VOL. 54 NO. 12, DEC. 2001 THE JOURNAL OF ANTIBIOTICS pp. 1045 — 1053

Goadsporin, a Chemical Substance which Promotes Secondary Metabolism and Morphogenesis in Streptomycetes

II. Structure Determination

YASUHIRO IGARASHI*, YUKIKO KAN[a], KIYONAGA FUJII[b], TSUYOSHI FUJITA[a], KEN-ICHI HARADA[b], HIDEO NAOKI[a], HIROKAZU TABATA, HIROYASU ONAKA and TAMOTSU FURUMAI

Biotechnology Research Center, Toyama Prefectural University
Kosugi, Toyama 939-0398, Japan
[a] Suntory Institute for Bioorganic Research
Mishima, Osaka 618-8503, Japan
[b] Faculty of Pharmacy, Meijo University
Tempaku, Nagoya 468-8503, Japan

(Received for publication July 30, 2001)

The structure of goadsporin was determined by using spectroscopic techniques. NMR analysis revealed that goadsporin consists of 19 amino acids, two of which are dehydroalanines (Deala), and six of which are cyclized to oxazoles (Oxz) and thiazoles (Thz) by dehydrative cyclization and dehydrogenation from serine, threonine and cysteine. NMR analysis established seven partial structures, and their sequence was determined by CID-MS/MS. Negative mode FAB-MS/MS gave product ions arising from charge-remote fragmentation that allowed determination of the sequence of the amino acid components as AcNH-Ala-MeOxz-Val-Deala-MeOxz-Ile-Leu-Thz-Ser-Gly-Gly-MeOxz-Leu-Deala-Oxz-Ala-Gly-Thz-Val-OH. The chiral amino acids were determined by the advanced Marfey's method to have L-configurations.

Goadsporin was discovered from the culture broth of *Streptomyces* sp. TP-A0584 as a chemical substance that promotes sporulation and secondary metabolism in actinomycetes. Fermentation of the producing strain and isolation and biological properties of goadsporin were described in the preceding paper[1]. We herein report on the physico-chemical properties and structure determination of goadsporin.

Results and Discussion

Physico-chemical Properties

Physico-chemical properties of goadsporin are summarized in Table 1.

NMR Analysis

NMR data for goadsporin measured in DMSO-d_6 are summarized in Table 2. The ^{13}C NMR spectrum revealed 72 carbon signals, and all one-bond ^{1}H-^{13}C connectivities were determined by editing-HSQC experiment. In the ^{1}H NMR spectrum, doublet and triplet methyl groups at 0.85~1.40 ppm, the α-protons of amino acid residues at 3.82~5.00 ppm and the exchangeable amide protons at 7.51~10.01 ppm were observed. In addition, three singlet methyl groups around 2.5 ppm and seven olefinic protons around 5.7~6.0 ppm and 8.2~8.7 ppm were detected. These spectral data suggested that goadsporin is an oligopeptide conjugated with unusual amino acids and/or aromatic residues. Further analyses by DQF-COSY and TOCSY experiments lead to the identification of the presence of three glycines, two alanines, two valines, two leucines, one isoleucine and one serine.

* Corresponding author: yas@pu-toyama.ac.jp

Table 1. Physico-chemical properties of goadsporin.

Appearance	Colorless powder
MP	172~174°C
$[\alpha]_D^{22}$	-21.0 (*c* 1.0, MeOH)
MALDI-TOFMS	
Found:	1634.6492 $[M+Na]^+$
Calcd:	1634.6491 for $C_{72}H_{97}N_{19}O_{20}S_2Na$
Molecular formula	$C_{72}H_{97}N_{19}O_{20}S_2$
UV λ_{max}^{MeOH} nm (log ε)	
in MeOH	204 (4.93), 226 (4.91)
in 0.01N HCl-MeOH (1:9)	226 (5.02)
in 0.01N NaOH-MeOH (1:9)	205 (5.29), 226 (4.90)
IR ν_{max} (cm^{-1})	3500, 2960, 1750, 1630
Solubility	
soluble in	MeOH, DMSO
slightly soluble in	$CHCl_3$, EtOAc
TLC[a] Rf	0.34
HPLC[b] Rt	13.4 min

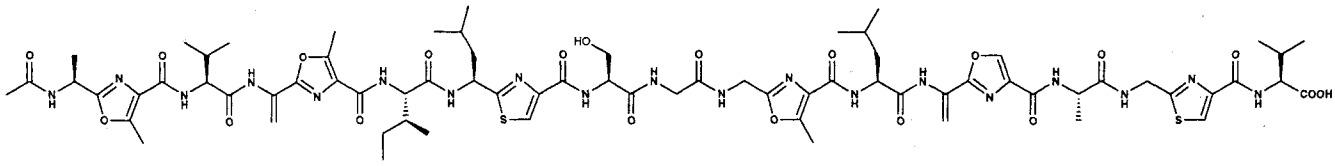
[a] Silica gel TLC (Merck Art 5715): ($CHCl_3$-MeOH=5:1)
[b] HPLC conditions: Cosmosil AR-II (250 × 4.6 mm, i.d.), mobile phase: CH_3CN-0.15% KH_2PO_4 (pH 3.5) (50:50), flow rate: 0.7 ml/min, detection: UV-254 nm.

