Bagremycin A and B, Novel Antibiotics from *Streptomyces* sp. Tü 4128†

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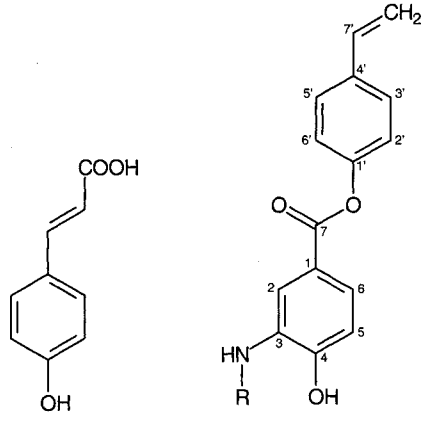
Two novel secondary metabolites, bagremycin A (**2**) and B (**3**), were detected in the culture filtrate of *Streptomyces* sp. Tü 4128 by HPLC-diode-array screening. They are phenol esters of 3-amino-4-hydroxybenzoic acid with a derivative of *p*-coumaric acid and show a moderate activity against Gram-positive bacteria and some fungi.

Our screening strategy for detection of novel secondary metabolites is based on a non-target approach, by analysing culture extracts of freshly isolated strains of actinomycetes with reversed-phase HPLC coupled with diode-array detection (HPLC-DAD) and a HPLC-UV-Vis-Database that contains about 600 reference compounds, mostly antibiotics[2]. Known metabolites can be identified and new compounds can be characterised by comparison of their retention times and UV-visible spectra. Strains were cultivated in two complex media which differed significantly both, from carbon and nitrogen sources and from the carbon-nitrogen ratio. The culture broth of each strain was extracted with ethyl acetate after 3, 6, 9 and 14 days, respectively, of incubation and investigated by HPLC-DAD for production of new secondary metabolites.

Strain Tü 4128 was isolated from a soil collected in Java, Indonesia. Using the HPLC-DAD technology, three peaks were detected in the extracts which were not in accordance with any of the reference compounds stored in the database. The compound with a retention time of 5.2 minutes was identified by HPLC-ESI-MS as *p*-coumaric acid (**1**). The compounds showing retention times of 7.9 minutes and 8.9 minutes, respectively, were characterised by NMR techniques as novel secondary metabolites and were named as bagremycin A (**2**) and B (**3**). Bagremycin B has not previously been described as a natural product, but is known as a product obtained by chemical degradation of the siderophore ferroverdin[3].

Fig. 1. Structures of *p*-coumaric acid (**1**), bagremycin A (**2**) and B (**3**), produced by *Streptomyces* sp. Tü 4128.



1

2: R = H (Bagremycin A)

3: R = CH_3CO (Bagremycin B)

† Art. No. 23 in 'Biosynthetic Capacities of Actinomycetes'. Art. No.22: See ref. 1.

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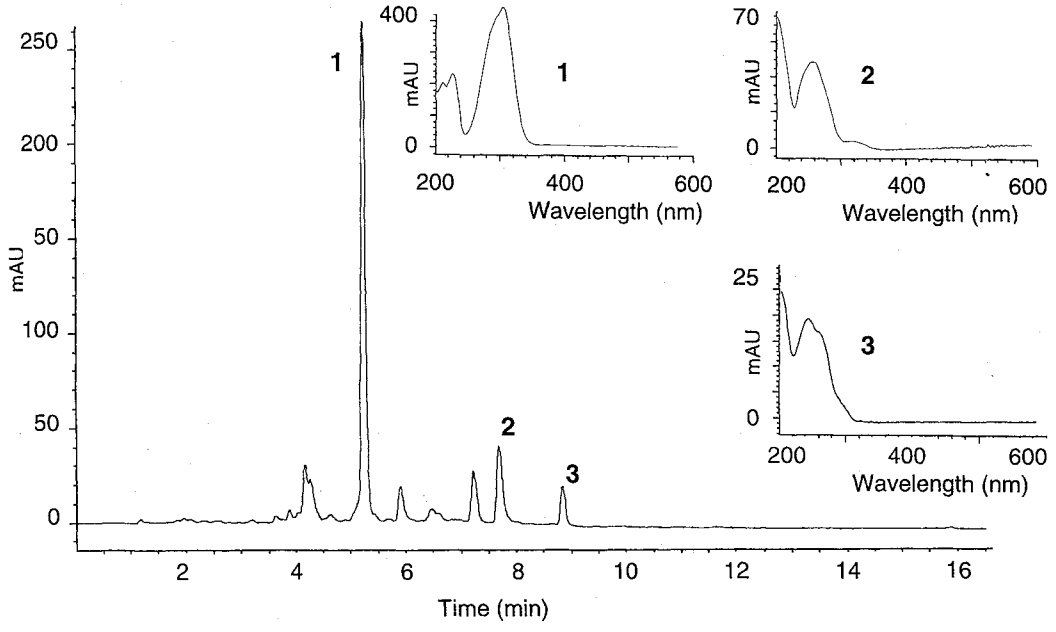
Fig. 2. HPLC analysis of culture filtrate extract from *Streptomyces* sp. Tü 4128 monitored at 280 nm, and overlayed UV spectra from *p*-coumaric acid (**1**), bagremycin A (**2**) and B (**3**).



