(–)-Agelasidine A from *Agelas clathrodes*

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(–)-Agelasidine A was identified from the methanol extract of the marine sponge *Agelas clathrodes* for the first time together with zooanemonin, 1-carboxymethylnicotinic acid, hymenidin, mukanadins A and C, monobromodispacamide, agelasidine D, 2-amide-4-bromopyrrole, *O*-methyltryptophan and an agelasines mixture. The structures were characterized by spectroscopic methods. (–)-Agelasidine A was tested for antibacterial and antifungal activities and shown to act as a bacteriostatic agent as it inhibited the growth of *Staphylococcus aureus* and partially the growth of other bacteria.

Key words: *Agelas clathrodes*, Hypotaurocyamine, (–)-Agelasidine A, Bacteriostatic Activity

Introduction

The compounds presently known from the marine sponge *Agelas clathrodes* have quite interesting structures namely containing adenine or hypotaurocyamine moieties (agelasine and agelasidine series), ceramide units (α-glycosphingolipids such as axiceramide A and B) and bromopyrrolic substructures (oroidin derivatives such as hymenidin).

The knowledge of *Agelas* metabolites structures has been the basis for further studies on chemotaxonomy (Braekman *et al*., 1992) and bioactivity. The later studies demonstrated that *Agelas* is an excellent source of novel biologically active compounds. *Agelas* metabolites proved to possess an inhibitory effect on the growth of microorganisms, contractile responses on smooth muscles and enzymatic reactions of Na,K-ATPase (Nakamura *et al*., 1985), and also showed potent and selective antagonistic *in vitro* activity against histaminergic receptors. (Cafieri *et al*., 1996).

Among the hypotaurocyamine compounds, the agelasidine A structure was published for the first time by Nakamura *et al*. (1983). This work along with others (Capon and Faulkner, 1983; Nakamura *et al*., 1985), that refer to agelasidine A, describes the metabolite as a dextrorotatory compound ($[\alpha]_D^{25} + 19.1°$ (*c* 1.0; methanol)). As a part of our ongoing search for bioactive substances from marine invertebrates we report the isolation of (–)-agelasidine A (**1**) from the methanol extract of *Agelas clathrodes* collected at Curaçao (Caribbean sea), together with zooanemonin, 1-carboxymethylnicotinic acid, hymenidin, mukanadins A and C, monobromodispacamide, agelasidine D, 2-amide-4-bromopyrrole, *O*-methyltryptophan and an agelasines mixture.

(–)-Agelasidine A was tested for its antifungal and antibacterial activities. From these bioassays (–)-agelasidine A showed no activity against fungi but proved to act as a bacteriostatic agent against *Staphylococcus aureus* and to be partially active against other bacteria.

Materials and Methods

Sponge material

A. clathrodes was collected at Curaçao (Caribbean sea) in 1998. A voucher specimen has been registered and incorporated in the collections of the Zoological Museum of the University of Amsterdam, The Netherlands (98/CU/MAY24/MK/143).

Extraction and isolation

After collection the sponge was preserved in methanol at 20 °C. The suspension was filtered.

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The sponge (12.71 g) was cut into small pieces and extracted successively with methanol and dichloromethane. The added solvents were evaporated under vacuum to obtain the extract (4.16 g) that was fractionated over Sephadex LH-20 to collect seven fractions *a*–*g*. From fraction *a* zooanemonin (398.2 mg) was identified. From fraction *b* 1-carboxymethylnicotinic acid (127.8 mg) was identified. Fraction *c* was composed of a mixture of agelasidines and agelasines (405.4 mg). Fraction *d* was mainly composed of hymenidin (278.4 mg). Fraction *e* was a mixture of mukanadins A and C, and monobromodispacamide (45.9 mg). Fraction *f* contained 2-amide-4-bromopyrrole (59.2 mg). Fraction *g* contained *O*-methyltryptophan (43.6 mg). The separation of agelasines from agelasidines was performed by flash chromatography over neutral alumina (I) using *n*-hexane/ethyl acetate/methanol mixtures as eluent resulting in the isolation of agelasines, (–)-agelasidine A (94.0 mg) and agelasidine D (21.4 mg).

