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Bhimamycin A~E and Bhimanone: Isolation, Structure Elucidation and Biological Activity of Novel Quinone Antibiotics from a Terrestrial Streptomycete

SERGE FOTSO[a], RAJENDRA P. MASKEY[a], IRIS GRÜN-WOLLNY[b], KLAUS-PETER SCHULZ[c], MORTON MUNK[c] and HARTMUT LAATSCH[a,*]

[a] Department of Organic Chemistry, University of Göttingen, Tammannstrasse 2, D-37077 Göttingen, Germany
[b] bioLeads GmbH Waldhofer Strasse 104, D-69123 Heidelberg, Germany
[c] Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287-1604

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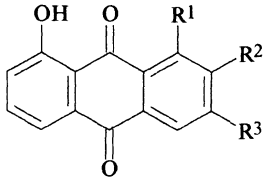
From the ethyl acetate extract of a terrestrial Streptomycete isolate, five new quinone antibiotics, bhimamycin A (**2a**), B (**2b**), C (**3c**), D (**5a**), E (**7**) and the new tetralone bhimanone (**8**) were isolated together with the known microbial products chrysophanol (**1a**), aloesaponarin II (**1b**), 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (**1c**), adenosine, 2′-deoxyadenosine, phenylacetamide, and 2-(*p*-hydroxyphenyl)ethanol. The structures of these natural products were deduced from the spectral data and confirmed by comparison with related compounds from the literature and by synthesis.

In our search for new biologically active components from microorganisms, the ethyl acetate extract of a terrestrial *Streptomyces* sp. isolate GW32/698 exhibited pronounced antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Streptomyces viridochromogenes*. In the TLC screening, several nonpolar yellow to orange zones were separated, which were shown by bioautography to be responsible for the antibacterial activity of the crude extract. In this paper we describe the taxonomy of the producing strain, the structure elucidation and the biological activities of the new antibiotics bhimamycin A (**2a**), B (**2b**), C (**3c**), D (**5a**), E (**7**) and of bhimanone (**8**).



	R^1	R^2	R^3
1a	OH	H	CH_3
1b	CH_3	H	OH
1c	CH_3	COOH	OH

Results and Discussion

The terrestrial *Streptomyces* sp. isolate GW32/698 was cultivated on M_2 medium and worked up using our standard procedure[1]. The crude extract was subjected to flash column chromatography on silica gel using a CH_2Cl_2/MeOH gradient. Further purification of the fractions by PTLC, HPLC, and size exclusion chromatography on Sephadex LH-20 yielded the new antibiotics bhimamycin A (**2a**), B (**2b**), C (**3c**) and D (**5a**) and the known natural products chrysophanol (**1a**)[2], aloesaponarin II (**1b**)[2], 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (**1c**)[3], adenosine[4], 2′-deoxyadenosine[5], phenylacetamide[6], and 2-(*p*-hydroxyphenyl)ethanol[7]. From a second fermentation of the strain on a bigger scale, we could isolate, in addition to the compounds mentioned above, bhimamycin E (**7**) and bhimanone (**8**).

Compound **2a** was obtained as a brown solid and gave a characteristic violet colour reaction with dilute sodium hydroxide, which is indicative of *peri*-hydroxy quinones. On spraying with anisaldehyde/sulphuric acid, the colour of

* Corresponding author: HLAATSC@gwdg.de

Fig. 1. Working up scheme of the strain *Streptomyces* sp. isolate GW32/698.

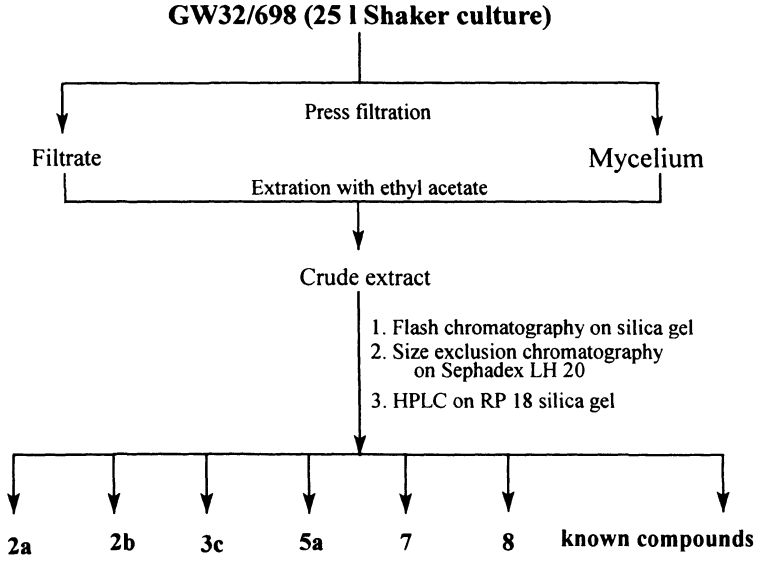
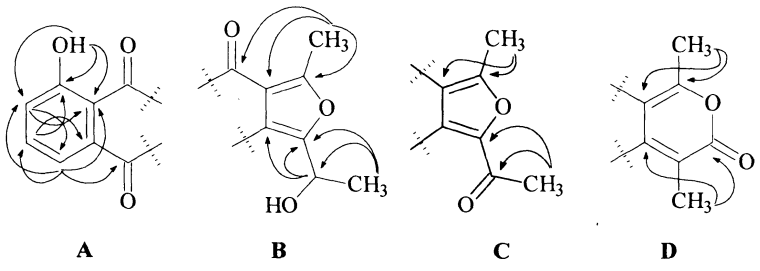


Fig. 2. Fragments of bhimamycin A (**2a**) and B (**2b**) from 1D and 2D NMR data.



the compound changed from yellow to grey. From the EI and CI mass spectra the molecular weight of *m/z* 272 was read, and EI HRMS led to the molecular formula $C_{15}H_{12}O_5$.

