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Langkolide, a 32-Membered Macrolactone Antibiotic Produced by *Streptomyces* sp. Acta 3062

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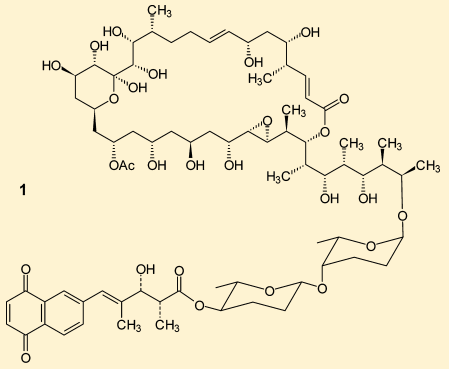
Supporting Information

ABSTRACT: A new 32-membered macrolactone antibiotic, named langkolide, was isolated from the mycelium of *Streptomyces* sp. Acta 3062. The langkolide structure was determined by HR-MS and 1D and 2D NMR as a 32-membered macrolactone connected from an overhanging polyketide tail to a naphthoquinone unit mediated by two carbohydrate moieties. The producing strain was isolated from a rhizosphere soil of *Clitorea* sp. collected at Burau Bay, Langkawi, Malaysia, and was characterized by its morphological and chemotaxonomic features in addition to its 16S rRNA gene sequence. It was identified as a member of the *Streptomyces galbus* clade. Langkolide exhibited various bioactivities including antimicrobial and antiproliferative activities. Furthermore, langkolide inhibited human recombinant phosphodiesterase 4 with an IC_{50} value of 0.48 μM.



Pristine Malaysian ecological niches were selected for the isolation of novel and unique actinomycetes strains for screening purposes to detect novel secondary metabolites for pharmaceutical applications, such as antibiotics, antitumor compounds, and enzyme inhibitors. The strains were cultivated in various complex media to stimulate their secondary metabolite production. Extracts from culture filtrates and mycelia were prepared, and their chemical diversity was characterized by HPLC-diode array monitoring (HPLC-DAD). Strain Acta 3062 was isolated from the rhizosphere soil collected from the roots of *Clitorea* sp. at Burau Bay, Langkawi, Malaysia. The strain seemed worth considering for closer inspection, because of a dominant peak in the HPLC chromatogram of the mycelium extract showing no spectral identity with more than 950 reference compounds, mostly antibiotics, stored in our in-house HPLC−UV−vis database.[1] Fermentation, isolation, and structure determination revealed a new 32-membered macrolactone antibiotic, which was named langkolide with reference to the collection site of the producing strain. Its structure is shown in Figure 1. Macrolides and macrolactones belong to a very important, structurally diverse, and manifold class of antibiotics, produced mainly by Gram-positive and Gram-negative bacteria and fungi. Not only are their structures and biological origin highly diverse, their biological activities are also. Small-membered ring macrolides (12 and 14 ring atoms) and

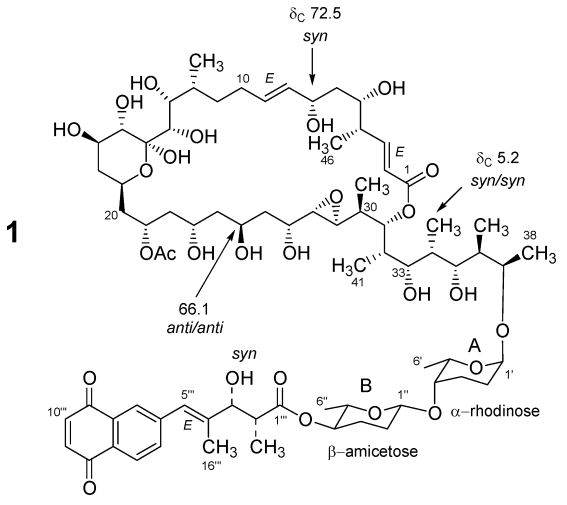


Figure 1. Relative stereochemistry of langkolide (**1**). Stereocenters marked with an asterisk were assigned on the basis of Kishi data sets; other stereocenters were assigned on the basis of NOESY-NMR data.

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medium-membered ring macrolides (16 ring atoms) are potent inhibitors of bacterial protein biosynthesis and are used for anti-infective treatment in medicine.[2] Larger macrolides (>16 ring atoms), often containing fused-ring systems and decorated with amino sugars, are distinguished by various interesting biological activities and exhibit antifungal, immunosuppressive, insecticide, and anthelmintic action.[3] This report describes the taxonomy of the langkolide-producing strain and its fermentation and the isolation, structure elucidation, and biological properties of langkolide.

