Pergamon

Tetrahedron 56 (2000) 8795–8798

TETRAHEDRON

Anti-Cryptococcal and Nitric Oxide Synthase Inhibitory Imidazole Alkaloids from the Calcareous Sponge *Leucetta* cf *chagosensis*

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Received 5 July 2000; accepted 29 August 2000

Abstract—Antifungal imidazole alkaloids were isolated from the Egyptian Red Sea sponge *Leucetta* cf *chagosensis* using HPLC. These compounds were the previously reported naamidine A, B, D and G and the unreported symmetric imidazole alkaloid naamine D. Naamine D possesses moderate antifungal and nitric oxide synthase inhibitory activity. The structure of naamine D was determined using 1D and 2D NMR experiments including $^{1}H-^{15}N$ HMBC and high resolution mass spectrometry. © 2000 Elsevier Science Ltd. All rights reserved.

A bright yellow sponge, *Leucetta* cf *chagosensis*, collected from the Egyptian Red Sea contained imidazole alkaloidal compounds that are active against the AIDS OI pathogen *Cryptococcus neoformans* (Table 1), and weak inhibitor of inducible nitric oxide synthase (iNOS). The identity of the isolated compounds was determined by examination of NMR and mass spectrometry data. The Marinlit™ database was searched using the HR-MS and ^{1}H NMR spectra of the isolated compounds.[1] The unreported guanidino containing alkaloid naamine D (**1**) was isolated along with four known alkaloids, naamidine A, B, D and G (Fig. 1).[2,3] Guanidino containing natural products have been shown to be an important class of biologically active compounds.[4] Naamines A and B, from a Red Sea *L. chagosensis* specimen were reported to have antifungal activity while the closely related leucettamine A is a leukotriene B4 receptor antagonist isolated from *Leucetta microraphis* collected from Palau.[5] Naamine C was reported from *L. chagosensis* collected in Micronesia.[6]

The presence of the unsubstituted imidazoline group in naamine D prompted us to evaluate its inhibitory potential against inducible nitric oxide synthase (iNOS), due to the wide variety of structurally unrelated guanidino-containing analogs that serve as inhibitors for this isoform.[7] Nitric oxide is catalytically synthesized from its endogenous precursor, L-arginine by a family of three distinct but related NADPH- and O_2-dependent nitric oxide synthase (NOS) enzymes.[8] Two constitutive Ca^{+2}/calmodulin dependent isozymes, neuronal NOS (nNOS) and endothelial NOS (eNOS), and one inducible NOS (iNOS) expressed primarily in macrophages in response to lipopolysaccharide (LPS) and various cytokines, have been cloned and expressed in a variety of cell types.[8,9] Nitric oxide

Table 1. Antifungal activity of alkaloids isolated from *L. chagosensis*

Compound	Molecular formula	Yield (mg) (% dry weight)	Antifungal MIC (μg/mL) *C. neoformans*
Naamidine A[a]	$C_{23}H_{23}N_5O_5$	5.0 (0.024)	12.5
Naamidine B[a]	$C_{24}H_{25}N_5O_5$	3.0 (0.014)	6.25
Naamidine D[a]	$C_{23}H_{23}N_5O_4$	2.5 (0.012)	Not tested
Naamidine G[b]	$C_{24}H_{25}N_5O_4$	4.1 (0.020)	12.5
Naamine D	$C_{19}H_{21}N_3O_2$	3.4 (0.016)	6.25

[a] Ref. 2.
[b] Ref. 3.

Keywords: antifungal; imidazole; marine; NMR.
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PII: S0040-4020(00)00821-8

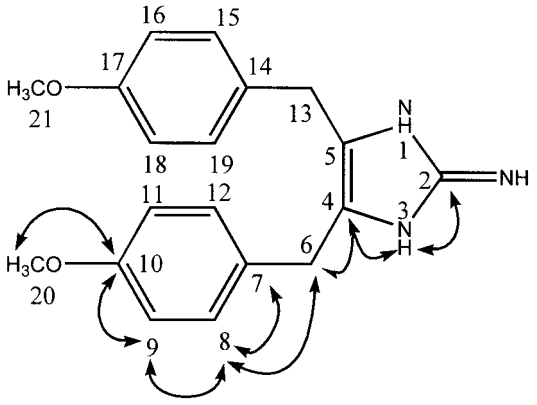
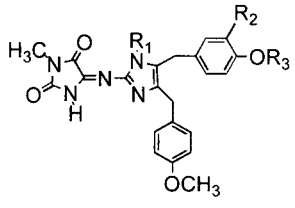


Figure 1. Numbering system and important HMBC correlations for naamine D (**1**).

