Headline Articles

Structure-Activity Relationship of Norzoanthamine Exhibiting Significant Inhibition of Osteoporosis

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Norzoanthamine is a zoanthamine-type alkaloid from the colonial zoanthid *Zoanthus* sp. Norzoanthamine hydrochloride, which suppresses the decrease in bone weight and strength in ovariectomized mice, could be a good candidate for an osteoporotic drug. Although the relative configuration of norzoanthamine was established based on an X-ray crystallographic analysis, the absolute stereochemistry of norzoanthamine remains unclear. To investigate their biogenesis and mechanisms of biological action, we conducted chemical and spectroscopic studies to determine the absolute configuration of norzoanthamines.

Over the past 30 years, there have been significant developments in separation techniques[1] and in methods of spectroscopic and X-ray crystallographic analyses.[2] Chemical synthetic methodology has also achieved notable successes.[3] Chemists in the field of marine natural products have benefited from these developments to discover an ever growing number of new molecular structures.[4—10] Scientists have been attracted to these compounds by their complexity, e.g., palytoxin or brevetoxin,[11—13] by their extraordinary biological activity, e.g., maitotoxin[14] and okadaic acid,[15] and by an interest in ecological phenomena,[16] such as medicinal resources for the discovery of new drugs or prototype of drugs.[17,18] We were also interested in investigations of such biologically active substances, especially IL-6 inhibitor, norzoanthamine (**1**).

In our continuing search for physiologically active substances from marine organisms, we found zoanthamine (**2**) and five novel cytotoxic alkaloids: norzoanthamine, oxyzoanthamine (**3**), norzoanthaminone (**4**), cyclozoanthamine (**5**), and epinorzoanthamine (**6**) from the genus *Zoanthus* sp.[19—23] on the Ayamaru coast of the Amami Islands (Fig. 1). Norzoanthamine inhibits IL-6 production. Furthermore, norzoanthamine and norzoanthamine hydrochloride, which suppress the decrease in bone weight and strength in ovariectomized mice, could be a good candidate for an osteoporotic drug.[24—27] The relative stereochemistry of norzoanthamines was determined by X-ray analysis (Fig. 1), although the absolute configuration remained unclear. To investigate their biogenesis and mechanisms of biological action, chemical and spectroscopic studies have been carried out. The absolute configuration of norzoanthamines was determined. We report here the absolute stereochemistry, biological activity, structure-activity relationship, and a proposed biogenesis of norzoanthamines.

Results and Discussion

The norzoanthamine which had been used for our studies was isolated by the following operation. The wet specimens (5.0 kg) which occurred as dense mats were minced by a Waring blender and extracted with ethanol. The ethanolic extract was filtered and concentrated in vacuo. The aqueous residue was partitioned between ethyl acetate and water, and the water layer was subsequently extracted twice with ethyl acetate. The lipid-soluble extracts were chromatographed on silica gel (eluted with chloroform containing methanol), then separated by preparative TLC on SiO_2 with acetonitrile, diethyl ether, or ethyl acetate as solvent, giving zoanthamine (32 mg, 6.4×10^{-4}%) and norzoanthamine as a colorless crystal [21 mg, 4.2×10^{-4}%, mp 282—285 °C, $[\alpha]_D$ 1.6° (*c* 1.0, $CHCl_3$)].

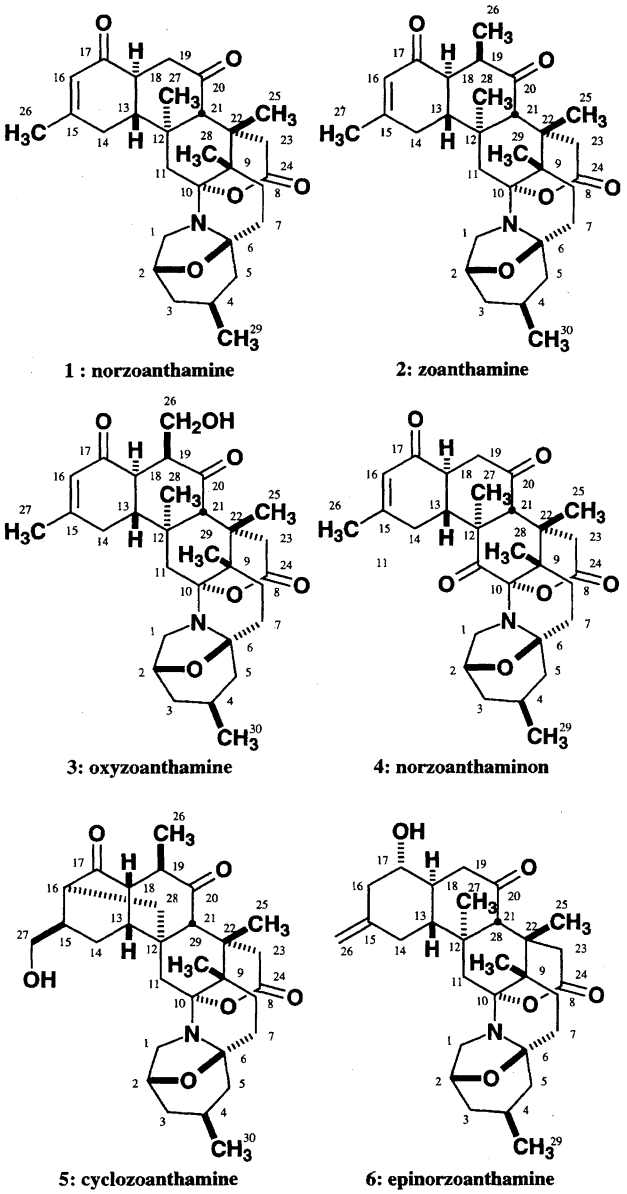

Fig. 1. Structures of zoanthamine family.

Reduction of Norzoanthamine. Norzoanthamine (**1**) was reduced with $NaBH_4$ (CH_3OH, r.t., 1 h) to give two derivatives, deoxynorzoanthamine (**7**) and deoxydihydronorzoanthamine (**8**). Interestingly, the ^{13}C NMR spectrum (Table 1) of **7** has revealed the presence of three ketonic carbonyls ($\delta_C = 198.7, 209.0, 211.5$), whereas **1** has two ketonic carbonyls [C17 ($\delta_C = 198.4$) and C20 ($\delta_C = 209.0$)] and one lactone carbonyl with carbon resonance at 172.4 ppm.[19] The molecular formula of **7** was assigned to be $C_{29}H_{39}NO_4$ from HREIMS of **7** (*m/z* 465.2889, Δ +1.2 mmu).

All of the signals in **7** were assigned by a detailed comparison of the NMR spectral data with those of **1** and by ^1H-^1H COSY and HMBC spectra. As shown in Fig. 2 and Fig. 3, the chemical shift of carbon networks among C1–C6 and C12–C24 of **7** closely resembled to those of **1**, except for the carbon resonance at C24 ($\delta_C = 211.5$). Carbon networks among C9–C12 were revealed by the observation

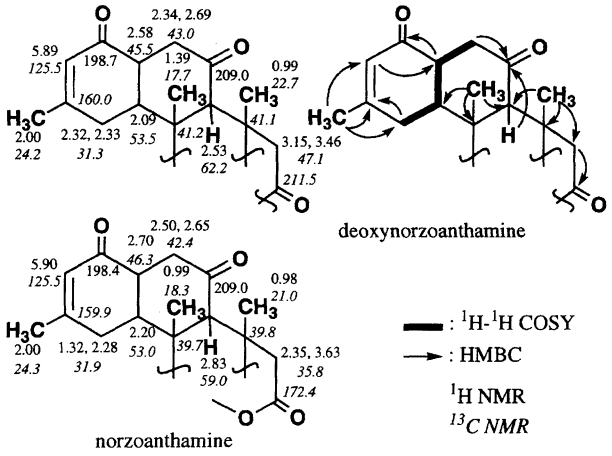

Fig. 2. Partial structures of norzoanthamine and deoxynorzoanthamine. (1)

