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ORIGINAL ARTICLE

New bhimamycins from *Streptomyces* sp. AK 671*

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From the wild-type strain *Steptomyces* sp. AK 671, three nitrogen-containing octaketides were isolated, bhimamycins F, H and I, besides the known azaanthraquinone utahmycin A and polyketide shunt products SEK 4, SEK 4b, mutactin, dehydromutactin and EM18. The structures were characterized by MS and NMR experiments. The hitherto unknown absolute configuration of the two enantiomers of EM18 was determined by online-CD spectroscopy and quantum-chemical CD calculations. Bhimamycins H and I show weak antibacterial activities, whereas the enzyme phosphodiesterase 4 is strongly inhibited by bhimamycins H and I, which has never been reported for nitrogen-containing octaketides. In addition, bhimamycin H inhibits the enzyme glycogen synthase kinase-3β.
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INTRODUCTION

The alkaliphilic strain *Streptomyces* sp. AK 671 is a potent producer of various intermediates and end-products in the biosynthetic pathway to aromatic polyketides.[2] It has been shown that the octaketide chrysophanol glucuronide (**4**) originates from one acetyl-CoA and seven malonyl-CoA units by an unusual folding mode S′ of the polyketide chain and cyclization to the bicyclic diketo precursor genoketide A1 (the Harris–Franck diketone, **1**),[3] then glucuronidation to genoketide A2 (**2**) and ring closure to prechrysophanol glucuronide (**3**),[4] as shown in Figure 1.

Astonishingly, strain *Streptomyces* sp. AK 671 accumulated the intermediates **1**–**3** in high concentrations, which permitted their isolation in substantial quantities.[2] Besides **1**–**4**, several other aromatic polyketides were produced by the strain depending on the cultivation conditions. In a previous publication,[2] we reported on the isolation and characterization of the polyketide synthase (PKS) shunt products BSM1 (**5**) and 3,8-dihydroxy-1-methylanthraquinone-carboxylic acid (DMAC, **6**), which were originally isolated from genetically engineered *Streptomyces* strains blocked in the biosynthesis of actinorhodin.[5,6] Continued studies with strain AK 671 resulted in the detection and characterization of a series of further PKS shunt products such as SEK 4 (**7**), SEK 4b (**8**), mutactin (**9**), dehydromutactin (**10**) and EM18 (**11**) as a mixture of all four possible stereoisomers. The structures of **5**–**11** are summarized in Figure 2.

In our previous studies, we isolated and characterized the main polyketides **1**–**4** and, furthermore, the octaketides bhimamycins A (**12**) and B (**13**) from the culture filtrate of strain AK 671. This extraordinary, biosynthetically talented wild-type *Streptomyces* strain additionally produced the known nitrogen-containing octaketidic compound utahmycin A (**14**),[7] the hitherto only synthetically prepared bhimamycin F (**15**),[8] and the new bhimamycins H (**16**) and I (**17**) with UV-visible absorption characteristics of anthraquinones. Their fermentation, isolation, structure determination and biological activities are reported in this publication. The structures of octaketides **12**–**17** are shown in Figure 3.

RESULTS

Fermentation and isolation

Bhimamycins F (**15**), H (**16**) and I (**17**) were isolated as minor congeners produced by strain AK 671 at a fermentation time of 72 h when the strain was grown in a 10-l fermentor in complex medium SGG. They were gained from the culture filtrate by ethyl acetate extraction and purified by column chromatography using diol-modified silica gel, Sephadex LH-20 and Toyopearl HW-40 F. Compounds **15**, **16** and **17** were obtained in amounts of 2 mg, 9 mg and 18 mg, respectively, all as yellowish powders after lyophilization.

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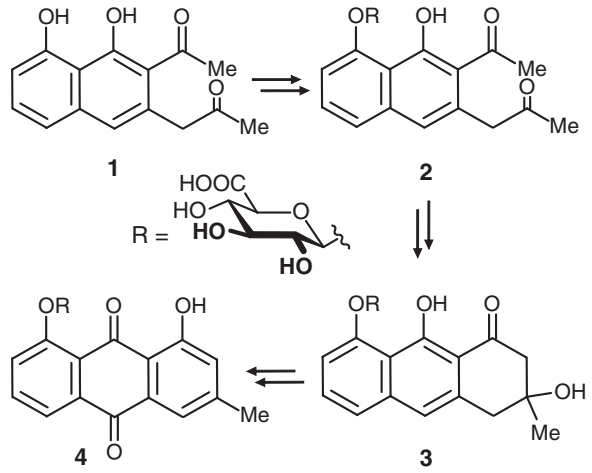

Figure 1 Conversion of intermediate genoketide A1 (**1**) *via* genoketide A2 (**2**) and prechrysophanol glucuronide (**3**) into chrysophanol glucuronide (**4**).

