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Marine sponge *Hymeniacidon* sp. amphilectane metabolites potently inhibit rat brain microglia thromboxane B₂ generation

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Abstract

The effects of five *Hymeniacidon* sp. amphilectane metabolites (**1–5**) and two semi-synthetic analogs (**6** and **7**) on thromboxane B₂ (TXB₂) and superoxide anion (O₂⁻) generation from *E. coli* LPS-activated rat brain microglia were investigated. All *Hymeniacidon* sp. metabolites and analogs potently inhibited TXB₂ (IC₅₀ = 0.20–4.69 μM) with low lactate dehydrogenase release and minimal mitochondrial dehydrogenase inhibition. While a lack of O₂⁻ inhibition would suggest that *Hymeniacidon* sp. metabolites and derivatives inhibit TXB₂ synthesis by a cyclooxygenase-dependent mechanism, their pharmacologic potency and limited *in vitro* cytotoxicity warrants further investigation to develop them as lead compounds to modulate enhanced TBX₂ release by activated microglia in neuroinflammatory disorders.

Keywords

marine; diterpene; microglia; neuroinflammation; thromboxane B2

1. Introduction

Neuroinflammation, a complex disease process involved in the pathology of several human brain diseases, including Alzheimer's and Parkinson's disease, multiple sclerosis, ischemic stroke, HIV-dementia, trauma and infections, has been hypothesized to involve activated microglia.¹ Microglia are central nervous system mononuclear phagocytes which respond to neuropathological conditions by becoming activated and releasing large amounts of proinflammatory and potentially neurotoxic mediators, i.e. proteolytic enzymes, reactive oxygen intermediates, cytokines and eicosanoids.² Increased release of the superoxide anion (O₂⁻) and the eicosanoid thromboxane A₂ (TXA₂), an arachidonic acid metabolite which is a vasoconstrictor and platelet aggregator³, have been observed in activated microglia⁴ as well as in neural trauma⁵ and neuroinflammation.⁶ Thus, the reduction or modulation of

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Supplementary material Copies of the NMR spectra (¹H and ¹³C) of compounds **1–7** and Figure 2 can be found in the online version.

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excessive O_2^- and TXB_2 generation by activated microglia has been hypothesized to contribute to the treatment and resolution of neuroinflammatory disorders.² Natural products isolated from marine sponges, in particular those diterpenes based on the amphilectane carbon skeleton bearing an isocyanide functionality, have been identified as potentially useful antimalarial metabolites.⁷ An increasing number of such molecules have also been reported to express antimicrobial,⁸ antifungal,⁹ cytotoxic,¹⁰ and antifouling activity.¹¹ Amphilectane-based diterpene isocyanides, on the other hand, which are typically isolated from sponges belonging to the order Halichondrida, are yet to be investigated as anti-inflammatory agents.

2. Results and discussion

The Caribbean marine sponge *Hymeniacidon* sp. was collected at Cabo Norte, Isla de Mona, at a depth of 89 feet during the summer of 2006. After the frozen sponge specimen was lyophilized, it was blended with a 1:1 mixture of $CHCl_3$ -MeOH and filtered *in vacuo*. The crude extract was concentrated and stored under vacuum to yield an orange thick paste (28.2 g) that was partitioned with hexane (4×500 mL) against H_2O ($1 \times L$). Preliminary bioassay screenings of the crude extract showed strong biological activity against *Plasmodium falciparum* ($IC_{50} < 0.08 \mu\text{g/mL}$) and *Mycobacterium tuberculosis* ($MIC < 16 \mu\text{g/mL}$). The hexane extract was purified by normal phase Si gel column chromatography using a gradient of increasing polarity of hexane-EtOAc (2%–50%) to afford the known diterpene isocyanides 7-isocyano-11(20)-15(16)-amphilectadiene (**1**),¹² (-)-8,15-diisocyano-11(20)-amphilectene (**2**),¹³ 7,15-diisocyano-11(20)-amphilectene (**3**),¹² 8-isocyano-11(20)-ene-15-amphilectaformamide (**4**),¹³ and monamphilectine A (**5**).¹⁴

Compounds **1–5** were screened for anti-neuroinflammatory activity in *E. coli* lipopolysaccharide-activated rat brain microglia *in vitro*, specifically for thromboxane B_2 (TXB_2) and superoxide anion (O_2^-) generation inhibition. During the course of this investigation it became clear to us that isocyanide amphilectanes **2** and **3** are in fact potent anti-neuroinflammatory agents. In order to reveal the basic structural features required for these marine diterpenes to display their biological activity, we sought to modify the molecular structure of the more abundant compound **2**.