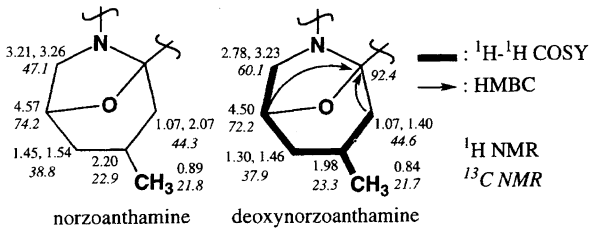

Fig. 3. Partial structures of norzoanthamine and deoxynorzoanthamine. (2)

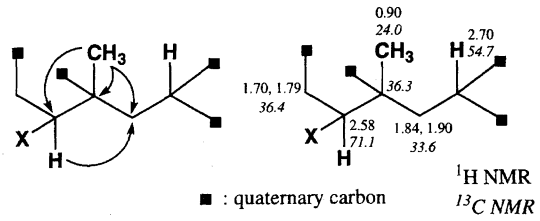

Fig. 4. Partial structures of deoxynorzoanthamine.

of cross-peaks in the ^1H-^1H COSY spectrum (H10/H11a and H10/H11b) and HMBC cross-peaks (28-CH_3/C9, 28-CH_3/C10, 27-CH_3/C11, and 27-CH_3/C12) (Fig. 4).

The HMBC cross-peaks between H1 and C10, and the chemical shift of C10 ($\delta_C = 71.1$) suggested connectivity among C1/N and N/C10. Finally, carbon connectivity between C7 and C24 was indicated by ^1H-^1H COSY cross-peaks (H7 and H8) and HMBC spectra (H7/C6, H7/C24, and H23/C7) (Fig. 5). As a result, the planar structure was proposed to be **7**, as shown in Fig. 5. The relative stereochemistry of **7** was the same as that of **1**, except at C7 and C10. The stereochemistry at C7 and C10 was deduced from the coupling constants of H10 ($J_{10,11a}$=4.5 Hz and $J_{10,11b}$=2.2 Hz), NOE between H10 and 28-CH_3 (Fig. 6), and the mechanism of rearrangement, as shown in Fig. 7.

The molecular formula of **8** was determined to be $C_{29}H_{41}NO_4$ from HREIMS of **8** (*m/z* 467.3011, Δ −2.1 mmu). The ^1H NMR spectral data of **8** (Table 1) resembled that for **7**, except at C17 ($\delta_H = 3.95$). The reduced compound **7** was treated with $NaBH_4$ to give compound **8**. All of the signals were assigned by a detailed comparison of the

Table 1. NMR spectral Data of Norzoanthamine, Deoxynorzoanthamine, and Deoxydihydronorzoanthamine[a)]

	Norzoanthamine		Deoxynorzoanthamine			Deoxydihydronorzoanthamine	
Position	^{1}H (J in Hz)	^{13}C (mult.)	^{1}H (J in Hz)	^{13}C (mult.)	HMBC	^{1}H (J in Hz)	^{13}C (mult.)
1	3.26 (d, 6.6)	47.1 (t)	2.78 (dd, 10.6, 7.3)	60.1 (t)	C2,C3,C4,C10	2.78 (dd, 10.7, 7.3)	60.1 (t)
	3.21 (dd, 6.6, 4.5)		3.23 (dd, 10.6, 1.5)			3.24 (dd, 10.7, 0.7)	71.1 (d)
2	4.57 (m)	74.2 (d)	4.50 (m)	72.2 (d)	C4,C6	4.50 (dddd, 7.3, 2.1, 1.9, 0.7)	71.1 (d)
3	1.45 (br. t, 11.9)	38.8 (t)	1.30 (ddd, 13.4, 11.0, 2.5)	37.9 (t)	C1,C2,C4,C5	1.33 (ddd, 13.3, 9.2, 2.1)	37.8 (t)
	1.54 (m)		1.46 (ddd, 13.4, 5.1, 3.6)			1.45 (ddd, 13.3, 4.8, 1.9)	
4	2.20 (m)	22.9 (d)	1.98 (m)	23.3 (d)	C2,C3,C5,C6,C29	1.98 (qdddd, 6.6, 12.3, 9.2, 5.5, 4.8)	23.2 (d)
5	1.07 (dd, 12.7, 11.5)	44.3 (t)	1.07 (dd, 13.2, 12.5)	44.6 (t)	C4,C6,C7,C29	1.09 (dd, 13.3, 12.3)	44.5 (t)
	2.07 (dd, 12.7, 4.8)		1.40 (dd, 12.5, 5.8)			1.44 (dd, 13.3, 5.5)	
6		90.0 (s)		92.4 (s)			92.5 (s)
7	1.75 (ddd, 13.3, 4.6, 3.5)	29.9 (t)	2.70 (dd, 3.3, 2.9)	54.7 (d)	C6,C24	2.71 (dd, 3.3, 2.7)	54.7 (d)
	1.67 (ddd, 13.3, 9.4, 3.8)						
8	1.87 (ddd, 13.5, 9.4, 4.6)	23.6 (t)	1.84 (dd, 14.1, 3.3)	33.6 (t)	C6,C7,C9,C10	1.85 (dd, 14.1, 2.7)	33.6 (t)
	1.55 (ddd, 13.5, 3.8, 3.5)		1.90 (dd, 14.1, 2.9)		C24,C28	1.95 (dd, 14.1, 3.3)	
9		36.4 (s)		36.3 (s)			36.1 (s)
10		101.5 (s)	2.58 (dd, 4.5, 2.2)	71.1 (d)	C9,C11,C12,C22	2.56 (m)	71.1 (d)
11	2.14 (d, 13.9)	41.8 (t)	1.70 (dd, 14.2, 4.5)	36.4 (t)	C10,C12,C13,C27	1.75 (m)	36.1 (t)
	1.89 (d, 13.9)		1.79 (dd, 14.2, 2.2)			1.79 (m)	
12		39.7 (s)		41.2 (s)			41.2 (s)
13	2.20 (m)	53.0 (d)	2.09 (ddd, 14.6, 8.5, 6.2)	53.5 (d)	C12,C14,C18,C21,C27	1.78 (m)	50.8 (d)
14	1.32 (m)	31.9 (t)	2.32 (dd, 13.5, 8.5)	31.3 (t)	C13,C15,C16	1.72 (m)	29.4 (t)
	2.28 (m)		2.33 (dd, 13.5, 6.2)			1.95 (m)	
15		159.9 (s)		160.0 (s)			133.0 (s)
16	5.90 (br. s)	125.5 (d)	5.89 (s)	125.5 (d)	C14,C15,C18,C26	5.38 (br. s)	124.8 (d)
17		198.4 (s)		198.7 (s)		3.95 (br. s)	75.8 (d)
18	2.70 (ddd, 11.8, 11.4, 6.6)	46.3 (d)	2.58 (ddd, 14.6, 11.8, 6.9)	45.5 (d)	C12,C13,C14,C17	1.75 (m)	42.5 (d)
19	2.65 (dd, 14.4, 6.2)	42.4 (t)	2.34 (dd, 13.1, 11.8)	43.0 (t)	C17,C18,C20,C21	2.09 (dd, 14.0, 4.6)	47.5 (t)
	2.50 (dd, 14.4, 11.4)		2.69 (dd, 13.1, 6.9)			2.83 (dd, 14.0, 10.3)	
20		209.0 (s)		209.0 (s)			210.0 (s)
21	2.83 (br. s)	59.0 (d)	2.53 (s)	62.2 (d)	C12,C20,C22,C23,C25	2.60 (s)	62.0 (d)
22		39.8 (s)		41.1 (s)			41.1 (s)
23	3.63 (d, 20.2)	35.8 (t)	3.15 (d, 14.2)	47.1 (t)	C7,C21,C22,C24,C25	3.18 (d, 13.9)	47.1 (t)
	2.35 (d, 20.2)		3.46 (d, 14.2)			3.53 (d, 13.9)	
24		172.4 (s)		211.5 (q)			212.0 (q)
25	0.98 (s)	21.0 (q)	0.99 (s)	22.7 (q)	C9,C21,C22,C23	1.01 (s)	22.8 (q)
26	2.00 (s)	24.3 (q)	2.00 (s)	24.2 (q)	C14,C15,C16	1.73 (s)	23.3 (q)
27	0.99 (s)	18.3 (d)	1.39 (s)	17.7 (q)	C11,C12,C13,C21	1.33 (s)	17.3 (q)
28	1.15 (s)	18.4 (t)	0.90 (s)	24.0 (q)	C8,C9,C10,C22	0.91 (s)	24.1 (q)
29	0.89 (d, 6.6)	21.8 (q)	0.84 (d, 6.6)	21.7 (q)	C3,C4,C5	0.85 (d, 6.6)	21.8 (q)

