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Specialized metabolites of the United States lichen *Niebla homalea* and their antiproliferative activities

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ABSTRACT

Three undescribed stictanes, nieblastictanes A–C, two flavicanes, nieblaflavicanes A and B, together with three already reported stictanes, along with the known compounds (+)-usnic acid, sekikaic acid, divaricatic acid, and divaricatinic acid methyl ester were isolated from an ethyl acetate extract of the western North American lichen *Niebla homalea*. The structures of the new and known compounds were established by spectroscopic methods including nuclear magnetic resonance spectroscopy, mass spectrometry and electronic circular dichroism. Among the compounds isolated, usnic acid exhibited moderately potent antiproliferative activities against the A2780 ovarian (IC_{50} 3.8 μM) and MCF-7 breast cancer (IC_{50} 6.8 μM) cell lines. A plausible mode of formation of the chlorine-containing compound nieblastictane C is provided and the contribution of the isolated compounds to the chemotaxonomy of United States lichen species of the genus *Niebla* is also discussed.

1. Introduction

Lichens are organisms formed by symbiotic associations of mycobionts (fungi) and one or many photobionts (algae and/or cyanobacteria). These symbiotic organisms are responsible for the biosynthesis of bioactive terpenoids and phenolic compounds (including depsides, depsidones, quinones, xanthones, and diphenyl ethers) present in lichens (Carpentier et al., 2017; Chin et al., 1973; Connolly et al., 1984; Corbett et al., 1976; Culberson, 1969; Elix et al., 1982; Gollapudi et al., 1994; Gonzalez et al., 1991; Gonzalez et al., 1974; Huneck, 1984; Krivoshchekova et al., 1982; Lai et al., 2013; Le et al., 2013; Le Pogam and Boustie, 2016; Li et al., 2015; Moroney et al., 1981; Nguyen et al., 2017; Yu et al., 2016). Apart from the important roles of lichen metabolites in symbiosis, they display various biological activities including anti-ultraviolet, antimicrobial, antiviral and cytotoxic effects (Carpentier et al., 2017; Gollapudi et al., 1994; Lai et al., 2013; Li et al., 2015; Yu et al., 2016). Our ongoing research project on developing new sources of antiproliferative compounds from endemic lichens to the United States and their associated microbes has focused on one species from the genus *Niebla* taxonomically identified as *Niebla homalea* (Ach.) Rundel & Bowler (family: Ramalinaceae). This species was chosen due to: (1) the lack of chemical and pharmacological investigations of its secondary metabolites; (2) the possible unique association of bioactive fungi (primarily *Niebla*) and algae that make this lichen organism endemic to the marine environment, and (3) the promising results obtained in a preliminary antiproliferative screening procedure against the hormone-dependent breast (MCF-7) and ovarian (A2780) cancer cell lines. We reported recently that a *Penicillium* fungus (*P. aurantiacobrunneun*) isolated from this species biosynthesized new and antiproliferative γ-pyrone and sterol derivatives (Anaya-Eugenio et al., 2020; Tan et al., 2019).

Species of the genus *Niebla* are fruticose lichens that grow on rocks and soil in the foggy ("niebla" means fog in Spanish) areas of the west coast of North America. The genus is reported to include 42 species distributed from Mendocino County in California south to Isla Santa Margarita in Baja California Sur (Spjut, 1996). Phytochemical investigations in the taxonomy of the genus have been limited to thin-layer chromatography (TLC) for identifying secondary metabolites as taxonomic characters to distinguish the species. Two chemotaxonomic *Niebla* groups are recognized: one contains triterpenoids with depsides and the other lacks triterpenoids with or without depsidones (Spjut, 1996). In the present contribution, we describe the isolation, structure determination and antiproliferative profile of taxonomically significant triterpenoid and depside constituents of an ethyl acetate-soluble extract of

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Table 1
^{1}H and ^{13}C NMR (400 MHz and 100 MHz, respectively) spectroscopic data for compounds **1**–**3**.

+Position	1		2		3	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	31.6	2.07 m	31.6	2.07 m	44.5	2.29
		1.43 m		1.43 m		d (7.76)
2	33.8	2.64 m	33.8	2.64 m	57.5	4.41 t
		2.27 m		2.27 m		(7.76)
3	220.4		220.4		210.5	
4	46.9		46.7		47.8	
5	43.3	1.44 m	43.3	1.43 m	45.8	2.33 m
6	20.5	1.43 m	20.5	1.43 m	20.1	1.53 m
		1.17 m		1.17 m		
7	33.8	2.00 m	33.8	2.00 m	32.7	1.96 m
		1.09 m		1.10 m		1.15 m
8	42.0		42.0		43.9	
9	47.4	2.04 m	47.4	2.04 m	43.1	1.61 m
10	36.1		36.1		36.5	
11	22.0	1.50 m	21.9	1.50 m	34.9	1.81 m
		1.36 m		1.29 m		1.40 m
12	21.3	1.61 m	21.3	1.61 m	70.8	3.98 m
		1.32 m		1.29 m		
13	48.8	1.35 m	48.7	1.30 m	54.1	1.39 m
14	42.2		42.1		41.6	
15	31.5	1.38 m	31.5	1.38 m	32.4	1.35 m
16	18.8	1.83 m	19.1	1.83 m	18.9	1.82 m
		1.23 m		1.23 m		
17	47.1	1.14 m	50.9	1.13 m	48.5	1.10 m
18	37.9		38.2		40.0	
19	33.8	1.57 m	36.2	1.57 m	37.4	2.34 m
		1.09 m		1.06 m		1.22 m
20	29.1	1.89 m	30.8	2.24 m	35.0	1.48 m
		1.68 m		1.40 m		1.23 m
21	47.4		48.3		35.4	
22	71.1	3.85	75.9	3.43	76.4	3.19
		d (11.0)		d (10.8)		d (10.8)
23	29.4	1.05 s	29.4	1.05 s	30.5	1.32 s
24	19.6	1.03 s	19.5	1.03 s	21.6	1.09 s
25	23.3	0.76 s	23.3	0.76 s	23.0	0.93 s
26	22.0	1.18 s	22.1	1.15 s	21.7	1.23 s
27	17.1	0.93 s	17.0	0.92 s	18.0	0.94 s
28	13.5	0.78 s	13.7	0.77 s	13.8	0.96 s
29	14.8	1.27 s	25.2	1.36 s	29.7	0.99 s
30	183.1		177.6		18.4	0.88 s

