JOURNAL OF NATURAL PRODUCTS

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Geranylphenazinediol, an Acetylcholinesterase Inhibitor Produced by a *Streptomyces* Species

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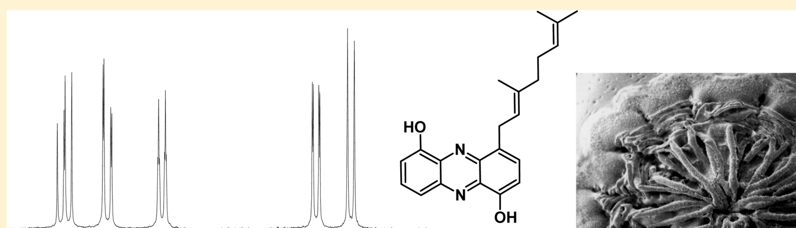
Supporting Information



ABSTRACT: Geranylphenazinediol (**1**), a new phenazine natural product, was produced by the *Streptomyces* sp. strain LB173, which was isolated from a marine sediment sample. The structure was established by analysis of NMR and MS data. **1** inhibited the enzyme acetylcholinesterase in the low micromolar range and showed weak antibacterial activity. In order to get a more detailed picture of the activity profile of **1**, its inhibitory potential was compared to that of related structures.

Phenazines are a group of compounds known since the early days of natural product research, with the first descriptions dating back to the 19th century. The first phenazines, pyocyanin and chlororaphin, were described as *Pseudomonas* pigments.[1] The extreme color spectrum of phenazines (reaching from blue and green to yellow) contributes to their attractiveness and possible is a reason for research on these compounds. In addition, the phenazine core structure offers different sites for derivatization, leading to a wide range of reported compounds, almost all of which have some kind of bioactivity, including highly active molecules such as endophenazines,[2,3] phenazinomycin,[4] D-alanylgriseoluteic acid,[5] and pelagiomicin A.[6] Geranylphenazinediol reported here is a new phenazine-derived natural product.

Inhibition of receptors and enzymes are two basic modes by which a drug can act, and depending on the type of receptor or enzyme that is affected, treatment of a variety of diseases can be envisioned. Acetylcholinesterase (AChE) is an enzyme responsible for the degradation of the neurotransmitter acetylcholine. Inhibitors of this enzyme such as galantamine or rivastigmine[7] enhance the amount of available acetylcholine and thereby improve cholinergic transmission. These compounds are used to alleviate the symptoms of Alzheimer's disease, which is associated with degeneration of cholinergic neurons and impaired transmission.[8]

In the course of our project aimed at establishing a compound library derived from marine microorganisms we screened different fungi and bacteria for the production of preferably new, but also known, secondary metabolites. One of the new metabolites is geranylphenazinediol (**1**) from *Streptomyces* sp. strain LB173. This strain was isolated from ambient sediment of a *Saccharina latissima* specimen growing in the Kiel Fjord.

Strain LB173 was grown in submerged culture in different complex media, and organic extracts were analyzed by HPLC-DAD/MS. This strain became of interest due to a prominent peak with a characteristic UV spectrum. For the production of the compound geranylphenzinediol (**1**), LB173 was cultivated in a starch-peptone medium.

1 was isolated as a yellow, amorphous powder. The compound had already been detected in the HPLC-DAD/MS chromatogram of the crude extract with a detected mass of 348 Da and a UV spectrum leading to the assumption of it being a phenazine. Most phenazines are colored compounds due to the heteroaromatic chromophore with an extended π-electron system. The phenazine core structure has a characteristic UV spectrum, and substitution with hydroxy groups leads to a bathochromic shift. With UV maxima at 204, 273, 354, 374, and 458 nm the UV spectrum of **1** was almost identical to that of 1,6-phenazinediol.[9] The structure of the new compound was established by interpretation of different NMR spectra (^{1}H, ^{13}C, DEPT, ^{1}H−^{13}C HSQC, ^{1}H−^{1}H COSY, and ^{1}H−^{13}C HMBC) and shown to be a phenazinediol substituted with an isoprenoid side chain (Figure 2). The molecular formula $C_{22}H_{24}N_2O_2$ was established by HRESIMS, which was congruent with the NMR data (Table 1). The ^{13}C spectrum comprised signals for 22 individual carbons, including nine quaternary olefinic carbons, seven aromatic/olefinic methines, three methylene groups, and three methyl groups. The signals of protons belonging to the phenazine substructure were shown