a) Spectra were recorded in $CDCl_3$. b) Multiplicity was determined by a DEPT experiment.

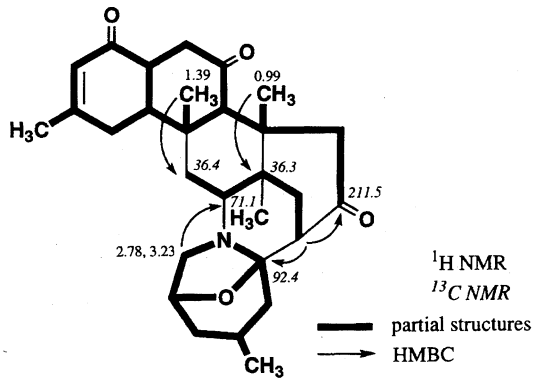

Fig. 5. Planar structure of deoxynorzoanthamine.

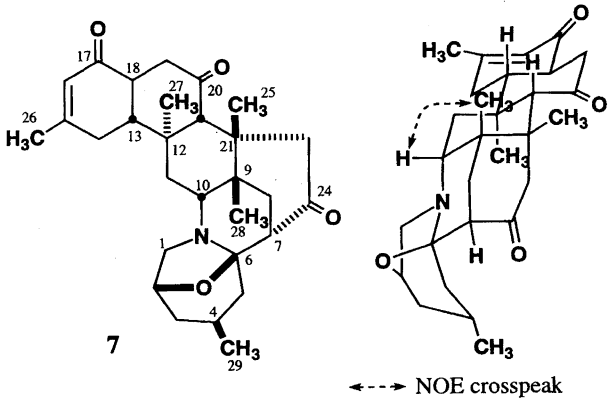

Fig. 6. Relative stereochemistry of deoxynorzoanthamine.

NMR spectral data (Table 1) with those of **7** and by ^{1}H-^{1}H COSY spectra. The chemical shift at C17 (δ_C = 75.2) and the molecular formula of **8** indicated the presence of a hydroxy group at C17. Furthermore, **8** was treated with acetic anhydride and pyridine, and the corresponding monoacetate (**9**) was obtained. The stereochemistry at C17 was suggested by the coupling constants of **9**. H17 was assigned to be axial, based on the relatively large coupling constants ($J_{17,18}$ = 8.8 Hz), as shown in Fig. 8. These data indicated the relative stereochemistry of **8**.

X-Ray Analysis of Deoxynorzoanthamine. As described before, the relative stereochemistry of norzoanthamine was clarified by X-ray analysis. Fortunately, recrystallization of deoxynorzoanthamine (**7**) from CH_3OH–$CHCl_3$ gave a well-formed crystal: $C_{29}H_{39}NO_4$, thin, prismatic needles belonging to the orthorhombic space group $P2_12_12_1$ with a = 13.424(8), b = 22.049(5), c = 8.392(2) Å; V = 2483.9(9) Å^3; Z = 4. Intensities of 2379 unique reflection were measured on a Mac Science MXC18 diffractometer.

A computer generated perspective drawing of the current X-ray model of **7** is shown in Fig. 9 (ORTEP drawing model). This ORTEP drawing model strongly supported the relative stereochemistry of **7** suggested by NMR spectral data. There-

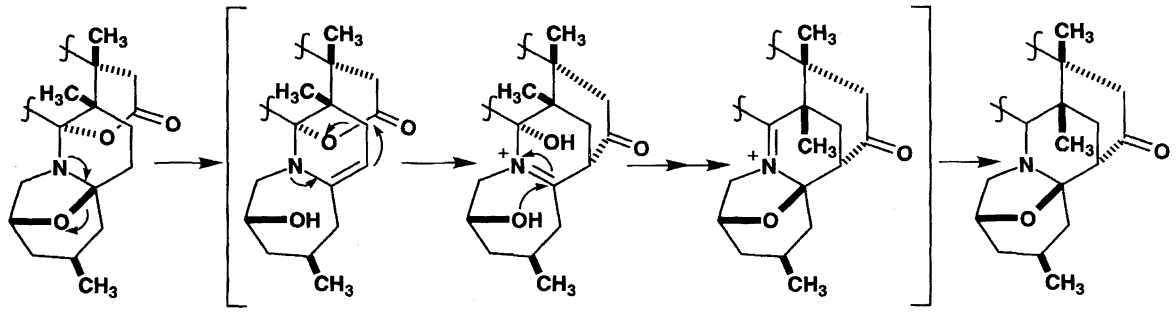

Fig. 7. Proposed mechanism of reductive formation of norzoanthamine.

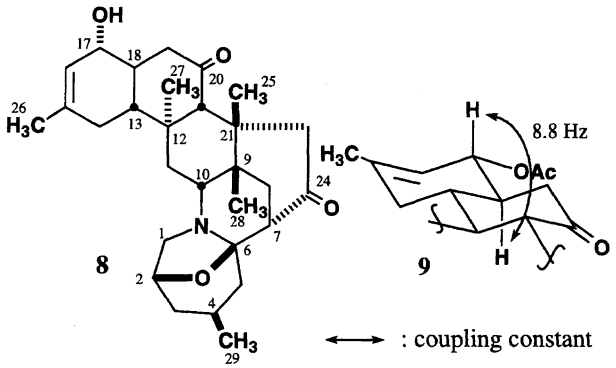

Fig. 8. Relative stereochemistry of deoxydihydronorzoanthamine.

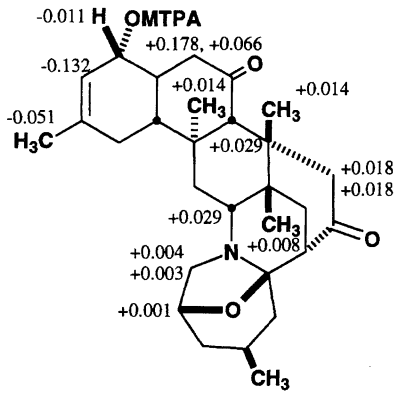

Fig. 10. $\Delta\delta$ Values [$\Delta\delta$ (in ppm) $= \delta_S - \delta_R$] obtained for (*S*)- and (*R*)-MTPA esters of deoxydihydronorzoanthamine.