In this report we describe the taxonomy of the producing strain, the fermentation, isolation, characterisation and structural elucidation as well as the biological activities of bagremycins.

Results

Taxonomy

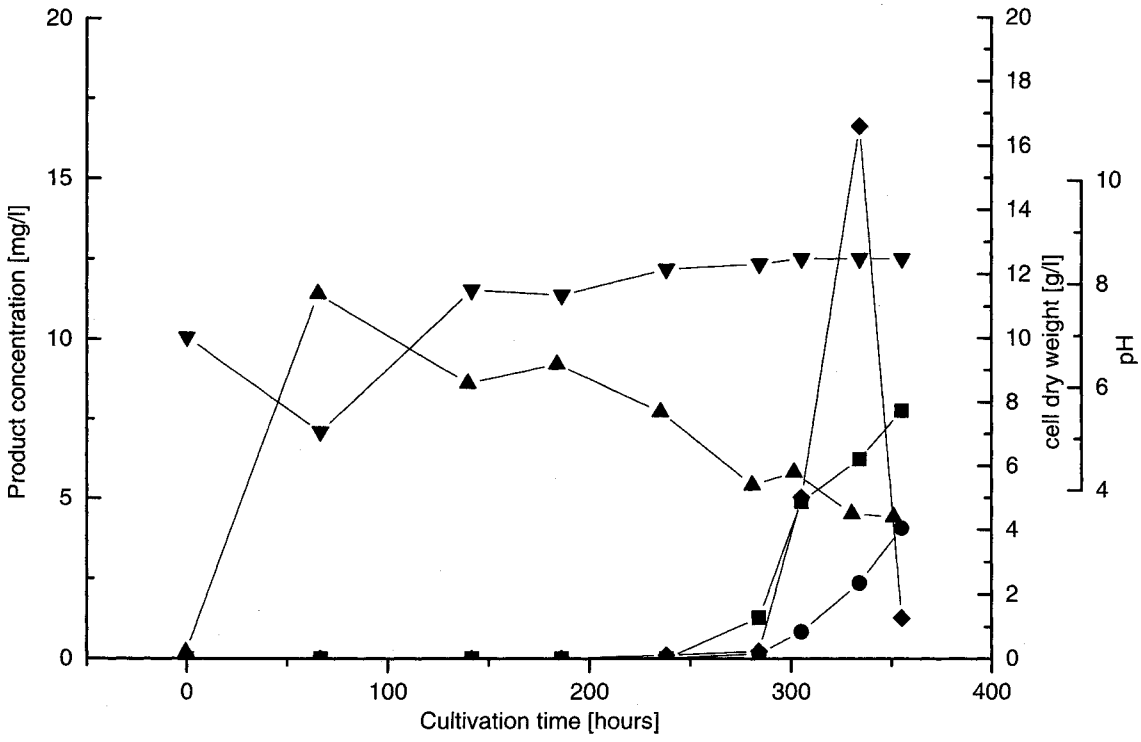
Strain Tü 4128 was assigned to the genus *Streptomyces* because of its morphological appearance and characteristic chemotaxonomic features, such as LL-diaminopimelic acid in the peptidoglycan, menaquinones MK-9(H_4), MK-9(H_6), MK-9(H_8), the typical pattern of saturated *iso*- and *anteiso*-branched fatty acids, and the cell wall sugars galactose, glucose and ribose. Partial sequencing of 16S rDNA confirmed the strain as a *Streptomyces* species showing a homology of 99.6% with type-strains of this genus.

