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ORIGINAL ARTICLE

Langkocyclines: novel angucycline antibiotics from *Streptomyces* sp. Acta 3034*

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Langkocyclines A1–A3 and B1 and B2, five new angucycline antibiotics produced by *Streptomyces* sp. Acta 3034, were detected in the course of our HPLC-diode array screening. The producing strain was isolated from the rhizospheric soil of a *Clitorea* sp. collected from Burau Bay, Langkawi, Malaysia, and was characterized by morphological, physiological and chemotaxonomic features in addition to 16S ribosomal RNA gene sequence information. Strain Acta 3034 is closely related to *Streptomyces psammoticus* NBRC 13971^T and *Streptomyces lanatus* NBRC 12787^T. Langkocyclines consist of an angular tetracyclic benz[*a*]anthracene skeleton and hydrolyzable *O*-glycosidic sugar moieties. The yellow-colored A-type langkocyclines differ in their aglycon from the blue-lilac-colored B-type langkocyclines. The A-type langkocycline aglycon is identical to that of aquayamycin and urdamycin A. The chemical structures of the langkocyclines were elucidated by HR-MS, 1D and 2D NMR experiments. They are biologically active against Gram-positive bacteria and exhibit a moderate antiproliferative activity against various human tumor cell lines.
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INTRODUCTION

A set of about 100 actinomycete strains was isolated from pristine Malaysian ecological niches with the aim of detecting novel secondary metabolites for pharmaceutical applications. The strains isolated from tropical rhizospheric and mangrove soils were found to be a rich source of secondary metabolites—one-third of the isolates were identified by our HPLC-diode array technique[2] as being producers of secondary metabolites. Besides known members of aromatic polyketides, oligolactones, macrolactones, polyen macrolides, macrolactams, phenazines and pyrrole antibiotics, some novel secondary metabolites were detected and characterized, such as gombapyrones produced by *Streptomyces griseoruber* Acta 3662,[3] langkolide from *Streptomyces* sp. Acta 3062,[4] nocardichelins from *Nocardia* sp. Acta 3026[5] and phenelfamycins from *Streptomyces albospinus* Acta 3619.[6] Strain Acta 3034 gained our special interest because of the presence of several peaks in the HPLC chromatogram of the culture filtrate extract, as shown in Figure 1. The comparison of their UV-Vis spectra and retention times with about 1000 reference compounds stored in our HPLC-UV-Vis database indicated an affiliation to the group of angucycline antibiotics, because of the similarity of their characteristic UV-Vis spectra to those of urdamycin A.[7] Fermentation, isolation and structure determination revealed new angucycline antibiotics of the urdamycin A-type with an altered and unusual glycosylation pattern. The new angucycline antibiotics were named langkocycline A1 (**1**), A2 (**2**) and A3 (**3**) and langkocycline B1 (**4**) and B2 (**5**), with reference to the collection site of the producing strain, the island Langkawi in the north-west of Malaysia. The classification and fermentation of the producing strain, as well as isolation, structural elucidation and biological activities of langkocyclines, are reported in this publication. The structures of langkocyclines **1–5** are shown in Figure 2.

RESULTS

Taxonomy of the producing strain

Strain Acta 3034 isolated from rhizospheric soil collected from the fine roots of *Clitorea* sp., a creeper, grew on a wide range of media, and its cultural characteristics are given in Table 1. Growth and sporulation of the aerial mycelium was observed on all examined agar media, except on ISP 2 and ISP 6. The scanning electron micrographs revealed that the aerial mycelium on ISP 4 agar medium produced 3–5 turns of tight spiral spore chains, and the spore surface showed a spiny ornamentation (Figure 3). Whole-cell hydrolysates of strain Acta 3034 showed the presence of LL-diaminopimelic acid in the peptidoglycan. The strain grew well in a temperature range of 27–35 °C and tolerated 1–2% NaCl concentrations (Table 2). Raffinose, arabinose, sucrose, lactose and inositol supported good growth

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of strain Acta 3034 compared with glucose, on which no aerial mycelium was formed. Further, xylose, rhamnose and sorbitol did not support the growth of the strain.

On the basis of 16S rRNA gene sequence data, strain Acta 3034 is closely related to *Streptomyces psammoticus* NBRC 13971^T and *Streptomyces lanatus* NBRC 12787^T, both having identical values of 98.7%. Phylogenetic analysis showed that strain Acta 3034 forms a distinct phyletic line in the *S. psammoticus* clade (Figure 4). However, the results of the comparison of the morphological characteristics and phenotypic properties of strain Acta 3034 with those of *S. psammoticus* (Table 3)[8] did not concur with the molecular information. Therefore, further investigations using DNA-DNA hybridizations are necessary to confirm a taxonomic novelty of strain Acta 3034.

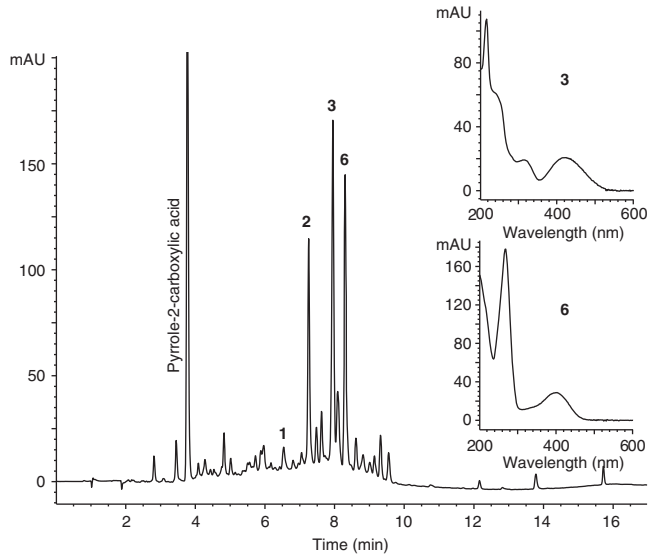

Figure 1 HPLC analysis of the culture filtrate extract from *Streptomyces* sp. Acta 3034 at a fermentation time of 70 h, monitored at $\lambda = 230$ nm. Inserts: UV-Vis spectrum of the main compound langkocycline A3 (**3**; **1** and **2** are nearly congruent) and of tetrangomycin (**6**).