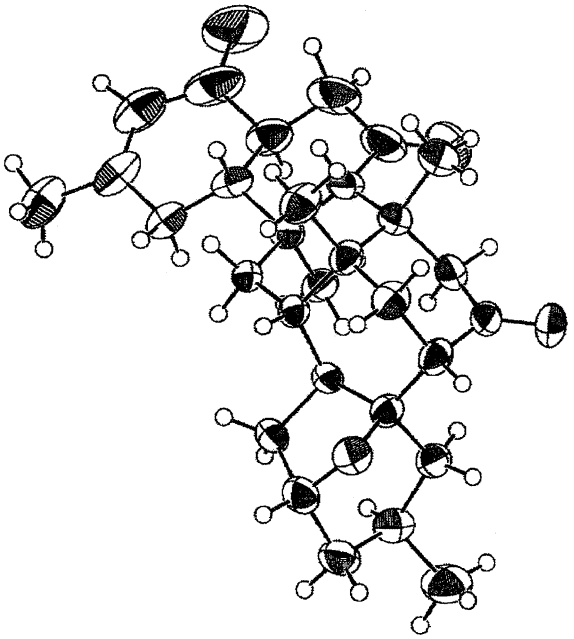

Fig. 9. ORTEP drawing model of deoxynorzoanthamine.

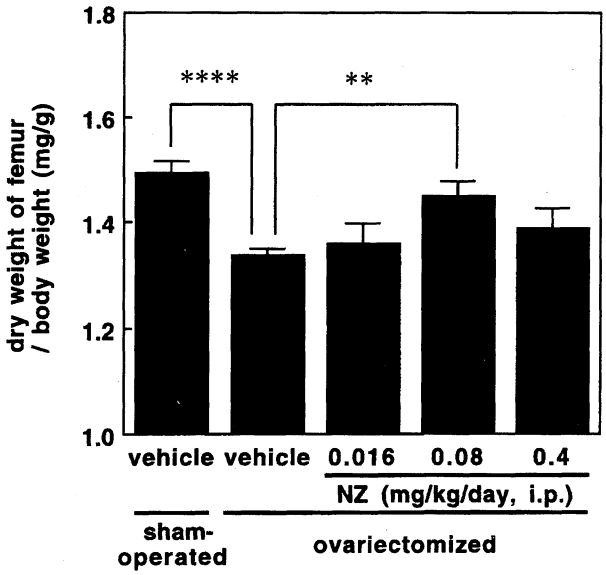

Fig. 11. Effect of norzoanthamine hydrochloride on the femoral weight in ovariectomized mice. Norzoanthamine hydrochloride (NZ) was administrated by i.p. for 4 weeks. Each point is the mean ±S.E., $n = 5$. *; $p < 0.05$, **; $p < 0.01$, vs. ovariectomized group treated by vehicle, by Student's *t*-test.

fore, the relative stereochemistry of **7** was determined to be 2*R**, 4*S**, 6*S**, 7*S**, 9*S**, 10*R**, 12*S**, 13*R**, 18*S**, 21*S**, and 22*S**. The complete $F_o - F_c$ data are deposited as Document No. 71018 at the Office of the Editor of Bull. Chem. Soc. Jpn.

Absolute Stereochemistry of Norzoanthamine. As described before, the relative stereochemistries of **7** and **8** were the same as that of **1** except for C7, C10, and C17. Therefore the absolute stereochemistry of **1** must be clarified by investigating that of **8**.

Treatment of **8** with (−)- and (+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPACl) gave the (*S*)- and (*R*)-MTPA esters (**10** and **11**), respectively.[28] The ^{1}H NMR

Table 2. $\Delta\delta$ Values [$\Delta\delta$ (in ppm) = $\delta_S - \delta_R$] Obtained for (*S*)- and (*R*)-MTPA Esters of Deoxydihydronorzoanthamine[a)]

	(*S*)-MTPA ester	(*R*)-MTPA ester	$\Delta\delta_{S-R}$ (in ppm)
H1a	2.7793	2.7752	0.0041
H1b	3.2293	3.2260	0.0033
H2	4.5012	4.5003	0.0009
H10	2.2901	2.2608	0.0293
H16	5.2586	5.3915	−0.1319
H17	5.4052	5.4158	−0.0106
H19a	2.1195	2.0535	0.0660
H19b	2.5585	2.3802	0.1783
H21	2.6029	2.5736	0.0293
H23a	3.1523	3.1335	0.0188
H23b	3.4822	3.4639	0.0183
H25	1.0111	0.9974	0.0137
H26	1.7094	1.7607	−0.0513
H27	1.3090	1.2952	0.0138
H28	0.9222	0.9140	0.0082

a) Spectra were recorded in $CDCl_3$.

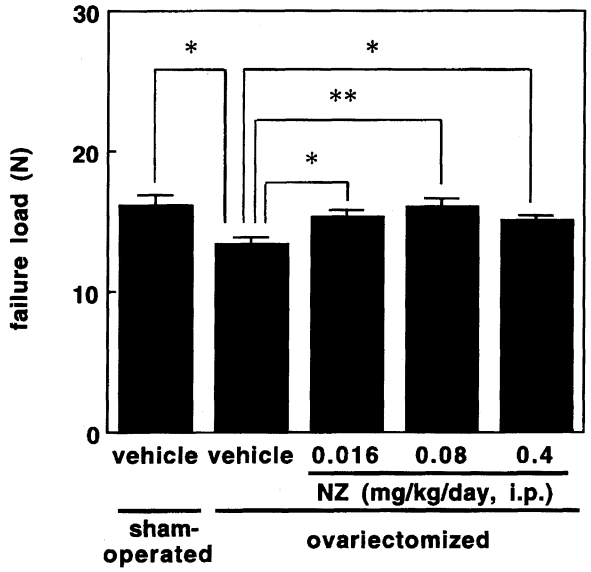

Fig. 12. Effect of norzoanthamine hydrochloride on the failure load in ovariectomized mice. Norzoanthamine hydrochloride (NZ) was administrated by i.p. for 4 weeks. Each point is the mean ±S.E., $n = 5$. *; $p < 0.05$, **; $p < 0.01$, vs. ovariectomized group treated by vehicle, by Student's *t*-test.

chemical shifts of **10** and **11** were assigned based on detailed analysis of ^{1}H-^{1}H COSY spectral data. The differences of the chemical shift ($\Delta\delta$; $\delta_S - \delta_R$) in the ^{1}H NMR spectra are shown in Table 2 and Fig. 10. Positive $\Delta\delta$ values were observed for H10, H19a, H19b, H21, H23a, H23b, H25, H27, and H28, whereas negative values were observed for H16, H17, and H26, indicating that the absolute configuration of **8** at C17 was *S*. These data and the relative stereochemistry of **8** suggested that the absolute configuration of reduced compound **8** at C2, C4, C6, C7, C9, C10, C12, C13, C17, C18, C21, and C22 was *R*, *S*, *R*, *S*, *S*, *R*, *S*, *R*, *S*, *S*, *S* and *S*. Therefore, the absolute stereochemistry of norzoanthamine

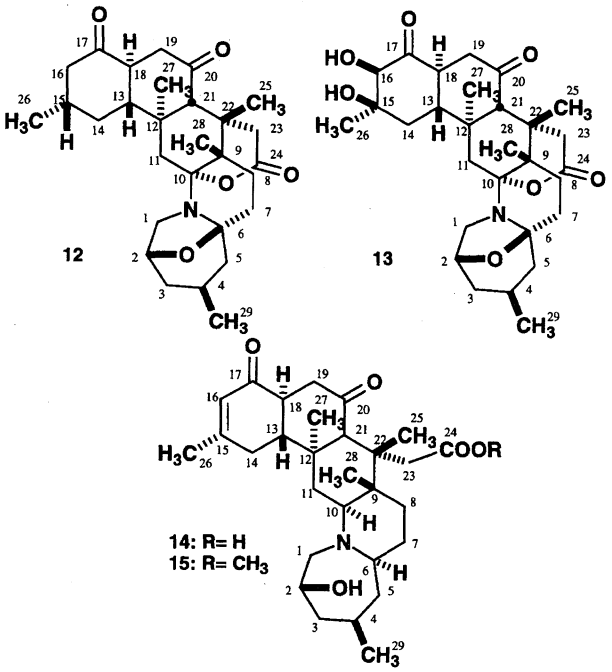

Fig. 13. Structures of norzoanthamine derivatives.

