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Grecoketides A and B: New Naphthoquinones from *Streptomyces* sp. Acta 1362[‡]

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Two novel naphthoquinones were produced by the streptomycete Acta 1362. It was determined by HPLC/diode-array screening that freshly isolated actinomycete strains from selected European ecosystems produce new compounds. Grecoketides A and B were isolated and their structures determined. Both compounds have the same aglycon grecoketidone with a sugar side-chain that differs in one of the two attached sugar units. Moreover, the biosynthesis of grecoketidone was studied by feeding singly and fully labelled acetate. The hexaketide formed is cyclized after s-mode folding.

Introduction

In the course of our HPLC/diode-array screening programme, we investigated freshly isolated actinomycete strains from selected European ecosystems with the aim of detecting novel compounds for pharmaceutical applications (http://www.actapharm.org). The strains were cultivated in shake flasks in various complex media. Extracts were generated from culture filtrates and mycelia over various fermentation times, and their secondary metabolite profiles were evaluated by HPLC/diode-array analysis. We have developed an HPLC/UV/Vis database.[1]

Strain Acta 1362, which was isolated from soil from the rhizosphere of an indigenous plant (*Pinus brutia*) of the island of Crete,[2] was of special interest because of the presence of a dominant peak in the culture filtrate extract at a retention time of 8.2 min and a minor congener with a retention time of 7.8 min in our standardized gradient elution profile. These two peaks correspond to grecoketide A (**1**) and grecoketide B (**2**), respectively (Figure 1). The nearly congruent UV/Vis spectra of the grecoketides were unlike those of all 867 reference compounds, mostly antibiotics, stored in our HPLC/UV/Vis database, but showed a close relationship to the UV/Vis spectra of anthraquinone-type compounds.

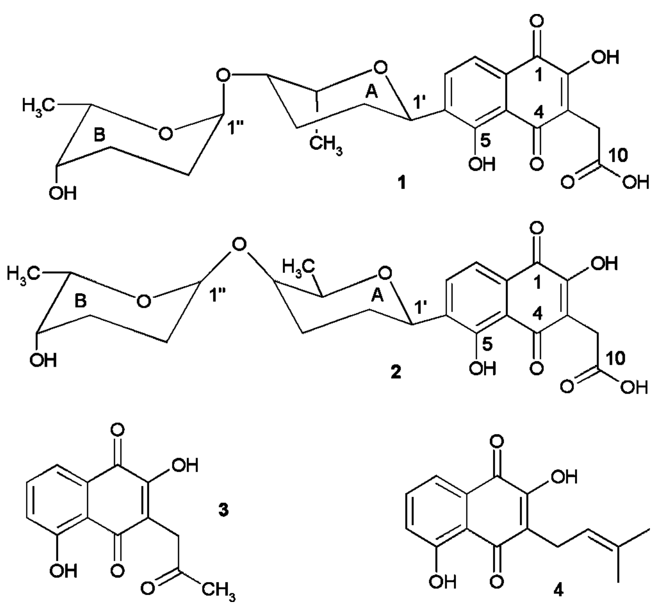

Figure 1. Structures of grecoketide A (**1**), B (**2**), gonioquinone (**3**) and hydroxylapachol (**4**).

Strain Acta 1362 was assigned to the genus *Streptomyces* on the basis of morphological and physiological properties known to be of value in streptomycete systematics[3] and of partial sequencing of the 16S rRNA gene, comparing the gene sequence with corresponding sequences of available *Streptomyces* type strains.[4]

Batch fermentations of strain Acta 1362 were carried out in a 20-L fermentor by using a complex medium. The production of grecoketides A and B started after 38 h, reaching

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a maximum amount of 13 and 4.7 mg L^{-1} grecoketides A (**1**) and B (**2**), respectively, at a fermentation time of 160 h. The grecoketides were isolated from the culture filtrate by extraction with ethyl acetate and were purified and separated by a succession of chromatographic steps using diol-modified silica gel and step-gradient elution with dichloromethane/methanol and Sephadex LH-20 and Toyopearl HW-40 column chromatography with methanol as eluent.

