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Grecocyclines: New Angucyclines from *Streptomyces* sp. Acta 1362[‡]

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Two novel angucyclines were isolated from the streptomycete Acta 1362. The strain was of particular interest regarding the production of characteristic metabolites that were detected by HPLC–diode array profiling of the extracts. Grecocycline A and B were isolated and their structures were determined. Grecocycline A shows cytotoxic activity and grecocycline B inhibits protein tyrosine phosphatase 1B. Moreover, shunt product grecocyline C was isolated and its structure was determined.

Introduction

Strain Acta 1362 was isolated from soil of rhizosphere of *Pinus brutia* on the island of Crete and was assigned to the genus *Streptomycetes* on the basis of morphological and physiological properties and on the basis of partial sequencing of the 16S rRNA gene.[1,2] In the course of our HPLC–diode array screening program with the aim to detect new drugs for pharmacological applications (http://www.actapharm.org), strain Acta 1362 was found to be of special interest due to the presence of dominant peaks in extracts of the culture filtrate. Two of the metabolites were grecoketide A and B, which are C-glycosylated naphthoquinones with an attached disaccharide unit.[3] Three further metabolites were detected in the culture filtrate, having different retention times but quinone-related UV/Vis spectra. Two compounds, grecocycline A (**1**) and B (**2**), were determined as new members of the angucycline group. The grecocyclines have an attached disaccharide side chain and an additional amino sugar attached to the aglycon. Grecocycline B (**2**) shows a free SH group in the C-6a position (Figure 1).

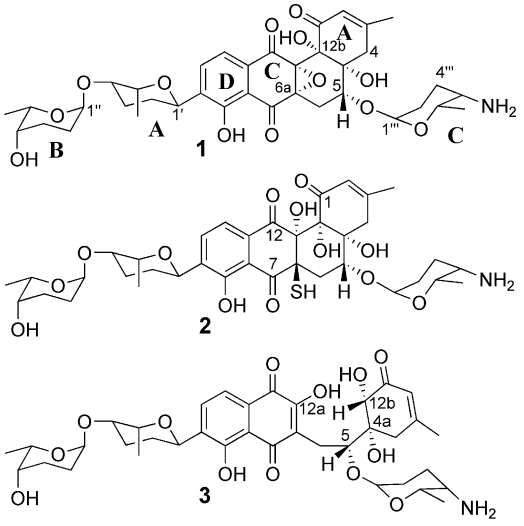

Figure 1. Structures of grecocycline A (**1**), B (**2**), and C (**3**).

The third metabolite grecocycline C (**3**) is a shunt product derived by ring opening of the B ring of the angucycline-type aglycon. Grecocycline B (**2**) exhibits a strong inhibitory activity against protein tyrosin phosphatase 1B with an IC_{50} value of 0.52 ± 0.17 μM. In addition, moderate cytotoxic and antimicrobial activities are also reported.

Results and Discussion

Batch fermentations of strain Acta 1362 were carried out in a 10-L fermentor by using a complex medium. The production of grecocycline A (**1**) and B (**2**) started after 20 h and reached a maximum value of 14.2 and 4.7 mg L^{-1} for **1** and **2**, respectively, at a fermentation time of 72 h. Grecocycline C (**3**; 2.2 mg) was isolated from the culture broth of a feeding experiment with fully labeled acetate for the investigation of grecoketide biosynthesis. After extraction

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Table 1. Physicochemical properties of grecocycline A (**1**), B (**2**), and C (**3**).

	1	**2**	**3**
Appearance	pale yellow	pale yellow	red
Molecular weight	713	747	715
Molecular formula	$C_{37}H_{47}NO_{13}$	$C_{37}H_{49}NO_{13}S$	$C_{37}H_{49}NO_{13}$
HRMS:[a] calcd.; found	714.3120; 714.3123 $[M + H]^+$	748.29973; 748.29974 $[M + H]^+$	716.32769; 716.32755 $[M + H]^+$
CD (MeOH):[b] λ [nm]	212 (–1847), 249 (+5181),	217 (9847), 243 (44626), 266 (–9113)	207 (–4334), 243 (1946), 266 (–4485)
θ [°]	328 (–1018), 380 (+347)	339 (6972), 382 (–2068)	304 (–2948), 374 (1140)
M.p. [°C]	187–189	201	181–183
UV (MeOH): λ [nm]	245, 290 (sh.), 370	236, 356	234, 273, 373
IR (KBr): $\tilde{\nu}$ [cm^{-1}]	3445, 2934, 1645, 1386, 1269, 1107, 1070, 1020	3436, 2930, 1661, 1436, 1384, 1264, 1111	3431, 2935, 1635, 1600, 1537, 1383, 1018

[a] **1**: HR from TOF system; **2** and **3**: HR from FTICR-MS. [b] **1** (c = 0.1 mg mL^{-1}), **2** and **3** (c = 0.25 mg mL^{-1}).

of the culture filtrate with ethyl acetate to separate the grecoketides, the aqueous residue was extracted with 1-butanol, and the grecocyclines were further purified by chromatography steps with the use of diol-modified silica gel and preparative reverse-phase HPLC.

LC-DAD-MS showed $[M + H]^+$ molecular ions at m/z = 714.4 for grecocycline A (**1**), m/z = 748.3 for B (**2**), and m/z = 716.4 for C (**3**) (Table 1). 1D NMR spectra indicate the grecocyclines to be members of the same natural product family. All grecocyclines have the same disaccharide unit as grecoketide A and showed the same amino sugar as a third sugar unit. Compounds **1** and **2** are members of the angucycline family, whereas **3** could be derived from an angucycline by opening of ring B.

