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Antifungal Diterpene Alkaloids from the Caribbean Sponge *Agelas citrina*: Unified Configurational Assignments of Agelasidines and Agelasines

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Abstract

Three new diterpene alkaloids – the hypotaurocyamines, (–)-agelasidines E and F (**5–6**), and the adeninium salt, agelasine N (**9**) – were isolated from the Caribbean sponge *Agelas citrina* along with six known natural products agelasines B–E (**7, 10–12**), 2-oxo-agelasine B (**8**), and (–)-agelasidine C (**3**). The chemical structures of **5**, **6** and **9** were elucidated by analysis of NMR spectra and mass spectrometry. This represents the first report of natural products from the sponge *A. citrina*.

Unified assignment of absolute configurations of the new compounds and known compounds were achieved by chemical correlation, quantitative measurements of molar rotations, and comparative analysis by van't Hoff's principle of optical superposition. (–)-Agelasidine C (**3**) exhibited potent antifungal and modest cytotoxic activity against human chronic lymphocytic leukemia (CLL) cells.

Keywords

marine natural product 1; alkaloid; specific rotation; circular dichroism; diterpene

Introduction

A growing class of nitrogenous diterpenes that include co-occurring diterpenoid hypotaurocyamines (e.g. **1–4**) and diterpenoid *N*⁹-adeninium alkaloids from marine sponges of the genus *Agelas*, have been shown to exhibit a wide range of biological activities, including antineoplastic,^[1] antimicrobial,^[2] and Na⁺,K⁺-adenosine triphosphatase (ATPase) inhibitory properties.^[3] As a part of ongoing investigations into bioactive chemical constituents of Caribbean sponges, we explored the constituents of the potentially antifungal extracts of the marine sponge *Agelas citrina* which inhibited the growth of several strains of *Candida* and the pernicious pathogens *Cryptococcus* var *grubii* and *Cryptococcus* var *gattii*. Here we report the isolation and structure elucidation of three new and six known alkaloid–diterpenes – two hypotaurocyamine–diterpenes, agelasidines E–F (**5–6**), one *N*⁹-

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Dedicated to Professor Ernesto Fattorusso on the occasion of his 75th birthday

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methyladeninium–diterpene agelasine N (**9**) – in addition known compounds agelasines B–E (**7**, **10–12**),^[3] 2-oxo-agelasine B (**8**),^[4] and the (–)-agelasidine C (**3**)^[5] (Figure 1). Compounds **5**, **6** and **9** expand the observed range of new oxidation motifs in the diterpenoid scaffold of hypotaurocyamine and adeninium alkaloids, respectively, and are the first natural products reported from *A. citrina*, a species endemic to the Caribbean Sea. A unifying chiroptical analysis of the natural products, by application of van't Hoff's principle of optical superposition, completed assignment of their absolute configurations.

Results and Discussion

A MeOH extract of the Caribbean sponge *A. citrina* was subjected to solvent partitioning and chromatography to yield compounds **3**, **5–12** (Figure 1); representing the first natural products reported from *A. citrina*.

Compound (–)-**3** (HRESIMS $[M+H]^+$ m/z 424.2993, $C_{23}H_{41}N_3O_2S$) displayed 1H NMR chemical shifts identical to the first reported literature values for (+)-agelasidine C ((+)-**3**),^[2a] but the sign of the specific rotation ($[\alpha]^{25}_D -12$ (c 2.0, MeOH)) was opposite ($[\alpha]^{25}_D +8.5$ (c 2.0, MeOH)).^[2a] Conversion of the (–)-**3** into the corresponding 4,6-dimethylpyrimidine derivative by reaction of the guanidine (pentane-2,4-dione, pyridine, see Scheme S1, Supporting Information), followed by reductive ozonolysis, according to the procedure of Nakamura and coworkers^[2a] gave derivatives from (–)-**3** (Supporting Information) that were enantiomeric with those obtained from (+)-**3**.^[2a] Consequently, the 5*R*,6*S* configuration was assigned to the sample of (–)-**3** obtained from *A. citrina*.