*Assignments based on the HSQC and HMBC spectra.

Niebla homalea. The contribution of the metabolites characterized to the relatively poorly described chemotaxonomy of the genus *Niebla* and the plausible pathway for the formation of the new chlorinated triterpene, nieblastictane C (**3**), are also discussed herein.

2. Results and discussion

The molecular formulas of nieblastictanes A and B (**1** and **2**, respectively) were both established as $C_{30}H_{48}O_4$ (accounting for 7 degrees of unsaturation) by high-resolution electrospray-ionization mass spectrometry (HRESIMS), which displayed sodiated molecular ion peaks at *m/z* 495.3447 $[M+Na]^+$ and *m/z* 495.3440 $[M+Na]^+$, respectively (calcd. for $C_{30}H_{48}O_4Na^+$, 495.3445). Their infrared (IR) spectra showed stretching and other bands characteristics of hydroxy and carbonyl functions. Apart from the mass spectra, the ^{1}H and ^{13}C NMR spectroscopic data of compounds **1** and **2** were also superimposable (Table 1). The ^{13}C NMR spectrum of **1** displayed carbon resonances for seven methyl groups (δ 13.5, 14.8, 17.1, 19.6, 22.0, 23.3, 29.4), ten methylenes (δ 18.8, 20.5, 21.3, 22.0, 29.1, 31.5, 31.6, 33.8, 33.8, 33.8), five methines (δ 43.3, 47.1, 47.4, 48.8, 71.1), six quaternary sp^3 carbons (δ 36.1, 37.9, 41.9, 42.2, 46.9, 47.4), a carboxylic acid (δ 183.1), and a keto carbonyl carbon (δ_C 220.4). Also, the ^{1}H NMR spectrum of **1** displayed signals corresponding to seven quaternary methyl groups (δ_H 0.76, 0.78, 0.93, 1.03, 1.05, 1.18, and 1.27, each a singlet and 3H) in the upfield region, one set of diastereotopic methylene protons (δ_H 2.64 and 2.27, each a multiplet, H-2a and H2b) adjacent to a carbonyl group, and an oxygen-bearing methine (δ_H 3.85, d, $J = 11.0$ Hz, 1H, H-22). A systematic literature review of compounds isolated from lichens showed that the above ^{1}H NMR and mass spectroscopic data closely resembled those of 22α-hydroxystictan-3-one (**7**), a compound that has been isolated previously from *Sticta coronata* Müll. Arg., *S. colensoi* C. Bab., *S. flavicans* Hook. f. & Taylor, and obtained also in the present study, except for the absence of one methyl group singlet in **1** (Chin et al., 1973). Examination of their ^{13}C NMR spectroscopic and mass spectrometric data suggested that a methyl group in **7** was replaced by a carboxylic acid unit in **1**. One- and two-dimensional NMR experiments were then performed to assign all methyl, methylene, methine and quaternary carbons present in **1** and thus to locate the carboxylic acid group at δ_C 183.1 ppm. The assignment of the keto carbonyl at C-3, the oxymethine at C-22, and the carboxylic acid function at C-30 on an identified stictane skeleton were substantiated by the interpretation of correlations observed in the COSY and HMBC spectra of **1**. The long-range HMBC correlations observed from the signal at δ_H 1.27 (CH_3-29) to C-30, C-20, and C-22 indicated the carboxylic acid group to be attached to C-21 (Fig. 1). The COSY spin network from the proton signals of H-1 to those of H-2 and the HMBC long-range correlations from the signals of H-1, H-2, H-23, and H-24 to C-3 (δ_C 220.4) were used to locate the keto carbonyl group at C-3. The hydroxymethine substituent at C-22 was confirmed by the HMBC long-range cross-peaks from H-29 to C-22, from H-22 to C-16, C-30 and the COSY correlations from H-22 through H-15 (Fig. 1). The chair-boat-chair-chair-chair conformation of the stictane skeleton (Corbett and Wilkins, 1976a) and the large coupling constant (11 Hz) of the doublet at 3.85 (H-22) demonstrated that the proton at C-22 is beta-oriented. From a consideration of all the above evidence, the structure of **1** was proposed as 22α-hydroxystictan-3-on-30-oic acid.