In feeding experiments with [1-^{13}C]acetate and [1,2-^{13}C]-acetate the strain was grown in 1-L fermentors by using the same complex medium as used for grecoketide production. The strain was fed with labelled acetate in 10 pulses over a period of 36 h starting at 40 h of fermentation. The cultures were harvested after 90 h of incubation, and the labelled grecoketides were isolated and purified by using the procedure described above.

Results and Discussion

The ESI-MS spectra of grecoketides A (**1**) and B (**2**) showed molecular ions at m/z = 475.3 ([M – H]$^-$), in accord with a molecular mass of 476 g mol^{-1} for both compounds (Table 1). The exact molecular masses were determined by high-resolution ESI-FT-ICR-MS and gave a molecular formula of $C_{24}H_{28}O_{10}$ for **1** and **2**. 1D NMR spectra of the two compounds were very similar with differences in one of the two sugar units (Table 2). Because of this similarity,

Table 1. Physicochemical properties of grecoketides A (**1**) and B (**2**).

	1	**2**
Appearance	orange-red solid	orange-red solid
Molecular mass	476	476
Empirical formula	$C_{24}H_{28}O_{10}$	$C_{24}H_{28}O_{10}$
HR-FT-ICR-MS [m/z]:		
Found [M – H]$^-$	475.160876	475.160779
Calcd. [M – H]$^-$	475.160971	475.160971
$[\alpha]_D^{20}$ (c = 0.05 mg mL^{-1}, MeOH)	+18.0	–42.0
CD: λ_{max} [nm] (θ) (c = 0.25 mg mL^{-1}, MeOH)	238 (+655), 294 (–1396), 387 (+1143)	227 (+651), 240 (–176), 295 (+1268), 385 (–1194)
Melting temperature [°C]	>250	>250
UV (MeOH): λ_{max} [nm]	220 (sh), 245, 290, 425	220 (sh), 245, 290, 425
IR (KBr): $\tilde{\nu}_{max}$ [cm^{-1}]	3433, 2929, 1626, 1348, 1387, 1274, 1215, 1069, 1019	3437, 2929, 1628, 1440, 1388, 1274, 1209, 1076, 1013

Table 2. NMR spectroscopic data for grecoketides A (**1**) and B (**2**) in [D$_6$]DMSO (600/150 MHz).

Pos.	**1**		**2**	
	δ_C [ppm]	δ_H [ppm] (J [Hz])	δ_C [ppm]	δ_H [ppm] (J [Hz])
1	182.0		183.7	
2	163.6		169.3	
3	114.5		111.8	
4	187.8		185.3	
4a	114.9		114.9	
5	156.8	13.98 (br. s, 5-OH)	156.6	14.93 (br. s, 5-OH)
6	138.9		138.7	
7	130.0	7.61 (d, 7.9)	128.6	7.49 (d, 7.8)
8	118.0	7.44 (d, 7.9)	117.4	7.38 (d, 7.8)
8a	129.7		129.6	
9	28.6	3.34 (s)	28.91	3.28 (s)
10	172.2		173.1	
1′	64.1	4.90 (dd, 11.1, 2.1)	72.9	4.70 (dd, 11.0, 1.2)
2′	31.2	2.06 (eq., ddd, 13.0, 5.2, 3.2)	26.7	1.78 (eq., m)
		1.34 (ax., ddd,13.0, 11.1, 4.9)		1.53 (ax., m)
3′	24.9	1.86 (eq., m)	29.7	1.98 (eq., ddd, 13.5, 5.6, 2.8)
		1.83 (ax., m)		1.83 (ax., ddd, 13.5, 6.4, 2.7)
4′	71.4	3.84 (ddd, 10.9, 6.2, 5.4)	73.6	3.46 (br. s)
5′	69.7	4.32 (dd, 6.7, 6.2)	75.2	3.68 (qd, 6.4, 1.1)
6′	11.2	1.23 (d, 6.7)	17.8	1.16 (d, 6.4)
1′′	94.9	4.86 (br. s)	98.8	4.77 (br. s)
2′′	23.6	1.91 (eq., m)	23.6	1.48 (eq., m)
		1.30 (ax., m)		1.91 (ax., m)
3′′	25.5	1.86 (eq., m)	25.8	1.90 (eq., m)
		1.52 (ax., m)		1.57 (ax., m)
4′′	65.3	3.39 (br. s)	65.3	3.40 (br. s)
		3.17 (br. s, 4′′-OH)		4.45 (br. s, 4′′-OH)
5′′	66.5	3.81 (q, 6.9)	66.7	3.82 (qd, 6.6, 1.1)
6′′	17.3	1.03 (d, 6.9)	17.3	1.00 (d, 6.6)

