Balmoralmycin, a New Angucyclinone, and Two Related Biosynthetic Shunt Products Containing a Novel Ring System

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A new angucyclinone, named balmoralmycin (**1**), was isolated as an inhibitor of protein kinase C-α (PKC-α) from the *Streptomyces* strain P6417. Chemical screening of extracts of the same strain resulted in the detection of two decaketides with unusual structural features (**2** and **3**). Both compounds belong to a recently described structural class of secondary metabolites which arises from engineered biosynthesis of a recombinant *Streptomyces* strain. The isolation of compounds of this class from a wild-type strain has never been reported before.

Intensively colored multicyclic aromatic polyketides like tetracyclines, anthracyclines or angucyclines are among the most powerful bioactive natural products[1,2]. In continuation of our screening project for bioactive secondary metabolites from microorganisms[3] we discovered a *Streptomyces* strain P6417, isolated from a soil sample collected at Balmoral's Castle, Scotland, producing an inhibitory activity against protein kinase C-α (PKC-α). This enzyme plays important roles in cellular transduction mechanisms[4]. By isolation and subsequent structural elucidation of the active component, the PKC-α inhibitory activity could be assigned to a new member of the rapidly growing group of angucyclinone antibiotics and was named balmoralmycin (**1**) (Scheme 1). Biogenetically balmoralmycin (**1**) can be classified to the urdamycin B-type angucyclines with a classical aglycone and a C-glycosidic moiety[2]. In addition we isolated two unusually arranged decaketides **2** and **3** (Scheme 1) consisting of a functionalized benzophenone substituted with a pyronyl methyl moiety. Like other unusually arranged aromatic polyketides the metabolites **2** and **3** are biosynthetic intermediates which help to understand the biogenetic formation of the tetracycline ring system of angucyclines, anthracyclines or tetracyclines. Recent investigations on producing organisms with genetically altered type-II polyketide synthases support the model of a successive formation of the four annealed rings in the family of the anthracyclines[5,6]. As a result of these findings the preparation of genetically engineered secondary metabolites of commercial interest is becoming a rapidly growing field[7]. This paper deals with the isolation and the characterization of the new compounds **1** and **2** and the related decaketide SEK 15 (**3**)[8], which has been described recently. A short discussion on the biosynthetic relationship of these compounds will be addressed.

Materials and Methods

General

The following instruments were used in this study: Bruker spectrometer IFS 48, ZAB-HF mass spectrometer (FISONS Instruments, Mainz-Kastel), NMR spectrometer Varian VXR-400 S. Chemical shifts are given in ppm using TMS as internal standard.

The enzyme assay for PKC-α was performed as described by MEYER *et al.*[9].

The chromatographic systems used for preparative normal-phase liquid chromatography with silica gel, for the analytical and preparative HPLC on reversed-phase silica gel are identical with those described by ROGGO *et al.*[3,10]. TLC-analysis was performed in analogy to the methods used by ROGGO *et al.*[3].

Culture Conditions and Purification

Streptomyces strain P6417 was isolated from a soil sample collected at a roadside near Balmoral's castle, Scotland. The strain was grown on agar slants containing potato starch (Blattmann) 1.0%, casein hydrolysate (Merck) 0.1%, yeast extract (Difco) 0.1%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and agar 2.0%. The pH

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was adjusted to 7.0 with 5N H_2SO_4 or 5N NaOH prior to sterilization. The culture was incubated at 28°C for ten days until complete sporulation had occured. Working stocks were stored at 4°C, whereas long-term storage was at −80°C and −169°C, respectively. A loopful of a mature slant culture of the P6417 was inoculated into an Erlenmeyer flask (500 ml) with 1 baffle containing 100 ml of a medium with the following composition: glucose 2.0%, Pharmamedia 1.5%, $(NH_4)_2SO_4$ 0.3%, $ZnSO_4 \cdot 7H_2O$ 0.003%, $CaCO_3$ 0.4%. Before sterilization the pH was adjusted to 7.0 with sulfuric acid (98%) or sodium hydroxide (30%). The culture was incubated on a rotary shaker (250 rpm) under aerobic conditions for 48 hours at 28°C. 5 ml of the seed culture was transferred into a second series of Erlenmeyer flasks (2,000 ml) with four baffles containing 500 ml of the seed medium each. This second seed culture was incubated under the same conditions as the previous one. 500 ml of this culture was then transferred into a 50-liter fermentor (Infors) containing 40 liters of the following production medium: mannitol 2.0% and soybean meal 2.0%. The fermentation was carried out at 28°C for 93 hours with an aeration rate of 40 liters/minute and an agitation rate of 400 rpm.