Received: December 12, 2011
Published: July 9, 2012

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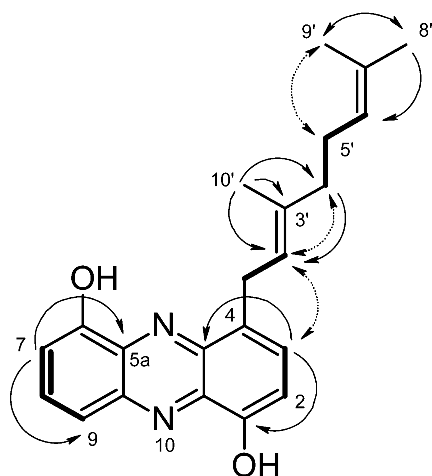

Figure 1. Selected COSY (bold), HMBC (arrows), and NOESY (dashed arrows) correlations pivotal for the structure elucidation of **1**.

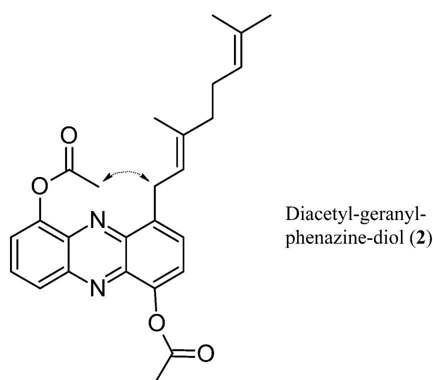

Figure 2. NOESY correlation essential for the determination of the position of the hydroxy groups.

to belong to either one of two separate aromatic spin systems. The first one consisted of the protons H-2 (δ_H 7.17) and H-3 (δ_H 7.61), which coupled with a coupling constant of $J = 7.7$ Hz, which gave further evidence of them being neighboring protons. Both of them had a ^{1}H−^{13}C HMBC correlation to the oxygen-bearing quaternary carbon C-1 (δ_C 151.7). H-2 also showed a strong correlation to C-4 (δ_C 131.3), making this the most probable site for the attachment of the isoprenoid chain. The COSY correlation of H-3 to the methylene group CH_2-1′ (δ_C 28.9, δ_H 4.05) of the side chain confirmed this. The first aromatic ring was completed by the quaternary carbons C-10a (δ_C 136.8) and C-4a (δ_C 141.3), whose signals had chemical shifts consistent with the proximity of nitrogen atoms. The second aromatic ring was the phenazine core structure, comprising the methine groups CH-7 (δ_C 110.5, δ_H 7.25) to CH-9 (δ_C 120.0, δ_H 7.73) and three quaternary carbons. The sequence CH-7 to CH-9 was unambiguous and proven by COSY and HMBC correlations as well as signal splitting patterns. HMBC correlations confirmed the position of the hydroxy group and completed the second aromatic ring, showing the connection to the nitrogen-bearing quaternary aromatic carbons C-5a (δ_C 135.3) and C-9a (δ_C 142.3). Thus, the phenazine substructure was established. The phenazine substructure explains 10 degrees of unsaturation, but the molecular formula of **1** demanded 12 degrees of unsaturation and two remained to be accounted for. The side chain had to include two double bonds. The methylene group CH_2-1′ was adjacent to the first double bond $\Delta^{2',3'}$, as shown by the coupling of H-1′ and H-2′ (δ_H 5.54) together with the appropriate long-range correlations. This double bond was substituted with the methyl group CH_3-10′ (δ_C 16.2, δ_H 1.80), whose HMBC data also showed the chain to start with the methylene group CH_2-4′ (δ_C 40.5, δ_H 2.10), which concluded the first isoprenoid building block of the side chain. As it was a trisubstituted double bond, the configuration of $\Delta^{2,3}$ could not be determined by analysis of coupling constants. The *E*-configuration of the double bond was deduced from NOESY correlations, which showed the proximity of CH_3-10′ and CH_2-1′ as well as CH-2′ and CH_2-4, but not CH-2′ and CH_3-10′. Starting with CH_2-5′ (δ_C 27.3, δ_H 2.13), which coupled to the neighboring CH_2-4′, a second isoprenoid building block, which terminated with the methylene groups CH_3-8′ (δ_C 25.8, δ_H 1.59) and CH_3-9′ (δ_C 17.7, δ_H 1.55), was identified.

