Antibiotic GE37468 A: A Novel Inhibitor of Bacterial Protein Synthesis

II. Structure Elucidation

P. FERRARI, L. COLOMBO, S. STELLA, E. SELVA and L. F. ZERILLI

Marion Merrell Dow Research Institute, Lepetit Research Center,
21040 Gerenzano (VA), Italy

(Received for publication July 17, 1995)

GE37468 A is a novel antibiotic produced by *Streptomyces* sp. ATCC 55365. It has molecular mass 1309.48 and formula $C_{59}H_{52}O_{12}N_{14}S_5$ and belongs to the thiazolyl peptide group of antibiotics. The structure was elucidated by ^1H and ^{13}C NMR and MS studies on intact molecule and its hydrolysis products. The antibiotic is a highly modified peptide containing a macrocycle and a side chain composed of a thiazole ring and two dehydroalanine units.

GE37468 A is a novel antibiotic[1] active against Gram-positive bacteria which inhibits procaryotic protein synthesis by acting on Elongation Factor Tu (EF-Tu). The antibiotic is similar in mode of action and spectrum of activity to the thiazolyl peptide antibiotic GE2270 A[2~5].

GE37468 A was produced by fermenting *Streptomyces* sp. ATCC 55365. The purified antibiotic is a white solid, soluble in organic solvents and sparingly soluble in water. The structure was determined by physico-chemical investigations applied to the intact molecule and to the main hydrolysis products.

Materials and Methods

UV absorption spectra were recorded with a Perkin-Elmer spectrophotometer mod. Lambda 16. The antibiotic was dissolved in methanol - acetonitrile - water 7:7:1 (v/v) as such and at extreme pH values obtained by adding a trace of HCl or KOH.

IR absorption spectra were obtained from a mineral oil suspension with a Bruker FT-IR spectrophotometer mod. ISF 48.

Acid-base titrations in aqueous medium were obtained with 0.01 N HCl after adding an excess of 0.01 N KOH to a solution of the sample in methylcellosolve - water 4:1 (v/v). Titrations in non-aqueous medium were carried out in glacial acetic acid with 0.01 N $HClO_4$, and in pyridine with tetrabutylammonium hydroxide.

FAB-MS spectra (low resolution positive and negative ion) were obtained on Finnigan MAT TSQ700 triple stage quadrupole mass spectrometer, using a saddle field atom gun (with Xe gas) at 8 kV voltage and 1 mA current. The instrument was previously calibrated both in negative and positive ion mode using CsI as reference compound up to m/z 2000. Samples were dissolved in dimethylsulphoxide (DMSO) - m-nitrobenzyl alcohol (m-NBA) 1:1 (v/v). High resolution exact mass measurements were obtained on Kratos MS50 mass spectrometer, using peak matching technique and operating in positive FAB mode.

^1H and ^{13}C NMR spectra were performed, using a solution of 10 mg of lyophilized GE37468 A in 0.5 ml of DMSO-d_6, on Bruker AM-500 spectrometer equipped with an Aspect 3000 computer, at 295 °K. ^{13}C NMR spectrum was obtained by composite pulse decoupling (CPD, Powgate microprogram). DEPT spectrum was obtained with 135° pulses for ^1H decoupler to select CH and CH_3 positive and CH_2 negative. Phase sensitive double quantum filter (PHDQ) ^1H-^1H COSY spectra were run using time-proportional-phase-incrementation in f1. HOHAHA (TOCSY) experiment (MLEV17PH microprogram) was based on Homonuclear Hartmann-Hann transfer with mixing by composite pulse cycle using inverse mode and phase sensitive (TPPI) optimized for long range couplings[6]. In ^1H-^1H COSY with delay[7] (COSYLR microprogram) the D2 delay was 100 ms. In the carbon-proton correlation through one bond[7~10] (XHCORRDC microprogram), D3 and D4 were 3.4 ms and 1.7 ms, respectively. HMBC experiment[11] was used for long range carbon-proton correlation in inverse mode. Evolution time for long range couplings was 60 ms.

Acid hydrolysis was carried out on 1 mg of GE37468 A at 100~120°C overnight with 6 N HCl containing 1% phenol. After cooling, the reaction mixture was diluted with water and the precipitate was filtered. The residue was washed with water and submitted to FAB-MS and FAB-MS/MS experiments. These measurements were performed on a Kratos MS50 and a Finnigan TSQ700. Samples were dissolved in NBA - DMSO 1:1 (v/v) immediately before analysis. Positive FAB-MS/MS spectra were recorded using argon as collision gas.