For the work up of cultures of strain P6417 the whole broth (40 liters) was rigorously mixed with EtOAc (80 liters). Subsequent filtration was performed using a filter aid (Celite; Celite Corp. U.S.A.). The aqueous and organic phases were separated, the aqueous phase was discarded, while the organic layer was washed with sodium chloride solution (20%, 5.25 liters) and concentrated *in vacuo*. The EtOAc-extract was partitioned between heptane (600 ml) and MeOH (400 ml). Evaporation of the MeOH phase yielded 13.3 g of a dark red crude extract. 9.8 g of this EtOAc-extract was suspended in CH_2Cl_2 (50 ml) and adsorbed on silica gel (15 g). This crude material was chromatographed at medium pressure over silica gel (Lichroprep Si 60, 15～25 μm, column with a diameter of 36 mm and a length of 460 mm) with stepwise elution of the following solvents (v/v): CH_2Cl_2, CH_2Cl_2-MeOH 99:1, 95:5, 90:10, 80:20, 60:40 and MeOH. The flow rate of the mobile phase was 30 ml/minute, the detection wavelength was set at 254 nm. The fractions were combined based on TLC-analysis. Crude balmoralmycin (**1**) (840 mg) was eluted with CH_2Cl_2-MeOH 95:5 (v/v), followed by crude **2** (730 mg) which was eluted with CH_2Cl_2-MeOH 80:20 (v/v). Compound **3** was subsequently eluted from the column with CH_2Cl_2-MeOH 60:40 (v/v).

Crude **1** (200 mg) was suspended in CH_2Cl_2 (5 ml). The precipitate was filtered off and taken up in MeOH (3 ml). Subsequent filtration and evaporation of the solvent yielded compound **1** (137 mg).

Crude **2** (730 mg) was further purified by gel filtration on Sephadex LH-20 (Pharmacia, 25～100 μm, column with a diameter of 25 mm and a length of 800 mm) using MeOH as mobile phase and a flow rate of 0.5 ml/minute to obtain the pure metabolite **2** (410 mg).

The isolation of pure **3** from 415 mg of crude material was successfully achieved by reversed-phase chromatography (Lichroprep RP-18, 15～25 μm, conical column with a diameter of 26～49 mm and a length of 230 mm) with a gradient using H_2O as mobile phase A and CH_3CN-H_2O 80:20 (v/v) as mobile phase B. The flow rate of the mobile phase was 30 ml/minute. The gradient was run from 30% to 100% phase B in 50 minutes, with the detection wavelength set at 235 nm. Final purification of **3** was performed with gel filtration on Sephadex LH-20 (Pharmacia, 25～100 μm, column with a diameter of 25 mm and a length of 800 mm) using MeOH as mobile phase and a flow rate of 0.5 ml/minute to obtain the pure metabolite **3** (26 mg).

Balmoralmycin (**1**)