RESULTS AND DISCUSSION

Strain Acta 3062 was isolated from rhizosphere soil collected from the roots of *Clitorea* sp., a creeper that grows abundantly above the intertidal zone of sandy beaches of Burau Bay in the main island of Langkawi, West Malaysia. Sporulation of the aerial mycelium was observed on all media studied except peptone-yeast extract agar. The spore mass was white in all media except tyrosine agar, which produced gray spores. The strain grew well in a temperature range of 17−27 °C and tolerated NaCl concentrations of 1−4%. The strain contained L,L-diaminopimelic acid in the cell wall. The scanning electron microscopic investigation revealed that aerial mycelium on ISP2 medium had 3−5 turns of tight spirals and the spore surface had sparse spiny ornamentations. On the basis of growth and chemotaxonomic characteristics, strain Acta 3062 was assigned to the genus *Streptomyces*, being similar to *S. galbus*, *S. longwoodensis*,[4] and *S. bungeonsis*.[5] On the basis of molecular data, the closest related validly described *Streptomyces* sp. to strain Acta 3062 is *S. galbus* DSM 40089^{T,6} and *S. longwoodensis* LMG 20096^{T}, with similarity values of 98.73% and 98.72%, respectively. Nevertheless, strain Acta 3062 seemed to form a distinct phyletic line from the *S. galbus* clade. Further work using DNA−DNA hybridization techniques will have to be done to determine the taxonomic integrity of strain Acta 3062.

Strain Acta 3062 was grown in 500 mL shake flasks in various complex media that differed in their carbon and nitrogen source as well as in nutrient-rich and -poor formulations suitable for the stimulation of secondary metabolite production.[7] Samples were taken between 48 and 144 h of incubation, and extracts were prepared from both the culture filtrate and the mycelium and were analyzed by HPLC-DAD. A high and characteristic production of an unknown metabolite with a retention time of 11.7 min was detected in the mycelium extract (Figure 2) when the strain was cultivated in nutrient-poor oatmeal medium. Strain Acta 3062 was cultured in a 20 L fermentor, and maximal growth was reached at 72 h with a biomass of 12 vol %, which corresponded to a DNA content of 30.5 mg/L. At the same fermentation time the production of langkolide reached a peak of 75 mg/L in the mycelium. Langkolide was isolated from the mycelium by extraction with methanol/acetone and re-extraction with ethyl acetate. The crude product was purified by chromatography on Sephadex LH-20 in the dark and preparative reversed-phase HPLC, resulting in pure langkolide.

Langkolide (**1**) was obtained as a yellow, amorphous powder. The ESIMS analysis established the molecular formula $C_{75}H_{114}O_{27}$. Further support in determining the molecular formula came from the interpretation of NMR data. Detailed analysis of the ^{13}C NMR data including the DEPT spectrum and the HSQC-NMR spectrum revealed the presence of 10 quaternary carbons (including a hemiacetal carbon at δ_C 100.5), 10 aromatic/olefinic carbons, 24 oxygenated methines (including two anomeric carbons at δ_C 97.3 and 104.8, representing

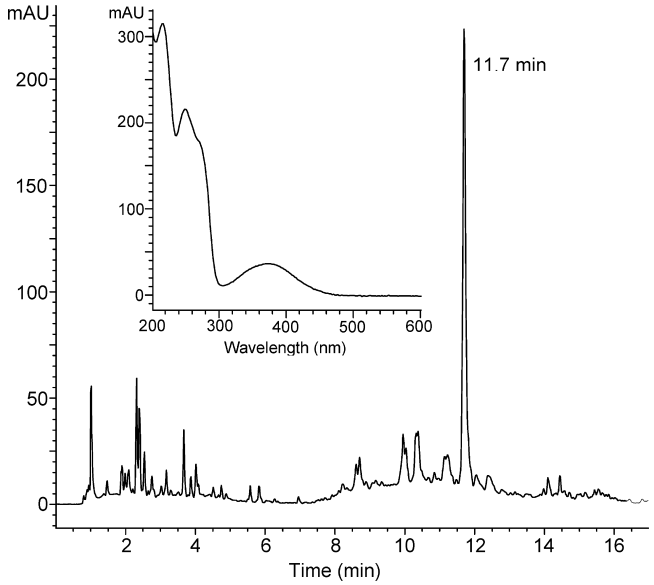

Figure 2. HPLC analysis of the mycelium extract from *Streptomyces* sp. Acta 3062 at a fermentation time of 72 h, monitored at λ = 230 nm. Inset: UV−visible spectrum of langkolide (**1**).

