Bioactive norditerpenoids from *Cephalotaxus fortunei* var. *alpina* and *C. lanceolata*

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ABSTRACT

Twenty-eight naturally occurring *Cephalotaxus* tropone analogues, including 19 previously undescribed ones, were identified from *Cephalotaxus fortunei* Hook. var. *alpina* H. L. Li and *C. lanceolata* K. M. Feng. The presence of the C20 cephinoids A–E revealed that these tropones were assigned to the norditerpenoids and were perhaps derived from labdane-type diterpenoids. These norditerpenoids showed excellent cytotoxicity against human cancer cells (IC$_{50}$, 20–0.1 μM) *in vitro*. The SAR (structure-activity relationship) analysis disclosed that the tropone moiety and the lactone ring were crucial structural features for the observed activities. Further SAR analyses led to a new candidate, cephinoid H, which demonstrated an inhibition of 49.0% by administration to zebrafish at a dose of 60.0 ng/mL, compared to cisplatin (DDP, 22.4%) at 15.0 μg/mL. These compounds might affect the NF-κB signaling pathway rather than binding to microtubules. Additionally, the isolated norditerpenoids showed almost equal anti-inflammatory activities compared to the positive control, MG132.

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1. Introduction

Research on natural products (NPs) plays an important role in the discovery of new and potential drugs. For example, in the area of cancer research, from the 1940s to the end of 2014, 175 small molecules were approved as medicinal drugs, and 85 (49%) of them were either NPs or directly derived from NPs (Newman and Cragg, 2012). In the treatment of human cancer, many of these drugs face problems of tolerance and selectivity; hence, the discovery of new drugs is an important issue. Currently, to detect potential drugs, a wide range of bioassays combined with analyses of the structure-activity relationships (SAR) of NPs represent a promising approach to identify prospective molecules. With the emergence of chemical ecology in recent years, it has become evident that many

of these NPs also fulfill important functions in the interaction between plants and their environment. Diterpenoids are derived from geranylgeranyl pyrophosphate and include a vast number of structurally highly variable compounds, including the famous medicinal drugs Taxol and ginkgolide. This class of compounds is distributed in plants, fungi, insects and marine organisms and many of them exhibited interesting bioactivities.

Phytochemicals from the species of *Cephalotaxus* Sieb. & Zucc. ex Endl. (Cephalotaxaceae) are of special interest due to the production of highly bioactive *Cephalotaxus* tropones (Abdelkafi and Nay, 2012). Powell et al. (1969, 1970) reported the anticancer activity of esters of cephalotaxine from *C. harringtonia* (Knight ex J. Forbes) Koch cv. *Fastigiata* Ohwi, and homoharringtonine was approved in anticancer therapy in 2012 by FDA. Buta et al. (1978) and Sun et al. (1979) identified the non-alkaloid harringtonolide (hainanolide), the first *Cephalotaxus* tropone, from *C. harringtonia* and *C. hainanensis* H. L. Li, respectively. Subsequently, hainanolidol (Xue et al., 1982), fortunolide A/B (Du et al., 1999), and 10-hydroxyhainanolidol (11-hydroxyhainanolidol) (Yoon et al., 2007) were isolated from *C. hainanensis*, *C. fortunei* Hook. var. *alpnia* H. L. Li, and *C. koreana* Nakai, respectively. The total synthesis of this type

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compounds has become one of the most attractive fields in organic synthesis (Abdelkafi et al., 2012; Frey et al., 1998; O'sullivan et al., 2007; Zhang et al., 2013, 2016; Zheng et al., 2016). The compounds bearing a C19 skeleton are assigned to *Cephalotaxus* tropones. Biosynthetically, Abdelkafi and Nay (2012) proposed the biosynthesis of tropones from a pimarane precursor. However, this hypothesis suffers from the scarce data available regarding potential precursors and intermediates derived from the *Cephalotaxus* species. The recent discoveries of tropone analogues bearing a C20 skeleton, known as mannolides A–C, showed that tropones are obviously derived from C20 diterpenoids directly (Ni et al., 2016; Xu et al., 2016). So far, seven norditerpenoids with a complete tropone skeleton were identified but only harringtonolide exhibited remarkable cytotoxicity against human cancer cells (Evanno et al., 2008). The exploration of bioactive plant constituents and the occurrence of *C. fortunei* var. *alpina* and *C. lanceolata* K. M. Feng in Yunnan steered us towards the investigation of these two species. The structural and functional similarities to colchicine, a strong inhibitor of cell proliferation by binding on microtubules (Slobodnick et al., 2015), attracted us to study the bioactivity of the isolated compounds.