Other components and partial structures were elucidated by HMBC and ROESY experiments. The amino group of the terminal alanine (Ala^1) is acetylated: the long-range couplings were observed from the methyl proton of the acetyl group and the α-proton of Ala^1 to the carbonyl carbon of the acetyl group. It was therefore determined that *N*-acetylalanine is located at the *N*-terminal of goadsporin. The singlet methyl group at 2.51 ppm is long-range coupled to 4- and 5-positions of oxazole ($MeOxz^2$) and the carbonyl carbon at 160.70 ppm, to which is long-range coupled from the α- and amide protons of valine (Val^3). The *exo*-methylene protons at 5.69 and 5.83 ppm were assigned to the dehydroalanine ($Deala^4$). The HMBC correlation from the *exo*-methylene protons to the α-carbon and an sp^2 carbon at 155.32 ppm, and that from the singlet amide proton at 10.01 ppm to the carbonyl carbon of Val^3 and the carbon at 155.32 ppm established partial structure **B**. The methyl group due to another methyloxazole ($MeOxz^5$) has long-range coupling to the carbonyl carbon at 160.32 ppm which is correlated with the α- and amide protons of isoleucine (Ile^6). The amide and the α-protons of leucine (Leu^7) have HMBC correlation to the carbonyl carbon of Ile^6. In addition, HMBC correlations were observed from the α- and β-protons of Leu^7 to the sp^2 carbon (174.56 ppm) at the 2-position of thiazole (Thz^8). Thiazoles could be distinguished from oxazoles on the basis of the carbon chemical shifts: C-5 of thiazoles appear upfield to C-5 of oxazoles whereas the C-2 of thiazoles are downfield to those of oxazoles in the ^{13}C NMR spectrum. Therefore, the presence of partial structure **C** was confirmed. Partial structure **D**, the sequence of Ser^9-Gly^{10}-Gly^{11}, was deduced from the HMBC correlations observed between the amino acids. Furthermore, substructure **E** was established by the long-range couplings from the methyl group at 2.53 ppm and the amide NH of Leu^{13} to the carbonyl carbon at 160.83 ppm, ROESY correlation between the α-proton of Leu^{13} and the amide proton of $Deala^{14}$, and the HMBC correlations from the H-5 of Oxz^{15} and the methylene and the amide protons of $Deala^{14}$ to the C-2 of Oxz^{15}. The presence of another thiazole (Thz^{18}) was confirmed by the HMBC correlation from H-5 to C-4 and C-2 (170.34 ppm), to which was coupled the methylene proton of Gly^{17} (partial structure **F**). HMBC correlations were detected from the α- and β-protons of Val^{19} to the carbonyl carbon at 172.59 ppm. If goadsporin is a linear peptide, the carbons at the C-1 positions of Ala^1, $Deala^4$, Gly^{11} and Val^{19} must be

Table 2. NMR data for goadsporin in DMSO-d_6.