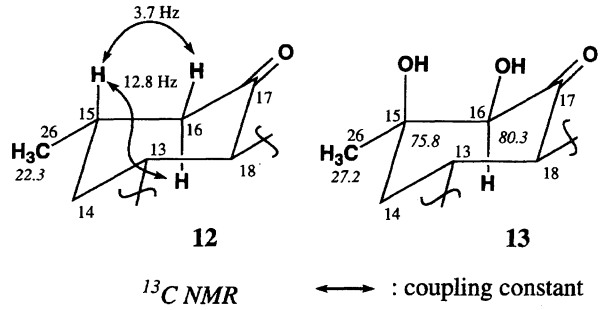

Fig. 14. The relative stereochemistry around the cyclohexanone moiety of **12** and **13**.

was determined to be 2*R*, 4*S*, 6*S*, 9*S*, 10*R*, 12*S*, 13*R*, 18*S*, 21*S*, and 22*S*.

Biological Activity of Norzoanthamine. In recent years, osteoporosis has become a serious disease for human health. Osteoporosis is caused by an imbalance between bone resorption and bone formation, which results in bone loss and fractures after mineral flux. Therefore, besides prevention of loss in bone mass, the effect on bone mechanical strength is a very important point for evaluation of osteoporotic drugs.[24,25)]

The effect of norzoanthamine hydrochloride on bone weight and strength was tested with ovariectomized mice, a postmenopausal osteoporosis model. Norzoanthamine hydrochloride (0.08 mg/kg/day, i.p. for 4 weeks) significantly suppressed the decrease of the femoral weight (Fig. 11) caused by ovariectomy without increase of the uterine weight.[29)] Such data suggested a mode of action of norzoanthamine hydrochloride alternative to that of estrogen. Furthermore, norzoanthamine hydrochloride also affected bone strength, measured by three-point bending test. As for the failure load of the femur, administrations of norzoanthamine

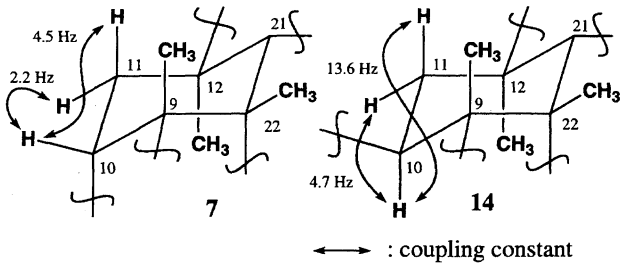

Fig. 15. The relative stereochemistry of around the cyclohexane moiety of **7** and **14**.

Table 3. Inhibitory Effect of IL-6 Induction of Norzoanthamine Derivatives

Sample	IC_{50} ($\mu g\,mL^{-1}$)
Norzoanthamine	13
7	ca. 25
8	30
9	23
12	45
13	35
14	42
17	>100

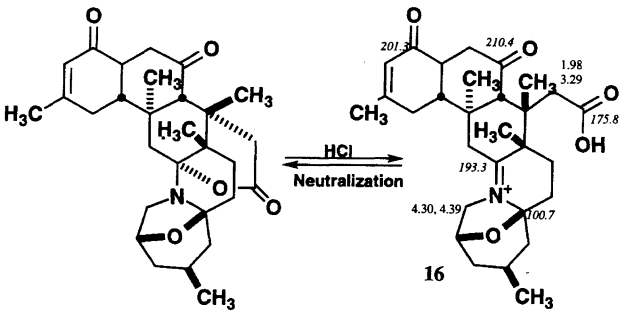

Fig. 16. Equilibrium of norzoanthamine hydrochloride.

hydrochloride significantly suppressed the reduction caused by ovariectomy at the dose of 0.016—0.4 mg/kg/day, i.p. (Fig. 12).

IL-6 is known as a stimulator of osteoclast formation, and suppression of IL-6 secretion can be effective for prevention of osteoporosis. Norzoanthamine and norzoanthamine hydrochloride exhibit the IL-6 induction at the value of 13 and 4.7 $\mu g\,ml^{-1}$, respectively. Therefore, the structure-activity relationship of norzoanthamine was examined for inhibitory effect of IL-6 production, as described below.[30]

Structure-Activity Relationship of Norzoanthamine. Studies on structure-activity relationship of norzoanthamine had been done. The presence of the double bond (C15–C16) and lactone moiety has attracted our attention. Therefore, norzoanthamine was transformed to several derivatives (**12**, **13**, **14**, and **15**) (Fig. 13). These derivatives were obtained in usual condition, as described before. Norzoanthamine (**1**) was reduced by Pd/C to obtain 15,16-dihydronorzoanthamine (**12**). Oxidation of **1** with OsO_4 followed by treatment with Na_2SO_3 gave the desired 15,16-dihydroxy-15,16-dihydronorzoanthamine (**13**). When norzoanthamine was treated

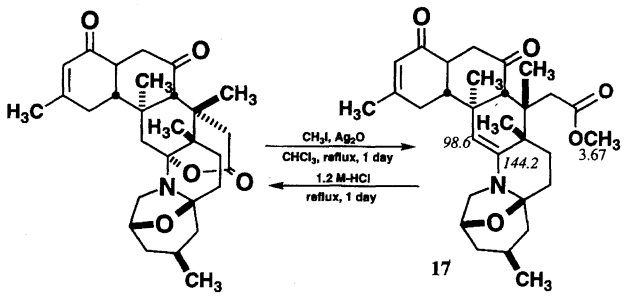

Fig. 17. Esterification of norzoanthamine.

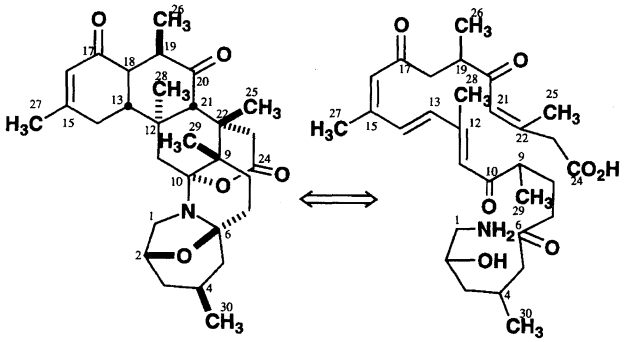

Fig. 18. Proposed biogenesis of zoanthamine.

with $NaBH_3CN$, the carboxylic acid (**14**) was obtained. Furthermore, **14** was transformed into the corresponding methyl ester **15** by treatment with diazomethane. These structures and stereochemistry of derivatives were deduced by NMR spectral data (Fig. 14). H15 of **12** was assigned to be axial based on the large coupling constant ($J_{15,16a} = 12.8$ Hz). Furthermore, the carbon chemical shift of 26-CH_3 ($\delta_C = 22.3$) indicated that methyl group is equatorial.[31] Therefore, the absolute stereochemistry of **12** at C15 was *S*. The structure of **13** was indicated by the NMR and EIMS (*m/z* 485) spectral data. The relative stereochemistry of **13** was also clarified by 2-D NMR spectral data. Interestingly, the ^1H NMR spectral data of **14**, where the proton resonance of C2 position was observed at $\delta_H = 3.86$ (1H, m) shifted to highfield from $\delta_H = 4.54$ (1H, m). Above data and the carbon chemical shift at C7 ($\delta_C = 68.9$) suggested the cleavage of the O–C6 bond. In the ^{13}C NMR spectral data of **14**, the carbon resonance of C10 was observed at $\delta_C = 63.2$. Furthermore, **14** was converted methyl ester **15**. These data indicated the cleavage of O–C10 bond. H10 of **14** was assigned to be axial based on the large coupling constant ($J_{10,11b} = 13.6$ Hz) (Fig. 15). Therefore, the structure of **14** was suggested to be as shown in Fig. 13.