2. Results and discussion

Chromatographic separation using normal and reversed phase of crude methanolic extracts of *C. fortunei* var. *alpina* and *C. lanceolata* led to the identification of 28 diterpenoids (Fig. 1). These *Cephalotaxus* tropones showed characteristic UV absorption at 241 and 320 nm (Buta et al., 1978; Du et al., 1999; Yoon et al., 2007; Ni et al., 2016) and were detected by spraying with 5% H_2SO_4 and Dragendorff's reagent on the TLC plate.

Compound **1** was obtained as a colorless powder with $[\alpha]_D^{25} = -153$ (*c* 0.11, MeOH). The UV absorption of **1**, with maximum absorption bands at 205 and 234 nm, indicated absence of conjugated system. The detected IR (KBr) absorption bands at 3479, 3439, 1743, and 1631 cm^{-1} resulted from hydroxyls, lactones, and olefinic bonds. The HRESIMS ($m/z = 399.1417$; calcd. for $C_{20}H_{24}O_7Na$ [M+Na]$^+$ 399.1420) and ^{1}H (Table 1) and ^{13}C (Table 2) NMR spectroscopic data of **1** established its molecular formula and indicated 9 degrees of unsaturation. The ^{13}C and DEPT NMR spectra showed 20 carbon signals, including seven quaternary carbons (δ_C 176.1, 175.5, 146.6, 126.4, 75.2, 44.2, and 44.0), four methylenes (δ_C 34.8, 34.5, 25.8, and 23.5), seven methines (δ_C 82.1, 80.0, 78.2, 75.0, 51.9, 33.9, and 33.0), and two methyls (δ_C 24.2 and 16.1). These data indicated that **1** might be a diterpenoid with an additional carbon compared to harringtonolide (**21**) (Buta et al., 1978). Harringtonolide was first reported as a *Cephalotaxus* tropone from the same plant species. Further comparison with the known hainanolidol (**25**) (He et al., 2015) by ^{13}C NMR revealed that **1** lacked two pairs of double bond signals approximately δ_C 140–160, suggesting a partially reduced tropone group. The HMBC correlations from two methylene signals at H-7 (δ_H 1.83 and 1.49) and H-15 (δ_H 2.65), with the additional ester carbonyl (δ_C 176.1), placed the carbonyl connected to C-8. Unlike hainanolidol (**25**), the molecular formula and the five carbons at ab. δ_C 70–85 for **1** revealed the presence of 3 additional oxygenated substitutions in **1**, a result that was further confirmed by related HMBC cross-peaks (Fig. 2). The 3-OH in the A-ring with the boat conformation was temporarily elucidated as β through the ROESY correlations of H-18/H-3. Additionally, ROESY correlations from H-16 to 3-OH and 13-OH and from 12-OH to H-13 placed the 12-OH and 13-OH at in α and β orientation, respectively. This novel five-membered lactone ring was assigned to α based on ROESY correlations from H-15β to H-16 and from 13-OH to H-14 as well as molecular constraints. Its absolute configuration was determined by a single-crystal X-ray diffraction study [Flack

parameter $= 0.05(16)$] (Flack and Bernardinelli, 2008) as (1*S*,2*R*,3*R*,4*S*,5*R*,8*S*,10*R*,12*S*,13*S*,14*R*)-cephinoid A (Fig. 2).

