Isolation and Structure Determination of Two Novel Phenazines from a *Streptomyces* with Inhibitory Activity against Metallo-enzymes, Including Metallo-β-lactamase

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Two novel metabolites, SB 212021 and SB 212305, have been isolated from a *Streptomyces* and shown to have molecular formulae of $C_{15}H_{10}N_2O_5$ and $C_{20}H_{17}N_3O_8S$, respectively. The structures were deduced by a combination of NMR techniques and mass spectral fragmentation patterns and shown to be novel members of the phenazine group of antibiotics. In the absence of added zinc, both compounds had IC_{50}'s of $1\sim75\,\mu M$ for the *Bacteroides fragilis* 262 CfiA and *Xanthomonas maltophilia* L-1 metallo-β-lactamases. The compounds also inhibited ACE with IC_{50}'s of 55 and 68 μM, respectively. Mode of action studies illustrate that the compounds inihibit some metalloenzymes by chelation of the active site metal ion. They exhibit poor antibacterial activity.

Metallo-β-lactamases, whilst not as prevalent in nature as their serine counterparts, do constitiute a possible threat to β-lactam chemotherapy due to the unavailability of effective inhibitors. Although only produced by a limited number of organisms they are potentially transferrable by plasmids and could therefore pose a wider threat[1]. The search for inhibitors of this enzyme is thus aimed at identifying structural types from natural product screening that could provide leads for chemical modification/synthesis.

We report on the isolation of two novel phenazines, designated SB 212021 (**1**) and SB 212305 (**2**), produced by an unidentified *Streptomyces* sp. and found to inhibit zinc-dependent metallo-β-lactamase from *Bacillus cereus*.

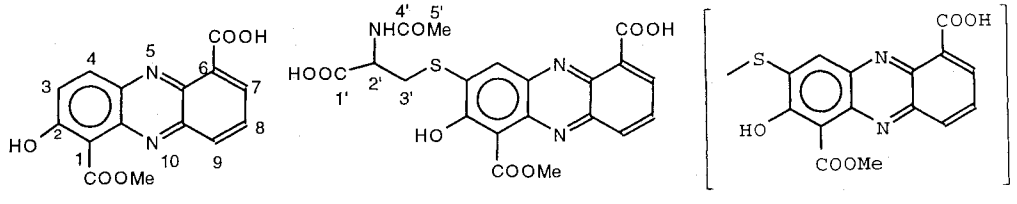
This paper describes the isolation, physico-chemical properties, structure determination and biological activity of SB 212021 and SB 212305.

Materials and Methods

Fermentation Conditions

The unidentified *Streptomyces* sp. was maintained as a vegetative cell suspension stored in glycerol 10% under liquid nitrogen. Vegetative cell and spore suspension (1 ml) was used to inoculate seed medium (100 ml) containing 1.5% agar. The seed stage medium consisted of yeast extract (Oxoid) 0.5%, malt extract (Oxoid) 1.0%, glycerol 1.0% and peptone soya (Oxoid) 0.5% dissolved in distilled water and adjusted to pH 6.5 before sterilisation in an autoclave at 121°C for 15 minutes. Four spiked 500 ml flasks, each containing 60 ml of seed stage medium as defined above, were inoculated with 3 plugs from a culture plate. The inoculated flasks were incubated on a gyratory shaking table at 240 rpm for 60 hours at 28°C and after this time were used to inoculate 70 spiked 500 ml flasks, each containing 60 ml of seed stage medium as defined above (inoculum level *ca.* 5%, 3 ml/flask). These inoculated flasks were shaken at 240 rpm for 4.5 days at 28°C. The harvested broth was

Fig. 1. Structures of SB 212021 and 212305.



SB 212021 SB 212305

(1) (2) (3)

clarified by centrifugation to afford 4.25 litres at pH 5.6.

Detection Methods

The phenazine inhibitors could be detected by HPLC in the culture broth of the unidentified *Streptomyces* sp. by monitoring at 300 nm. A C18 Spherisorb S 10 ODS 2 column (250 × 4.6 mm) (PhaseSep, Deeside Industrial Estate, Queensferry, Clwyd, U.K.) was used for the separation and eluted with 0.05 M ammonium acetate buffer at pH 6.5 in 30% methanol. At a flow rate of 1.5 ml/minutes SB 212021 had a Rt of 9.6 minutes and SB 212305 had a Rt of 6.5 minutes. A Waters 600 multisolvent delivery system was used and monitoring was by a Waters Lambda Max Model 481 LC spectrophotometer.

The extraction and purification of the phenazines could also be followed by monitoring inhibitory activity against *Bacillus cereus* II metallo-β-lactamase (Porton Products Ltd., Maidenhead, Berks., U.K.). Nitrocefin was used as reporter substrate in an assay which has been previously described[2]. IC_{50}'s were determined following a 15 minute pre-incubation of enzyme and inhibitor.