Two-dimensional Nuclear Overhauser Effect (NOE) and COSY experiments as well as analyses of H–H coupling constants were performed to investigate the relative configuration of **1**. NOESY correlations between CH_3-25 and CH_3-24, CH_3-24 and H-9, H-9 and CH_3-27, CH_3-27 and CH_3-28, and CH_3-28 and H-22 confirmed the fusion and conformation of rings A through D. In addition, the equatorial substitution of the C-21 carboxylic acid group was corroborated by the presence of a NOESY correlation between H-17 and CH_3-29. The configuration at C-10 of **1** was elucidated as *R*, since the ECD spectrum of **1** disclosed a characteristic positive Cotton effect at 294.5 nm (Δε +15.38, the n→π* transition) (Snatzke, 1968). Consequently, the chemical structure of **1** was characterized as (8*S*,9*S*,10*R*,14*S*,17*R*,18*S*,21*R*,22*R*)-22α-hydroxystictan-3-on-30-oic acid, and it has been given the trivial name nieblastictane A.

Comparison of the ^{13}C NMR data of **1** and **2** (Table 1) led to the conclusion that these two compounds differ from each other only in the chemical shifts arising from ring E and their respective carboxylic acid substituents (δ_C 183.1 vs. 177.6 ppm). In order to elucidate the structural difference between these two compounds, coupling constant analyses and NOESY experiments were carried out. The coupling constant of H-22 (δ_C 3.43, d, $J = 10.8$ Hz) as well as the NOESY correlation observed from H-22 to CH_3-28 and CH_3-30, and CH_3-27 and CH_3-28, prompted the assignment of the carboxylic acid group of **2** to be at C-21 and axially oriented, and the hydroxy group at C-22 to be alpha-oriented. Since compound **2** (nieblastictane B) displayed an ECD spectrum similar to **1**, its complete structure was determined as (8*S*,9*S*,10*R*,14*S*,17*R*,18*S*,21*S*,22*R*)-22α-hydroxystictan-3-on-29-oic acid (**2**).

The molecular formula of nieblastictane C (**3**; isolated as a white amorphous powder) was determined to be $C_{30}H_{49}O_3Cl$ by HRESIMS, which showed peaks characteristic of a chlorine-containing atom at *m/z* 515.3268 $[M+Na]^+$ (calcd. for $C_{30}H_{49}O_3ClNa^+$, 515.3262) and accounted for six degrees of unsaturation. Its IR spectrum showed the presence of carbonyl stretching (ν_{max} 1709 cm^{-1}) and a band corresponding to a hydroxy functionality (ν_{max} 3411 cm^{-1}). The ^{1}H NMR spectrum of **3** displayed signals of eight quaternary methyl groups in the