Structure elucidation

The structures of the known compounds, **5–14**, were determined by mass and NMR spectra, and by comparison with the spectroscopic and physical data from the literature. Interestingly, the shunt product EM18 (**11**, Figure 2) was detected 'twice', in a ratio of ca. 6:1 (41 mg, **11a**, and 6.5 mg, **11b**), in the culture filtrate, eluting baseline separated during HPLC on an achiral reverse-phase adsorbant. The nearly identical 1D- and 2D-NMR data (for the complete NMR data see Supplementary Tables S1 and S2) delivered the same constitution for both substances. EM18, bearing two stereogenic centers at C-6 and C-15, has been known for a long time,[9] and its biosynthetic origin has been intensely studied,[5,10,11] but never has the relative or the absolute configuration been discussed. Obviously *Streptomyces* sp. AK 671 produced both diastereomeric forms of EM18, the *unlike*-diastereomer[12] (that is, *R*/*S* or *S*/*R*) and the *like*-diastereomer (that is, *R*/*R* or *S*/*S*). As standard NOE experiments (1 sec mixing time) only delivered quite identical results for both diastereomers, extended NOE experiments were performed to provide information on the relative configuration. Transient NOE enhancements permit estimation of internuclear distances by variation of the mixing time.[13] In our case, multiple NOE spectra with increasing mixing times (50, 100, 200 and 400 ms) were taken. Compound **11a** had an NOE correlation between H-6 and H_a-14 at all mixing times, whereas compound **11b** showed this correlation only in the case of the longer mixing times (200 and 400 ms). This correlation can only be seen when the respective protons (in yellow) are both axial, which means that the pyranone substituent must occupy an equatorial orientation (Figure 4).

It seems that in case of compound **11b**, this equatorial position is energetically less favored and, hence, less populated in solution than the axial orientation. As a consequence, the NOE correlation can only be seen in longer mixing times. By contrast, this is not the case for **11a**, where the correlation can be found at all mixing times. A conformational analysis using quantum-chemical calculations (SCS-MP2/def2-TZVP//B97D/SVP and COSMO[acetone]) of all possible diastereomers of **11** showed that significant conformational differences are to be expected between the *like* and the *unlike* configurations. In the *like* configuration, the axial position of the pyranone is highly favored and the equatorial position is nearly not populated (94% axial, only 6% equatorial), in contrast to the *unlike* configuration, where the equatorial orientation is significantly more populated (79% axial, 21% equatorial). Consequently, compound **11a** is either *R*/*S*- or *S*/*R*-configured (*unlike*) and **11b** *R*/*R* or *S*/*S*.

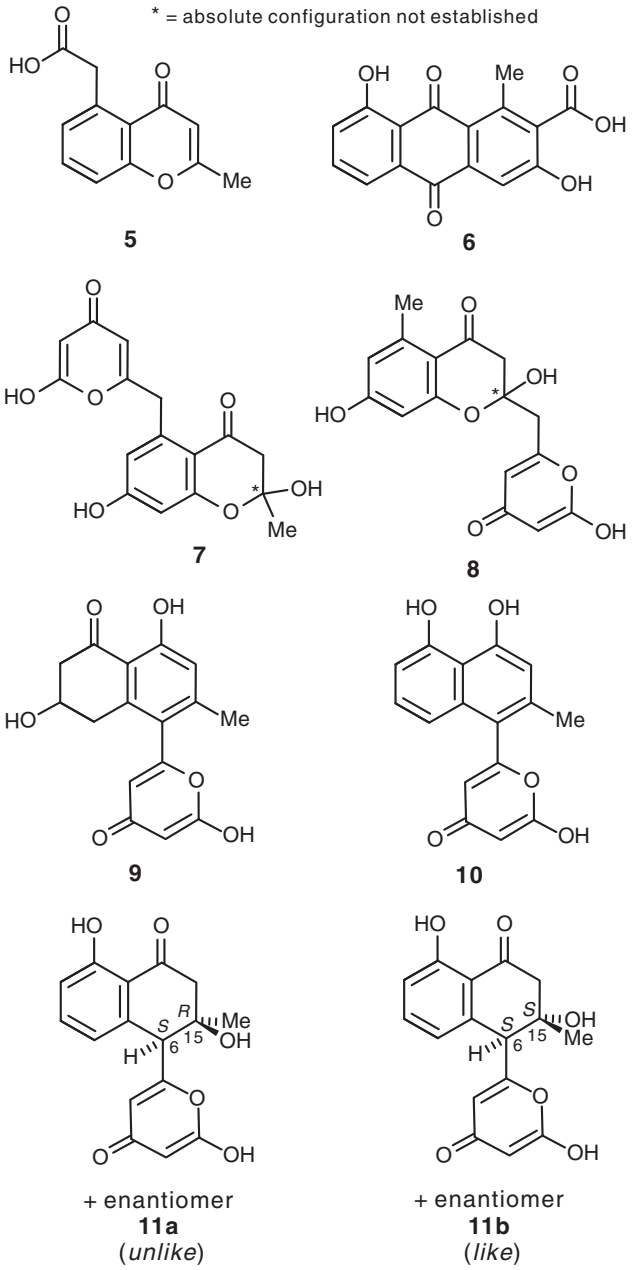

Figure 2 The polyketide shunt products BSM1 (**5**), DMAC (**6**), SEK 4 (**7**), SEK 4b (**8**), mutactin (**9**), dehydromutactin (**10**) and the (racemic) diastereomers of EM18, **11a** and **11b**, produced by *Streptomyces* sp. AK 671.