Mass spectrometric measurements of agelasidine E (**5**), isolated as colorless oil, gave an HRESIMS $[M+H]^+$ m/z of 440.2940, and suggested a molecular formula of $C_{23}H_{41}N_3O_3S$; one oxygen more than agelasines B–C (**2–3**). Analysis of the 1H and ^{13}C NMR spectra of **5** (Table S3) identified a terminal methylene at H₂-15 (δ_H 5.50, 5.57) and a quaternary C-13 (δ_C 68.9), and supported the branched hypotaurocyamine moiety similar to **2**. Lack of one of the methyl singlets and appearance of low-field diastereotopic methylene proton signals (δ_H 3.97, 4.07) was consistent with an oxidation product of **2** formed the replacement of a CH₃ group with a CH₂OH group. The location of the OH group in **5** was secured through a detailed examination of TOCSY and HMBC spectra (Figure 2). Interpretation of collective long-range 1H – ^{13}C correlations from H₂-16 to C-3 (δ_C 124.7) and C-4 (δ_C 143.6); H₃-18 (δ_H 0.95) to C-4, C-5 (δ_C 40.4), C-6 (δ_C 34.4), and C-7 (δ_C 36.1); and H-3 (δ_H 5.79) to C-2 (δ_C 25.7) and C-5 supported the placement of the hydroxyl group at C-16 (δ_C 63.1).

Unlike **5**, which showed no absorption in the UV-vis spectrum above 210 nm, agelasidine F (**6**) contained a chromophore (λ_{max} 240 nm). Analysis of HRESIMS data ($[M+H]^+$ m/z 438.2786, $C_{23}H_{39}N_3O_3S$) and 1H NMR signals of **6** (Table S3) suggested the presence of a conjugated olefin associated with a new downfield proton signal (δ_H 9.32, s) that was readily assigned to an α,β -unsaturated aldehyde (δ_C 196.8). Differences in the NMR chemical shifts between **5** and **6** near the cyclohexene ring (Table S3) and similarities of the remaining COSY and HMBC correlations allowed placement of the CH=O group at C-16.

Stereochemical assignments at C-13 for **5–6** were made by chemical degradation and comparison of the products with known compounds (Scheme 1a). Ozonolysis of either **5** or **6** (O_3 , –78 °C, MeOH), followed by reductive workup ($NaBH_4$) and acetylation (Ac_2O , pyridine), gave the same tetraacetyl derivative (+)-**13** ($[\alpha]^{25}_D +6.7$ (c 0.2, $CHCl_3$)), reported earlier from degradation of **2**.^[2a] Consequently, both **5** and **6** are 13*R*.

The C5, C6 configurations in the cyclohexene segments of **5** and **6** were solved independently by global estimations of molar rotation, $[\phi]$ by employing the principle of

optical superposition, first proposed by van't Hoff^[12] based on canonical observations by Le Bel. The power of the method has been nicely demonstrated by Wipf and Beratan in applications to natural product assignments of absolute and relative configurations of relatively complex natural products.^{[13a,c],[14]} In its simplest interpretation, the molar rotation is the summation of independent atomic contributions to optical rotatory power – both positive and negative angles – from isolated centers of asymmetry, which, in turn, are dependent upon polarizability. Molar rotations can be calculated by DFT methods,^[13b] but it is not trivial and requires formidable computational power. More conveniently, global contributions to molar rotation $[\phi]_D$, measured at the sodium D-line emission, can be approximated from the measured molar rotations of simpler analogs (derived from the $[\alpha]_D$ according to the simple Eq. 1, where M is the molar mass), each containing isolated chiral molecular segments. In practice, this empirical approach is valid and van't Hoff's rule can be applied if stereocenters are separated by one or more methylene units and non-bonded interactions are negligible.”^[13a]

$$[\phi]_D = \frac{[\alpha]_D \times M}{100} \quad (\text{Eq. 1})$$

In order to test the applicability of the method in the context of **5** and **6**, we calculated the values of all four stereoisomers of agelasidine B (**2**) (Table 1, entries 6–9) by superposition of combinations of both enantiomers of **1** and **3** and compared them to measured value (Table 1, entry 2, $[\phi]_D = -11.5 \text{ deg. mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$). A good fit of the calculated value (Table 1, entry 9, $[\phi]_D = -19.7$) was obtained only for the known (5*S*,6*R*,13*R*) configuration of (–)-**2**, independently derived by Nakamura using chemical degradation.^[2a]