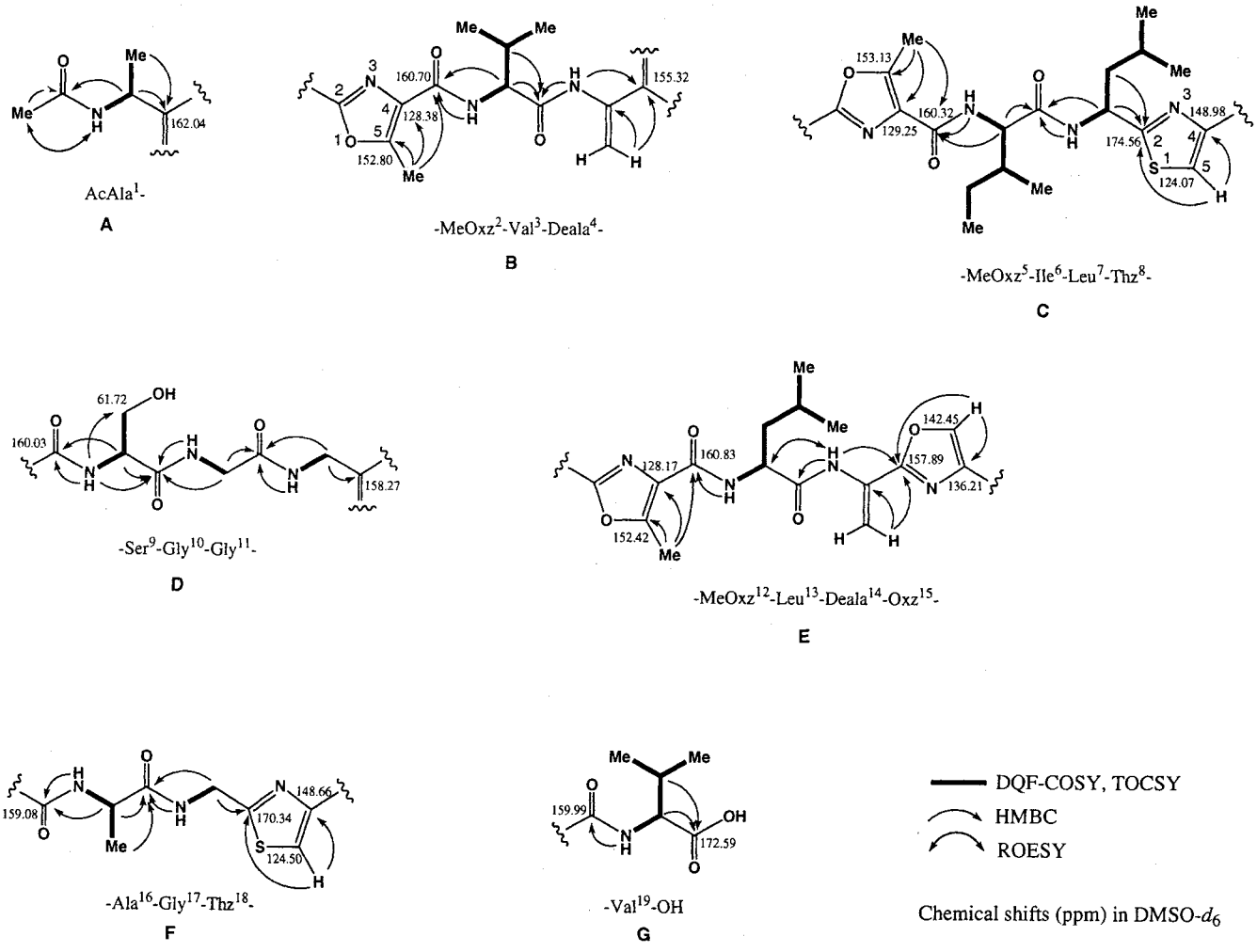
Position	^{1}H	^{13}C	Position	^{1}H	^{13}C
AcAla[1]			Gly[10]		
α	5.00 (quint, 7.3)	42.18	α	3.82 (dd, 5.9, 16.9)	42.08
βCH$_3$	1.40 (d, 7.1)	18.70		3.75 (dd, 5.5, 16.9)	
NH	8.50 (d, 7.8)		CO		169.11
Ac-CO		168.74	NH	8.46 (t, 5.2)	
Ac-CH$_3$	1.83 (s)	22.40	Gly[11]		
MeOxz[2]			α	4.39 (dd, 6.0, 17.9)	35.69
2		162.04		4.37 (dd, 5.3, 17.4)	
4		128.38	NH	8.53 (t, 5.6)	
5		152.80	MeOxz[12]		
5-CH$_3$	2.51 (s)	11.27	2		158.27
CO		160.70	4		128.17
Val[3]			5		153.13
α	4.62 (m)	56.94	5-CH$_3$	2.53 (s)	11.29
β	2.17 (m)	31.31	CO		160.83
γCH$_3$	0.96 (d, 6.7)	19.09	Leu[13]		
γCH$_3$	0.91 (d, 7.0)	17.83	α	4.72 (dt, 3.8, 9.4)	51.15
CO		170.66	βCH$_2$	1.74 (m)	40.67
NH	7.55 (d, 9.1)			1.58 (m)	
Deala[4]			γ	1.62 (m)	24.42
α		128.95	δCH$_3$	0.89 (d)*	23.02
βCH$_2$	5.69 (s)	109.20	δCH$_3$	0.89 (d)*	21.47
	5.83 (s)		CO		171.84
NH	10.01 (s)		NH	7.94 (d, 8.6)	
MeOxz[5]			Deala[14]		
2		155.32	α		128.95
4		129.25	βCH$_2$	5.96 (s)	108.02
5		153.13		5.70 (s)	
5-CH$_3$	2.58 (s)	11.39	NH	9.88 (s)	
CO		160.32	Oxz[15]		
Ile[6]			2		157.89
α	4.49 (m)	56.08	4		136.21
β	1.87 (m)	37.39	5	8.69 (s)	142.45
γCH$_2$	1.06 (m)	24.03	CO		159.08
	1.43 (m)		Ala[16]		
γCH$_3$	0.88 (d, 5.9)	15.57	α	4.53 (quint, 7.2)	48.02
δCH$_3$	0.82 (t, 7.3)	10.87	βCH$_3$	1.37 (d, 7.1)	18.34
CO		170.75	CO		172.36
NH	7.51 (d, 9.3)		NH	8.06 (d, 7.6)	
Leu[7]			Gly[17]		
α	5.19 (q, 7.7)	49.30	α	4.65 (dd, 6.3, 16.5)	40.48
βCH$_2$	1.79 (t, 7.5)	43.07		4.58 (dd, 5.7, 16.5)	
γ	1.68 (sep, 6.7)	24.26	NH	9.06 (t, 5.6)	
δCH$_3$	0.92 (d, 6.3)	22.91	Thz[18]		
δCH$_3$	0.86 (d, 6.5)	21.10	2		170.34
NH	9.02 (d, 8.1)		4		148.66
Thz[8]			5	8.21 (s)	124.50
2		174.56	CO		159.99
4		148.98	Val[19]		
5	8.19 (s)	124.07	α	4.32 (br.t, 4)	57.09
CO		160.03	β	2.18 (m)	30.34
Ser[9]			γCH$_3$	0.90 (d)*	19.11
α	4.50 (m)	54.85	γCH$_3$	0.89 (d)*	17.90
βCH$_2$	3.76 (dd, 4.9, 10.7)	61.72	CO		172.59
	3.68 (dd, 4.5, 11.0)		NH	7.86 (d, 8.7)	
CO		169.99			
NH	8.07 (d, 7.7)				

Fig. 1. Structure of goadsporin.



