Namenamicin, a New Enediyne Antitumor Antibiotic from the Marine Ascidian *Polysyncraton lithostrotum*

Leonard A. McDonald,[†] Todd L. Capson,[†]
Girija Krishnamurthy,[‡] Wei-Dong Ding,[‡]
George A. Ellestad,[‡] Valerie S. Bernan,[‡]
William M. Maiese,[‡] Piotr Lassota,[‡] Carolyn Discafani,[‡]
Robert A. Kramer,[‡] and Chris M. Ireland*,[†]

Department of Medicinal Chemistry, University of Utah
Salt Lake City, Utah 84112
Wyeth Ayerst Research
Pearl River, New York 10965

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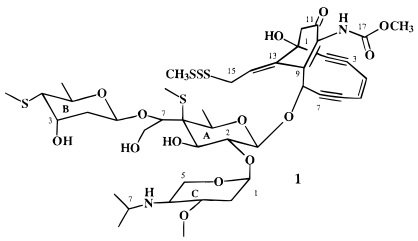
Marine ascidians belonging to the family Didemnidae have proven a remarkable source of chemically diverse natural products with potent biological properties. Prominent among these are the didemnins,[1] bistramide A,[2] varacin,[3] and the patellazoles.[4] As part of our continuing investigation of marine invertebrates from the Fiji Islands, we collected the thin encrusting orange ascidian *Polysyncraton lithostrotum* (Order: Aplousobranchia, Family: Didemnidae)[5] from Namenalala Island. A methanolic extract of the organism showed induction of SOS response in an agar based biochemical induction assay (BIA)[6] and a similar cytotoxicity profile to calicheamicin in a 26 cell line human tumor panel. These combined data were suggestive of a DNA cleavage agent. Bioautography directed fractionation of the extract using the BIA assay gave a new enediyne antitumor antibiotic namenamicin (**1**) in 10^{-4}% yield (1 mg from 1 kg of frozen tissue). Namenamicin contains the



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same "enediyne warhead" as the calicheamicins;[7] however, the attached carbohydrate moiety differs in replacement of the N−O sugar linkage between the A and B sugars with a C−O, an *S*-methyl substituent at the 4 position of the A sugar, and the absence of a benzoate ring appended to the B sugar.[8] Namenamicin exhibited potent *in vitro* cytotoxicity with a mean IC_{50} of 3.5 ng/mL and *in vivo* antitumor activity in a P388 leukemia model in mice (ILS 40% @ 3 μg/kg). Namenamicin also showed potent antimicrobial activity as shown in Table 1. DNA cleavage experiments indicated that namenamicin cleaved DNA with a slightly different recognition pattern than calicheamicin γ_1^I.

Table 1. Antimicrobial Activity (MIC, μg/mL)[a]

organism	namenamicin	calicheamicin	penicillin G
Bacillus subtilis	0.03	0.00005	0.25
Staphylococcus aureus	0.001	0.000001	0.015
Enterococcus faecium	0.03	0.00012	128
Escherichia coli	0.12	0.12	32
Klebsiella pneumoniae	0.06	0.25	128
Candida albicans	0.25	0.03	>128
Ustilago maydis	0.004	0.001	>128
Saccharomyces cerevisiae	0.06	0.008	>128
Neurospora crassa	0.25	0.06	>128

[a] Broth dilution method; inoculum, $1-5 \times 10^5$ (10^4 for fungi) cfu/mL; incubation, 37 °C (28 °C for fungi) for 36 h.

A molecular formula of $C_{43}H_{62}N_2O_{14}S_5$ was suggested by a prominent ion in the electrospray mass spectrum at *m/z* 991, corresponding to $(MH)^+$. However, repeated attempts to generate this ion for high resolution measurement using FAB, CI, or EI ionization methods failed.[9] In fact, the only other ion consistently observed under any ionization technique was at *m/z* 172, corresponding to a fragment generated by cleavage of the glycosidic bond between the A and C sugars. This ion was also reported for the esperamicins which have an identical carbohydrate unit.[10] Detailed analysis of data from the ^1H and ^{13}C NMR spectra, a suite of 2D correlation experiments, and comparison with the calicheamicins and esperamicins readily allowed complete assignment of the aglycone (Supporting Information). All assignments were completely analogous with the calicheamicins.[11] Our assignments for carbons 3 and 6, though consistent with the calicheamicins are opposed to those of the esperamicins.[12] A strong HMBC correlation between H8 (6.25 ppm) and a carbon at 83.44 ppm supports our assignment of this acetylene resonance to C6.