The aqueous solution from acid hydrolysis was lyophilized, suspended in 3 ml of methanol and divided in 3 equal portions which were evaporated to dryness

Table 1. UV bands and molar absorbances (ε_{mol}) of GE37468 A in solutions of methanol-acetonitrile-water, 7:7:1 at different pH values.

	λ_{max} (nm)	ε_{mol} (1 cm, 1 mol/liter)	λ_{max} (nm)	ε_{mol} (1 cm, 1 mol/liter)	λ_{max} (nm)	ε_{mol} (1 cm, 1 mol/liter)
Solvent	250(sh)	43000	305	34400	340(sh)	18300
Solvent + HCl	250(sh)	43000	307	33200	340(sh)	17700
Solvent + KOH	250(sh)	51700	302	36200	340(sh)	19700

under nitrogen. Portions were submitted to the following derivatization procedures[1,2]: a) methylation with 2.4 N HCl in CH_3OH and acylation with trifluoroacetic anhydride (TFA)-CH_2Cl_2, 1:1 (v/v); b) esterification with 2.4 N HCl in isopropanol and acylation with TFA-CH_2Cl_2, 1:1 (v/v); c) esterification with 2.4 N HCl in isopropanol and acylation with pentafluoropropionic anhydride (PFA)-CH_2Cl_2, 1:1 (v/v). Solvents were evaporated, residues were dissolved in n-hexane and then analyzed by GC-MS using Finnigan TSQ700 instrument. GC: capillary column (HP-5, 25 m × 0.32 mm i.d., 0.17 μm film tickness); helium as carrier gas (column pressure 8 psi, split vent 50 ml/minute, split mode injection). Injector and interface temperatures were set at 270 and 280°C, respectively. Oven temperature was set at 60°C for 2 minutes and increased at 8°C/minute to 260°C. EI-MS: source temperature 150°C, electron energy 70 eV. CI-MS (positive ion chemical ionization): source temperature 120°C, electron energy 120 eV, ionization gas CH_4, manifold pressure $6 \cdot 10^{-6}$ torr.

Peak identification was obtained, when possible, by comparison with NBS reference spectra.

Results and Discussion

Basic structure information was obtained from the physico-chemical characteristics and by comparison with antibiotic GE2270 A.

The UV spectra of antibiotic GE37468 (Table 1) and of GE2270 A[2,3] were very similar. The spectra of GE37468 A showed a small hyperchromic shift at basic pH which indicated the presence of a phenol. IR spectrum (Tab. 2) was typical of a peptide. Absorption at about 1533 cm^{-1} corresponded to heterocycles and those at 1615 and 1630 cm^{-1} to double bonds. Shoulder at about 1720 cm^{-1} indicated the presence of a free carboxyl group. Ionization studies in water and in pyridine confirmed the presence of an ionizable function of medium strength. A weak basic function was detected in acetic acid. A weak basic function, related to a pyridine moiety, was also present in antibiotic GE2270 A.[2,3].

Positive FAB-MS spectrum (Fig. 1) showed the lowest isotope of the main peak at 1291 u. This peak derived from protonated molecule m/z 1309 by loss of water, as

Table 2. Assignments of the main absorption bands (cm^{-1}) of the IR spectrum of GE37468 A in nujol mull.

3600÷3100	νNH & νOH
1720 (sh)	carboxylic νC=O
1653	νC=O (amide I)
1630, 1615, 1582	ethylenic and aromatic νC=C
1533	heterocyclic νC=C & νC=N
1514	δNH (amide II)
1269, 1200	aromatic δCH
1024, 1007	νC-O & νC-O-C
920	heterocyclic γCH
808	para-substituted aromatic γCH
758, 703	mono-substituted aromatic γCH

demonstrated by the peaks of Na^+ and K^+ adducts shifted 22 and 38 units, respectively. Isotope pattern of these protonated species indicated a molecular formula containing higher mass isotopes, *e.g.*, 5~8 sulphur atoms. Accurate mass was determined using high resolution (r.p. = 10.000; 5% valley) peak matching. The lowest isotope of the more abundant peak resulted 1291.240 ± 0.01 u (mean ± s.d.). Negative FAB-MS spectrum confirmed the attribution of molecular mass by showing a $(M-H)^-$ ion at m/z 1307. Considering that NMR analyses indicated the presence of 59 carbon and 52 hydrogen atoms, the most likely molecular formula resulted $C_{59}H_{52}N_{14}O_{12}S_5$, which has a theoretical molecular mass (lowest isotope) of 1308.24927.

