

Guadinomines, Type III Secretion System Inhibitors, Produced by *Streptomyces* sp. K01-0509

II. Physico-chemical Properties and Structure Elucidation

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Dedicated to the late Prof. Shigeo Iwasaki

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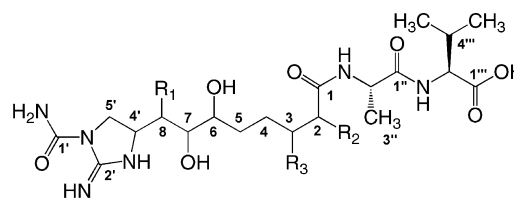
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Abstract The structures of guadinomines, new inhibitors of a bacterial Type III secretion system produced by *Streptomyces* sp. K01-0509, were elucidated by spectroscopic studies including various NMR experiments. Guadinomines A, B, C₁, C₂ and D consist of a carbamoylated cyclic guanidinyll moiety, an alkyl chain moiety and an L-Ala-L-Val moiety in common, while guadinomic acid is a smaller molecule consisting of a carbamoylated cyclic guanidinyll moiety and a hydroxyl hexanoate moiety.

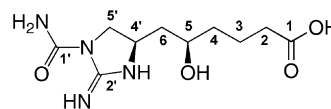
Keywords guadinomines, Type III secretion system, *Streptomyces* sp. K01-0509, EPEC

Introduction

Type III secretion system (TTSS) is a common virulence system present in many Gram-negative bacteria. As described in the preceding study, a conventional assay system was conducted by using TTSS-induced hemolysis in screen for TTSS inhibitors from microbial metabolites [1]. As a result, we discovered six new compounds designated guadinomines A (1) to D (5) and guadinomic acid (6) (Fig.



	R ₁	R ₂	R ₃
Guadinomine A (1)	OH	NH ₂	NH ₂
Guadinomine B (2)	H	NH ₂	NH ₂
Guadinomines C ₁ (3) and C ₂ (4) (diastereomers at C-6')	H		
Guadinomine D (5)	H		NH ₂



Guadinomic acid (6)
(K01-0509 B)

Fig. 1 Structures of guadinomines A (1), B (2), C₁ (3), C₂ (4), D (5) and guadinomic acid (6).

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1), from the culture broth of *Streptomyces* sp. K01-0509. Guadinomines have a carbamoyl cyclic guanidine moiety in common. The taxonomy, fermentation, isolation, and biological properties were described in a preceding paper [2]. In this study, the physico-chemical properties and structure elucidation of guadinomines and guadinomic acid are described.

Materials and Methods

General Experiment

NMR spectra were measured on a Varian XL-400 spectrometer or a Varian Inova 600 spectrometer with ^1H -NMR at 400 or 600 MHz and ^{13}C -NMR at 100 or 150 MHz in D_2O or $\text{D}_2\text{O}/1\%$ TFA. The chemical shifts are expressed in ppm and are referenced to HDO (4.76 ppm) in the ^1H -NMR spectra and the end of both field (0, 200 ppm) in the ^{13}C -NMR spectra. FAB-MS spectra were measured on a JEOL JMS AX-505 HA mass spectrometer. IR spectra (KBr) were taken on a Horiba FT-210 Fourier transform Infrared spectrometer. UV spectra were measured with a Beckman DU640 spectrophotometer. Optical rotation was measured on a JASCO model DIP-181 polarimeter.

Amino Acid Analysis

Guadinomines A (**1**) to D (**5**) ($50\ \mu\text{g}$) were completely hydrolyzed in a gas phase of 6 N HCl ($198\ \mu\text{l}$) and phenol ($2.0\ \mu\text{l}$) at 110°C for 18 hours in a reaction vial in which air was replaced by N_2 gas using the Pico-Tag work station (Waters). In the amino acid analysis, an alkalization reagent

($20\ \mu\text{l}$, EtOH/water/triethylamine; 2 : 2 : 1) was added to the hydrolysates and the mixture was dried *in vacuo*. The hydrolysates were derivatized with a derivatization reagent ($50\ \mu\text{l}$, EtOH/water/triethylamine/phenyl isothiocyanate; 7 : 1 : 1 : 1) at room temperature for 20 minutes and dried *in vacuo*. The PTC-derivatized amino acids were analyzed by HPLC on a Pico-Tag column ($3.9\ \text{i.d.} \times 150\ \text{mm}$, Waters) as described in [3]. HPLC was carried out using HP-1100 systems (Hewlett Packard). To determine the absolute configuration of amino acids in guadinomines, the hydrolysates were analyzed by HPLC using a SUMICHIRAL column ($4.6\ \text{i.d.} \times 250\ \text{mm}$, Sumitomo Chemical Co.) according to an established method [4].

