VOL. 53 NO. 4, APR. 2000 THE JOURNAL OF ANTIBIOTICS pp. 364 — 372

SB-203207 and SB-203208, Two Novel Isoleucyl tRNA Synthetase Inhibitors from a *Streptomyces* sp.

II. Structure Determination

CATHERINE S. V. HOUGE-FRYDRYCH*, MARTIN L. GILPIN, PETER W. SKETT and JOHN W. TYLER

SmithKline Beecham Pharmaceuticals,
New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW, UK

(Received for publication November 15, 1999)

Two novel isoleucyl tRNA synthetase inhibitors, SB-203207 and SB-203208 have been isolated from a *Streptomyces* sp. and found to be structurally related to altemicidin. Structures of SB-203207 and SB-203208 have been deduced by a combination of spectroscopic techniques, derivatisation, hydrolysis studies and found to be 4-(aminocarbonyl)-7-{[(2-amino-3-methylpentanoyl)aminosulphonyl]acetamido}-2,4a,5,6,7,7a-hexahydro-6-hydroxy-2-methyl-1*H*-2-pyrindine-7-carboxylic acid (**1**) and 4-(aminocarbonyl)-7-{[(2-amino-3-methyl pentanoyl)-aminosulphonyl]acetamido}-2,4a,5,6,7,7a-hexahydro-6-(2-amino-3-phenylbutanoyl oxy)-2-methyl-1*H*-2-pyrindine-7-carboxylic acid (**2**), respectively.

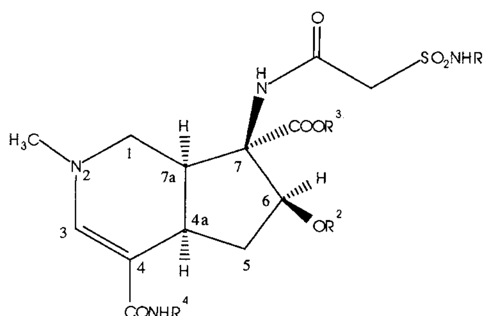
SB-203207 (**1**) and SB-203208 (**2**), isolated from a fermentation broth of *Streptomyces* sp. NCIMB 40513, are new analogues of altemicidin (**3**)[1~4] having isoleucyl tRNA synthetase inhibition activity. The preceding paper describes the fermentation, isolation, physico-chemical and biological properties of (**1**) and (**2**)[5]. The structure determination studies of these compounds are reported in this paper. The structures of (**1**), (**2**) and (**3**) as well as the structures of (**4**) and (**5**), two triacetylated analogues of (**2**), are shown in Fig. 1.

Results and Discussion

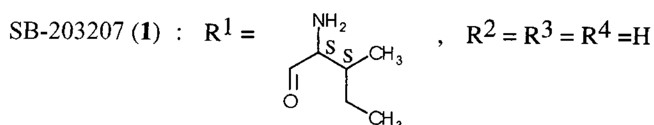
The molecular formulae of (**1**) and (**2**) were established as $C_{19}H_{31}N_5O_8S$ and $C_{29}H_{42}N_6O_9S$, respectively, by high resolution fast atom bombardment mass spectrometry (HRFAB-MS)[5] and ^{13}C NMR spectra. Spectroscopic data obtained on (**1**) and (**2**) indicated that their structures were closely related to altemicidin: for example, the IR spectra of (**1**) and (**2**) both showed the presence of amide absorption bands (1636, 1509 and 1637, 1526 cm^{-1}) and a sulphonyl group (1338, 1117 and 1350, 1112 cm^{-1}). The UV absorption maxima of (**1**) and (**2**) at 299 and 297 nm, respectively, were in agreement with the presence of a chromophore such as 3-aminocarbonyl-1,4,5,6-tetrahydropyridine[1]. In addition, certain features of the ^1H NMR spectra of (**1**) and (**2**) [an olefinic proton (s, ~7.4), *N*-methyl protons (s, ~3.1), isolated methylene protons (AB system, ~4.2 and ~4.35) which were easily deuterated in D_2O] were similar to those reported in the ^1H NMR spectrum of altemicidin (Tables 1 and 2)[2]. It was postulated that the difference in molecular weight between altemicidin (MW=376) and (**1**) (MW=489) or (**2**) (MW=650) would be due to the addition of substituent(s) attached to the altemicidin nucleus.