To establish the absolute configuration, CD spectra were taken of the two diastereomers, but neither **11a** nor **11b** delivered any CD effect, suggesting that both compounds were racemic mixtures. A successful resolution by HPLC on a chiral column revealed that each diastereomer consisted of two enantiomers in an approximately 1:1 ratio and permitted measurement of the CD spectra of all of the enantiomers online, in the stopped-flow mode. By application of the Exciton Chirality method,[14] it was now possible to assign the absolute configuration of all four stereoisomers of **11**. The CD spectra of the enantiomers of **11a** and **11b** show all an exciton couplet at 270 nm. This couplet arises from an interaction of the pyranone chromophore with the phenolic one. In the case of a 6*S*-configuration, a positive

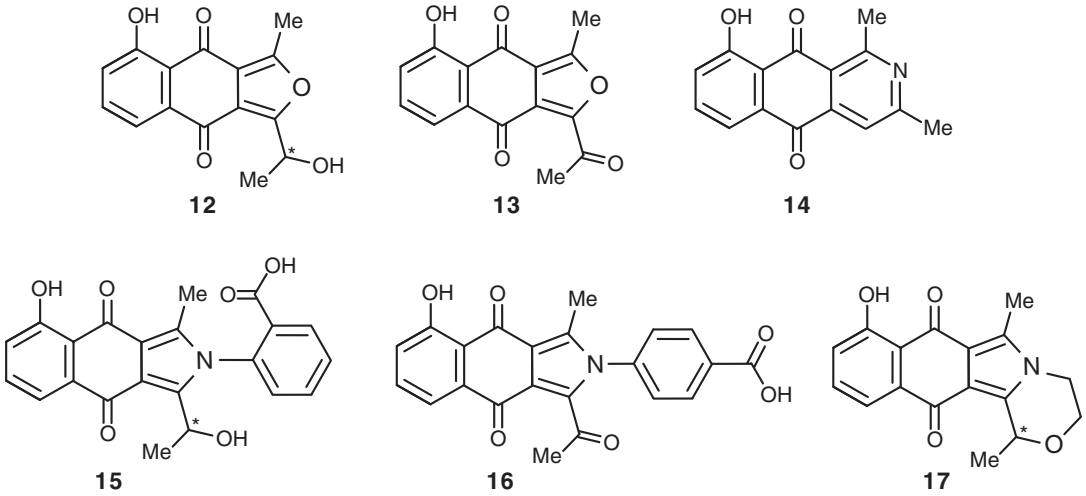

Figure 3 Structures of bhimamycins A (**12**) and B (**13**), utahmycin A (**14**), and of the new nitrogen-containing natural products bhimamycins F (**15**), H (**16**) and I (**17**) produced by *Streptomyces* sp. AK 671.

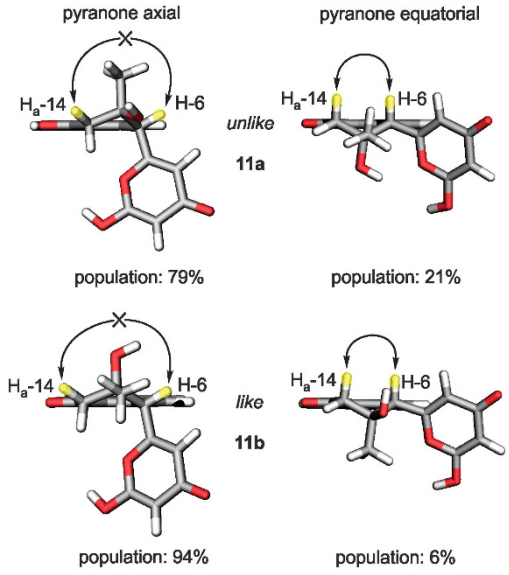

Figure 4 NOESY correlations between H_a-14 and H-6 in the diastereomers of EM18, **11a** (rapid, *unlike*, above), and **11b** (slow, *like*, below). Arbitrarily the 6*S*, 15*R*-enantiomer for *unlike* and the 6*S*, 15*S*-enantiomer for *like* are shown. Conformational analyses were done by SCS-MP2/def2-TZVP//B97D/SVP and COSMO calculations.

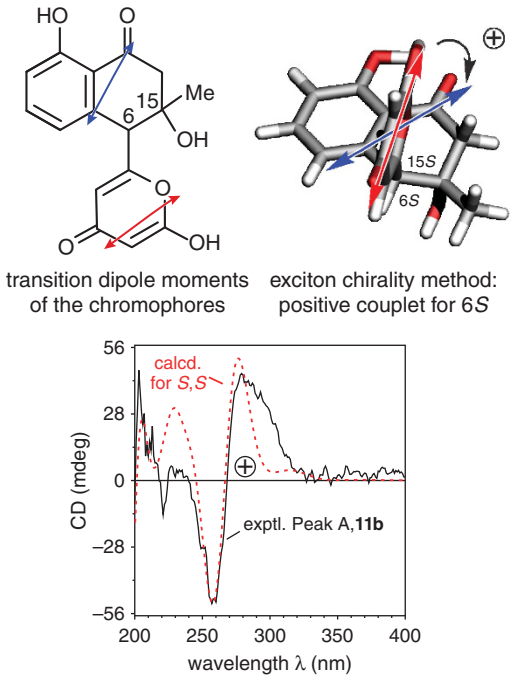

Figure 5 Determination of the absolute configurations of the two diastereomers of EM18 using the Exciton Chirality method, exemplarily shown for the 6*S*, 15*S*-isomer (the faster eluting enantiomer of **11b**), which was arbitrarily chosen for the calculations.

