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Bifunctionalized Amphilectane Diterpenes from the Sponge *Stylissa* cf. *massa*

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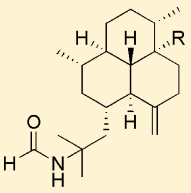
Supporting Information

ABSTRACT: Two new amphilectane-type diterpenes, 8-isocyanato-15-formamidoamphilect-11(20)-ene (**1**) and 8-isothiocyanato-15-formamidoamphilect-11(20)-ene (**2**), along with two known derivatives, 8-isocyano-15-formamidoamphilect-11(20)-ene (**3**) and 7-formamidoamphilect-11(20),15-diene (**4**), were isolated from the sponge *Stylissa* cf. *massa*. Diterpenes bearing two different isonitrile-related functionalities, as in **1**−**3**, are rare. The coexistence of these compounds, all of which possess the identical carbon skeleton, in the same sponge specimen suggests interconversion among them. All the isolated compounds were tested for antimalarial activity. Compound **3** proved approximately 10 times more active than **1** and **2**, indicating the importance of the isonitrile moiety to antimalarial activity versus the isocyanate and isothiocyanate groups, respectively. Compound **4**, which contains only the formamide group, was inactive at the highest concentration tested.

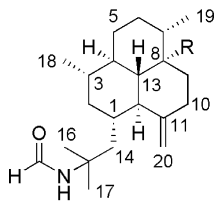


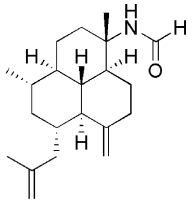
1; R = NCO
2; R = NCS

Sesquiterpenes and diterpenes possessing isonitrile and related functionalities are unique and exclusively marine-derived natural products. Terpene skeletons, which include the spiroaxane and pupukeanane types for the sesquiterpenes and the amphilectane and cycloamphilectane for the diterpenes, are rare in nature. Such skeletons are also associated closely with certain sponge genera reported as sources of terpenes in this class. Specifically, diterpenes of the amphilectane type bearing isonitrile, isocyanate, isothiocynate, and formamide functionalities have been isolated from only a few sponge genera, including *Hymeniacidon*,[1] *Cribochalina*,[2] *Stylissa*,[3] and *Cymbastela*.[4] It has been reported that such functionalities, especially isonitrile, contribute to the biological activities, particularly for those compounds that are reported to have antimalarial activity.[4b,5]

Recently we reported the isolation and antimalarial activities of a series of isonitrile amphilectenes from the sponge *Stylissa* cf. *massa* (Carter) (then identified as belonging to the genus *Ciocalapata*).[6] Extended investigation of the remaining extract fractions led to the isolation of two new bifunctionalized amphilectenes, 8-isocyanato-15-formamidoamphilect-11(20)-ene (**1**) and 8-isothiocyanato-15-formamidoamphilect-11(20)-ene (**2**), and two known derivatives, 8-isocyano-15-formamidoamphilect-11(20)-ene (**3**) and 7-formamidoamphilect-11(20),15-diene (**4**). The chemical structures of the two known diterpenes were identified by spectroscopic analysis, including MS, UV, IR, and NMR spectra, and by comparison with data from previously published reports.[1,4b]

Compound **1** is proposed to have a molecular formula of $C_{22}H_{34}N_2O_2$ based on the high-resolution ESI mass of 359.2713 $[M + H]^+$. The proposed molecular formula led to the unsaturation degree of seven. Four of these were attributed to a carbonyl at δ 159.6 (15-NCHO), an isocyanate at δ 123.0