Results

Physico-chemical Properties of Guadinomines

The physico-chemical properties of guadinomines are summarized in Table 1. The strong IR absorption at 1682 or

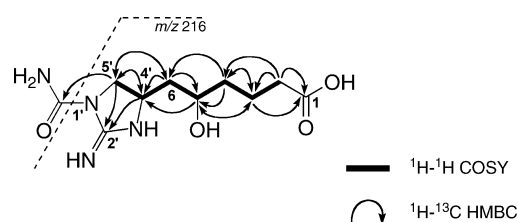


Fig. 2 Selected ^1H - ^1H COSY and HMBC correlations and key mass fragmentation in guadinomic acid (**6**).

Table 1 Physico-chemical properties of guadinomines A (**1**), B (**2**), C₁ (**3**), C₂ (**4**), D (**5**) and guadinomic acid (**6**)

	Guadinomine					Guadinomic acid (6)
	A (1)	B (2)	C ₁ (3)	C ₂ (4)	D (5)	
Appearance	Pale brown powder	Pale brown powder	Colorless powder	Colorless powder	Pale brown powder	Colorless powder
Molecular formula	$\text{C}_{20}\text{H}_{38}\text{N}_8\text{O}_8$	$\text{C}_{20}\text{H}_{38}\text{N}_8\text{O}_7$	$\text{C}_{23}\text{H}_{40}\text{N}_8\text{O}_8$	$\text{C}_{23}\text{H}_{40}\text{N}_8\text{O}_8$	$\text{C}_{22}\text{H}_{40}\text{N}_8\text{O}_8$	$\text{C}_{10}\text{H}_{18}\text{N}_4\text{O}_4$
Molecular weight	518	502	556	556	544	258
FAB-MS (<i>m/z</i>)						
positive	519 [M+H] ⁺ 541 [M+Na] ⁺	503 [M+H] ⁺	557 [M+H] ⁺	557 [M+H] ⁺	545 [M+H] ⁺	259 [M+H] ⁺
HRFAB-MS (<i>m/z</i>)						
calcd.	519.2897	503.2941	557.3047	557.3047	545.3048	259.1406
found [M+H] ⁺	519.2897	503.2956	557.3052	557.3023	545.3055	259.1410
$[\alpha]_D^{26}$ (c 0.1, MeOH)	+14.0°	+11.3°	-7.84°	-5.00°	+4.28°	+31.9°
UV $\lambda_{\text{max}}^{50\% \text{ MeOH}}$ nm (ϵ)	End absorption	End absorption	End absorption	End absorption	End absorption	End absorption
IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1}	1560, 1683	1560, 1683	1564, 1682	1564, 1682	1564, 1682	1564, 1682

1683 cm^{-1} suggested the presence of a primary or secondary amide group. These are readily soluble in water, MeOH and DMSO, but insoluble in CHCl_3 and EtOAc. UV spectra showed end absorbance. All guadinomines turned a characteristic deep-purple color with Fearon reagent [5], suggesting the presence of a ureido or guanidinyll moiety ($-\text{N}-\text{C}(=\text{X})-\text{N}-$, $\text{X}=\text{O}$ or N) in the structures. Furthermore, guadinomines (except for **6**) were positive in the ninhydrin and Rydon-Smith color reaction, suggesting the presence of a primary amino group and a peptide unit. Similarity in physico-chemical properties among guadinomines strongly suggested that they are structurally related.

Amino Acid Analysis of Guadinomines A (1) to D (5)

The Pico-Tag analysis of PTC derivatives of acid hydrolysates of guadinomines (except for **6**) revealed the existence of Ala and Val. Moreover, the stereochemistry was determined to be L-Ala and L-Val by HPLC using a chiral column. Therefore, **1** to **5** have L-Ala and L-Val in the structures.