Structure Elucidation

The high-resolution MS (ESI) of grecocycline B (**2**) led to the molecular formula $C_{37}H_{49}NO_{13}S$. The ^{13}C NMR spectrum of **2** showed 35 signals representing 37 carbon atoms. Combination of the ^{13}C NMR spectrum with HSQC indicates 4 methyl signals, 8 methylene signals, 13 methine signals, and 12 quaternary carbon atoms. Nineteen carbon atoms were assigned to an angucycline system, with eighteen carbon atoms to three sugar units.

The constitution of sugar B was determined as 2,3,6-tridesoxyhexose by use of COSY and HMBC couplings. The 1″-H and 4″-H protons show no axial–axial coupling and must be equatorial. ROESY coupling between 4″-H and 6″-CH_3 shows 6″-CH_3 to be equatorial. The ^1H and ^{13}C NMR signals are very similar to those of the terminal sugar α-L-rhodinose in grecoketide A.[3]

The constitution of sugar A was determined from COSY and HMBC spectra to be a 2,3,6-tridesoxyhexose. The anomeric 1′-H proton has an axial–axial coupling (J = 11.0 Hz) that is typical for a β-sugar. The 4′-H proton has an axial position shown by the coupling constants (J = 8.2 and 5.3 Hz), supported by ROESY correlations between 4′-H and 2′-H_{ax}. The ROESY couplings 6′-CH_3/3′-H_{ax} and 3-H_{ax}/1′-H prove 6′-CH_3 to be axial. The ^1H and ^{13}C NMR spectroscopic data of sugar A in **2** is quite similar to the NMR spectroscopic data of the α-L-rhodinose in the $^4C_1$ conformation that is directly attached to the aglycon in grecoketide A.[3]

A third sugar was found to be an amino sugar. The constitution was established as 2,3,4,6-tetradesoxy-4-aminohexose by COSY, HMBC, and ^1H–^{15}N HMBC. The 1‴-H and 4‴-H protons show no axial–axial coupling and are therefore in the equatorial position. The proton–carbon coupling constant C-1‴/1‴-H was measured as $^1J_{C,H}$ = 170 Hz (coupled HSQC), which proves that the sugar C is an α-sugar. Two ROESY couplings, 5‴-H/3‴-H_{ax} and 6‴-CH_3/4‴-H, prove that 6‴-CH_3 is equatorial and that 4‴-NH_2 is axial. The ^1H and ^{13}C NMR signals of sugar C in **2** are very similar to those reported for the amino sugar in BU-4514N, 4-epi-α-L-tolyposamine (Figure 2).[4]

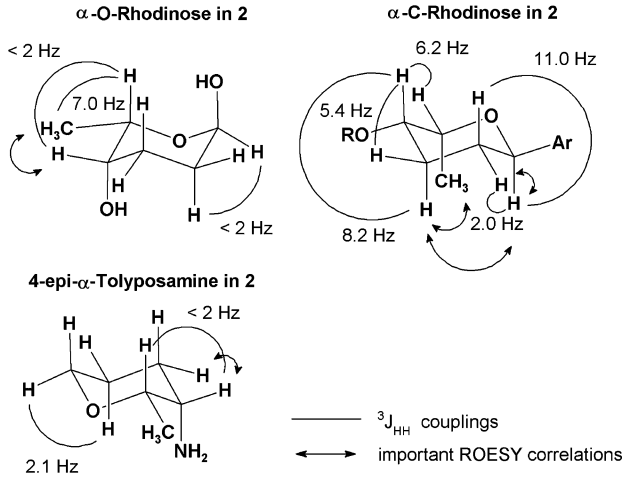

Figure 2. Structure elucidation of the sugars in grecocycline B (**2**).

The angucycline-type aglycon contains three carbonyl groups, one methylene group, four methine groups, and nine quaternary carbon atoms. Moreover, the aglycon is substituted with four hydroxy groups, one SH group, and one methyl group. Ring D of the angucycline shows two aromatic methine protons that are in the *ortho* position (J = 7.9 Hz in the ^1H NMR spectrum) to each other. HMBC analysis proves the substitution pattern of the aglycon. Ring D is substituted with a hydroxy group at the C-8 position and with sugar A at the C-9 position. The aromatic methine groups are at the C-10 and C-11 positions. Ring C is a quinone with ^{13}C NMR chemical shifts of δ_C = 190.2 (C-12) and 192.7 (C-7) ppm, which are typical for a

quinone-type ring without double bonds. Ring B contains an O–CH–CH_2-fragment in the C-5–C-6 position and the C-5-methine is indicated by the C-5/4-H_2 HMBC correlation. Ring A contains a carbonyl group in the C-1 position (δ_C = 196.9 ppm) and a methyl-substituted double-bond in the C2–C3–CH_3 position. The angucycline has hydroxy groups in the C-12a position and in the C-4a and C-12b angularic positions with typical carbon chemical shifts (78.8, 78.8, 78.1 ppm). The C-6a position is substituted with a SH group, indicated by its chemical shift δ_C = 62.8 ppm, which is high-field shifted relative to the chemical shift of hydroxy group substitution (Figure 3).

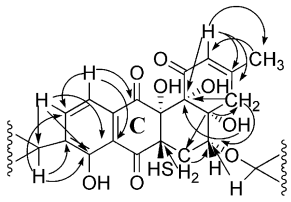

Figure 3. HMBC couplings in the aglycon of grecocycline B (**2**).