The ^{1}H NMR spectrum indicated a methyl doublet at δ 1.61 and a methine quartet at δ 5.10 suggesting the presence of a CH_3–CH–O fragment in the molecule. The aliphatic region of the spectrum delivered singlets at δ 2.74 and δ 5.47 for an aromate-bound methyl group and an acidic proton, respectively. Furthermore, the spectrum exhibited an ABC pattern of three adjacent aromatic protons between δ 7.76~7.26, and the signal of a chelated hydroxyl group at δ 12.81. Extensive interpretation of the ^{1}H and ^{13}C NMR data, H,H COSY, HMQC, and HMBC couplings combined with the molecular formula resulted in fragments **A** and **B** which have one carbonyl group in common. These substructures can be connected in two different manners, however, as the methyl singlet at δ 2.74 showed a weak ^4J-coupling with the signal of the chelated carbonyl group at δ 186.6 and the *peri*-proton at δ 7.76 showed a ^3J-coupling to the nonchelated carbonyl at δ 180.6, the methyl group and the chelated hydroxy group must be placed *syn*-periplanar resulting in **2a** as the only possible structure for bhimamycin A.

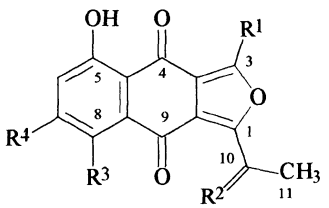
Compound **2b** was a brown *peri*-hydroxy quinone as well which turned on TLC from yellow to blue-violet on spraying with anisaldehyde/sulphuric acid. The yellow fluorescence under 366 nm and the similarity of the UV/VIS spectrum with that of bhimamycin A (**2a**) indicated **2b** probably to possess the same chromophore. The EI mass spectrum showed a molecular peak at *m/z* 270 whose high resolution delivered the molecular formula $C_{15}H_{10}O_5$.

The ^{1}H NMR spectrum showed again relatively few

Table 1. ^{1}H NMR (300 MHz, $CDCl_3$) data of bhimamycin A (**2a**), B (**2b**), C (**3c**) and D (**5a**) (δ values, [J] in Hz).

C No.	2a	2b	3c	5a[a]
6	7.26 (dd, 8.3, 1.2)	7.31 (dd, 8.3, 1.2)	7.16 (dd, (8.3, 1.1)	7.02 (m)
7	7.62 (dd, 8.3, 7.6)	7.68 (t, 8.0)	7.53 (t, 8.0)	7.02 (m)
8	7.76 (dd, 7.6, 1.2)	7.83 (dd, 7.6, 1.2)	7.66 (dd, 7.6, 1.1)	7.79 (d, 6.4)
10	5.10 (q, 6.8)	-	5.05 (q, 6.8)	-
11	1.61 (d, 6.8)	2.86 (s)	1.54 (d, 6.8)	2.76 (s)
12	2.74 (s)	2.88 (s)	2.70 (s)	2.10 (s)
1'	-	-	4.19-3.99 (m)	-
2'	-	-	3.90 (m)	-
3'	-	-	-	6.56 (d, 7.2)
4'	-	-	-	6.90 (t, 7.2)
5'	-	-	-	6.84 (t, 7.2)
6'	-	-	-	7.86 (d, 7.2)
5-OH	12.81 (s)	12.61 (s)	13.12 (s)	13.45 (s)
10-OH	5.47 (s)	-	6.67 (s br)	-

[a] in C_6D_6



	R1	R2	R3	R4
2a	CH_3	OH, H	H	H
2b	CH_3	O	H	H
2c	H	OH, H	OH	OCH_3

signals which were similar to those of **2a** in the deep field region. In the aliphatic region, instead of one singlet at δ 2.74 in **2a**, two at δ 2.86 and 2.88 for methyl groups attached to sp^2 carbon were visible. The signals for the CH_3CHO– group of **2a** were missing in the spectrum of **2b**. In the ^{13}C NMR spectrum, 15 signals were observed, which represented, according to the APT NMR spectrum, two methyl, three sp^2 methine groups, seven aromatic or olefinic quaternary carbon atoms, two quinone carbonyls and an additional carbonyl group (δ 187.2). The latter could be due to a lactone or conjugated ketone. Careful interpretation of the H,H COSY, HMQC and HMBC couplings delivered the fragments **A** and **C** or **D** (Fig. 1). Fragment **D** with the lactone structure could be ruled out, as other than in the related anhydrofusarubin lactone[8], no IR signal above 1700 cm^{-1} was visible.

Connection of the fragments A and C resulted in two possible structures with the chelated hydroxyl group at C-5 or 8 which were indistinguishable on the basis of the available NMR data. As the structurally related **2a** was isolated from the same strain, it can be assumed, however, that both compounds originated on the same biosynthetic pathway. In this case, the OH group must be at C-5 resulting in the final structure **2b** for bhimamycin B. This structure was finally confirmed by the synthesis of **5a** from **2b** (see below).