production from NOS is involved in a multitude of physiological and pathophysiological processes, including blood pressure regulation, inflammation, apoptosis, platelet adhesion, neurotransmission, and host-defense mechanisms.[10] Of the three known isozymes, the inducible isozyme is perhaps the most scrutinized due to its involvement in a collection of diseases, including septic- and cytokine-induced shock, immune-type diabetes, rheumatoid arthritis, tissue damage, inflammation, and inflammatory bowel disease.[11]

The high degree of symmetry for the unreported naamine D provided a deceptively simple ^{1}H NMR and ^{13}C NMR spectra. The symmetrical nature of the structure is supported by the HR-MS data (M+H=324.1704), which is within 0.8 mmu of the molecular formula $C_{19}H_{22}N_3O_2$. The proton spectrum indicates a substituted benzene ring containing a methoxy group (δ 3.76) *para* to a benzylic methylene group (δ 3.80). The methoxy proton signal δ 3.76 and the aromatic proton signal at δ 7.09 show correlations to the quaternary carbon at δ 160.4. The proton signal at δ 7.09 shows a COSY correlation to δ 6.86 and an HMBC correlation to the carbon at δ 115.4. The proton doublet at δ 6.86 also shows an HMBC correlation to a quaternary carbon at δ 131.0 and this carbon has an HMBC correlation to the benzylic protons at δ 3.80, which in turn show a long-range correlation to the carbon at δ 123.4. This carbon (δ 123.4) shows an HMBC correlation to the NH protons (δ 11.35) and these NH protons also have an HMBC correlation to a carbon at δ 146.6. Carbon δ 146.6 was not observed in the 1D spectrum due to its reduced abundance and long relaxation time. The increased sensitivity of inverse detected 2D experiments and the fact that they rely on proton relaxation instead of slower ^{13}C relaxation made assignment of C-2 possible.



Naamidine	R_1	R_2	R_3
A	CH_3	H	H
B	CH_3	OH	CH_3
D	H	H	CH_3
G	CH_3	H	CH_3

Figure 2. Previously reported naamidine alkaloids isolated from *Leucetta* cf *chagosensis*.

The $^{1}H-^{15}N$ HMBC shows a one-bond correlation between the downfield proton (δ 11.35) and an ^{15}N at δ 135.2. This nitrogen also has a long-range correlation to the benzylic protons (δ 3.80). This ^{15}N chemical shift can therefore be assigned to the 1 and 3 positions of naamine D.

Naamine D demonstrated a 50% reduction in the rate of NO production when tested at a concentration of 1.0 mM. Naamine D has an MIC of 6.25 μg/mL (Table 1) against *Cryptococcus neoformans*. The naamidine alkaloids isolated from this sponge (Fig. 2) also showed similar anti-cryptococcal activity (Table 1).

Although naamine D is a modest inhibitor of iNOS, this natural product can serve as a viable template for the design and optimization of NOS inhibitors and anti-cryptococcal compounds.

Experimental

General experimental

NMR spectra were recorded as reported previously.[12] IR spectra were recorded on an ATI Mattson FTIR. UV–VIS spectra were recorded using a Hewlett–Packard 8452A diode array spectrophotometer.

Sponge material

The sponge was collected from the Red Sea near Hurghada, Egypt, in 1995. It is massive, bulbous, quite firm with a smooth undulating surface which 'catches' to the touch. The sponge is slightly elastic, the texture compressible, easily torn. The color in live organisms is intense lemon yellow throughout. This sample was identified as *Leucetta* cf *chagosensis* Dendy 1913 (Class Calcarea, Order Clathrinida, Family Leucettidae), morphologically closely comparable to the type species, but differs from it in the size of the equiangular triactinal spicules.[13]

Extraction and isolation

The freeze-dried sample (21 g) was extracted three times with ethyl acetate/isopropanol (1:1) to provide a lipophilic extract. A hydrophilic extract was obtained by extracting the marc three more times with ethanol/water (1:1). The solvents were dried under vacuum and a small portion of each extract was removed for biological evaluation. The lipophilic extract, which showed antifungal activity, was fractionated on a silica gel flash column using a block gradient. Due to the small amount of starting material, biological evaluation could not be carried out at each step of the isolation. Instead, ^{1}H NMR spectra were recorded to guide the isolation. The ^{1}H NMR spectrum of the hexane/