A dark red amorphous powder. IR ν_{max} (KBr) cm^{-1} 3474, 2950, 2930, 2860, 1690, 1640, 1580; UV λ_{max} nm ($E^{1\%}_{1cm}$) 265 (1,208), 495 (251); ^1H NMR (400 MHz, pyridine-d_5) δ 15.08 (1OH, br s), 13.97 (1OH, s), 13.30 (1OH, br s), 9.23 (1H, s, 10-H), 8.06 (2H, br s, 3-H and 4-H), 7.93 (1H, s, 9-H), 7.60 (1H, dd, $J_{2'',3''}=15.3$ Hz, $J_{3'',4''}=10.8$ Hz, 3″-H), 7.18 (1H, s, 7-H), 6.22 (1H, dd, $J_{4'',5''}=15.5$ Hz, $J_{3'',4''}=10.8$ Hz, 4″-H), 6.09 (1H, d, $J_{2'',3''}=15.3$ Hz, 2″-H), 6.09 (1H, m, 5″-H), 5.42 (1H, t, $J=9.4$ Hz, 4′-H), 5.23 (1H, br d, $J=11.1$ Hz, 1′-H), 4.46 (1H, m, 3′-H), 3.89 (1H, m, 5′-H), 3.03 (1H, ddd, $J=$ 13.4, 5.0, 2.1 Hz, 2′-H_a), 2.36 (3H, s, 8-Me), 2.16～1.96 (3H, m, 2′-H_b and 6″-H_2), 1.51 (3H, d, $J_{5'',6''}=6.1$ Hz, 6′-H_3), 1.36～1.14 (6H, m, 7″-H_2, 8″-H_2 and 9″-H_2), 0.85 (3H, t, $J_{9'',10''}=6.8$ Hz, 10″-H_3); ^{13}C NMR (100 MHz, pyridine-d_5) δ 188.0 (s, C-5 or C-12), 186.8 (s, C-5 or C-12), 166.8 (s, C-1″), 163.3 (s, C-1 or C-6 or C-11), 159.1 (s, C-1 or C-6 or C-11), 157.3 (s, C-1 or C-6 or C-11), 145.5 (d, C-3″), 144.9 (d, C-5″), 141.9, 137.5, 132.9, 129.3, 125.8, 125.5, 116.7, 109.4 (8s, C-2, C-4a, C-5a, C-6a, C-8, C-9a, C-11a, C-12a), 132.9 (d, C-3), 128.4 (d, C-4″), 119.4 (d, C-2″), 118.6 (d, C-4), 117.9 (d, C-10), 116.5 (d, C-7), 115.0 (d, C-9), 78.6 (d, C-4′), 74.8 (d, C-5′), 71.9 (d, C-1′), 70.3 (d, C-3′), 40.8 (t, C-2′), 32.7 (t, C-6″), 31.1 (t, C-8″), 28.2 (t, C-7″), 22.3 (t, C-9″), 21.8 (q, 8-Me), 18.1 (q, C-6′), 13.7 (q, C-10″); FAB-MS *m/z* 601 $(M+H)^+$; HRDCI-MS *m/z* 601.2442 ($C_{35}H_{37}O_9$, δ_m 0.5 mmu).

Anal Calcd for $C_{35}H_{36}O_9$: C 69.98, H 6.04.
Found: C 69.96, H 6.17.

2-[(2,4-Dihydroxy-6-methylbenzoyl)-3-hydroxyphenylmethyl]-6-hydroxy-4*H*-pyran-4-one (**2**)

A slightly yellow amorphous powder: IR ν_{max} (KBr) cm^{-1} 3330, 3180, 1690, 1670, 1620, 1460; UV λ_{max} nm ($E^{1\%}_{1cm}$) 291 (522), 350 sh; ^1H NMR (400 MHz, MeOH-d_4) δ 7.25 (1H, dd, $J_{8,9}=7.8$ Hz, $J_{9,10}=8.2$ Hz, 9-H), 6.87 (1H, d, $J_{8,9}=7.8$ Hz, 8-H), 6.81 (1H, d, $J_{9,10}=8.2$ Hz, 10-H), 6.17 (1H, d, $J_{16,18}=2.5$ Hz, 16-H), 6.07 (1H, d, $J_{16,18}=2.5$ Hz, 18-H), 5.68 (1H, br s, 4-H), 5.21 (1H, br s, slowly exchanging, 2-H), 3.63 (2H, br s, 6-H_2), 1.82 (3H, s, 20-H_3); ^{13}C NMR (100 MHz, MeOH-d_4) δ 202.2 (s,

C-13), 173.1 (s, C-3), 168.3 (s, C-15 or C-17), 168.0 (s, C-1), 165.7 (s, C-17 or C-15), 165.0 (s, C-5), 155.2 (s, C-11), 145.3 (s, C-19), 133.4 (s, C-7), 132.3 (s, C-12), 131.6 (d, C-9), 123.2 (d, C-8), 116.5 (s, C-14), 116.1 (d, C-10), 113.3 (d, C-18), 103.2 (d, C-4), 102.1 (d, C-16), 89.9 (d, C-2), 38.1 (t, C-6), 22.4 (q, C-20); FAB-MS *m/z* 369 $(M+H)^+$; HREI-MS *m/z* 368.0895 ($C_{20}H_{16}O_7$, δ_m 0.1 mmu).

2-[(2,4-Dihydroxy-6-methylbenzoyl)-3,5-dihydroxyphenylmethyl]-6-hydroxy-4*H*-pyran-4-one (**3**)

A slightly yellow amorphous powder; spectroscopic data identical with those reported for SEK 15[8].