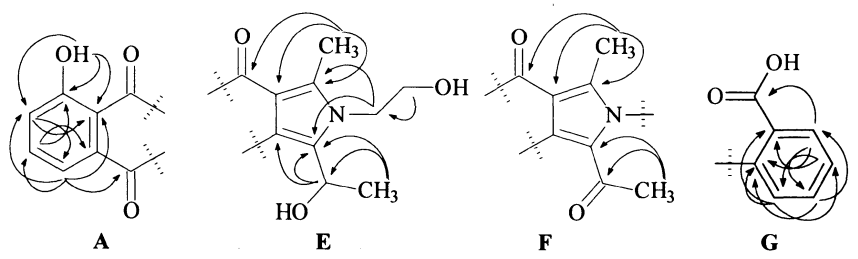
Bhimamycin A (**2a**) and B (**2b**) are the first compounds with the rare naphtho[2,3-c]furan-4,9-dione chromophore which have been isolated from bacteria so far. From fungi, however, three compounds of this type have been described, *e.g.* nectriafurone (**2c**) and its monomethyl ether from *Nectria haematococca*[8]. Further furanoquinones have been obtained from plants, *e.g.* the closely related 5-hydroxy-3-methylnaphtho[2,3-c]furan-4,9-dione from *Bulbine capitata*[9] and *Aloe ferox*[10]. These compounds and further naphtho[2,3-c]furan-4,9-diones from the roots of *Bulbine capitata* showed antioxidant and weak antiplasmodial

Table 2. ^{13}C NMR data (75.5 MHz, $CDCl_3$) of **2a**, **2b**, **3c**, and **5a** (δ values).

C No.	2a	2b	3c[a]	5a[b]	C No.	2a	2b	3c[a]	5a[b]
1	163.9	148.9	147.4	134.2	9a	116.9	123.2	117.7	122.1
3	158.1	161.9	140.6	141.4	10	63.9	187.2	64.1	192.9
3a	117.1	118.6	118.1	117.2	11	21.2	29.4	23.4	30.7
4	186.6	186.0	188.2	187.5	12	13.7	14.4	11.9	11.8
4a	117.6	116.9	118.8	117.6	1'	-	-	47.9	128.5[a]
5	162.8	162.9	163.9	163.2	2'	-	-	61.9	137.3
6	124.6	124.6	124.6	123.7	3'	-	-	-	129.5
7	136.1	136.7	136.4	135.6	4'	-	-	-	133.2
8	119.5	120.2	119.9	119.3	5'	-	-	-	129.4
8a	135.2	135.8	137.1	136.3	6'	-	-	-	132.4
9	180.6	178.3	182.4	179.9	7'	-	-	-	167.8

[a] in CD_3OD, [b] C_6D_6

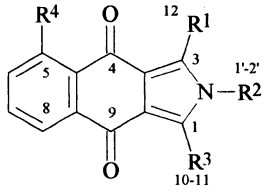
Fig. 3. Fragments of bhimamycin C (**3c**) and D (**5a**) from 1D and 2D NMR data.



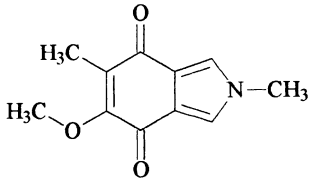
activities[11]. The only two antibiotics with the isomeric naphtho[2,3-b]furan-4,9-dione chromophore from bacteria, α-rubromycin and γ-isorubromycin have been reported from *Streptomyces collinus* and *Streptomyces antibioticus*[12]. Compounds of this type are more abundant in plants and show antifungal and antibacterial activity[13] as well. For 2-(1*R*)-1-hydroxyethylnaphtho[2,3-b]furan-4,9-dione, also anti-trypanosomal activity was reported[14].

The yellow compound **3c** afforded EI and ESI mass spectra corresponding to a molecular weight of 315, and EI HRMS led to the molecular formula of $C_{17}H_{17}NO_5$. The compound showed an orange fluorescence and turned pink with anisaldehyde/sulphuric acid. The ^1H NMR spectrum was again very similar to that of bhimamycin A (**2a**) showing the signal of a chelated OH group at δ 13.12, three aromatic protons of a 1,2,3-substituted aromatic system, a quartet for an aliphatic methine proton connected to oxygen, a singlet at δ 2.70 for an aromatic methyl and a methyl doublet at δ 1.54. The evident difference between both spectra was the appearance of two additional methylene multiplets at δ 4.19~3.99 and 3.90. Comparison of the sp^2 carbon signals with those of **2a** confirmed the close similarity except for the signals of C-1 and C-3, which were shifted from δ 163.9 and 158.1 in **2a** to δ 147.4 and 140.6 in **3c**, respectively. It followed that these carbon atoms are more likely connected to nitrogen and not to oxygen as in **2a**. Correspondingly, the ethylidene fragment with signals at δ 61.9 and 47.9 must be connected with oxygen and nitrogen, respectively, giving a $-NCH_2CH_2O-$ group. The 1D and 2D NMR data in combination with the molecular formula delivered fragments A and E, which because of the cross peak of the methyl signal at δ 2.70

with the signal of the chelated carbonyl group at δ 188.8 had to be connected to structure **3c**, a new natural product with 2*H*-benzo[f]isoindole-4,9-dione chromophore which we named bhimamycin C. As furanes are easily transformed into pyrroles by reaction with primary amines, **3c** may be a follow-up product of **2a**.