Lack of fragmentation in positive FAB-MS spectrum was consistent with presence of a peptide macrocycle, as observed for antibiotic GE2270 A[4]. Negative FAB spectrum showed high mass fragments with losses of 71 (m/z 1237) and 140 u (m/z 1168). These were consistent with an extracyclic chain constituted by two dehydroalanine residues terminating with a free carboxylic group.

^1H and ^{13}C NMR analyses provided additional information through ^1H-^1H homonuclear and ^{13}C-^1H heteronuclear short and long range coupling experiments. Heteronuclear experiments on GE37468 A in its unprotonated form were obtained in normal and inverse modes. Assignments are reported in Table 3.

Two dehydroalanines were indicated by two negative DEPT signals in the olefinic region (100.6 and

Fig. 1. Positive ion FAB-MS spectrum of GE37468 A in m-NBA.

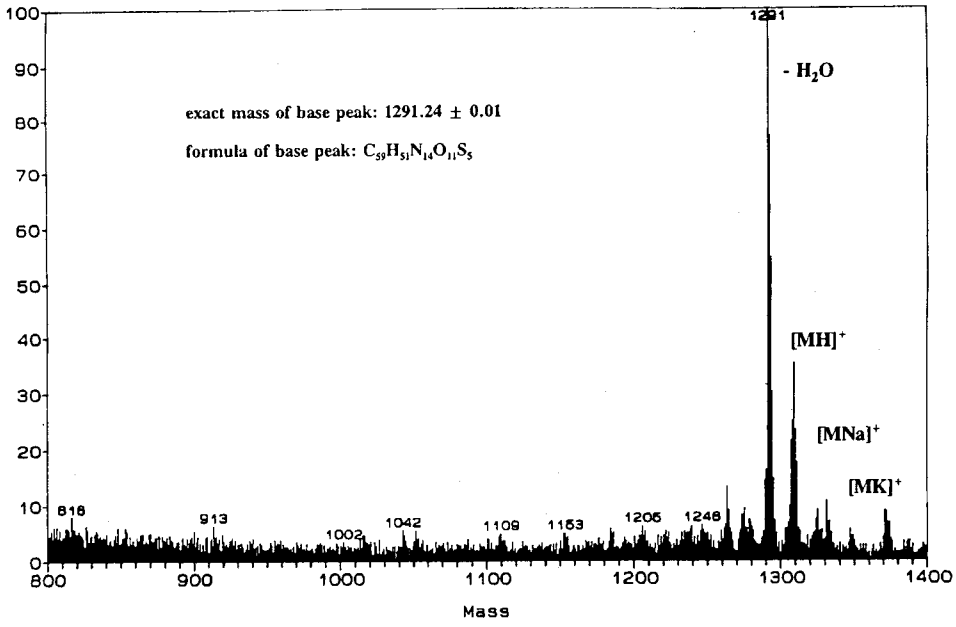


Table 3. NMR assignments of GE37468 A in the unprotonated form (DMSO-d_6, TMS internal standard, δ ppm).