Specifically, we wanted to scrutinize the plausible structure-activity relationships of **2** by modifying the isocyanide groups at C-8 and C-15 as well as its exocyclic alkene functionality at C-11(20). Thus, we hydrolyzed the isocyanide groups of **2** to the corresponding formamides and, in a separate experiment, oxidatively cleaved the exocyclic double bond of **2** upon ozonolysis. The structures of derivatives **6** and **7**, which were prepared using well-established procedures as shown in Scheme 1, were unambiguously demonstrated by a combination of IR, NMR (1H and ^{13}C) and mass spectrometric measurements of the purified compounds. Inspection of the NMR data indicated that **7** actually consisted of an inseparable mixture of all of the four possible rotational isomers **7a–d** (Scheme 2).

Derivatives **6** and **7** along with natural products **1–5** were screened in order to determine the effect of these compounds on *E. coli* LPS-activated microglia TXB_2 and O_2^- generation *in vitro*. O_2^- and TXB_2 release, as well as short and long term cell viability, were assessed as described in Methods. As shown in Table 1 and supplementary Figure 2A, the results were as follows: all *Hymeniacidon* sp. metabolites and analogs prepared (**1–7**) potently inhibited TXB_2 generation ($IC_{50}=0.20$ – $4.69 \mu\text{M}$) but demonstrated minimal effect on O_2^- release ($IC_{50}>10 \mu\text{M}$). Furthermore the compounds showed low short-term toxicity ($LDH_{50}>10 \mu\text{M}$, supplementary Figure 2A) and reduced long-term toxicity (WST-1 assay, supplemental Figure 2B). Thus, in our *in vitro* experimental conditions, it appeared that

inhibition of microglia TXB₂ generation resulted from a pharmacologic rather than a toxic effect of compounds (**1-7**) on the microglia cells.

3. Conclusion

Comparison of the IC₅₀'s of closely related amphilectane diterpenes **2** (IC₅₀~0.23 μM) and **3** (IC₅₀~0.20 μM) supports the observation that the observed bioactivity is associated with presence of the two isonitrile groups. However, the amphilectane diterpenoid skeleton does play a significant role, as suggested by the comparison between the IC₅₀'s of these two compounds and derivative **7** (IC₅₀~3.14 μM), whereby each isonitrile group was replaced by a formamide functionality. Within the series of natural products **1-5**, analogs possessing an isocyanide functionality at C-15 display the highest anti-neuroinflammatory activity, and the presence of a second isocyanide moiety within the same amphilectane core seems to further potentiate its biological activity. However, substitution at C-15 with bulkier functional groups, as observed in compound **5**, appear to lower the activity. The presence of an exocyclic alkene at C-11(20) also seems to play a role in potentiating the observed pharmacological activity. Lack of O₂⁻ inhibition would appear to suggest that all *Hymeniacidon* sp. metabolites and their derivatives inhibit TXB₂ synthesis through a cyclooxygenase-dependent mechanism. In addition to their pharmacologic effects on enhanced TXB₂ generation and their reduced cytotoxic effects, it is perhaps of interest to note that the *Hymeniacidon* sp. compounds are more potent inhibitors of rat microglia TXB₂ generation than acetylsalicylic acid (aspirin) (IC₅₀= 3.12–10.0 μM)^{15,16} and flurbiprofen (apparent IC₅₀= 100 nM),¹⁷ which are two clinically used NSAIDs. Thus, taken together, our present results appear to support the hypothesis that marine *Hymeniacidon* sp. metabolites and derivatives could become lead compounds for the development of novel agents to modulate excessive release of TXB₂ by activated microglia cells in neuroinflammatory disorders. This possibility remains to be investigated in *in vivo* pharmacologic and toxicologic investigations with these compounds.