Thus, the structure of **1** could be unequivocally proven with one exception: from the spectroscopic data it was impossible to discriminate between a 1,6-phenazinediol and a 1,9-phenazinediol core structure. A similar problem has been encountered by researchers describing related structures. In the case of JBIR-46-48[10] the problem could be solved by NOESY correlations from the hydroxy groups to methylene groups of the isoprenoid chains which were connected to the other aromatic ring. However, comparable NOESY correlations were not observed for **1**. In order to determine the constitution, **1** was acetylated using pyridine-acetic anhydride, which yielded compound **2**. As expected, two additional signals appeared in the ^{1}H NMR spectrum accounting for one acetylmethyl group each. The respective NOESY spectrum showed no correlation of the two methyl signals to each other, but a correlation from one of the signals to CH_2-1′ of the isoprenoid side chain (Figure 2). Thus, we concluded the core structure to be 1,6-phenazinediol, a substitution pattern that is congruent with that of other naturally occurring phenazines.[9−11]

Phenazines can be subdivided into different groups, in which the core structure can be substituted with hydroxy, methyl, or carboxy groups or with larger residues.[1] Terpenoidal phenazines belong to the more complex phenazines, which are not as widespread as the simple ones and typically produced by *Streptomyces* rather than by *Pseudomonas* species.[12]

Geranylphenazinediol (**1**) was moderately active against human acetylcholinesterase, with an IC_{50} value of 2.62 μM (Table 2). **1** and **2** were weakly active against *Bacillus subtilis* and inactive against fibroblasts (see Supporting Information). Interestingly, the derivative diacetylgeranylphenazinediol (**2**) showed a similar activity spectrum (Table 2). Neither **1** nor **2** was active against *Xanthomonas campestris* or *Candida albicans*.

Of the compounds tested, only **1**, **2**, and 1,6-phenazinediol inhibited human acetylcholinesterase, suggesting that the 1,6-hydroxylation of the phenazine core structure is important for this activity.

Compared to the reference huperzine A, the inhibitory potential of **1** is very weak (Table 2). On the other hand, this certainly does not mean that its pharmacological potential is not of interest. Galantamine, one of the newer anticholinergic drugs on the market, inhibits AChE with an IC_{50} value of only about 1 μM,[13] which is in a range comparable to the inhibitory activity of geranylphenazinediol. The inhibitory activity of this new phenazine against acetylcholineserase justifies further research.

Table 1. NMR Spectroscopic Data for 1 and 2 in Acetone-d_6 (500 MHz)

	geranylphenazinediol (1)						diacetylgeranylphenazinediol (2)	
position	δ_C, type	δ_H (J in Hz)	COSY	HMBC	NOESY		δ_H (J in Hz)	NOESY
1	151.7, C							
2	110.3, CH	7.17, d (7.7)	3	1, 4, 4a, 10a	3		7.61, d (7.7)	3
3	130.4, CH	7.61, dt (7.7, 1.0)	2, 1′	1, 2, 4a, 1′	2, 1′, 2′		7.76, dt (7.7, 1.0)	2, 1′, 2′
4	131.3, C							
4a	141.3; C							
5a	135.3, C							
6	153.8, C							
7	110.5, CH	7.25, dd (7.7, 1.0)	8, 9	5a, 6, 9, 9a	8		7.71, dd (7.7, 1.0)	8
8	132.4, CH	7.82, dd (8.9, 7.7)	7, 9	9a, 7, 6, 5a	7		7.97, dd (8.9, 7.7)	7, 9
9	120.0, CH	7.73, dd (8.9, 1.0)	7, 8	6, 7, 8, 5a			8.14, dd (8.9, 1.0)	8
9a	142.3, C							
10a	136.8, C							
1′	28.9, CH_2	4.05, br d (7.4)	3, 2′, 4′, 10′	2,, 3, 4, 4a, 2′, 3′, 4′, 5′	3, 2′, 10′		4.06, br d (7.4)	3, 2′, 10′, 6-acetyl-CH_3
2′	123.9, CH	5.54, tq (7.4, 1.2)	1′, 4′, 10′	1′, 4′, 10′	3, 1′, 4′		5.61, tq (7.4, 1.2)	3, 1′, 4′
3′	136.9, C							
4′	40.5, CH_2	2.10, m	1′, 2′, 5′	2′, 3′, 5′, 6′, 10′	2′		2.08, m	2′, 6′
5	27.3, CH_2	2.13, m	4′, 6′, 8′, 9′	3′, 4′, 6′, 7′	6′		2.12, m	9′, 10′
6′	125.1, CH	5.10, tsept. (6.8, 1.4)	5′, 8′, 9′	5′, 8′, 9′	5′, 8′		5.09, tsept. (6.8, 1.4)	4′, 8′
7′	131.8, C							
8′	25.8, CH_3	1.59, br s	5′, 6′	6′, 7′, 9′	6′		1.58, br s	6′
9′	17.7, CH_3	1.55, br s	5′, 6′	6′, 7′, 8′			1.54, br s	5′
10′	16.2, CH_3	1.80, br s	1′, 2′	2′, 3′, 4′	1′		1.82, br s	1′, 5′
OH		9.01				1-acetyl-CH_3	2.48, s	
OH		9.20				6-acetyl-CH_3	2.52, s	1′