Table 2. ^{1}H, ^{13}C and ^{15}N NMR assignments of **1**

Position	^{13}C	DEPT	^{1}H	Multiplicity	^{15}N
2	146.6	s			
4, 5	123.4	s			
6, 13	29.6	t	3.80	s	
7, 14	131.0	s			
8, 12, 15, 19	115.4	d	6.86	d, 8.76	
9, 11, 16, 18	130.6	d	7.09	d, 8.76	
10, 17	160.4	s			
20, 21	55.9	q	3.76	s	
1, 3			11.35		135.2

ethyl acetate (4:1) fraction contained methoxy, *N*-methyl and aromatic signals which indicated the presence of secondary metabolites. This fraction was then subjected to reverse phase C-18 HPLC (Alltech Econosil, 21×250 mm, 10 mL/min) using gradient elution from 100% H_2O to 100% acetonitrile (CH_3CN). Five alkaloids were eluted with CH_3CN/H_2O (3:2). Four alkaloids, naamidine A, B, D and G (Fig. 1), were purified using the same column and an isocratic solvent system consisting of CH_3CN/H_2O (3:2). The unreported alkaloid naamine D was purified with CH_3CN/H_2O (1:1) on a Phenomonex Ultracarb C18 column to yield 3.4 mg (0.016% dry weight) of **1**.

Naamine D (1). ^{13}C NMR (Table 2, 125 MHz, $CDCl_3$), ^{15}N (Table 2, 50 MHz, $CDCl_3$) and ^{1}H NMR (Table 2, 500 MHz, $CDCl_3$); IR ν_{max} (neat, NaCl 3051 (w), 2980 (s), 1662(m), 1612(m), 1402(m) cm^{-1}; UV λ_{max} (ϵ)($CHCl_3$) 230 (15400), 262 (1290), 299 (3300) nm. HRFABMS *m/z* (fragment) 324.1704(M^++1, 100), calcd for $C_{19}H_{22}N_3O_2$, 324.1712 (Δ−0.8 mmu).

Enzymology – NO synthesis assay

Methods and Materials: Hewlett–Packard 8453 Diode-Array Biochemical Analysis Spectrophotometric System, with a multicell transport accessory and a Neslab Variable-Temperature Circulating Water Bath. Recombinant inducible nitric oxide synthase was purchased from Alexis Biochemicals, San Diego, CA. The cofactors and biochemical reagents used in this study were purchased either from Sigma or Alexis.

The ability of these compounds to serve as inducible nitric oxide synthase substrates or inhibitors was measured using the protocol described by Salter and Knowles, with slight modification.[14] Enzyme activity was characterized under initial rate conditions by measuring NO generation spectrophotometrically at 37°C based on the rapid and quantitative oxidation of oxy- to methemoglobin by NO, derived from L-arginine. The specific activity of iNOS used in this study was 7 nmol NO/min/mg protein. None of the compounds tested served as substrates for iNOS when incubated at 1 mM concentrations in the absence of L-arginine. For inhibition studies, incubation mixtures (total volume= 400 μL) containing final concentrations of $MgCl_2$ (1 mM), dithiothreitol (170 μM), tetrahydrobiopterin (12 μM), HbO_2 (16 μM; freshly prepared from sodium dithionite reduction followed by passage through a Sephadex G-25 column), FAD (4 μM), FMN (4 μM), L-arginine (500 μM), iNOS (3 μL) and namine D (1 mM, stock solution 25% DMSO-75% water) in 50 mM potassium phosphate buffer (pH=7.2) were preincubated for 30 s at 37°C. NADPH (1 mM) was added, and the reaction was monitored spectrophotometrically at 401 nm (ϵ=19,700 $M^{-1}cm^{-1}$).

Naamine D was initially screened against murine macrophage iNOS at a concentration of 1.0 mM (solubilized in 20% DMSO-water), using the well established continuous spectrophotometric assay, monitoring the production of methemoglobin from oxyhemoglobin mediated by nitric oxide (Salter reference). There was 50% reduction in nitric oxide production at 1.0 mM concentration of naamine D (control incubation mixture contained all components minus Naamine D). The inhibition appears to be competitive, since addition of 1.0 mM L-arginine to the incubation cuvette fully regenerated NO production in a time-dependant manner.

Antifungal assay

Minimum inhibitory concentrations against *Cryptococcus neoformans* were measured using the two-fold serial dilution technique in a 96 well microtiter plate with 200 μL per well. Mycophil™ broth was used as the growth medium and cultures were incubated 48 h at 25°. The bioassay broth was inoculated from a 48 h broth and a growth control was included to demonstrate the viability of the inoculum. Amphotericin B was included as a positive control and had an MIC of 0.39 μg/mL.

Acknowledgements

The authors gratefully acknowledge the National Institutes of Health grants R29A136596 (MTH) and K02AI01502 (MTH) and RO1AI27094 (AMC), Mississippi-Alabama Sea Grant and Monsanto/G.D. Searle for financial support.

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