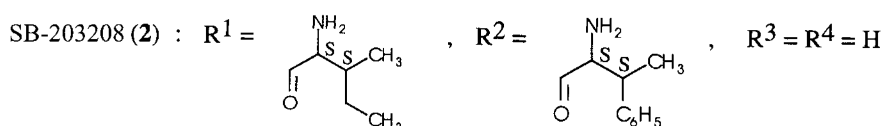
The preceding paper described the controlled hydrolysis of (**2**) to (**1**) with loss of β-methylphenylalanine[5]. An erythro relative stereochemistry for this amino acid was deduced from comparison of HPLC retention times and ^1H NMR spectra with an authentic sample of β-methylphenylalanine containing a 1:2 erythro:threo mixture. The absolute configuration of the two chiral centres was found to be (2*S*,3*S*) based on the optical rotation ($[\alpha]_D^{20}$ $-27.1°$ (c 8.5×10^{-4}, H_2O)) and comparison with literature data[6,7].

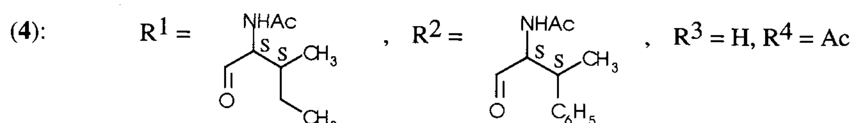
Fig. 1. Structures of SB-203207 (**1**), SB-203208 (**2**), altemicidin (**3**), (**4**) and (**5**).

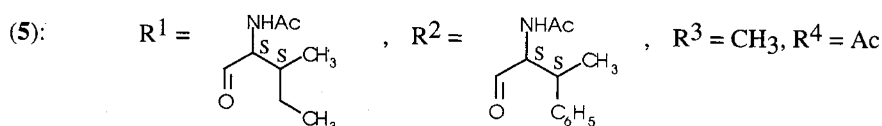


Altemicidin (**3**) : $R^1 = R^2 = R^3 = R^4 = H$







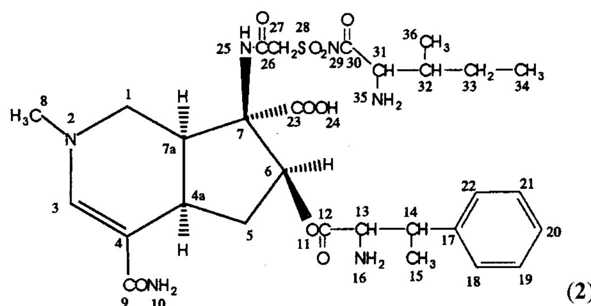


Signals present in the ^1H NMR spectra of (**1**) and (**2**) suggested the presence of an isoleucyl fragment (Table 1). To confirm this hypothesis, (**2**) was subjected to strong hydrolysis (HCl 5 N, 100°C, 18 hours) and the resulting reaction mixture analysed by TLC and chiral HPLC. The presence of isoleucine was confirmed and the absolute configuration assigned as 2*S*,3*S* *i.e.* L-isoleucine. Amino acid analysis on (**2**) agreed with the presence of L-isoleucine and (2*S*,3*S*)-*β*-methylphenylalanine. Addition of an isoleucyl fragment to altemicidin increases its molecular weight to 489, the observed molecular weight of (**1**) and further addition of a *β*-methylphenylalanyl fragment increases the molecular weight to 650, that determined for (**2**).

With the identity of the nucleus of (**1**) and (**2**) established and the nature of the substituents determined, the last step in the structure elucidation was to establish how and where the isoleucyl and *β*-methylphenylalanyl substituents were linked to the altemicidin nucleus. This information could not be obtained by NMR spectroscopy despite the use of advanced techniques such as HMBC. Indeed, a major problem with the structure elucidation was the number of spectroscopically (^1H NMR) silent centres (heteroatoms and quaternary carbons) from which no information could be gained as to their immediate environment. Additionally, overlapping ^1H NMR signals (H-1, H-4a, H-5 at δ 2.86 ppm) made the interpretation of the spectra ambiguous. Mass spectrometric deuteration experiments had indicated that (**1**) and (**2**) contained large numbers of exchangeable protons (8 and 9 respectively). NMR spectroscopic studies were therefore attempted using aprotic solvents. However, the ^1H NMR spectra of (**1**) and (**2**) in DMSO-d_6, CD_3NO_2 or C_5D_5N were broader or more complex than those in D_2O.

Mass spectroscopy gave valuable information on the

Table 1. ^{1}H NMR data of SB-203207 (1), SB-203208 (2) and altemicidin (3).