The linkage between the four fragments was determined from HMBC correlations. The HMBC signal C-5/1′′′-H confirmed the *O*-glycosidic attachment of C-1′′′ of 4-epi-tolyposamin to C-5 of the aglycon. The HMBC cross-peaks C-8/1′-H, C-9/1′-H, C-10/1′-H, and C-1′/10-H show a C-glycosidic binding of sugar A to C-9 of the aglycon. HMBC signals C-1′′/4′-H and C-4′/1′′-H prove the (4–1) glycosidic binding between sugars A and B (disaccharide unit). The structure of grecocycline B is determined as **2** (Figure 4).

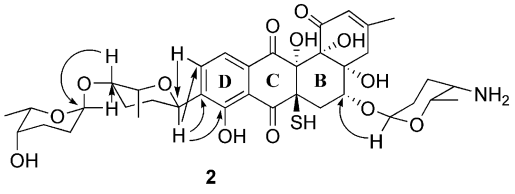

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Figure 4. The linking between the fragments in grecocycline B (**2**), as determined by HMBC NMR couplings.

The ROESY NMR spectrum shows a strong signal from 2-H to 5-H caused by the small distance of these protons. As these protons refer to ring A and ring B of the angucycline, this ROESY signal led to two possible configurations, (4a*R*,5*R*,6a*R*,12a*S*,12b*R*) or (4a*S*,5*S*,6a*S*,12a*R*,12b*S*). To determine the absolute configuration of the angucycline, theoretical CD spectra for both enantiomers (without sugars) were predicted by using quantum chemical calculations at the B3LYB level (Figure 5) and led to the (4a*R*,5*R*,6a*R*,12a*S*,12b*R*)-configuration of grecocycline B (**2**).

Nevertheless, the ROESY NMR spectrum of **2** shows couplings between the amino sugar and ring A of the angucycline system. Correlations 5′′′-H/4-CH_2, 5′′′-H/3-CH_3, and 6′′′-CH_3/3-CH_3 indicate that this sugar is close to ring A. Integration of the 2D ROESY peaks allow calculation of the proton distances. Comparison to distances from theoretical calculations for **2**, including 4-epi-α-D-tolyposamine or 4-epi-α-L-tolyposamine, show better agreement for the L-sugar (Table 2). This proves the amino sugar in grecocyline B (**2**) is 4-epi-α-L-tolyposamine.

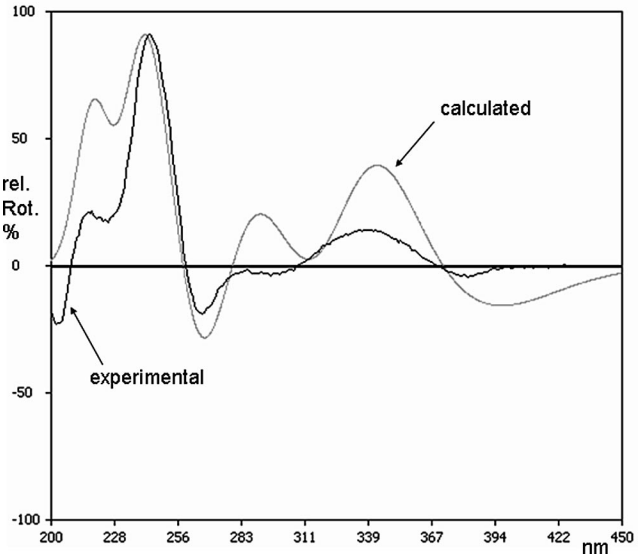

Figure 5. Comparison of the calculated CD (4a*R*,5*R*,6a*R*,12a*S*,12b*R*) and measured CD spectra of grecocycline B (**2**).

Table 2. Calculation of proton distances from the ROESY NMR spectrum of grecocycline B (**2**) and theoretical calculations for **2**, including 4-epi-α-D-tolyposamine and 4-epi-α-L-tolyposamine.

Protons	ROESY distances [Å]	Calcd. including 4-epi-α-D-tolyposamine [Å]	Calcd. including 4-epi-α-L-tolyposamine [Å]
3-CH_3/5′′′-H	3.0	3.9	4.3
3-CH_3/6′′′-CH_3	3.0	4.9	3.7
4-CH_2/5′′′-H	2.9	3.7	3.0
4-CH_2/4′′′-H	no signal	5.8	5.4
4-CH_2/6′′′-CH_3	no signal	4.1	4.1
5-H/1′′′-H	2.6	3.6	2.2
6-H_a/1′′′-H	2.6	2.2	2.7
6-H_b/1′′′-H	no signal	3.2	3.8
2-H/5-H	3.7	3.4	3.5
3-CH_3/5-H	4.5	3.9	3.8
4-CH_2/5-H	3.6	3.4	3.3

LC-DAD-MS showed $[M + H]^+$ molecular ions at *m*/*z* = 713.4 for grecocycline A (**1**) and *m*/*z* = 716.4 for C (**3**). The exact molecular masses were determined by high-resolution MS (ESI) and led to the molecular formula $C_{37}H_{47}NO_{13}$ for **1** and $C_{37}H_{49}NO_{13}$ for **3** (Table 1). 1D NMR spectra indicate the grecocyclines to be members of the same natural product family. All grecocyclines have the same disaccharide unit as grecoketide A and show the same amino sugar as the third sugar unit. Grecocycline A (**1**) and B (**2**) are members of the angucycline family; grecocycline C (**3**) could be derived from an angucycline by opening of ring B.