two sugar moieties), seven aliphatic methines, 12 methylenes, and 12 methyl carbons. From 19 double-bond equivalents deduced from the molecular formula, eight were assigned to a naphthoquinone moiety, three to olefinic double bonds, and three to carbonyls (δ_C 168.8, 173.5, 176.6). This suggests that **1** contains either five ring systems or double bonds. The $^{1}H-^{1}H$-COSY NMR spectrum of **1** showed a series of correlations establishing the structure of the H2−H14 spin system (Table 1 and Figure 3). In addition, $^{1}H-^{13}C$-HMBC correlations were observed from H2/H3 to C1 (δ_C 168.98)/C4 (δ_C 44.0), further corroborating the structure of the C1−C14 backbone (Figure 3, a). Furthermore, COSY correlations between all subsequent adjacent protons established the structure comprising C16−C38. A set of HMBC correlations established the polyketide moiety C16−C38 with four methyl groups (C39−C42) and an acetoxy group (C43/C44) attached to C21. Additionally, HMBC correlations from H13 to C15 (δ_C 100.5), from H14 (δ_H 3.68) to C15/C16, and from H16 to C15 allowed the construction of the linear polyketide chain from C1 to C38. Finally, one long-range coupling from H31 to C1 formally constructed a 32-membered macrolide ring (Figure 3, a). Moreover, residual COSY and HMBC data of langkolide revealed two 2,3,6-trideoxyhexose moieties, C1′−C6′ and C1″−C6″ (Figure 3, b and c), and a monosubstituted naphthoquinone moiety, C1‴−C17‴ (Figure 3, d). The three glycosidic linkages were established on the basis of the HMBC correlations from H1′ to C37 (from H37 to C1′), from H1″ to C4′ (H4′ to C1″), and from H4″ to C-1‴. Consequently, 1D- and 2D-NMR data established the constitutional formula of langkolide (**1**) as a 32-membered macrolide (Figure 1).

Langkolide (**1**) consists of six stereoclusters involving 32 stereocenters. The stereoclusters comprise the substructures C4−C7, C12−C19, C21−C37, C1′−C6′, C1″−C6″, and C2‴−C3‴. Several crystallization attempts to obtain a single crystal of **1** in order to facilitate elucidation of the relative and absolute configuration were unsuccessful. Therefore we used NMR spectroscopy in order to derive relative stereochemical data. An approach for stereochemical assignment of unknown polyketides based on universal NMR databases was reported by

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Table 1. NMR Spectroscopic Data for Langkolide (1) in MeOH-d_4 (500 MHz)

