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Shishijimicins A−C, Novel Enediyne Antitumor Antibiotics from the Ascidian *Didemnum proliferum*[1]

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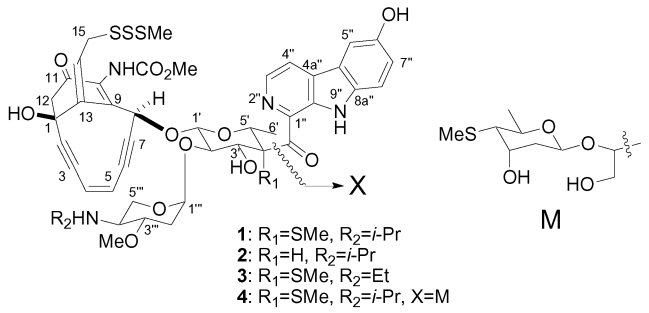
The enediyne family of antibiotics are potent antitumor agents with a unique mode of action, and clinical promise and success in cancer chemotherapy have been reported.[2] Namenamicin (**4**) isolated from the Fijian tunicate *Polysyncraton lithostrotum* is the only marine natural product of this family.[3] As part of our continuing search for bioactive metabolites from Japanese marine invertebrates, we found that a lipophilic extract of the thin encrusting orange ascidian *Didemnum proliferum* collected in southern Japan showed unique activity in the 3Y1 cell morphology assay.[4] Bioassay-guided isolation furnished four active compounds, one of which was readily identified as namenamicin (**4**) by spectral data. The other three were new compounds of the enediyne class named shishijimicins A−C (**1**−**3**), which showed extremely potent cytotoxicity. In this communication, we report isolation, structure elucidation, and biological activities of shishijimicins.

The MeOH and EtOH extracts of the tunicate were combined, evaporated, and subjected to the solvent partitioning scheme to afford the active aqueous MeOH fraction, which was separated by centrifugal partition chromatography followed by reversed-phase HPLC to afford shishijimicins A (**1**), B (**2**), and C (**3**), together with the know namenamicin (**4**).



1: R_1=SMe, R_2=*i*-Pr
2: R_1=H, R_2=*i*-Pr
3: R_1=SMe, R_2=Et
4: R_1=SMe, R_2=*i*-Pr, X=M

Shishijimicin A (**1**) had the molecular formula of $C_{46}H_{52}N_4O_{12}S_4$ as determined by HRFABMS. An initial analysis of the ^{1}H and ^{13}C NMR data in conjunction with the HMQC spectra in CD_3OD and DMSO-d_6 showed the presence of three aliphatic doublet methyls, two aromatic or *S*-methyls, two *O*-methyls, several methylene and methine carbons substituted by oxygen or nitrogen, two acetals, three olefinic protons, and five aromatic protons. As for the nonprotonated carbons, there were six resonances between δ 73 and 102 which were assigned as being oxygenated or acetylenic, eight signals between δ 122 and 158, and two signals at δ 194 and 198 which were ascribed to conjugated ketones. A notable feature was the presence of highly deshielded oxygenated methine (δ_H 6.35; δ_C 70.8) and broad *O*-methyl (δ 3.74) proton signals.