Grecocycline A (**1**) was unstable in terms of showing changes in the NMR spectra in [D_6]DMSO after a short period of time. We were not able to obtain good spectra from long experiment times. To overcome this problem we ran all NMR spectra in CD_3OD at *T* = –20 °C. Most NMR

signals are broad, and for this reason we did determine the structure on the basis of comparison to the NMR spectroscopic data of **2**, supported by 1D and 2D NMR spectra of **1**.

The structure of grecocycline A (**1**) is very similar to the structure of grecocycline B (**2**). 1D and 2D NMR spectra confirmed that both compounds have the same three sugar units attached at the same positions at the aglycon. The molecular formula $C_{37}H_{47}NO_{13}$ of **1** shows two protons and one sulfur less than the molecular formula of **2**, indicating changes in the structure of aglycon. The most important difference in the ^{13}C NMR spectroscopic data is the chemical shift of C-12a (δ_C = 66.3 ppm) in **1** in comparison to C-12a (δ_C = 78.8 ppm) in **2**. C-12a and C-6a (δ_C = 63.3 ppm) show typical carbon chemical shifts for epoxides in this position of angucyclines.

As in grecocycline B (**2**), the ROESY spectrum of grecocycline A (**1**) shows a strong signal from 2-H to 5-H, which led to four possible configurations of the aglycon: (4a*R*,5*R*,6a*S*,12a*R*,12b*R*), (4a*S*,5*S*,6a*R*,12a*S*,12b*S*), (4a*R*,5*R*,6a*R*,12a*S*,12b*R*), or (4a*S*,5*S*,6a*R*,12a*S*,12b*S*). Comparison of measured and calculated CD spectra (calculation without sugars) led to the (4a*R*,5*R*,6a*S*,12a*R*,12b*R*)-configuration of grecocycline A (**1**; Supporting Information, Figure S20). The structure of grecocycline A is determined as **1**.

Moreover, a third compound, named grecocycline C (**3**) was isolated from a feeding experiment with fully labeled acetate that was carried out to investigate the biosynthetic pathway of grecoketides.[3] An amount of 2.7 mg of this compound was isolated from only this fermentation. High-resolution mass spectrometry led to the molecular formula $C_{37}H_{49}NO_{13}$, which is two mass units higher than **1** and one sulfur less than **2**. The ^1H and ^{13}C NMR spectra show this compound to have high similarity to **1** and **2**. Because of the incorporation of uniformly labeled acetate, the carbon signals of the aglycon show carbon–carbon couplings that support the structure elucidated (Figure 1). Grecocycline C (**3**) contains the same three sugar units as **1** and **2** with one major difference in the aglycon. HMBC analysis gave 2-hydroxyjuglone and 3-methyl-5,6-dihydroxycyclohex-2-en-1-one as substructures of **3**. The 2-hydroxyjuglone part is C-glycosylated at C-6 and substituted with a CH_2-CH bridge where 4-epi-α-L-tolyposamine and 3-methyl-5,6-dihydroxycyclohex-2-en-1-one are attached at the methine group. ROESY analysis gave two possible configurations, (4a*R*,5*R*,12b*S*) or (4a*S*,5S,12b*R*), from cross-peaks 12b-H/4-H_{ax}, 12b-H/6-H_2, and 4-H_{eq}/5-H. Comparison of measured and calculated CD led to the (4a*R*,5*R*,12b*S*)-configuration of grecocycline C (**3**; Supporting Information, Figure S46). The structure of grecocycline C is determined as **3**.

Biological Activity

Grecocyclines A (**1**) and B (**2**) were tested against Gram-positive and Gram-negative bacteria. Compound **1** showed a weak growth inhibitory effect against *Ralstonia solanacearum* that causes Granville wilt on tobacco plants. Furthermore, **1** exhibited moderate cytotoxic activity against the mouse fibroblast cell line NIH-3T3 and the human cancer cell lines HepG2 and HT-29 (IC_{50} = 14.6 ± 1.0, 11.1 ± 0.9, and 20.2 ± 4.3 μM, respectively). In contrast, **2** showed growth inhibition activity that was about fivefold smaller. Interestingly, compound **2** inhibited protein tyrosin phosphatase B1 in an in vitro assay with an IC_{50} value of 0.52 ± 0.17 μM. The protein tyrosine phosphatase PTP1B is a promising target for the treatment of type 2 diabetes, obesity, and cancer.[5,6] The free SH group in the C-6a position is supposed to be responsible for the irreversible inhibition of PTP1B.

Conclusions

Grecocycline A (**1**) and B (**2**) are members of the angucycline group. Compound **1** contains an epoxide at the C-6a–C-12a position. Epoxides at this position are described for elmycin C and SF2315B, but in both compounds, C-12 is reduced to an alcohol functionality.[7,8] C-6a–C-12a epoxides in angucyclic anthraquinone systems are reported as intermediate products in the syntheses of angucyclines.[9,10] Grecocycline A (**1**) is the major product of *Streptomyces* sp. Acta 1362, but it is unstable. It is thinkable that the C-6a–C-12a epoxide in **1** is opened by attack of a sulfur compound to give grecocycline B (**2**); this assumption is supported by the elucidated absolute configurations of **1** and **2**.