(–)-Agelasidine A (**1**) was identified by its spectroscopic data (IR, UV, ^{1}H NMR, ^{13}C NMR, HMBC, HMQC, ESI-MS/MS) and by comparison with literature data of its enantiomer (Nakamura *et al*., 1983). The other compounds were also identified by their physical and spectroscopic data and comparison with literature data: zooanemonin (Hattori *et al*., 2001), 1-carboxymethylnicotinic acid (Matsunaga *et al.*, 1998), hymenidin (Kobayashi *et al*., 1986), mukanadins A and C (Uemoto *et al*., 1999), monobromodispacamide (Cafieri *et al*., 1996), agelasidine D (Nakamura *et al*., 1983), 2-amide-4-bromopyrrole (Braekman *et al*., 1992) and *O*-methyltryptophan (Ovenden and Capon, 1999).

Physical and spectroscopic measurements

FTIR spectra were recorded on a Perkin Elmer Spectrum 1725X apparatus. The UV spectra were recorded on a UV-Vis spectrophotometer Spectronic Helios Y. NMR spectra were recorded on a Bruker DRX 500 and on a Bruker ARX 400 instrument. The ^{1}H NMR and ^{13}C NMR spectra were recorded in CD_3OD and referenced to the methanol residual signal (δ 3.30 ppm and 49.0 ppm). Chemical shifts are presented as δ values in ppm. ESI-MS/MS were recorded on a Bruker Esquire 3000 apparatus and the HR mass spectrum (ESI positive) was recorded on a VG Autospec. M. instrument. Sephadex LH-20 (Fluka, Buchs SG, Switzerland) and neutral alumina (I) (Merck, Darmstadt, Germany) were used for chromatography separations.

*(–)-Agelasidine A (***1***)*

Colourless oil (94.0 mg). – $[\alpha]_D^{20}$ –14.5° (*c* 1.55; methanol). – FTIR (NaCl): υ_{max} = 3359, 3188, 1633, 1460, 1373, 1129, 945 cm^{-1}. – UV (MeOH): $\lambda_{max} < 200$ nm. – ^{1}H NMR and ^{13}C NMR: see Table I. – ESI-MS/MS: *m/z* = 356 (100) $[M+1]^+$, 152 (36). – HRESIMS $[M+1]^+$: *m/z* = 356.22749 (calcd. for $C_{18}H_{33}N_3O_2S$: 355.22935).

Microorganisms and culture media

The microorganisms are from the Culture Collection of Industrial Microbiology of the Laboratory of Industrial Microbiology (INETI, Lisbon, Portugal). The set of the test bacteria was: *Bacillus subtilis* CCMI 355, *Escherichia coli* CCMI 270, *Mycobacterium smegmatis* CCMI 690, *Salmonella enteritides* CCMI 859, *Staphylococcus aureus* CCMI 335, *Streptococcus faecium* CCMI 338, *Streptococcus mutans* CCMI 1022. The yeast was: *Candida albicans* CCMI 209. The filamentous fungi were: *Cladosporium cucumerinum* CCMI 206, *Trichoderma harzianum* CCMI 783, *Trichoderma koningii* CCMI 877, *Trichoderma koningii* CCMI 304. Brain Heart Infusion (Merck, Darmstadt, Germany) was used to grow bacteria and malt extract agar (Merck) was used for fungi.

Qualitative assay

The disc diffusion method was performed according to Gadd (1989). 1 ml of a 10^8 cfu ml^{-1} microorganism suspension was incorporated to the molten culture medium at 45 °C in Petri dishes. Bacteria cell counting took place in a spectrophotometer UNICAM 8700 at 620 nm. The fungi cells were counted in a hematocytometer. Filter paper discs (Macherey-Nagel, Durin, Germany) were impregnated with 0.02 ml solution containing 0.1 mg of the test compounds and distributed on the solidified culture medium. After 24–48 h incubation, the plates were observed for antimicrobial activity indicated by the formation of a clear zone around the discs, measured in mm. 5-Fluorocytosine (Sigma, NJ, USA), was used as a positive control to *C. albicans*. Rifampicin (Sigma) was used as a positive control for bacteria and carbendazim® for filamentous fungi. The positive controls were tested at 0.020 mg/disc.

Quantitative bioassay

The minimum inhibitory concentration (MIC) of the active compounds was determined by the broth dilution method (Muroi and Kubo, 1996). A 10^5 cfu ml^{-1} suspension was obtained by measurement of the cell concentration in a spectrophotometer (UNICAM 8700) at 620 nm and successive dilutions in saline solution. The following concentrations ($\mu g\ ml^{-1}$) were tested: 200, 100, 50, 25, 12.5. After incubation for 24 h at 30–37 °C the microbial growth was examined and the MIC was defined as the lowest concentration of the test compound yielding no visible growth. The MIC of each compound was determined in duplicate. Samples with no visible growth were inoculated into the culture medium in order to detect bactericidal/bacteriostatic activity. Rifampicin (Sigma) was used as a positive control.