couplet results from the orientation of the respective transition dipole moments (Figure 5), whereas a 6*R*-configuration gives a negative couplet. Thus, peak A of **11a** with its negative couplet is 6*R*,15*S* and peak B, with the positive Cotton effect is 6*S*,15*R*-configured and for **11b** peak A is 6*S*,15*S*-configured (Figure 5), whereas peak B has the 6*R*,15*R* configuration.

For compound **15**, a molecular formula of $C_{22}H_{17}NO_6$ was cald from its exact mass of 390.09821 ($[M\text{-}H]^-$). In the ^1H NMR, the signals for seven aromatic protons, one proton in the shift range of an R_2-C*H*-O fragment and two methyl groups were visible. The aromatic protons were assigned by ^1H-^1H-COSY and NOESY correlations (Figure 6b) to correspond to an ABC spin system and to a further system of four consecutive aromatic protons. The ^{13}C NMR data gave hints at 16 sp^2 carbons, two keto functions, one carboxy function, one saturated carbon attached to a heteroatom and two methyl groups (Table 1; for the complete NMR data, see Supplementary Table S3). In a series of HMBC experiments, a structure consisting of a utahmycin A-related part and a benzoic acid derivative part was deduced (Figure 6a). In comparison with utahmycin A (**14**, Figure 3),[7] the NMR signals of one aromatic carbon and one proton in the three-ring skeleton of compound **15** were missing, suggesting that one of the aromatic rings was only five-membered. As the three protons and the hydroxy function of the western part were preserved, the aromatic system containing the nitrogen had to be different. By comparison

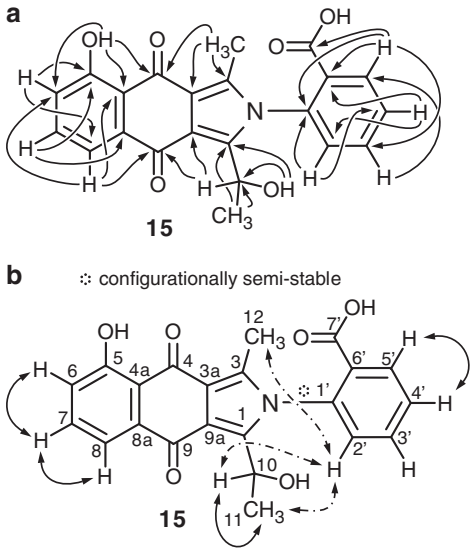

Figure 6 Important HMBC (**a**, ↷), COSY (**b**, ⟷) and NOESY (**b**, ⇢) correlations used for the structural elucidation of bhimamycin F (**15**).

Table 1 NMR spectroscopic data of bhimamycin F (15) in (D6) acetone (^{1}H: 600 MHz; ^{13}C: 150 MHz; 20 °C)

Pos.	δ_C	δ_H (*J* Hz)
1	148.1	–
2	–	–
3	139.2	–
3a	117.9	–
4	188.0	–
4a	118.4	–
5	163.6, 163.9	13.20 s
6	124.6, 124.7	7.28 m
7	136.6, 136.7	7.74 dd (8.4, 7.5)
8	119.8	7.78 dd (8.4, 1.2) 7.79 dd (8.4, 1.2)
8a	136.6, 136.7	–
9	182.0	–
9a	117.6	–
10	63.8	4.51 q (6.6)
11	23.8	1.40 d (6.6)
12	12.3	2.43 s
1′	140.0	–
2′, 4′	129.2, 129.5	7.70–7.72 m
3′, 5′	132.1, 132.3	8.31–8.33 m
6′	133.5	
7′	166.7	

with data from the literature, compound **15** was identified to belong to the class of the bhimamycins (Figure 3).[8]

As compound **15** carried a hydroxy group at C-10 (Figure 6a), it seemed to be a follow-up product of bhimamycin A (**12**). The attached benzoic acid derivative was found to be anthranilic acid, linked to the 2*H*-benzo[f]isoindole-4,9-dione at the *ortho* position. Due to the increased steric hindrance of the carboxylic acid moiety the *N*,*C*-axis is rotationally somewhat hindered, yet slowly rotating, creating two sets of co-occurring proton and carbon signals for the two configurationally semi-stable atropo-diastereomers **15** in the NMR spectrum (Table 1). Compound **15**, previously obtained as a side product in the chemical synthesis of bhimamycin D, has now been isolated for the first time as a natural product from a living organism. It was named bhimamycin F.[8]

Table 2 NMR spectroscopic data of bhimamycin H (16) in (D5) pyridine (^{1}H: 600 MHz; ^{13}C: 150 MHz; 20 °C)