The method was applied next to agelasidine E [(–)-**5**] by combinations of $[\phi]_D$ for **1** and **15** (Figure 1, $[\alpha]_D -8.2$) prepared in optically pure form reported by Paquette and coworkers as an intermediate in the synthesis of (+)-cleomeolide A.^[15] Again, the best fit for the measured molar rotation of (–)-**5** ($[\phi]_D = -44.3$) was obtained for the calculated value (**5d**, $[\phi]_D = -56.3$) of the (5*S*,6*R*,13*R*) configuration of the natural product. This also matches agelasidine F [(–)-**6**], ($[\phi]_D = -59.7$), although, strictly, the molecular structure comparison here is not as valid as with (–)-**5**. In any case, as co-occurrence of (–)-**5** with (–)-**6** likely results from the latter being the oxidation product of the former; both are expected to share the same absolute configuration. Comparisons of $[\phi]_D$ using a different synthetic analog **14**^[15a] and a synthetic compound^[15b] lacking the allylic OH group (*c.f.* (–)-**3**) gave a poor fit ($[\phi]_D = -9.4$ and $+1.8$, respectively, see Table S1, Supporting Information). Despite the levorotatory nature of (–)-**3**, (–)-**5** and (–)-**6** obtained from the same sample of *Agelas citrina*, the C5, C6 configurations of the latter two are *opposite* to the former, which reveals the dominant rotatory power of the allylic C-13 quaternary hypotaurocyamine group. These examples serve to illustrate the power of optical superposition and molar rotations^[13] and underscore a reminder of the inherent risk of over-reliance on the sign of $[\alpha]_D$ alone.^[4]

Agelasine N (**9**), an amorphous white solid, revealed a formula of C₂₆H₄₂N₅O from HRESIMS data ($[\text{M}+\text{H}]^+ m/z 440.3386$) and upon comparison with those of the known agelasines **7**, **10–12**, indicated the addition of H₂O. The ¹H NMR data of **9** were consistent with a hydroxy-substituted clerodane ring system. HMBC correlations from both H₃-17 (δ_{H} 0.94) and H₃-19 (δ_{H} 0.89) to C-10 (δ_{C} 80.6) supported a tertiary alcohol by placement of the OH group at C-10 (Figure 2). NOE correlations of **9** allowed assignment of the relative configuration at all stereogenic centers in a *trans*-fused clerodane bicyclic ring system through observation of *syn*-facial NOEs (Scheme 1b). The paucity of available sample (~0.5 mg, **9**) precluded determination of absolute configuration; consequently the depicted configuration of **9** is arbitrary.

The reported absolute configurations of agelasines A–F were determined through step-wise chemical degradation, followed by comparison of the products to known compounds by optical rotation and CD.^[3] Total syntheses of agelasines A,^[6] B,^[7] D,^[8] and E^[9] supported the assignments, or – in the case of synthesis of agelasine C (**10**)^[10] – lead to revision of the originally assigned absolute configuration. Subsequent configurational assignments of new agelasines were heavily reliant upon comparisons of $[\alpha]_D$ with those of known agelasines (Table 2), despite significant structural differences, including variable counterions (HCO_2^- , CF_3CO_2^- , Cl^-) that result from purification of quaternary *N*⁹-methyl adeninium or guanidinium salts under different protocols, or use of different solvents for optical rotation measurements.^[4,11] The inherent danger of mis-assignment of absolute configuration through reliance on optical rotation alone, is well-known, but may be compounded further by variables in measurement of α , especially highly charged or polar compounds. For example, the $[\alpha]_D$ of sceptrin, a pyrrole-aminoimidazole alkaloid from several different *Agelas* spp., was shown to be highly variable and dependent – both in sign and magnitude – on concentration and, particularly, on the nature of the counterion.^[16]

In order to resolve lingering concerns of assignments of configuration in the agelasine/agelasidine family of adeninium alkaloids based on optical activity, we undertook measurements of $[\alpha]_D$ of (–)-agelasine B (**7**) and (–)-agelasidine C (**3**) after ion exchange with Cl^- or HCOO^- while using a fixed concentration, *c*, close to the literature reported values,^[2,3] or varying *c* over a range of values (Supporting Information). Neither the counter ion (Cl^- , HCOO^-) or variation of *c* across the range 0.03–3.0 g/100 mL appeared to affect the sign or magnitude of $[\alpha]_D$, within experimental error (Table S2).