Proton	Chemical shifts (δ value in ppm) and coupling constants (Hz) in D_2O (ref. HDO; δ 4.80)					
	SB-203207 (1)		SB-203208 (2)		altemicidin (3)[2,3]	
1	2.93	br s	2.86	m	2.86	m
		s	3.22	dd, J = 5.0, 12.6		
3	7.45	s	~7.40	obscured	7.39	s
4a	2.96	m	2.86	m	~2.93	m
5	1.32	m	1.05	m	1.26	ddd, J = 9.0, 12.6, 12.6
	2.75	ddd, J = 7.0, 7.0, 13.0	2.86	m	2.67	ddd, J = 7.6, 7.6, 12.6
6	4.35	dd, J = 7.0, 7.0	5.65	dd, J = 3.5, 7.1	4.28	dd, J = 7.6, 9.0
7a	2.93	br s	2.64	ddd, J = 5.0, 5.0, 10.0	2.86	m
8	3.05	s	3.10	s	2.98	s
13	-	-	4.34	d, J = 5.0	-	-
14	-	-	3.51	dq, J = 5.0, 7.0	-	-
15	-	-	1.52	d, J = 7.0	-	-
18 → 22	-	-	7.3 - 7.5	m	-	-
27	4.21 & 4.42	AB system, J = 14.0	4.21 & 4.31	AB system, J = 13.6	4.29 & 4.39	AB system, J = 14.0
31	3.79	d, J = 4.2	3.74	d, J = 4.0	-	-
32	2.12	m	2.10	m	-	-
33	1.32	m	1.30	m	-	-
	1.60	m	1.53	m	-	-
34	1.00	t, J = 7.3	0.98	t, J = 7.3	-	-
36	1.10	d, J = 7.0	1.09	d, J = 7.0	-	-

nature of the linkage of the two amino acids to the altemicidin nucleus. The mass spectrum (Flow FAB, −ve ion) of (**2**) contains two major peaks in the low mass region of the spectrum at 129 and 178 mass units. The peak at 178 corresponds to a *β*-methylphenylalanyl ion which could be attached to the rest of the molecule through either the amino or the acid function (Scheme 1). An isoleucyl ion would be expected to give a peak at 130 instead of the observed 129 mass units. This can be explained by the presence of an isoleucylamide ion connected to the nucleus *via* its amino or amide function (Scheme 1). Mass spectral data on the triacetylated analogues of (**2**), namely (**4**) and (**5**), showed fragments at 220 (178+42) and 171 (129+42) mass units corresponding to the *N*-acetylated *β*-methylphenylalanyl and the *N*-acetylated isoleucylamide ions. These results indicated that the *β*-methylphenylalanyl moiety is linked to the altemicidin structure through an ester bond and the isoleucylamide moiety is linked *via* its amide function. The presence of an ester group in (**2**) is confirmed by IR spectroscopy (1744 cm^{-1}).

Mass spectrometry was also the technique of choice to determine the position of attachment of the two amino acid moieties to the altemicidin nucleus. Only the 6-position, bearing a hydroxy group, is available to link the *β*-methylphenylalanyl fragment *via* an ester bond onto the altemicidin backbone. This regiochemistry was supported by NMR data, namely the substantial downfield shift observed for H-6 in (**2**) compared to altemicidin, and confirmed by mass data using the MS/MS technique (Fig. 2). The major peak on the mass spectrum (Flow FAB, −ve ion) of (**2**) is found at 426 mass units. The same peak was absent in the mass spectrum of (**1**) but appeared when the $[M-H]^-$ ion was subjected to collisional fragmentation during an MS/MS experiment (FAB). These observations indicate that both compounds (**1**) and (**2**) fragment to give a peak at 426 mass units, but the fragmentation process is more facile for (**2**) than for (**1**). Loss of 62 mass units from (**1**) to produce a fragment with *m/z* 426 corresponds to the

Table 2. ^{13}C NMR data of SB-203207 (**1**), SB-203208 (**2**) and altemicidin (**3**) in D_2O.

Carbon	Chemical shifts (ppm)		
	SB-203207 (1)	SB-203208 (2)	altemicidin (3)[2,3]
1	45.65	45.07	45.5
3	147.37	146.90	147.3
4	97.30	98.48	97.1
4a	31.80	31.12	31.7
5	41.50	40.02	40.8
6	76.20	79.77	76.0
7	69.00	70.70	69.1
7a	40.89	42.28	41.4
8	43.38	43.32	43.2
9	174.23	173.8	174.2
12	-	167.49	-
13	-	59.54	-
14	-	41.36	-
15	-	17.42	-
17	-	139.32	-
18,19,21,22	-	128.67, 130.11	-
20	-	129.24	-
23	179.47	176.45	179.7
26	164.90	164.97	164.4
27	58.34	57.99	60.3
30	176.27	177.01	-
31	61.09	61.02	-
32	37.13	37.11	-
33	24.86	24.92	-
34	11.61	11.81	-
36	15.46	15.42	-

Scheme 1. Nature of linkage of the amino acids to the altemicidin nucleus.