position	δ_C	δ_H, mult. (J in Hz)	COSY	HMBC	NOESY
1	168.8, C				
2	122.5, CH	5.97, d (15.6)	3	1, 4	4, 46
3	153.9, CH	7.04, dd (15.6, 7.1)	2, 4	1, 4, 5, 46	5, 46
4	44.0, CH	2.50, m	3, 5, 46	2, 3, 5, 46	2, 5, 7
5	73.1, CH	3.71, m	4, 6	7	3, 4, 7
6	42.4, CH_2	1.63, 1.72, m	5, 6a, 7	4, 8	
7	72.5, CH	4.27,[a] m	6, 8	5, 9	4, 5, 8, 9
8	133.2, CH	5.49, dd (15.3, 7.4)	7, 9	6, 10	7
9	134.2, CH	5.78, dt (15.3, 6.4)	8, 10	7, 11	7
10	30.5, CH_2	2.08, 2.22, m	9, 10a, 11	8, 11	
11	33.9, CH_2	1.36, 1.66, m	10, 11a, 12	9, 13, 45	
12	37.8, CH	1.81, m	11, 13, 45	13, 45	13, 14
13	72.9, CH	3.86, d (6.8)	12, 14	11, 12, 15, 45	12, 14, 16
14	72.6, CH	3.68, s	13	15, 16	12, 13, 16
15	100.5, C				
16	74.7, CH	3.72, d (9.3)	17	14, 15, 17	13, 14
17	69.9, CH	3.91, dt (15.6, 5.1)	16, 18	16	19
18	40.8, CH_2	1.37, 1.92, m	17, 18a, 19	17, 19	18b
19	66.7, CH	4.22, t (10.8)	18, 20	17, 20	17, 18b, 20a
20	44.6, CH_2	1.57, 1.64, m	19, 20a, 21	19	19, 21
21	70.4, CH	5.27, m	20, 21	43	20b, 23, 27
22	44.3, CH_2	1.71, 1.79, m	21, 22a, 23	21, 23	
23	66.2, CH	3.84,[a] m	22, 24	25	21
24	46.0, CH_2	1.56, m	23, 25	23, 25	
25	66.1, CH	3.98, m	24, 26		
26	39.2, CH_2	1.64, 1.74, m	25, 26a, 27	25, 27, 28	
27	68.2, CH	3.49[a], m	26, 28	28	21, 27, 28
28	63.4, CH	2.80, d (4.5)	27, 29	27, 29	27, 31
29	60.7, CH	2.78, d (9.3)	28, 30	28, 30, 31, 42	27, 30, 42
30	40.6, CH	1.61,[b] m	29, 31, 42	29, 42	29
31	75.1, CH	5.36, d (10.3)	30, 32	1, 29, 30, 32, 33, 41	28, 32, 33, 42
32	38.5, CH	1.98,[b] m	31, 33, 41	33, 41	31
33	78.2, CH	3.42, d (9.6)	32, 34	31, 35, 40, 41	31, 34, 35
34	35.9, CH	1.86,[b] m	33, 35, 40	33, 35, 40	33, 35
35	79.7, CH	3.51[a], m	34, 36	33, 34, 36, 37, 39, 40	33, 34, 39
36	40.7, CH	1.99, m	35, 37, 39	35, 37, 39	37
37	74.3, CH	4.09, m	36, 38	36, 38, 39, 1′	36, 1′
38	15.7, CH_3	1.15, d (6.1)	37	36, 37	35, 37, 39
39	10.2, CH_3	0.84, d (6.8)	36	35, 36, 37	35, 38
40	5.2, CH_3	0.92, d (6.9)	34	33, 34, 35	36
41	9.5, CH_3	0.90, d (6.7)	32	31, 32, 33	30
42	14.5, CH_3	1.11, d (7.0)	30	29, 30, 31	29, 31
43	173.5, C				
44	21.4, CH_3	2.10, s		21, 43	
45	15.6, CH_3	0.97, d (6.6)	12	11, 12, 13	14
46	14.6, CH_3	1.15, d (6.1)	4	3, 4, 5	2, 3
Sugar A, α-rhodinose					
1′	97.3, CH	4.90,[a] d (1.6)	2′	37, 2′, 3′, 5′	37, 2′
2′	26.0, CH_2	1.43, 2.03,[b] m	1′, 2′a, 3′	1′, 3′, 4′	1′
3′	25.8, CH_2	2.01, m	2′, 4′	1′, 2′, 5′	
4′	77.5, CH	3.58, brs	3′, 5′	2′, 3′, 1″	5′, 1″
5′	67.8, CH	4.05, dq (6.2, 1.5)	4′, 6′	1′, 3′, 6′	4′, 1″
6′	17.4, CH_3	1.16, d (5.4)	5′	4′, 5′	
Sugar B, β-amicetose					
1″	104.8, CH	4.62, d (8.4)	2″	4′, 2″, 3″, 5″	4′, 5′, 5″
2″	31.5, CH_2	1.70, 2.00, m	1″, 2″a, 3″	1″, 2″, 4″	
3″	28.7, CH_2	1.65, 2.14, m	2″, 3″, 4″	1″, 2″, 5″	
4″	74.2, CH	4.52, ddd (9.9, 10.0, 4.6)	3″, 5″	2″, 6″, 1‴	6″
5″	74.6, CH	3.61, dq (11.2, 6.3)	4″, 6″	1″, 3″, 6″	1″
6″	18.7, CH_3	1.26, d (6.3)	5″	4″, 5″	4″
1‴	176.6, C				
2‴	45.5, CH	2.76, m	3‴, 16‴		16‴
3‴	81.2, CH	4.31, d (9.4)	2‴	1‴, 2‴, 5‴,16‴, 17‴	5‴
4‴	143.9, C				
5‴	127.9, CH	6.68, s		3‴, 7‴, 16‴	3‴
6‴	145.1, C				
7‴	127.3, CH	8.02, s		5‴, 9‴, 13, 15‴	
8‴	133.2, C				
9‴	186.8, C				
10‴	139.8, CH	7.05, s		8‴, 12‴	
11‴	140.1, CH	7.05, s		9‴, 13‴	
12‴	186.4, C				
13‴	131.8, C				
14‴	127.4, CH	8.08, d (8.0)	15‴	6‴, 8‴, 12‴	15‴
15‴	135.4, CH	7.78, d (8.0)	14‴	5‴, 7‴, 13‴	14‴
16‴	13.0, CH_3	1.96, s		3‴, 4‴, 5‴	2‴, 17‴
17‴	14.8, CH_3	1.11, d (7.0)	2‴	1‴, 2‴, 3‴	16‴

[a]Partially obscured signals. [b]Overlapping signals, δ in ppm.

Kobayashi et al.,[8] showing that steric and/or stereoelectronic interactions between the structural motifs connected either directly or with a CH_2 bridge are significant for stereochemical assignment, whereas interactions between the structural motifs connected by two or more CH_2 bridges are almost negligible. On this basis, they determined that the central C atom of acyclic 1,3,5-triols exhibits a distinctive ^{13}C chemical shift that is dependent on the 1,3- and 3,5-relative configuration, but is independent from the functionalities present outside this structural motif.[8] These NMR characteristics were used in the stereochemical assignments of several natural products, e.g., linear mycolactones A and B,[9] linear tetrafibricin,[10] and the