3a: $R^1 = R^2 = R^3 = R^4 = H$
3b: $R^1 = R^3 = R^4 = H$, $R^2 = CH_3$
3c: $R^1 = CH_3$, $R^2 = CH_2CH_2OH$, $R^3 = CHOHCH_3$, $R^4 = OH$



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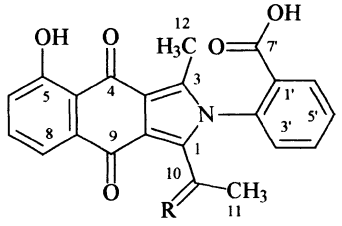
Compound **5a** was obtained as a yellow solid with a molecular formula of $C_{22}H_{17}NO_6$ (EI HRMS 389.0909). It turned red-violet with anisaldehyde/sulphuric acid and showed an orange fluorescence at 366 nm similar as bhimamycin C (**3c**). The ^1H NMR spectrum showed seven sp^2 protons which could be assigned by H,H COSY data to three adjacent aromatic protons as in fragment A of **2a**, **2b** and **3c**, and to four consecutive protons either of an olefinic or of an electron rich aromatic system (Table 1). Additionally, the spectrum exhibited the signal of a chelated hydroxyl group and two methyl singlets at δ 2.80 and 2.20. The ^{13}C NMR spectrum exhibited signals of 20 sp^2 carbons and two methyl groups. Beside two quinone carbonyl signals at 187.5 and 179.9, two additional carbonyl signals were visible. The first one at δ 192.9 was assigned to an aromatic or α,β-unsaturated ketone, and a second signal at δ 167.8 could be due to an acid group. This was confirmed indeed by the facile formation of a methyl ester by treatment of **5a** with diazo methane.

By interpretation of the 2D correlation and by comparison of the NMR data with those of bhimamycin A (**2a**), B (**2b**) and C (**3c**), the fragments A, F and G were obtained. Connection of A, F and G resulted in bhimamycin D (**5a**), which is a new natural product and obviously a follow-up product of **2b** with anthranilic acid.

It should be emphasized that with the spectra interpreter SESAMI[15] several further structures were found which were in full agreement with the spectroscopic data, one of them being an indole instead of the isoindole. Although the structures of the potential precursor **2b** and the related isoindole **3c** were unequivocally established, we tried additionally to prove **5a** by partial synthesis. Since we had **2a** in larger amount, this was treated first with anthranilic acid in a model reaction to find out if the oxygen of the furan ring can be replaced by nitrogen. Indeed, a mixture of **5b** and **6** was obtained, when **2a** was refluxed with anthranilic acid in ethanol. Finally the structure **5a** was confirmed by the synthesis from **2b** in the same way. It should be mentioned that the ^1H NMR shifts of the aromatic protons in **5a** depend strongly on the purity of the sample. Shift differences up to $\Delta\delta$ 0.38 were observed between pure and impure products. Compounds **5b** and **6** should deliver diastereomers due to atrop-isomerisms and a second stereo centre. The line form in the ^1H NMR spectra and the behaviour on HPLC gave, however, no hint that these isomers are stable.

Isoindolequinones are very rare in nature as well. Compounds with 2*H*-benzo[f]isoindole-4,9-dione chromophore have not been reported as natural products. The only related natural metabolite reported so far is 5-methoxy-2,6-dimethyl-2*H*-isoindole-4,7-dione (**4**) from the sponge *Reniera* sp.[16] having antimicrobial activity. 2*H*-Benzo[f]isoindole-4,9-dione (**3a**)[17] and 2-methyl-2*H*-benzo[f]isoindole-4,9-dione (**3b**)[18] are two compounds which possess the same chromophore as **3c**, however, are of synthetic origin.

Compound **7** was obtained as a yellow solid with the molecular weight of *m/z* 246 ($C_{13}H_{10}O_5$ by HR). The ^1H NMR spectrum exhibited signals of three adjacent protons as in **2a**, **2b**, **3c** and **5a**, a singlet for a methylene group connected to an oxygen atom and an acetate methyl singlet. The spectrum also contained two acidic proton signals at δ 11.08 and 7.38. The ^{13}C NMR spectrum delivered 13 carbon signals as demanded by its molecular formula, two quinone carbonyls, a conjugated ketone, eight aromatic



5a: R = O
5b: R = H, OH

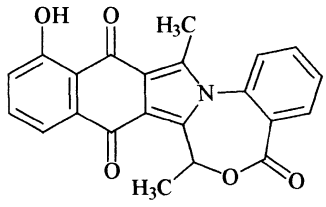


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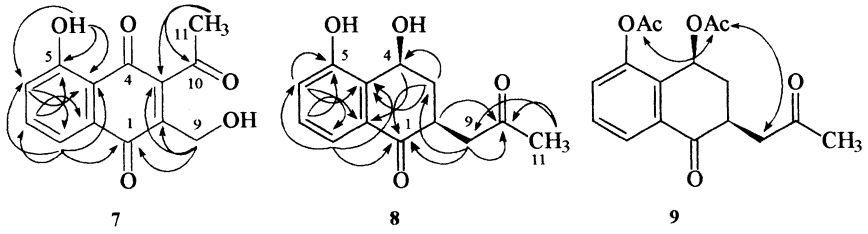
Table 3. Physochemical properties of bhimamycin A (**2a**), B (**2b**), C (**3c**), and D (**5a**).