Extraction and Isolation

The clarified broth was acidified to pH 3 by addition of 5 M HCl and extracted with butanol. The dark butanol layer was then back-extracted with water maintained at a pH of 7.5. The aqueous layer afforded a red powder (3.6 g) on freeze-drying. Ion-exchange chromatography on IRA 458 (Cl^- form) (Rohm and Haas, Philadelphia, U.S.A.) and elution with 0.5 M NaCl gave a purer product. Desalting on Diaion HP-20 styrene divinyl benzene cross-linked polymeric adsorbent (supplied by Mitsubishi Chemical Industries Limited, Tokyo, Japan) afforded two active fractions; Fraction 1 (0.61 g) which eluted from the column with water, and Fraction 2 (0.35 g) which eluted with neat methanol.

Fraction 2 was further chromatographed on silica gel, eluting with n-butanol - ethanol - water (4 : 1 : 1) to give red powder (69 mg) and subsequently purified by HPLC on a Dynamax 150 A preparative C18 column (300 × 10 mm, fitted with pre-column) (supplied by Rainin Instruments Co. Inc., Mack Road, Woburn, Massachusetts, U.S.A.), eluting with 27.5% MeOH in 0.05 M ammonium acetate buffer and monitoring at 300 nm. This yielded 10 mg of SB 212021. The Rt under the above conditions was 18 minutes.

Fraction 1 was further chromatographed on silica gel, eluting with n-butanol - ethanol - water (2 : 1 : 1) to give red powder (0.29 g) and subsequently purified by HPLC on a Dynamax preparative C18 column (details above), eluting with a linear gradient of 100% water to 50% methanol over 30 minutes. Under these conditions, and monitoring at 300 nm, SB 212305 (47 mg) was isolated with Rt 19 minutes. A further component, SB 210767 (49 mg), was also isolated with Rt 21 minutes. This latter compound appeared to have very similar spectroscopic properties to SB 212305 and the same mass spectrum. We believe that it may be a chelated form of SB 212305.

Biological Evaluation

The IC_{50}'s of each compound for *Bacillus cereus* II[3], *Bacteroides fragilis* 262 CfiA[4] and *Xanthomonas maltophilia* L-1[5] metallo-β-lactamases were determined as above. Each IC_{50} was measured both in the absence of added zinc and in the presence of 1 mM zinc sulphate. The reporter substrate for the zinc-dependant angiotensin-converting enzyme (ACE) IC_{50}'s was *N*-(3-[2-furyl]acryloyl-Phe-Gly-Gly (FaFGG)[6]. Assays were performed in 0.05 M Hepes, 0.3 M NaCl at pH 7.5 at 37°C. A range of inhibitor concentrations were incubated for 5 minutes with purified ACE (Sigma Chemical Company, Poole, Dorset, U.K.) and the assay initiated by the addition of 0.35 mM FaFGG (final concentration). Percent inhibition of ACE activity relative to an untreated control was calculated for each concentration and the IC_{50} calculated.

Mechanism of Inhibition of *B. cereus* II

B. cereus II metallo-β-lactamase was incubated with a sufficient concentration of SB 212021, SB 212305 and EDTA (for comparison) to ensure >90% inhibition. These samples were then passed down a PD10 (Pharmacia Biotech Europe, Brussels, Belgium) gel filtration column. Fractions (0.5 ml) were collected and examined for β-lactamase activity by diluting 50 μl of sample into 3 ml of 25 mM Pipes buffer (pH 7.0) containing 1 mM $ZnSO_4$ and 333 μM ampicillin. The rate of hydrolysis of ampicillin was measured at 235 nm. A control sample was also subjected to this treatment. The amount of inhibitor in each fraction was estimated by reading the absorbance at 290 nm, 305 nm and 229 nm for SB 212021, SB 212305 and EDTA respectively.

Spectroscopic Methods

NMR spectra obtained on SB 212021 and SB 212305 were run at 25°C on a Bruker AM 400 operating at 400 MHz. IonSpray MS were obtained on a Sciex (Toronto) API-III triple-quadrupole mass spectrometer.

Results and Discussion

Isolation

Culture broth (4.25 litres) from an unidentified *Streptomyces* sp., when treated as outlined above, afforded 10 mg of SB 212021 and 47 mg of SB 212305.

Properties of SB 212021 and SB 212305

The physico-chemical properties of the two phenazines, SB 212021 and SB 212305, are shown in Table 1. NMR data is presented in Tables 2 and 3.