1; R = NCO
2; R = NCS
3; R = NC

4

(8-NCO), and an olefin at δ 150.9 (C-11) and 106.0 (C-20); thus, three rings were deduced. The NMR (500 MHz for ^{1}H and 125 MHz for ^{13}C, C_6D_6, Table 1) spectra of **1** showed two sets of signals in a 3:2 ratio characteristic of two formamide rotamers. For brevity, the discussion throughout this report refers to the resonances of the major rotameric species. Both spectra are almost identical to those of **3** (see Supporting Information, p S6), indicating that they share a similar amphilec-11(20)-ene skeleton. The major difference between the two compounds is the presence of an isocyanate functionality, characterized by the ^{13}C resonance at δ 123.0 and the IR absorption band at ν 2250 cm^{-1}, which replaces the isonitrile functional group of **3**. The signals assigned for C-8 of both compounds, although resonating at comparable chemical shifts (δ 65.7 for **1**, 66.7 for **3**), change from the isonitrile triplet in **3** (^{CN}J = 1.4 Hz) to a singlet for the isocyanate in **1**; this change therefore indicates that the substitution of the newly observed isocyanate group is at C-8. The structure elucidation of **1** was confirmed by analysis of COSY and HMBC spectra

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Table 1. ^{1}H and ^{13}C NMR Chemical Shifts for 1 and 2 (500 MHz for ^{1}H, 125 MHz for ^{13}C; C_6D_6)[a]

	1		2	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	33.4 [32.8], CH	1.66 [1.58][b]	33.4 [32.8], CH	1.66[b]
2	41.2 [41.3], CH_2	1.78;[b] 0.60[b]	41.3 [41.9], CH_2	1.78[b]; 0.60, dd (14.0, 10.0)
3	35.9 [35.8], CH	0.79, d (5.6)	35.9 [35.8], CH	0.76[b]
4	43.1 [43.2], CH	0.92[b]	42.6 [42.5], CH	0.90[b]
5	30.1 [30.2], CH_2	1.75;[b] 0.54, dddd (12.9, 12.2, 8.0, 4.6)	30.1 [30.17], CH_2	1.76;[b] 0.48, m
6	30.5 [30.2], CH_2	1.32;[b] 1.21[b]	30.5 [30.2], CH_2	1.32;[b] 1.22[b]
7	42.6 [42.5], CH	0.92[b]	43.3 [43.4], CH	0.92[b]
8	65.7, C		69.6 [69.5], C	
9	41.4, CH_2	1.84;[b] 0.95[b]	41.1 [40.2], CH_2	1.88 [1.93], dddd (13.1, 4.6, 2.6, 1.9); 0.85[b]
10	34.3 [34.2], CH_2	2.11, ddd (13.4, 13.1, 4.6); 1.98[b]	34.3 [34.2], CH_2	2.12 [2.44], ddd (13.1, 13.1, 4.7); 1.95 [2.31], ddd (13.1, 2.6, 2.4)
11	150.9 [151.1], C		150.2 [150.5], C	
12	47.1 [47.0], CH	1.68 [1.58][b]	47.2 [47.1], CH	1.71, dd (10.5, 10.0)
13	57.1 [57.0], CH	0.72, br d (13.9)	56.9 [56.8], CH	0.70 [0.67], dd (10.9, 10.8)
14	45.4 [48.2], CH_2	2.01, br d (13.6); 1.21 [1.13][b]	45.7 [48.1], CH_2	2.02 [1.96], dd (14.6, 1.7); 1.03 [0.97], dd (14.6, 10.0)
15	53.6 [52.1], C		53.6 [52.2], C	
16	27.7 [30.7], CH_3	1.22, s	27.7 [30.6], CH_3	1.25 [1.35], s
17	29.1 [28.7], CH_3	1.26, s	29.0 [28.8], CH_3	1.24 [1.35], s
18	20.1 [20.0], CH_3	0.79, br s	20.0 [19.9], CH_3	0.75 [0.82], d (5.6)
19	15.9 [14.3], CH_3	0.75 [0.74], d (6.3)	16.0 [14.3], CH_3	0.76 [0.82], d (6.1)
20	106.0 [105.8], CH_2	4.81 [4.75], s; 4.68 [4.50], s	106.5 [106.4], CH_2	4.79 [4.72], s; 4.70 [4.49], s
8-NCX[c]	123.0, C		131.0, C	
15-N*H*		4.07, br s [5.67, br d (12.0)]		4.06, br s [5.68, br d (12.0)]
15-NC*H*O	159.6 [162.1], CH	7.67, d (1.7) [8.17, d (12.0)]	159.6 [162.1], CH	7.69, d (1.7) [8.16, d (12.0)]