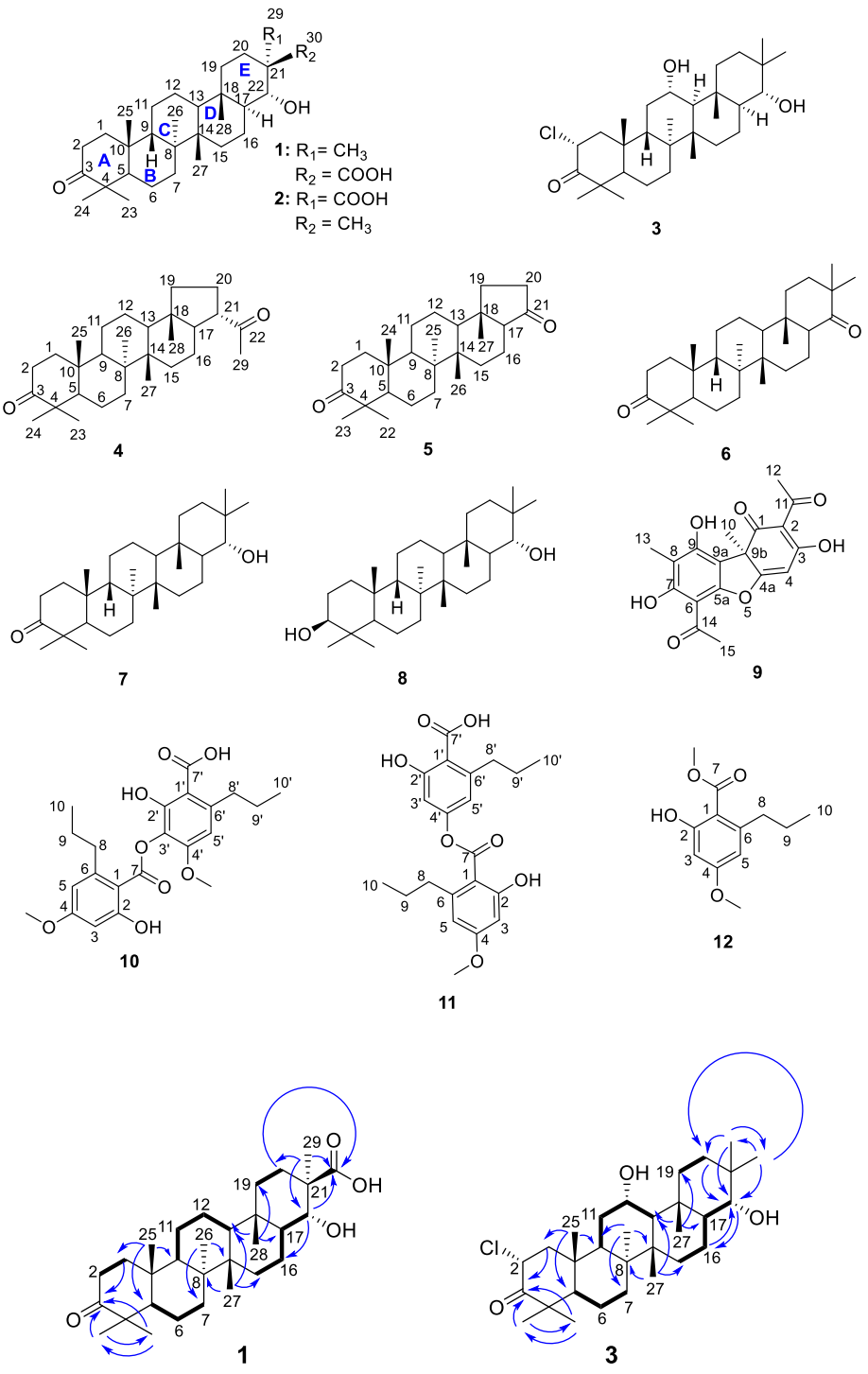

Fig. 1. Key ^{1}H–^{1}H COSY(—) and HMBC (H→C) correlations of **1** and **3**.

upfield region (δ_H 0.88, 0.93, 0.94, 0.96, 0.99, 1.09, 1.23, 1.32; each a singlet), and seven methines, of which three were downfield (δ_H 4.41 t, J = 7.76 Hz; 3.98, m; and 3.19, d, J = 10.8 Hz). The ^{13}C NMR spectra showed 30 carbon signals assignable to the above-mentioned eight methyl groups (δ_C 13.8, 18.0, 18.4, 21.6, 21.7, 23.0, 29.7, 30.5), eight methylenes (δ_C 18.8, 20.1, 32.4, 32.7, 34.9, 35.0, 37.4, 44.5), seven methines (δ_C 43.1, 45.8, 48.5, 54.1, 57.5, 70.8, 76.4), and seven quaternary carbons, including a keto carbonyl resonance (δ 210.5). Inspection of its ^{1}H and ^{13}C NMR and two-dimensional NMR spectra, suggested that **3** was also a carbonyl group-bearing pentacyclic stictane-type triterpene with a planar structure similar to that **1**. Comparison of the NMR spectroscopic data of **1** and **3** revealed that the only differences between the two compounds were from the occurrence of two methine groups (δ_H 4.41 t, J = 7.76 Hz, δ_C 57.5 and δ_H 3.98, m, δ_C 70.8) in **3**, which replaced the signals corresponding to two methylenes at H-2 and H-12 of **1**. The chemical shift values of the newly observed methine groups in **3** together with the interpretation of long-range HMBC spectrum (Fig. 1), led to the conclusion that C-2 and C-12 must be chlorine- and oxygen-bearing methines, respectively. In the HMBC spectrum, correlations from CH_3-23/CH_3-24 to C-3 and C-5; CH_3-25 to C-1, C-5, and C-9; CH_3-26 to C-7, C-9, and C-14; CH_3-27 to C-8, C-13, and C-15; CH_3-28 to C-13, C-19, and C-17; H-22 to C-16; CH_3-29/CH_3-30 to C-20,

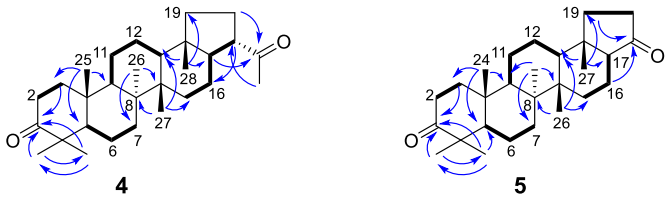

Fig. 2. Key ^{1}H–^{1}H COSY(—) and HMBC (H→C) correlations of **4**, **5**.

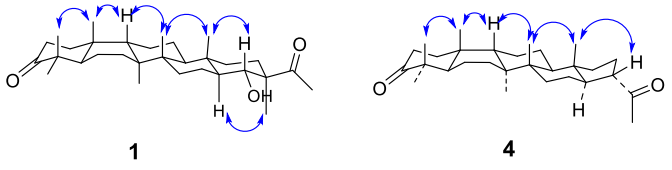

Fig. 3. Key NOESY (↔) correlations of **1** and **4**.

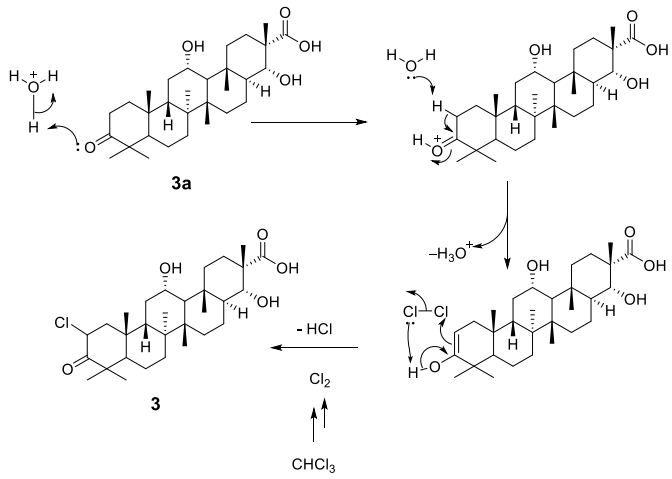

Fig. 4. Proposed chemical conversion pathway to produce compound **3**.