Nevertheless, an examination of the reported specific rotations of the same compound (Table 2) isolated from different *Agelas* specimens from different geographic locations reveals variable optical activity.

Agelasines B (**7**), C (**10**), and 2-oxo-agelasine B (**8**) from *Agelas citrina* displayed specific rotations consistent with earlier reports;^[3a,4] however, agelasines D (**11**) and E (**12**) were both opposite in sign with different magnitudes (Table 2, entries 7/8 and 9/10, respectively), and consequently, are antipodal to the originally reported natural products. It is also of interest to note that the $[\alpha]_D$ of (–)-agelasidine C (**3**) originally reported from *A. clathrodes*,^[5] has approximately half the magnitude of our observed value for (–)-**3** (entries 22 and 23, respectively) suggesting, not only the occurrence of enantiomeric modifications from different geographically disparate sources, but the possibility of heterogeneous composition (% ee) as non-racemic mixtures of enantiomers.

However, enantiomeric heterogeneity is still rare among marine natural products. The origins of optical purity in marine natural products have been the subject of discussion and speculation.^{[17],[18]} One explanation for biosynthesis of antipodal C5, C6 configurations between (–)-**3** and (–)-**5/6** is that both enantiomers of **3** may be formed with less stringent asymmetric control (a promiscuous terpene cyclase, or two independent cyclases, as seen with independent α - and β -pinene biosynthesis in *Salvia officinalis*^[19]) and that **5** and **6** arise through an independent oxidoreductase that effects kinetic resolution during allylic hydroxylation under more stringent control.

Natural products **3**, **5–8**, **10**, **12** were tested for activity against the fungal pathogen *Candida albicans* and human chronic lymphocytic leukemia (CLL) cells. (–)-Agelasidine C (**3**) exhibited modest cytotoxicity against CLL (IC₅₀ value of 10 3M) and potent antifungal activity (MIC of 0.5 $\mu\text{g/mL}$). Other agelasidines showed less potent antifungal activity (**5**, 8.0 and **6**, 4.0 $\mu\text{g/mL}$) while the other compounds were less active (**8** and **10** >32; **12**, 16.0 $\mu\text{g/mL}$).

Conclusions

In conclusion, we describe three new alkaloid–diterpenes, (–)-agelasidines E–F (**5–6**) and (+)-agelasine N (**9**), providing the first report of natural products from the Caribbean sponge *Agelas citrina*. Experimental evidence suggests counter ion and *c* have negligible effects on sign and magnitude of $[\alpha]_D$ for members in this family of nitrogenous diterpenoid natural products.

Experimental Section

General Experimental Procedures

General experimental procedures are described elsewhere.^[17a]

(–)-**Agelasidine C (3)**: pale yellow oil; $[\alpha]_D^{25} -12$ (*c* 2.0, MeOH); FTIR (ATR, ZnSe) $\nu_{\max}$ 3234 (br), 2966, 2935, 1633, 1572, 1450, 1380, 1297, 1120 cm^{-1} ; HRESIMS $[\text{M}+\text{H}]^+ m/z$ 424.2993 (calcd for $\text{C}_{23}\text{H}_{42}\text{N}_3\text{O}_2\text{S}$ 424.2998); NMR spectroscopic data are in agreement with the literature;^[2a,5] $[\alpha]_D$ is opposite in sign to the enantiomer reported by Nakamura and coworkers from *Agelas mauritiana*,^[2a] but the same as that reported by Rodriguez^[5] from a sample of *A. clathrodes* (see Table 2).