Results and Discussion

(–)-Agelasidine A (**1**) isolated from *A. clathrodes*, $C_{18}H_{33}N_3O_2S$ (*m/z* 356 $[M+1]^+$ in ESI-MS/MS, and confirmed by HRESIMS) was obtained as a colorless viscous oil. The ^1H NMR spectrum of **1** (see Table I) exhibited four methyl signals at δ 1.66 (d, $J_{12,10}$ = 1.1 Hz, 3H), δ 1.59 (sl, 6H) and δ 1.52 (sl, 3H), two characteristic signals of a terminal methylene group of a monosubstituted double bond at δ 5.57 (d, $J_{1A,2}$ = 10.7 Hz) and δ 5.50 (d, $J_{1B,2}$ = 17.5 Hz), three olefinic protons signals at δ 6.01 (dd, $J_{2,1A}$ = 10.8 Hz and $J_{2,1B}$ = 17.4 Hz), δ 5.13 (br t, $J_{6,5}$ = 7.0 Hz), and at δ 5.08 (th, $J_{10,9}$ = 7.0 Hz, $J_{10,12}$ = 1.4 Hz, $J_{10,15}$ = 1.4 Hz). The ^{13}C NMR spectrum (see Table I) confirmed the presence of four methyl groups at 25.9 (q), 17.4 (q), 16.3 (q) and 16.0 (q) and the presence of six olefinic carbon atoms at 137.4 (d), 136.4 (s), 132.3 (s), 125.3 (d), 124.3 (d), and δ 121.9 (t). The ^1H NMR spectrum (see Table I) also showed two other methylene down shift signals at δ 3.71 (tl, J = 6.1 Hz, 2H) and at δ 3.28 (m, 2H) that were in accordance with the signals in the ^{13}C NMR spectrum (Table I) at 35.8 (t) and δ 46.4 (t). The assignments of the NMR signals presented above were established by the analysis of the correlations observed on the bidimensional COSY, HMQC and HMBC spectra as shown in Table I.

(–)-Agelasidine A (**1**) gave positive tests with Dragendorff and molybdophosphoric acid reagents which confirmed the presence of nitrogen and terpenoid moieties in its structure. The FTIR spectrum showed a band at 1633 cm^{-1} characteristic of a guanidine moiety and bands at 3359 cm^{-1} and 1460 cm^{-1} due to N-H and N-C-N vibrations, respectively. Further analysis of the ^{13}C NMR spectrum established the presence of a quaternary carbon atom (δ 69.3 ppm) and a methylene carbon atom (δ 46.4 ppm) both bound to a sulfur atom. The two bands observed in the FTIR spectrum at 1373 cm^{-1} and 1129 cm^{-1} proved the presence of

Table I. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectral data of (–)-agelasidine A (**1**) [CD_3OD, coupling constants (*J*) in Hz], COSY (^1H-^1H) and HMBC correlations.

C	^{13}C NMR (ppm)	^1H NMR δ [multiplicity, *J* (Hz)]	COSY (^1H-^1H)	HMBC (^1H-^{13}C)
1	121.9	A 5.57 (d, $J_{1A,2}$ = 10.7)	H-1B, H-2	C-2, C-3
		B 5.50 (d, $J_{1B,2}$ = 17.5)	H-1A, H-2	
2	137.4	6.01 (dd, $J_{2,1A}$ = 10.8; $J_{2,1B}$ = 17.4)	H-1A, H-1B	C-3, C-13
3	69.3	–	–	–
4	23.1	1.90 (m)	–	–
5	33.1	1.90 (m)	H-6	C-6, C-7
6	124.3	5.13 (br t, $J_{6,5}$ = 7.0)	H-5, H-14	C-8, C-14
7	136.4	–	–	–
8	40.7	2.09 (m)		C-9, C-14
9	27.6	2.09 (m)	H-10, H-12	–
10	125.3	5.08	H-9, H-14	–
		(th, $J_{10,9}$ = 7.0; $J_{10,12}$ = 1.4; $J_{10,15}$ = 1.4)		
11	132.3	–	–	–
12	25.9	1.66 (d, $J_{12,10}$ = 1.1)	H-10	C-10, C-11
13	16.0	1.59 (sl)	–	C-2, C-3
14	16.3	1.59 (sl)	H-6	C-6, C-7, C-8
15	17.4	1.52 (sl)	–	C-10, C-11
1′	46.4	3.28 (m)*	H-2′A, H-2′B	C-2′, C-3
2′	35.8	3.71 (tl, J = 6.1)	H-1′A, H-1′B	C-1′, C-3′
3′	158.6	–	–	–