only the structure elucidation of grecoketide B (**2**) and the difference in the structure of the sugar unit in grecoketide A (**1**) will be discussed in detail. All 1D and 2D NMR spectra were measured in two different solvents ([D$_6$]DMSO and CD$_3$OD; see Tables S1–S4 of the Supporting Information).

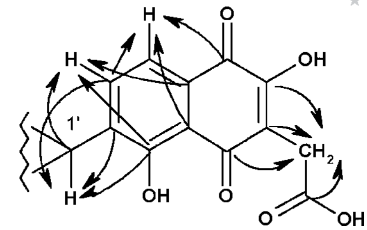

Figure 2. HMBC couplings in the aglycon part of **2**.

Structure Elucidation

The ^{13}C NMR spectra of **2** showed two methyl signals, and signals of five methylene groups, eight methine groups and nine quaternary carbon atoms. Twelve carbon atoms were assigned to the aglycon and twelve carbon atoms to two sugar units (Table 2).

The aglycon contains a naphthoquinone core with one enol unit and an attached acetate side-chain with juglone-like carbon chemical shifts. The naphthoquinone core shows three carbonyl/enol groups, one phenolic carbon atom, two aromatic methine groups and four additional quaternary carbon atoms. The acetate side-chain shows one methylene signal and one carboxy signal. According to the coupling pattern, the two aromatic protons are in an *ortho* position relative to each other. The acetate methylene group was observed as a singlet. The phenolic hydroxy signal is observed as a broad singlet.

HMBC analysis showed a juglone core with the two aromatic methine groups in the 7- and 8-positions, based on the crosspeak C-1/8-H. The acetate group is attached at C-3, as evidenced by the crosspeaks C-2/9-H$_2$, C-3/9-H$_2$, C-4/9-H$_2$ and C-10/9-H$_2$ (Figure 2). The aglycon structure (grecoketidone **5**, see Figure 7) is very similar to those of the known natural compounds gonioquinone (**3**) and hydroxylapachol (**4**).[5,6]

The constitution of sugar A was determined by COSY and HMBC spectra, which showed a 2,3,6-tridesoxyhexose, rhodinose or amicetose. This sugar is bound as a *C*-glycoside at the 6-position of the aglycon. Sugar A is identified as a β-sugar because of the proton–proton coupling constant (J = 11.0 Hz) of 1′-H, which is typical for an axial–axial coupling. Proton 4′-H shows no axial–axial coupling and is therefore in an equatorial orientation. In the ROESY NMR spectrum a crosspeak between 5′-H and 1′-H is visible and proves the axial position of 5′-H. Thus, sugar A in **2** is determined to be β-rhodinose and has the expected conformation (Figure 3). Comparison of the ^{1}H and ^{13}C NMR spectroscopic data of sugar A in **2** with that of the *C*-glycosidic-bound β-D-rhodinose in urdamycin R led to the assumption that sugar A in **2** is β-D-rhodinose.[7]