C-21, and C-22 were observed (Fig. 1). Moreover, a ^{2}J correlation between the chlorine-bearing methine (H-2) and the keto carbonyl signal (C-3) was observed. The complete assignments of all proton and carbon signals of **3** were accomplished by interpretation of the COSY and HSQC spectroscopic data. Thus, the planar structure of nieblastictane C (**3**) was assigned as 2-chloro-12,22-dihydroxystict-3-one.

Analyses of the ^{1}H NMR coupling constant data and the results obtained from the interpretation of the NOESY spectra permitted the determination of the relative configuration of **3**. NOESY correlations from CH_3-25 to CH_3-24 and H-9; CH_3-27 to H-9 and CH_3-28; H-2 to CH_3-25; H-12 to CH_3-28; and from H-13 to CH_3-26 allowed an assignment of the hydroxy group at C-12 as alpha-oriented. In addition, the similarity of the coupling constant of the H-22 resonances of **1** and **3** (11 Hz vs. 10.8 Hz), together with the overall match of the chemical shifts values arising from rings B, C and D of compound **3** with those of **1**, confirmed the beta-orientation of H-22 and the same relative configurations of rings B, C and D as compound **1**. Moreover, the presence of NOESY cross-peaks from CH_3-27 to CH_3-28; CH_3-28 to H-22; and CH_3-29 to H-17 supported the relative configurations of rings D and E proposed and confirmed the orientation of H-22. The absolute configuration at C-10 was concluded to be *R* in the same manner as described for **1**. From this evidence, the chemical structure of nieblastictane C (**3**) was characterized as (2*R*,8*S*,9*S*,10*R*,12*S*,13*S*,14*S*,17*R*, 18*S*,21R,22*R*)-2-chloro-12α,22α-dihydroxystictan-3-one.

Compound **3** is the first C-2 chlorinated stictane to have been obtained a from lichen organism. The presence of chlorine gas in chloroform as a product of photodecomposition has been reported (Kuwahara et al., 2012). Since chloroform was used during the isolation, we must assume that an acid-catalyzed alpha chlorination of a related compound (**3a**) yielded compound **3** (Fig. 4).

Compound **4** was isolated as thin white needles with a molecular formula of $C_{29}H_{46}O_2$, as substantiated by the sodiated molecular ion peak in the HRESIMS at *m/z* 449.3394 $[M + Na]^+$ (calcd. for 449.3396, $C_{29}H_{46}O_2Na$). The presence of signals of two keto carbonyl groups, as confirmed by the IR combined with the NMR spectra, and the seven degrees of unsaturation deduced from the mass spectrometric data obtained, suggested that **4** is a pentacyclic nor-triterpenoid. The ^{1}H NMR spectrum disclosed signals for seven quaternary methyl groups (0.68, 0.76, 0.90, 1.03, 1.05, 1.18, and 2.15, each equivalent to three protons and singlets), of which one was found to be attached to a carbonyl group. HMBC experiments showing long-range correlations from H_3-29 to C-21, from H-17/H-20 to C-22 (Fig. 2), and interpretation of the 2D NMR spectra, led to the assignments of all proton and carbon signals of **4**. The ^{13}C NMR chemical shifts arising from rings A, B, C, and D were very similar to those of **1** and **2**, and the presence of a downfield methyl resonance ascribable to a methyl carbonyl group and the absence of the signal for a C-22 carbinol suggested the E ring to be five-membered. The chair-boat-chair-chair conformation of the rings A, B, C, and D, the presence of a five-membered ring E, as well as the nor-flavicane instead of the nor-hopane nature of **4**, were confirmed by systematic analyses of the HMBC and NOESY spectroscopic data (Fig. 3).

The allocation of a keto carbonyl group at C-3 and an acetyl group at C-21 in **4** was carried out in the following manner. The NOESY correlations from the resonance at δ 1.03 (CH_3-24) to the signals arising from CH_3-25 (δ 0.76), CH_3-23 (δ 1.05), and H-9 (δ 2.03) as well as those from CH_3-27 to H-9 and CH_3-28 confirmed the configuration of the first four rings of compound **4**. In addition, the HMBC correlation between the methyl proton at δ 2.15, CH_3-29) and C-21 (δ 53.6) and the NOESY correlation between H-21 and CH_3-28 enabled the conclusion that an alpha-attached acetyl group occurred at C-21. The observation of HMBC correlations from H-1 to C-3, and from CH_3-23 and CH_3-24 to C-3, C-4, and C-5 confirmed the assignment of the C-3 carbonyl group. From the above data, the structure of **4** was elucidated as 29-*nor*-21βH-flavican-3,22-dione.