Pos.	δ_C	δ_H (*J* Hz)
1	134.9	–
2	–	–
3	141.3	–
3a	117.9	–
4	187.7	–
4a	118.0	–
5	163.4	–
6	124.4	7.40 dd (8.4, 1.2)
7	136.5	7.64 dd (8.4, 7.5)
8	119.7	7.95 d (7.5)
8a	136.5	–
9	180.1	–
9a	122.2	–
10	194.0	–
11	31.3	2.92 s
12	12.4	2.48 s
1′	140.6	–
2′, 6′	128.2	7.66 d (8.6)
3′, 5′	131.7	8.55 d (8.6)
4′	134.0	
7′	168.4	

Compound **16** exhibited an exact mass of 390.09596 ($[M+H]^+$), corresponding to an elemental composition of $C_{22}H_{15}NO_6$. The proton NMR experiments evidenced the presence of three neighboring aromatic protons and two methyl groups, and an additional A_2B_2 spin system. In the ^{13}C NMR spectrum, the signals of 14 aromatic carbons, two methyl groups, three keto functions and one carboxy function were observed. The intensities of two of the aromatic carbon signals were twice as high as those of the others, possibly representing two chemically identical carbons, each (Table 2; for the complete NMR data see Supplementary Table S4). From the similarity of its NMR data with those of bhimamycin F (**15**), compound **16** might be related to bhimamycin B because of its keto function at C-10. As the mass was similar to that of **15**, the attached aromatic unit had to be anthranilic acid or a close analog, like *para*-aminobenzoic acid. The reduced set of signals in the ^{1}H and ^{13}C NMR spectra indicated a symmetric structure in the phenyl ring, thus excluding anthranilic acid. The complete structure was assigned by NOE and HMBC experiments (Figure 7a and b), evidencing **16** to be a product of bhimamycin B (**13**) and *para*-aminobenzoic acid. It was thus a new compound, now named bhimamycin H.

For compound **17**, a molecular formula of $C_{17}H_{15}NO_4$ was deduced by high-resolution ESI MS ($[M+Na]^+$ *m/z* 320.08911). In the ^{1}H NMR spectrum signals for three neighboring aromatic protons, one alkoxy or acetalic proton, four alkyl protons, forming high-order multiplets, and two methyl groups were observed. The ^{13}C NMR and the DEPT spectra showed the presence of two keto groups, seven quaternary sp^2-carbon atoms, six aromatic CH functions, two methylene units, one alkoxy carbon and two methyl groups (Table 3; for the complete NMR data, see Supplementary Table S5). Again, a bhimamycin-like structure was assumed, in this case resembling that

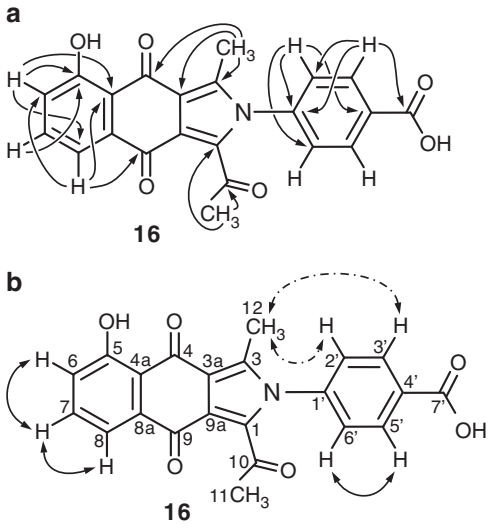

Figure 7 Important HMBC (**a**,↷), COSY (**b**,↷), and NOESY (**b**,⇢) correlations used for the structural elucidation of bhimamycin H (**16**).

Table 3 NMR spectroscopic data of bhimamycin I (17) in (D6) acetone (^{1}H: 600 MHz; ^{13}C: 150 MHz; 20 °C)

Pos.	δ_C	δ_H (*J* Hz)
1	138.6	–
2	–	–
3	137.7	–
3a	117.3	–
4	188.2	–
4a	118.5	–
5	163.3	–
6	123.4	7.17 dd (6.3, 6.6)
7	136.2	7.65 m
8	118.9	7.65 m
8a	137.2	–
9	179.0	–
9a	115.9	–
10	70.4	5.25 q (6.5)
11	18.9	1.66 s
12	10.7	2.65 s
1′	43.3	4.05 m
2′	60.3	H_a 4.01 m
		H_b 4.26 m

of the known bhimamycin C (**18**, Figure 8),[8] previously isolated as a natural product from a *Streptomyces* strain.

In contrast to bhimamycin C the 2′-methylene unit of **17** carried two diastereotopic protons. As **18** had a mass of 315 kDa, which is the MW of **17** (298 kDa) plus that of water ($H_2O = 18$ kDa), the new compound was proposed to be the condensed cyclic form of bhimamycin C. This assumption was unambiguously confirmed by HMBC and NOESY experiments (Figure 9a and b). Of significance were the HMBC correlations from H_a-2′ and H_b-2′ to C-10, and the NOESY interactions of H_a-2′ and H_b-2′ with H-10 and Me-11. Bhimamycin I (**17**) did not deliver a measurable α_D or CD effect (for the physicochemical properties of compounds **15–17**, see Table 4), leaving the assumption that it might be a racemic mixture. However, resolution of the enantiomers on a chiral phase did not succeed.