Screening, fermentation and isolation

Freshly isolated actinomycetes strains were grown in 500-ml Erlenmeyer flasks as submerged cultures in various complex media,[9] and extracts were prepared from mycelia and culture filtrates at various fermentation times. The extracts were analyzed by HPLC-diode array monitoring in combination with our in-house HPLC-UV-Vis database.[2] Strain Acta 3034 was of interest because of the presence of various peaks in the culture filtrate extract (Figure 1); one of them was identified as pyrrole-2-carboxylic acid, a common and

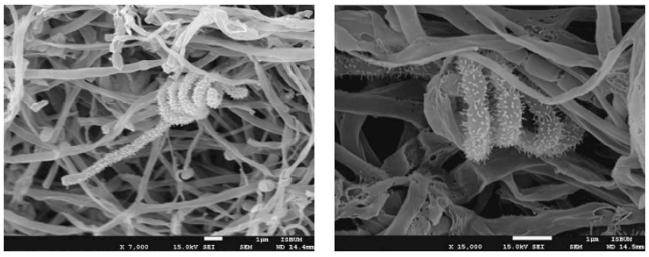

Figure 3 Micrographs of the aerial mycelium of strain Acta 3034 (grown on ISP 4 agar medium) with 3–4 turns of spiny ornamented spore chains classified in *spira* and *rectiflexibiles*; scale bar: 1 mm.

Table 1 Cultural characteristics of strain Acta 3034. Cultures were grown at 28 ± 2 °C for 14 d on various ISP agar media

Agar medium	*Growth*	*Aerial mycelium*	*Substrate mycelium*	*Soluble pigment*
Yeast extract–malt extract (ISP 2)	poor	—	yolk-yellow	—
Oat meal (ISP 3)	very good	white	brown	dark brown
Inorganic salts–starch (ISP 4)	very good	white	white	none
Glycerol–asparagine (ISP 5)	moderate	white	yellowish-brown	light brown
ISP 6	no growth	—	—	—
ISP 7	moderate	white	yolk-yellow	light brown

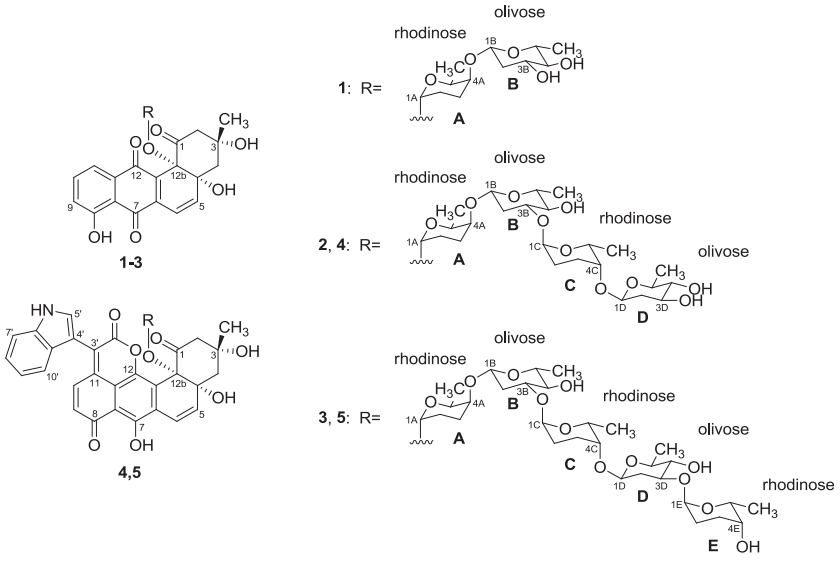

Figure 2 Structures of langkocycline A1 (**1**), A2 (**2**), A3 (**3**d), B1 (**4**) and B2 (**5**).

widespread metabolite of streptomycetes.[10] Three peaks with retention times of 6.6 min (**1**), 7.3 min (**2**) and 7.9 min (**3**) were assigned to urdamycin A-type antibiotics because of their nearly identical UV-Vis spectra. Another peak with retention time 8.3 min (**6**) was identified after preparative isolation by ESI-MS analysis as the angucyclinone antibiotic tetrangomycin having a molecular mass of 322 Da. Tetrangomycin was first described by Dann *et al.*[11] and was produced by a mutant strain of *Streptomyces rimosus*.

Table 2 Phenotypic properties of strain Acta 3034

	Growth[a]
pH	
4	± (no aerial mycelium)
5	+ (no aerial mycelium)
6	+ +
7	+ +
8	+ +
9	+
Temperature	
4 °C	–
14 °C	± (no aerial mycelium)
27 °C	+ +
35 °C	+ +
45 °C	–
Salinity	Growth[a]
1%	+ +
3%	± (no aerial mycelium)
4%	± (very poor growth)
5%	–
Other physiological properties[b]	
Casein degradation	+
Starch degradation	+
Coagulation of milk	–
Liquefaction of gelatine	+
Nitrate reduction	–

[a] + +, good growth; +, moderate growth; ±,poor growth; –, no growth
[b] +, positive reaction; –, negative reaction

Strain Acta 3034 was cultured in a 10-l fermentor and maximal growth was reached at 70 h with a biomass of 9 vol-% that corresponded to a maximal concentration of langkocyclines A1–A3 (**1–3**) in the culture filtrate, reaching amounts of 3 mg l^{-1} for compound **1**, 17 mg l^{-1} for compound **2** and 30 mg l^{-1} for compound **3**. The minor congeners **4** and **5** were not quantified in the culture filtrate because of their occurrence in lesser amounts. Compounds **1–5** were isolated from the culture filtrate by separation on an Amberlite XAD-16 column and were purified by subsequent chromatography steps on silica gel 60, Sephadex LH-20 and Toyopearl HW-40F columns. Langkocyclines A1–A3 (**1–3**) were obtained as yellow powders after lyophilization in amounts of 8 mg for **1**, 58 mg for **2** and 21 mg for **3**, respectively. In the course of the purification procedure, two blue-lilac-colored elution bands were seen during chromatography on Sephadex LH-20 and Toyopearl HW-40F, respectively, showing a UV-Vis spectrum similar to that of urdamycin D. These minor congeners, langkocyclines B1 (**4**) and B2 (**5**), were obtained in amounts of 1.4 and 2.1 mg, respectively.