The constitution of sugar B was determined as 2,3,6-tridesoxyhexose (rhodinose or amicetose) from the couplings in the COSY and HMBC spectra. The protons 1′′-H and 4′′-H show no axial–axial coupling and must be equatorial. The proton–carbon coupling constant C-1′′/1′′-H was measured as J_{CH} = 169 Hz (coupled HSQC), which proves sugar B to be an α-sugar. ROESY coupling between 4′′-H and 6′′-CH$_3$ shows 6′′-CH$_3$ to be equatorial. Thus, sugar B is determined as α-rhodinose (Figure 3). The ^{1}H and ^{13}C NMR signals of sugar B in **2** are very similar to those of the terminal α-L-rhodinose of saquayamycin Z. Thus, sugar B in **2** should be the α-L-rhodinose.[8]

The positions of attachment of the three fragments, the aglycon and two sugars, were determined by HMBC correlations. The HMBC signals C-5/1′-H, C-6/1′-H, C-7/1′-H and C-1′/7-H confirmed the attachment of C-6 to C-1′ of sugar A. The HMBC crosspeaks C-1′′/4′-H and C-4′/1′′-H prove a (4–1) glycosidic binding between the two sugar units (Figure 4). The structure of grecoketide B is determined as **2**.

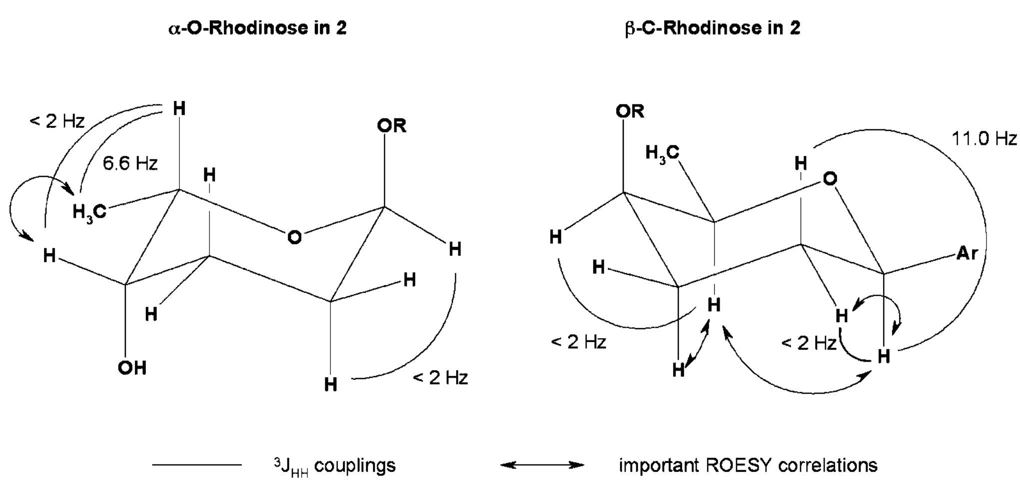

Figure 3. Structure elucidation of the rhodinoses in grecoketide B (**2**).

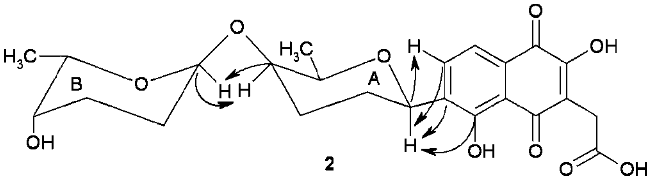

Figure 4. The linking of the aglycon and the two sugar units in grecoketide B (**2**), as determined by HMBC NMR couplings.

The structure of grecoketide A (**1**) is very similar to that of **2**. Both compounds have the same molecular formula, and the 1D and 2D NMR spectra confirmed that **1** and **2** have the same naphthoquinone aglycon and an identical sugar B (α-L-rhodinose), but differ in sugar A. Binding between the fragments in **1** is similar to that in **2**.