Screening, Fermentation and Isolation

Strain Tü 4128 attracted attention in the course of our HPLC-DAD screening because of the production of metabolites that could not be identified or characterised by means of our HPLC-UV-Vis-Database. The production of these metabolites occured in shaking flask cultures only in nutrient-rich complex media that consisted of a mixture of carbon sources like glucose, starch and glyerol, and a mixture of various nitrogen sources. In contrast to other new isolated strains of streptomycetes, the production of secondary metabolites started relatively late, more than 10 days after inoculation. The HPLC analysis of an ethyl acetate extract of the whole culture broth is shown in Fig. 2.

Batch fermentations of strain Tü 4128 were carried out in 20-liter fermenters equipped with an intersor system, using a complex medium that consisted of glucose 1%, glycerol 1%, starch 1%, cornsteep powder 0.25%, peptone 0.5%, yeast extract 0.2%, NaCl 0.1%, $CaCO_3$ 0.3%. Production of *p*-coumaric acid (**1**) started at about 225 hours after inoculation and reached a maximal value of 17 mg/liter after 334 hours. Bagremycin production started 21 hours later and reached maximal values of 8 mg/liter bagremycin A (**2**) and 4 mg/liter bagremycin B (**3**) at a fermentation time of 355 hours. At this time, a significant decrease of *p*-coumaric acid (**1**) was observed in the fermentation broth. A few hours after the maximal production phase, the bagremycins were quickly decomposed within a short time. The time course of a representative fermentation for production of bagremycins is shown in Fig. 3.

Fig. 3. 20-liter batch fermentation of *Streptomyces* sp. Tü 4128.



▲ biomass (dry weight); ▼ pH; ◆ *p*-coumaric acid (**1**); ■ bagremycin A (**2**); ● bagremycin B (**3**).

Bagremycins were isolated from the culture filtrate by extraction with ethyl acetate, and from the mycelium by extraction with MeOH and re-extraction with ethyl acetate. The combined extracts were concentrated *in vacuo* and purified by subsequent silica gel and Sephadex LH-20 column chromatography. Pure bagremycins were obtained after preparative reversed-phase HPLC using Nucleosil-100 C-18 and gradient elution as white powders. Rf values of bagremycins in TLC and colour reaction with anisaldehyde-H_2SO_4 staining reagent are given in Table 1.

Table 1. Rf values and colour reactions of bagremycins.

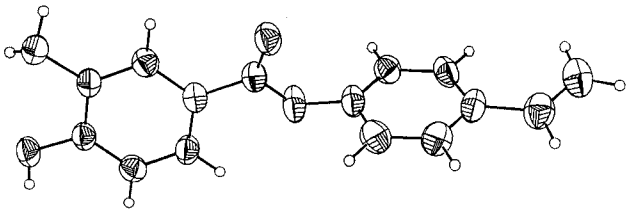
I: $CHCl_3$ - MeOH (9 : 1), II: acetone - cyclohexane (2 : 1).

Compound	I	II	Anisaldehyde-H_2SO_4
2	0.61	0.50	red
3	0.65	0.40	pink

Structure Elucidation

The structure of the bagremycin A (**2**) was elucidated using both, 2D-NMR measurements and X-ray analysis. The ^1H and ^{13}C NMR spectra of **2** exhibit 13 proton and 15 carbon signals. The APT spectrum and 2D-NMR data indicate the presence of one olefinic methylene and one olefinic methine group, seven aromatic methine groups, one carbonyl group and five further quaternary sp^2 carbon atoms. The molecular formula $C_{15}H_{13}NO_3$ was deduced from an ESI mass spectrum (m/z=256 $[M+H]^+$). By COSY and HMBC experiments two substructures could be assigned: One *para* substituted aromatic ring with an oxygen and a vinyl group attached and a second aromatic ring, a benzoic acid derivative with a hydroxy and an amino group in *para* and *meta* position, respectively. The aromatic rings are connected *via* an ester group. The X-ray analysis (Figure 4) gave evidence of the connection and the substitution pattern of the moieties. Thus, bagremycin A (**2**) could be identified as 4-vinylphenyl 3-amino-4-hydroxybenzoate (**2**). The molecular formula of