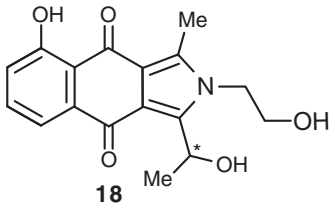

Figure 8 Bhimamycin C (**18**), previously isolated from a *Streptomyces* strain.

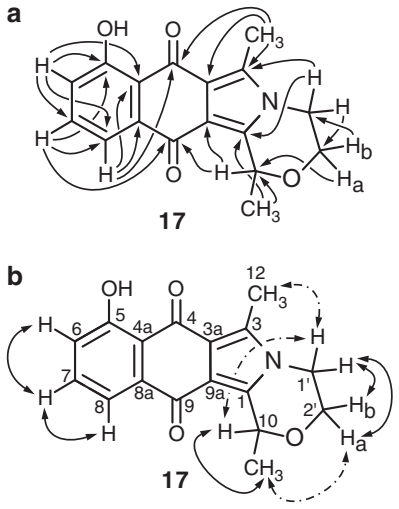

Figure 9 Important HMBC (**a**,↷), COSY (**b**,↷) and NOESY (**b**,⇢) correlations used for the structural elucidation of bhimamycin I (**17**).

Biological activities

Compounds **14**, **16** and **17** showed weak activities against *S. epidermidis* with IC_{50} values of 11 μM, 14.6 μM and 88.9 μM, respectively. Interestingly, only compound **16** affected the growth of methicillin-resistant *Staphylococcus aureus*, with an IC_{50} value of 13.5 μM. The activity of the enzyme GSK-3β[15,16] was inhibited by compound **16** (IC_{50} value of 18 μM). The IC_{50} values of the inhibitory activity against phosphodiesterase 4 of compounds **14**, **16** and **17** were >30 μM, 7.65 μM and 6.05 μM, respectively.

DISCUSSION

The pattern of polyketide metabolites from *Streptomyces* sp. AK 671 changed significantly in dependence on the fermentation conditions, in particular on the medium constituents and the type of cultivation vessel. The conversion of the octaketidic intermediate genoketide A1 (**1**) via genoketide A2 (**2**) and prechrysophanol glucuronide (**3**) to the end product chrysophanol glucuronide (**4**) was observed preferably in the complex medium SGG. Interestingly, the strain accumulated the three intermediates in high amounts and then converted them one by one, eventually producing **4**.[2] Besides compounds **1–4** as the main polyketide products, strain AK 671 additionally produced seven members of so-called 'metabolic shunt products', **5–11**, as minor compounds.

Polyketide shunt products are usually found as spontaneously cyclized intermediates that cannot be enzymatically transformed to their biosynthetic end-product (for example, to actinorhodin), as a consequence of the inactivation of genes in the biosynthetic

Table 4 Physicochemical properties of bhimamycins F (15), H (16), and I (17)

	15	*16*	*17*
Appearance	Yellow powder	Yellow powder	Yellow powder
Formula	$C_{22}H_{17}NO_6$	$C_{22}H_{15}NO_6$	$C_{17}H_{15}NO_4$
HR-ESI-MS (m/z)			
Found	390.09821 $[M\text{-}H]^-$	390.09596 $[M+H]^+$	320.08911 $[M+Na]^+$
Calcd.	390.09831 $[M\text{-}H]^-$	390.09721 $[M+H]^+$	320.08933 $[M+Na]^+$
UV λ_{max} (ε) nm (MeOH)	247 (4.73), 275 (4.35), 405 (4.18)	250 (4.81), 409 (4.21)	242 (4.60), 267 (4.20), 275 (4.15), 405 (4.15)
$[\alpha]_D^{20}$ ($\mu g\,ml^{-1}$, MeOH)	−4.5° (c 0.2)	–	0° (c 0.2)[a]

[a]Optical rotation oscillates between positive and negative values.

pathway.[17] The truncated hexaketide BSM1 (**5**) was described as a shunt product and not catabolic metabolite from the actinorhodin-deficient recombinant host *S. lividans* K4-114.[5] The octaketides SEK 4 (**7**) and SEK 4b (**8**) were discovered in *act*III ketoreductase mutants of *S. coelicolor* A3(2),[18] whereas mutactin (**9**), dehydromutactin (**10**) and EM18 (**11**) were found to be accumulated in *act*VII aromatase deficient mutants of *S. coelicolor* A3(2).[5,9] The fully aromatized octaketide DMAC (**6**) was isolated from *act*IV mutants, and a spontaneous cyclization of the third carbocyclic ring was postulated to build up the anthraquinone system of DMAC followed by a decarboxylation step to yield aloesaponarin II.[19] Even though DMAC was detected in strain AK 671,[2] no aloesaponarin II was detected under any cultivation condition. It can be speculated that the huge number of polyketide shunt products in cultures of the wild-type *Streptomyces* strain AK 671 is caused by a high production level and accumulation of non- or partially-aromatized intermediates leading to genoketide A1 (**1**) up to a concentration of $390\,mg\,l^{-1}$.[2] This precursor is then, at a slow rate, enzymatically transformed *via* **2** and **3** to the octaketidic end-product chrysophanol glucuronide (**4**).