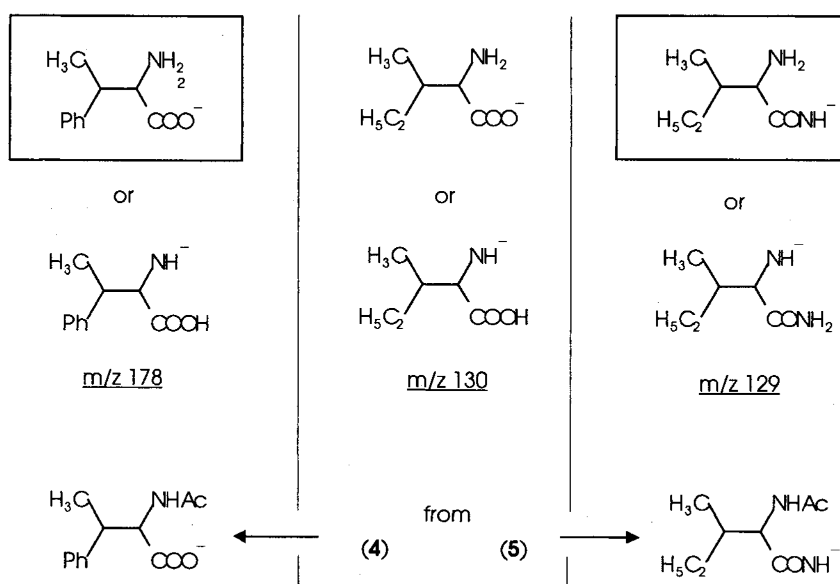
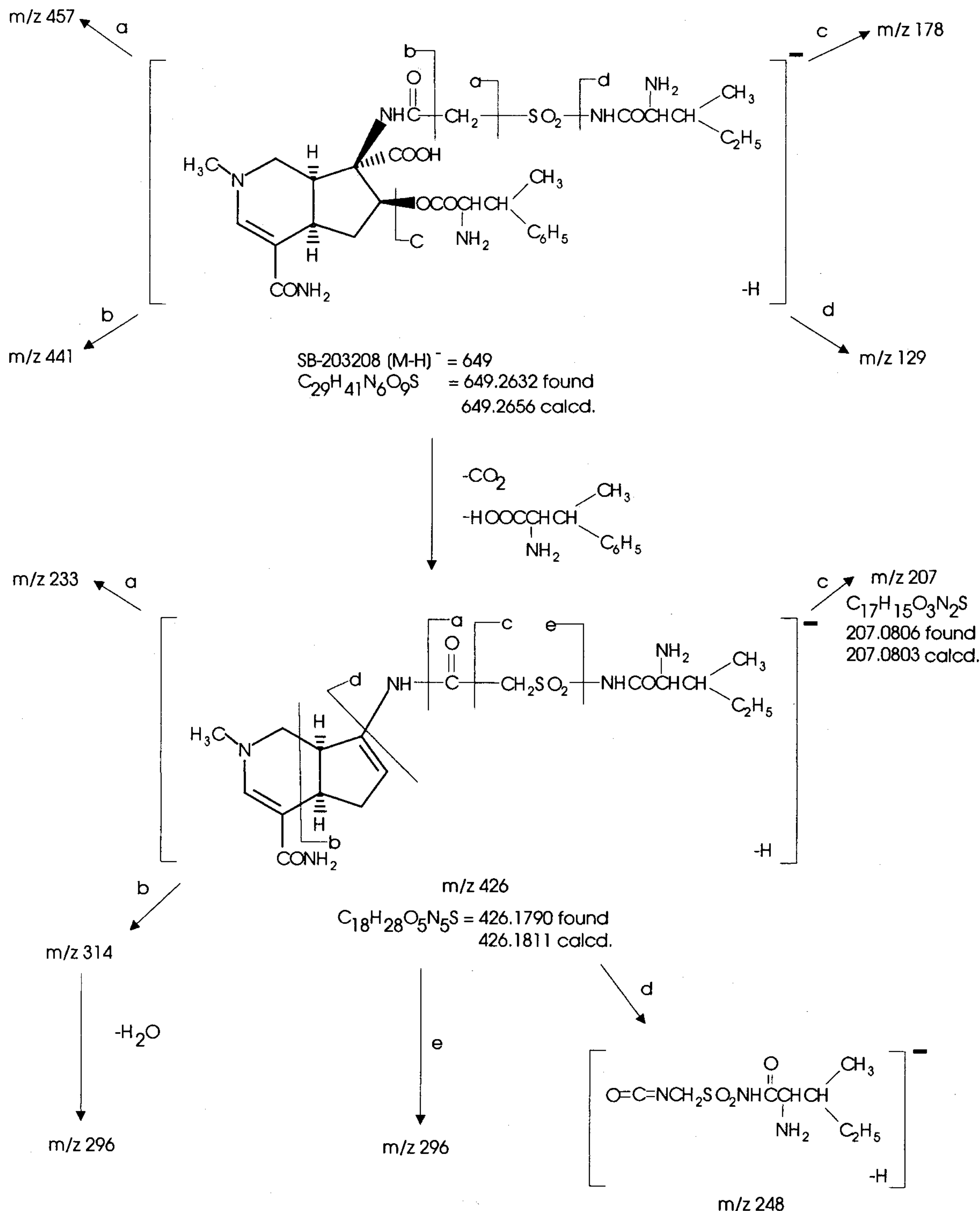