The identities of the sugars and the glycosidic linkages between the sugars and the enediyne aglycon were established based on chemical shift, $^1J_{CH}$, and $^3J_{HH}$ coupling constants and comparison with calicheamicin β_1^I and esperamicin A_1. The chemical shift and $^1J_{CH}$ of carbon A1 (δ 100.33, $^1J_{CH}$ = 163 Hz) is consistent with a β glycosidic linkage.[13] The upfield shift of the proton at A1 (H-A1, 4.53, d, J = 8 Hz) and the large 8 Hz coupling between H-A1 and H-A2 (3.84, dd, J = 10, 8 Hz) further supports a β glycosidic linkage between the A ring sugar and the aglycon. H-A3 (4.08, d, J = 10 Hz) is axial based on its coupling to H-A2. The intervening quaternary carbon A4 precluded further analysis of the conformation of the A ring using scalar coupling constants. However, the very strong nOe correlation between H-A1 and H-A5 (3.97, q, J = 6 Hz) defined a 1,3-diaxial relationship between these protons. The $CH(CH_2OH)$-O linkage between A4 and B1 was defined by oxygen bearing carbon resonances at δ 84.46 and 61.56 assigned as A7 and A8, respectively, and the corresponding protons at δ 3.55 (HA7, dd, J = 7, 7 Hz), 3.78 (HA8, dd, J = 13, 7 Hz) and 3.94 (HA8, dd, J = 13, 7 Hz). The orientation and placement of this unit between the A and B sugars was

[†] University of Utah.
[‡] Wyeth Ayerst Research.

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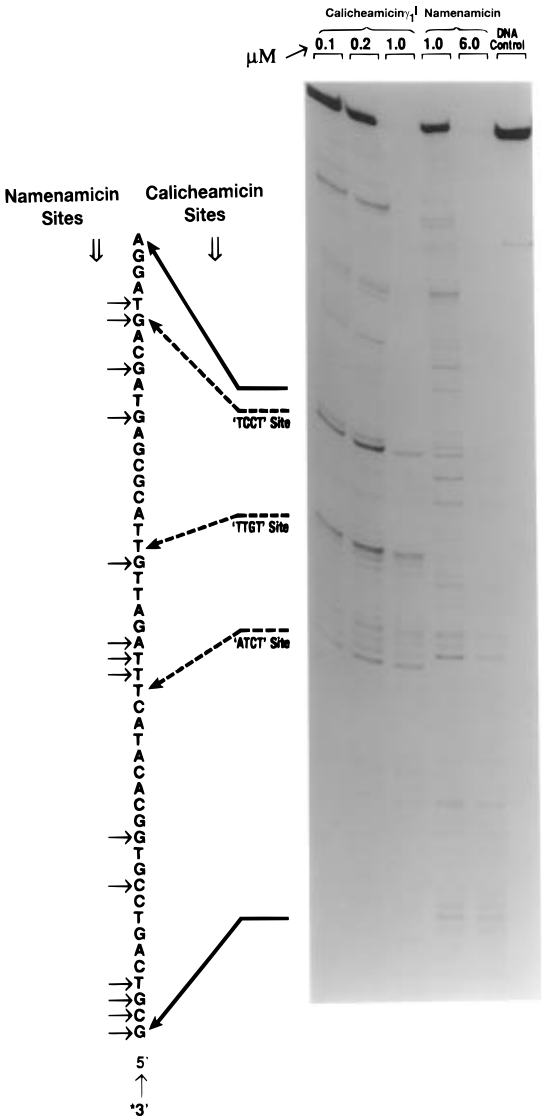

Figure 1. Site selective DNA cleavage due to calicheamicin γ_1^I and namenamicin. 3′-End labeled DNA fragment was reacted with the indicated concentrations of calicheamicin or namenamicin for 1 h at 37 °C in the presence of 50 mM NaCl according to the procedure described in ref 14. The three major calicheamicin cleavage segments are identified by dashed lines, and the namenamicin cleavage sites are identified by solid arrows.