AcAla-MeOxz-Val-Deala-MeOxz-Ile-Leu-Thz-Ser-Gly-Gly-MeOxz-Leu-Deala-Oxz-Ala-Gly-Thz-Val-OH

Fig. 2. Partial structures of goadsporin elucidated by NMR analysis.



assigned to the 2-positions of three methyloxazoles and the *C*-terminal carboxyl carbon. Since the chemical shift of the carbonyl carbon of Val^{19} was too far downfield for the C-2 carbon of an oxazole, Val^{19} was determined to be the *C*-terminal. Evidence for this conclusion was provided by the HMBC experiment with the methyl ester of goadsporin, which was prepared by the treatment of goadsporin with methyl iodide and 1,8-diazabicyclo[5.4.0]undecene in

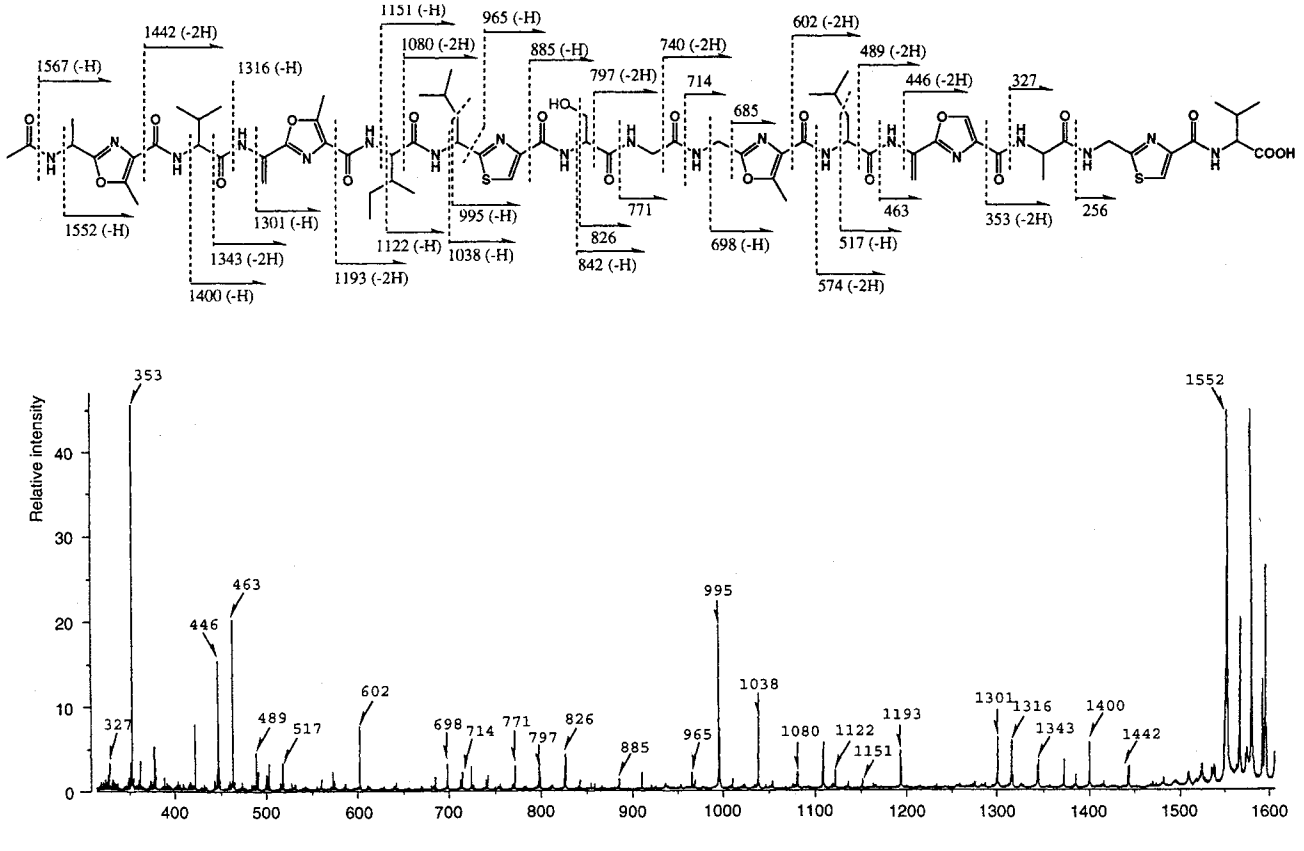
acetonitrile: long-range coupling was detected from the α-proton of Val[19] and the methyl proton of the methyl ester to the carbonyl carbon of Val[19] (data not shown). Though the *N*-terminal AcAla[1], *C*-terminal Val[19] and five peptide fragments were identified and their sequence was deduced to be linear, their order of connection was not established by NMR experiments including ^{1}H-^{15}N HMBC.

MS/MS Analysis

To elucidate the sequence of partial structures, goadsporin was subjected to collision-induced dissociation tandem mass spectrometry (CID-MS/MS). Both positive and negative FAB ionization modes were examined. In each measurement, $[M+H]^+$ at *m/z* 1612 and $[M-H]^-$ at *m/z* 1610 was chosen as the precursor ion for the MS/MS experiment.