[a]Detectable chemical shifts of the minor components, for both ^{1}H and ^{13}C, are presented in brackets. [b]Signals are overlapped, and the multiplicity and coupling constants cannot be determined. [c]X = O for **1**; X = S for **2**.

(Figure 1) to furnish its structure as a new amphilectene analogue, 8-isocyanato-15-formamidoamphilect-11(20)-ene.

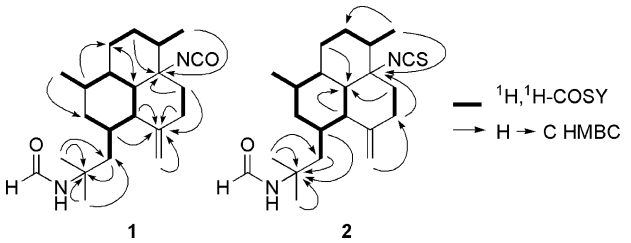

Figure 1. ^{1}H,^{1}H-COSYand key HMBC correlations of **1** and **2**.

The NOEDS experiments on **1** showed dipolar couplings among H-1, H-3, H-5a (δ 0.54), and H-13 and indicated that all resided on the same face of the molecule. Although the clustered signals of the alicyclic methylenes and methines did not facilitate more extensive NOE experiments, particularly from H-4, H-7, and H-12, the implication of the observed NOEs and the similarity between the NMR spectra of **1** and **3** allowed us to propose that **1** adopts the relative configuration of an all-*trans* perhydrophenalene skeleton similar to **3**.[1] Note that the chemical shifts of two equatorial methyls (C-18, δ 20.1; C-19, δ 15.9) are atypically upfield for such an orientation. The chemical shifts of both carbons result from the shielding effects of the solvent C_6D_6. A similar phenomenon was also observed in our previous report.[6] For the geometry of the formamide group, the coupling constants of 15-NHC*H*O (δ 7.67, d, J = 1.7 Hz, for the major, and δ 8.18, d, J = 12.0 Hz for the minor rotamers) indicated the *cisoid* conformation to be the dominant species. This is in agreement with the observation made by Wright and Lang-Unnasch.[4b]

The molecular formula of **2** is proposed to be $C_{22}H_{34}N_2OS$, also on the basis of HRESIMS data. The proposed molecular formula indicates that one of the oxygens present in the structure of **1** is replaced by a sulfur. This is evident as the IR absorption band of the isocyanate functionality of **1** (ν 2250 cm^{-1}) is replaced by that of an isothiocyanate (ν 2075 cm^{-1}) in the spectrum of **2**. The ^{13}C chemical shift of the isocyanate carbon (δ 123.0; 8-NCO) of **1** also shifted downfield, resonating in the characteristic range for the isothiocyanate (δ 131.0; 8-NCS). The NMR spectra of **1** and **2**, apart from the resonances assigned for the isocyanate and isothiocyanate carbons, were almost identical (Table 1). This included the characteristic features of a 3:2 rotameric mixture. The structure of **2**, determined in a similar fashion to that of **1** (Figure 1), was proposed to be a new 8-isothiocyanate analogue of **1**. Also similar to that of **1** was the relative configuration of **2**, which was determined by means of the same experiments and assumption as described earlier.

