m*/*z* 711.3146 $[2M + Na]^+$ (calcd 711.3145). Analysis of the ^1H and ^{13}C NMR data (Tables S1 and S3, SI) with DEPT experiments revealed the presence of two ester carbonyls (δ_C 176.0 and 182.7), a trisubstituted double bond (δ_C 124.7, 147.7; δ_H 5.79, 1H, d, *J* = 7.0 Hz), two methyl, four methylene, eight sp^3 methine, and two sp^3 quaternary carbons. Comparing the NMR data of **2** with those of **1** showed great similarity, and the main differences occurred at the C-9 and C-20, suggesting that they are structural analogues with different substitutes at these two positions. The only hydroxy group in **2** was located at C-20 by the chemical shifts of H-20 at δ_H 4.40 (1H, t, *J* = 4.5 Hz) and C-20 at δ_C 72.5, which was confirmed by the spin-coupling sequence of H-1, H-9 to H-11, and H-20 in the $^1H-^1H$ COSY spectrum, as well as the key HMBC correlations from OH-20 to C-1, C-11, and C-20 (Figures S2, S16, and S18, SI). The relative configuration of **2** was assigned to be consistent with that of **1** by ROESY data (Figures S2 and S19, SI). Compound **2** gave fine crystals in pyridine at rt. The structure of **2** was finally confirmed with the determination of absolute configuration [Flack parameter = −0.09(6)][8] by a single-crystal X-ray diffraction study (Figure 2).

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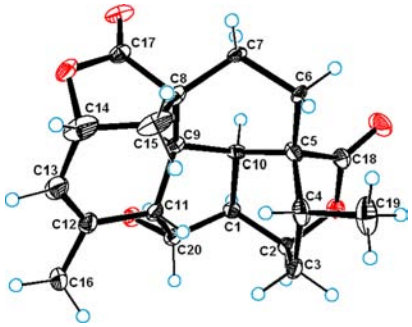

Figure 2. ORTEP drawing of compound **2**.

Mannolide C (**3**) was assigned a molecular formula of $C_{20}H_{24}O_4$, which contains one less oxygen atom than **1** and **2**, based on the (+)-HRESIMS ion at *m/z* 679.3258 $[2M + Na]^+$ (calcd 679.3247) with nine DBEs. The NMR data of **3** (Tables S1 and S3, SI) revealed signals (rings B−E, Figure S3, SI) similar to those of **1**, and the major differences between the two compounds were attributable to the structural changes within ring A. Compared with those of **1**, the NMR data of **3** showed the presence of extra resonances for a C-9 methine (δ_H 2.26, m; δ_C 49.0) in place of an oxygenated quaternary carbon for C-9 of **1**. This assignment was supported by the spin-coupling sequence of H-1, H-9 to H-11, and H_2-20 in the $^1H-^1H$ COSY spectrum and the correlations from H-7, H-10, and H_2-15 to C-9 in the HMBC spectrum (Figures S3, S24, and S26, SI). The relative configuration of **3** was assigned by ROESY data analysis (Figures S3 and S27, SI). Compound **3** yielded fine crystals in MeOH at rt. The structure of **3** was finally confirmed with the determination of absolute stereochemistry [Flack parameter = 0.02(7)][8] by an X-ray crystallography study (Figure 3).

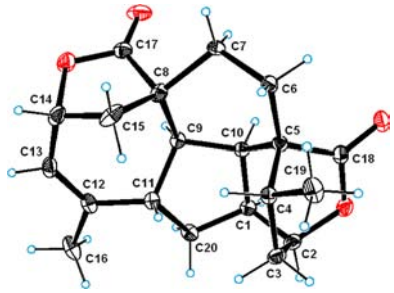

Figure 3. ORTEP drawing of compound **3**.

6-en-Harringtonolide (**4**) had a molecular formula of $C_{19}H_{16}O_4$ as determined via the (+)-HRESIMS ion at *m/z* 309.1127 $[M + H]^+$ (calcd 309.1127) indicative of a didehydro congener of harringtonolide.[2] Analysis of the NMR data (Tables S2 and S3, SI) of **4** supported this hypothesis with diagnostic signals for a Δ^6 double bond (CH-6: δ_H 6.70, δ_C 134.3; CH-7: δ_H 6.42, δ_C 131.3) replacing those with two methylenes in harringtonolide.[2] This structural variation was supported by the HMBC correlations from H-15 (δ_H 6.62) to C-7 (δ_C 131.3) and from H-4 (δ_H 2.21) and H-10 (δ_H 3.62) to C-6 (δ_C 134.3). The structure of **4** was further supported by HMBC and ROESY data (Figures S34 and S35, SI) and was confirmed with the assignment of absolute configuration [Flack parameter = 0.01(10)][8] by a single-crystal X-ray crystallography study (Figure 4). The crystals were obtained from recrystallization in MeOH at rt.

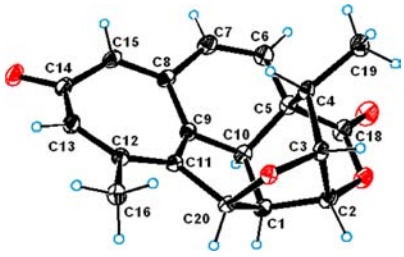

Figure 4. ORTEP drawing of compound **4**.

