Nocapyrones A–D, γ-Pyrones from a Nocardiopsis Strain Isolated from the Marine Sponge Halichondria panicea

Imke Schneemann, Birgit Ohlendorf, Heidi Zinecker, Kerstin Nagel, Jutta Wiese, and Johannes F. Imhoff*

Kieler Wirkstoff-Zentrum (KiWiZ) at the Leibniz-Institute of Marine Sciences (IFM-GEOMAR), Am Kiel-Kanal 44, 24106 Kiel, Germany

Received May 11, 2010

Four new γ-pyrones, nocapyrones A–D (1–4), were isolated from an organic extract of the Nocardiopsis strain HB383, which was isolated from the marine sponge Halichondria panicea. These are the first γ-pyrones reported from a Nocardiopsis strain. The structures were elucidated on the basis of one- and two-dimensional NMR experiments and supported by HPLC-UV/MS and HRESIMS analyses. The biosynthesis of nocapyrone A was investigated by feeding experiments with 13C-labeled compounds. In addition, one diketopiperazine, which was only known as a synthetic compound before, was isolated. The bioactivities of 1, 2, and the diketopiperazine were evaluated in a panel of assays.

Nocardiopsis belongs to the Actinobacteria and was designated as a distinct genus in 1976.1 It is set apart from similar genera such as Actinomadura, Nocardia, and Streptomyces by characteristics such as type of sporulation, morphology of aerial and substrate mycelium, cell wall lipid composition, and susceptibility to lysozyme.1 Due to the wide distribution of members of the genus, it is not surprising that it has already been isolated from marine sponges as well. Considering the facts that Actinobacteria are exceptionally potent producers of secondary metabolites and that the rediscovery rates of known compounds are high in intensively studied genera such as Streptomyces species, strategies such as focusing on less common taxa, e.g., Nocardiopsis species, and isolating bacteria from extraordinary and promising habitats such as sponges have become more and more popular.2,3 Halichondria panicea is a sponge species suitable for such an approach, because it has been reported to harbor a vast number of different microorganisms, among them also several Actinobacteria.4 The majority of Actinobacteria isolated from H. panicea belong to the well-studied genus Streptomyces. Among 46 actinobacterial isolates from H. panicea only one was classified as belonging to the genus Nocardiopsis.5

Nocardiopsis strain HB383 was chosen for further investigations due to unidentified peaks in the LC-UV/MS chromatogram and antibacterial activity of the organic extract. This strain was selected in a study focusing on the discovery of new secondary metabolites from marine Actinobacteria associated with the sponge H. panicea.6 By 16S rRNA gene sequence analysis this strain was identified as a member of the genus Nocardiopsis with 99.8% similarity to Nocardiopsis alba DSM 43377. The HPLC-MS analysis of the EtOAc extract displayed four main peaks in the UV chromatogram, which could be assigned to the masses 334, 320, 286, and 268. On the basis of UV, mass, and NMR data the first three peaks presumably belonged to a number of diketopiperazines that have not yet been described for Nocardiopsis species. The fourth peak belonged to a new γ-pyron, which, together with three derivatives, will be introduced in this paper.

With the aim of obtaining the new metabolites, the extract was separated using preparative reversed-phase (C18) HPLC, which yielded nocapyrones A (1) and B (2). Another HPLC fraction yielded the derivatives nocapyrones C (3) and D (4) after further purification.