Structure Elucidation of Guadinomic Acid (6)

Structure elucidation was first carried out for **6**, the simplest compound among the guadinomines. The molecular formula of **6** was determined by HR-FAB-MS to be $\text{C}_{10}\text{H}_{18}\text{N}_4\text{O}_4$, requiring four degrees of unsaturation. The ^1H - and ^{13}C -NMR spectral data of **6** are listed in Table 2. The ^{13}C -NMR and HMQC spectra indicated 10 carbons which were classified into one carboxyl carbon at δ_{C} 183.5, two ureido or guanidinyll carbons at δ_{C} 156.5 and 156.0, one nitrogenated sp^3 methine carbon at δ_{C} 51.5, one oxygenated sp^3 methine carbon at δ_{C} 68.8, four sp^3 methylene carbons, and one nitrogenated methylene carbon at δ_{C} 51.0, thus accounting for three degrees of unsaturation. Therefore, the remaining degree of unsaturation should be due to a ring structure. As shown by the bold lines for **6** in Fig. 2, a proton spin network from H_2-2 (δ_{H} 2.19) to H_2-5' (δ_{H} 3.80, 4.22) became clear from the ^1H - ^1H COSY spectra. On the basis of ^1H - ^{13}C HMBC experiments, the correlations from H_2-2 and H_2-3 (δ_{H} 1.60) to C-1 (δ_{C} 183.5) indicated the carboxyl group is linked to C-2. Moreover, the correlations from H-4' (δ_{H} 4.24) and H_2-5' to C-2' (δ_{C} 156.0) and the geminal coupling constant at C-5' ($^2J_{\text{H-H}}$ 8.5 Hz) in ^1H -NMR spectra indicated the presence of a five-membered cyclic ureido or guanidinyll moiety. On the other hand, the correlations from H_2-5' to C-1' (δ_{C} 156.5) indicated that another ureido or guanidinyll group is connected to N-1'. To confirm this point, the characteristic fragment ion m/z 216 $[\text{M}-(\text{CONH}_2)]^+$ in FAB mass spectrum proved that the carbamoyl group is

Table 2 ^1H - and ^{13}C -NMR spectral data of guadinomic acid (**6**)

Position	6	
	^{13}C	^1H (J in Hz)
1	183.5	—
2	37.1	2.19 t (7.2)
3	22.5	1.60 m
4	36.8	1.48 m
5	68.8	3.75 m
6	41.2	1.82 m
1'-CO	156.5	—
2'	156.0	—
4'	51.5	4.24 m
5'	51.0	4.22 dd (8.5, 5.7) 3.80 dd (8.5, 5.6)

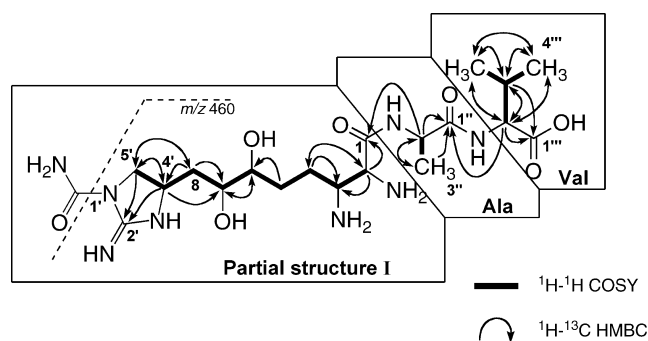


Fig. 3 Selected ^1H - ^1H COSY and HMBC correlations and key mass fragmentation in guadinomine B (**2**).

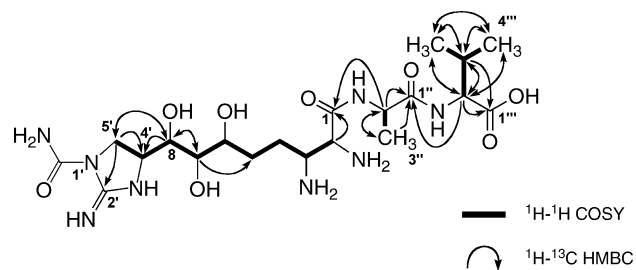


Fig. 4 Selected ^1H - ^1H COSY and HMBC correlations in guadinomine A (**1**).

connected to N-1' and the five-membered cyclic guanidinyll ring is built up. From all the observations described above, the structure of **6** was elucidated as shown in Fig. 1.