(–)-**Agelasidine E (5)**: colorless oil; $[\alpha]_D^{25} -9.3$ (*c* 1.0, MeOH); FTIR (ATR, ZnSe) $\nu_{\max}$ 3350 (br), 3173 (br), 2958, 2928, 1672, 1633, 1458, 1373, 1281, 1135, 1082, 1005 cm^{-1} ; NMR spectroscopic data, see Supporting Information; HRESIMS $[\text{M}+\text{H}]^+ m/z$ 440.2940 (calcd for $\text{C}_{23}\text{H}_{42}\text{N}_3\text{O}_3\text{S}$ 440.2941).

(–)-**Agelasidine F (6)**: colorless oil; $[\alpha]_D^{25} -12.6$ (*c* 2.0, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 229 (4.60), 273 (3.60) nm; CD (MeOH) λ 320 ($\Delta\epsilon$ +0.78), λ 220 ($\Delta\epsilon$ –4.4); FTIR (ATR, ZnSe) $\nu_{\max}$ 3350 (br), 3165 (br), 2965, 2928, 1680, 1625, 1572, 1458, 1373, 1343, 1289, 1135 cm^{-1} ; HRESIMS $[\text{M}+\text{H}]^+ m/z$ 438.2786 (calcd for $\text{C}_{23}\text{H}_{40}\text{N}_3\text{O}_3\text{S}$ 438.2785).

(–)-**Agelasine B (7)**: white amorphous powder; $[\alpha]_D^{25} -20$ (*c* 1.5, MeOH); NMR spectroscopic data are in agreement with the literature.^[3a]

(–)-**2-Oxo-agelasine B (8)**: pale yellow oil; $[\alpha]_D^{25} -7.4$ (*c* 0.5, MeOH); NMR spectroscopic data are in agreement with the literature.^[4]

(+)-**Agelasine N (9)**: amorphous white solid; $[\alpha]_D^{25} +5.6$ (*c* 0.5, MeOH); UV (MeOH) $\lambda_{\max}$ (ϵ) 210 (4.03), 273 (3.67) nm; FTIR (ATR, ZnSe) $\nu_{\max}$ 2952, 2925, 2357, 1658, 1586, 1462, 1384, 1344 cm^{-1} ; NMR spectroscopic data, see Supporting Information; HRESIMS $[\text{M}+\text{H}]^+ m/z$ 440.3386 (calcd for $\text{C}_{26}\text{H}_{43}\text{N}_5\text{O}$ 440.3389).

(–)-**Agelasine C (10)**: pale yellow oil; $[\alpha]_D^{25} -12$ (*c* 1.0, MeOH); NMR spectroscopic data are in agreement with the literature.^[3a]

(–)-**Agelasine D (11)**: amorphous solid; $[\alpha]_D^{25} -5.1$ (*c* 1.0, MeOH); NMR spectroscopic data are in agreement with the literature.^[3a]

(+)-**Agelasine E (12)**: amorphous solid; $[\alpha]_D^{25} +1.7$ (*c* 2.0, MeOH); NMR spectroscopic data are in agreement with the literature.^[3b]

Supporting Information (see footnote on the first page of this article): 1- and 2-D NMR spectra of **5**, **6** and **9**, along with key experimental details for degradation correlation of **3** and **5**.

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Figure 1. Agelasidines A–F (**1–6**); agelasines B (**7**), C (**10**), D (**11**), and E (**12**), (–)-2-oxo-agelasine B (**8**), and (+)-agelasine N (**9**); and synthetic compounds **14–15**.^[15a] Guanidinium groups are depicted as a free-base.

Figure 2. Key ^1H - ^1H COSY/TOCSY (bold line) and HMBC (arrow) correlations for agelasidine E (**5**) and agelasine N (**9**).

Scheme 1.

a) Chemical degradation of agelasidine E (**5**) to *R*-**13**^[2a]; b) Key NOEs (double-headed arrows) are shown for (+)-agelasine N (**9**).