Fig. 2. Proposed mass spectral fragmentation for (**2**) $[M-H]^-$ *m/z* 649 and its daughter ion $[M-H]^-$ *m/z* 426 by FAB-MS/MS.



loss of CO_2 and H_2O. In the case of (**2**) loss of 223 mass units to provide fragment *m/z* 426 arises from the loss of CO_2 and β-methylphenylalanine, a better leaving group than H_2O (Fig. 2). In both cases, the fragments corresponding to the individual losses of CO_2, H_2O or β-methylphenylalanine are not observed which suggests that the losses may be concerted in a manner analogous to the fragmentation of maduramicin and related compounds[8].

These results are in good agreement with the vicinal spacial arrangement of the two substituents (COOH and OR).

The isoleucyl fragment could be linked to the altemicidin backbone through an amide bond formed between its carbonyl group and the amino group of either the carboxamide function or the sulfonamide function of altemicidin. Using the FAB technique in the negative ion mode, MS/MS experiments on the parent ion $[M-H]^-$ of (**2**) at *m/z* 649 and on its daughter ion at *m/z* 426 produced anions at 457, 441, 314, 296, 296, 248, 233, 207, 178 and 129, some of which (314, 296, 248, 233 and 207) can only be explained from the latter regioisomer (Fig. 2). Where possible (*i.e.* where sufficient signal was available in the FAB-MS spectrum without collisional activation), accurate mass measurements were performed on fragment ions (*m/z* 426 and 207) confirming their proposed molecular

Fig. 3. Proposed mass spectral fragmentation for (**2**) $[M+H]^+$ *m/z* 651 by electrospray - MS/MS.