indicated by HMBC correlations from A4 to both H-A7 and -A8, whereas B1 only showed correlations to H-A7. The conformation of the B ring sugar was established by analysis of proton coupling patterns and nOe interactions. The $^1J_{CH}$ (162 Hz) and the coupling of H-B1 (4.82, dd, $J = 10$, 2 Hz) to H-B2, diastereotopic methylene protons at δ 2.27 and 1.73 indicate that H-B1 is axial and again supports a β glycosidic linkage. The relatively small couplings between the H-B2 protons and H-B3 (4.13, ddd, $J = 5$, 5, 3 Hz) suggests H-B3 is equatorial. Since H-B4 (2.49, dd, $J = 11$, 3 Hz) and H-B5 (3.80, dd, $J = 11$, 6 Hz) couple very strongly and H-B5 shows a strong 1,3-diaxial nOe interaction with H-B1, the conformation of ring B was established as shown and is identical to the corresponding sugar in the calicheamicins aside from the substituent on sulfur at B4. The NMR data for the C ring sugar was essentially identical to the E ring of calicheamicin β_1^I, which has the same isopropyl amine substituent at the 4 position. Interestingly, the coupling pattern between H-C1 and the H_2-C2 protons along with the observation of nOe coupling between H-C1 and H-C4 suggest that this ring is flattened relative to the corresponding ring in the calicheamicin γ series. The flattening of this ring is probably due to steric interactions between C4 isopropyl amine (versus an ethyl amine in the calicheamicin γ series) and the A ring and warhead units. The NMR data for the B and C rings of namenamicin were also identical to the corresponding rings of esperamicin A1.[12]

The sequence specific DNA interactions of namenamicin were mapped on a 142 base pair pBR322 restriction fragment (Figure 1) and compared to calicheamicin γ_1^I.[14] The comparison is significant because the warheads are identical, but there are a few key differences in the carbohydrate domains of the two molecules. First, namenamicin lacks the rhamnose sugar (D ring) and the thiobenzoate moiety. The second difference is the substitution of a C−O for the N−O glycosidic linkage between the A and B sugars. Thirdly, the A sugar bears a *S*-methyl group in the 4 position. It is widely recognized that both the thiobenzoate and D sugar contribute to the overall strength of site specific interactions of the calicheamicins. In the DNA mapping experiments, namenamicin produced fewer high specificity cleavage sites than calicheamicin. At comparable concentrations, namenamicin cleaved DNA less efficiently than calicheamicin. There were some similarities in the sequence specific recognition patterns between the two compounds and several distinct differences. For example, TCCT, the primary recognition site for calicheamicin was cleaved, but with greatly diminished cleavage intensity. The primary recognition site for namenamicin in this restriction fragment was a 5′TTT segment which overlaps the "ATCT" recognition site of calicheamicin. Both calicheamicin and namenamicin cleave within this region. Interestingly, TTGT a strong cleavage site in the case of calicheamicin was not cleaved by namenamicin. The observations that namenamicin produced fewer high affinity cleavage sites and a slightly altered recognition pattern may be due to its truncated structure. The cleavage pattern due to namenamicin also bears some similarity to the cleavage pattern reported for esperamicin C.[15] For example both namenamicin and esperamicin recognize and cleave TCCT residues as well as a run of pyrimidines (5′TTT). The lowered cleavage efficiency and the altered selectivity may be attributed to several structural features of the carbohydrate moiety, namely, absence of the rhamnose sugar and thiobenzoate ring, presence of the *S*-methyl group in the A sugar, and more importantly the change in glycosidic linkage between the A and B ring sugars.

It has been well-established that bacteria form highly specific symbiotic relationships with marine plants and animals which leads one to speculate about the true biosynthetic origin of namenamicin. The fact that all of the enediyne antitumor antibiotics previously isolated have been products of actinomycetes and namenamicin's extremely low and variable yield from the ascidian lend support to the hypothesis of a microbial origin for this natural product. In order to address the question of compound origin, isolation experiments have been carried out in search of a possible producing microorganism. To date, 16 Micromonosporas have been isolated from the tissue of *Polysyncraton lithostrotum*. Three of these Micromonospora do produce potent DNA-damaging compounds, and structure identification studies are ongoing.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, and COSY spectra and NMR data for namenamicin (4 pages). See any current masthead page for ordering and Internet access instructions.

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