Table 2. IC_{50} Values [μM] for Inhibition of Acetylcholinesterase Activity

	AChE IC_{50} [μM]
geranylphenazinediol (**1**)	2.62 ± 0.35
diacetylgeranylphenazinediol (**2**)	2.01 ± 0.02
1,6-phenazinediol	4.52 ± 0.46
2-hydroxyphenazine	>50
phenazine-1-carboxylic acid	>50
endophenazine A	>50
endophenazine B	>50
huperzine A	0.012 ± 0.001

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were obtained on a NanoVue (GE Healthcare) spectrometer. NMR spectra were recorded on a Bruker DRX500 spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively), using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 2.04 and δ_C 29.8 ppm for acetone-d_6). High-resolution mass spectra were acquired on a benchtop time-of-flight spectrometer (MicrOTOF-II, Bruker Daltonics) with positive electrospray ionization. Analytical reversed-phase HPLC-UV/MS experiments were performed using a C_{18} column (Phenomenex Onyx Monolithic C_{18}, 100 × 3.00 mm) applying an H_2O (A)/CH_3CN (B) gradient with 0.1% HCOOH added to both solvents (gradient: 0 min 5% B, 4 min 60% B, 6 min 100% B; flow 2 mL min^{-1}) on a VWR Hitachi Elite LaChrom system (VWR International GmbH, Darmstadt, Germany) coupled to an ESI-ion trap detector (Esquire 4000, Bruker Daltonics).

Preparative HPLC was carried out using a VWR Hitachi system consisting of an L-7150 pump, an L-2200 autosampler, and an L-2450 diode array detector.

Acetylation of 1. A 0.8 mg amount of **1** was dissolved in 250 μL of pyridine and cooled. To this solution was added 250 μL of acetic anhydride, and the resulting solution was mixed and left at room temperature overnight. The solvents were removed under a stream of nitrogen, so that only the acetylated product, diacetylgeranylphenazinediol (**2**), remained.

Isolation and Identification of *Streptomyces* sp. LB173. For isolation of the strain LB173, a sample was taken from the ambient sediment of *Saccharina latissima* rhizoids growing in the Kiel Fjord, Baltic Sea (Germany). The sediment sample was collected by scuba diving in January 2010 at a depth of approximately 6 m. The sample was suspended in sterile seawater and homogenized. The suspension was diluted with sterile seawater, plated on medium P according to Kalinovskaya et al.,[14] and modified by supplementing with 18 g of Tropic Marin sea salt (Dr. Biener GmbH, Wartenberg, Germany) and 15 g of Bacto agar in 1 L of deionized water, pH 7.6. A colony of strain LB173 was picked from the agar plate after 11 days of incubation at 20 °C. Pure cultures were obtained by subsequent plating steps on GYM4 agar plates (10 g of glucose, 4 g of yeast extract, 4 g of malt extract, and 15 g of Bacto agar in 1 L of deionized water, pH 7.2). The pure cultures were stored at −100 °C using the Cryobank System (Mast Diagnostica GmbH, Reinfeld, Germany) according to the manufacturer's instructions.