4. Methods

4.1. General methods

Optical rotations were obtained with an Autopol IV automatic polarimeter. Infrared spectra were obtained with a Nicolet Magna FT-IR 750 spectrometer. 1D- and 2D-NMR spectra were recorded with a Bruker DRX-500 FT-NMR spectrometer. Mass spectrometric data were generated at the Mass Spectrometry Laboratory of the University of Illinois at Urbana-Champaign. Column chromatography was performed using Si gel (35–75 mesh), and TLC analysis was carried out using glass pre-coated Si gel plates and the spots were visualized using a UV lamp at λ = 254 nm or by exposure to I₂ vapor. All solvents used were either spectral grade or were distilled from glass prior to use. The identification of **1-5** was accomplished through rigorous comparisons with the physical and chemical data previously reported for these compounds.¹²⁻¹⁴ The purity of each test compound was determined by NMR (¹H and ¹³C) and high resolution mass spectrometric analysis or HPLC (95% > purity; see Supporting Information).

4.2. Collection, extraction, and isolation

Fresh specimens of the sponge *Hymeniacidon* sp. (Phylum Porifera; Class Demospongiae) were collected by hand using SCUBA at a depth of 89 ft off Mona Island (Cabo Norte), Puerto Rico in July 2006. A voucher specimen (No. IM06-04) is stored at the Chemistry Department of the University of Puerto Rico, Río Piedras Campus. The organism was partially air dried, frozen, and lyophilized prior to its extraction. The dry sponge (200 g) was cut into small pieces and blended using a mixture of CHCl₃-MeOH (1:1) (4 × 1L). After

filtration, the crude extract was concentrated and stored under vacuum to yield an orange thick paste (28 g) that was suspended in H₂O (700 mL) and extracted with *n*-hexane (4 × 500 mL). The resulting hexane extract was concentrated *in vacuo* to yield 7.6 g of an orange oil that was purified by Si gel (170 g) column chromatography using a gradient of increasing polarity with *n*-hexane/EtOAc (98:2–1:1) as mobile phase and separated into 37 fractions on the basis of TLC and ¹H NMR analyses. Fraction 4 consisted of a colorless crystalline solid that was subsequently identified as known 7-isocyano-11(20)-15(16)-amphilectadiene (**1**) (10.2 mg, 0.005%).¹² Likewise, fraction 11 consisted of a colorless crystalline solid that was identified as known (–)-8,15-diisocyano-11(20)-amphilectene (**2**) (528 mg, 0.27%) after X-ray crystallographic analysis.¹³ Fractions 13 and 27 were subsequently identified as known compounds 7,15-diisocyano-11(20)-amphilectene (**3**) (188.6 mg, 0.09%) and 8-isocyano-11(20)-ene-15-amphilectaformamide (**4**) (204.3 mg, 0.10%), respectively.^{12,13} Fraction 36 (13.1 mg) was re-chromatographed over Si gel (1.0 g) with 20% EtOAc in *n*-hexane to afford pure monamphilectene A (**5**) (3.0 mg, 0.002%).¹⁴

4.3. Ozonolysis of (–)-8,15-diisocyano-11(20)-amphilectene (**2**)

Crystals of compound **2** (20 mg, 0.062 mmol) were dissolved in freshly distilled MeOH (20 mL) and the resulting solution was cooled to –78 °C and bubbled with O₂/O₃ for 2 min. The O₃ generator was turned off and O₂ was bubbled through the solution to remove excess ozone. The reaction was stirred for another 30 min before it was allowed to slowly warm up to 25 °C. After evaporation of the solvent, the amorphous solid left over was purified over a short column of Si gel (1.5 g) using 20% EtOAc/hexane to afford 15.6 mg (77%) of 8,15-diisocyano-11-amphilectone (**6**). Although this compound was prepared before by Ciavatta et. al.,¹² the physical and chemical properties of **6** were not described.