Biological Properties

SB 212021 showed some antibacterial activity against certain strains of *Streptococcus pneumoniae* and *En-*

Table 1. Physico-chemical properties of SB 212021 and SB 212305.

	SB 212021	SB 212305
Molecular formula	$C_{15}H_{10}N_2O_5$	$C_{20}H_{17}N_3O_8S$
MS (Ionspray) $(M+H)^+$	299	460
$(M-H)^-$	297	458
UV λ_{max} nm (ε)	MeOH	H_2O
	210 (23,563), 247 (11,608), 294 (17,672), 370 (2,840), 520 (1,178)	212 (25,422), 249 (18,754), 305 (33,757), 380 (5,000), 530 (2,084)
	MeOH+HCl	H_2O+HCl
	203 (26,145), 248 (16,421), 268 (17,914), 369 (5,938), 431 (2,582)	212 (22,088), 251 (22,921), 278 (23,755), 378 (7,084), 440 (2,917)
	MeOH+NaOH	H_2O+NaOH
	210 (73,271), 246 (17,554), 298 (19,778), 370 (4,000), 520 (2,528)	249 (18,754), 305 (36,674), 370 (2,917) 515 (2,500)
IR (KBr) cm^{-1}	3395, 1691, 1616, 1580, 1536, 1476, 1424, 1382, 1341, 1269, 1237, 1205, 1134, 1093, 1049	3389, 1690, 1622, 1580, 1533, 1506, 1472, 1433, 1403, 1313, 1232, 1206, 1147, 1118, 1053

Table 2. ^1H and ^{13}C NMR chemical shifts and assignments for SB 212021 in CD_3OD.

Position	δ_H	δ_C
1	—	110.1
2	—	168.3
3	6.83, d, *J* 8.6	110.8
4	8.60, d, *J* 8.6	143.1
6	—	131.5
7	8.82, d, *J* 8.6	137.3
8	7.99, dd, *J* 8.6, 8.6	131.5
9	8.52, d, *J* 8.6	134.5
1-COOMe	—	171.1
6-COOH	—	171.1
Me	3.97, s	52.0
4a	—	137.9
5a	—	137.6
9a	—	146.0
10a	—	145.6

Table 3. ^1H and ^{13}C NMR chemical shifts and assignments for SB 212305 in D_2O.

Position	δ_H	δ_C
1	—	108.3
2	—	169.4
3	—	113.3
4	8.36, s	146.6
6	—	130.2
7	8.60, d, *J* 7.8	137.4
8	7.90, dd, *J* 8.3, 7.8	131.6
9	8.20, d, *J* 8.3	133.1
1-COOMe	—	167.9
6-COOH	—	171.7
OMe	3.99, s	53.0
1′	—	177.9
2′	4.03, dd, *J* 9.0, 3.7	55.2
3′	3.57, dd, *J* 14.0, 3.7, 2.98, dd, *J* 14.0, 9.0	34.7
4′	—	174.1
5′	1.80, s	22.5
4a	—	136.0
5a	—	136.8
9a	—	143.9
10a	—	143.1

terococcus faecalis but was otherwise devoid of activity against a range of Gram-positive and Gram-negative organisms. SB 212305 was inactive against all bacterial strains tested.

The IC_{50}'s of the two metabolites (plus the chelator EDTA as a control) against three metallo-β-lactamase enzymes and the metallo-enzyme ACE are shown in Table 4. Both SB212021 and SB212305 were poor inhibitors of *B. fragilis* 262 CfiA with and without added zinc. In the absence of added zinc both compounds and EDTA had IC_{50} values of $< 100\ \mu M$ for the *X. maltophilia* L-1 and *B. cereus* II metallo-β-lactamases, but when these assays were performed in the presence of 1 mM zinc the IC_{50}s increased by >10 fold. Therefore, inhibition of metallo-β-lactamases by the phenazines, like EDTA, was dependent on the zinc concentration. To examine the mechanism of inhibition further, *B. cereus* II metallo-β-lactamase inhibited by SB212021, SB212305 and EDTA was subjected to gel filtration. In each case this treatment separated excess inhibitor from the metallo-β-lactamase. When the fractions containing enzyme were assayed in excess zinc $>80\%$ of enzyme activity was recovered. Therefore, enzyme inhibited by these agents, like EDTA, could be reversed by the addition of zinc. Also, both the phenazines and EDTA inhibited ACE with IC_{50} values of $50 \sim 68\ \mu M$, illustrating the low specificity exhibited by these agents. These data have shown that, like EDTA, SB212021 and SB212305 inhibit metallo-β-lactamases by chelation of the active site zinc. By definition such agents should exhibit low specificity. This is illustrated by their activity against two unrelated metalloenzymes.