Bifunctionalized diterpenes possessing two different isonitrile-related functionalities, although readily documented,[2,4b,5b,c] are rare. Recent reports from Garson's group proved that sponges had the ability to incorporate cyanide and thiocyanate as sources of isonitrile and isothiocyanate functionalities as well as to interconvert the two groups biosynthetically,[7] whereas the formamide was shown to be derived chemically from hydrolysis of the isonitrile,[7b,8] but has never been proven to be formed biosynthetically. The biogenetic origin of the least common

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isocyanate has not been addressed experimentally. The coexistence of **1**–**3**, each of which bears the identical structural scaffold and differs from each other only at the nitrogenous substitutions on C-8, extends the biosynthetic connection from the isonitrile and isothiocyanate to the isocyanate, presumably through peroxidase in a related manner to that mentioned by Simpson and Garson.[7b] The presence of the bifunctionalized diterpenes possessing a formamide group along with other nitrogenous functionalities either indicates that the formamide functional group genuinely exists in the sponge or is merely evidence for the chemical transformation from a precursor that is more sterically accessible at C-15 on the side chain rather than on the sterically hindered C-8.

Sponges of the genus *Stylissa* from various collecting sites have been reported as sources of biologically active metabolites, among which cyclic peptides[9] and bromoimidazole and bromopyrrole alkaloids[10] were common. However, to our knowledge, the report by Mitome et al. on the Okinawan specimen was the one that focused on the isonitrile-containing amphilectane diterpenes and related derivatives isolated from this genus.[3] Bearing the same skeletons, diterpenes isolated from the Thai specimen reported here and in our previous work[6] have extended the range of nitrogenous diterpenes from the *Stylissa* sponges toward multifunctionalized derivatives.

The antimalarial activities of all four isolated compounds were assessed to confirm that the isonitrile functionality of **3**, through the linear nonbonded sp orbital, is the most crucial for such bioactivity (IC_{50} 0.52 μM). The isocyanate- and isothiocyanate-containing compounds, although presumably reacting through their linear functionalities but with the less active lone-pair electrons, were approximately 10-fold less effective (IC_{50}'s 8.85 and 8.07 μM for **1** and **2**, respectively). Compound **4**, with only a single formamide group, was inactive at the highest concentration tested (10 mg/mL). Such observations agreed well with a previous report by Wright and Lang-Unnasch[4b] and complemented the heme-isonitrile coordination mechanism also proposed by Wright et al.[5d] Cytotoxicity against MCF-7 breast adenocarcinoma cells was also examined. All of the isolated compounds were inactive, inhibiting less than 50% of the tested cells at the highest concentration of 25 mg/mL.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were obtained from a Shimadzu UV-160A spectrophotometer, and IR spectra were from a Jasco IR-810 spectrophotometer. ^{1}H and ^{13}C NMR experiments were performed on an FT-NMR Varian Unity Inova 500 spectrometer, using C_6D_6 as an operating solvent. The chemical shifts were recorded referencing the signals of residual C_6HD_5 (7.15 ppm) for ^{1}H and of C_6D_6 (128 ppm) for ^{13}C as an internal standard. Mass spectra were obtained from a Thermo-Finnigan MAT 95 XL mass spectrometer. HPLC separations were perform on a Water 600E multisolvent delivery system, connected to a Water 484 tunable UV detector, a Rheodyne 7125 injector port, and a Jasco 807-IT integrator.

Animal Materials. The sponge specimen investigated in this work was identified to be *Stylissa* cf. *massa* (Carter), family Dictyonellidae (van Soest, Diaz & Pomponi, 1990), by Dr. Sumaitt Putchakarn, Marine Science Institute, Burapha University, Chonburi, Thailand. The specimen was assigned to the genus *Ciocalapata* in our previous report;[6] however, re-examination of the specimen led to the revised identification as reported here. The specimen was collected by means of scuba from the vicinity of Koh-Tao, Surat-Thani Province, Thailand (10°7.569′ N, 99°48.665′ E), at the depth of 15–20 m, in April 2002. Morphologically, the specimen is described as an irregular-shaped, encrusting colonial sponge. The external color upon surfacing is purplish maroon, with pale khaki inner color. The specimen turned white-brown in preserving spirits. For extended microscopic descriptions, including remarks on deviations from curated specimens lodged at Marine Science Institute, Burapha University, see Supporting Information (p S8).