In the negative mode, most of the ions were assigned to the fragments containing the *C*-terminal carboxylic acid generated by charge-remote fragmentation (Fig. 3). The observed ions generally arose from bond dissociation between the carbonyl carbon and the amide nitrogen (CO–NH), between the amide nitrogen and the α-carbon (CONH–CH), or between the α-carbon and the amide carbonyl carbon (CH–CONH), as is often observed with peptide compounds. By analyzing the mass of the detected fragments in referring to the possible alignment of the partial structures, the total structure of goadsporin was determined as follows. The carbon at C-1 of Ala[1] (162.04 ppm) must be the one at the 2-position of MeOxz in the partial structures **B**, **C** or **E** because the carbonyl carbons at the *N*-terminal side in **D** (160.03 ppm), **F** (159.08 ppm) or **G** (159.99 ppm) did not coincide with it. Among the three possible sequences, **A-B** was the most probable since the fragment ions at *m/z* 1400 and 1343, which could be generated by the cleavage at both sides of the α-carbon of Val, were observed. By similar reasoning, the C-1 carbon of Deala[4] should be the one at C-2 of MeOxz in **C** or **E**. Though the ions at *m/z* 1080 and 1038 suggested either possibility, the fragments at *m/z* 995 and 885 were likely generated from the partial structure **A-B-C**-. The observation of the ions at *m/z* 797, 771, 740, 714 and 698 supported the sequence of **A-B-C**-Ser-Gly-Gly-. Partial structure **E** has to be linked at the *C*-terminal side of Gly in

Fig. 3. Negative mode product ion spectrum and charge remote fragmentation of goadsporin.



D as confirmed by the observation of *m/z* 574, 517, 463 and 446. Consequently, the remaining fragments must complete the total sequence of the partial structures as **A-B-C-D-E-F-G**. Ions at *m/z* 995 and 517 were assigned to fragments derived from the side chain cleavage of isopropyl groups in leucines. Assignable fragmentations in the oxazole or thiazole rings were not observed. In the mass range less than *m/z* 500~600, ion peaks derived from secondary collision induced dissociation of the first-generated fragments were observed.

The positive-ion mode MS/MS experiment provided additional evidence to support the proposed structure (Fig. 4). Relative intensities of the ion peaks indicated that dominant fragmentation took place at the amide bonds between Val[3] and Deala[4], Ile[6] and Leu[7], and Leu[13] and Deala[14], giving fragment ions containing the *N*-terminal and *C*-terminal at *m/z* 294 and 1319, *m/z* 557 and 1056, and *m/z* 1148 and 465, respectively. Among these major fragment ions, those at *m/z* 1319 and 1056 were likely further cleaved into smaller fragments by CID. Ions at *m/z* 827 and 855 were considered to be derived from the fragment ion at *m/z* 1319 and those at *m/z* 564 and 592

Fig. 4. Positive mode product ion spectrum and fragmentation of goadsporin.