Compounds **2**–**5** shared similar UV absorption values to **1**, attributing them to the same type. The ^{1}H and ^{13}C NMR (Tables 1 and 2) data of compound **2** were highly similar to those of **1**, with the exception that a methine in **1** was substituted by a methylene in **2**. The molecular formula of **2**, $C_{20}H_{24}O_6$ (HRESIMS $m/z = 383.1468$ [M+Na]$^+$), was 16 Da less than **1**, indicating the absence of 3- or 13-OH of **1** in **2**. The HMBC correlations between H-18 (δ_H 0.86) and C-3 (δ_C 30.5, t) suggested the absence of OH from C-3. Compound **3** had the same molecular formula as **1** based on the HRESIMS ($C_{20}H_{24}O_7$). The HMBC correlations from H-2 (δ_H 4.62) to C-19 (δ_C 175.7) and from H-14 (δ_H 4.36) to C-17 (δ_C 178.1) suggested the presence of both lactone rings, similar to those in **1** and **2**. Comparison of the ^{13}C NMR spectra of **1** with **2** and **3** showed that **3** did not contain the double bond at C-9/C-11. However, the appearance of a hemiketal group (δ_C 104.7), together with the degrees of unsaturation, indicated an oxygen bridge between C-9 and C-13, which was supported by the indirect HMBC correlations from H-15 (δ_H 2.15) to C-13 (δ_C 104.7) and the downshift δ_C 88.6 of C-9. The ROESY correlations between H-16 and 13-OH supported the β orientation. The same molecular formula ($C_{20}H_{24}O_8$) and similar NMR spectra of compounds **4** and **5** indicated that both compounds contained an additional hydroxyl compared to **3**. The HMBC correlations between the H-18 (δ_H 0.69) and CH-3 (δ_C 64.2) in **4** confirmed the presence of the OH group at C-3. Unlike **4**, the absence of a hemiketal in **5** indicated the absence of the hydroxyl at C-13. The new oxygenated quaternary carbon (δ_C 91.5) of **5**, together with its HMBC correlations from H-16 suggested the presence of an OH at C-11. The configuration of 11-OH in **5** was assigned as α based on the constraints imposed by the molecular models. The 3-OH was elucidated as β by ROESY correlations of H-18/H-3. The skeletal configurations of **2**–**5** were the same as those in **1** based on the ROESY correlations, coupling constants, and biosynthesis. Thus, **2**–**5** were subsequently named cephinoids B–E, respectively.

The maximum UV absorption bands at 244 and 320 nm of **6** were the same as those in the known compound fortunolide B (**22**) (Du et al., 1999). The molecular formula of **6**, deduced from HRESIMS (m/z 327.1227 [M+H]$^+$) data, was elucidated as $C_{19}H_{18}O_5$, which is identical to known compounds fortunolide B (**22**) (Du et al., 1999) and 10-hydroxyharringtonolide (**23**) (Ni et al., 2016). Compound **6** shared similar NMR spectrum (Tables 1 and 2) to fortunolide B (**22**), except that a quaternary carbon δ_C 82.0 (C-1) and methylene (ab. δ_C 32.3) in **22** (Du et al., 1999) were substituted with two methines (δ_C 42.1 and 74.1) in **6**. These differences indicated the hydroxyl connection at C-7 of **6**, which was confirmed by the HMBC correlations of H-15 (δ_H 6.82)/δ_C 74.1 (C-7). Although there were no available ROESY correlations from H-7 or OH-7 to the other protons, the coupling constant (5.4 Hz) of H-7 in the six-membered ring with the boat-conformation supported the presence of the 7-OH in **6** in the α position. Compound **7** also displayed similar NMR data to **22**, with the exception of an additional oxygenated quaternary carbon replaced by a methine in **22**, in accordance with the $C_{19}H_{18}O_5$ formula derived from HRESIMS data ($m/z = 341.1028$ [M − H]$^+$). The additional hydroxy group was assigned at C-10 based on the new quaternary carbon δ_C 85.7 and its HMBC correlations from H-2 and H-20. Based on the molecular constraints, both OH-1 and OH-10 in **7** were determined as α. Compounds **6**–**7** were thus named cephinoids F–G, respectively.

Compounds **8**–**13** displayed similar UV absorption bands to **6**–**7**. The molecular formula of **8**, $C_{19}H_{20}O_3$, was determined from the HRESIMS data at m/z 319.1307 [M+Na]$^+$. The comparison of mass spectra with the known hainanolidol (**25**) (Xue et al., 1982) and fortunolide A (**26**) (Du et al., 1999) indicated the absence of a hydroxyl group in **8**. The pattern of the NMR signals of **8** was more