4.3.1. 8,15-Diisocyano-11-amphilectone (6**)**—White solid; [α]²⁰_D –20.3 (*c* 1.4, CHCl₃); IR (neat) ν_{max} 2968, 2925, 2870, 2127, 1714, 1460, 1444, 1371, 1166, 1149, 1116, 1087, 1060, 977, 914, 883 cm^{–1}; ¹H NMR (CDCl₃, 500 MHz) δ 2.66 (1H, td, *J* = 13.5 and 6.0 Hz, H-9a), 2.42 (1H, dd, *J* = 13.7 and 5.2 Hz, H-10a), 2.36 (1H, t, *J* = 11.3 Hz, H-12), 2.24 (1H, m, H-9b), 2.19 (1H, dt, *J* = 13.5 and 3.5 Hz, H-2a), 1.95 (1H, m, H-6a), 1.90 (1H, m, H-1), 1.51 (1H, m, H-10b), 1.49 (1H, m, H-6b), 1.47 (1H, m, H-14a), 1.44 (3H, s, H-16), 1.38 (1H, m, H-5a), 1.33 (3H, s, H-17), 1.31 (1H, m, H-7), 1.27 (1H, dd, *J* = 13.8 and 9.3 Hz, H-14b), 1.19 (1H, m, H-13), 1.11 (1H, qd, *J* = 10.7 and 3.2 Hz, H-4), 0.99 (1H, m, H-3), 0.96 (3H, d, *J* = 6.1 Hz, H-19), 0.82 (3H, d, *J* = 6.4 Hz, H-18), 0.75 (1H, m, H-2b), 0.74 (1H, m, H-5b); ¹³C NMR (CDCl₃, 125 MHz) δ 210.8 (C, C-11), 157.9 (C, t, *J* = 4.3 Hz, C-20), 154.2 (C, t, *J* = 4.6 Hz, C-21), 65.7 (C, t, *J* = 4.6 Hz, C-8), 56.5 (C, t, *J* = 4.6 Hz, C-15), 55.0 (CH, C-13), 53.5 (CH, C-12), 46.7 (CH₂, C-14), 43.3 (CH, C-4), 40.4 (CH, C-7), 40.3 (CH₂, C-2), 38.5 (CH₂, C-9), 38.2 (CH₂, C-10), 35.6 (CH, C-3), 32.2 (CH₃, C-17), 31.4 (CH, C-1), 29.7 (CH₂, C-6), 29.6 (CH₂, C-5), 28.2 (CH₃, C-16), 19.6 (CH₃, C-18), 15.8 (CH₃, C-19); HRESIMS *m/z* [M + Na]⁺ 349.2237 (calcd for C₂₁H₃₀N₂ONa, 349.2256). Recrystallization of **6** by slow evaporation from acetone gave crystals of excellent quality that were amenable to X-ray crystallographic analysis. The crystallographic data for **6** have been deposited at the Cambridge Crystallographic Data Centre, under the reference number CCDC 842072. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

4.4. Acid hydrolysis of (–)-8,15-diisocyano-11(20)-amphilectene (**2**)

Compound **2** (10 mg, 0.031 mmol) was dissolved in freshly distilled MeOH (10 mL), after which a few drops of glacial AcOH were added. The solution was stirred and allowed to stand at ~25 °C until all of the starting material disappeared on the basis of TLC analysis (24 h). Thereafter, the solvent was removed by vacuum distillation and the resulting solid

residue was purified by flash Si gel column chromatography with 5% MeOH(NH₃)/CHCl₃ to afford 7.2 mg (65%) of 11(20)-ene-8,15-amphilectodiformamide (**7**). This compound consisted of an inseparable mixture of four rotational isomers about the formamide moieties, i.e. **7a–d** (Scheme 2). Thus, a complete assignment of the ¹H and ¹³C NMR signals was not feasible.

4.4.1. 11(20)-ene-8,15-Amphilectodiformamide (7)—Colorless oil; [α]_D²⁰ −18.0 (*c* 1.0, CHCl₃); IR (neat) ν_{\max} 3294, 3080, 3055, 2964, 2935, 2916, 2856, 1676, 1531, 1456, 1384, 1317, 1267, 891, 754, 665 cm^{−1}; HRESIMS *m/z* [M + H]⁺ 383.2749 (calcd for C₂₂H₃₆N₂O₂Na 383.2674).

4.5 Anti-neuroinflammatory assay

The experimental protocol to study the effect of *Hymeniacidon* sp. metabolites and semisynthetic derivatives on microglia release of O₂[−] and TXB₂ was as follows. Rat neonatal microglia (2 × 10⁵ cells) were activated *in vitro* by seeding the cells into each well of 24-well flat-bottom culture clusters and stimulating them with *E. coli* lipopolysaccharide (LPS) (0.3 ng/mL) for 17 h in Dulbecco's modified Eagle medium + 10% fetal bovine serum + 120 U/mL penicillin + 12 μg/mL streptomycin in a humidified 5% CO₂ incubator at 35.9° C.² Media was then removed, microglia washed with warm (37° C) Hanks' balanced salt solution (HBSS) and then microglia pre-incubated with compounds **1–7** (0.01–10 μM) or vehicle (DMSO) for 15 min prior to stimulation with phorbol 12-myristate 13-acetate (PMA) (1 μM) for 70 min. All experimental treatments were run in triplicate and in a final volume of 1 mL. After PMA stimulation HBSS was aspirated from each well and O₂[−] determined via superoxide dismutase-inhibitable reduction of ferri-cytochrome C and TXB₂ by EIA as described.² Table 1 and supplementary Figure 2A show data for each compound from 2–4 representative experiments and is expressed as the compound's inhibitory concentration 50% [IC₅₀] for O₂[−] and TXB₂ generation.