Grecocycline B (**2**) is an angucycline with a thiol substitution at C-6a. Sulfur substitution at C-6a is reported for potent endothelin receptor antagonists WS009A and B (FR901366 and FR901367), where an *N*-acetylcysteine moiety is present at C-6a.[11,12] Moreover, the angucycline part of **2** only differs from WS009B in the hydroxylation pattern: in WS009B the C-6 position is hydroxylated, and in **2** the C-5 position bears the oxygen atom and sugar moieties are attached. The structures of WS009A and B do not contain any sugar units and the absolute configuration is not reported.[11]

The disaccharide unit in grecocyclines, α-L-rhodinose-α-L-rhodinose with C-glycosidic bound α-L-rhodinose in the $^4C_1$ conformation is reported for urdamycin S and grecoketide A.[3,13] Finally, the amino sugar 4-epi-α-L-tolyposamine was found to be present in BU-4514N.[14]

Grecocycline C (**3**) was isolated from a feeding experiment with fully labeled acetate and shows the typical incorporation pattern for an angucycline with intact rings. This led to the assumption that an opening of ring B of an unknown angucyclic precursor occurs to give grecocycline C (**3**).

Experimental Section

General: NMR spectra were measured with a Varian VNMR-S 600 MHz spectrometer equipped with 3 mm triple resonance inverse and 3 mm dual broadband probe heads. Spectra are recorded

in 150 μL [D$_6$]DMSO or 30 μL CD_3OD for structure elucidation (3 mm tube and 1.7 mm capillary). Solvent signals were used as internal standard ([D$_6$]DMSO: δ_H = 2.50 ppm, δ_C = 39.5 ppm. CD_3OD: δ_H = 3.30 ppm, δ_C = 49.0 ppm), δ_N values are taken from ^1H–^{15}N HMBC spectrum with spectrometer reference based on ^2H (lock) using Varians setref-macro. NMR spectra in [D$_6$]DMSO are recorded at T = 35 °C and in CD_3OD at T = –20 °C (lower temperature limit of our 3 mm inverse triple resonance probe).

ESI-FT-ICR mass spectra were performed with an Apex II FTICR mass spectrometer (4.7 T, Bruker Daltonics) and HR-ESI mass spectra with a Bruker micoTOF spectrometer. CD spectra were recorded with an Applied Photophysics Chirascan spectrometer equipped with 0.2 and 1.0 cm cuvettes. IR spectra were measured with a Bruker Tensor 27 spectrometer.

HPLC-DAD-ESI-MS analysis was done with an Agilent 1200 HPLC series equipped with a binary HPLC pump, autosampler and diode array detector, and an Agilent LC/MSD Ultra Trap System XCT 6330. The samples (2.5 μL) were injected onto an HPLC column (Nucleosil-100 C-18, 3 μm, 100 × 2 mm) and separated by 0.1% aqueous HCOOH and CH_3CN (linear gradient from 10 to 100% CH_3CN over 17 min, flow rate 400 μL min^{-1}).

Fermentation and Isolation: Strain Acta 1362 was cultivated in a 10-L stirred-tank fermentor (Biostat E, B. Braun) by using a production medium (pH 7.3) that consisted of oatmeal (20 g) and trace element solution (5 mL) in tap water (1 L). The fermentor was inoculated with 5 vol.-% of shake flask cultures, 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 (g L^{-1} tap water) glucose 10, glycerol 10, oatmeal 5, soybean meal 10, yeast extract 5, Bacto casamino acids 5, and $CaCO_3$ 1. The fermentation was carried out for 72 h at 27 °C with an aeration rate of 0.5 vvm and an agitation of 250 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 (7 L) was adjusted to pH 4 (1 M HCl) and extracted with EtOAc (2 × 1 L). The aqueous residue was extracted with 1-butanol (2 × 1.5 L). The combined butanol 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 (35 × 2.6 cm, LiChroprep Diol, E. Merck), the separation was accomplished by a step gradient from CH_2Cl_2 to 2, 5, 10, and 15% MeOH, respectively, at a flow rate of 8.5 mL min^{-1}. Fractions containing **1** and **2** were purified by preparative RP-HPLC (ReproSil Pur Basic, 10 μm, 250 × 20 mm, Maisch) with H_2O/CH_3CN (gradient from 30 to 70% CH_3CN over 20 min) at a flow rate of 24 mL min^{-1}. After concentration to dryness in vacuo, grecocyclines were obtained as pale-yellow powders.