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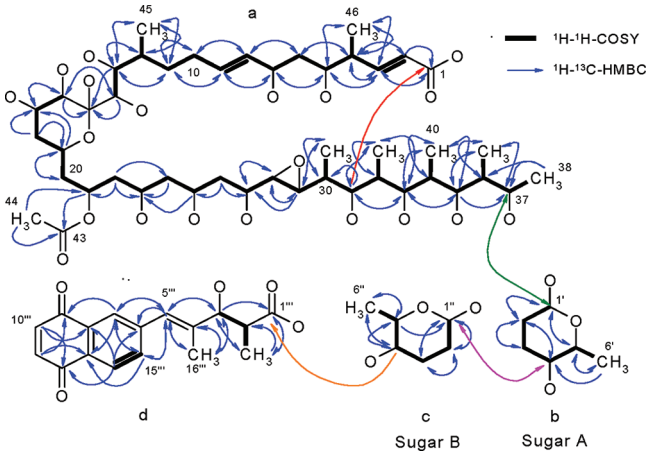

Figure 3. Structural moieties of langkolide (**1**) established from 2D NMR data (C1−C37 (a), C1′−C6′ (b), C1″−C6″ (c), and C1‴−C17‴ (d)) and 2D NMR correlations.

cyclic desertomycin/oasomycin class of natural products.[11] We used these data sets[8,10,11] for the stereochemical assignment of the 1,3,5-triol structural motifs (C23−C27) of langkolide and the 1,3-diol C5−C7. The ^{13}C NMR chemical shift of the central C atom of the C23−C27 portion (C25, δ_C 66.1, CD_3OD) matched the central C atom in data set A[10,11] with an anti/anti-configuration (δ_C 66.3, CD_3OD) (Figure 4). This suggested the relative configuration of the structural motif C23−C27 to be in anti/anti-configuration. Similarly, the structural motif C5−C7 was assigned to have a syn-configuration on the basis of the ^{13}C chemical shift of C7 (δ_C 72.5, CD_3OD), which is also in good agreement with the chemical shift of the C atom in data set B[10,11] with syn-configuration (δ_C 71.7, CD_3OD) (Figure 4). Likewise, NMR data sets for the stereochemical assignment of 2-methyl-1,3-diols were also reported by Kobayashi et al.[10,11] On the basis of data set C,[10,11] the relative configuration of the structural motif C33−C35 was assigned to a syn/syn-configuration (C40, δ_C 5.2, CD_3OD). The significant upfield ^{13}C NMR chemical shift of the methyl group is characteristic for a syn/syn-configuration.[10,11] NOESY NMR experiments were performed for the assignment of the remaining 24 stereogenic centers of **1**. As a proof of the suggested configuration from the Kishi NMR data sets,[8,10,11] NOE correlations from H19 to H20a, H20b to H21, H37 to H-1′, and H-1″ to H-4′/H-5′ allowed the four independent stereoclusters involving the C12−C19, C21−C37, C1′−C6′, and C1″−C6″ portions to be treated as a single stereocluster C12−C6″, which reduces the above-mentioned six independent stereoclusters into three stereoclusters (C4−C7, C12−C6″, and C1‴−C3‴, Figure 5). Furthermore, NOE correlations

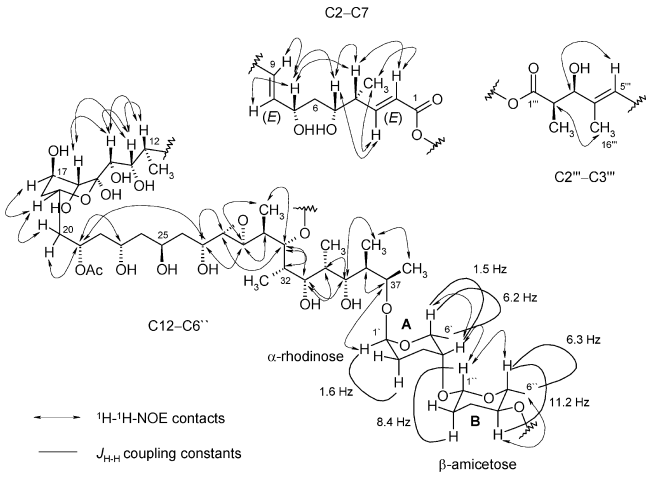

Figure 5. NOE correlations of the three stereoclusters of langkolide (C2−C7, C12−C6″, and C2‴-C3‴) and coupling constants of the carbohydrate residues α-rhodinose and β-amicetose.

observed between H4 and H2/H5/H7, H5 and H3/H7/H9, and H7 and H8/H9 revealed a syn/syn-configuration of the stereocluster C4−C7, which is also supported by the data of the Kishi data set. NOE correlations between H12 and H13/H14; H13/H14 and H16; H17 and H19, H19, and H20a; H21 and H20b/H23/H27; H27 and H28/H29; H28 and H31, H29, and H42/H30; H31 and H32/H33/H42; H33 and H34/H35; H35 and H39, H36, and H37; and H38 and H39 established the relative configuration of the C12−C37 portion (Figure 5). The above-mentioned NOE correlation between H37 and the anomeric proton H1′ of sugar A was the starting point for the assignment of the relative configuration of the 2,3,6-trideoxyhexose moieties found in langkolide. The NOE contact between H37 and H1′ together with the coupling constant of H1′ (J < 2 Hz) proved the equatorial position of H1′. Furthermore, a NOE correlation from H4′ to H5′ shows H6′ (CH_3) to be equatorial. The signal for H5′ with a coupling constant of J = 1.5 Hz to H4′ is indicative of an axial OH group. Thus, sugar A in langkolide was determined as an α-rhodinose.[12] Likewise, the coupling constant of the anomeric proton H1″ (δ_H 4.62, d, J = 8.4 Hz) of sugar B together with the NOE correlations with H4′/H5′ revealed an axial position of H1″. Further NOE correlations between H3″ and H5″ and between H4″ and H6″ together with the axial−axial coupling constant of H5″ (J = 11.2 Hz to H4″) revealed the equatorial