Nocapyrones A (1) and B (2) were amorphous, colorless solids with molecular weights of m/z 269.1847 [M + H]+ and 253.1805 [M + H]+, respectively. These results of the high-resolution mass measurements yielded the molecular formulas of C15H23O4 for 1 and C15H23O3 for 2, which were entirely consistent with the spectroscopic data. The structure elucidation of the two compounds was mainly based on the NMR spectra of 1. The respective 13C NMR spectrum displayed 14 distinct signals. Not only was the signal of C-11/12 (δC 29.4) much more intense than the other carbon signals, but the corresponding 1H signal additionally integrated to six protons, which evidenced the presence of two magnetically equivalent methyl groups. Three additional methyl groups, C-13 (δC 10.3), C-14 (δC 7.2), and C-15 (δC 56.5), were present, with the shift of C-15 proving it to be a methoxy group. Furthermore, the molecule comprised a chain of four methylene groups, CH2–6 to CH2–9 (δC 31.7, δH 2.72; δC 28.8, δH 1.71; δC 25.0, δH 1.46; and δC 44.5, δH 1.50), and four olefinic quaternary carbons, C-1 (δC 164.8), C-2 (δC 100.2), C-4 (δC 119.3), and C-5 (δC 161.4). Two of these quaternary carbons, C-1 and C-5, were located adjacent to oxygen atoms as proven by their 13C resonances of 164.8 and 161.4 ppm. Finally, the signal with a chemical shift of 183.4 ppm gave evidence of a carbonyl function being present. A 1H–13C HSQC spectrum correlated all proton resonances to the corresponding 13C NMR resonances of directly bonded carbon atoms. A 1H–1H COSY spectrum revealed that the methylene groups constitute the only coherent spin system, which later was identified as belonging to the side chain of a γ-pyron core structure. The final structure of the molecule was elucidated with the help of a 1H–13C HMBC spectrum (Figure 1).

The molecular formula C15H23O4 implied four degrees of unsaturation, meaning that apart from the carbonyl group and two double bonds, one ring had to be part of the structure. This was in good agreement with a γ-pyron moiety being the core structure of the molecule. This deduction was additionally supported by the UV maxima (253 and 215 nm), which fit nicely with literature data of γ-pyrones.9 Consequently, the γ-pyron skeleton consisted of the olefinic carbons and the carbonyl group, C-1 to C-5, with C-1 and C-5 being connected via an oxygen atom. The 1H chemical shifts of CH2–13 and CH2–14 as well as their 1H–13C HMBC correlations to the quaternary carbon atoms clearly showed that they were located at positions 2 and 4 of the γ-pyron ring (HMBC
correlations from CH3-11 (δH 1.94) to C-3, C-4, C-5 and from CH2-14 (δH 1.81) to C-1, C-2, and C-3. The methoxy group OCH3-13 was also directly attached to the pyrone ring, as shown by the correlation of the resonance of H-15 (δH 4.03) to the resonance of C-1. Therefore, the methoxy group had to be attached to C-1. The H-6 signal correlated to the signals of C-7 and C-8, as well as to those of C-4 and C-5. As mentioned above, the CH2 groups CH2-6 to CH2-9 formed a spin system, as shown by strong COSY correlations and the resulting multiplicities of the signals. The methylene chain terminates with the quaternary carbon C-10 (δC 71.4), which is substituted with the equivalent methyl groups CH3-11 and CH3-12 (δC 29.4, δH 1.17) as well as a hydroxy group. All spectroscopic data are in good accordance with the determined structure.

Overall, the 1H NMR chemical shifts of nocapyrone B (2) were close to being identical to those of nocapyrone A (1). The only differences were the appearance of an additional signal for H-10 (δH 1.60) and the upfield shift of the signal of the methyl protons H2-11 and H2-12 (δH 0.93). The upfield shift already indicated the loss of the hydroxy group. This assumption was further supported by the mass difference of 16 and the fact that the signal of the methyl groups in question now appeared as a doublet due to coupling with H-10. Thus, the structure of nocapyrone B (2) was established.