Table 3 ^1H - and ^{13}C -NMR spectral data of guadinomines A (**1**), B (**2**), C₁ (**3**), C₂ (**4**) and D (**5**)

Position	1		2		3		4		5	
	^{13}C	^1H (J/in Hz)	^{13}C	^1H (J/in Hz)	^{13}C	^1H (J/in Hz)	^{13}C	^1H (J/in Hz)	^{13}C	^1H (J/in Hz)
1	170.5	—	167.9	—	168.4	—	168.3	—	176.6	—
2	56.4	4.10 m	56.2	4.23 d (3.4)	56.0	4.43 d (3.4)	59.8	4.50 d (3.4)	55.8	4.81 d (3.4)
3	55.5	3.75 m	54.7	3.75 m	53.8	3.97 m	53.8	3.99 m	53.8	3.73 m
4	27.9	2.03 m	28.5	1.92 m	30.3	1.71 m	30.4	1.71 m	25.2	1.82 m
		1.82 m		1.73 m		1.57 m		1.57 m		1.69 m
5	29.6	1.82 m	29.8	1.67 m	29.6	1.76 m	30.1	1.76 m	22.5	1.66 m
		1.58 m		1.40 m		1.44 m		1.44 m		1.42 m
6	75.4	3.78 m	76.2	3.42 m	76.7	3.48 m	76.8	3.48 m	72.6	3.44 m
7	76.6	3.61 dd (7.0, 6.8)	74.3	3.52 m	74.1	3.61 m	74.2	3.61 m	72.0	3.57 m
8	74.4	3.88 dd (7.0, 2.1)	38.8	1.83 m	38.8	1.93 ddd (14, 5.6, 2.1)	38.8	1.93 ddd (14, 5.6, 2.1)	37.0	1.90 ddd (14, 5.6, 2.1)
				1.70 m		1.77 m		1.77 m		1.78 m
1'-CO	158.4	—	158.4	—	158.9	—	158.9	—	156.1	—
2'	158.4	—	159.0	—	158.9	—	158.9	—	157.2	—
4'	56.9	4.40 m	53.9	4.22 m	54.0	4.23 m	54.0	4.23 m	52.2	4.22 m
5'	49.4	4.11 m	53.1	4.11 dd (8.4, 8.4)	53.1	4.23 dd (8.4, 8.4)	53.2	4.23 dd (8.4, 8.4)	53.5	4.18 dd (8.4, 8.4)
		4.10 m		3.65 dd (8.5, 5.6)		3.79 dd (8.5, 5.6)		3.79 dd (8.5, 5.6)		3.77 dd (8.5, 5.6)
6'	—	—	—	—	53.3	4.17 q (7.0)	54.9	4.09 q (7.0)	175.7	—
7'	—	—	—	—	172.1	—	172.1	—	22.7	2.03 s
6''-CH ₃	—	—	—	—	18.2	1.55 d (6.9)	17.0	1.53 d (6.9)	—	—
Ala										
1''	176.4	—	177.3	—	176.6	—	176.6	—	175.6	—
2''	52.9	4.46 q (7.0)	52.5	4.73 q (7.0)	52.2	4.44 q (7.0)	52.2	4.44 q (7.0)	50.6	4.35 q (7.0)
3''	19.2	1.43 d (7.0)	19.2	1.32 d (7.0)	19.1	1.39 d (7.0)	19.2	1.39 d (7.0)	17.2	1.35 d (7.0)
Val										
1'''	180.4	—	177.7	—	179.2	—	179.0	—	176.6	—
2'''	63.7	4.05 d (6.0)	61.5	4.09 d (6.0)	62.4	4.07 d (7.0)	62.4	4.07 d (7.0)	59.9	4.10 d (6.0)
3'''	32.9	2.07 dsep (6.9, 6.5)	32.3	2.07 dsep (6.9, 6.5)	32.5	2.11 dsep (6.9, 6.5)	32.6	2.12 dsep (6.9, 6.5)	30.6	2.12 dsep (6.9, 6.5)
3'''-CH ₃	19.4	0.93 d (6.9)	20.0	0.89 d (6.9)	20.1	0.92 d (6.9)	20.1	0.92 d (6.9)	17.5	0.89 d (6.9)
3'''-CH ₃	21.0	0.92 d (6.9)	20.9	0.89 d (6.9)	21.2	0.92 d (6.9)	21.1	0.92 d (6.9)	19.2	0.91 d (6.9)

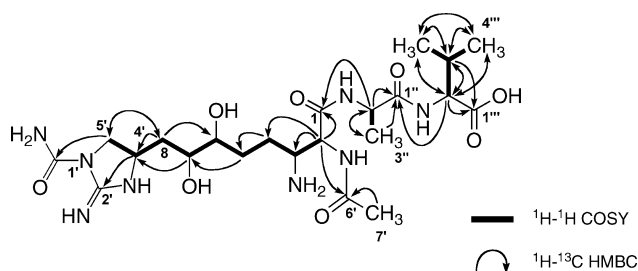


Fig. 5 Selected ^1H - ^1H COSY and HMBC correlations in guadinomine D (**5**).