Structure elucidation

The physicochemical properties of langkocyclines **1–5** are summarized in Table 4. The molecular masses were determined by high-resolution Orbitrap-ESI-MS establishing the molecular formulae $C_{31}H_{36}O_{12}$ (**1**), $C_{43}H_{56}O_{17}$ (**2**), $C_{49}H_{66}O_{19}$ (**3**), $C_{53}H_{61}O_{18}N$ (**4**) and $C_{59}H_{71}O_{20}N$ (**5**). The chemical structures of **1–5** as shown in Figure 2 were elucidated by extensive 1D and 2D NMR (Supplementary Tables 1–4, Supplementary Figures 1–13) analyses exemplarily described for derivative **3** as follows. The ^{1}H-NMR spectrum of **3** (Supplementary Figures 1-3) showed 16 signals in the high-field aliphatic region, 17 signals in the oxygenated aliphatic region and five signals in the olefinic region (Table 5). Five signals were characteristic of a hydroxy group. Detailed analysis of the ^{13}C-NMR data including the APT-NMR spectrum (Supplementary Figure 4) and the HSQC-NMR spectrum (Supplementary Figure 7) revealed the presence of six methyl carbons, 10 methylenes, five aromatic/olefinic methines, 17 oxygenated methines (including five anomeric carbons at δ_C

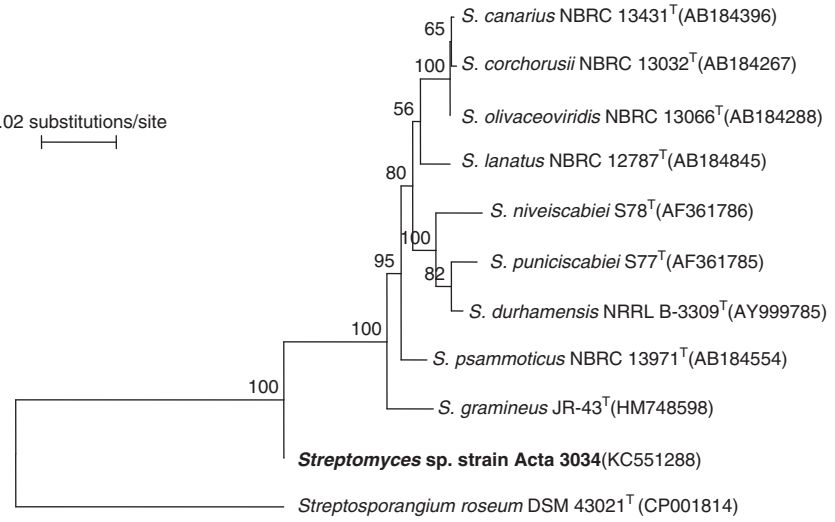

Figure 4 Neighbor-joining tree based on partial 16S rRNA gene sequences (at least 1330 nucleotides) showing relationships between strain Acta 3034 and representatives of closely related *Streptomyces* species. *Streptosporangium roseum* DSM 43021^{T} (CP001814) was used as the outgroup. The numbers at the nodes indicate the level of bootstrap support (%) based on the analysis of 1000 resampled data sets; the bar represents substitutions per nucleotide position.

94.8, 97.7, 98.0, 101.7 and 101.9, representing five sugar moieties) and 11 quaternary carbons. HSQC-NMR data established all $^{1}J^{1}H$-^{13}C connectivities (Table 5). The ^{1}H-^{1}H-COSY spectrum (Supplementary Figure 5) revealed seven spin systems for **3** (H-5-H-6, H-9-H-10-H-11, H-1A-H-2A-H-3A-H-4A-H-5A-H-6A, H-1B-H-2B-H-3B-H-4B-H-5B-H-6B, H-1C-H-2C-H-3C-H-4C-H-5C-H-6C, H-1D-H-2D-H-3D-H-4D-H-5D-H-6D and H-1E-H-2E-H-3E-H-4E-H-5E-H-6E) (Figure 5).

The ^{1}H-^{13}C long-range couplings from H-2 (δ_H 2.66, 2.62) to C-1 (δ_C 201.7), C-3 (δ_C 73.8), C-4 (δ_C 46.3), C-12b (δ_C 80.6), C-13 (δ_C 28.8), from H-4_{eq} (δ_H 2.13) to C-4a (δ_C 77.9), C-5 (δ_C 147.0), C-12b (δ_C 80.6), from H-5 (δ_H 6.40) to C-6 (δ_C 116.8), C-6a (δ_C 137.9), C-12a (δ_C 139.3), C-12b (δ_C 80.6), from H-6 (δ_H 6.86) to C-7 (δ_C 188.6), from H-9 (δ_H 7.29) to C-7 (δ_C 188.6), C-7a (δ_C 114.9), C-8 (δ_C 162.2), C-11 (δ_C 120.4), from H-10 (δ_H 7.68) to C-8 (δ_C 162.2), C-11a (δ_C 132.4) and from H-11 (δ_H 7.63) to C-7a (δ_C 114.9), C-9 (δ_C 125.0), C-12 (δ_C 183.3) gave rise to an angucycline backbone. The chemical shifts assigned to the aglycon were in good accordance with the data given in the literature.[12]

The constitutions of the sugars were determined from COSY and HMBC NMR spectra (Supplementary Figures 5 and 8) and revealed three 2,3,6-tridesoxyhexose moieties and two 2,6-didesoxyhexose moieties. The five glycosidic linkages were established on the basis of the HMBC correlations from H-1A to C-12b, from H-1B to C-4A (from H-4A to C-1B), from H-1C to C-3B (from H-3B to C-1C), from H-1D to C-4C (from H-4C to C-1D) and from H-1E to C-3D (from H-3D to C-1E), resulting in the structure of **3** (Figure 5).