Structure Determination

FAB, EI and CI mass spectroscopic techniques failed to give any MW information on SB 212021 or SB 212305.

Table 4. IC_{50} values for the inhibition of metallo-β-lactamases *Xanthomonas maltophilia* L-1, *Bacteroides fragilis* 262 CfiA and *Bacillus cereus* II and metallo-protease ACE.

	IC_{50} (μM)			
	X. maltophilia L-1	*B. fragilis* 262 CfiA	*B. cereus* II	ACE
SB 212021	19 (>1000)	820 (>1000)	37 (>1000)	55
SB 212305	1 (235)	>1000 (>1000)	75 (978)	68
EDTA	7 (>1000)	37 (>1000)	7 (>1000)	50

Figures in parenthesis at $[Zn^{2+}]$ of 1 mM.

Ionspray, on the other hand, afforded a peak at 299 in +ve ion mode (after acidification of the sample) and 297 in −ve ion mode for SB 212021, indicating a MW of 298. SB 212305 similarly afforded a molecular ion at 458 in −ve ion mode under ionspray conditions, together with a major fragment at 329. In +ve ion, peaks at 460, 477 and 482 were observed corresponding to protonated, ammoniated and sodiated molecules, respectively. A MW of 459 was established.

Consideration of the UV data (see Table 1) obtained on the two inhibitors, coupled with the characteristic low-field chemical shifts of the aromatic protons (Tables 2 and 3) rapidly established these compounds as members of the phenazine class of antibiotics.[7~9]

The ^1H NMR of SB 212021 (Table 2) indicated two possible substitution patterns; either a 1,2,6- or a 1,4,6-trisubstitution. The high field shift (δ 6.83) of one of the aromatic protons indicated that it must be *ortho* to a hydroxyl or ether function. Further support for the presence of an oxygen substituent came from the ^{13}C NMR which indicated two high field signals (110.1 and 110.8) corresponding to the two *ortho* carbon atoms. It has been reported[10,11] that bridgehead carbon atoms in the phenazine ring system are particularly insensitive to adjacent hydroxyl substitution and do not display the usual upfield shift expected for *ortho* carbons. This being so, the fact that two such high field signals are observed in the ^{13}C NMR spectrum of SB 212021 indicates the presence of a 2-hydroxy (or methoxy) rather than a 1- or 4-hydroxy substituent.

The observed MW for SB 212021 was readily satisfied by the inclusion into the structure of two carboxy substituents at positions 1 and 6. This was supported spectroscopically by carboxyl absorption bands at 1691 cm^{-1} in the IR spectrum and by 2 signals in the ^{13}C NMR at 171.1 ppm. It is conceivable that the two carboxyl functions could be in a 1,9-arrangement and NMR techniques would not be expected to distinguish between these two possibilities. However, work by HOLLIMAN *et al.* and others[12,13] has established that phenazine-1,6-dicarboxylic acid is a universal precursor for microbial phenazines and therefore it is reasonable to assume this regiochemistry for SB 212021.

Thus, the structure of SB 212021 would appear to be based on 2-hydroxy phenazine-1,6-dicarboxylic acid. The methyl group can be shown to be present as a methyl ester rather than a methyl ether. Evidence for this comes from the methyl signal in the ^1H NMR spectrum which did not exhibit measurable nuclear Overhauser enhancement with any other proton in the molecule. A 1-carboxymethyl substituent neatly explains this lack of interaction, as indicated in structure (**1**). Further evidence to support this placing of the methyl group is presented below.

Consideration of the ^1H NMR data (Tables 2 and 3) leads to the conclusion that SB 212305 is a substituted derivative of SB 212021 where the C-3H is replaced with a $-CH_2CH-$ unit. The ionspray mass spectrum (−ve ion) of SB 212305 gives a major fragment at 329 and, since no corresponding loss is seen in the spectrum of SB 212021, the fragmentation must be associated with the C-3 side-chain. That being so, the peak observed at 329 is readily explained in terms of the phenazine nucleus carrying a sulphur atom at C-3 (**3**).

The ^1H and ^{13}C chemical shifts corresponding to the C-3 side chain in SB 212305 strongly suggest the presence of an amino acid and signals were consistent with an *N*-acetyl cysteine moiety. There is good agreement between literature[14,15] and observed chemical shifts for this group. Furthermore, the presence of sulphur in SB 212305 was confirmed by microanalysis.

Finally, the presence of a methyl ester in SB 212305 was confirmed by MS/MS experiments resulting in peaks corresponding to the loss of 44 (CO_2) and 59 (COOMe). In addition, potentiometric titration of this compound indicated the presence of a phenolic group with pKa 9.1+/−0.25.

The assignments in Tables 2 and 3 are based on the

results of COSY and HMBC NMR experiments.

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