In contrast to the well-investigated hexaketidic naphthylisoquinoline alkaloids from tropical plants,[20,21] only a few members of nitrogen-containing octaketides have so far been described as natural products. While several 3-methyl-2-azaanthraquinone derivatives have been isolated from lichens and fungi, utahmycin A (**14**) is the only natural precedent of the 1,3-dimethyl-substituted 2-azaanthraquinones, previously isolated from cultures of *Streptomyces albus* transformed with an environmental DNA clone by Bauer *et al.*[7] They postulated that utahmycin A may arise from erdacin polyketide intermediates originating through a folding mode S of the octaketidic chain (actually mode S', as defined earlier[4]), that is, in a *Streptomyces*-specific way.[7,22] In *Streptomyces* AK 671, utahmycin A (**14**) might be formed from the known chrysophanol precursor genoketide A1 (**1**; Figure 1) just by nitrogen incorporation and oxidation to the azaanthraquinone. The diketone **1** (like also compounds **2**–**4**) are formed through the S′-type folding, so that compound **14** should originate from the S′-mode, too, as also postulated by Bauer *et al.*[7] However, the question whether utahmycin A is a shunt product in the polyketide biosynthesis or not—or even an artefact, given the easy spontaneous incorporation of ammonia into monocyclic diketones related to **1**[23]—remains open.

Bhimamycins F (**15**), H (**16**) and I (**17**) are characterized by a benz[g]isoindolo-4,9-dione chromophore, which was first described in bhimamycins C and D. These metabolites have so far been isolated from *Streptomyces* sp. GW32/698 besides bhimamycins A and B.[8] As reported earlier, bhimamycins A and B were also found and isolated from *Streptomyces* strain AK 671.[2] Bhimamycin F (**15**) can be considered as a product of bhimamycin A and anthranilic acid, and bhimamycin H (**16**) as the product of bhimamycin B and *para*-aminobenzoic acid. Furthermore, compound **17** could represent the condensed cyclic form of bhimamycin C. Whether or not compounds **15**–**17** are true natural products cannot be proven. Due to the low quantities, they are neither detectable in the culture filtrate nor in the mycelium extracts, in contrast to bhimamycins A and B. The presence of anthranilic acid and *para*-aminobenzoic acid as constituents of the production medium was excluded by HPLC-DAD analysis, and thus, a chemical reaction with bhimamycins A or B is considered to be unlikely. A further argument for the natural origin of the two compounds is the fact that the other products—bhimamycin A and *para*-amino benzoic acid, and bhimamycin B and anthranilic acid—were not found. If the formation of **15** and **16** were the result of random chemistry in the fermentation broth, the mentioned combination products should occur in comparable amounts, too.

The origin of compound **17** as a genuine natural product, by contrast, is indeed questionable. If bhimamycin C (**18**, Figure 8) is produced by the *Streptomyces* strain, the conditions during workup—aqueous, acidic—will favor the spontaneous formation of a stabilized heterobenzylic cation, and, subsequently, the cyclization to bhimamycin I, which possesses a stable six-membered ring structure (see Supplementary Information: considerations on the native origin of bhimamycin I).

Utahmycin A (**14**) and bhimamycin H (**16**) showed weak, bhimamycin I (**17**) very weak activities against *S. epidermidis*, a human-pathogenic bacterium. The methicillin-resistant *S. aureus* strain was inhibited only by bhimamycin H (**16**). In an earlier study, antibacterial activity against *S. aureus* had been observed for bhimamycins A (**12**), B (**13**) and E at a concentration of 20 μg per disc,[8] whereas genoketides A1 (**1**) and A2 (**2**) had shown no antibacterial activities up to concentrations of $1\,mg\,ml^{-1}$.[2] Utahmycin A (**14**), bhimamycin H (**16**) and bhimamycin I (**17**) were active against the enzyme phosphodiesterase 4 (PDE4), bhimamycin H (**16**) inhibited the activity of the glycogen synthase kinase-3β (GSK-3β). To our knowledge, this is the first report on nitrogen-containing octaketides showing an inhibition of PDE4. Investigations on further, similar compounds, like derivatives of bhimamycins H and I, could be of interest for the discovery of new drugs for the treatment of the chronic obstructive pulmonary disease.[24]

The diversity and multitude of aromatic polyketides and polyketide shunt products isolated from the alkaliphilic *Streptomyces* AK 671 are unprecedented for a wild-type *Streptomyces* strain. The fermentation and growth conditions strongly influenced the accumulation of metabolites, resulting in the isolation and structure elucidation of new polyketides related to chrysophanol and bhimamycins. The described chrysophanol-related compounds gave unique insight into the biosynthesis of aromatic octaketides including final products from main metabolic pathways and side routes. The secondary metabolites from

the bhimamycin class showed the first reported inhibition of PDE4 by a nitrogen-containing octaketide. The results encourage the search for further novel bioactive natural compounds produced by bacteria of the order *Actinomycetales*, in particular of the genus *Streptomyces*.