Fig. 4. Perspective view of bagremycin A (**2**).



bagremycin B (**3**) was established as $C_{17}H_{15}NO_4$ by HREI-MS (m/z=297 [M^+]). It differs from **2** in an acetyl group, only. In accordance with the NMR spectra verifying the acetylation of the amino group of **2** bagremycin B was established as 4-vinylphenyl 3-*N*-acetyl-4-hydroxy-benzoate (**3**).

Biological Properties

The antimicrobial spectra of the bagremycins were examined by an agar plate diffusion assay (Table 1) and the minimal inhibition concentration by the broth dilution method (Table 2). Both compounds were not active against Gram-negative bacteria at a concentration up to 1 mg/ml, but showed a moderate activity against Gram-positive bacteria such as *Bacillus subtilis* DSM 10, *Streptomyces viridochromogenes* Tü 57 and *Arthrobacter aurescens* DSM 20166. The producing strain *Streptomyces* sp. Tü 4128 was not inhibited by its own antibiotics. Other Gram-positive bacteria such as *Micrococcus luteus* ATCC 381, *Mycobacterium phlei* DSM 750 and *Staphylococcus aureus* DSM 20231 were not sensitive. Bagremycin A (**2**) exhibited a weak activity against *Saccharomyces cerevisiae* ATCC 9080 and *Candida albicans* Tü 164, whereas bagremycin B (**3**) against *Botrytis cinerea* Tü 157 only.

Table 2. Antimicrobial spectra of bagremycin A (**2**) and bagremycin B (**3**) determined by the agar plate diffusion assay at a concentration of 1 mg/ml (inhibition zones in mm).

Test organims	**2**	**3**
Arthrobacter aurescens ATCC 13344	18	8
Bacillus brevis ATCC 9999	-	-
Bacillus brevis ATCC 9999[a]	-	-
Bacillus subtilis DSM 10	-	-
Bacillus subtilis DSM 10[a]	19	10
Streptomyces viridochromogenes Tü 57	19	11
Candida albicans Tü 164	8	-
Saccharomyces cerevisiae ATCC 9080	9	-
Botrytis cinerea Tü 157[b]	-	10

[a] Chemically defined medium (per liter): glucose 5 g, tri-Na-citrate×$2H_2O$ 0.5 g, KH_2PO_4 3 g, K_2HPO_4 7 g, $MgSO_4$×$7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1 g, Bacto agar 15 g.
[b] Test on giant colony

Table 3. Minimal inhibition concentrations (μg/ml) of bagremycin A (**2**) and bagremycin B (**3**) as determined by the broth dilution method.

Test organisms	**2**	**3**
Arthrobacter aurescens DSM 20166	3	30
Bacillus subtilis DSM 10[a]	>100	>100
Streptomyces viridochromogenes Tü 57	30	30
Streptomyces sp. Tü 4128	>100	>100
Saccharomyces cerevisae ATCC 9080	100	-

[a] Chemically defined medium

Discussion

Bagremycins were detected in a screening programme that was adjusted to slow growing actinomycetes strains, *e.g.* rare actinomycetes, and to a late production phase for secondary metabolites. Even strain Tü 4128 showed a typical growth behaviour as observed in case of 'common' streptomycetes, reaching the stationary phase at about 66 hours, the production of *p*-coumaric acid (**1**) and bagremycins (**2**, **3**) started not before 10 days with a close maximum between 14 and 15 days after inoculation.