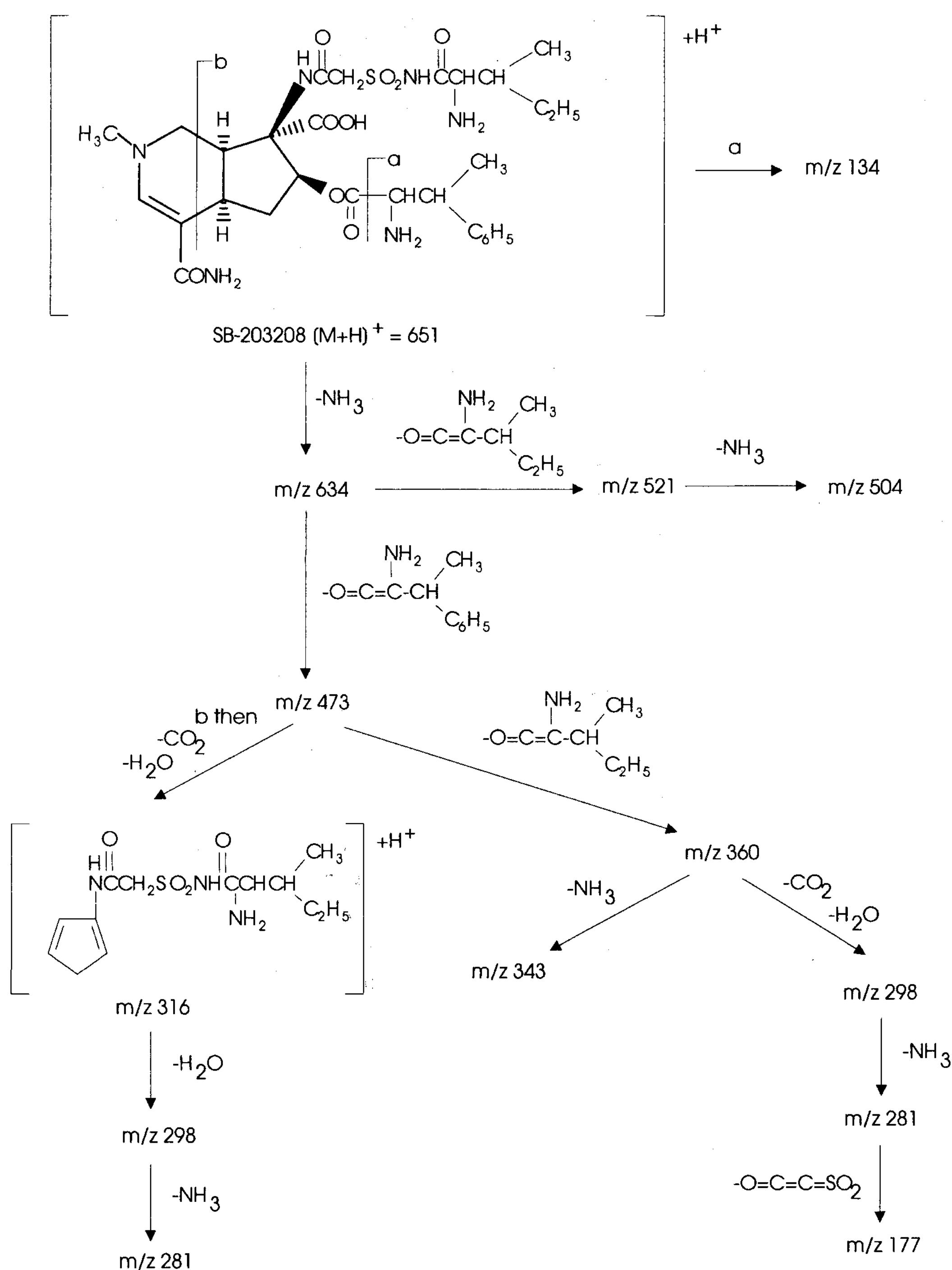


Table 3. Physico-chemical properties of (**4**) and (**5**).

	(**4**)	(**5**)
Appearance	white powder	white powder
Molecular formula	$C_{35}H_{48}O_{12}N_6S$	$C_{36}H_{50}O_{12}N_6S$
FAB-MS ([M-H]$^-$, m/z)	775	789
UV $\lambda^{H_2O}_{max}$ nm(ε)	322 (11,320)	320 (10,344)
IR ν_{max} (KBr) cm^{-1}	3383, 1740, 1654, 1619, 1538, 1373, 1257, 1186, 1117, 1063	3424, 1735, 1655, 1622, 1585, 1374, 1244, 1170, 1145
$[\alpha]_D^{25}$ (H_2O)	+ 18.0° (c 1.8×10^{-3})	-27.0° (c 5×10^{-4})
HPLC (Rt minutes)^a	14	29

^{a} Column: Hypersil ODS 5μm (0.46x25cm); mobile phase: 60% NH_4OAc 0.05M, 40% MeOH; detection: UV 220nm; flow rate: 2ml/minute

formulae. Using the electrospray technique, the cation of (**2**) was generated. An MS/MS experiment on the positive molecular ion *m/z* 651 provided further useful fragments (Fig. 3). The most abundant cation at *m/z* 634 corresponds to loss of ammonia. Some of the fragment ions 316, 298 and 281 can again only be explained from the regioisomer with the isoleucyl group linked to the sulfonamide function.

Comparison of the UV data obtained on (**2**) ($\lambda^{H_2O}_{max}$ 297 nm) with that of the triacetyl derivatives (**4**) (322 nm) and (**5**) (320 nm) indicated a bathochromic shift of 25 and 23 nm respectively. This observation is in agreement with the presence of an unsubstituted carboxamide function in (**2**) which, in addition to the two amino groups, would react readily with acetic anhydride-pyridine to provide (**4**) and (**5**) in which the chromophore has been extended by an acetyl group[9]. The esterification in the case of (**5**) is rationalised as occurring during the work-up by reaction of the putative mixed anhydride intermediate with methanol added during this procedure. The physico-chemical properties of (**4**) and (**5**) are summarised in Table 3 and the ^{1}H NMR data are given in Table 4.