The determination of the absolute stereostructure of **4** to be as depicted was achieved from the positive Cotton effect at observed at 294 nm (Δε +12.91, the n→π* transition) in the ECD spectrum and the results of a NOESY experiment showing the arrangement of the A, B, C, D, and rings. Therefore, the chemical structure of **4** was characterized as (8*S*,9*S*,10*R*,14*S*,17*R*,18*S*,21*S*)-30-*nor*-21βH-flavican-3,22-dione, and it has been named nieblaflavicane A.

Flavicanes are triterpenoids found in lichens and have structures very similar to those of hopanes (Chin et al., 1973; Connolly, 1977; Corbett and Wilkins, 1976a, b; Wilkins et al., 1989). Due to the difference in the fusion of the rings in both skeletons, they may be differentiated readily by their NMR spectroscopic data. Natural flavicanes are very rare and the present study has shown that lichens of the genus *Niebla* should be added as a source of this group of compounds.

Compound **5** (obtained as thin white needles) gave a molecular formula of $C_{27}H_{42}O_2$, with seven degrees of unsaturation, as determined from the HRESIMS, which exhibited a sodiated molecular ion peak at *m/z* 421.3088 $[M + Na]^+$ (calcd. for $C_{27}H_{42}O_2Na^+$: 421.3083). The ^{1}H NMR spectrum of **5** showed resonances of five singlet signals equivalent to six quaternary methyl groups (δ_H 0.78, 0.97, 1.03, 1.05, 1.05, 1.16). The ^{13}C NMR and HSQC spectra of **5** showed 27 signals representative of six quaternary methyl groups, ten methylenes, four methines, and seven quaternary carbons (δ_C 36.1, 41.5, 41.6, 41.7, 46.9, 220.2, 221.2). The HMBC correlations observed (Fig. 2) from CH_3-22/CH_3-23 to C-3, C-5, C-23/C-22; CH_3-24 to C-1, C-5, C-9; CH_3-25 to C-7, C-5, C-14; H_3-26 to C-8, C-13, C-15; CH_3-27 to C-13, C-19, C-17, and the COSY and HSQC correlations led to the assignments of all of the proton and carbon signals. Analysis of the above data together with the IR data and the seven degrees of unsaturation indicated by HRESIMS suggested that **5** is also a

pentacyclic compound bearing two carbonyl groups. Inspection of its one- and two-dimensional NMR spectroscopic data led to the conclusion that **5** has the same planar structure as **4**, but is without the C-21 methine and the acetyl group. As confirmed by the ^{13}C NMR and HMBC spectroscopic data, a more downfield keto carbonyl signal at 221.2 ppm (C-21), due the breakdown of the sidechain of **4** was observed in **5**. The structure of **5** was finally established as 22,29,30-*trinor*-flavican-3,21-dione. In this contribution, we report the NMR spectroscopic data of compound **5** for the first time, since its structure was proposed tentatively previously without any NMR data (Tabacchi et al., 1987).

Since compound **5** has two carbonyl carbons in its structure, its absolute configuration as shown was determined by the positive Cotton effect observed at 282.5 nm ($\Delta\varepsilon$ +3.97, the n→π* transition (Snatzke, 1968), and the negative Cotton effect at 316.5 nm ($\Delta\varepsilon$ −4.15, the n→π* transition) in the ECD spectrum (Kirk and Klyne, 1976). Consequently, the chemical structure of **5** (nieblaflavicane B) was characterized as (8*S*, 9*S*,10*R*,14*S*,17*R*,18*S*)-22,29,30-*trinor*-flavican-3,21-dione.

Three known triterpenoids were identified as stictane-3,22-dione (**6**), 22α-hydroxystictan-3-one (**7**), and stictane-3β,22α-diol (**8**), with usnic acid (**9**), sekikaic acid (**10**), divaricatic acid (**11**), and divaricatinic acid methyl ester (**12**) also isolated and identified during this investigation. Their structures were determined by comparison of their spectroscopic data with those published in the literature (Lubbe et al., 2008; Mie et al., 2014; Wilkins et al., 1989; Yu et al., 2016).

Among the compounds isolated, only the known cytotoxic lichen metabolite (+)-usnic acid (**9**) (Brisdelli et al., 2013) exhibited moderate antiproliferative activity against the two cell lines used (IC_{50} values of 3.8 and 6.8 μM against A2780 ovarian and MCF-7 breast cancer, respectively).

The phylogeny of the fruticose Ramalinaceae (Spjut et al., in press) supports the taxonomy of Spjut (1996) based on the specialized metabolites present. Within *Niebla*, two major chemotaxonomic clades are recognized as previously mentioned: one with triterpenes with depsides, and the other without triterpenes, with or without ß-orcinol-depsidones. The depside species are interpreted as belonging to a polytomy of four divaricatic acid clades and a fifth, a relatively short divaricatic acid with a subterminal larger clade of sekikaic acid species among which are species that may contain a lower occurrence of divaricatic acid (Spjut et al., in press).

The species within the depside clades remain unresolved. For example, *Niebla homalea*, the type species for the genus, originally monotypic, is narrowly circumscribed morphologically by Spjut, 1996, but found in three clades, two in northern California and one in Baja California (Spjut et al., in press). Our present report of the biologically active constituents is from one of the two California species, both appearing endemic to California but are referred to *N. homalea* based on the only name currently available for our collection.