A molecular formula of $C_{19}H_{18}O_5$ for 10-hydroxyharringtonolide (**5**) was assigned by (+)-HRESIMS analysis at *m/z* 327.1233 $[M + H]^+$ (calcd 327.1232) suggestive of an oxygenated analogue of harringtonolide.[2] Examination of the NMR data (Tables S2 and S3, SI) of **5** corroborated this deduction with characteristic resonances for an oxygenated sp^3 quaternary carbon (δ_C 87.9, C-10) and a hydroxy group (δ_H 8.41, 10-O<u>H</u>) instead of those for a methine (CH-10) in harringtonolide,[2] which was further supported by the HMBC correlations of 10-O<u>H</u>/C-5 and C-10 (Figure S43, SI). The 10-OH was assigned to be coplanar with H-1 and H-6α and thus to be α-directed via a pyridine-induced solvent effect on the chemical shifts of the latter two protons,[9] in which the H-1 and H-6α measured in C_5D_5N were significantly shifted downfield by $\Delta\delta_H$ = 0.29 and 0.35, respectively, compared with those measured in CD_3OD (Figure S40, SI). The structure of **5** was also confirmed by X-ray diffraction analysis (Figure 5), and the absolute stereochemistry was thus established as shown [Flack parameter = −0.05(4)]. Recrystallization of **5** in pyridine at rt gave the fine crystals.

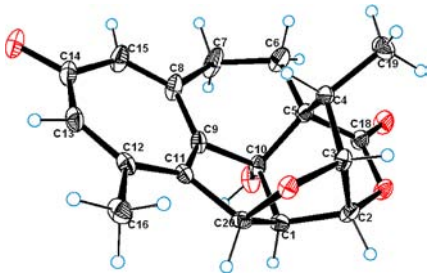

Figure 5. ORTEP drawing of compound **5**.

The biogenesis of the *Cephalotaxus* troponoids is of great interest because of their characteristic multicyclic framework and especially the potent antitumor activity. Recently, a biosynthetic pathway (Scheme 1, route B) was proposed that hypothesized that the troponoids were derived from pimaranes,[1] whereas the lack of a real co-occurring compound possessing the hypothetical carbon skeleton of the key intermediate **iib** made this proposal less convincing. Discovery of compounds **1**−**3** in the current study sheds new light on the biogenesis of the *Cephalotaxus* troponoids. On the basis of a prudent structural analysis, it is evident that the troponoid skeleton from *Cephalotaxus* plants is derived from the cephalotane backbone by the loss of the C-17 via oxidative decarboxylation (Scheme 1, route A).[10]

Our current hypothesis (Scheme 1, route A; Scheme S1, SI) is that the cephalotane-type diterpenoids have a de novo biosynthetic pathway entirely different from that of any other known diterpenoids, where unidentified cyclases are predicated to be responsible for the annulation procedures to produce

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Table 1. Cytotoxicity of 4 and 5 against Tumor Cell Lines (IC_{50} in μM)

	A549	KB	HL-60	HT-29
4	7.804 ± 3.797	5.115 ± 0.148	2.319 ± 0.247	4.890 ± 0.622
5	3.683 ± 0.947	2.325 ± 0.040	1.038 ± 0.002	2.108 ± 0.108
Adr.[a]	0.481 ± 0.201	0.570 ± 0.072	0.076 ± 0.008	0.559 ± 0.097

[a]Adr. (adriamycin) was used as a positive control.

cephalotanes. Starting from the initial diterpenoid precursor geranylgeranyl pyrophosphate (GGPP), cyclization of C-4/C-5, C-8/C-9, C-9/C-11, and C-1/C-20 would generate the rigid tetracyclic ring system. Subsequent Wagner−Meerwein 1,2-hydride and 1,2-methyl migration terminating in proton loss[11] would yield the new cephalotane skeleton **ia** (as represented by compounds **1**−**3**), which after a cascade of oxidative processes would yield a key intermediate **iia**. The Cephalotaxus troponoids would be finally produced by decarboxylation at C-8, aromatization of ring A, and further biosynthetic modifications of the key intermediate **iia**.

All isolates were evaluated for their cytotoxicity against a panel of human tumor cell lines, A549 and KB using the SRB method[12] and HL-60 and HT-29 with MTT method.[13] The assay results (Table 1) revealed that norditerpenoids **4** and **5** with a tropone motif displayed remarkable cytotoxic activities against the tested tumor cell lines, whereas the diterpenoids without the tropone moiety were inactive. The biological testing also demonstrated that structural variation such as extension of the conjugation system in **4** and the presence of 10-OH in **5** did not obviously change the activity. The tested results and literature-reported antitumor activity for *Cephalotaxus* troponoids, such as harringtonolide, indicated that the tropone motif is the antitumor bullet.

In conclusion, we have discovered a novel class of diterpenoids and proposed that the cephalotanes are real biosynthetic precursors of the antitumor *Cephalotaxus* troponoids. We, moreover, proposed for the cephalotanes a new biosynthetic pathway that distinguishes them from other known diterpenoids. Our bioassays provide further evidence that the *Cephalotaxus* troponoids have significant cytotoxic activities against human cancer cells and are potentially promising leads for the development of new antitumor drugs. A brief SAR (structure−activity relationship) discussion also revealed that the tropone motif is essential for the activity. Therefore, the exploration of cephalotanes as the biosynthetic key intermediates for these troponoids is of great importance.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00653.

Selected key 2DNMR correlations, Detailed biogenetic proposals, Tabulated NMR data, Experimental section, X-ray crystallographic data, 1D and 2D NMR, MS and IR spectra of compounds **1**−**5** (PDF)

X-ray data for **1** (CIF)

X-ray data for **2** (CIF)

X-ray data for **3** (CIF)

X-ray data for **4** (CIF)

X-ray data for **5** (CIF)

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Notes

The authors declare no competing financial interest.

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