In conclusion this paper describes the structural elucidation of two potent isoleucyl t-RNA synthetase inhibitors SB-203207 and SB-203208. These inhibitors isolated from a *Streptomyces* sp. are of a novel structural type related to altemicidin.

Experimental

FAB-MS and HRFAB-MS were carried out on VGZAB IF and JEOL SX-102 spectrometers. Mass spectral deuteration experiments and FAB-MS/MS were run on a Finnigan MAT TSQ 70 spectrometer. Electrospray-MS data were obtained on a Sciex API III triple quadrupole spectrometer. Optical rotations were determined with an Optical Activity Ltd AA-1000 and a Perkin Elmer 241 polarimeters. IR spectra were measured on a Perkin Elmer 983 IR spectrophotometer. UV spectra were taken with a Beckman DU68 UV-visible spectrophotometer. NMR spectra were recorded on a Bruker AM400 spectrometer equipped with a dual ^{1}H/^{13}C 5 mm probe at 300°K referenced to external HOD. TLC was performed on precoated plates, Merck Kieselgel 60 F_{254}. Packed columns of the Hypersil ODS and the Spherisorb ODS were purchased from Jones Chromatography.

Table 4. ^{1}H NMR data of (4) and (5).

Proton	Chemical shifts (δ value in ppm) and coupling constants (Hz) in D_2O (ref. HDO; δ 4.80)					
	(4)			(5)		
1	3.02	m	-	3.12	dd	J = 9.4, 13.5
	3.25	obscured	-	3.24	dd	J = 5.6, 13.5
3	7.70	s	-	7.67	s	-
4a	3.02	m	-	3.02	bq	J = 8.0
5	0.62	ddd	J = 5.0, 9.8, 14.5	1.00	obscured	-
	2.67	ddd	J = 7.0, 8.0, 14.5	2.70	ddd	J = 7.0, 8.0, 14.2
6	5.28	dd	J = 5.0, 7.0	5.26	dd	J = 7.0, 7.2
7a	2.80	ddd	J = 5.8, 5.8, 10.9	2.93	m	-
8	3.25	s	-	3.18	s	-
13	~4.8	obscured	-	4.73	d	J = 8.9
14	3.29	m	-	3.32	qd	J = 7.0, 8.1
15	1.33	d	J = 7.0	1.36	d	J = 7.0
18→22	7.25-7.41	m	-	7.30-7.42	m	-
24-OMe	-	-	-	3.74	s	-
27	4.30 & 4.35	AB system	J = 14.2	4.28 & 4.35	AB system	J = 14.0
31	4.21	d	J = 6.3	4.21	d	J = 6.4
32	1.95	m	-	1.95	m	-
33	1.23	m	-	1.22	m	-
	1.52	m	-	1.51	m	-
34	0.92	t	J = 7.4	0.92	t	J = 7.4
36	1.00	d	J = 6.8	0.99	d	J = 6.9
10, 16, 35 N-Ac	2.08, 2.1, 2.23	3s	-	2.08, 2.09, 2.23	3s	-

Hydrolysis of SB-203208 (2)

SB-203208 (**2**) (2 mg) was hydrolysed with 5 N HCl (1 ml) at 100°C for 18 hours in a sealed tube. The resulting solution was concentrated to dryness, redissolved in H_2O and evaporated to dryness to remove HCl before TLC analysis (SiO_2) using *n*-Butanol : Ethanol : Water-1 : 1 : 1 and chiral HPLC analysis.

Determination of the Absolute Configuration by Chiral HPLC

An aliquot of the above hydrolysis mixture (concentration ~0.1 mM) was dissolved in buffer (1 ml of 0.04 M Li_2CO_3 adjusted to pH 9.5 with 1 M HCl) and 0.011 M dansyl chloride solution in acetonitrile (1 ml) was added. After gentle shaking, the mixture was allowed to react at room temperature for 15 minutes. A 1 to 4 dilution of the mixture with mobile phase (0.5 mM copper acetate, 0.5 mM L-aspartyl-L-phenylalanine methyl ester, 0.05 M ammonium acetate pH 7 in 25% acetonitrile) was carried out before injection. The HPLC system comprised a reverse phase column (Spherisorb ODS 5 μm (0.46×25 cm)) with a flow rate of 1.0 ml/minute. Detection was carried out by UV at 320 nm. The retention times of dansyl amino acids were as follows (minutes): Dansyl-L-Ile: 30.0, Dansyl-D-Ile: 25.5, Dansyl-L-allo-Ile: 25.5, Dansyl-D-allo-Ile: 20.1.