Table 3 shows the inhibitory effect of IL-6 induction of norzoanthamine derivatives. As shown in Table 3, the inhibitory effect of norzoanthamine was reduced by transformation. This data indicated the importance of the double bond (C15–C16) and lactone moiety in the appearance of bioactivity.

Furthermore, we have carried out experiments of equilibration between lactone structure and iminium structure. The NMR spectrum of norzoanthamine hydrochloride in CD_3OD implied the presence of iminium structure (**16**) but not lac-

tone structure (**1**) of norzoanthamine. The ^{13}C NMR spectral data of **16**, the carbon resonance of C10 was observed at $\delta_C = 193.3$ shifted to lowfield from $\delta_C = 101.5$. Furthermore, the carbon resonances of C1 and C6 also shifted to lowfield. These data indicated the structure of **16** as shown in Fig. 16. The iminium structure was further proved by transformation into methyl ester (**17**) which was obtained by treatment of **1** with CH_3I–Ag_2O. The NMR and MS spectral data suggested the structure of **17**. On the other hand, hydrolysis of **17** with 1.2 M HCl (1 M = 1 mol dm^{-3}) gave norzoanthamine, as shown in Fig. 17.

Biogenesis of Zoanthamines. Although zoanthamines have been regarded as terpenoids based on their molecular formulas, the biogenetic pathway of zoanthamines is unclear. Since marine organisms usually produce super carbon chain molecules with terminus amino group, e.g., palytoxin[32] and pinnatoxin,[33] we propose here a polyketide biogenetic pathway for zoanthamines, as shown in Fig. 18.

Conclusion

The novel alkaloid norzoanthamines were isolated from the colonial *Zoanthus* sp. collected on the Ayamaru coast of the Amami Islands. As described above, the absolute configurations of norzoanthamines were determined to be 2*R*, 4*S*, 6*S*, 9*S*, 10*R*, 12*S*, 13*R*, 18*S*, 21*S*, and 22*S*. Furthermore, the biological activities of norzoanthamines were clarified. Decrease in the failure load and the yield energy caused by ovariectomy were significantly suppressed by administration of norzoanthamine hydrochloride. Since the purpose of osteoporosis therapy is prevention of bone fracture, norzoanthamine hydrochloride that suppressed the loss of the bone weight and strength could be a good candidate for osteoporosis. The mechanism of action and the in vivo behaviors of the samples are currently under investigation. Further studies on the detailed chemistry of norzoanthamine, including the biogenetic pathway and structure-activity relationship, are underway in our laboratory. Synthetic studies of the complex molecule are also interesting.

Experimental

The ^1H and ^{13}C NMR spectra were taken on a JEOL GSX 400 spectrometer, using $CDCl_3$ as a solvent. IR spectra were taken on JASCO IR-81O. Mass spectra were measured on JEOL JMS-DX303HF.

Isolation of Norzoanthamine: The genus *Zoanthus* sp. (5.0 kg) which was collected on the Ayamaru coast of the Amami Islands, Kagoshima prefecture, Japan was minced in 10 L of ethanol with a Waring blender. It was soaked in the methanol at low temperature for 2—3 d, and then the solid matter was removed by filtration.

The resulting filtrate was concentrated by an evaporator at a temperature lower than 45 °C to produce about 1 L of the aqueous extract. This residue was extracted three times with each 1 L of EtOAc and concentrated under reduced pressure. The oily matter thus obtained was suspended in a small amount of chloroform, and then charged to a column (3.5 ID×50 cm) packed with silica gel (No. 7734 silica gel 60, manufactured by E. Merck). This column was developed with 100 ml of chloroform, 100 ml of 5% methanol–chloroform, 100 ml of 10% methanol–chloroform, 100 ml of 25% methanol–chloroform, and 100 ml of methanol to collect fractions containing norzoanthamine followed by concentrating under reduced pressure to produce an oily material. Finally, the oily material was purified by preparative thin layer chromatography (PTLC) (No. 13895 Silica Gel 60F 254, manufactured by E. Merck) using ether as a developing solvent to obtain norzoanthamine (21 mg, R_f 0.06).

Detection of norzoanthamine at each purification step was effected by means of HPTLC (No. 13728 Silica Gel 60F 254S, manufactured by E. Merck) using acetonitrile as a developing solvent and an anisaldehyde–sulfuric acid color developing agent, and Draggendorf's reagent.

Biological Activity Tests of Norzoanthamines: 1) Recovery Effect of Norzoanthamines on Bone Weight and Strength in Ovariectomized Mice: Four-weeks-old female ddY mice were obtained from Japan SLC Inc. Ovariectomies and sham operations were carried out under ketamine hydrochloride anesthesia. The mice were divided into 5 groups: sham-operated, ovariectomized control, and sample-treated ovariectomized groups (0.016, 0.08, 0.4 mg/kg/day, i.p.). Administration of vehicle or norzoanthamine hydrochloride dissolved in sterilized water were started from the next day of operations and continued 5 d a week for 4 weeks. After 4 weeks' administration, all the mice were weighed and killed to take their femurs and uterus for measurements. The right femur was used for the measurements of length and dry weight, and the left femur used for biomechanical parameter, failure load. Measurement of bone biomechanical parameter was performed using a bone strength tester (Model TK-252C, Muromachi Kikai Co., Ltd.). The femurs were tested in three-point bending until failure.

2) Suppressive Activity of IL-6 Production: MC3T3-E1 cells (1×10^4 cells per well) were cultivated in wells of 96-well test plate for 3 d until the cell growth reached became confluent. Test drug and 20 ng ml^{-1} of parathyroid hormone (PTH) were added, and the cultivation was further continued for 24 h. Then, the activity of IL-6 secreted in the medium was measured by bioassay using MH-60 cells.

Reduction of Norzoanthamine (1): To a CH_3OH (1 ml) solution of **1** (50.2 mg, 156 μmol) cooled at 0 °C, $NaBH_4$ (10.0 mg, 2.70 μmol) was added, and the mixture was stirred at r.t. for 1 h. The reaction mixture was partitioned between $CHCl_3$ and H_2O, and the organic extract was dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by preparative TLC (CH_3OH : $CHCl_3$ = 3 : 47), and provide **7** (20.9 mg, 44.0 μmol, R_f 0.32) and **8** (16.8 mg, 36.0 μmol, R_f 0.15): ^1H NMR (Table 1), ^{13}C NMR (Table 1).

Reduction of Deoxynorzoanthamine (7): To a CH_3OH (0.5 ml) solution of **7** (2.0 mg, 4.3 μmol) cooled at 0 °C, $NaBH_4$ (0.3 mg, 8 μmol) was added, and the mixture was stirred at r.t. for 1 h. The reaction mixture was partitioned between $CHCl_3$ and H_2O, and the organic extract was dried over Na_2SO_4. After evaporation, the residue was purified by preparative TLC (CH_3OH : $CHCl_3$=3 : 47), furnishing **8** (1.6 mg, 3.4 μmol, R_f 0.15, yield 76%).