The type specimen for *Niebla homalea* was collected by Archibald Menzies from rocks in California (Spjut, 1996; Supporting information), most likely during November 1792, judging from text of his diary provided by Alice Eastwood (Eastwood, 1924). Menzies had visited Presidio in San Francisco, Bodega Bay, and Tomales Bay. The type specimen is most similar morphologically and chemically (divaricatic acid plus triterpenes) to a collection by William Weber from Point Reyes shown in Spjut (Spjut, 1996; Supporting information).

Our collection (Spjut 17806) is from Point Reyes. However, from DNA phylogeny of the genus *Niebla* (Spjut et al., in press), which included multiple specimens from Point Reyes containing divaricatic acid, it is indicated that two divaricatic acid species occur there (Spjut 17806, 17807), one undoubtedly *N. homalea*, the other an undescribed cryptic species (LaGreca et al., 2020). The species may have evolved in isolation as a result of continental drift northwards of an island segment (Point Reyes) of the Pacific Plate during the past 1.8 million years, and the two species may have come together along the San Andreas Fault system when the Pacific Plate came into contact with the North American Plate (Jachens et al., 1998). Although we could likely identify chemical differences between these species, the type specimen of that cryptic species is not likely to be available for an in-depth chemical extraction.

3. Experimental

3.1. General experimental procedures

All chemical solvents were purchased from Thermo Fisher Scientific (Columbus, OH, USA). Column chromatography was carried out with SiliaFlash® P60 (230–400 mesh), purchased from Parc-Technologique BLVD (Quebec City, Canada) as the normal stationary phase and octadecyl-functionalized (C_{18}, 17%) silica gel (230–400 mesh), purchased from SiliCycle (Quebec, Canada). Analytical thin-layer chromatography was performed on aluminum-backed TLC plates (250 mm thickness) also purchased from SiliCycle. Analytical reversed-phase thin-layer chromatography was conducted on aluminum-backed TLC (250 μm thickness C_{18}): (SiliCycle). Size-exclusion chromatography was executed with SephadexTM LH-20. Optical rotation data were measured on PerkinElmer 343 polarimeter. UV spectra were obtained on a Hitachi U-2910 UV/vis double-beam spectrophotometer. IR spectra were recorded on Thermo-Nicolet 6700 Fourier-transform IR spectrophotometer on a KBr ground crystal window from Sigma-Aldrich (catalog number: Z267635-1 EA). NMR spectra were recorded at 25 °C with a Bruker Avance III 400 HD NMR spectrometer. High-resolution mass spectra (HRMS) were acquired with a Thermo LTQ Orbitrap (analyzer: ITMS and FTMS, mass range: 50–4000 *m/z*, resolution: 7500−100,000).

3.2. Lichen material

The lichen *Niebla homalea* (Ach.) Rundel & Bowler (Ramalinaceae) was collected on October 30, 2017 from coastal scrub on sand with rocky outcrops, just east of a lighthouse on a narrow peninsula with strong cross winds in Marin County, Point Reyes, CA, USA (N 37°53′09.50″, W 122°37′34.83″), by one of the authors (R. W. S.). Voucher specimens of the lichen (Spjut 17806) were deposited in a herbarium at the Université de Liège Département de Botanique, Belgium (LG), where DNA was also extracted from fresh material (Sérusiaux, LG 6251) for a phylogenetic study of the Ramalinaceae family, and also at the World Botanical Associates (WBA). An associated voucher specimen of the lichen was also deposited at the Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University as A1-3.

3.3. Extraction and isolation of secondary metabolites of Niebla homalea

The lichen *Niebla homalea* (Ach.) Rundel & Bowler (62 g) was macerated for 1 week with 3 L of ethyl acetate. The extract was filtered and concentrated *in vacuo* to yield 4 g of a brown residue. This ethyl acetate residue was subjected to passage over a silica gel column [SiliaFlash® P60 (230–400 mesh)], and eluted sequentially with hexane containing increasing amounts of ethyl acetate (1:0, 20:1, 10:1, 5:1, 2:1, 1:1, and 0:1), to afford ten major fractions labeled N1 to N10. Fraction N2 (20 mg) yielded compound **6** (5 mg) by precipitation in chloroform ($CHCl_3$). Fractions N3 (6 mg) and N4 were washed with chloroform to afford compounds **4** (1 mg) and **7** (6 mg), respectively. Fraction N5 (40 mg) was purified using a C_{18} column (CH_3CN–H_2O, 7:3) to give **5** (3 mg). Fraction N6 (40 mg) was purified also via a C_{18} column (CH_3CN–H_2O, 3:2) to yield compound **9** (3 mg). Fraction N7 (120 mg) was chromatographed over a Sephadex LH-20 column with hexane-$CHCl_3$-MeOH (5:5:1) as solvent and washed with hexanes-$CHCl_3$-MeOH (5:5:1) to afford **11** (6 mg). Fraction N8 (170 mg) was chromatographed over a Sephadex LH-20 column, eluted with hexanes-$CHCl_3$-MeOH (5:5:1), and further purified via silica gel column chromatography (hexanes-EtOAc, 5:1) to yield **12** (4 mg) and **8** (4 mg). Fraction N9 (130 mg) was chromatographed over a Sephadex LH-20 column with hexanes-$CHCl_3$-MeOH (5:5:1) and further purified via a C_{18} column (CH_3CN–H_2O, 7:3)

Table 2
^{1}H and ^{13}C NMR (400 MHz and 100 MHz, respectively) spectroscopic data for compounds **4** and **5**.