Grecoketides A (**1**) and B (**2**) show optical rotation values of $[\alpha]_D^{20} = +18$ for **1** and $[\alpha]_D^{20} = -42$ for **2**, which indicates that different sugar units are bound to the achiral aglycon. The CD spectra of **1** and **2** show curves with maxima at $\lambda \approx 240$, 295 and 385 nm but with opposite signs and support the assumption that the sugars attached directly to the chromophore are enantiomers (Figure 5).

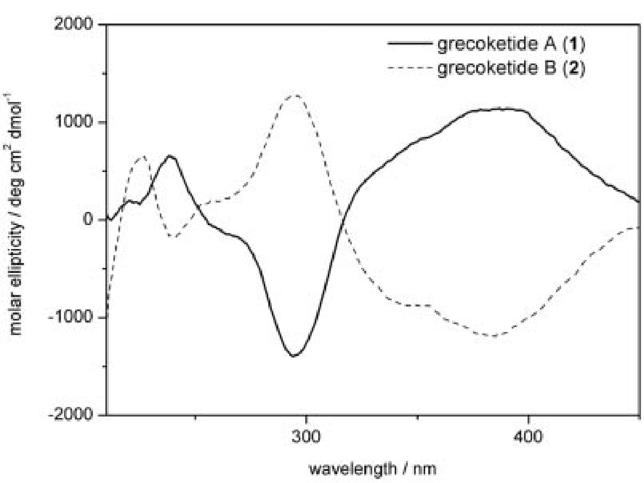

Figure 5. Overlay of CD spectra of grecoketides A (**1**) and B (**2**).

The constitution of sugar A in **1** was determined from COSY and HMBC spectra, which revealed a 2,3,6-tridesoxyhexose moiety (rhodinose or amicetose). The anomeric proton 1′-H has an axial–axial coupling (J = 11.1 Hz) that is typical of a β-orientation of the C–C bond. The coupling pattern of 4′-H (J = 10.9, 6.2 and 5.4 Hz) revealed an axial position of 4′-H. Two ROESY couplings, 6′-H_3/3′-H_{ax} and 6′-CH_3/1′-H (weak in [D_6]DMSO), prove 6′-CH_3 to be axial. This sugar could not be β-rhodinose as in **2** because of the difference in the NMR chemical shifts of this sugar in **1** compared with those of **2**. It has been assigned as α-rhodinose with a different chair structure so that the aglycon, as a large substituent, is in the equatorial position (Figure 6). A *C*-glycosidically bound α-L-rhodinose, which exhibits NMR spectroscopic data very similar to sugar A in **1**, has been reported for urdamycin S, which shows the same atypical conformation of α-L-rhodinose as grecoketide A (**1**).[7] Thus, both sugars in grecoketide A (**1**) should be α-L-rhodinose. The structure of grecoketide A is determined as **1**.

Biosynthesis

The juglone-like algycone of grecoketides A (**1**) and B (**2**) and grecoketidone (**5**) appears to be a hexaketide constructed along the polyketide pathway, and this was proved by feeding experiments with singly and uniformly labelled acetate as precursors. Due to a higher amount of grecoketide A (**1**), the conclusions drawn from biosynthesis experiments are based on data derived from labelled **1** after feeding with [1-^{13}C]acetate and [1,2-$^{13}C_2$]acetate. The addition of [^{2}H]hydrochloric acid led to better line shapes in the ^{13}C NMR spectra of grecoketides A (**1**) and B (**2**), necessary to determine the incorporation rates at low concentrations (Table 3).