Interpretation of the COSY, HOHAHA, and HMQC data allowed for the assignment of units A−G (Figure S1). Unit A was assigned as 2,4-dideoxy-4-isopropylaminopentopyranose. Large vicinal coupling constants, H-2a‴/H-3‴ (10.1 Hz), H-3‴/H-4‴ (9.0), and H-4‴/H-5b‴ (10.5), and small coupling constants of H-1‴ (2.8 and 3.2 Hz) suggested that these protons were accommodated on a six-membered ring, in which H-3‴ and H-4‴ were both axial, while H-1‴ was equatorial. Unit B consisted of two spin systems which were separated by a quaternary carbon (C-4′) to which was attached an *S*-methyl group (δ_H 2.46; δ_C 15.2). Weak but clear cross-peaks observed from the *S*-Me signal to H-3′ and H-5′ as well as large vicinal coupling constants ($J_{1',2'}$ = 7.7 Hz and $J_{2',3'}$ = 8.9 Hz) suggested *trans*-orientations of the *S*-Me, H-3′, and H-5′, indicating that they were on a six-membered ring. Three aromatic protons in unit C (C-4b″−C-8a″) could be placed on a 1,2,4-trisubstituted benzene moiety on the basis of the magnitudes of *ortho*- and *meta*-couplings (8.8 and 2.4 Hz, respectively). The substituent on C-6″ was suggested to be an oxygen on the basis of the chemical shifts of C-5″ (δ 106.8) and C-7″ (120.2). A vicinal coupling constant of 4.9 Hz between H-3″ (δ 8.41) and H-4″ (8.21) as well as their low-field chemical shift values indicated that unit D (C-1″−C-4a″, C-9a″) was a part of a 2,3,4-trisubstituted pyridine moiety. Unit E contained a *cis*-olefin ($J_{4,5}$ = 9.3 Hz), in which H-5 was coupled to the highly deshielded oxymethine (H-8) by 1.7 Hz, while unit F (C-14, C-15) consisted of an allylic methylene bearing a heteroatom (δ_H 3.97, 4.18; δ_C 41.2), which was connected to a trisubstituted olefin (δ_H 6.51; δ_C 128.6). Unit G (C-12) was an isolated methylene (δ_H 2.71, 3.02; δ_C 54.7).

Units A−G were further elaborated by interpretation of the HMBC data. C-3‴ of unit A was shown to be methoxylated, while C-1‴ was attached to C-2′ of unit B through a glycosidic bond which was evident from a cross-peak H-1‴/C-2′. H-3′ of unit B correlated with a carbonyl at δ 197.9 (1′-CO), which was attached to C-4′ resonating at δ 74.7. Chemical shift values of all of the carbons in units C and D were unambiguously determined, while a linkage between C-4a″ and C-4b″ was demonstrated by the cross-peaks, H-4″/C-4b″ and H-5″/C-4a″. C-8a″ and C-9a″ could be connected through 9″-NH on the basis of cross-peaks 9″-NH/4b″ and 9″-NH/4a″. Therefore, the presence of a 6-hydroxy-9*H*-β-carbolin-1-yl group was indicated. The remaining portion, which comprised units E, F, and G and nonprotonated carbons at δ 73.1 (C-1), 83.8 (C-3), 89.2 (C-6), 98.7 (C-7), 102.1 (C-2), 132.6 (C-10), 138.5 (C-13), 149.3 (C-9), and 194.2 (C-11), contained elements of $C_{18}H_{16}NO_5S_3$ requiring 11° of unsaturation. Intrepretation of the HMBC data permitted us to assign the carbon skeleton of the aglycone (Figure 1a). The unassigned portion corresponding to elements of $C_3H_7NO_2S_3$ included an *S*-methyl (δ_H 2.56; δ_C 23.0), a methoxycarbonyl (δ_H 3.74; δ_C 53.7 and 157.8), and an exchangeable proton (δ 8.49). Chemical shift values of C-10, H_2-15, and C-15 inferred the presence of an *O*-methylcarbamate on C-10 and a methyl trisulfide on C-15, thus assigning a gross structure identical to that of calicheamicinone.[5,6] Moreover, an HMBC cross-peak H-1′/C-8 placed the thio-sugar at C-8. Finally, the remaining carbonyl

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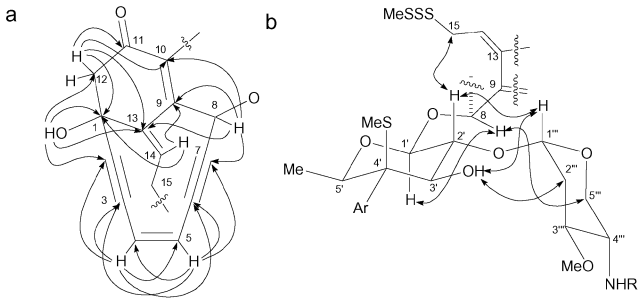

Figure 1. (a) Key HMBC correlations for the aglycone and (b) NOESY correlations for the glycoside in **1**.

carbon (C-1′) was connected to C-1″ of the β-carboline unit by a process of elimination.