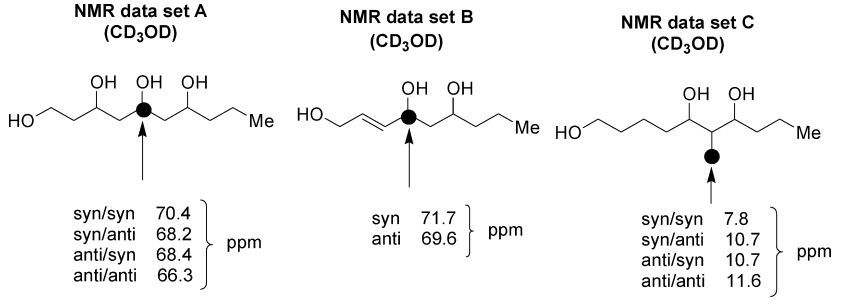

Figure 4. Kishi NMR data sets for elucidation of the relative configuration of 1,3,5-triols, 1,3-diols, and 2-methy-1,3-diols (for comparison: ^{13}C chemical shifts of data set C in $CDCl_3$ syn/syn (4.40 ppm), syn/anti (11.7 ppm), anti/syn (11.6 ppm), and anti/anti (12.8 ppm)).[10,11]

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positions of the methyl group and the OH group. Ultimately this established sugar B as β-amicetose, the 4-epimer of rhodinose.[12]

The ^{1}H and ^{13}C NMR spectroscopic data of sugar A are in accordance with those of the α-L-rhodinose in saquayamycin Z[13] and landomycin C.[14] Likewise, the ^{1}H and ^{13}C NMR spectroscopic data of sugar B are very similar to those of β-D-amicetose of aranciamycin F[15] and landomycin X.[16] Hydrolysis of langkolide and subsequent derivatization and carbohydrate analytics proved unsuccessful. Since most examples of rhodinose-containing natural products are α-L-rhodinoses, we suggest the presence of an α-L-rhodinose in langkolide. Likewise, sugar B in langkolide is suggested to be β-D-amicetose. The configuration of the stereocluster C12−C6″ was established as shown in Figure 5. Finally, NOE correlations between H3‴ and H2‴/H5‴ and between H2‴ and H16‴ established the relative configuration of the stereocluster C2‴−C3‴. The geometries of the double bonds C2/C3, C8/C9, and C4‴/C5‴ were all assigned *E*-configuration, based on the ^{1}H−^{1}H coupling constants values and NOESY data (see Table 1). In conclusion, on the basis of NOESY data as well as Kishi NMR data sets we have assigned the relative stereochemistry of **1** (Figure 1). Experiments to crystallize langkolide in order to obtain supportive data on the relative or even the absolute stereochemistry were unsuccessful. Likewise, various attempts of degradation or derivatization of langkolide failed due to the sensitivity of the molecule. Therefore an assignment of the absolute stereoconfiguration currently must remain unsolved.