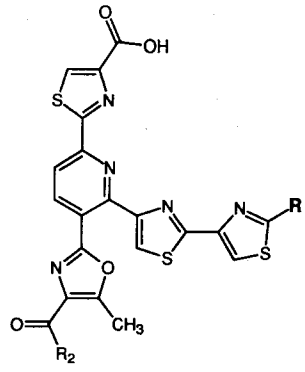
^{13}C	^{1}H	Assignment	^{13}C	^{1}H	Assignment
174.4		C2	129.0	8.67	A5
173.9		D2	128.4	7.29	Phe3,5
173.0		Cys2	127.5		Tyr1
171.6	7.18, 6.78	Asn CO–NH_2, Tyr C=O	126.8	7.20	Phe4
171.4			123.9	7.91	D5
169.3		Cys C=O	123.1		Pyr3
167.6		A2	122.3	8.18	B5
165.4		Dha1 CO_2H	118.7	8.38	Pyr5
161.3		B2	116.7	7.75	C5
161.2		Oxa C=O	115.1	6.59	Tyr3,5
160.9		D C=O, Dha2 C=O	103.7	6.45, 5.80	Dha 2β
159.0		A C=O	100.6	6.06, 5.80	Dha 1β
156.0	9.03	Tyr C–OH	81.9	5.79 and 6.45	Pro δ and OH
155.7		Oxa2	77.5	4.93	Cys4
153.8		Oxa4	66.7	4.64	Pro α
153.3		B4	54.3	4.87 and 8.57	Phe α and NH
152.1		Pyr2	52.3	5.07 and 7.29	Tyr α and NH
150.5		A4+Pyr6	48.5	5.27 and 8.64	Asnα and NH
149.3		D4	42.8	2.12, 1.91	Pro γ
147.4		C4	39.0	2.63	Pro β
140.1	8.54	Pyr4	38.4	3.33	Phe β
138.1	9.59	Dha 1α and NH	38.1	2.66, 2.00	Asn β
137.3		Phe1	37.0	3.35, 2.95	Tyr β
135.6	10.08	Dha 2α and NH	36.4	3.63, 3.19	Cys5
130.6	7.06	Tyr2,6	16.0	1.14	Pro CH_3
129.8		Oxa5	11.7	2.73	Oxa CH_3
129.5	7.29	Phe2,6			

A, B, C, and D=thiazole rings; Dha=dehydroalanine; Pyr=pyridine; Pro=proline; Tyr=tyrosine; Cys=thiazoline; Phe=phenylalanine; Asn=asparagine; Oxa= oxazole (see Fig. 3).

103.7 ppm). They showed couplings with the corresponding β-NH singlets, which in turn were coupled with carbonyl groups of respective amino acids.

Because of the presence of five sulfur atoms, as deduced by exact mass measurements and hydrolysis data (Table 4), the four protonic singlets in the δ 7.7~8.7 ppm region were confidently attributed to four thiazoles. In HMBC spectra, each of them was coupled to three carbon atoms

Table 4. Interpretation of FAB-MS spectrum of the residue from the hydrolysis of GE37468 A.



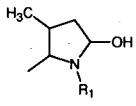
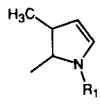
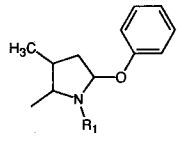
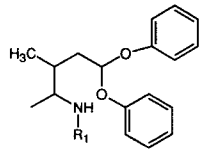
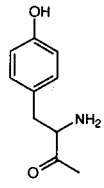
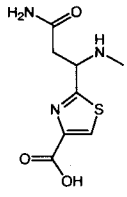
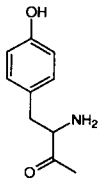
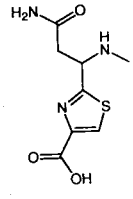
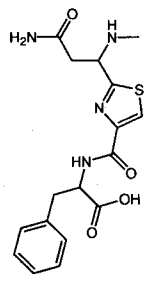
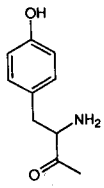
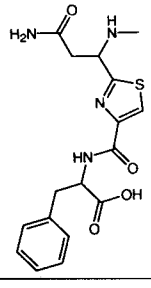
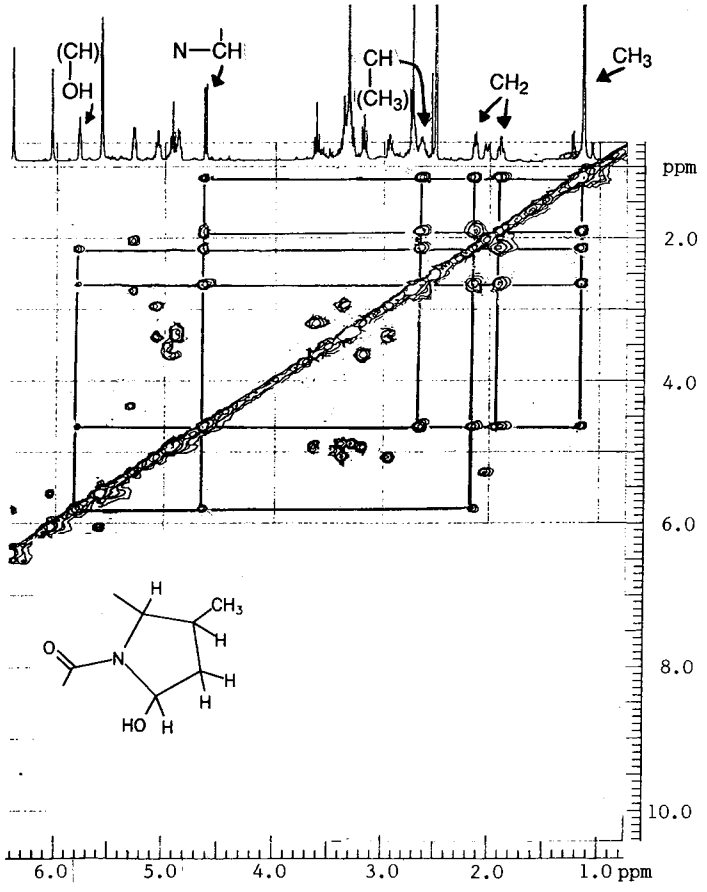
					R				
1	R_1=	H	R_2=	OH	597	579	673	767
2	R_1=		R_2=	OH	760	742	836	930
3	R_1=	H	R_2=		795	777	871	965
4	R_1=		R_2=		958	940	1034	1128
5	R_1=	H	R_2=		942	924	1018	1112
6	R_1'=		R_2=		1105	1087	1181	1275