EXPERIMENTAL SECTION

HPLC-diode array analysis

The HPLC-DAD system consisted of an HP 1090M LC equipped with a diode array detector, an HP Kayak XM 600 ChemStation and HPLC software revision A.08.03 (Agilent Technologies, Waldbronn, Germany). Multiple-wavelength monitoring was performed at 210, 230, 260, 280, 310, 435 and 500 nm, and UV-visible spectra were measured from 200 to 600 nm. Sample preparation and chromatographic conditions were performed as described earlier.[25] Evaluation of the chromatograms was done by means of an in-house HPLC-UV-Vis database containing about 960 entries, mostly antibiotics.[26]

Fermentation and isolation

For production of compounds **5–14**, strain AK 671 was cultivated in a 10-l stirred tank fermentor (Biostat S, B. Braun, Melsungen, Germany) in the complex SGG medium that consisted of starch soluble 10 g, glucose 10 g, glycerol 10 g, Bacto peptone 5 g (Becton Dickinson, Franklin Lakes, NJ, USA), corn steep powder 2.5 g (Marcor, Carlstadt, NJ, USA), yeast extract 2 g (Ohly Kat, Deutsche Hefewerke, Hamburg, Germany), NaCl 1 g in 1 l tap water. The pH was adjusted to 7.3 (5 M NaOH) prior to sterilization. The fermentation was carried out at 27 °C for 96 h with an aeration rate of 0.5 volume air per volume per min, and an agitation of 250 r.p.m. The isolation of compounds **5–14** from the culture filtrate was done in a succession of ethyl acetate extraction and size-exclusion column chromatography on Sephadex LH-20 (Amersham, Freiburg, Germany) and Toyopearl HW-40-F (Toyo Biosep, Stuttgart, Germany) in MeOH. For production of compounds **15–17**, batch fermentations of strain AK 671 were carried out in a 10-l stirred tank fermenter in SGG medium. The fermentor was inoculated with 5 vol-% of a shake pre-culture grown in the same medium in 500-ml Erlenmeyer flasks with one baffle for 40 h on a rotary shaker at 120 r.p.m. at 27 °C. The fermentation was carried out for 72 h at 27 °C with an agitation of 300 r.p.m. and an aeration of 0.5 volume air per volume per min. Hyflo Super-cel (2%) was added to the fermentation broth, which was separated by multiple sheet filtration; the mycelium was discarded. The culture filtrate (8.5 l) was adjusted to pH 5.0 (1 M HCl) and extracted three times with EtOAc. The organic extracts were combined and concentrated to dryness *in vacuo*. The crude extract was subjected to a diol-modified silica gel column (40 cm × 3.6 cm; LiChroprep Diol; E. Merck, Darmstadt, Germany) and separation was accomplished by a linear gradient from CH_2Cl_2 to CH_2Cl_2-MeOH (9:1) within 4 h at a flow rate of 9 ml min^{-1}. Fractions containing **15**, **16** and **17** were combined and purified by chromatography on Sephadex LH-20 (90 cm × 2.5 cm) and Toyopearl HW-40-F (90 cm × 2.5 cm) using MeOH as the eluent.

Structure elucidation

NMR spectra were recorded on a Bruker DMX 600 spectrometer (Bruker, Rheinstetten, Germany) at ambient temperature. The chemical shifts are given in δ units (p.p.m.) taking the signals of the deuterated solvents as internal reference for ^{1}H and ^{13}C NMR spectroscopy; the coupling constants J are given in Hertz (Hz). For basic NOESY experiments the mixing time was set to 1 s, for extended NOESY experiments the mixing time was set to 50, 100, 200 and 400 ms. Proton-detected, heteronuclear correlations were analyzed using HSQC (optimized for $^1J_{HC} = 145$ Hz) and HMBC (optimized for $^nJ_{HC} = 7$ Hz). Mass spectral analysis was achieved on a time-of-flight mass detector micrOTOF II focus (Bruker Daltonics, Bremen, Germany).

HPLC on a chiral stationary phase was carried out on a Lux Cellulose-1 column (0.5 µm; 250 mm × 4.6 mm; Phenomenex, Torrance, CA, USA) with a flow rate of 0.8 ml min^{-1} and an isocratic solvent system: solvent (A) H_2O (+0.05% trifluoroacetic acid), and solvent (B) CH_3CN (+0.05% trifluoroacetic acid), 18% of solvent A. Online-CD spectra were recorded in the stopped-flow mode on a JASCO J-715 circular-dichroism spectrometer (JASCO, Gross-Umstadt, Germany) equipped with a 5-mm flow cell and a BESTA motor valve (BESTA-Technik, Wilhelmsfeld, Germany).

Biological assays

The antimicrobial activity of compounds **14**, **16** and **17** against *S. epidermidis* DSM 20044 and methicillin-resistant *S. aureus* DSM 18827 was measured according to Schulz *et al.*[27] The cytotoxic activity against the cell line HepG2 and the determination of the phosphodiesterase (PDE4-4B2) inhibitory activity of compounds **14**, **16** and **17** were performed according to Kim *et al.*[28] Glycogen synthase kinase-3β (GSK-3β) inhibition was determined in an *in vitro* assay adapted from a luminescent assay described by Baki *et al.*[29] To determine the IC_{50} values of the enzyme-inhibitory activities concentrations ranging from 0.1 µM to 50 µM were analyzed twice in duplicates.

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