As the hydrogens at position 1 and 4 of the 2,3,6-tridesoxyhexose sugars showed broad singlets in the ^{1}H NMR spectrum it was not possible to determine the exact coupling constants of these protons. Because of the peak-width-at-half-heights of hydrogen 1 (6-7 Hz) and hydrogen 4 (~7 Hz) of the 2,3,6-tridesoxyhexose sugars, an axial–axial coupling in sugars A, C and E was excluded. Comparison of the ^{1}H and ^{13}C NMR data from the literature,[13] together with the presence of the NOESY correlation between H-$3A_{ax}$ and H-5A (Figure 6), led to the assumption that the 2,3,6-tridesoxyhexose sugars are α-rhodinoses. With regard to the coupling constants $J_{1B,2Beq} = 1.8$ Hz ($J_{1D,2Deq} = 1.8$ Hz), $J_{1B,2Bax} = 9.9$ Hz ($J_{1D,2Dax} = 9.8$ Hz), $J_{3B,4B} = 8.7$ Hz ($J_{3D,4D} \sim 8.7$ Hz) and $J_{4B,5B} = 8.7$ Hz ($J_{4D,5D} \sim 8.7$ Hz), the presence of three axial–axial couplings in sugars B and D was confirmed (Figure 6). Considering these results, along with the results of the comparison of the ^{1}H and ^{13}C NMR data in the literature,[7] the 2,6-didesoxyhexose sugars were assigned as β-olivoses. On the basis of Klyne's rule,[14,15] which gives an estimate for the assignment of α-configuration to L-sugars and that of β-configuration to D-sugars, we suggest α-L-rhodinoses and β-D-olivoses to be present in the herein reported langkocyclines. Compound **2** differs from compound **3** in terms of the absence of sugar E, and compound **1** is missing sugars D and E. Compared with **3,** compound **5** has an additional indole-based moiety in its angucycline backbone (Table 6), which is comparable to that of urdamycin D.[16] Similar to **1**,

Table 3 Comparison of phenotypic properties of strain Acta 3034 with those of *S. psammoticus*[8]

	Acta 3034	*S. psammoticus*
Spore-chain morphology	3-4 turns of spiral spore chains	*Rectiflexibiles*
Spore-surface ornamentation (shape)	cylindrical spores with spines on surface	smooth cylindrical shaped spores
Color of aerial mycelium (media)	white (ISP 4)	beige-green (ISP 4)
Color of substrate mycelium	white (ISP 4)	beige-green (ISP4)
Carbon source utilization		
L-Arabinose	+	±
D-Glucose	+	+
meso-Inositol	+	−
Raffinose	+	−
L-Rhamnose	−	−
Sucrose	+	+
D-Xylose	−	−
Salinity tolerance (%)	4	5
pH for growth	4	NA
ISP 6	−	−
ISP 7	−	−

NA, not available; +, positive growth; −, negative growth

Table 4 Physicochemical properties of langkocycline A1 (1), A2 (2), A3 (3), B1 (4) and B2 (5)

	***1** (A1)*	***2** (A2)*	***3** (A3)*	***4** (B1)*	***5** (B2)*
Appearance	yellow powder	yellow powder	yellow powder	blue-lilac powder	blue-lilac powder
Molecular mass	600.2	844.3	958.4	999.4	1113.5
Molecular formula	$C_{31}H_{36}O_{12}$	$C_{43}H_{56}O_{17}$	$C_{49}H_{66}O_{19}$	$C_{53}H_{61}O_{18}N$	$C_{59}H_{71}O_{20}N$
HR-Orbitrap-ESI-MS [*m/z*]:					
Found $[M-H]^-$	599.21201	843.34222	957.41075	998.38175	1112.44816
Calcd. $[M-H]^-$	599.21340	843.34447	957.41255	998.38159	1112.44967
Δ p.p.m.	−0.489	−1.371	−0.737	1.262	−0.368
UV (MeOH): δ_{max} [nm]	422, 313	422, 313	422, 313	562, 318	562, 318
$[\alpha]_D^{23}$ (MeOH): (*c*, (mg ml^{-1}))	+95.9 (c, 0.8)	+38.0 (c, 1.0)	+1.8 (c, 0.65)	+251.3 (c, 2.5)	+353.5 (c, 2.8)
CD (MeOH): δ_{max} (nm) (θ) ($c = 0.2$ mg ml^{-1})	459 (+0,9); 400 (−2,1); 325 (+7,7); 278 (+0,2); 244 (+7,0)	459 (+0,9); 400 (−2,0); 326 (+7,6); 278 (+0,3); 244 (+7,0)	457 (+1,3); 400 (−3,0); 324 (+11,5); 277 (+0,4); 243 (+10,6)	440 (+0,1); 400 (−0,1); 355 (+1,5); 321 (−2,6); 296 (−1,2); 284 (−3,0); 230 (+12,2)	441 (+0,2); 400 (−0,3); 355 (+1,2); 320 (−1,7); 296 (−0,7); 284 (−1,6); 257 (+3,9); 246 (+2,6); 230 (+9,4)