4.6 Microglia cell viability assays

Short-term cell viability (90 min) was assessed by lactate dehydrogenase (LDH) release from microglia as described elsewhere.² Microglia LDH was expressed as percent of total LDH. Total LDH resulted from Triton X-100 (0.1%)-treated (and 100% lysed) microglia cells (intracellular LDH) plus LDH released to the extracellular medium. Table 1 and supplementary Figure 2A show data for each compound from 3–4 representative experiments. *Long-term* cell viability (2.5–19 h) was determined by the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]1,3-benzene disulfonate) colorimetric assay, based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]1,3-benzene disulfonate) (slightly red) to formazan (red) by mitochondrial dehydrogenases in viable cells.¹⁸ Briefly, rat neonatal microglia (10,000 cells/well) were plated in LPS-free 96-well cell culture clusters in 0.2 mL DMEM without FBS containing 120 U/mL P + 12 μg/mL S. Thereafter, each well received 2 μL of compounds **1–7** (0.1 μM–10 μM final concentration) or vehicle (DMSO) and plates were incubated in a humidified 5% CO₂ incubator at 36° C for 18 h. Thereafter, 20 μL of the tetrazolium salt WST-1 (Roche Diagnostics Indianapolis, IN) was added to wells, plates were incubated in a humidified 5% CO₂ incubator at 36° C for 2 h, and the reduction of the WST-1 reagent to formazan was measured at 450 nM with a reference at 620 nM, with a microtiter plate (ELISA) reader. Supplementary Figure 2B illustrates data for compounds **1–7** from one representative experiment in triplicate.