	2a	**2b**	**3c**	**5a**
Appearance	brown powder	brown powder	yellow powder	yellow powder
R_f (CH_2Cl_2/8 % MeOH	0.89	0.97	0.57	0.63
Formula	$C_{15}H_{12}O_5$	$C_{15}H_{10}O_5$	$C_{17}H_{17}NO_5$	$C_{22}H_{15}NO_6$
EI-MS: *m/z* (%)	272 (M^+, 80), 257 (96), 230 (100), 200 (8), 173 (8), 121 (12), 115 (8)	270 (M^+, 100), 255 (15), 242 (68), 227 (8), 199 (20), 171(8), 115 (12),	315 (M^+, 100), 272 (96), 270 (36), 252 (24), 228 (12), 141 (4), 121 (4), 45 (4),	389 (M^+, 100), 344 (85), 330 (21), 254 (44), 210 (4), 121 (3), 43 (6)
EI-HRMS	272.0694 (calc.272.068473)	270.0539 (calc.270.052823)	315.1107 (calc.315.110672)	389.0909 (calc.389.089937)
(+)-ESI-MS: *m/z* (%)	-	-	653([2M+Na]$^+$, 100), 338([M+Na]$^+$, 20)	801([2M+Na]$^+$, 100), 412([M+Na]$^+$, 12)
(-)-ESI-MS: *m/z* (%)	-	-	314 ([M-H]$^-$)	388 ([M-H]$^-$)
CI-MS (NH_3): *m/z* (%)	273 ([M+H]$^+$, 100), 290 ([M+NH_4]$^+$, 28), 562 ([2M+ NH_4]$^+$, 4)	271 ([M+H]$^+$, 72), 288 ([M+NH_4]$^+$, 100), 558 ([2M+ NH_4]$^+$, 20)	-	-
IR (KBr): ν cm^{-1}	3372, 2980, 2926, 2850, 1636, 1604, 1454, 1419, 1350, 1256, 1163, 1109, 1072, 1011, 892, 860, 836, 769, 717	3429, 2922, 2852, 1681, 1636, 1586, 1535, 1456, 1417, 1373, 1346, 1253, 1223, 1169, 1149, 1103, 1057, 991, 896, 853, 783 709, 651, 611	3424, 2924, 2853, 1656, 1620, 1458, 1410, 1368, 1262, 1238, 1168, 1080, 1098, 1056, 808, 785, 777, 713	3394, 2923, 2852, 1737, 1646, 1463, 1430, 1382, 1266, 1236, 1165, 1120, 1095, 1028,
UV/VIS (MeOH): λ_{max} (lg ε)	244 (4.26), 302 (3.45), 384 (3.92)	224 (4.22) 284 (3.68), 390 (3.77)	380 (3.39)	248 (4.43), 404 (3.92)
$[\alpha]^{20}_D$ (mg/ml, $CHCl_3$)	+55.6° (c 0.450)	-	-23.4° (c 0.555)	-2.5° (c 0.790)



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carbons, a methylene and a methyl signal. The structure of the compound was derived as **7** by comparison of the NMR data with those of bhimamycin A~D and by interpretation of the 1D and 2D spectra and named bhimamycin E.

Compound **8** was obtained as colourless needles from dichloromethane/methanol with a molecular weight of *m/z*

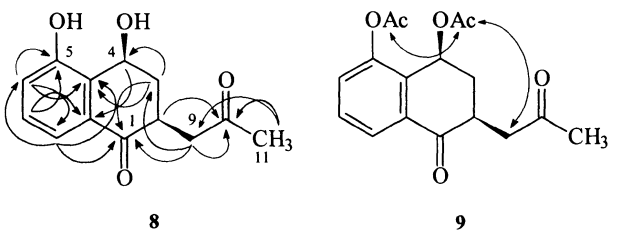
Table 4. ^{1}H (300 MHz, DMSO-d_6) and ^{13}C (75.5 MHz, DMSO-d_6) NMR data of bhimamycin E (**7**), bhimanone (**8**), and bhimanone diacetate (**9**) (δ values, [J] in Hz).

	7[a]		8		9	
C No.	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	-	183.1	-	199.0	-	197.3
2	-	153.7	3.50 (m)	37.2	3.56 (m)	37.9
3	-	119.9	2.10 (m)	36.5	2.24 (m)	33.5
4	-	184.3	5.05 (t, 3.0)	58.9	6.32 (t, 3.0)	62.4
4a	-	113.0	-	130.9	-	131.2
5	11.08 (s, OH)	161.4	-	155.2	-	148.6
6	7.21 (m)	123.4	7.05 (d, 8.0)	120.2	7.35 (d, 8.1)	128.1
7	7.64 (m)	137.8	7.20 (t, 8.0)	128.5	7.52 (t, 8.1)	130.2
8	7.64 (m)	119.9	7.27 (d, 8.0)	116.5	7.94 (d, 8.1)	124.9
8a	-	132.3	-	132.1	-	133.7
9	3.76 (s)	38.1	2.90 (dd, 17.5, 5.9), 2.50 (dd, 17.5, 5.3)	43.2	3.07 (dd, 17.7, 6.0), 2.58 (dd, 17.7, 5.2)	43.0
10	-	203.5	-	206.7	-	206.4
11	2.34 (s)	30.1	2.15 (s)	30.0	2.09 (s)	30.3
OH	7.38 (s, 9-OH)		9.80 (s)	-	-	-
4-$COCH_3$	-		-	-	-	170.2[b]
4-$COCH_3$	-		-	-	2.31 (s)	21.0[c]
5-$COCH_3$	-		-	-	-	169.2[b]
5-$COCH_3$	-		-	-	2.27 (s)	20.7[c]