Grecocycline A (1): ^1H NMR (600 MHz, [D$_4$]methanol, –20 °C): δ = 1.06 (d, J = 5.7 Hz, 3 H, 6‴-H$_3$), 1.15 (d, J = 6.2 Hz, 3 H, 6″-H$_3$), 1.29 (d, J = 6.4 Hz, 3 H, 6′-H$_3$), 1.29 (m, 1 H, 2′-H$_{ax}$), 1.43 (m, 1 H, 2″-H$_{ax}$), 1.67 (m, 1 H, 3″-H$_{ax}$), 1.68 (m, 1 H, 2‴-H$_{ax}$), 1.87 (m, 1 H, 3′-H$_{ax}$), 1.91 (m, 1 H, 2‴-H$_{eq}$), 1.92 (m, 1 H, 3‴-H$_{eq}$), 1.98 (m, 1 H, 3′-H$_{eq}$), 1.99 (m, 2 H, 2″-H$_{eq}$, 3″-H$_{eq}$), 2.01 (s, 3 H, 3-CH$_3$), 2.14 (m, 1 H, 2′-H$_{eq}$), 2.16 (m, 1 H, 6-H$_a$), 2.23 (m, 1 H, 3‴-H$_{ax}$), 2.87 (s, 2 H, 4-H$_2$), 3.31 (m, 1 H, 6-H$_b$), 3.38 (br. s, 1 H, 4‴-H), 3.54 (br. s, 1 H, 4″-H), 3.71 (t, J = 8.3 Hz, 1 H, 5-H), 3.79 (m, 1 H, 5‴-H), 3.90 (m, 1 H, 4′-H), 3.95 (q, J = 6.5 Hz, 1 H, 5″-H), 4.38 (m, 1 H, 5′-H), 4.91 (br. s, 1 H, 1″-H), 5.00 (d, J = 10.7 Hz, 1 H, 1′-H), 5.12 (br. s, 1 H, 1‴-H), 5.90 (s, 2-H), 7.67 (d, J = 7.7 Hz, 1 H, 11-H), 7.87 (d, J = 7.7 Hz, 1 H, 10-H) ppm. ^{13}C NMR (150 MHz, [D$_4$]methanol, –20 °C): δ = 11.1 (q, C-6′), 17.4 (q, C-6‴), 17.7 (q, C-6″), 22.8 (t, C-6), 23.1 (t, C-2‴), 23.7 (t, C-2‴), 24.0 (q, 3-CH$_3$), 24.8 (t, C-2″), 26.2 (t, C-3′), 26.5 (t, C-3″), 32.6 (t, C-2′), 38.1 (t, C-4), 49.7 (d, C-4‴), 63.3 (s, C-6a), 65.0 (d, C-5‴), 65.6 (d, C-1′), 66.2 (s, C-12a), 67.7 (d, C-4″), 67.8 (d, C-5″), 67.9 (d, C-5), 72.0 (d, C-5′), 73.6 (d, C-4′), 78.4 (s, C-4a), 78.7 (s, C-12b), 93.9 (d, C-1‴), 97.2 (d, C-1″), 113.8 (s, C-7a), 120.9 (d, C-11), 123.3 (d, C-2), 130.8 (s, C-11a), 135.1 (d, C-10), 140.8 (s, C-9), 159.6 (s, C-8), 162.1 (s, C-3), 190.6 (s, C-12), 196.8 (s, C-7), 199.3 (s, C-1) ppm. ^{15}N NMR (50 MHz, [D$_4$]methanol, –20 °C): δ = –353.1 (4‴-NH$_2$) ppm.

Grecocycline B (2): ^1H NMR (600 MHz, [D$_6$]DMSO, 35 °C): δ = 1.05 (d, J = 7.0 Hz, 3 H, 6″-H$_3$), 1.07 (d, J = 6.6 Hz, 3 H, 6‴-H$_3$), 1.24 (br. s, 3 H, 6′-H$_3$), 1.32 (dm, J_m = 11.8 Hz, 1 H, 2″-H$_{ax}$), 1.45 (m, 1 H, 2′-H$_{ax}$), 1.55 (m, 1 H, 3″-H$_{ax}$), 1.80 (m, 1 H, 3‴-H$_{eq}$), 1.85 (m, 2 H, 3′-H$_2$), 1.86 (m, 1 H, 2‴-H$_{ax}$), 1.89 (m, 1 H, 3″-H$_{eq}$), 1.92 (m, 1 H, 2″-H$_{eq}$), 1.97 (s, 3 H, 3-CH$_3$), 2.03 (m, 1 H, 2′-H$_{eq}$), 2.11 (ddd, J = 13.5, 10.7, 3.5 Hz, 1 H, 3‴-H$_{ax}$), 2.34 (dd, J = 13.1, 12.2 Hz, 1 H, 6-H$_a$), 2.75 (s, 2 H, 4-H$_2$), 3.26 (dd, J = 13.1, 3.5 Hz, 1 H, 6-H$_b$), 3.29 (br. s, 1 H, 4‴-H), 3.41 (br. s, 1 H, 4″-H), 3.84 (q, J = 7.0 Hz, 1 H, 5″-H), 3.86 (dd, J = 8.2, 5.6 Hz, 1 H, 4′-H), 4.00 (q, J = 7.0 Hz, 1 H, 5‴-H), 4.10 (dd, J = 11.4, 3.5 Hz, 1 H, 5-H), 4.33 (dq, J = 6.5, 6.2 Hz, 1 H, 5′-H), 4.40 (br. s, 1 H, 4″-OH), 4.86 (br. s, 1 H, 1″-H), 4.94 (dd, J = 11.0, 2.0 Hz, 1 H, 1′-H), 5.06 (d, J = 2.1 Hz, 1 H, 1‴-H), 5.59 (s, 1 H, 4a-OH), 5.86 (s, 1 H, 2-H), 6.62 (br. s, 1 H, not assigned), 7.44 (br. s, 1 H, not assigned), 7.52 (d, J = 7.9 Hz, 1 H, 11-H), 7.74 (d, J = 7.9 Hz, 1 H, 10-H), 7.92 (br. s, 1 H, not assigned), 10.9 (s, 1 H, 8-OH) ppm. ^{13}C NMR (150 MHz, [D$_6$]DMSO, 35 °C): δ = 11.3 (q, C-6′), 17.3 (q, C-6″, C-6‴), 21.6 (t, C-3‴), 23.0 (t, C-6, C-2‴), 23.3 (q, 3-CH$_3$), 23.7 (t, C-2″), 25.0 (t, C-3′), 25.5 (t, C-3″), 30.9 (t, C-2′), 35.8 (t, C-4), 48.1 (d, C-4‴), 63.8 (d, C-5‴), 64.1 (d, C-1′), 65.4 (d, C-4″), 66.5 (d, C-5″), 69.7 (d, C-5), 70.1 (d, C-5′), 71.7 (d, C-4′), 76.1 (s, C-12b), 78.8 (s, C-12a), 78.8 (s, C-4a), 82.8 (s, C-6a), 93.2 (d, C-1‴), 95.1 (d, C-1″), 116.5 (s, C-7a), 117.9 (d, C-11), 123.5 (d, C-2), 130.1 (s, C-11a), 131.4 (d, C-10), 137.6 (s, C-9), 156.2 (s, C-8), 157.9 (s, C-3), 190.2 (s, C-12), 196.6 (s, C-1), 197.7 (s, C-7) ppm. ^{15}N NMR (50 MHz, [D$_6$]DMSO, 35 °C): δ = –347.1 (4‴-NH$_2$) ppm.