Table 1

Measured and Calculated Molar Rotations^[a] $[\phi]_{\text{D}}$ ($\text{deg} \cdot \text{mol}^{-1} \text{dm}^3 \text{cm}^{-1}$) by van't Hoff's Principle of Optical Superposition.^{[12],[13]}

Entry	Compound	$[\phi]_{\text{D meas}}^{[b]}$	$[\phi]_{\text{D calcd}}^{[b]}$
1	(+)-(13 <i>S</i>)- 1	74.9	
2	(-)-(5 <i>S</i> ,6 <i>R</i> ,13 <i>R</i>)- 2	-11.5	
3	(-)-(5 <i>R</i> ,6 <i>S</i>)- 14 ^[c]	-15.9	
4	(-)-(5 <i>R</i> ,6 <i>S</i> ,13 <i>R</i>)- 5	-44.3	
5	(-)-(5 <i>R</i> ,6 <i>S</i> ,13 <i>R</i>)- 6	-59.7	
6	(5 <i>R</i> ,6 <i>S</i> ,13 <i>S</i>)- 2a		+19.7
7	(5 <i>R</i> ,6 <i>S</i> ,13 <i>R</i>)- 2b		-130.1
8	(5 <i>S</i> ,6 <i>R</i> ,13 <i>S</i>)- 2c		+130.1
9	(5 <i>S</i> ,6 <i>R</i> ,13 <i>R</i>)- 2d		-19.7
10	(5 <i>R</i> ,6 <i>S</i> ,13 <i>S</i>)- 5a		+56.3
11	(5 <i>R</i> ,6 <i>S</i> ,13 <i>R</i>)- 5b		-93.4
12	(5 <i>S</i> ,6 <i>R</i> ,13 <i>S</i>)- 5c		+93.4
13	(5 <i>S</i> ,6 <i>R</i> ,13 <i>R</i>)- 5d		-56.3

^[a] Calculated from $[\alpha]_{\text{D}}$ values (MeOH, see Table 2) of the corresponding Cl^- salts;

^[b] units: $\text{deg} \cdot \text{mol}^{-1} \text{dm}^3 \text{cm}^{-1}$

^[c] see Ref. [15a].

Table 2

[α]_D (MeOH) of salts of agelasines A–N and agelasidines A–F.

	Compound	[α] _D	Counterion	<i>c</i> (g/100 mL)	Ref
1	agelasine A	–31.3	Cl [–]	0.59	[3a]
2	agelasine B	(–) 7	Cl [–]	1.0	[3a]
3		(–) 7	HCO ₂ [–]	1.5	[a]
4	2-oxo- 7	(–) 8	HCO ₂ [–]	0.3	[4]
5			HCO ₂ [–]	0.5	[a]
6	agelasine C	(–) 10	Cl [–]	2.04	[3a]
7	agelasine D	(+) 11	Cl [–]	1.1	[3a]
8		(–) 11	HCO ₂ [–]	1.0	[a]
9	agelasine E	(–) 12	Cl [–]	1.88	[3b]
10		(+) 12	HCO ₂ [–]	1.7	[a]
11	agelasine F ^[b]	–5.5	Cl [–]	2.55	[3b]
12	agelasine G	–85	Cl [–]	0.02	[1]
13	agelasine H	–63.9	HCO ₂ [–]	0.36	[11a]
14	agelasine I	–2.5	HCO ₂ [–]	0.2	[11a]
15	agelasine J	+14	HCO ₂ [–]	0.46	[11b]
16	agelasine K	+60	HCO ₂ [–]	0.11	[11b]
17	agelasine L	–3.2	HCO ₂ [–]	1.0	[11b]
18	agelasine M	+208	HCO ₂ [–]	0.8	[4]
19	agelasine N	(+) 9	Cl [–]	0.5	[a]
20		(+) 9	HCO ₂ [–]	0.5	[a]
21	agelasidine A	(+) 1	Cl [–]	1.0	[2a]
22	agelasidine B	(–) 2	Cl [–]	0.43	[2a]
23	agelasidine C	(+) 3	Cl [–]	2.0	[2a]
24		(–) 3	Cl [–]	7.2	[5]
25		(–) 3	Cl [–]	2.0	[a]
26		(–) 3	HCO ₂ [–]	2.0	[a]

	Compound	$[\alpha]_D$	Counterion	c (g/100 mL)	Ref
27	agelasidine D	(-)- 4	Cl ⁻	2.75	[5]
28	agelasidine E	(-)- 5	Cl ⁻	1.0	[a]
29	agelasidine F	(-)- 6	HCO ₃ ⁻	2.0	[a]

[a] This report.

[b] ageline A