Acetylation of Deoxydihydronorzoanthamine (8): A suspension of **8** (1.0 mg, 2.1 μmol) in pyridine (1.0 ml) was stirred at room temperature for 15 min. To the reaction solution, acetic anhydride (0.5 ml) was added and the mixture was stirred for 4 h. The reaction mixture was evaporated with toluene in vacuo to give a residue. After complete evaporation, the residue was purified by preparative TLC (CH_3OH : $CHCl_3$ = 1.5 : 98.5), giving monoacetate **9** (1.0 mg, 1.8 μmol, R_f 0.6, yield 85%): ^1H NMR (CD_3OD) δ = 5.22 (1H, m, H17), 2.04 (–OCO<u>CH</u>$_3$); EIMS *m/z* 535 (M^+).

Reaction of Deoxydihydronorzoanthamine (8) with (−)-

and (+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl Chloride (MTPACl): To a pyridine solution (0.5 mol) containing **8** (2.0 mg, 4.2 μmol), (−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (No. 15526-7, manufactured by Aldrich) (20 μL) was added. After addition, the reaction mixture was stirred for 13 h. To the reaction mixture, 3-(dimethylamino)propylamine (10 μL) was added. The solvent was evaporated off in vacuo and the residue was purified by PTLC (CH_3OH : $CHCl_3$ = 1.5 : 98.5, R_f 0.54) to obtain (*R*)-MTPA ester **10** (1.4 mg, 2.3 μmol): ^{1}H NMR (Table 2).

(*S*)-MTPA ester **11** (1.3 mg, 2.1 μmol) was also obtained form (+)-MTPA chloride: ^{1}H NMR (Table 2).

15,16-Dihydronorzoanthamine (12): Palladium on carbon (10%, 5 mg) was suspended in a CH_3OH solution (3 ml) of **1** (12.3 mg, 27.7 μmol). The slurry was stirred at r.t. under 1 atm of H_2 for 3 h. Removal of the palladium catalyst by filtration through Celite, followed by evaporation of the filtrate under reduced pressure, afforded an oily material. This residue was purified by PTLC (CH_3OH : $CHCl_3$ = 5 : 95, R_f 0.29) to obtain **12** (10.0 mg, 20.7 μmol, yield 74.7%): ^{1}H NMR δ = 3.22 (1H, dd, *J* = 7.0, 6.2 Hz, H-1a), 3.26 (1H, d, *J* = 6.2 Hz, H-1b), 4.52 (1H, m, H-2), 1.44 (1H, ddd, *J* = 13.6, 13.6, 3.1 Hz, H-3a), 1.53 (1H, m, H-3b), 2.27 (1H, m, H-4), 1.07 (1H, dd, *J* = 12.9, 10.9 Hz, H-5a), 2.07 (1H, dd, *J* = 12.9, 3.7 Hz, H-5b), 1.75 (1H, ddd, *J* = 12.5, 3.5, 2.5 Hz, H-7a), 1.78 (1H, m, H-7b), 1.54 (1H, m, H-8a), 1.66 (1H, ddd, *J* = 13.1, 13.1, 3.5 Hz, H-8b), 2.16 (1H, d, *J* = 13.9 Hz, H-11a), 1.86 (1H, d, *J* = 13.9 Hz, H-11b), 1.39 (1H, m, H-13), 1.24 (1H, m, H-14a), 1.84 (1H, m, H-14b), 1.93 (1H, m, H-15), 2.05 (1H, dd, *J* = 12.8, 12.8 Hz, H-$16_{ax.}$), 2.46 (1H, dd, *J* = 3.7, 12.8 Hz, H-$16_{eq.}$), 2.74 (1H, ddd, *J* = 12.1, 12.0, 5.1 Hz, H-18), 2.32 (1H, dd, *J* = 11.8, 5.1 Hz, H-19a), 2.67 (1H, dd, *J* = 12.8, 11.8 Hz, H-19b), 2.79 (1H, S, H-21), 2.34 (1H, d, *J* = 20.1 Hz, H-23a), 3.60 (1H, d, *J* = 20.1 Hz, H-23b), 0.97 (3H, s, 25-CH_3), 1.10 (3H, d, *J* = 6.8 Hz, 26-CH_3), 0.96 (3H, s, 27-CH_3), 1.13 (3H, s, 28-CH_3), 0.89 (3H, d, *J* = 6.6 Hz, 29-CH_3); ^{13}C NMR δ = 22.3 (26-CH_3), 44.3 (C-15), 49.2 (C-16); EIMS *m/z* 483 (M^+).

15,16-Dihydroxy-15,16-dihydronorzoanthamine (13): Osmium(VIII) oxide solution (100 μL, H_2O, 2 wt%) was added to the flask containing **1** (15.2 mg, 31.6 μmol), 4-methylmorpholine *N*-oxide (20.0 mg, 171 μmol), H_2O (0.5 mL), and CH_3OH (1 mL). The reaction mixture was stirred at r.t. for 3 h. Saturated Na_2SO_3 solution (1 mL, H_2O) was added to the reaction mixture, and the mixture was stirred for 30 min. The reaction mixture was partitioned between $CHCl_3$ and H_2O, and organic extract was dried over Na_2SO_4. After evaporation, the residue was purified by PTLC (CH_3OH : $CHCl_3$ = 5 : 95, R_f 0.72), and provide **13** (14.8 mg, 28.7 μmol, yield 90.8%): ^{1}H NMR δ = 4.03 (1H, dd, *J* = 8.4, 8.4 Hz, H-1a), 2.99 (1H, m, H-1b), 4.51 (1H, m, H-2), 1.44 (1H, ddd, *J* = 10.6, 10.6, 3.3 Hz, H-3a), 1.52 (1H, m, H-3b), 2.29 (1H, m, H-4), 1.08 (1H, dd, *J* = 13.2, 11.7 Hz, H-5a), 1.97 (1H, dd, *J* = 13.2, 4.8 Hz, H-5b), 1.78 (1H, ddd, *J* = 19.4, 4.8, 4.8 Hz, H-7a), 1.84 (1H, m, H-7b), 1.59 (1H, m, H-8a), 1.86 (1H, m, H-8b), 1.55 (1H, d, *J* = 13.3 Hz, H-11a), 1.58 (1H, d, *J* = 13.3 Hz, H-11b), 2.75 (1H, m, H-13), 1.52 (1H, m, H-14a), 3.01 (1H, dd, *J* = 13.6, 2.2, H-14b), 3.96 (1H, s, H-16), 2.78 (1H, m, H-18), 2.78 (1H, m, H-19a), 2.32 (1H, m, H-19b), 3.01 (1H, S, H-21), 2.54 (1H, d, *J* = 20.5 Hz, H-23a), 4.04 (1H, d, *J* = 20.5 Hz, H-23b), 1.00 (3H, s, 25-CH_3), 1.46 (3H, s, 26-CH_3), 11.28 (3H, s, 27-CH_3), 1.06 (3H, s, 28-CH_3), 0.89 (3H, d, *J* = 6.6 Hz, 29-CH_3); ^{13}C NMR δ = 27.3 (25-CH_3), 75.8 (C-16), 80.3 (C-15); EIMS *m/z* 515 (M^+).