The precursors of bagremycin A (**2**) are presumably *p*-coumaric acid and 3-amino-4-hydroxybenzoic acid (3,4-AHBA). As a cinnamic acid derivative, *p*-coumaric acid is derived from phenylalanine which is supplied from

shikimate pathways. Cinnamic acid is the building block of most abundant plant constituents like lignin, lignans, coumarins and flavonoids, but the general assumption was that cinnamic acid is no intermediate in biosynthetical pathways of microorganisms. Only recently there has been a reference, that cinnamic acid is a precursor of enterocin produced by *Streptomyces maritimus*[4]. Thus, bagremycin A (**2**) is the second example for a natural product derived *via* a plant-like biosynthesis of *p*-coumaric acid. The other moiety of **2**, 3,4-AHBA is derived *via* a condensation of a three-carbon unit with a four-carbon unit as in manumycin, asukamycin and the michigazones[5,6].

Experimental

General

Melting points were determined on a Reichert hot-stage microscope and are not corrected. NMR spectra were measured with Varian Unity 300 (300 MHz) and Varian Inova 500 (500 MHz) instruments. Chemical shifts are expressed in δ values (ppm) with solvents as internal standards. The mass spectra were taken by Finnigan MAT 95 (EI-MS: 70 eV, high resolution with perflourkerosine as internal standard) and by Finnigan LQC. IR spectra in pressed KBr discs were recorded on a Perkin Elmer FT IR-1600 spectrometer and the UV spectra on a Kontron Uvikon 860 spectrophotometer. TLC was carried out on silica gel 60 F_{254} plates (Merck, 0.25 mm) and Rf values were determined on 20×20 cm plates, the evaluation length was 10 cm. Compounds were viewed under UV lamp at 254 nm and sprayed with anisaldehyde-H_2SO_4 followed by heating.

Microorganisms

Strain Tü 4128 was isolated from a soil collected in Java, Indonesia, using HV-medium[7] with addition of nalidixic acid and tunicamycin, and was characterised by morphological and chemotaxonomic methods[8], and by 16S rDNA partial sequencing[9]. It is deposited in the culture collection of our laboratory.

The standard strains for testing the biological activity spectrum were obtained from DSMZ, ATCC and the stock collection of our laboratory.

Fermentation

Strain Tü 4128 was cultivated in a 20-liter fermenter (b-20, intensor system; Giovanola) using a production medium consisting of glucose 1%, glycerol 1%, starch 1%, cornsteep powder 0.25%, peptone 0.5%, yeast extract 0.2%, NaCl 0.1%, $CaCO_3$ 0.3% (pH 7.2, adjusted with 1 N NaOH). The fermenter was inoculated with 5 vol-% of shaking cultures grown for 48 hours in 500 ml-Erlenmeyer flasks with one baffle on a rotary shaker at 120 rpm and 27°C in the same medium. For production of bagremycins, the fermentation was carried out at 27°C for 355 hours with an aeration rate of 0.5 v/v/m and an agitation rate of 1000 rpm.

Isolation

Hyflo Super-cel (3%) was added to the fermentation broth which was separated by multiple sheet filtration into culture filtrate and mycelium cake. The culture filtrate was adjusted to pH 5 and extracted 3 times with ethyl acetate. The biomass was extracted 3 times with MeOH, combined and were concentrated *in vacuo* to the aqueous residue, which was re-extracted 3 times with ethyl acetate. The ethyl acetate extracts from culture filtrate and mycelium were combined and concentrated *in vacuo* to dryness. The raw product was dissolved in CH_2Cl_2, subjected to a silica gel column (32×2.5 cm, silica gel 60, 63~200 μm; Merck), and eluted by a linear gradient starting with CH_2Cl_2 to a MeOH content of 20% within 4 hours and a flow rate of 600 ml/hour (LKB 2249 Gradient Pump). **2** and **3** containing fractions were combined and concentrated to dryness. Further purification was done by Sephadex LH-20 chromatography (90×2.5 cm) using MeOH as eluent. Finally, pure **2** and **3** were obtained by preparative reversed-phase HPLC using stainless steel columns (250×16 mm; Maisch). **2** was separated on 10-μm Nucleosil-100 C-18 and a linear gradient with H_2O - MeOH, starting from 50% MeOH to 100% MeOH within 20 minutes and a flow rate of 20 ml/minute. **3** was separated on 10-μm LiChrospher RP-Select B and a linear gradient with 0.1% HCOOH-MeOH, starting from 55% MeOH to 90% MeOH within 16 minutes and a flow rate of 24 ml/minute. The preparative HPLC system consisted of two high-pressure pumps (Sepapress HPP-200/100: Kronwald), gradient unit (Sepacon GCU-311), and a Valco preparative injection valve (6UW; VICI) with a 5-ml sample loop. The UV absorbance of the eluate was monitored simultaneously at 260 nm and 310 nm by a Gilson spectrophotometer Mod. 116 equipped with a preparative cell.