For taxonomical characterization the DNA extraction was performed with the QIAGEN DNeasy tissue kit (QIAGEN, Hilden, Germany). The digestion of the bacterial cell wall was achieved with an enzymatic lysis buffer, containing 20 mg mL^{-1} lysozyme (Sigma Life Science, München, Germany). For amplification of the 16S rRNA gene the universal eubacterial primers 27F and 1492R[15] (MWG Biotech AG, Ebersberg, Germany) and the DreamTaq Green PCR Master Mix (Fermentas, St. Leon-Rot, Germany) were used. The resulting sequences were compared with sequences in the EMBL nucleotide database available online at the European Bioinformatics Institute homepage using the Basic Local Alignment Search Tool (nucleotide BLAST)[16] and the RDP-II Project homepage.[17]

A well-grown agar plate of strain LB173 was prepared by critical point drying and subsequently sputter-coated with gold/palladium. Micrographs were made with a Zeiss DSM940 scanning electron microscope (Zeiss, Jena, Germany).

dx.doi.org/10.1021/np2009626 | J. Nat. Prod. 2012, 75, 1400−1404

The strain *Streptomyces* sp. LB173 is deposited at Kieler Wirkstoff-Zentrum (KiWiZ) at the Helmholtz-Zentrum für Ozeanforschung (GEOMAR, Am Kiel-Kanal 44, 24106 Kiel, Germany).

Fermentation and Isolation. Strain LB173 was grown in SPS medium consisting of starch 10 g, peptone from soymeal (Merck, Darmstadt, Germany) 5 g, and artificial sea salt (Tropic Marin, Dr. Biener GmbH, Wartenberg, Germany) 10 g per liter of deionized water. Two-liter flasks with a single baffle were used for the fermentation. Each flask was filled with 1 L of SPS medium and inoculated with a piece of well-grown agar plate. The fermentation was carried out for 7 days on a rotary shaker at 120 rpm and 28 °C.

For the isolation of **1**, a 10 L fermentation in SPS medium was harvested. Culture broth and cell mass were separated by centrifugation. To the wet cell mass (approximately 0.75 L) was added 2 L of EtOH, and the mixture was homogenized. The cells were separated from the liquid by centrifugation and homogenized again with another 2 L of EtOH. After further centrifugation the organic supernatants were combined and concentrated *in vacuo* until an aqueous phase remained. This was extracted twice with 500 mL of EtOAc. The organic phases were combined and dried *in vacuo* to give 1.98 g of crude extract, which was fractionated by column chromatography on Sephadex LH-20 (3 × 120 cm, MeOH). Fractions containing **1** were pooled, concentrated, and purified by preparative RP-HPLC (Phenomenex Gemini C_{18} 110A AXIA, 100 × 21.20 mm, Phenomenex, Aschaffenburg, Germany) with CH_3CN−0.1% HCOOH(aq) as mobile phase with a gradient from 80% to 100% CH_3CN in 15 min. This yielded 6 mg of **1** with a retention time of 5.7 min.

Assays for Antimicrobial Activity. Assays were performed using *Bacillus subtilis* DSM 347, *Xanthomonas campestris* DSM 2405, and *Candida albicans* DSM 1386. TSB medium (1.2% tryptic soy broth, 0.5% NaCl) was used for the cultivation of the bacteria, and M186/3 (0.1% yeast extract, 0.1% malt extract, 0.17% peptone from soybeans, 0.33% glucose) for cultivation of the yeast. Overnight cultures of the test organisms were diluted with medium to an optical density (600 nm) of 0.01−0.05. Test substances, dissolved in DMSO, were transferred into a 96-well microtiter plate, and 200 μL of the cell suspension cultures was added to each well. After the microtiter plates were incubated for 5 h at 37 °C (*B. subtilis and C. albicans*) or 6 h at 28 °C (*X. campestris*), 10 μL of a resazurin solution (0.2 mg mL^{-1} in phosphate-buffered saline) was added, and the plates were incubated for another 5−60 min. To evaluate cell viability, the reduction of resazurin to resorufin was assessed by measuring the absorbance at 600 nm (reference 690 nm). The resulting values were compared with a positive (10 μM chloramphenicol for bacteria; 10 μM nystatin for the yeast) and a negative control (no compound) on the same plate.