Carboxylic Acid (14): $NaBH_3CN$ (5.7 mg, 80 μmol) was added to the $CHCl_3$–CH_3OH solution (10.3 mL, pH 5, $CHCl_3$: CH_3OH = 10 : 0.3) containing **1** (8.5 mg, 18 μmol). The reaction mixture was stirred at r.t. for 30 min. The mixture was partitioned between $CHCl_3$ and H_2O, and the organic extract was dried over Na_2SO_4. After evaporation, the residue was purified by PTLC (CH_3OH : $CHCl_3$ = 1 : 9, R_f 0.27), and provided **14** (6.8 mg, 14 μmol, yield 79%): ^{1}H NMR δ = 2.78 (1H, m, H-1a), 3.18 (1H, dd, *J* = 6.8, 6.8 Hz, H-1b), 3.86 (1H, m, H-2), 1.04 (1H, m, H-3a), 1.95 (1H, m, H-3b), 1.75 (1H, m, H-4), 1.43 (1H, m, H-5a), 0.95 (1H, m, H-5b), 2.80 (1H, m, H-6), 1.39 (1H, m, H-7a), 1.92 (1H, m, H-7b), 1.64 (1H, m, H-8a), 1.94 (1H, m, H-8b), 2.69 (dd, *J* = 13.6, 4.7 Hz, H-10), 1.97 (1H, dd, *J* = 14.9, 4.7 Hz, H-11a), 1.86 (1H, dd, *J* = 14.9, 13.6 Hz, H-11b), 2.51 (1H, ddd, *J* = 14.3, 11.8, 5.2 Hz, H-13), 2.32 (1H, dd, *J* = 16.8, 11.8 Hz, H-14a), 2.58 (1H, dd, *J* = 16.8, 5.2 Hz, H-14b), 5.89 (1H, s, H-16), 2.80 (1H, m, H-18), 2.40 (1H, dd, *J* = 14.8, 6.7 Hz, H-19a), 2.55 (1H, m, H-19b), 2.79 (1H, S, H-21), 2.34 (1H, d, *J* = 20.1 Hz, H-23a), 3.60 (1H, d, *J* = 20.1 Hz, H-23b), 0.97 (3H, s, 25-CH_3), 1.10 (3H, d, *J* = 6.8 Hz, 26-CH_3), 0.96 (3H, s, 27-CH_3), 1.13 (3H, s, 28-CH_3), 0.89 (3H, d, *J* = 6.6 Hz, 29-CH_3), 3.86 (1H, m, H-2), 2.59 (1H, m, H-7); ^{13}C NMR δ = 63.2 (C-10), 68.9 (C-7), 71.6 (C-2), 178.0 (C-24); EIMS *m/z* 485 (M^+).

Methyl Ester (15): A solution of *N*-methyl-*N'*-nitrosoguanidine (10 mg, 68 μmol) in 10% aq NaOH (3 mL) and diethyl ether (3 mL) was kept at room temperature. To a suspension of carboxylic acid (**14**) (2.7 mg, 5.6 μmol) in $CHCl_3$–CH_3OH solution, previous diazomethane solution (1 mL) was added, and the mixture was stirred at r.t. for 15 min. The resulting mixture was evaporated in vacuo, and the residue was purified by preparative TLC (CH_3OH–$CHCl_3$ = 4 : 96) to give **8** (2.7 mg, 5.4 μmol, yield 97%): ^{1}H NMR δ = 3.60 (3H, s, –$COOCH_3$); EIMS *m/z* 499 (M^+).

Iminium Salt (16): To a CH_3OH (0.5 mL) solution of **1** (4.8 mg, 10 μmol), 1.2 MHCl (3 drops) was added, and the mixture was stirred at r.t. for 1 h. The reaction mixture was concentrated to obtain iminium salt **16** under reduced pressure: ^{1}H NMR δ = 4.33 (1H, dd *J* = 13.4, 6.2 Hz, H-1a), 4.42 (1H, d, *J* = 13.4 Hz, H-1b), 4.96 (1H, m, H-2), 1.65 (1H, ddd, *J* = 13.9, 11.7, 2.6 Hz, H-3a), 1.92 (1H, m, H-3b), 1.97 (1H, m, H-4), 1.48 (1H, dd, 14.3, 12.5 Hz, H-5a), 2.58 (1H, m, H-5b), 1.94 (1H, m, H-7a), 2.31 (1H, ddd, *J* = 13.9, 3.7, 3.5 Hz, H-7b), 1.64 (1H, ddd, *J* = 14.7, 3.5, 3.3 Hz, H-8a), 2.54 (1H, m, H-8b), 2.95 (1H, d, *J* = 15.8 Hz, H-11a), 3.23 (1H, d, *J* = 15.8 Hz, H-11b), 2.57 (1H, m, H-13), 2.48 (1H, m, H-14a), 2.69 (1H, m, H-14b), 5.90 (1H, s, H-16), 2.73 (1H, m, H-18), 2.61 (1H, m, H-19a), 2.70 (1H, m, H-19b), 3.34 (1H, s, H-21), 1.98 (1H, d, *J* = 15.0 Hz, H-23a), 3.30 (1H, d, *J* = 15.0 Hz, H-23b), 1.35 (3H, s, 25-CH_3), 2.07 (3H, s, 26-CH_3), 1.02 (3H, s, 27-CH_3), 1.50 (3H, s, 28-CH_3), 1.03 (3H, d, *J* = 6.6 Hz, 29-CH_3); ^{13}C NMR δ = 44.2 (C-11), 57.4 (C-1), 100.7 (C-210), 175.8 (C-24), 193.3 (C-10).

Methyl Ester (17): CH_3I (100 μL) was added to the flask containing **1** (9.7 mg, 20.0 μmol), and Ag_2O (28.3 mg, 120 μmol), in $CHCl_3$ (1 mL). The reaction mixture was stirred at reflux temperature for 35 h. After filtration, the filtrate was partitioned between $CHCl_3$ and H_2O, and the organic extract was evaporated under reduced pressure, and affording an oily material. This residue was purified by PTLC (CH_3OH : $CHCl_3$ = 2 : 98, R_f 0.75) to obtain methyl ester **17** (7.1 mg, 14.1 μmol, yield 71.1%): ^{1}H NMR δ = 3.13 (1H, dd, *J* = 8.4, 5.9 Hz, H-1a), 4.42 (1H, dd, *J* = 8.4, 7.9 Hz, H-1b), 4.69 (1H, dddd, *J* = 7.9, 5.9, 2.4, 0.7 Hz, H-2), 1.65 (1H, ddd, *J* = 14.0, 13.1, 2.4 Hz, H-3a), 1.92 (1H, ddd, *J* = 13.1, 5.2, 0.7 Hz, H-3b), 1.97 (1H, qdddd, *J* = 6.4, 14.0, 12.5, 5.2, 5.0 Hz, H-4), 1.48 (1H, dd, *J* = 12.5, 12.5 Hz, H-5a), 2.58 (1H, dd, *J* = 12.5, 5.0 Hz, H-5b), 1.80 (1H, m, H-7a), 0.92 (1H, m, H-7b), 1.80 (1H, m, H-8a), 1.51 (1H, m, H-8b), 4.10 (1H, s, H-11), 2.13 (1H, ddd, *J* = 11.6, 11.5, 4.2 Hz, H-13), 2.29 (1H, dd, *J* = 17.8, 11.5 Hz, H-14a), 2.42 (1H, dd, *J* = 17.8, 4.2 Hz, H-14b), 5.90 (1H, s, H-16), 2.62 (1H,

m, H-18), 2.52 (1H, m, H-19a), 2.64 (1H, m, H-19b), 2.88 (1H, s, H-21), 2.52 (1H, d, J = 14.3 Hz, H-23a), 3.22 (1H, d, J=14.3 Hz, H-23b), 1.22 (3H, s, 25-CH_3), 2.00 (3H, s, 26-CH_3), 1.00 (3H, s, 27-CH_3), 1.14 (3H, s, 28-CH_3), 0.91 (3H, d, J = 6.4 Hz, 29-CH_3), 3.67 (3H, s, –$COOCH_3$); ^{13}C NMR δ = 98.6 (C-11), 142.2 (C-10); EIMS m/z 495 (M^+).

Hydrolysis of Methyl Ester 17: To a dioxane (0.5 mL) solution of methyl ester **17** (3.1 mg, 6.2 μmol), 1.2 M HCl (1.5 mL) was added, and the mixture was stirred at reflux temperature for 12 h. After evaporation, the residue was purified by PTLC (CH_3OH : $CHCl_3$ = 1 : 9, R_f 0.27) to provid **1**.

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