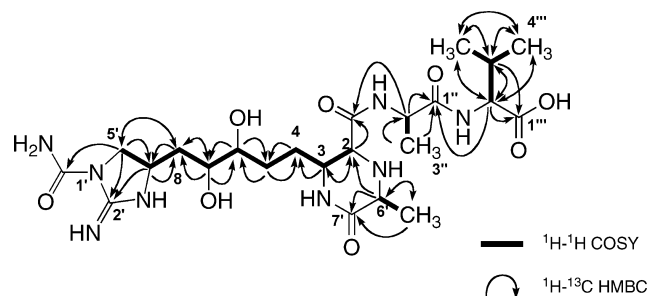


Fig. 6 Selected ^1H - ^1H COSY and HMBC correlations in guadinomine C₁ (**3**).

Structure Elucidation of Guadinomine B (**2**)

The molecular formula of **2** was determined by HR-FAB-MS to be $\text{C}_{20}\text{H}_{38}\text{N}_8\text{O}_7$, requiring six degrees of unsaturation. The ^1H - and ^{13}C -NMR spectral data of **2** are shown in Table 3. The ^{13}C -NMR and HMQC spectra indicated 20 carbons, which were classified into one carboxyl carbon at δ_{C} 177.7, two amide carbonyl carbons at δ_{C} 177.3 and 167.9, two ureido or guanidiny carbons at δ_{C} 158.4 and 159.0, three methylene carbons, eight sp^3 methine carbons, one nitrogenated methylene carbon and three methyl carbons. The ^1H - and ^{13}C -NMR spectral data (Table 3) resembled those of **6** (Table 2) in part. As shown in Fig. 3, the partial structure I containing the whole structure **6** became clear from the 2D NMR spectra. Furthermore, the ^1H - ^1H COSY experiments revealed a proton spin network from H-2 (δ_{H} 4.23) to H₂-5' (δ_{H} 4.11, 3.65), and the correlation from H-2 to C-1 (δ_{C} 167.9) in ^1H - ^{13}C HMBC spectra indicated that the carbonyl group is linked to C-2 (Fig. 3). The connectivity of the partial structure I and two amino acid residues was shown from the observation of the HMBC correlations. The correlation from H-2'' (δ_{H} 4.73) to C-1 and from H-2'', H₃-3'' (δ_{H} 1.32) and H-2''' (δ_{H} 4.09) to C-1'' (δ_{C} 177.3) indicated the linkage of I-L-Ala-L-Val (Fig. 3). Moreover, the chemical shifts and the degrees of unsaturation indicated that two amino residues are attached to C-2 (δ_{C} 56.2) and C-3 (δ_{C} 54.7) and that two hydroxyl groups are attached to C-6 (δ_{C} 76.2) and C-7 (δ_{C} 74.3). From all the observations described above, the structure of **2** was elucidated as shown in Fig. 1.

Structure Elucidation of Guadinomine A (**1**)

The molecular formula of **1** was determined by HR-FAB-MS to be $\text{C}_{20}\text{H}_{38}\text{N}_8\text{O}_8$, indicating the presence of one additional oxygen atom compared with that of **2**. The ^1H - and ^{13}C -NMR spectral data (Table 3) resembled those of **2** except for the proton and carbon signals of C-8. The differences are explained below. In the HMBC spectrum of **1**, the correlations from H-4' (δ_{H} 4.40), H₂-5' (δ_{H} 4.11,

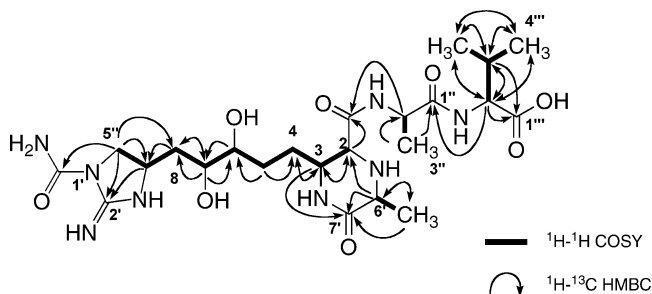


Fig. 7 Selected ^1H - ^1H COSY and HMBC correlations in guadinomine C₂ (**4**).

4.10) and H₂-7 (δ_{H} 3.61) to C-8 (δ_{C} 74.4) and from H-8 (δ_{H} 3.88) to C-4' (δ_{C} 56.9), C-5' (δ_{C} 49.4) and C-7 (δ_{C} 76.6) indicated that a hydroxyl group is connected to C-8 of **2** (Fig. 4). From these observations described above, the structure of **1** was elucidated as shown in Fig. 1.