Table 5 ^{1}H-, ^{13}C-, HMBC- and COSY signals of 3 measured in CD_2Cl_2-d_2

Position	δ (^{1}H) (p.p.m.) J in Hz	δ (^{13}C) (p.p.m.)	HMBC	COSY
1	—	201.7	—	—
2_{eq}	2.66 (1H, dd, 11.9, 2.3)	54.9	1, 3, 4, 12b, 13	4_{eq}
2_{ax}	2.62 (1H, d, 11.9)	54.9	1, 3, 4, 12b, 13	13
3	—	73.8	—	—
3-OH	1.76 (1H, s)	—	2, 3, 4, 13	—
4_{eq}	2.13 (1H, dd, 14.6, 2.3)	46.3	2, 3, 4a, 5,12b, 13	2_{eq}, 4_{ax}
4_{ax}	1.89 (1H, overlap)	46.3	3, 13	4_{eq}
4a	—	77.9	—	—
4a-OH	3.18 (1H, s)	—	4, 4a, 12b	—
5	6.40 (1H, d, 9.8)	147.0	6, 6a, 12a, 12b	6
6	6.86 (1H, d, 9.8)	116.8	4a, 7, 12a	5
6a	—	137.9	—	—
7	—	188.6	—	—
7a	—	114.9	—	—
8	—	162.2	—	—
8-OH	11.90 (1H, s)	—	7a, 8, 9	—
9	7.29 (1H, dd, 8.2, 1.3)	125.0	7, 7a, 8, 11	10
10	7.68 (1H, pt, 7.6, 8.2)	137.8	8, 11a	9, 11
11	7.63 (1H, dd, 7.6, 1.3)	120.4	7a, 9, 12	10
11a	—	132.4	—	—
12	—	183.3	—	—
12a	—	139.3	—	—
12b	—	80.6	—	—
13	1.39 (3H, s)	28.8	2, 3, 4	2_{ax}
Sugar signals				
1A	5.27 (1H, br. s)	94.8	12b, 3A, 5A	$2A_{eq}$, $2A_{ax}$
$2A_{eq}$	1.89 (1H, m)	24.3	1A, 3A, 4A, 5A	1A, $2A_{ax}$
$2A_{ax}$	1.72 (1H, m)	24.3	3A, 5A	1A, $2A_{eq}$
$3A_{eq}$	1.97 (1H, m)	24.8	—	$3A_{ax}$
$3A_{ax}$	1.81 (1H, m)	24.8	—	$2A_{eq}$, $2A_{ax}$, 4A
4A	3.32 (1H, br. s)	76.4	2A, 1B	5A
5A	3.56 (1H, q, 6.6)	67.3	1A, 4A, 6A	4A, 6A
6A	0.49 (3H, d, 6.5)	16.6	4A, 5A	5A
1B	4.32 (1H, dd, 9.9, 1.8)	101.9	4A, 2B	$2B_{eq}$, $2B_{ax}$
$2B_{eq}$	2.04 (1H, ddd, 12.8, 5.2, 1.8)	37.4	1B, 3B, 4B	1B, $2B_{ax}$, 3B
$2B_{ax}$	1.47 (1H, m)	37.4	1B, 3B	1B, $2B_{eq}$, 3B
3B	3.36 (1H, m)	81.2	4B, 1C	$2B_{eq}$, $2B_{ax}$, 4B
4B	2.96 (1H, pt, 8.7, 8.7)	76.1	3B, 5B, 6B	3B, 4B-OH, 5B
4B-OH	4.21 (1H, s)	—	3B, 4B, 5B	4B
5B	3.14 (1H, m)	72.0	1B, 3B, 4B, 6B	4B, 6B
6B	1.25 (3H, d, 6.1)	18.2	4B, 5B	5B
1C	4.84 (1H, br. s)	98.0	3B, 3C, 5C	$2C_{eq}$, $2C_{ax}$
$2C_{eq}$	1.98 (1H, m)	25.4	—	1C, $2C_{ax}$, 3C
$2C_{ax}$	1.46 (1H, m)	25.4	3B	1C, $2C_{eq}$, 3C
3C	1.90 (2H, m)	24.9	1C, 2C, 4C	$2C_{ax}$, 4C
4C	3.49 (1H, br. s)	76.4	2C, 1D	3C, 5C
5C	4.02 (1H, q, 6.6)	67.9	1C, 4C, 6C	4C, 6C
6C	1.11 (3H, d, 6.6)	17.1	4C, 5C	5C
1D	4.45 (1H, dd, 9.8, 1.8)	101.7	4C, 2D, 5D	$2D_{eq}$, $2D_{ax}$
$2D_{eq}$	2.21 (1H, ddd, 12.6, 5.2, 1.8)	37.4	1D, 3D, 4D	1D, $2D_{ax}$, 3D
$2D_{ax}$	1.56 (1H, m)	37.4	1D, 3D	1D, $2D_{eq}$, 3D
3D	3.46 (1H, m)	81.1	4D, 1E	$2D_{eq}$, $2D_{ax}$, 4D
4D	3.03 (1H, pt, 8.6, 8.8)	76.1	3D, 5D, 6D	3D, 5D
4D-OH	4.11 (1H, overlap)	—	3D, 4D, 5D	—
5D	3.21 (1H, m)	71.9	1D, 3D, 6D	4D, 6D
6D	1.28 (3H, d, 6.1)	18.2	4D, 5D	5D
1E	4.91 (1H, br. s)	97.7	3D, 3E, 5E	$2E_{eq}$, $2E_{ax}$
$2E_{eq}$	2.00 (1H, m)	24.5	—	1E, $2E_{ax}$, 3E
$2E_{ax}$	1.54 (1H, m)	24.5	1E, 5E	1E, $2E_{eq}$, 3E
3E	1.71 (2H, m)	26.1	1E, 5E	$2E_{eq}$, $2E_{ax}$
4E	3.58 (1H, br. s)	67.7	2E	3E, 5E
5E	4.10 (1H, q, overlap)	68.1	1E, 4E, 6E	4E, 6E
6E	1.16 (3H, d, 6.6)	17.2	5E	5E

Abbreviation: pt, pseudo-triplet.