4.7 Statistical analysis of the data

Data were analyzed with the Prism[®] software package purchased from GraphPad (San Diego, CA.). One-way analysis of variance followed by Dunnett's test was performed on all sets of data. *Hymeniacidon* sp. metabolites-treated groups were compared with the vehicle-treated group, shown as 0 or control in the corresponding figures. Differences were considered statistically significant at $p < 0.05$ and reported in each figure legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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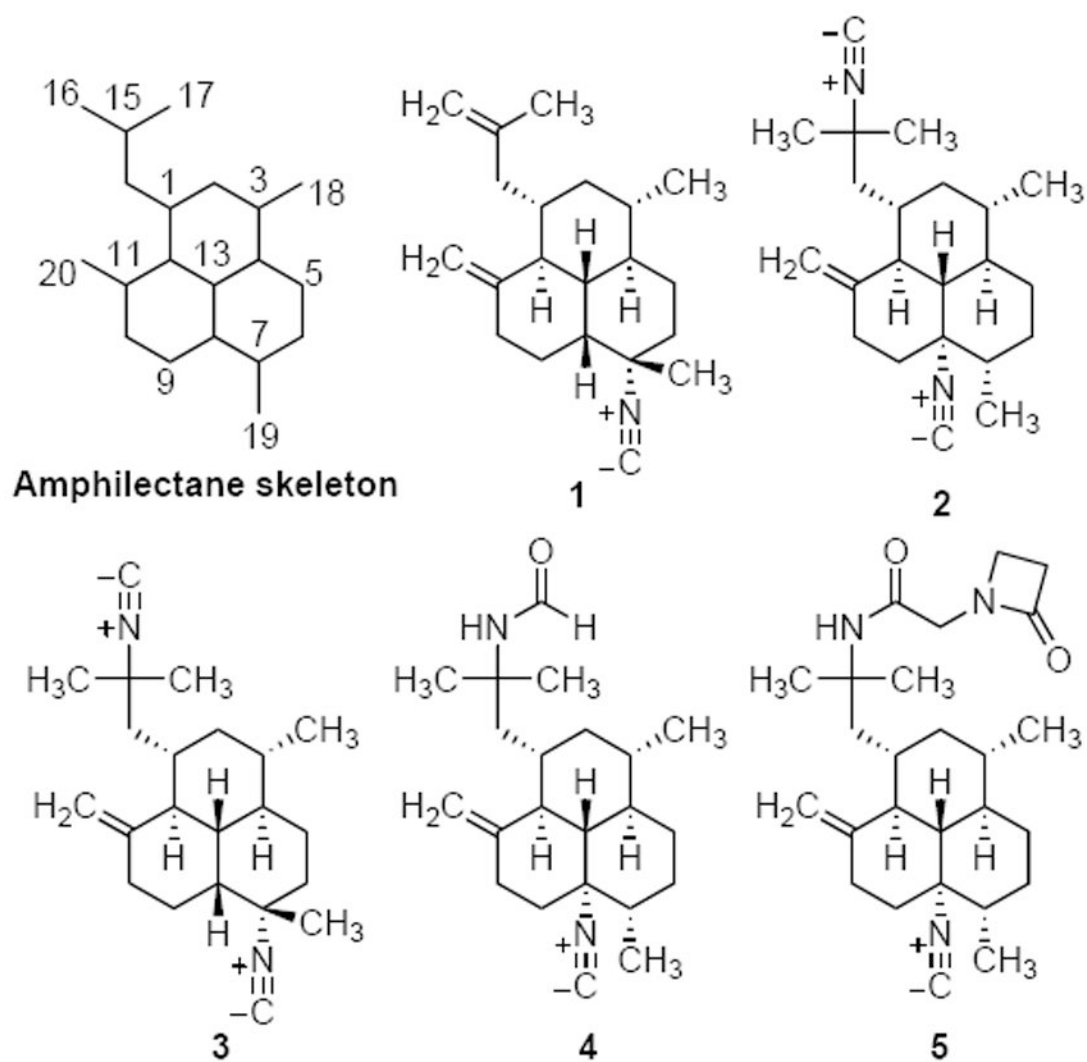
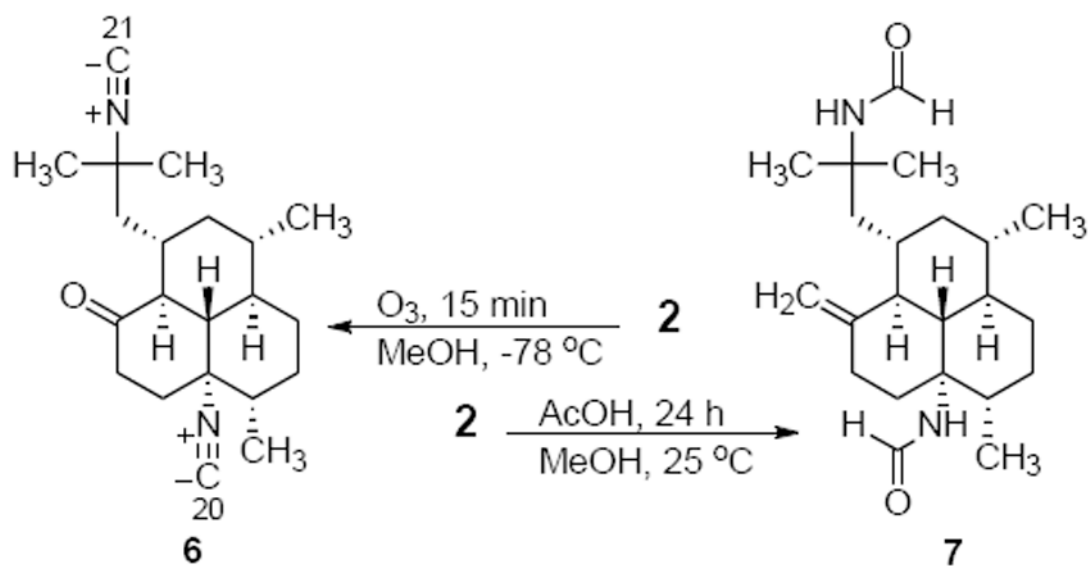
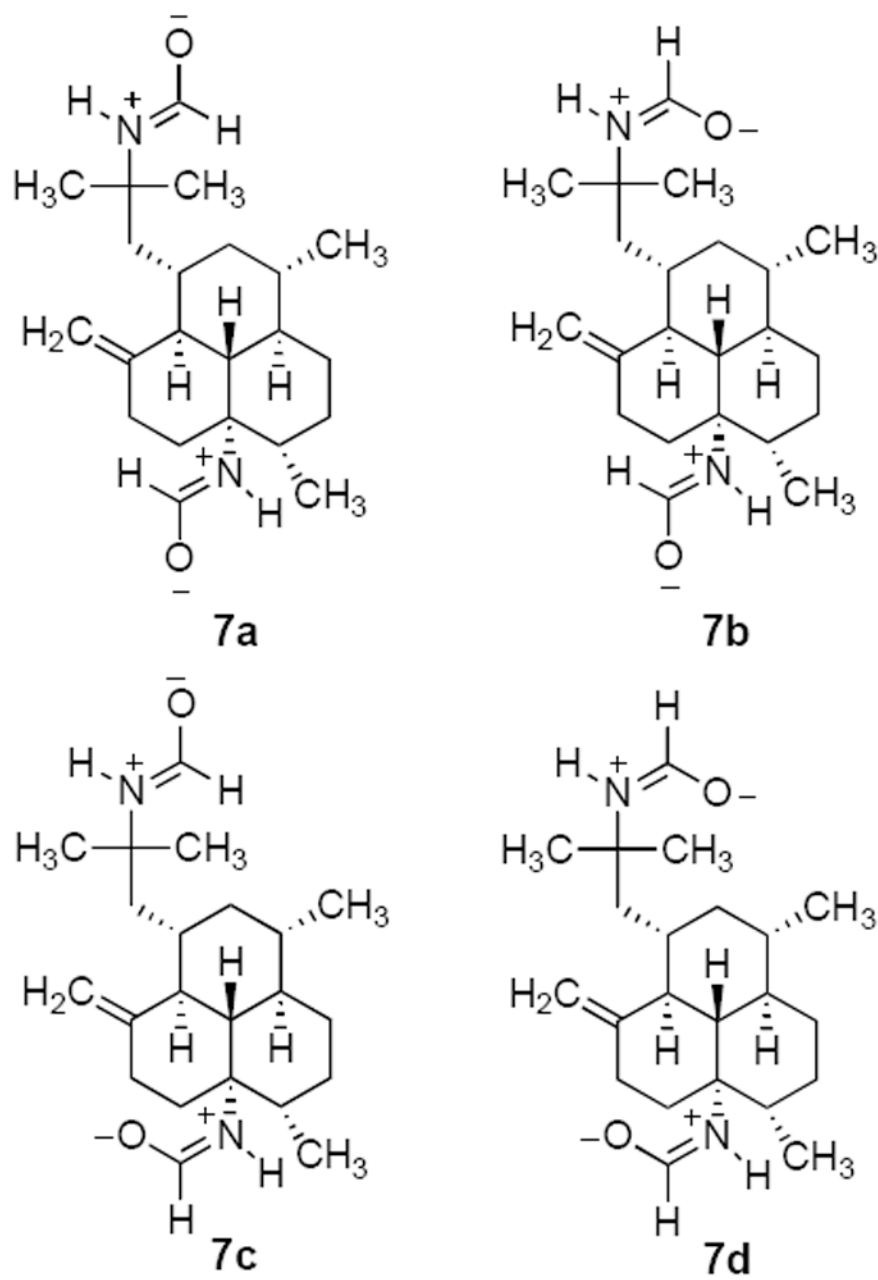


Figure 1.
Amphilectane-based isocyanides isolated from the Caribbean marine sponge *Hymeniacidon* sp.

**Scheme 1.**

Preparation of semi-synthetic analogs **6** and **7** from (-)-8-15-diisocyano-11(20)-amphilectene (**2**).

**Scheme 2.**

Structures for the four possible rotational isomers for compound **7** detected by NMR spectroscopy.

Table 1Anti-neuroinflammatory activity of *Hymeniacidon* sp. amphilectane metabolites^a

Compound	O ₂ ⁻ IC ₅₀ [μM]	TXB ₂ IC ₅₀ [μM]	LDH ₅₀ ^b [μM]
1	>10	1.72	>10
2	>10	0.23	>10
3	>10	0.20	>10
4	>10	1.48	>10
5	>10	4.69	>10
6	>10	1.43	>10
7	>10	3.14	>10

^aEffect on rat microglia PMA [1μM]-stimulated release of O₂⁻, TXB₂ and LDH. The anti-neuroinflammatory assay is described in Methods. Data corresponds to 2–4 independent experiments.

^bLDH₅₀ represents the concentration of the compound that caused 50% release of the total LDH content of microglia cells. LDH was measured as described in Methods.