Fig. 2. HOHAHA spectral region showing the coupling pattern of β-methyl-δ-hydroxyproline.

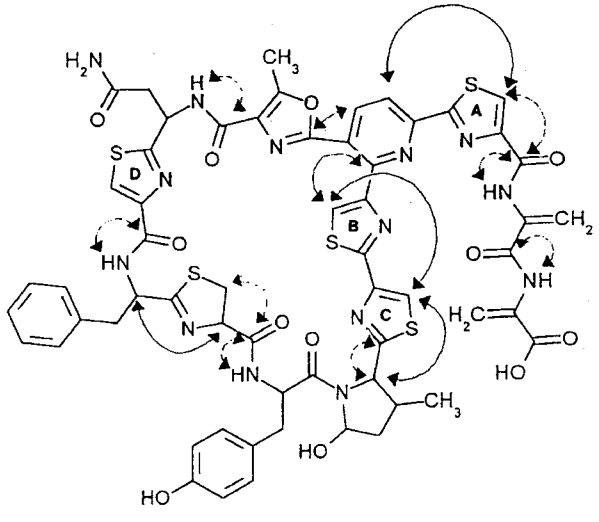


in the corresponding ring. One methyloxazole was also present as indicated by protonic methyl singlet at 2.73 ppm and by its coupling with two carbons in the ring. Chemical shifts were consistent with literature data of methyloxazoles[13].

A 2,3,6-substituted pyridine was indicated by two protonic doublets around 8.5 ppm. Each proton showed an HMBC coupling through three bonds with the three quaternary pyridine carbons. These data were in accord with ionization results and with UV spectrum, in indicating that the chromophore, in analogy to antibiotics GE2270 A[3~5] and amythiamicin D[14] was a pyridine ring conjugated to other heterocycles. In fact, the characteristic UV spectrum (Table 1, λ_{max} 250 nm (sh), 305 nm, 340 nm (sh)) and ^{1}H and ^{13}C NMR spectral data (Table 3) were in good agreement with those of the chromophores contained in GE2270 A and amythiamicin D.

Other building blocks were revealed by NMR studies. One β-methyl-δ-hydroxyproline was revealed by the pattern of the numerous couplings through three bonds in the PHDQ-COSY experiment and through more than three bonds in the TOCSY experiment (Fig. 2). The consequent attributions were confirmed by heteronuclear intra-unit HMBC couplings. The tyrosine unit was assigned analogously. Thiazoline ring was indicated by

Fig. 3. Structure of GE37468 A. The arrows indicate the sequencing process by COSY with delay (full arrows) and HMBC (dotted arrows).



the two signals at 3.19 and 3.63 ppm attributed to the ring CH_2 coupled with adjacent CH (4.93 ppm). This attribution was confirmed by heteronuclear couplings in HMBC spectrum. One phenylalanine and one asparagine residue were identified. These amino acids were also found as hydrolysis products.