Biological Assay

An agar-plate diffusion assay was used to determine the antibacterial and antifungal spectrum of **2** and **3**. Ten μl of the samples were applied to filter disks (6 mm diameter). The test plates were incubated for 24 hours at a temperature that permitted optimal growth of the test organisms.

A broth dilution method was used to determine the minimal inhibition concentrations of **2** and **3**. The antibiotics were dissolved in MeOH, giving a MeOH concentration in the cultures of not more than 5%. 10^6 cells/ml were used as inoculum for the media and growth inhibition was evaluated after incubation for 24 and 48 hours at 37°C on a rotary shaker.

HPLC-DAD-Analysis

The chromatographic system consisted of a HP 1090M liquid chromatograph and diode-array detector, HP 3D-DOS ChemStation and software revision A.02.02 (Hewlett-Packard). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360 and 435 nm. The spectrum range was 200~600 nm in steps of 2 nm and sampling intervals of 640 mseconds.

The culture broth (50 ml) was adjusted to pH 5 and extracted with the same volume of ethyl acetate. The organic layer was concentrated to dryness and dissolved in 5 ml MeOH. Ten μl of the samples were injected onto an HPLC column (125×4.6 mm), fitted with a guard column (20×4.6 mm) which was packed with 5-μm Nucleosil-100 C-18 (Maisch). The samples were analysed by linear gradient elution using 0.1% phosphoric acid as solvent A and acetronitrile as solvent B at a flow rate of 2 ml/minute. The gradient was from 0% to 100% solvent B in 15 minutes with a 1-minute hold at 100% B, followed by a 5-minute post-time at initial conditions.

Bagremycin A (2)

MP 176°C; UV (MeOH) λ_{max} nm (ε) 311 (7600), 298 (7000), 240 (31900), 221 (20900), 207 (32900); IR ν_{max} (KBr) cm^{-1} 3405, 1730, 1604, 1506, 1386, 1290, 1190, 1138, 1053; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.15 (s, 2H, NH_2), 5.25 (d, J=11.0 Hz, J_{ab}<1.0 Hz, 1H, 8′-H_a), 5.80 (d, J=18.0 Hz, J_{ab}<1.0 Hz, 1H, 8′-H_b), 6.72 (dd, J=18.0, 11.0 Hz, 1H, 7′-H), 6.78 (dd, J=8.0 Hz, 1H, 5-H), 7.16 (d, J=8.0 Hz, 2H, 2′-H, 6′-H), 7.27 (dd, J=8.0, 2.0 Hz, 1H, 6-H), 7.38 (d, J=2.0 Hz, 1H, 2-H), 7.52 (d, J=8.0 Hz, 2H, 3′-H, 5′-H), (OH not detectable); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 113.9 (d, C-5), 114.4 (t, C-8′), 115.2 (d, C-2), 119.7 (s, C-1), 119.8 (d, C-6), 122.2 (d, C-2′, C-6′), 127.2 (d, C-3′, C-5′), 134.7 (s, C-4′), 135.9 (d, C-7′), 136.9 (s, C-3), 149.5 (s, C-4), 150.6 (s, C-1′), 165.0 (s, C-7); ESI-MS (positive mode) m/z (%) 256 $[M+H]^+$ (34), 278 $[M+Na]^+$ (49), 533 $[2M+H]^+$ (100).