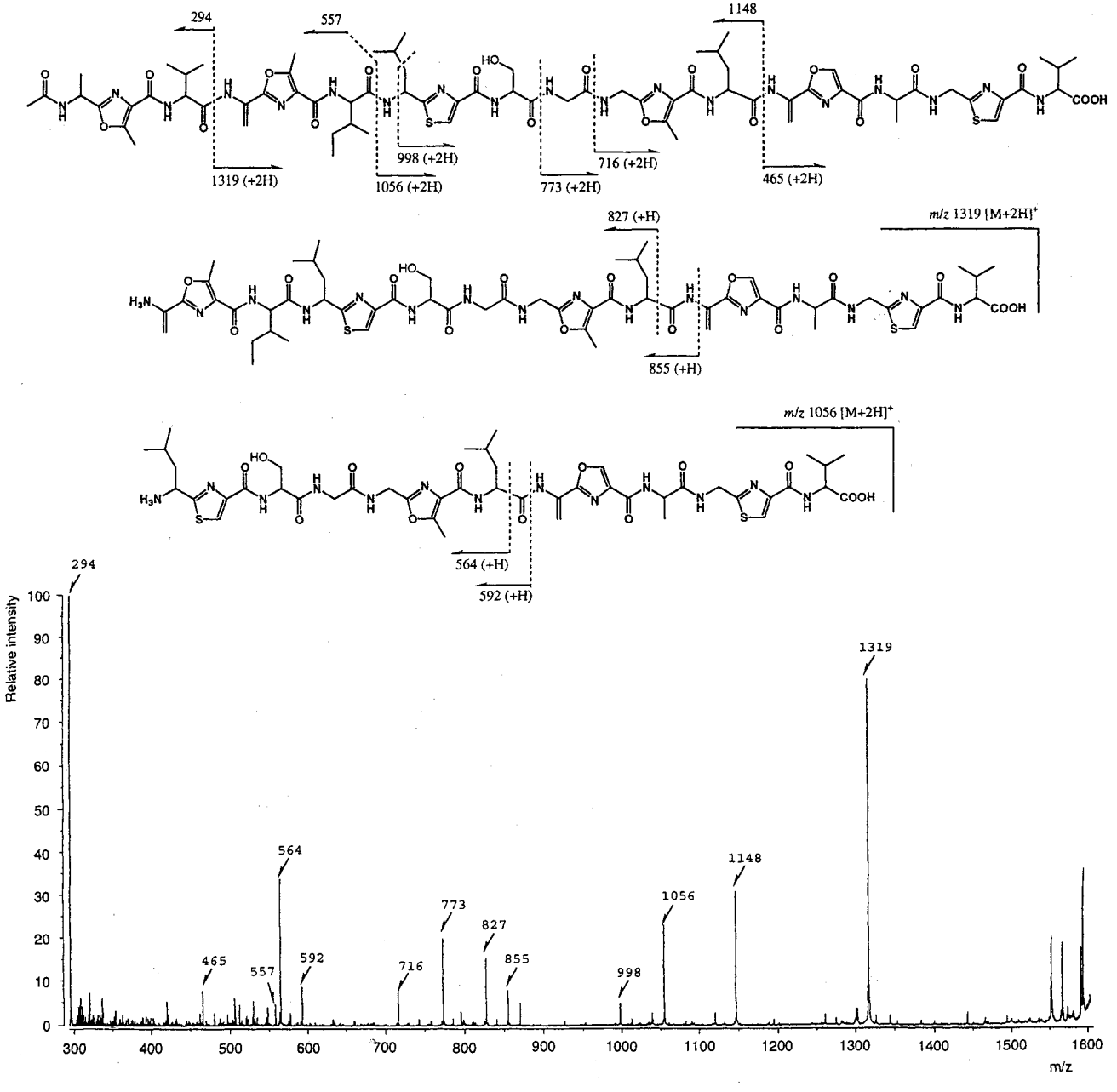
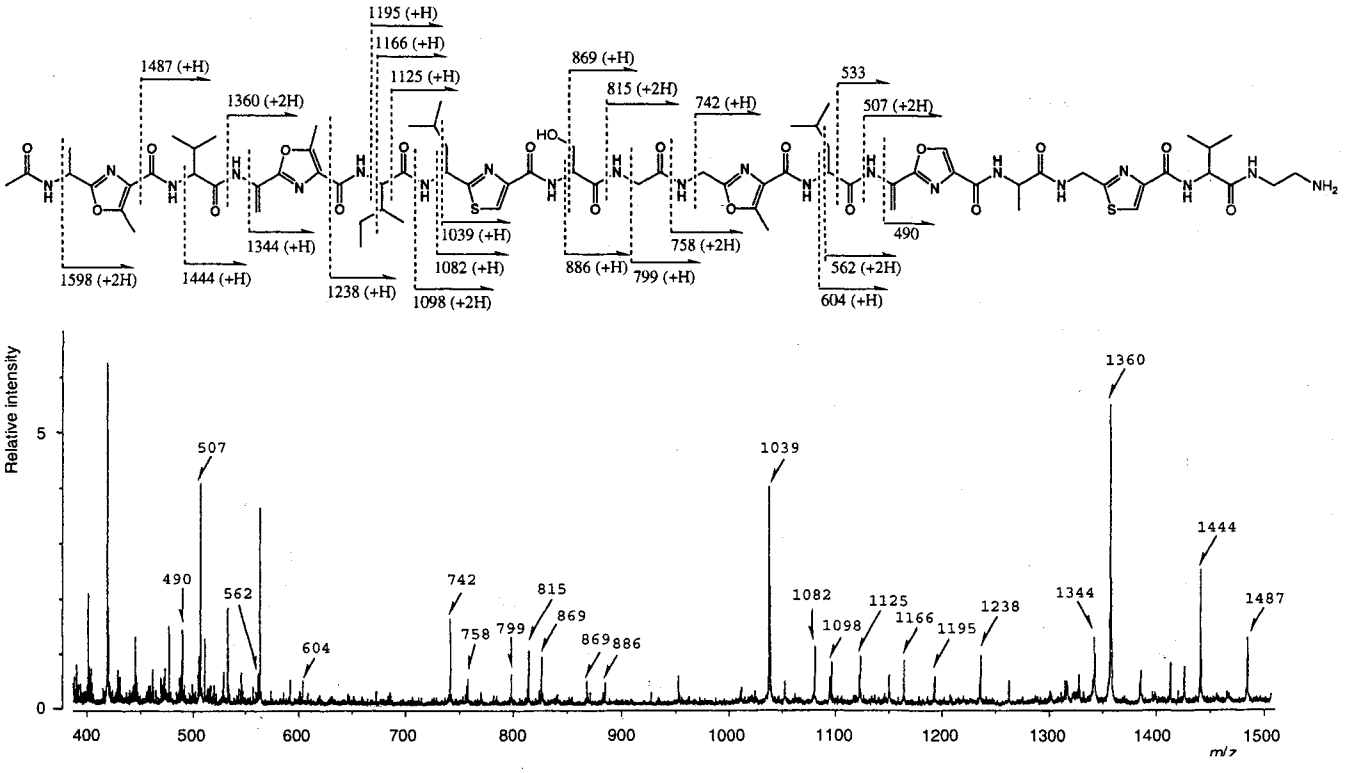


Fig. 5. Product ion spectrum and charge remote fragmentation of the diaminoethane derivative of goadsporin.



from the one at *m/z* 1056. Some of the detected ion peaks could not be assigned to any appropriate structures.