[a] $CDCl_3$, [b,c] assignment may be reversed

234 ($C_{13}H_{14}O_4$ by HRMS). The proton NMR spectrum of the compound contained signals for three aromatic protons of a 1,2,3-substituted benzene ring, two aliphatic methines, two methylenes, a methyl and an OH group. The ^{13}C NMR spectrum showed two ketone, six aromatic and five aliphatic carbon signals. The structure was derived by H,H COSY, HSQC and HMBC correlations. Since the HMBC spectrum was not delivering enough information to fix the position of the OH and the carbonyl group in the ring, **8** was converted into the diacetate. The NOE couplings of the diacetate (**9**) confirmed the structure and the relative stereochemistry of bhimanone (**8**), a novel new natural product.

Fig. 4. 2D correlations in **8** and **9**.



8 **9**

Arrows indicate HMBC (**8**, →) and NOE (**9**, ↔) couplings.

Table 5. Physochemical properties of bhimamycin E (**7**), bhimanone (**8**), and bhimanone diacetate (**9**).

	Bhimamycin E (**7**)	Bhimanone (**8**)	Bhimanone diacetate (**9**)
Appearance	yellow solid	colourless needles	colourless solid
R_f (CH_2Cl_2/5 % MeOH)	0.54	0.31	0.66
molecular formula	$C_{13}H_{10}O_5$	$C_{13}H_{14}O_4$	$C_{17}H_{18}O_6$
EI-MS: *m/z* (%)	246 (M^+, 16), 204 (100), 176 (17), 147 (8), 121 (12), 102 (6), 43 (64)	234 (M^+, 12), 216 (12), 198 (13), 176 (100), 173 (75), 145 (32), 131 (13), 127 (14), 121 (2), 115 (19), 91 (8), 77 (8), 65 (12), 43 (44)	318 (M^+, 4), 276 (2), 258 (5), 216 (63), 198 (9), 173 (100), 145 (11), 131 (6), 127 (5), 115 (7), 91 (4), 77 (3), 43 (52)
EI-HRMS	246.0532(calc. 246.052823)	234.0893(calc. 234.089209)	318.1089(calc. 318.110338)
(+)-ESI-MS: *m/z* (%)	291 ([M+2Na-H]$^+$, 85), 269 ([M+Na]$^+$], 100)	491([2M+Na]$^+$, 50), 257 ([M+Na]$^+$, 100)	-
(-)-ESI-MS: *m/z* (%)	513 ([2M+Na-2H]$^-$, 60), 245 ([M-H]$^-$, 100)	233 ([M-H]$^-$, 100)	-
IR (KBr): ν cm^{-1}	3409, 3272, 2924, 2854, 1706, 1636, 1600, 1578, 1482, 1458, 1411, 1363, 1300, 1207, 1173, 1159, 1073, 1053, 875, 823, 748, 699, 678, 599, 577	3504, 3165, 2951, 2893, 1702, 1681, 1589, 1472, 1446, 1414, 1363, 1336, 1290, 1241, 1219, 1202, 1168, 1119, 1096, 1054, 1034, 1017, 973, 955, 918, 901, 809, 749, 594, 564, 532, 518, 502	3426, 3085, 2930, 1770, 1736, 1692, 1654, 1604, 1582, 1466, 1582, 1371, 1315, 1231, 1194, 1122, 1021, 954, 917, 890, 869, 803, 751, 597, 549, 530, 501, 473, 424
UV/VIS (MeOH): λ_{max} (lg ε)	226 (4.35), 280 (4.24), 404 (3.79)	220 (4.12), 256 (3.65), 316 (3.28)	206 (4.42), 244 (4.01), 287 (3.28)
$[\alpha]^{20}_D$	-	+65.5° (c 0.550 mg/ml, MeOH)	-

Biological Activity

Antibacterial and antifungal activities were qualitatively determined using the agar diffusion method with 9 mm paper discs with 20 and 40 μg of the bhimamycins. Bhimamycin A (**2a**) and B (**2b**) showed moderate activity against *Bacillus subtilis* and *Escherichia coli*, bhimamycin E (**7**) selectively inhibited the growth of *Staphylococcus aureus*, whereas *Streptomyces viridochromogenes* (Tü 57), *Candida albicans*, *Mucor miehei*, *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus* were not affected at all (Table 6). Bhimamycin C (**3c**), D (**5a**), **5a**-methyl ester, bhimanone (**8**) and bhimanone diacetate (**9**) were inactive against all the test organisms mentioned above. They were also inactive in antitumor and antiviral tests.

Table 6. Antibacterial activities of bhimamycin A (**2a**), B (**2b**), and E (**7**) in the agar diffusion test with 20 and 40 μg/disc (i.d. mm).