X-Ray Crystallography of Bagremycin A (2)

A single colourless crystal of **2** was obtained by recrystallization from acetone/MeOH. A block-shaped fragment of dimensions 0.5*0.2*0.1 mm^3 (half the crystal size) was mounted inside a nylon cryo-loop (Hampton Research) using perfluorated polyether oil[10] and cooled to 133°K at the low-temperature device. Crystal data: $C_{15}H_{13}NO_3$, M=255.26, orthorhombic, space group $P2_12_12_1$ (#19), a=7.478(3) Å, b=10.583(4) Å, c=15.958(4) Å, V=1262.9(8) $Å^3$, Z=4, D_{calc}=1.343 Mgm^{-3}, F(000)=536, μ(Mo-Kα)=0.094. All measurements were made using a four-circle diffractometer equipped with a Stoe fine-focus sealed tube X-Ray generator (graphite monochromated Mo-Kα radiation), Siemens CCD area detector, Huber goniometer, and low-temperature device. The diffractometer was controlled using the SMART program[11]. Intensities were measured by means of φ- and ω-scans with a step width of 0.5° and exposition time of 60 seconds/frame. Frame integration was carried out using the SAINT program[12]. Of the 15880 reflections measured, 8192 were used to determine the cell parameters. Within the Θ range of 2.31~25.05° (0≤h≤8, 0≤k≤12, 0≤l≤19, d_{max}=0.84 Å), 1306 independent reflections were observed, representing 99.8% of the unique data. Due to the absence of atoms heavier than oxygen, Friedel-Pairs were merged. The structure was solved by direct methods and refined by full-matrix least-squares against F^2 using the programs SHELXS97 and SHELXL97, respectively[13,14]. Anisotropic displacement parameters were refined for all non-hydrogen atoms. Carbon-bound hydrogen atoms were placed at geometrically calculated positions and refined by a riding model, whereas the coordinates of those attached to heteroatoms were refined with distance restraints. All hydrogen atoms were refined isotropically with displacement parameters constrained to multiple U_{eq}-values of the attached atoms. In total, 185 structure parameters were refined using 3 restraints, leading to a final R1 of 0.0395 (1144 reflections with $|F_o|>4\sigma(F_o)$), wR2 of 0.1043 (all 1306 data) and a restrained goodness of fit of 1.212. The residual electron density after the final difference Fourier synthesis was observed between −0.152 and 0.355 $eÅ^{-3}$ with a root mean square deviation of 0.040 $eÅ^{-3}$. Average estimated standard deviations are 0.004 Å for the C–C bonds and 0.3° for the C–C–C angles.

Bagremycin B (3)

MP 300°C; UV (MeOH) λ_{max} nm (ε)244 (22200), 221 (12200), 204 (23000); IR ν_{max} (KBr) cm^{-1} 3378, 1716, 1650, 1588, 1542, 1504, 1438, 1317, 1278, 1211, 1191, 1125, 1072, 1013; ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.10 (s, 3H, NHCOCH_3), 5.26 (d, J=11.0 Hz, 1H, 8′-H_a), 5.80 (d, J=18.0 Hz, 1H, 8′-H_b), 6.73 (dd, J=18.0, 11.0 Hz, 1H, 7′-H), 7.00 (d, J=8.0 Hz, 1H, 5-H), 7.20 (d, J=8.0 Hz, 2H,

2′-H, 6′-H), 7.53 (d, J=8.0 Hz, 2H, 3′-H, 5′-H), 7.74 (dd, J=8.0, 2.0 Hz, 1H, 6-H), 8.60 (d, J=2.0 Hz, 1H, 2-H), 9.32 (s (br), NH); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 23.7 (q, $NHCOCH_3$), 114.4 (t, C-8′), 115.4 (d, C-5), 118.9 (s, C-1), 122.1 (d, C-2′, C-6′), 123.8 (d, C-2), 126.7 (s, C-3), 127.1 (d, C-6), 127.2 (d, C-3′, C-5′), 134.8 (s, C-4′), 135.8 (d, C-7′), 150.4 (s, C-1′), 153.3 (s, C-4), 164.4 (s, C-7), 169.1 (s, $NHCOCH_3$); EI-MS m/z (%) 297 [M^+, calcd. for $C_{17}H_{15}NO_4$ and found] (19), 178 (100), 136 (100).

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