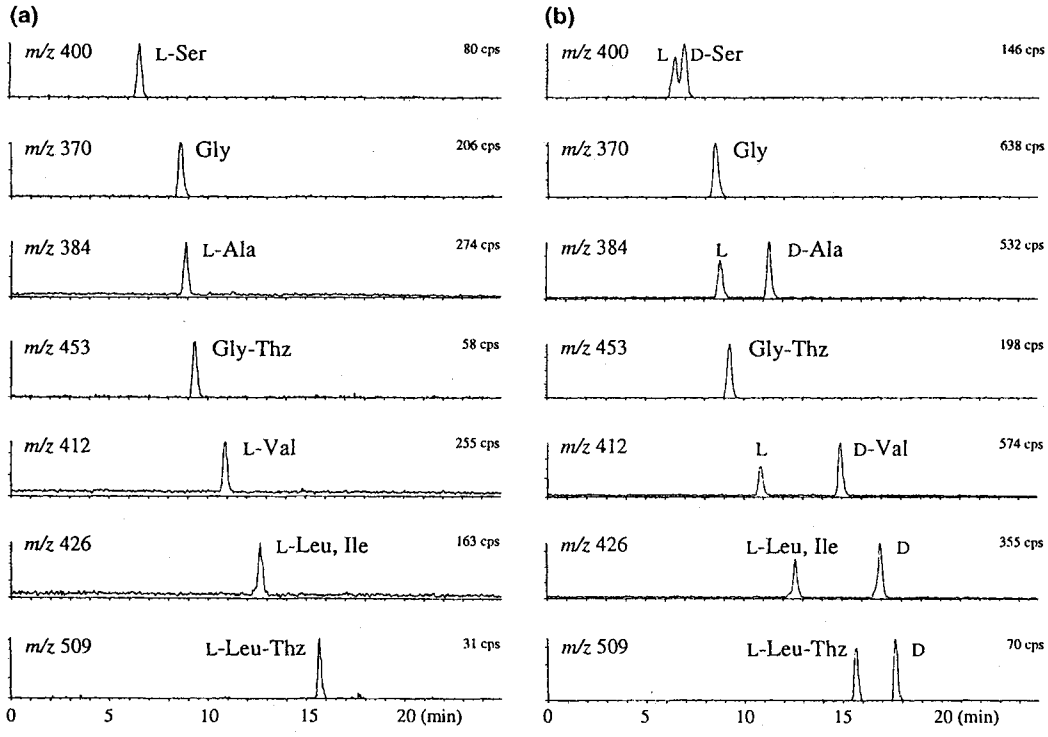
In order to confirm the proposed structure of goadsporin, a derivative was prepared to observe the charge remote fragmentation in the positive-ion mode. Diaminoethane was coupled to the *C*-terminal valine to generate the positive charge at the *C*-terminal. The positive-ion mode CID-MS/MS spectrum of the derivative showed the CID-derived fragment ion peaks containing the *C*-terminal amino residue which satisfactorily explained the proposed structure (Fig. 5).

Stereochemistry

The absolute configuration of the amino acids was determined by the advanced Marfey's method[2,3]. Goadsporin was hydrolyzed in 6 N HCl at 110°C for 1 hour, and the hydrolysate was derivatized with L- and D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide) and analyzed by LC/MS. Fig. 6 shows the mass chromatograms obtained by monitoring at *m/z* values for the protonated ions $[M+H]^+$ of the FDLA-derivatized amino acid constituents. The expected peaks of L-FDLA derivatives were detected in each chromatogram when monitored at the respective *m/z* values (Fig. 6a). In the mass chromatograms of the mixture of D- and L-FDLA derivatives of the hydrolysate, both of the diastereomers were detected except for Gly and Gly-Thz (Fig. 6b). It is reported that L-amino acid-L-FDLA derivatives are generally eluted faster than the D-amino acid-L-FDLA derivatives or L-amino acid-D-FDLA derivatives under reversed-phase conditions with a few exceptions[2,4]. This rule has been shown to be applicable to the FDLA derivatives of 2-(1-aminoalkyl)-4-thiazolecarboxylic acid such as Leu-Thz (K. FUJII *et al.*, manuscript in preparation). As shown in Fig. 6, all of the constituent chiral amino acids in goadsporin were unambiguously determined to be L-isomers since no peaks due to the D-isomers were detected. In addition, Ile[6] was determined to be L-isoleucine, not L-*allo*-isoleucine, by comparing the HPLC retention time with the authentic samples (data not shown). Therefore, the structure of goadsporin was established as shown in Fig. 1.

Goadsporin is a linear oligopeptide consisting of 19 amino acid residues, eight of which are dehydrated and/or

Fig. 6. Mass chromatograms of the L-FDLA derivatives (a) and the mixture of L- and D-FDLA derivatives (b) of goadsporin hydrolysate.



oxidized to form dehydroalanines, thiazoles and oxazoles. Linear peptide antibiotics as large as goadsporin are not often found from microorganisms, compared with cyclic ones. Although the molecular size of goadsporin is close to that of gramicidin A, a linear normal peptide antibiotic from *Bacillus brevis*, some of the amino acid residues in goadsporin are modified as described. To our best knowledge, analogous molecules have not been reported so far. Goadsporin can thus be classified as a new class of peptide secondary metabolite with novel bioactivity.