Detection of Amino Acids

SB-203207 (**1**) and SB-203208 (**2**) (~1 mg) were hydrolysed using a vapour phase method with 6 N HCl-1% phenol for 24 hours at 110°C *in vacuo*. The amino acid(s) released were derivatised with PITC at room temperature for 20 minutes and the reaction mixtures then evaporated to dryness. The resulting PTC-amino acids were chromatographed on a C18 reverse phase column [WATERS Nova-Pak, 60 Å, 4 μm (0.39×15 cm)] at a flow rate of 1.0 ml/minute using as solvents: 0.14 M sodium acetate trihydrate / 0.05% triethylamine / 6% acetonitrile adjusted to pH 6.4 with phosphoric acid and 60% acetonitrile / 40% water. Detection was carried out by UV at 254 nm. Retention times of PTC amino acids were as follows (minutes): PTC-Ile: 9.0, PTC-β-MePh: 10.97.

Synthesis of (4) and (5)

SB-203208 (**2**) (100 mg) was dissolved in pyridine (10 ml) and acetic anhydride (10 ml) was added. The solution

was stirred for 4 hours at room temperature. Methanol (10 ml) was then added dropwise. After one hour, the reaction mixture was evaporated *in vacuo*. The residue was purified by HPLC to give (**4**) (18 mg) (15%) and (**5**) (20 mg) (15%). The physico-chemical and ^{1}H NMR data of (**4**) and (**5**) are summarised in Tables 3 and 4.

Acknowledgements

The authors wish to thank D. BELL, D. BRYANT, G. RISBRIDGER, and A. WEST for mass spectral data, J. WHITE for NMR measurement, E. A. CUTMORE for IR and UV spectra, B. NEVILLE for amino acid analysis and K. JENNINGS for chiral HPLC work. We also express our thanks to D. BRYANT, E. A. CUTMORE, N. HIRD and P. MILNER for helpful discussions. We also thank the Institute of Microbial Chemistry, Tokyo, for kindly sending us a sample of altemicidin.

References

1) TAKAHASHI, A.; S. KURASAWA, D. IKEDA, Y. OKAMI & T. TAKEUCHI: Altemicidin, a new acaricidal and antitumour substance. I. Taxonomy, fermentation, isolation and physico-chemical and biological properties. J. Antibiotics 42: 1556~1561, 1989
2) TAKAHASHI, A.; D. IKEDA, H. NAKAMURA, H. NAGANAWA, S. KURASAWA, Y. OKAMI, T. TAKEUCHI & Y. IITAKA: Altemicidin, a new acaricidal and antitumour substance. II. Structure determination. J. Antibiotics 42: 1562~1566, 1989
3) TAKAHASHI, A.; H. NAGANAWA, D. IKEDA & Y. OKAMI: Structure determination of altemicidin by NMR spectroscopic analysis. Tetrahedron 47: 3621~3632, 1991
4) KENDE, A.; K. LIU & K. BRANDS: Total synthesis of (−) altemicidin: A novel exploitation of the Potier-Polonovski rearrangement. J. Am. Chem. Soc. 117: 10597~10598, 1995
5) STEFANSKA, A.; R. CASSELS, S. READY & S. WARR: SB-203207 and SB-203208, two novel tRNA synthetase inhibitors from a *Streptomyces* sp. I. Fermentation, isolation and properties. J. Antibiotics 53: 357~363, 2000
6) KATAOKA, Y.; Y. SETO, M. YAMAMOTO, T. YAMADA, S. KUWAKA & H. WATANABE: Studies of unusual amino acids and their peptides. VI. The synthesis and the optical resolutions of β-methylphenylalanine and its dipeptide present in bottromycin. Bull. Chem. Soc. Japan 49: 1081~1084, 1976
7) TSUCHIHASHI, G.; S. MITAMURA & K. OGURA: Synthesis of (*S,S*)-2-amino-3-phenylbutyric acid. Bull. Chem. Soc. Japan 52: 2167~2168, 1979
8) SIEGEL, M.; W. MCGAHREN, K. TOMER & T. CHANG: Applications of fast atom bombardment mass spectrometry and fast atom bombardment mass spectrometry—mass spectrometry to the maduramicins and other polyether antibiotics. Biomedical and Environmental Mass Spectrometry 14: 29~38, 1987
9) BIRNBAUM, G.; E. DE CLERCQ, P. HATFIELD & M. ROBINS: Synthesis and conformational studies of 4-carbamoyl-1-[(2-hydroxyethoxy)methyl]-5-(methylamino)imidazole and derivatives as new acyclonucleoside analogues. Heterocycles 25: 493~506, 1987