The sequence of building blocks was investigated by COSY with delay and by HMBC experiments. Their results are indicated in Fig. 3 with solid arrows and dotted arrows, respectively. Starting point was the C terminal

Fig. 4. FAB-MS spectrum of the residue from the hydrolysis of GE37468 A.

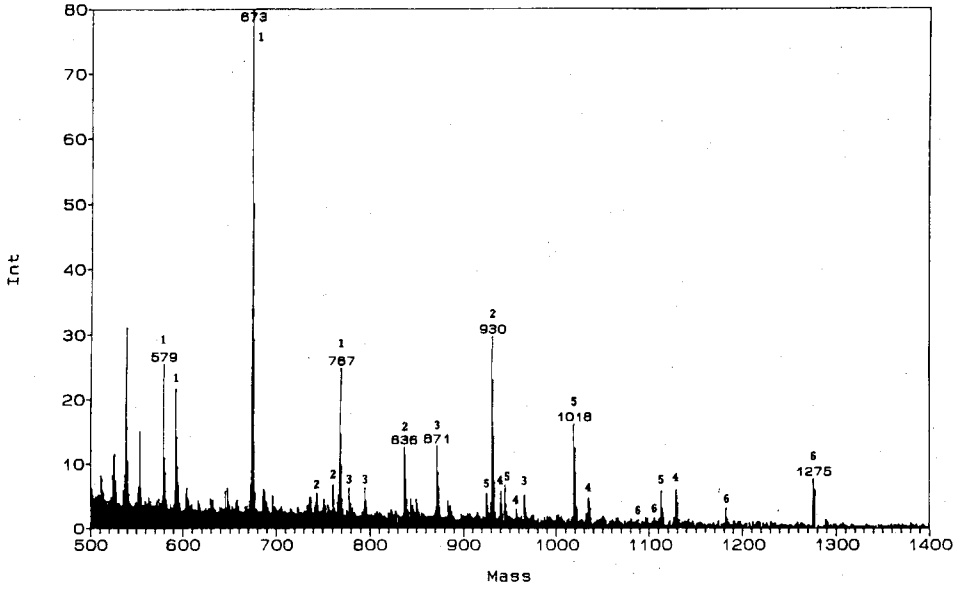


Fig. 5. FAB-MS/MS spectrum of the main product of the hydrolysis residue.