Grecocycline C (3): ^1H NMR (600 MHz, [D$_6$]DMSO, –20 °C): δ = 0.96 (d, J = 6.8 Hz, 3 H, 3‴-H$_3$), 1.05 (d, J = 6.8 Hz, 3 H, 6″-H$_3$), 1.23 (d, J = 6.8 Hz, 3 H, 1′-H$_3$), 1.31 (m, 1 H, 3″-H$_{ax}$), 1.35 (m, 1 H, 3‴-H$_{ax}$), 1.37 (m, 1 H, 2′-H$_{ax}$), 1.54 (m, 1 H, 2″-H$_{ax}$), 1.65 (m, 1 H, 2‴-H$_{ax}$), 1.66 (m, 1 H, 3‴-H$_{eq}$), 1.83 (m, 2 H, 3′-H$_2$), 1.88 (m, 1 H, 2″-H$_{eq}$), 1.90 (m, 1 H, 2‴-H$_{eq}$), 1.91 (s, 3 H, 3-CH$_3$), 2.02 (m, 1 H, 2′-H$_{eq}$), 2.45 (d, J = 19.0 Hz, 1 H, 4-Ha), 2.61 (d, J = 19.0 Hz, 1 H, 4-H), 2.65 (d, J = 4.3 Hz, 2 H, 6-H$_2$), 3.11 (br. s, 1 H, 4‴-H), 3.40 (br. s, 1 H, 4″-H), 3.82 (m, 1 H, 5″-H), 3.84 (m, 1 H, 4′-H), 3.86 (d, J = 4.3 Hz, 1 H, 12b-H), 4.06 (t, J = 4.6 Hz, 1 H, 5-H), 4.11 (br. q, J = 6.8 Hz, 1 H, 5‴-H), 4.32 (dq, J = 6.8, 5.9 Hz, 1 H, 5′-H), 4.86 (br. s, 1 H, 1″-H), 4.88 (dd, J = 11.3, 2.0 Hz, 1 H, 1′-H), 5.05 (d, J = 2.0 Hz, 1 H, 1‴-H), 5.72 (d, J = 1.2 Hz, 1 H, 2-H), 7.25 (d, J = 7.9 Hz, 1 H, 11-H), 7.42 (d, J = 7.9 Hz, 1 H, 10-H), 15.53 (s, 1 H, 8-OH) ppm. ^{13}C NMR (150 MHz, [D$_6$]DMSO, 35 °C): δ = 11.2 (q, C-6′), 17.0 (q, C-6‴), 17.2 (q, C-6″), 21.5 (t, C-2‴), 22.9 (t, $J_{C,C}$ = 39 Hz, C-6), 22.9 (t, C-3‴), 23.6 (t, C-3″), 23.8 (q, $J_{C,C}$ = 40 Hz, 3-CH$_3$), 24.9 (t, C-3′), 25.5 (t, C-2″), 31.1 (t, C-2′), 38.7 (t, $J_{C,C}$ = 39 Hz, C-4), 48.2 (d, C-4‴), 63.0 (d, C-5‴), 64.1 (d, C-1′), 65.3 (d, C-4″), 66.4 (d, C-5″), 69.8 (d, C-5′), 71.6 (d, C-4′), 75.8 (d, $J_{C,C}$ = 44 Hz, C-12b), 77.4 (s, $J_{C,C}$ = 39 Hz, C-4a), 77.4 (s, $J_{C,C}$ = 39 Hz, C-5), 93.7 (d, C-1‴), 94.9 (d, C-1″), 115.5 (s, $J_{C,C}$ = 63 Hz, C-6a), 115.6 (s, $J_{C,C}$ = 64 Hz, C-7a), 116.7 (d, $J_{C,C}$ = 61 Hz, C-11), 122.8 (d, C-2), 127.8 (d, $J_{C,C}$ = 60 Hz, C-10), 130.0 (8s, $J_{C,C}$ = 61 Hz, C-11a), 137.8 (s, $J_{C,C}$ = 60 Hz, C-9), 158.6 (s, $J_{C,C}$ = 64 Hz, C-8), 158.8 (s, $J_{C,C}$ =

40 Hz, C-3), 172.5 (s, $J_{C,C}$ = 49 Hz, C-12a), 183.8 (s, $J_{C,C}$ = 63 Hz, C-7), 184.0 (s, $J_{C,C}$ = 49 Hz, C-12), 197.9 (s, $J_{C,C}$ = 44 Hz, C-1) ppm. ^{15}N NMR (50 MHz, [D$_6$]DMSO, 35 °C): δ = –348.0 (4‴-NH_2) ppm.

Other physicochemical properties of **1**, **2**, and **3** are given in Table 1.