2 and **3,** compounds **4** and **5** vary in terms of the length of the sugar chain.

The CD spectrum of compound **3** showed absorption bands at $\lambda_{max} \approx 457$ (+1.3), 400 (−3.0), 324 (+11.5), 277 (+0.4) and 243 (+10.6) nm (Table 4), which were in good accordance with the absorption bands of the CD spectrum of urdamycin A ($\lambda_{max} \approx 459$ (+0.5), 402 (−1.4), 327 (+3.8), 289 (+1.2), 260 (−0.7) and 232 (+4.9) nm) measured as a reference substance under the same conditions. Further, the CD curve maxima of urdamycin A reported in the literature[7] were nearly identical to those of **1–3,** corroborating the assignment of the same stereochemistry of the chromophore. The CD peak maxima of **5** (Table 4) were also in good accordance with the peak maxima of urdamycin D in the literature.[7] Concluding from the CD spectra, the resultant stereochemistry of the polyketide backbones is derived and is shown in Figure 2.

Biological activity

Compounds **1**, **2**, **3**, **5** and **6** showed antibacterial and cytotoxic activity (Table 7). In detail, compounds **1**, **2**, **3** and **6** inhibited the growth of the Gram-positive *Bacillus subtilis* with IC_{50} values of 40.7 (±4.2), 4.07 (±0.47), 2.17 and 4.1 (±0.31) μM, respectively. The growth of the yeast *Candida albicans* was not inhibited by the compounds **1**, **2**, **3** and **6**. A moderate cytotoxic activity against the human hepatocellular liver carcinoma cell line HepG2 was observed for compounds **1**, **2**, **3** and **6**. The IC_{50} values were 26.5 (±3.4) μM, 13.6 (±3.4) μM, in the range of 2.5–5.0 μM and in the range of 5.0–10.0 μM, respectively. The growth of the cell line NIH 3T3 was inhibited in a similar range as the cell line HepG2 with IC_{50} values of 9.9 (±3.3) μM, 11.75 (±2.85) μM, 2.5–5.0 μM and 29.2 (±6.7) μM for compounds **1**, **2**, **3** and **5.**

DISCUSSION

The term angucycline/angucyclinone antibiotics was introduced in the literature by Drautz *et al.*[7] with reference to the angle (lat.: *angus*) that is a characteristic feature in the tetracyclic benz[*a*]anthracene skeleton. Angucyclinones are biosynthetically derived from a

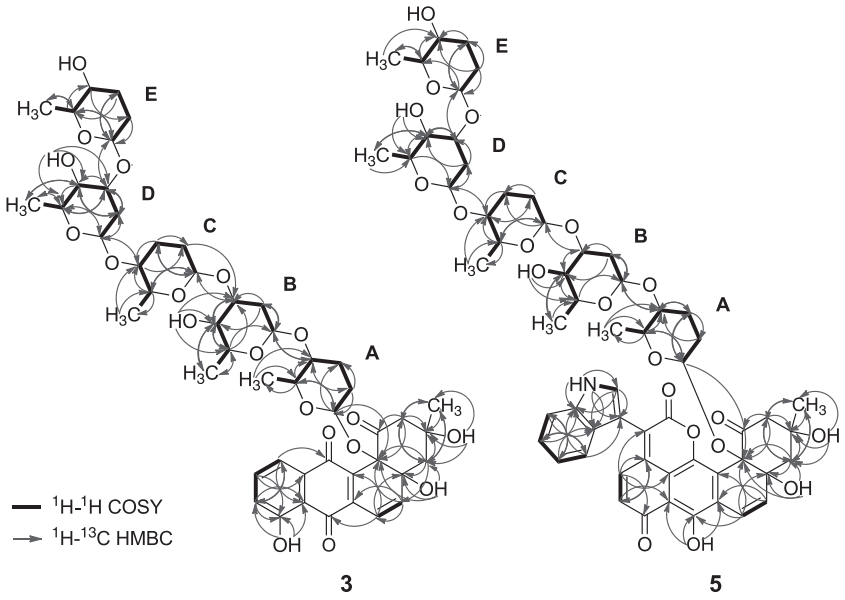

Figure 5 ^{1}H-^{1}H COSY and HMBC correlations of langkocycline A3 (**3**) and langkocycline B2 (**5**). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

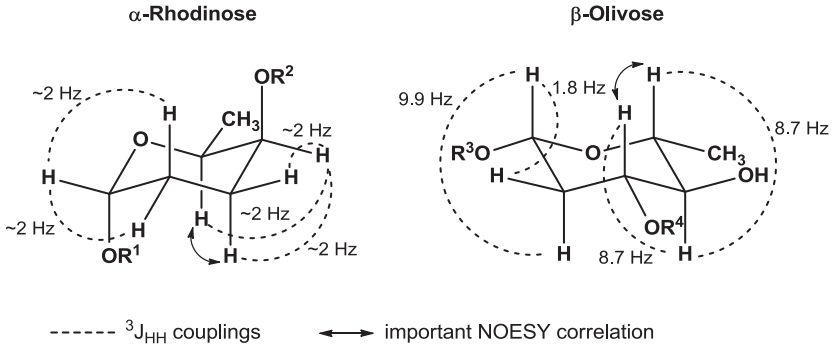

Figure 6 Structure elucidation of the rhodinoses and olivoses by compiling coupling constants.

decaketide chain formed *via* the polyketide biosynthetic pathway, whereas angucyclines consist of the angucyclinone aglycon and hydrolyzable *O*-glycosidic sugar moieties.[17] The first member of this large and ever-growing group of secondary metabolites was tetrangomycin.[11] All producers of angucyclines/angucyclinones belong to Gram-positive bacteria of the order *Actinomycetales*, and the majority of metabolites were isolated from the genus *Streptomyces*, as the producer of langkocyclines described in this report. *Streptomyces* sp. Acta 3034 was included in our HPLC-diode array screening program and was found to produce besides the angucyclinone antibiotic tetrangomycin (**6**) five new members of the angucycline group of antibiotics, langkocyclines A1–A3 (**1–3**) and the minor congeners langkocyclines B1 (**4**) and B2 (**5**).