Position	4		5	
	δ_C	δ_H	δ_C	δ_H
1	31.6	2.08 m	31.5	2.06 m
		1.44 m		1.43 m
2	33.8	2.65 m	33.7	2.65 m
		2.27 m		2.27 m
3	220.4		220.2	
4	46.9		46.9	
5	43.5	1.42 m	43.6	1.49 m
6	20.6	1.44 m	20.3	1.43 m
		1.16 m		1.16 m
7	34.2	2.00 m	33.6	1.96 m
		1.09 m		1.05 m
8	41.8		41.6	
9	47.4	2.03 m	47.2	2.03 m
10	36.2	109.6	36.1	
11	21.9	1.46 m	23.5	1.63 m
12	21.2	1.51 m	22.3	1.55 m
		1.34 m		1.36 m
13	48.7	1.47 m	38.8	1.34 m
14	42.7		41.7	
15	32.4	1.37 m	27.0	1.14 m
		1.33 m		1.10 m
16	24.2	1.46 m	35.6	2.27 m
				2.23 m
17	54.3	1.35 m	57.5	1.87 m
18	45.1		41.5	
19	39.8	1.54 m	34.5	2.10 m
		1.16 m		1.35 m
20	25.6	1.94 m	17.2	2.02 m
		1.71 m		1.66 m
21	53.6	2.65 m	221.2	
22	212.6		29.4	1.05 s
23	29.4	1.05 s	19.6	1.03 s
24	19.6	1.03 s	23.3	0.78 s
25	23.3	0.76 s	21.7	1.05 s
26	22.2	1.18 s	15.1	0.97 s
27	17.0	0.90 s	24.3	1.16 s
28	14.9	0.68 s		
29	30.3	2.15 s		

*Assignments based on HSQC and HMBC spectra.

to yield compounds **2** (2 mg) and **3** (2 mg). Fraction N10 (70 mg) was chromatographed over a Sephadex LH-20 column with hexanes-$CHCl_3$-MeOH (5:5:1) and washed with hexanes-$CHCl_3$-MeOH (5:5:1) to yield 3 mg of **1** and 4 mg of **10**.

*Nieblastictane A (8S,9S,10R,14S,17R,18S,21R, 22R)-22α-hydroxystictan-3-on-30-oic acid, **1**)*: white amorphous powder; $[\alpha]^{20}_D$ +66.0 (*c* 0.001, MeOH); IR (KBr) ν_{max}: 3465, 2956, 2875, 1699, 1471, 1385, 1241, 754, 667 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data, see Table 1; HRESIMS *m/z* 495.3447 $[M + Na]^+$ (calcd. for $C_{30}H_{48}O_4Na^+$: 495.3445).

*Nieblastictane B (8S,9S,10R,14S,17R,18S,21S,22R)-22α-Hydroxystictа-3-on-29 oic acid, **2**)*: white amorphous powder; $[\alpha]^{20}_D$ +24.0 (*c* 0.001, MeOH); IR (KBr) ν_{max} 3390, 2956, 2873, 1702, 1457, 1385, 1230, 1057, 755 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data, see Table 1; HRESIMS *m/z* 495.3440 $[M + Na]^+$ (calcd. for $C_{30}H_{48}O_4Na^+$: 495.3445).

*Nieblastictane C (2R,8S,9S,10R,12S,13S,14S,17R,18S,21R,22R)-2-chloro-12, 22-dihydroxystictan-3-one (**3**)*: white amorphous powder; $[\alpha]^{20}_D$ −1.00 (*c* 0.001, MeOH); IR (KBr) ν_{max} 3411, 2945, 2873, 1709, 1458, 1386, 1217, 1059, 1022, 755 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data, see Table 1; HRESIMS *m/z* 515.3268 $[M + Na]^+$ (calcd. for $C_{30}H_{49}O_3ClNa$, 515.3262).

*Nieblaflavicane A (8S,9S,10R,14S,17R,18S,21S)-30-nor-21βH-flavican-3,22-dione, **4**)*: thin white needles (CH_2Cl_2–CH_3OH); $[\alpha]^{20}_D$ −127.2 (*c* 0.001, MeOH); IR (KBr) ν_{max} 2928, 1704, 1383 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data, see Table 2; HRESIMS *m/z* 449.3394 $[M + Na]^+$ (calcd. for 449.3396, $C_{29}H_{46}O_2Na$).

*Nieblaflavicane B (8S,9S,10R,14S,17R,18S)-22,29,30-trinor-flavican-3,21-dione, **5**)*: white needles (CH_2Cl_2–CH_3OH); $[\alpha]^{20}_D$ +3.0 (*c* 0.001, MeOH); IR (KBr) ν_{max} 2928, 2869, 1734, 1705, 1461, 1384, 1110 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data, see Table 2; HRESIMS *m/z* 421.3088 $[M + Na]^+$ (calcd. for 421.3083, $C_{27}H_{42}O_2Na$).

3.4. Cytotoxicity assay

The cytotoxic activities of the isolates were evaluated against human hormone-dependent breast (MCF-7) and ovarian (A2780) cancer cell lines, according to a previously described protocol and paclitaxel and camptothecin were used as positive controls (Tan et al., 2019).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112521.

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