Experimental

General

NMR experiments were performed on a Bruker DMX-750 NMR spectrometer in DMSO-d_6. The DMSO-d_6 signals (2.50 ppm for ^{1}H; 39.5 ppm for ^{13}C) were used as references. Tandem mass spectra (CID-MS/MS) were recorded on a JEOL JMS HX-110/HX-110 tandem mass spectrometer. LC/MS was measured on an Applied Biosystems QSTAR (ESI-TOFMS) with an Agilent HP1100 HPLC system. MALDI-TOFMS was measured on an Applied Biosystems Voyager. UV spectra were recorded on a BECKMAN DU 640 spectrophotometer. The IR spectrum was recorded on a Shimadzu FT IR-300 spectrophotometer. The optical rotation was measured on a Horiba SEPA-300 polarimeter.

CID-MS/MS Measurements

Goadsporin dissolved in methanol was mixed with 2,2′-dithiodiethanol and subjected to MS/MS measurements in the negative- and positive-ion FAB ionization modes. Helium was introduced to cause dissociation at a pressure that reduced the intensity of the precursor ions to 30%.

Preparation of the Diaminoethane Derivative of Goadsporin

Goadsporin (2 mg, 1.2 μmol) was dissolved in dry DMF (0.8 ml) containing BOP reagent[5] (2 mg, 4.5 μmol), 1-hydroxybenzotriazole (1 mg, 7.4 μmol) and *N-t*-butoxycarbonyldiaminoethane (1 mg, 6.3 μmol). To the solution was added *N*,*N*-diisopropylethylamine (1 mg, 7.7 μmol), and the mixture was stirred for 18 hours at room

temperature. The reaction mixture was poured into ice-water and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by preparative TLC with chloroform-methanol (10:1) to give the *N*-protected product (1.2 mg). This compound was dissolved in 1 N HCl-methanol (1 ml) to deprotect the amino group. After stirring for 3 hours at room temperature, the solution was neutralized with 1 N NaOH and evaporated, and the remaining aqueous solution was extracted with ethyl acetate. After drying over Na_2SO_4, the organic phase was concentrated and the residue was purified by preparative TLC with chloroform-methanol (5:1) to give the derivative (0.3 mg). Although the NMR assignment of the diaminoethane derivative was not completed due to the sample amount, several characteristic signals due to the olefinic and aromatic regions and the methyl groups were confirmed in the ^1H NMR spectrum. In the CID-MS/MS experiment, the monoisotopic mass ion at *m/z* 1654.70 ($[M+H]^+$, $C_{74}H_{104}N_{21}O_{19}S_2$) was introduced as the precursor ion.

Hydrolysis of Goadsporin and Derivatization with FDLA Reagent

Goadsporin (500 μg) was dissolved in 6 N HCl (500 μl) in a sealed tube. The reaction mixture was heated at 110°C for 1 hour and evaporated to dryness. The residue was dissolved in water (100 μl). To each a half portion (50 μl) were added 1 M $NaHCO_3$ (20 μl) and 1% L- or D-FDLA[2] acetone solution (100 μl). After incubation at 40°C for 1 hour, the reactions were quenched with 1 N HCl (20 μl) and diluted with acetonitrile (260 μl). One μl of each solution was injected for LC/MS.

LC/MS Analysis of FDLA Derivatives of the Hydrolysate

The analysis of the L- and DL-FDLA derivatives was performed on an Agilent HP1100 HPLC system with a column of TSKgel Super-ODS (2 μm, 100×2.0 mm, i.d.) at 40°C with a flow rate of 0.2 ml/minute. The solvent was 1% formic acid-acetonitrile with a linear gradient from 30% to 80% acetonitrile over 25 minutes. An Applied Biosystem QSTAR mass spectrometer was used for detection in the ESI (positive) mode. The effluent from the HPLC was split at a ratio of 1:40 for introduction into the mass spectrometer at a flow rate of 5 μl/minure.

References

1) ONAKA, H.; H. TABATA, Y. IGARASHI, Y. SATO & T. FURUMAI: Goadsporin, a chemical substance which promotes secondary metabolism and morphogenesis in streptomycetes. I. Purification and characterization. J. Antibiotics 54: 1036~1044, 2001
2) FUJII, K.; Y. IKAI, H. OKA, M. SUZUKI & K. HARADA: A nonempirical method using LC/MS for determination of the absolute configuration of constituent amino acids in a peptide: elucidation of limitations of Marfey's method and of its separation mechanism. Anal. Chem. 69: 3346~3352, 1997
3) FUJII, K.; Y. IKAI, H. OKA, M. SUZUKI & K. HARADA: A nonempirical method using LC/MS for determination of the absolute configuration of constituent amino acids in a peptide: combination of Marfey's method with mass spectrometry and its practical application. Anal. Chem. 69: 5146~5151, 1997
4) HARADA, K.; A. MATSUI, Y. SHIMIZU, R. IKEMOTO & K. FUJII: Abnormal elution behavior of ornitine derivatized with FDLA in advanced Marfey's method. J. Chromatography A 921:187~195, 2001
5) CASTRO, B.; J-R. DORMOY, B. DOURTGLOU, G. EVIN, C. SELVE & J-C. ZIEGLER: Peptide coupling reagents VI. A novel, cheaper preparation of benzotriazolyloxytris[dimethylamino]phosphonium hexafluorophosphate (BOP reagent). Synthesis: 751~752, 1976