The specimen was preserved in an ice-chest (0 °C) upon surfacing and at −20 °C in the lab. The voucher specimens are lodged at Marine Science Institute, Burapha University, Chonburi, Thailand (BIMS-I2001), and at Marine Natural Products Research Unit, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand (AP02-006-02).

Extraction and Isolation. The sponge specimen was freeze-dried (279 g) and extracted exhaustively with hexane, CH_2Cl_2, and MeOH, to yield extracts from each solvent of 7, 6, and 63 g, respectively. The fractionation of the antimalarial-active hexane fraction (IC_{50} 0.05 mg/mL) led to a series of fractional pools, from which the less polar fractions were investigated and reported by us.[6] Here, the more polar fraction from the pool (723 mg) was successively chromatographed over Sephadex LH20 (EtOAc/hexane, 1:1) and SiO_2 (two steps; EtOAc/hexane, 1:1, then EtOAc/hexane/THF, 2:8:1). Finally, separation using reversed-phase HPLC (VertiSep C_{18}; 10 × 250 mm, 10 μm; CH_3CN/H_2O, 98:2; flow rate 3.0 mL/min) consecutively yielded compounds **3** (7 mg; t_R 10.8 min), **1** (4 mg; t_R 13.9 min), **2** (4 mg; t_R 15.4 min), and **4** (3 mg; t_R 16.6 min) in a single step.

8-Isocyanato-15-formamidoamphlect-11(20)-ene (1): yellow oil; $[\alpha]_D$ −17 (*c* 0.28, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (log ε) 252 (2.00) nm; IR (thin film) ν 3280, 2250, 1670 cm^{-1}; ^{1}H and ^{13}C NMR see Table 1; ESIMS *m*/*z* (% relative intensity) 381 ([M + Na]$^+$, 35), 359 ([M + H]$^+$, 100); HRESIMS *m*/*z* 359.2713 (calcd for $C_{22}H_{35}N_2O_2$, 359.2690).

8-Isothiocyanato-15-formamidoamphlect-11(20)-ene (2): yellow oil; $[\alpha]_D$ −23 (*c* 0.19, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (log ε) 250 (2.12) nm; IR (thin film) ν 3280, 2075, 1670 cm^{-1}; ^{1}H and ^{13}C NMR see Table 1; ESIMS *m*/*z* (% relative intensity) 397 ([M + Na]$^+$, 12), 375 ([M + H]$^+$, 100); HRESIMS *m*/*z* 375.2474 (calcd for $C_{22}H_{35}N_2OS$ 375.2472).

8-Isocyano-15-formamidoamphilect-11(20)-ene (3): $[\alpha]_D$ −49 (*c* 0.075, CH_2Cl_2); lit. −24 (*c* 1.0, $CHCl_3$).[1]

7-Formamidoamphilect-11(20),15-diene (4): $[\alpha]_D$ +13 (*c* 0.12, CH_2Cl_2); lit. +15.5 (*c* 0.22 $CHCl_3$).[4b]

Antimalarial Activity. The antimalarial activity was determined by the Central Bioassay Lab, BIOTEC, Thailand. The assay was performed using the microculture radioisotope techniques,[11] targeting *Plasmodium falciparum* K1 as the tested parasite. Dihydroartemisinin was used as a reference (IC_{50} 3.8 nM).

Cytotoxicity. The cytotoxicity determination utilized an assay protocol modified from the sulphorhodamine B assay techniques,[12] targeting MCF-7 breast adenocarcinoma cell lines. Camptothecin was used as a reference (IC_{50} 1.6 nM).

ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR spectra of **1** and **2**, spectroscopic data of **3** and **4**, taxonomic description of the investigated specimen. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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