Compounds **1**, **2** and **3** show a structural relationship to urdamycin A, an angucycline antibiotic isolated from *Streptomyces fradiae* Tü 2717.[7] The only difference is in the linking position of the sugar chain to the polyketide backbone. Instead of the *C*-glycosidic sugar chain of urdamycin A the langkocyclines show a hydrogen in place. Comparable to urdamycin K and sakyomicin C the sugar chain of langkocyclines **1–5** is located at carbon C-12b.[12,18] The same analogy is given between the two compounds **4** and **5** and urdamycin D. As in urdamycin A and D, the 2,3,6-tridesoxyhexose sugars of **1–5** could be assigned as α-L-rhodinoses, and the 2,6-didesoxyhexose sugars as β-D-olivoses. According to the absorption bands of the CD-spectra, the polyketide backbones of the langkocyclines show the same stereochemistry as the chromophores of urdamycin A and D.

Angucyclines are known for their antibiotic and cytotoxic activities.[17] Kerriamycins A–C showed a minimal inhibition concentration of 12.5 μg ml^{-1} against *Bacillus subtilis* and *Staphylococcus aureus*; Gram-negative bacteria and fungal strains were not inhibited by kerriamycins, whereas an inhibitory activity on Ehrlich mouse ascites carcinoma was observed.[19] Further, urdamycins A–E exhibited an antibacterial activity against *B. subtilis* and an antiproliferative activity. The most active compounds in the proliferation assay using L1210 leukemia cells were urdamycins A and E with IC_{50} values of 2.4 and 1.7 μg ml^{-1}, respectively.[7] Similar activities were reported for aquayamycin (IC_{50} value of 2.8 μg ml^{-1}),[7] an angucycline antibiotic described by Sezaki *et al.*[20] As langkocycline A3 and the angucyclinone tetrangomycin showed the most potent antimicrobial and cytotoxic activities among the langkocyclines, we assume that there are two different mechanisms of action as in the case of landomycins.[13] It is possible that the mode of action of the sugar-free

Table 6 ^{1}H-, ^{13}C-, HMBC- and COSY signals of the polyketide backbone of 5 measured in CD_2Cl_2-d_2

Position	δ (^{1}H) (p.p.m.) J in Hz	δ (^{13}C) (p.p.m.)	HMBC	COSY
1	—	203.2	—	—
2_{eq}	2.97 (1H, d, 12.0)	54.8	1, 3, 13	2_{ax}
2_{ax}	2.77 (1H, dd, 12.0, 2.7)	54.8	1, 3, 4, 12b, 13	2_{eq}
3	—	73.0	—	—
3-OH	1.67 (1H, s)	—	2, 3, 4, 13	
4_{eq}	2.17 (1H, dd, 14.1, 2.7)	46.4	2, 3, 4a,12b	4_{ax}
4_{ax}	2.02 (1H, overlap)	46.4	3, 13	4_{eq}
4a	—	77.9	—	—
4a-OH	3.19 (1H, overlap)	—	4, 4a, 12b	—
5	6.09 (1H, d, 9.9)	138.9	6a, 12b	6
6	7.04 (1H, d, 9.9)	118.6	4a, 6a, 7,12a	5
6a	—	124.2	—	—
7	—	156.2	—	—
7-OH	13.14 (1H, s)	—	6a, 7, 7a	—
7a	—	112.9	—	—
8	—	188.9	—	—
9	6.74 (1H, d, 10.1)	132.1	7a, 11	10
10	7.89 (1H, d, 10.1)	140.2	8, 11, 11a, 3′	9
11	—	132.2	—	—
11a	—	116.6	—	—
12	—	142.7	—	—
12a	—	127.1	—	—
12b	—	82.5	—	—
13	1.46 (3H, s)	29.5	2, 3, 4	—
2′	—	159.2	—	—
3′	—	127.1	—	—
4′	—	108.9	—	—
5′	7.82 (1H, d, 2.8)	131.2	4′, 6′a, 10′a	6′
6′	8.99 (1H, s)	—	4′, 5′, 6′a, 10′a	5′
6′a	—	136.6	—	—
7′	7.53 (1H, d, 7.8, overlap with 7.55)	112.4	9′, 10′a	8′
8′	7.30 (1H, pt, 7.5, 7.8)	124.0	6′a, 10′	7′, 9′
9′	7.22 (1H, pt, 7.5, 7.7)	122.2	7′, 10′a	8′, 10′
10′	7.55 (1H, d, 7.7, overlap with 7.53)	120.6	4′, 6′a, 8′	9′
10′a	—	127.5	—	—

Abbreviation: pt, pseudotriplet

Table 7 Biological activity of compounds 1–6; IC_{50} values are given in μM

	1	2	3	4	5	6
B. subtilis	40.7 (±4.2)	4.07 (±0.47)	2.17	n.d.	n.d.	4.1 (±0.31)
C. albicans	not active	not active	not active	n.d.	n.d.	not active
HepG2	26.5 (±3.4)	13.6 (±3.4)	2.5–5.0[a]	n.d.	n.d.	5.0–10.0[a]
NIH 3T3	9.9 (±3.3)	11.75 (±2.85)	2.5–5.0[a]	n.d.	29.2 (±6.7)	n.d.

n.d., not determined because of insufficient amounts of compounds.
[a]The range is given because of insufficient amounts of compounds for further analyses.

tetrangomycin is based on DNA intercalation,[13] and the langkocyclines with longer saccharide chains are inhibiting the thymidylate synthase.[21] In conclusion, the activity patterns of langkocyclines A1–A3 (**1**–**3**) and tetrangomycin (**6**) as described in this report correspond to the activity profiles of angucycline antibiotics from other studies.