Results and Discussion

Cultures of *Streptomyces* strain P6417 were worked up after 93 hours of fermentation time. Separation and isolation of the individual components from the EtOAc extract of cultures of strain P6417 were achieved by using the following successive purification steps: (a) solvent partitioning between *n*-heptane and MeOH, (b) medium pressure chromatography of the MeOH soluble fraction on a normal-phase silica gel column (Lichroprep Si 60) yielding crude secondary metabolites **1**, **2** and **3**, (c) precipitation of **1** in CH_2Cl_2, (d) gel chromatography of crude **2** on Sephadex LH-20 with MeOH as mobile phase and (e) medium pressure reversed-phase chromatography (Lichroprep RP-18) of crude **3**, which was purified by (f) chromatography on Sephadex LH-20 with MeOH as mobile phase. All involved steps of the purification procedure were monitored by HPLC-DAD or bioassay analysis of the respective fractions.

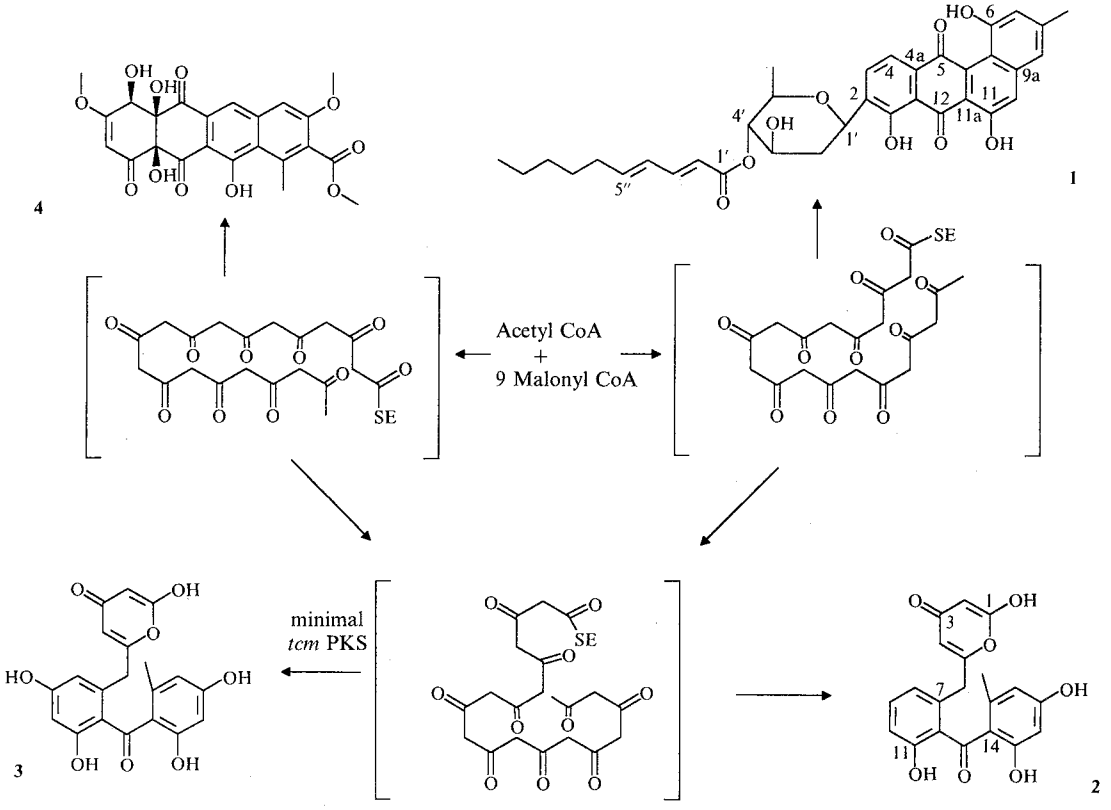
Balmoralmycin (**1**) was isolated as a red amorphous powder. Its positive-ion FAB-MS displayed a pseudomolecular $(M+H)^+$ cation at *m/z* 601 and in combination with the results of the elemental analysis its molecular formula of $C_{35}H_{36}O_9$ was determined. The IR spectrum of balmoralmycin (**1**) exhibited carbonyl bands at 1710 (ester), 1638 and 1614 (chelated quinone groups) and intensive bands at 3470 cm^{-1} (hydroxy). Furthermore the UV-spectrum showed intensive absorption maxima at 265 and 495 nm. These data indicated the presence of an anthraquinone core structure. The typical quinone signals at 188.0 and 186.3 ppm were recorded in the ^{13}C NMR spectrum of **1**. Detailed analysis of the 35 signals in the ^{13}C NMR spectrum of balmoralmycin (**1**) revealed a strong relationship to capoamycin[11], an angucyclinone-type antibiotic with a C-glycosidic bound olivose, whose 4-hydroxy group is acylated with (*E,E*)-2,4-decadienoic acid. Further interpretation of the H-H COSY and C-H COSY spectra as well as intensive NOE measurements revealed that **1** in contrast to capoamycin has no angular oxygens. Ring A of the angucyclinone moiety of **1** is aromatized as in the case of the anthracyclinone antibiotic described by ARNONE *et al.*[12]. In contrast to the compound mentioned above and to capoamycin, balmoralmycin (**1**) has an additional hydroxy group at C-6 in ring B. Since anthraquinones proved to be inhibitors of protein kinases[13], it was not surprising that the angucycline **1** turned out to be the active principle of strain P6417 exhibiting a moderate IC_{50} value of 50 μM in the PKC-α assay[9].