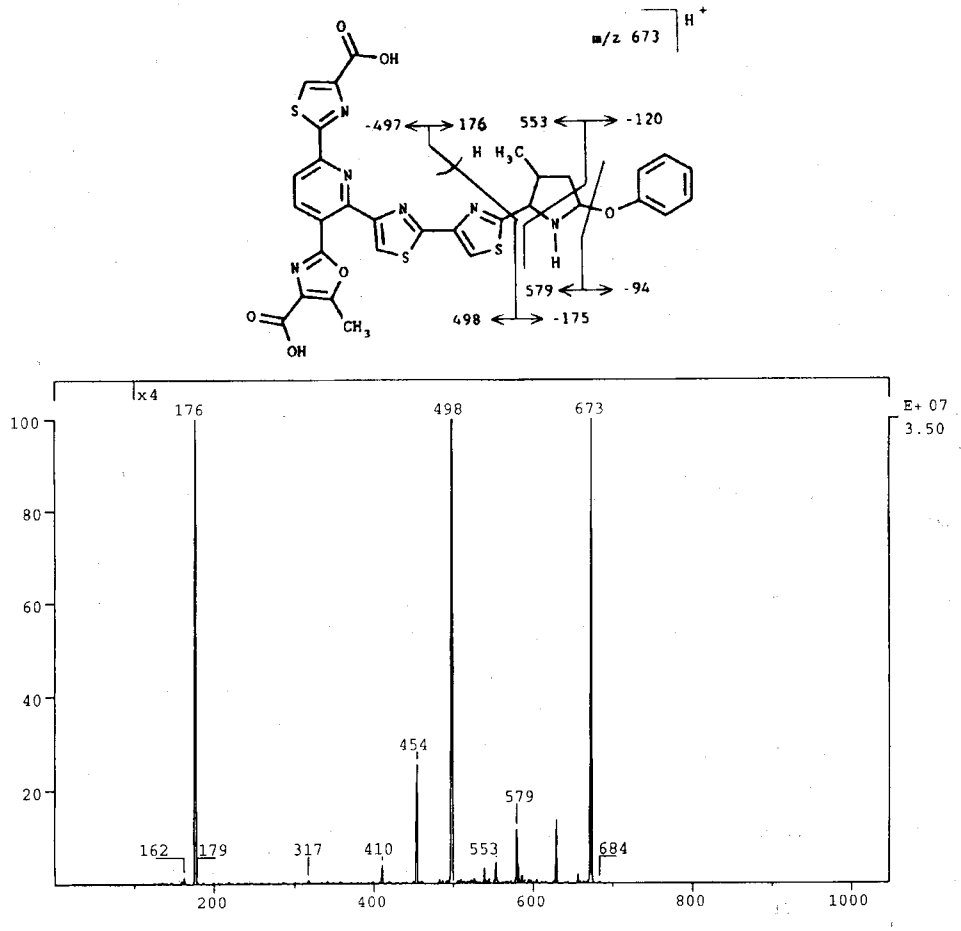
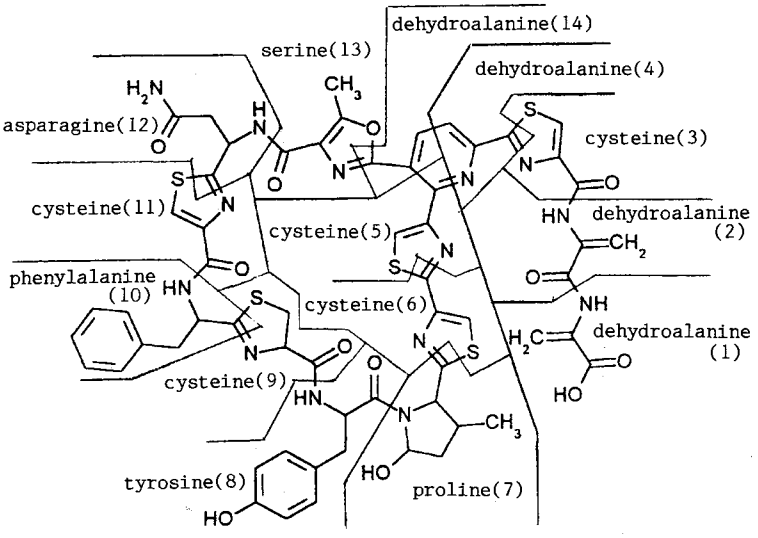


Fig. 6. Structure of GE37468 A, showing also the original amino acids of the initial linear peptide precursor.



of dehydroalanine, whose free carboxylic function conferred pH sensitivity to carbonyl ^{13}C signal (165.4 ppm as reported in Table 3 for the unprotonated form, and 164.9 ppm as found in the case of protonated form). Three thiazoles and methyloxazole were allocated as substituents of pyridine in the chromophore. Remaining connections were established by GC-MS and FAB-MS of hydrolysis products. Acid hydrolysis of GE37468 A produced one soluble fraction and a precipitate. The soluble fraction was divided in three portions which, after derivatization, were analyzed by GC-MS.

Tyrosine, thiazolyl-aspartic acid, phenylalanine and cysteine with its by-products were identified by GC-MS of the soluble fraction. Phenylalanine and cysteine were generated from phenylalanyl-thiazoline, which is unstable under the hydrolysis conditions. Thiazolyl-aspartic acid is stable and was found intact by EI-MS and CI-MS analyses.

The precipitate was analyzed by FAB-MS as shown in Fig. 4. It resulted a mixture of products originated from α-hydroxy-proline connected to chromophore. This group is a cyclic aminal which, upon acidic treatment, eliminated water or generated hemiacetal and acetal as derivatives with phenol present in hydrolysis mixture. FAB-MS/MS, with collisional-induced dissociation of most abundant ion *m/z* 673, confirmed that hemiacetal structure derived from aldehydic group and phenol. The daughter ion spectrum and the interpretation is shown in Fig. 5. The precipitate was constituted of incomplete hydrolysis products. Six main hydrolysis products were found as clusters of four derivatives (Table 4). Cluster No. 2, for example, referred to a hydrolysis product containing chromophore and tyrosine. The peak at *m/z* 760 corresponded to an ion which retained intact the α-hydroxyproline moiety, and the peak at *m/z* 742 to the previous ion after loss of H_2O and formation of one double bond. Two peaks at *m/z* 836 and 930 derived from the reactions with one or two phenols, respectively.