EXPERIMENTAL SECTION

General experimental procedures

The HPLC-DAD system consisted of an HP 1090M liquid chromatograph equipped with a diode-array detector and an HP Kayak XM 600 workstation (Agilent Technologies, Waldbronn, Germany). Multiple wavelength monitoring was performed at $\lambda = 210$, 230, 260, 280, 310, 360, 435 and 500 nm, and UV-Vis spectra were measured from 200 to 600 nm. Sample preparation was carried out and chromatographic conditions were set up as described earlier.[22]

Producing organism

Rhizosphere soil samples were collected from the fine roots of the creeper *Clitorea* sp., growing on the sandy beach at Burau Bay, main Langkawi Island, Malaysia. The soil sample was air-dried for 3 days before dry heat treatment at 60 °C for 40 min.[5] The sample was then diluted in 0.9% (w/v) NaCl, and 0.1 ml of the 10^{-3} diluted sample was then plated on to starch–casein agar and humic acid–vitamin agar incorporated with antifungal agents (nystatin and cycloheximide, each at 50 μg ml^{-1}) and antibacterial agent nalidixic acid (20 μg ml^{-1}). The inoculated plates were then incubated at 27 ± 2 °C and observed periodically for putative actinomycete colonies. The axenic culture of strain Acta 3034 was transferred to yeast extract–malt extract agar (ISP 2). Standard protocols were then used for the observation of growth characteristics and identification of the strain.[3,8] Color grouping was based on fluorescent light observation and categorization according to color codes in the Meuthen Handbook of Colors.[23] The culture grown on ISP 2 medium for 14 d was observed for aerial and substrate mycelium and spore chain morphology by scanning electron microscopy (Phillips SEM 15; FEI, Singapore). The culture was exposed to osmium tetroxide vapor for 4 h and then mounted on aluminum stubs prior to coating with gold.

Genomic DNA extraction and PCR amplification of the 16S rRNA gene of strain Acta 3034 were carried out as described previously.[24] The 16S rRNA gene sequence of the strain was manually aligned against closely related corresponding sequences of representative *Streptomyces* species retrieved from the GenBank and EzTaxon-e databases,[25] using the PHYDIT program.[26] A neighbor-joining tree was inferred with the Jukes & Cantor algorithm,[27] and the topology of the resultant tree was evaluated by a bootstrap analysis in the TREECON program.[28] The 16S rRNA gene sequence was deposited in GenBank under accession number KC551288.

Fermentation and isolation

Batch fermentations of strain Acta 3034 were carried out in a 10-l stirred tank fermentor (Biostat S, B. Braun, Melsungen, Germany) in SGG medium that consisted of soluble starch 1%, glucose 1%, glycerol 1%, Bacto peptone 0.5% (Becton Dickinson, Franklin Lake, NJ, USA), cornsteep powder 0.25% (Marcor, Carlstadt, NJ, USA), yeast extract 0.2% (Ohly Kat, Deutsche Hefewerke, Hamburg, Germany), NaCl 0.1% and $CaCO_3$ 0.3% in tap water. The pH was adjusted to 7.3 (5 M HCl) prior to sterilization. The fermentor was inoculated with 5% by volume of a shake flask culture grown in the same medium at 27 °C in 500 ml Erlenmeyer flasks with a single baffle for 72 h on a rotary shaker at 120 r.p.m. The fermentation was carried out for 70 h at 27 °C with an aeration rate of 0.5 volume air per volume per min and agitation at 250 r.p.m.

Hyphlo Super-cel (2%; Johns-Manville, Denver, CO, USA) was added to the fermentation broth, which was separated by multiple sheet filtration into culture filtrate and mycelium. Compounds **1**–**5** were isolated from the culture filtrate (8 l) by Amberlite XAD-16 (Rohm and Haas, Frankfurt, Germany) column chromatography (resin volume 800 ml) and were eluted with MeOH. The eluate was adjusted to pH 4.0, extracted twice with EtOAc and concentrated *in vacuo* to dryness (1.0 g). The crude product was dissolved in CH_2Cl_2 and applied to a silica gel 60 column (40 × 2.6 cm; E. Merck, Darmstadt, Germany). Compounds **1**–**5** were separated by a linear gradient from CH_2Cl_2 to CH_2Cl_2-MeOH (9:1). Pure compounds were obtained by subsequent chromatography on Sephadex LH-20 (Amersham, Freiburg,

Germany) and Toyopearl HW-40F (Tosoh Biosep, Stuttgart, Germany) with MeOH as eluent (each column 90 × 2.5 cm). After concentration to dryness *in vacuo* compounds **1–3** were obtained as yellow powders and compounds **4** and **5** as blue-lilac powders.

Structure determination

ESI-MS and high-resolution ESI-MS mass spectra were recorded using a LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany). NMR spectra were recorded on a DRX 500 spectrometer (Bruker, Karlsruhe, Germany) and a DRX 700 spectrometer (Bruker, Karlsruhe, Germany). The chemical shifts are given in p.p.m. referred to CD_2Cl_2-*d2* as 5.32 p.p.m. (^{1}H) and 54.00 p.p.m. (^{13}C). CD-spectroscopy was performed using a JASCO J-815-150S CD spectrometer (Jasco, Groß-Umstadt, Germany). Optical rotation was recorded on a JASCO P-2000 polarimeter using a 50 mm path-length cell at 589 nm.

Biological assays

The antimicrobial activity of compounds **1**, **2**, **3** and **6** against the bacterium *Bacillus subtilis* DSM 347 and the yeast *Candida albicans* DSM 1386, as well as the cytotoxic activity of compounds **1**, **2**, **3**, **5** and **6** against HepG2 (human hepatocellular liver carcinoma cell line) and NIH 3T3 (mouse embryonic fibroblast cell line), was determined according to the method described by Schulz *et al.*[29]

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