Cytotoxicity Assays. The sensitivity of the human fibroblast cell line KIF5 (kindly provided by Prof. H. Kalthoff, Institute for Experimental Cancer Research, Division of Molecular Oncology, University Hospital Schleswig Holstein, Kiel) to the compounds was evaluated by monitoring the metabolic activity using the CellTiterBlue cell viability assay (Promega, Mannheim, Germany).

KIF5 cells were maintained in RPMI 1640 medium (Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Life Technologies, Darmstadt, Germany), 100 IU mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Promocell, Heidelberg, Germany). The cultures were maintained at 37 °C under a humidified atmosphere and 5% CO_2. The cell lines were transferred every 3 or 4 days.

For the cytotoxicity assays, cells were seeded in 96-well plates at a concentration of 5000 cells per well. After 24 h incubation, the medium was removed, and 100 μL of each sample (adjusted to final concentrations of 10 and 50 μM by diluting in growth medium) was added to the cells. Each sample was prepared in triplicate. Tamoxifen (25 μM) was used as a positive control. Following compound addition, plates were incubated for 24 h at 37 °C. Afterward, the assay was performed according to the manufacturer's instructions. Cells were incubated once more for 2 h at 37 °C. Fluorescence was measured using the microplate reader Infinite M200 with an excitation wavelength of 560 nm and an emission of 590 nm.

Acetylcholinesterase Assay. To measure the activity of the test compounds against the human acetylcholinesterase, a modified Ellman's method[18] was applied. The enzyme (50 mU mL^{-1}), the co-substrate 5,5-dithiobis-2-nitrobenzoic acid (150 μM), and the test sample were incubated for 10 min at 37 °C. Everything was dissolved in phosphate buffer (100 mM Na_2HPO_4, 100 mM NaH_2PO_4, pH = 7.4) to a final volume of 180 μL per well. The reaction was started with 20 μL of acetylthiocholine iodide (140 μM). After 5 min the absorbance at 412 nm was measured. Huperzine A (0.1 and 1 μM) was used as a positive control for the inhibition of AChE. All substances were ordered from Sigma (Munich, Germany).

Phenazine Samples. Phenazine-1-carboxylic acid and 2-hydroxyphenazine were isolated from the culture broth of *Pseudomonas chlororaphis* subsp. *aurantiaca* DSM19603, the endophenazines were obtained from *Streptomyces cinnamonensis* DSM1042, and 1,6-phenazinediol was obtained from *Xenorhabdus szentirmaii* DSM16338^T. Most of the compounds have already been reported from these strains. The identity of each compound was verified by HPLC-DAD/MS, and for phenazine-1-carboxylic acid and 2-hydroxyphenazine also by ^{1}H NMR. Purity of the compounds was controlled by HPLC-DAD/MS. The structure of 1,6-phenazinediol was not absolutely verified, but like iodinin, an N-hydroxylated derivative of 1,6-phenazinediol, it is reported to be produced by *Xenorhabdus*.[19] Further verification was not deemed necessary.

*Geranylphenazinediol (**1**):* yellow, amorphous solid; UV (MeOH + 25% H_2O) λ_{max} (log ε) 204 (4.29), 273 (4.67), 354 (3.47), 373 (3.52), 458 (3.40) nm; for 1D and 2D NMR data see Table 1; HRESIMS *m/z* 349.1917 (calcd for $C_{22}H_{25}N_2O_2$, 349.1911 $[M + H]^+$).

*Diacetylgeranylphenazinediol (**2**):* yellow, amorphous solid; UV (MeOH + 25% H_2O) λ_{max} (log ε) 217 (4.26), 259 (4.77), 364 (3.96), 410 (3.60) nm; for ^{1}H NMR and NOESY data see Table 1; HRESIMS *m/z* 433.2123 (calcd for $C_{26}H_{29}N_2O_4$, 433.2122 $[M + H]^+$).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and selected 2D NMR data for compounds **1** and **2**, as well as a description of the strain. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors gratefully thank R. Schmaljohann for microscopy, and G. Kohlmeyer-Yilmaz, M. Höftmann, and Dr. F. Sönnichsen for running and processing NMR experiments. This study is from the Kieler Wirkstoff-Zentrum KiWiZ, which was supported by the Ministry of Science, Economic Affairs and Transport of the State of Schleswig-Holstein (Germany), in the framework of the "Future Program for Economy", which is co-financed by the European Union (EFRE).

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