δ values for **1** are referenced to the signal of residual CD_3OD (δ 3.30 ppm and 49.0 ppm).
* Overlapped signal with the residual methanol signal.

Microorganism	Disc diffusion method [mm]		Broth dilution method MIC [μg/ml]	
	(−)-Agelasidine A 0.10 mg/disc	Rifampicin 0.02 mg/disc	(−)-Agelasidine A	Rifampicin
B. subtilis	±	45	50[a]	0.001 [b]
E. coli	±	15	100[b]	1.00 [b]
M. smegmatis	±	14	100[b]	0.50[b]
S. enteritides	–	35	n.t.	n.t.
S. aureus	15	40	50[a]	0.001 [b]
S. faecium	±	30	100[b]	0.25 [b]
S. mutans	±	40	100[b]	0.25 [b]

Table II. Results of antibacterial activity tests of (−)-agelasidine A (**1**) by the disc diffusion method and determination of the MIC values by the broth dilution method. The assays were performed in duplicate. No differences between the means were observed in the results obtained.

±, Partial inhibition of the bacterial growth; –, no inhibition; n.t., not tested.
[a] Bacteriostatic activity. [b] Bactericidal activity.

a sulfone group (asymmetric and symmetric deformations of the SO_2 group, respectively). All these data were in accordance with those found in the literature for (+)-agelasidine A (Nakamura *et al.*, 1983). Nevertheless the specific optical rotation of compound **1** was negative ($[\alpha]_D^{20}$ −14.5° (*c* 1.55; methanol)) meaning that (−)-agelasidine A [(*3R*)-*N*-[2-(3,7,11-trimethyldodeca-1,6,10-triene-3-sulfonyl)-ethyl]-guanidine] was isolated for the first time (Fig. 1).

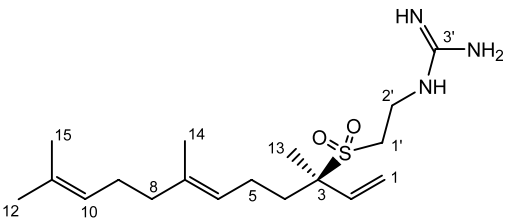

Fig. 1. Chemical structure of (−)-agelasidine A (**1**).

(−)-Agelasidine A was tested against the opportunistic bacteria *B. subtilis* and *M. smegmatis*, and human pathogenic *E. coli*, *S. enteritides* and *S. aureus*. The antifungal activity was also tested against a blue-stain fungus, *C. cucumerinum*, and the wood surface contaminants *T. harzianum* and *T. koningii*. The human yeast pathogenic *C. albicans* was also tested.

The disc diffusion method shows the level of inhibition on a microorganism growing in agar culture media induced by a test compound. Complementarily, the broth dilution method indicates the concentration, at which the test compound produces either microstatic activity or microcidal activity. Results presented in Table II show that the test compound completely inhibited the growth of *S. aureus* and partially the growth of the other bacteria (except for *S. enteritides*) indicating that (−)-agelasidine A acts preferably as a bacteriostatic agent rather than a bactericidal agent (disc diffusion method). (−)-Agelasidine A did not inhibit fungal growth that was tested at 0.5 mg/disc.

The MIC results obtained by the broth dilution method have shown that the bacterial growth was completely inhibited at concentrations between 50 and 100 μg ml^{-1}.

Capon and Faulkner (1983) reported the antimicrobial activity of (+)-agelasidine A against *C. albicans*, *B. subtilis* and *S. aureus*. These results agree with those obtained in the present work, in which growth inhibition of *B. subtilis* and *S. aureus* was observed.

Overall, tests of antifungal and antibacterial activities showed that (−)-agelasidine A has no activity against fungi but acts as bacteriostatic agent against *S. aureus* and is partially active against other bacteria.

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