Compounds	μg/disc	BS[a]	SV[b]	SA[c]	EC[d]
Bhimamycin A (**2a**)	20	0	0	13	11
	40	14	0	15	11
Bhimamycin B (**2b**)	20	11	0	17	0
	40	12	0	20	11
Bhimamycin E (**7**)	20	0	0	15	0
	40	0	0	20	0

[a] *Bacillus subtilis*, [b] *Streptomyces viridochromogenes* (Tü 57),[c] *Staphylococcus aureus*, [d] *Escherichia coli*.

Experimental

Materials and Methods

NMR spectra were measured on AMX 300 (300.135 MHz), Varian Unity 300 (300.145 MHz) and Varian Inova 500 (499.876 MHz) spectrometers. ESI-MS was recorded on a Finnigan LCQ with quaternary pump Rheos 4000 (Flux Instrument). EI-MS spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluorkerosine as reference substance for EI-HRMS. IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer from KBr pellets. Preparative HPLC was performed using an RP18 column (Eurochrom Eurospher RP 100-C18, 5 μm) at 202 nm detector wavelength (Knauer variable wavelength monitor). Flash chromatography was carried out on silica gel (230~400 mesh). R_f-values were measured on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.) with dichloromethane/8% methanol when not stated otherwise. Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia).

Taxonomy

The actinomycete strain GW32/698 was obtained from the strain collection of bioLeads in Heidelberg, Germany. It was Gram-positive, non-acid fast, and grew aerobically with substrate and aerial mycelium. The well developed aerial mycelium carried typical streptomycete-like long spiral chains of arthrospores. Neither aerial hyphae nor substrate mycelium showed fragmentation. Other morphological features such as sporangia, or motile spores, were not observed. The colour of the aerial spore mass was light pink on yeast extract-malt agar and white on soil extract agar, the substrate mycelium was brown on these media. Melanin pigments were not produced on tyrosine agar. The diaminopimelic acid isomer and the sugar composition of the whole cell hydrolysate indicated that the strain had cell walls of type I and belongs to the genus *Streptomyces*. The strain is deposited in the culture collection of actinomycetes at bioLeads GmbH, Waldhofer Strasse 104, D-69123 Heidelberg, Germany.

Malt Extract/Yeast Extract/Glucose-medium

Malt extract (10 g), yeast extract (4 g) and glucose (4 g) were dissolved in 1 liter of tap water and the medium was adjusted to pH 7.8 with 2 N NaOH and sterilized for 33 minutes at 121°C. After sterilization an end pH 7.0 of the medium is attained.

Fermentation, Extraction and Isolation

With a well grown agar culture of the terrestrial *Streptomyces* sp. GW32/698, 100 of 1 liter-Erlenmeyer flasks each containing 250 ml of M_2 medium were inoculated and incubated for 3 days at 28°C on a linear shaker (110 rpm). The 25-liter culture broth was mixed with *ca.* 1 kg celite and filtered through a press filter to separate mycelia and water phase. The mycelial cake and the filtrate were separately extracted each three times with ethyl acetate (*ca.* 2 liters each time). Since the chemical compositions of both organic phases were similar, they were combined and concentrated under reduced pressure to yield 3 g of a dark yellow oily extract. The crude extract was dissolved in methanol (150 ml), extracted with cyclohexane (150 ml) and both phases were evaporated separately.

The cyclohexane phase was separated by PTLC (20×20 cm, 5 plates, hexane/10% ethyl acetate) to yield chrysophanol (**1**, 3.6 mg, R_f 0.98) as a yellow solid. On further purification by CC with CH_2Cl_2, a second orange more polar zone afforded 3.5 mg of bhimamycin A (**2a**).

The methanol phase was pre-separated by column chromatography on silica gel (3×30 cm, 240 g) with a CH_2Cl_2/MeOH gradient into nine fractions. Purification of fraction 1 on a silica gel column (1×10 cm, 15 g, CH_2Cl_2) resulted in bhimamycin B (**2b**, 4 mg). The HPLC purification of fraction 6 using MeCN/90% H_2O afforded aloesaponarin II (**2**, 2 mg, R_f 0.69). On trituration of fraction 8 with dichloromethane/10% methanol (20 ml), an orange solid remained undissolved which was separated by centrifugation to yield 15 mg of 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (**3**, R_f 0.06). Purification of the soluble part of fraction 8 by HPLC gave adenosine (4 mg) and 2′-deoxy-adenosine (3 mg).

Chromatography of fraction 9 on Sephadex LH-20 using CH_2Cl_2/60% MeOH followed by HPLC and PTLC gave another 3 mg of aleosaponarin II (**2**) as a brown powder, bhimamycin C (**3c**, 2 mg) and bhimamycin D (**5a**, 5 mg) both as yellow solids.

The crude extract obtained from a second fermentation on a 50-liter scale was separated on silica gel (CH_2Cl_2/MeOH gradient) into six fractions. Bhimamycin B (**2b**, 50 mg), bhimamycin A (**2a**, 70 mg) and 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (**3**, 50 mg) were obtained from fractions I, II and VI, respectively, after PTLC (CH_2Cl_2/5% MeOH) and Sephadex LH-20 (CH_2Cl_2/60% MeOH). Fraction IV delivered in a similar way aleosaponarin II (**2**, 3 mg), bhimamycin C (**3c**, 7 mg) and D (**5a**, 17 mg). After separation on Sephadex LH-20 (CH_2Cl_2/60% MeOH) followed by PTLC (CH_2Cl_2/10%

MeOH), fraction III gave bhimamycin E (**7**, 10 mg). On crystallisation from dichloromethane/methanol, fraction V yielded 60 mg of bhimanone (**8**) as colourless needles.