Feeding of [1-^{13}C]acetate resulted in signal enhancements for **1** in positions C-2, C-4, C-5, C-7, C-8a and C-9 (Table 3). The biosynthesis of the polyketide chain was established through a feeding experiment with [1,2-$^{13}C_2$]acetate as the precursor, leading to six intact acetate units in the aglycon by strong coupling of the following pairs:

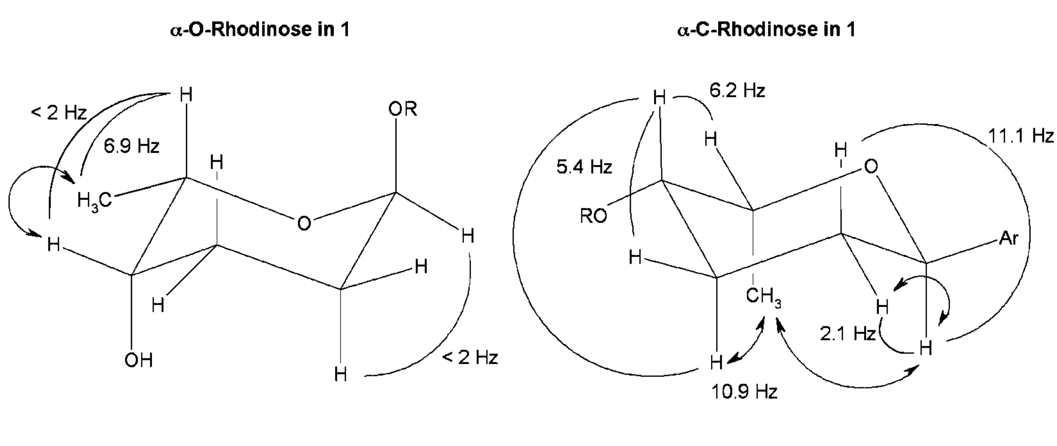

Figure 6. Structure elucidation of the rhodinoses in grecoketide A (**1**).

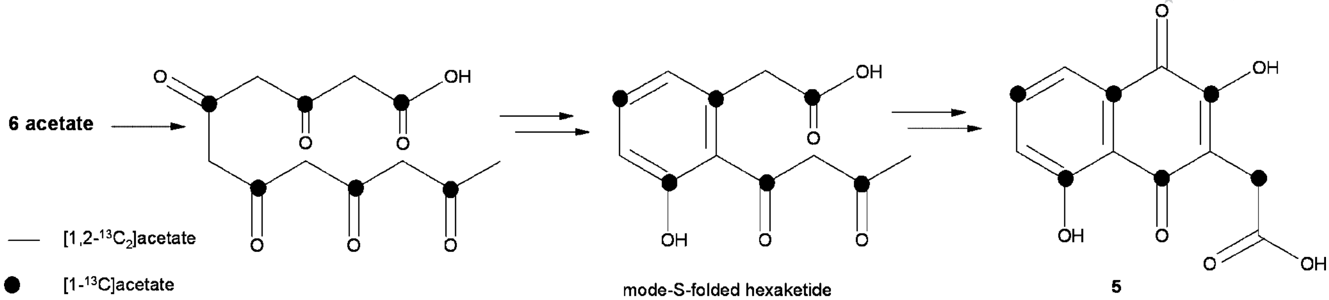

Figure 7. Biosynthesis of the carbon atoms in the grecoketidone (**5**).

Table 3. ^{13}C NMR signals of grecoketides A (**1**) and B (**2**) together with specific incorporations and coupling constants after feeding with [1-^{13}C]acetate (I) and [1,2-$^{13}C_2$]acetate (II).

Pos.	**1**			**2**		
	δ_C [ppm][a]	I[b,c]	II ($^1J_{CC}$/Hz)[c]	δ_C [ppm][a]	I[b,c]	II ($^1J_{CC}$/Hz)[c]
1	181.1	0	52	181.5	*	52
2	158.4	1.7	52	158.6	0.1	52
3	117.5	0.4	58	117.7	–0.1	58
4	191.6	1.4	58	192.0	1.8	58
4a	114.4	–0.2	65	114.6	0	64
5	158.6	2.0	65	159.7	0.8	64
6	140.9	–0.6	60	139.6	*	60
7	132.7	1.5	60	133.2	2.5	60
8	119.9	–0.2	61	119.9	–0.5	61
8a	129.9	1.2	61	127.9	2.7	61
9	28.8	6.2	59	28.8	1.4	59
10	172.8	–0.4	59	172.9	–0.3	59

[a] Chemical shifts in CD_3OD/DCl, 95:5. [b] Relative enrichments were normalized to the peak intensity of the C-1′ signal. [c] Relative enrichments/coupling constants based on ^{13}C NMR data obtained in CD_3OD/DCl, 95:5. * Signal missing in the labelled compound.