Compound **2** was isolated by chemical screening methods[14] due to its interesting HPLC-DAD spectrum with absorption maxima at 291 and 350 nm and an unusual slight orange color formation after staining with anisaldehyde/H_2SO_4 on TLC plates. Its molecular formula of $C_{20}H_{16}O_7$ was determined by HREI-MS based on the molecular ion peak at *m/z* 368. The data of the ^1H NMR and ^{13}C NMR spectra were consistent with the presence of a γ-pyrone with a C-linked exchangeable hydrogen and two hydroxylated benzene rings. The constitution of the three building blocks and their unusual connection as shown in compound **2** (Scheme 1) could be determined by NOE investigations and low power selective decoupling experiments. The observed NOE effects between the methylene protons and 8-H (19%) and 4-H (18%), respectively, corroborate the structure of **2** as shown in Scheme 1.

TLC color reaction and UV absorption maxima of the minor compound **3** were nearly identical with those described for **2**, thus suggesting a nearly unchanged chromophore. The data from the EI-MS of **3** indicated the presence of an additional hydroxyl group which could be assigned to the C-9 position of the decaketide on the basis of NOE experiments.

The structural arrangement of **2** and **3** with its hydroxylated benzophenone moieties represent novel ring systems, which are unique for naturally derived secondary metabolites. They are related to mutactin, isolated from a mutant of an actinorhodin producing *Streptomyces coelicolor* strain[15]. Compound **3** is identical with the recently described SEK 15[8] and only differs from component **2** by having an additional hydroxy group at the C-9 position. FU *et al.*[8] isolated the decaketide **3** as the product of an engineered biosynthesis, namely from a recombinant strain with minimal tetracenomycin polyketide synthase (*tcm* PKS). Based on the well established biosynthesis of angucyclinones[2] and in analogy to the biological formation of SEK 15 (**3**) and tetracenomycin (**4**) described by FU *et*

Scheme 1. Chemical structures of balmoralmycin (**1**), compound **2**, SEK 15 (**3**) and tetracenomycin (**4**) and the biosynthetic relationship of these four secondary metabolites.



–SE: enzyme linked *via* thioester bond.

al.[8] the secondary metabolite **2** seems to arise by an alternate cyclization of the same precursor decaketide which gives rise to the aglycone of balmoralmycin (**1**) (Scheme 1). In accordance with FU's results one can hypothetize that the chain formation and the first cyclization of the aglycone of **1** and the component **2** are determined by a minimal balmoralmycin PKS. Subsequently, the biosynthesis of both compounds has to involve a C-9 ketoreductase before the second cyclization takes place. A cyclase activity is needed in the further biosynthesis of **1**, whereas the remaining cyclizations of **2** seem to proceed non enzymatically. In conclusion the isolation of **2** and **3** as natural products from a wild-type strain supports the concept, that aromatic, multicyclic polyketides are formed by a stepwise ring closure of an almost unmodified polyketide chain. The C-9 carbonyl reduction represents one of the first transformation reactions following the formation of the complete polyketide chain.

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