Methylation of Bhimamycin D (**5a**)

To the solution of bhimamycin D (**5a**, 5 mg) in dichloromethane (1 ml), a few drops of a etherial diazomethane solution were added at 20°C and the mixture was evaporated to dryness after mixing for about 5 seconds to yield bhimamycin D methyl ester (5.2 mg, 100%) as a yellow solid, R_f 0.87 (CH_2Cl_2/7% MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 12.98 (s br, 1H, OH), 8.17 (d, J=7.2 Hz, 1H, CH), 7.72 (m, 2H, 2CH), 7.59 (m, 2H, 2CH), 7.22 (m, 2H, 2CH), 3.84 (s, 3H, OCH_3), 2.68 (s, 3H, $COCH_3$), 2.35 (s, 3H, Ar-CH_3); EI MS: m/z (%) 403 (M^+, 84), 374 (8), 360 (20), 344 (100), 329 (28), 316 (16), 301 (13), 272 (10), 254 (36), 244 (16), 77 (20), 45 (36).

Partial Synthesis of Bhimamycin F (**5b**) and G (**6**)

Bhimamycin A (**2a**, 17 mg) and anthranilic acid (35 mg) were dissolved in ethanol (5 ml) and refluxed for 12 hours and then the solvent was evaporated under vacuum. Purification of reaction mixture on Sephadex LH-20 (CH_2Cl_2/60% MeOH) yielded 6 mg of bhimamycin F (**5b**, 24.5%) and 3 mg of bhimamycin G (**6**, 12.8%).

Bhimamycin F (**5b**) was obtained as a yellow solid, R_f 0.40 (CH_2Cl_2/7% MeOH); ^{1}H NMR (DMSO-d_6): δ 13.10 (s br, 1H, OH), 7.91 (d, J=7.2 Hz, 1H, CH), 7.65 (m, 2H, 2CH), 7.52 (m, 2H, 2CH), 7.28 (d, J=7.2 Hz, 1H, CH), 7.20 (d, J=7.2 Hz, 1H, CH), 6.01 (s br, 1H, OH), 4.58 (q, J=6.5 Hz, 1H, CH), 2.23 (s, 3H, CH_3), 1.33 (d, J=6.5 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 125.7 MHz): δ 186.5 (CO), 179.6 (CO), 169.2 (COOH), 162.6 (C_q), 146. 9 (C_q), 139.9 (C_q), 138.5 (C_q), 135.7 (C_q), 135.7 (CH), 132.5 (C_q), 130.4 (CH), 129.7 (CH), 129.3 (CH), 128.8 (CH), 123.2 (CH), 118.5 (CH), 117.1 (C_q), 115.9 (C_q), 115.5 (C_q), 61.9 (CH), 21.7 (CH_3), 12.0 (CH_3); (+)-ESI MS: m/z (%) 805 ([2M+Na]$^+$, 100), 414 ([M+Na]$^+$, 13); (−)-ESI MS: m/z (%) 390 ([M−H]$^{-1}$, 100).

Bhimamycin G (**6**) formed a yellow solid, R_f 0.42 (CH_2Cl_2/7% MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 13.21 (s, 1H, OH), 8.25 (d, J=7.2 Hz, 1H, CH), 7.79 (m, 2H, 2CH), 7.69 (t, J=7.2 Hz, 1H, CH), 7.59 (t, J=7.2 Hz, 1H, CH), 7.41 (d, J=7.2 Hz, 1H, CH), 7.22 (d, J=7.2 Hz, 1H, CH), 4.35 (q, J=6.4 Hz, 1H, CH), 2.36 (s, 3H, Ar-CH_3), 1.32 (d, J=6.4 Hz, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 187.2 (CO), 181.7 (CO), 166.3 (COO), 162.9 (C_q), 146.5 (C_q), 138.8, 135.6, 135.4, 135.3, 134.8, 133.0, 130.8, 130.0, 127.9, 124.1, 119.3, 117.8, 117.3, 117.3, 63.4 (CH), 22.8 (CH_3), 12.0 (CH_3); EI MS: m/z (%) 373 (M^+, 100), 328 (80), 240 (22), 208 (4), 77 (6).

Synthesis of Bhimamycin D (**5a**)

Bhimamycin B (**2b**, 30 mg) and anthranilic acid (50 mg) were dissolved in ethanol (5 ml) and refluxed for 18 hours and then the solvent was evaporated under vacuum. Purification of reaction mixture on Sepahdex LH-20 (CH_2Cl_2/60% MeOH) and finally on HPLC yielded 1 mg of bhimamycin D (**5a**, 2.3%).

Acetylation of Bhimanone (**8**)

To the solution of bhimanone (**8**, 6 mg) in pyridine (0.5 ml), acetic acid anhydride (0.5 ml) and a crystal of 4-dimethylaminopyridine (DMAP) was added. The reaction mixture was left at room temperature for 2 hours and worked up to yield bhimanone diacetate (**9**, 8 mg, 98%).

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