Interpretation of the NOESY data (Figure 1b) allowed us to assign both the relative stereochemistry and the conformation of shishijimicin A (**1**). A cross-peak 1-OH/H-14 indicated the *E*-geometry for the $\Delta^{13,14}$-olefin, while a cross-peak 10-NH/H-8 demonstrated the *syn*-relationship of the two oxygen atoms on C-1 and C-8 as the case of calicheamicins.[2] Disposition and the stereochemical relationship of the thio-sugar unit with respect to the aglycone were delineated by the cross-peaks, H-8/H-1′, H-8/H-2′, and H_2-15/H-2′, while those between the thio-sugar and the nitrogenous sugar units were derived from the cross-peaks, H-1‴/H-2′, H-2b‴/3′-OH, and H_2-5‴/H-8. The orientation of the β-carboline unit with respect to the thio-sugar was inferred on the basis of cross-peaks, H-3″/H-3′ and H-3″/H-5′. It should be noted that the NOESY correlations were consistent with the proposed conformation of the aglycone and A and E sugars in calicheamicin γ_1^I bound to DNA.[7−10] The CD spectrum of shishijimicin A exhibited a negative Cotton effect at 325 nm ($\Delta\epsilon$ −5.0) and a negative exciton split [272 ($\Delta\epsilon$ +7.1) and 239 nm (−3.1)] due to the interaction of the enediyne and dienone chromophores (Figure S2): the positive Cotton effect at around 270 nm was in agreement with the value reported for calicheamicin γ_1^I.[11] Therefore, the absolute stereochemistry of the aglycone of **1** was identical to that of calicheamicins and that of the saccharide portion, as shown.

Shishijimicin B (**2**) had a molecular formula smaller than that of **1** by a CH_2S unit. The NMR spectrum exhibited no signal for the *S*-methyl group on the thio-sugar moiety in **2**, which was replaced by a proton signal at δ 4.56, dd, $J = 9.8$ and 11.0 Hz; the axial nature of this methine was consistent with NMR data. Therefore, shishijimicin B (**2**) was the des-4′-methylthio derivative of **1**. Shishijimicin B (**2**) exhibited the CD spectrum almost identical to that of **1**, thus suggesting their identical absolute stereochemistry.

Shishijimicin C (**3**) had the molecular formula of $C_{45}H_{50}N_4O_{12}S_4$, which was smaller than **1** by a methylene unit. The NMR spectra were almost superimposable on that of **1** except for the presence of an ethylamino group [δ_H 1.23 (3H, t) and 2.96 (2H, m)] instead of the isopropylamino group in **1**. Therefore, **3** is the desmethyl analogue of **1**. The CD spectrum of **3** was almost identical to that of **1**, again indicating the same absolute stereochemistry.

Shishijimicins are highly cytotoxic, as is namenamicin (Table 1). However, shishijimicin A was almost 10 times more active than

Table 1. Cytotoxicity of **1**−**4** (IC_{50}, pg/mL)

	1	2	3	4	adriamycin
3Y1	2.0	3.1	4.8	13	13 000
HeLa	1.8	3.3	6.3	34	17 000
P388	0.47	2.0	1.7	3.3	52 000

4 in the three cell-lines tested. It is highly likely that **1**−**3** cleave DNA as in the case of other enediyne antibiotics including namenamicin.[2] The sequence specificity of calicheamicin γ_1^I was reported to be ascribed to the presence of the aryl-substituted saccharides.[2] Because β-carboline not only intercalates DNA,[14] but also the β-carboline unit in shishijimicin A (**1**) occupies the position similar to that of the aryl group in calicheamicin γ_1^I, it may exhibit a different pattern of DNA sequence recognition from those of other enediynes.

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Supporting Information Available: Experimental section, table of NMR data, and NMR spectra of **1**−**3** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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