The closest related structure to langkolide is that of antibiotic A 77951 described in a patent of Sankyo Ltd.[17] The 32-membered macrolactone antibiotic is produced by *Streptomyces* sp. SANK 66797 and showed a pronounced antifungal and immunosuppressive activity. Langkolide and antibiotic A 77951 comprise the same backbone and the same sugar moieties. The structural modifications of langkolide include two additional substitutions in the macrolide ring (hydroxy and methyl groups). In addition, langkolide lacks the thiomethyl group that is attached to the naphthoquinone ring of antibiotic A 77951. Another strongly related polyketide is brasilinolide C from *Nocardia brasiliensis*,[18,19] which also is a 32-membered macrolide for which the absolute stereochemistry is known.[20] Remarkably, the brasilinolides and antibiotic A 77951 all show potent immunosuppressive activity. Other structurally related 32-membered macrolactones produced by actinomycetes comprise the antifungal niphithricin A (synonymous with copiamycin) from *Streptomyces violaceoniger*[21] and mycoticin (synonymous with flavofungin and faerifungin) produced by *Streptomyces ruber*.[22] On the basis of the various bioactivities of known macrolides and macrolactones a variety of bioassays were performed using **1**. While **1** showed neither Gram-positive nor Gram-negative antibacterial effects, it exhibited a moderate growth inhibitory effect against *Candida glabrata* and *Candida albicans*, with IC_{50} values of 1.00 ± 0.02 and 1.23 ± 0.10 μM, respectively. Furthermore, **1** exhibited a remarkable antiproliferative activity against the mouse fibroblast cell line NIH-3T3 as well as the human cancer cell lines HepG2 and HT-29 (IC_{50} = 0.49 ± 0.11, 2.41 ± 0.03, and 1.38 ± 0.25 μM, respectively). Interestingly, **1** inhibited phosphodiesterase 4 (PDE4) in an *in vitro* enzyme activity assay with an IC_{50} value of 0.48 ± 0.16 μM. PDE4 is a promising drug target for the treatment of chronic inflammatory diseases such as chronic obstructive pulmonary disease (COPD). Furthermore, **1** showed weak inhibitory activity against glycogen synthase kinase 3β (GSK-3β) and inhibited the recombinant enzyme in an *in vitro* enzyme activity assay with an IC_{50} = 2.17 ± 0.23 μM. The serine/threonine GSK-3β plays a pivotal role in numerous signaling pathways such as the Wnt signaling, and is therefore an emerging drug target for several diseases including Alzheimer's disease, cancer, and type II diabetes. Langkolide (**1**) is the first 32-membered macrolactone reported to affect the enzyme activity of recombinant PDE4 and GSK-3β. Similar activities are not known from other macrolide and macrolactone compounds. Potent inhibitors of both enzymes are of great interest for drug discovery programs.

EXPERIMENTAL SECTION

General Experimental Procedures. LC-MS experiments were performed on a Q-Trap 2000 (Applied Biosystems, Germany) coupled to an Agilent 1100 HPLC system (Agilent Techologies, Germany). High-resolution ESIMS mass spectra were recorded using a LTQ-Orbitrap XL (Thermo Scientific, Germany) coupled to an Agilent 1260 HPLC system (Agilent Technologies, Germany). NMR experiments were performed using 14.4 mg of **1** on a DRX 500 NMR spectrometer (Bruker, Germany) equipped with a broadband inverse detection probe head with *z* gradients. Methanol-d_4 was used as a solvent for NMR experiments, and chemical shifts were referenced to the solvent peaks (δ_H 3.35, 4.78 and δ_C 49.3).

Producing Organism. A rhizosphere soil sample was collected from a creeper, *Clitorea* sp., at the sandy Burau Bay on the main Langkawi Island, Malaysia. The soil sample was air-dried for three days prior to moist heat treatment.[23] The sample was then diluted in 0.9% NaCl, and 0.1 mL of the 10^{-3} sample was plated on starch-casein agar incorporated with antifungal and antibacterial agents. The inoculated plates were then incubated at 27 ± 2 °C and observed periodically for putative actinomycete strains.[24] The axenic culture of strain Acta 3062 was transferred to yeast extract-malt extract agar (ISP2). Standard protocols were then used for the observation of growth characteristics and identification of the strain.[25,26] Color grouping was based on fluorescent light observation and categorization according to color codes of Meuthen Handbook of Colors.[27] The culture grown on ISP2 media 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.

The hydrolysate of whole cells of strain Acta 3619 grown on ISP2 medium was analyzed for diaminopimelic acid.[28] The carbohydrate utilization pattern and physiological characteristics were studied on the basis of standard protocols.[25,28] Genomic DNA extraction and PCR amplification of the 16S rRNA gene of strain Acta 3062 were carried out as described previously.[29] The 16S rRNA gene sequence of the strain was manually aligned against closely related corresponding sequences of representative *Streptomyces* spp. retrieved from the GenBank and EzTaxon databases[30] using the PHYDIT program.[31] A neighbor-joining tree was inferred with the Jukes and Cantor algorithm,[32] and the topology of the resultant tree was evaluated by using a bootstrap analysis using the TREECON program.[33] The 16S rRNA gene sequence was deposited in GenBank with the accession number JX040470.

Screening, Fermentation, and Isolation. Sample preparation of the culture broth and HPLC diode array analysis of the culture filtrate and mycelium extracts were performed according to the protocol described by Fiedler.[1] Batch fermentations of strain Acta 3062 were carried out in a 20 L fermentor equipped with a turbine impeller system and intensor (b20; B. Braun, Germany) in a complex medium that consisted of (per liter of tap water) oatmeal (Holo Hafergold, Neuform, Germany) (20 g) and trace element solution (5 mL), which was composed of (per liter of deionized water) $CaCl_2{\cdot}2H_2O$ (3 g), iron(III) citrate (1 g), $MnSO_4{\cdot}H_2O$ (200 mg), $ZnCl_2$ (100 mg), $CuSO_4{\cdot}5H_2O$ (25 mg), $Na_2B_4O_7{\cdot}10H_2O$ (20 mg), $CoCl_2{\cdot}6H_2O$ (4 mg), and $Na_2MoO_4{\cdot}2H_2O$ (10 mg); the pH was adjusted to 7.3 (5 M