Antimicrobial Assays: The antimicrobial assays were performed using *Bacillus subtilis* (DSM 347), *Staphylococcus epidermidis* (DSM 20044), *Staphylococcus lentus* (DSM 6672), *Erwinia amylovora* (DSM 50901), *Escherichia coli* K12 (DSM 498), *Pseudomonas fluorescens* (NCIMB 10586), *Propionibacterium acnes* (DSM 1897), *Pseudomonas aeruginosa* (DSM 50071), *Pseudomonas syringae pv. aptata* (DSM 50252), *Ralstonia solanacearum* (DSM 9544), *Xanthomonas campestris* (DSM 2405), and the *yeast Candida glabrata* (DSM 6425). The assays were prepared by transferring 50 μL of a 2 mM solution of the sample compounds dissolved in DMSO into one well of a 96-well microtiter plate and evaporating the solvent in a vacuum centrifuge. Overnight cultures of the test organisms in tryptic soy broth were diluted to an OD600 of 0.02–0.06, and 200 μL of the resulting suspension was added to the wells. After the microtiter plates were incubated for 14–16 h at 28 °C, 10 μL of a resazurin solution (0.2 mg mL-1 PBS) was added to each well and the plates were incubated at 28 °C for 30 min. To evaluate cell viability, the reduction of resazurin to resorufin was assessed by measuring the intensity of fluorescence at 560Ex/590Em nm. The resulting values were compared to a positive (50 mg chloramphenicol for bacteria; 50 mg nystatin for the yeast) and a negative control (no compound) on the same plate. *Propionibacterium acnes* was grown anaerobically (Anaerocult A mini, Merck, Darmstadt, Germany) in peptone-yeast extract-glucose (PYG)-medium (modified DSMZ-medium 104) at 37 °C for 24–48 h. The bacterial culture was diluted to an OD600 of 0.03, 200 μL of the inoculum was added to each well and the microtiter plate was incubated anaerobically at 37 °C for 48 h.

Cytotoxicity Assays: The sensitivity of the cell lines NIH-3T3, HepG2, and HT-29 to the isolated compounds was evaluated by monitoring the metabolic activity by using the CellTiter-Blue®Cell Viability Assay (Promega, Mannheim, Germany). The mouse fibroblast cell line was kindly provided by G. Rimbach (University of Kiel, Germany). The human hepatocellular carcinoma cell line HepG2 and the human colon adenocarcinom cell line HT-29 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). HepG2 and NIH-3T3 cells were maintained in RPMI 1640 medium, and HT-29 cells were cultured in Mc Coy's 5A medium. Media were supplemented with 10% fetal bovine serum (Promocell, Heidelberg, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The cultures were maintained at 37 °C under a humidified atmosphere and 5% CO_2. The cell lines were transferred every 3 or 4 d. For experimental procedures, cells were seeded in 96-well plates at concentrations of 7,500 cells (NIH-3T3) and 10,000 cells (HepG2 and HT-29) per well. After 24 h incubation, the medium was removed and 100 μL of the test sample adjusted to final concentrations of 10 μM by diluting in growth medium were added to the cells. Each sample was prepared in triplicate. Tamoxifen, as a standard therapeutic drug was used as a positive control. Following compound addition, plates were cultured for 24 h at 37 °C. Afterwards, the assay was performed according to the instructions of the manufacturer. Cells were incubated for 2 h at 37 °C. Fluorescence was measured by using the microplate reader Infinite® M200 (Tecan, Männedorf, Switzerland) at excitation 560 nm and emission 590 nm.

PTP1B Enzyme Activity Assay: Putative inhibitory activities against human recombinant protein tyrosin phosphatase N1 (PTP1B) were tested using the Biomol Green™ PTP1B tyrosin phosphatase drug discovery kit (catalogue number AK822-0001, Biomol, Hamburg, Germany). IC_{50} values were calculated by measuring the enzymatic rate at different inhibitor concentrations and plotting the resulting values vs. inhibitor concentration. Each data point was converted to percent inhibition with respect to positive and negative controls and represents duplicates (mean ± SD) from two independent experiments. The resulting IC_{50} values were calculated using the GraphPad Prism 4 software.

Calculation of CD Spectra and Molecule Geometry: The geometry optimization of grecocyclines was carried out at the DFT/B3LYP/6-31G* level in vacuo by Gaussian03 software.[15] All the UV/CD computations have been carried out by Gaussian03 software employing the TDDFT approach, the B3LYP functional, and the TZVP basis set. Grecocycline structures are used without sugars for calculations of CD spectra and with sugars for calculation of atomic distances. Overlay of measured and calculated CD spectra are done with SpecDis 1.42.[16] Distance measurements from calculations are done with Chemcraft Lite.[17]

Calculation of Atomic Distances: Calculation of atomic distances from 2D ROESY spectra were done by integrating the 2D peaks with Varian VNMRJ2.2C peak picking routine by using the following equation:[18]

$r_{AB} = [(r_{10\text{-}H/11\text{-}H})^6 \times Int_{10\text{-}H/11\text{-}H}/Int_{AB}]^{-6}$; with r_{AB} = distance between proton A and B, Int_{AB} = Integral over 2D ROESY peak between signals of proton A and B, $r_{10\text{-}H/11\text{-}H}$ = 0.25 nm (distance 10-H/11-H, as reference), $Int_{10\text{-}H/11\text{-}H}$ = Integral over 2D ROESY peak 10-H/11-H (reference signal).

Supporting Information (see footnote on the first page of this article): 1D and 2D NMR spectroscopic data and spectra; UV and IR spectra; quantum chemical calculations of CD spectra and atomic distances.

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