Products from incomplete hydrolysis demonstrated connections between methyl-hydroxyproline and tyrosine, between phenylalanine and thiazolasparagine, between thiazolasparagine and methyloxazole, and confirmed other connections as revealed by NMR studies.

Structure of GE37468 A was thus elucidated as reported in Fig. 6. Structural similarity with other thiazolyl peptide antibiotics[15] suggests a common biogenesis, as discussed in the accompanying paper. The putative peptide precursor of GE37468 is visualized from Fig. 6.

References

1) STELLA, S.; N. MONTANINI, F. LE MONNIER, P. FERRARI, L. COLOMBO, P. LANDINI, I. CICILIATO, B. P. GOLDSTEIN, E. SELVA & M. DENARO: Antibiotic GE37468 A: A novel inhibitor of bacterial protein synthesis. I. Isolation and characterization. J. Antibiotics 48: 780~786, 1995

2) SELVA, E.; G. BERETTA, N. MONTANINI, L. GASTALDO, P. FERRARI, R. LORENZETTI, B. P. GOLDSTEIN, M. BERTI, L. MONTANARO & M. DENARO: Antibiotic GE2270 A: A novel inhibitor of bacterial protein synthesis. I. Isolation and characterization. J. Antibiotics 44: 693~701, 1991

3) KETTENRING, J.; L. COLOMBO, P. FERRARI, P. TAVECCHIA, M. NEBULONI, K. VÉKEY, G. G. GALLO & E. SELVA: Antibiotic GE2270 A: a novel inhibitor of bacterial

protein synthesis. II. Structure elucidation. J. Antibiotics 44: 702~715, 1991

4) COLOMBO, L.; P. TAVECCHIA, E. SELVA, G. G. GALLO & L. F. ZERILLI: Contribution of mass spectrometric techniques to the structure elucidation of antibiotic GE2270 A, a novel inhibitor of bacterial protein synthesis. Org. Mass Spectrom. 27: 219~225, 1992
5) TAVECCHIA, P.; P. GENTILI, M. KURZ, C. SOTTANI, R. BONFICHI, S. LOCIURO & E. SELVA: Revised structure of the antibiotic GE2270 A. J. Antibiotics 47: 1564~1567, 1994
6) BAX, A. & D. G. DAVIES: MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. J. Magn. Res. 65: 355~360, 1985
7) BAX, A. & R. FREEMAN: Investigation of complex networks of spin-spin coupling by two-dimensional NMR. J. Magn. Res. 44: 542~561, 1981
8) BAX, A.: Broadband homonuclear decoupling in heteronuclear shift correlation NMR spectroscopy. J. Magn. Res. 53: 517~520, 1983
9) RUTAR, V.: Suppression of homonuclear J coupling in ^{1}H-^{13}C chemical-shift correlation maps. J. Magn. Res. 58: 306~310, 1984
10) WILDE, J. A. & P. H. BOLTON: Suppression of homonuclear couplings in heteronuclear two-dimensional spectroscopy. J. Magn. Res. 59: 343~346, 1984
11) BAX, A. & M. F. SUMMERS: ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108: 2093~2094, 1986
12) KNAPP, D. R.: Amino acids and peptides. In Handbook of analytical derivatization reactions. *Ed.*, D. R. KNAPP, pp. 242~337, John Wiley & Sons, 1979
13) ABE, H.; K. KUSHIDA, Y. SHIOBARA & M. KODAMA: The structures of sulfomycin I and berninamycin A. Tetrahedron Letters 29: 1401~1404, 1988
14) SHIMANAKA, K.; Y. TAKAHASHI, H. IINUMA, H. NAGANAWA & T. TAKEUCHI: Novel antibiotics amythiamicin. II. Structure elucidation of amythiamicin B. J. Antibiotics 47: 1145~1152, 1994
15) BÉRDI, J.: Thiazolyl peptides. *In* Handbook of antibiotic compounds. Vol.IV/I. *Ed.*, J. BÉRDI *et al.*, pp. 389~417, CRC Press Inc., 1980