C-10/C-9, C-1/C-2, C-3/C-4, C-4a/C-5, C-6/C-7 and C-8/C-8a (named in the direction of the biosynthetic pathway beginning with the starter unit, Table 3). The starter unit C-10/C-9 is modified towards the end of the biosynthesis by reduction of acetate-C1 and oxidation of acetate-C2. All the carbon atoms in the two sugar units are unlabelled.

Finally, the biosynthesis of grecoketidone (**5**) occurs along a typical polyketide pathway by mode-S folding[9] (Figure 7).

Biological Activity

The grecoketides did not show growth inhibitory effects against Gram-positive and -negative bacteria, yeasts or filamentous fungi. No cytostatic effects were observed against various human tumour cell lines, for example, gastric adenocarcinoma, breast carcinoma and hepatocellular carcinoma, at a concentration of 10 μg mL^{-1}.

Conclusion

Grecoketides A (**1**) and B (**2**) are novel *C*-glycosylated naphthoquinones derived from *Streptomyces* sp. Acta 1362. The aglycon grecoketidone (**5**) is structurally very similar to the known naphthoquinones gonioquinone (**3**) and hydroxylapachol (**4**) with different side-chains at the 3-position of the naphthoquinone core.[5,6] At the 6-position of grecoketidone (**5**) a disaccharide is attached. This disaccharide differs for **1** and **2**. In grecoketide A (**1**), α-L-rhodinose is *C*-glycosidically bound at C-1′ of the first sugar, the second α-L-rhodinose is attached as an α-(1–4)-*O*-glycoside. The *C*-glycosidically bound α-L-rhodinose in **1** changes its conformation to bring the aglycon into the equatorial position. In grecoketide B (**2**), β-D-rhodinose instead of α-L-rhodinose was found to be the sugar A.

A similar situation has been reported for urdamycins R and S.[7] Urdamycins R and S are produced from the genetically modified strain *Streptomyces fradiae*. In urdamycin R the disaccharide β-D-rhodinose-α-L-rhodinose and in urdamycin S the disaccharide α-L-rhodinose-α-L-rhodinose are *C*-glycosidically bound to the angucyclinone. The linkage between the two rhodinoses is established as a (1–4)-*O*-glycoside link. Moreover, a change of conformation is reported for the *C*-glycosidically bound α-L-rhodinose in urdamycin S. NMR spectroscopic data for the disaccharides in urdamycins R and S are in very good accord with our data for grecoketides A (**1**) and B (**2**).

Grecoketidone (**5**) was generated through the polyketide pathway as expected. Feeding experiments with singly and fully ^{13}C-labelled acetate proved grecoketidone (**5**) to be a hexaketide constructed by mode-S folding.

The grecoketides showed no anti-infective or cytostatic activity in different test systems. The related naphthoquinones, gonioquinone (**3**) and hydroxylapachol (**4**), are produced from plants. Gonioquinone (**3**) was found by bioassay-guided isolation against mouse lymphocytic leukemia cells and hydroxylapachol (**4**) is reported to be cytotoxic to brine shrimp.[5,6]

Experimental Section

General: NMR spectra were recorded with a Varian VNMR-S 600 MHz spectrometer equipped with 3-mm triple-resonance inverse and 5-mm dual-broadband probe heads. Spectra were recorded in 150 μL of [D$_6$]DMSO or CD_3OD for structure elucidation and in 150 μL of CD_3OD/DCl (95:5) for biosynthetic studies (3-mm tubes). Solvent signals were used as internal standards ([D$_6$]-DMSO: δ_H = 2.50, δ_C = 39.5 ppm; CD_3OD: δ_H = 3.30, δ_C = 49.0 ppm). All spectra were recorded at T = 25 °C. Mass spectra were recorded with a Finnigan LCQ Deca mass spectrometer. ESI-