HCl) prior to sterilization. The fermenter 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 rpm. The shake flasks were inoculated with 4 vol % of a seed culture grown for 72 h on a rotary shaker at 120 rpm in a seed medium that consisted of glucose (10 g), glycerol (10 g), oatmeal (5 g), soybean meal (Schoenenberger, Germany) (10 g), yeast extract (Ohly Kat; Deutsche Hefewerke, Germany) (5 g), Bacto casamino acids (5 g), and $CaCO_3$ (1 g) in 1 L of tap water. The fermentation was carried out for 96 h with an aeration rate of 0.5 vol/vol/min and agitation at 1000 rpm. Hyphlo Supercel (2%) was added to the fermentation broth, which was separated by multiple sheet filtration into culture filtrate and mycelium. The culture filtrate was discarded. The mycelium was extracted five times (each 1.5 L) with MeOH/acetone (1:1), and the extracts were combined, concentrated *in vacuo* to an aqueous residue, and re-extracted three times (each 500 mL) with EtOAc. The extracts were combined and concentrated *in vacuo* to dryness (6 g). The crude extract was dissolved in CH_2Cl_2, and an aliquot was applied to a silica gel column (55 × 3.5 cm, silica gel SI 60; E. Merck, Germany). **1** was separated by a linear gradient of CH_2Cl_2/MeOH at a flow rate of 5 mL/min. Fractions containing **1** (601 mg) were further purified on a Sephadex LH-20 column (65 × 4.5 cm) using MeOH as eluent at a flow rate of 0.25 mL/min. To obtain pure compound, fractions containing **1** (fractions 81−91, 266 mg) were subjected to preparative reversed-phase HPLC using a C_{18} column (Grom-Sil 300 ODS-5 ST, 10 μm, 250 × 20 mm; Alltech Grom, Germany) and elution with MeOH/H_2O (83% MeOH) at a flow rate of 15 mL/min. After concentration to dryness *in vacuo*, 14.4 mg of **1** was obtained as a yellow, amorphous powder.

Structure Determination. The ion peaks at *m/z* 1447.8 $[M + H]^+$ and 1469.9 $[M + Na]^+$ in the ESIMS spectrum of langkolide (**1**, $[\alpha]_D^{23}$ +14.6, *c* 0.1 in MeOH) revealed a molecular mass of 1446.8 g/mol. The molecular formula of **1** was established as $C_{75}H_{114}O_{27}$ by determining the exact molecular mass *m/z* 1469.74756 $[M + Na]^+$ (calcd *m/z* 1469.74397, Δ*m* 2.44 ppm) derived from the high-resolution Orbitrap-ESIMS spectrum.

Biological Activity. *Antimicrobial Assays.* *Bacillus subtilis* DSM 347, *Propionibacterium acnes* DSM 1897, *Staphylococcus lentus* DSM 6672, *Xanthomonas campestris* DSM 2405, and the yeasts *Candida glabrata* DSM 6425 and *Candida albicans* DSM 1386 were used for antimicrobial assays as recently described by Schneemann et al.[34] The resulting values were compared to a positive control (10 μM chloramphenicol for bacteria; 10 μM nystatin for *Candida glabrata*) and a negative control (no compound) on the same plate. Because of no inhibitory activity against bacteria, MIC values were not determined.

Cytotoxic Assays. The sensitivity of the cell lines NIH-3T3, HepG2, and HT-29 to the isolated compounds was evaluated by monitoring of the metabolic activity using the CellTiter-Blue cell viability assay (Promega, Germany). The cultivation of the cell lines and the bioassays were performed as described by Schneemann et al.[34] Tamoxifen (25 μM), as a standard therapeutic drug, was applied as positive control.

Enzyme Inhibition Assays. The activity of phosphodiesterase 4 was measured using the PDE Light HTS cAMP kit (Lonza, USA) according to the manufacturer's instructions. Five units of human recombinant PDE-4B2 (Cat. No. 60042-BPS Biocat, Germany) was added per reaction. Rolipram (1 and 10 μM) (Cat. No. 557330, E. Merck Bioscience, Germany) was used as positive controls for inhibition. GSK-3β inhibition was determined in an *in vitro* assay adapted from a luminescent assay described by Baki et al.[35] TDZD-8, a selective, non-ATP competitive inhibitor of GSK-3β, was used as positive control. To determine the IC_{50} values of the enzyme inhibitory activities, concentrations ranging from 0.1 to 50 μM were analyzed twice in duplicates. The calculation of the IC_{50} values was performed using the software GraphPad Prism 5 (GraphPad software, USA).

■ ASSOCIATED CONTENT

Supporting Information

ESIMS, high-resolution Orbitrap-ESIMS, and ^1H, ^{13}C, and 2D NMR spectra of langkolide (**1**) and scanning electron micrographs of strain Acta 3062. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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