Structure Elucidation of Guadinomine D (**5**)

The molecular formula of **5** was determined by HR-FAB-MS to be $\text{C}_{22}\text{H}_{40}\text{N}_8\text{O}_8$, indicating the presence of an additional $\text{C}_2\text{H}_2\text{O}$ unit compared with that of **2**. The ^1H - and ^{13}C -NMR spectral data (Table 3) resembled those of **2** except for the proton signal of H-2 (δ_{H} 4.81). In the HMBC spectrum of **5**, the correlations from H-7' (δ_{H} 2.03) and H-2 (δ_{H} 4.81) to C-6' (δ_{C} 175.7) indicated that an *N*-acetyl group is connected to the C-2 of **2** (Fig. 5). These results were supported by the down-field shifts of H-2 (δ_{H} 4.23 to δ_{H} 4.81). From these observations described above, the structure of **5** was elucidated as shown in Fig. 1.

Structure Elucidation of Guadinomines C₁ (**3**) and C₂ (**4**)

The same molecular formulas of **3** and **4** were revealed by HR-FAB-MS to be $\text{C}_{23}\text{H}_{40}\text{N}_8\text{O}_8$, thus requiring one more degree of unsaturation compared with that of **2**. The similarity in the ^{13}C -NMR spectra (Table 3) and ^1H - ^{13}C HMBC correlations (Fig. 6 and 7) of **3** and **4** strongly

suggested that they have the same planar structure. The ^{13}C -NMR spectral data of **3** and **4** resembled those of **2** (Table 3) except for the signals of a methyl carbon (δ_{C} 18.2), a methine carbon (δ_{C} 53.3) and a carboxyl carbon (δ_{C} 172.1). As shown by the bold lines for **3** in Fig. 6, the spin network of C-6'-CH₃ (δ_{H} 1.55) and H-6' (δ_{H} 4.17) was shown from the ^1H - ^1H COSY spectra. In the HMBC spectrum of **3** (Fig. 6), the correlations from H-6' (δ_{H} 4.17) to C-2 (δ_{C} 56.0) and C-7' (δ_{C} 172.1) and from C-6'-CH₃ to C-7' were observed. Furthermore, taking the degree of unsaturation into consideration, it was concluded that a 2-oxo-3-methylpiperazine ring was formed. From all the observations described above, the identical planar structure of **3** and **4** was elucidated as shown in Fig. 1.

Discussion

Guadinomines A, B, C₁, C₂ and D consist of a carbamoylated cyclic guanidinyll moiety, an alkyl chain moiety having several hydroxyl and amino groups and a dipeptide moiety. The relative stereochemistry of six stereogenic centers in the side chain was not elucidated. Guadinomic acid is the simplest molecule having a carbamoylated cyclic guanidinyll moiety and a hydroxyl hexanoate moiety. This compound was synthesized asymmetrically by Tsuchiya *et al.* as K01-0509B and they confirmed the connected position of carbamoyl group and its stereochemistry as shown in Fig. 1 [6]. The common substructure of the cyclic guanidinyll moiety is very unique and only a few natural products have been reported to share the substructure, namely microbial NA22598A1 [7],

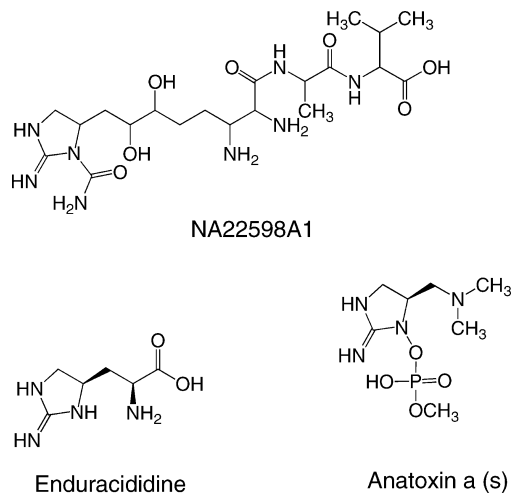


Fig. 8 Structures of natural compounds having cyclic guanidinyll moiety.

enduracidin [8], mannopeptimycin [9], enduracididine of plant origin [10], and anatoxin a(s) of algal origin [11] (Fig. 8). The planar structure of NA22598A1 produced by *Streptomyces* sp. was very similar to that of guadinomine B except for the position of the carbamoyl group.

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