FT-ICR mass spectra were recorded with an Apex II FTICR mass spectrometer (4.7 T, Bruker Daltonics). Optical rotations were measured with a Perkin-Elmer 241 instrument. CD spectra were recorded with an Applied Photophysics Chirascan spectrometer equipped with a 0.2-cm cuvette. IR spectra were recorded with a Bruker Tensor 27 spectrometer.

Fermentation and Isolation: Strain Acta 1362 was cultivated in a 20-L fermentor equipped with a turbine impellor system by using a production medium (pH = 7.3) that consisted of oatmeal (20 g) and a trace solution (5 mL) in tap water (1 L). The fermentor was inoculated with shake flask cultures (5 vol-%) grown for 48 h in 500-mL Erlenmeyer flasks with one baffle on a rotary shaker at 120 rpm at 27 °C in a seed medium (pH = 7.0) that consisted of glucose (10 $g L^{-1}$), glycerol (10 $g L^{-1}$), oatmeal (5 $g L^{-1}$), soybean meal (10 $g L^{-1}$), yeast extract (5 $g L^{-1}$), Bacto casamino acids (5 $g L^{-1}$) and $CaCO_3$ (1 $g L^{-1}$) in tap water. The fermentation was carried out for 160 h at 27 °C at an aeration rate of 0.5 vvm and an agitation of 1000 rpm. Hyflo Super-cel (2%) was added to the culture broth, which was separated by multiple-sheet filtration into culture filtrate and mycelium. The culture filtrate (15 L) was adjusted to pH = 4 (1 M HCl) and extracted twice with EtOAc (each 3 L). The combined organic extracts were concentrated to dryness in vacuo. The crude product was dissolved in CH_2Cl_2 and added to a diol-modified silica gel column (45 × 2.6 cm, LiChroprep Diol, Merck). The separation was accomplished by a step gradient from CH_2Cl_2 to 1 and 2% MeOH, respectively, at a flow rate of 5 $mL min^{-1}$. Fractions containing **1** and **2** were purified by Sephadex LH-20 and Toyopearl TSK HW-40 chromatography (each column 90 × 2.5 cm) using MeOH as eluent. After concentration to dryness in vacuo, the grecoketides were obtained as red powders.

Feeding Experiments: Strain Acta 1362 was grown in a 1-L fermentor (Biostat S; B. Braun International, Germany) by using the same production medium. The fermentor was inoculated with shake flask cultures (10 vol-%) grown for 48 h in 500-mL Erlenmeyer flasks with one baffle on a rotary shaker at 120 rpm at 27 °C in the seed medium. The fermentation was carried out for 90 h at 27 °C with an aeration rate of 0.5 vvm and an agitation of 250 rpm. Sterile filtered solutions of 6.0 $mmol L^{-1}$ sodium [1-^{13}C]acetate and [1,2-^{13}C]acetate (each 99% ^{13}C atom purity), respectively, were fed to the cultures following the pulse feeding method in 5-mL portions at 40, 43, 46, 49, 52, 64, 67, 70, 73 and 76 h after incubation. The labelled grecoketides were isolated and purified by the same procedure as for grecoketide production, which resulted in 3.8 mg of singly labelled and 1.6 mg of doubly labelled **1**, and in 1.0 mg of singly labelled and 1.6 mg of doubly labelled **2**.

Supporting Information (see footnote on the first page of this article): 1D and 2D NMR spectroscopic data, ^{1}H and ^{13}C NMR, IR, UV, and CD spectra of grecoketides of **1** and **2**. ^{13}C NMR spectra of labeled **1**.

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