The UV spectra of nocapyrones C (3) and D (4) were almost identical to those of the other two derivatives. Consequently, the chromophore of the structures was very likely to be the same. This was proven by analyses of one- and two-dimensional NMR spectra (Table 2 and SI). The 1H NMR and 13C NMR shifts of the methyl groups CH3-13, CH3-14, and CH3-15 as well as those of 1H-13C HMBC correlations fit perfectly with the assigned γ-pyrones substructure. Thus, similar to 1 and 2, the structures of 3 and 4 differed only in the side chain of the molecule. The molecular formulas C16H26O4 for 3 and C17H28O4 for 4 proved the chain to be longer than those of the nocapyrones A (1) and B (2). Due to uniformity in the UV and MS data of compound 3, but doubled signals in NMR measurements, we presume the presence of two isomers of compound 3. Like 1, nocapyrone C had two methyl groups, CH2-12 (δH 20.0, δC 1.11) and CH2-16 (δC 14.9, δH 0.88), included in the aliphatic side chain, each of them forming a doublet in the 1H NMR spectrum. The terminal methyl group CH3-12 showed a 1H-1H COSY correlation to H-11 (δH 3.62) and additionally a 1H-15C HMBC correlation to C-10 (δC 40.8), which established the sequence from C-12 to C-10. The shift of CH-11 (δH 3.62, δC 71.8) proved it to be an oxygen-bearing methine, whereas CH-10 (δH 1.45) was the methine group located adjacent to CH2-16. The side chain was completed by the methyl groups CH2-6 (δH 2.72, δC 31.5) to CH2-9 (δH 1.3–1.5, δC 33.3). Compared to 3, in the 1H NMR spectrum of nocapyrone D (4) only one of the side chain methyl groups, CH2-16 (δH 1.08, δC 16.5), appeared as a doublet. The other one appeared as a singlet at δH 2.14 and δC 28.3 ppm. These data suggested the terminal methyl group to be connected to a carbonyl carbon. Compared to nocapyrone C, there is no oxygen-bearing methine group in 4. Instead C-10 is a quaternary carbon with a shift of 215.3 ppm, which showed it to be a keto group. Thus, the only difference between the nocapyrones C and D is the hydroxy group in the former that is replaced by a keto group in the latter.

Although these are the first γ-pyrone derivatives described from a Nocardiodisps strain, natural products based on such a substructure are widespread and constitute a large class of biologically active compounds.7,8 Compounds containing the same dimethylated and methoxylated pyrone substructure as the nocapyrones are relatively common, with examples being aureothin9 and actinopyrone,6 which was first reported to have vasodilating and antibacterial activities. Further studies complemented this activity spectrum with promising activities against Helicobacter pylori.10

According to the widespread nature of γ-pyrones, the biosynthesis of structures related to the nocapyrones has been studied before. Molecules belonging to this compound class have been reported to be synthesized via different pathways including type I, type II, and type III PKSs.11 A bacterial γ-pyrene with a well-explored biosynthesis is the Streptomyces metabolite aureothin.9 Aureothin is a type I PKS product generated by the assembly of a p-nitrobenzoate starter unit, one acetate, and four propionate building blocks.

By analogy, we suggest nocapyrone A to be a polyketide composed of an isobutyrate starter unit, two acetates, and two propionate building blocks. Results of feeding experiments with 13C-labeled acetate, propionate, and methionine (see Figure 2 and SI) support this assumption; that is, feeding of 1-13C-propionate led to an enhancement of the 13C NMR signals of C-1 and C-3. The analogous experiment with 1-13C-acetate resulted in an intensification of the carbon resonances of C-5 and C-7. Finally, the signal of C-15 became enhanced after feeding with [Me1-13C] methionine, which demonstrated the methyl group to be S-adenosylmethionine (SAM)-derived. Therefore, in a similar fashion to that described for aureothin we propose the formation of an α-pyrene that is transformed to the γ-pyrene in the course of the methylation by an O-methyltransferase.

Despite the inhibition of the Gram-positive bacteria Staphylococcus lentus and Bacillus subtilis by the extract, the isolated nocapyrones A (1) and B (2) showed no bioactivities against a range of Gram-positive and Gram-negative bacteria and the yeast Candida glabrata. Furthermore, in vitro cytotoxicity analyses revealed no activity against the mouse fibroblast cell line NIH-3T3 as well as the human hepatocellular carcinoma cell line HepG2 and the human colon adenocarcinoma cell line HT-29. Additionally, 1 and 2 did not inhibit phosphodiesterase 4, protein tyrosine phosphatase 1B, acetylcarnitine, acetylcholinesterase, reverse transcriptase, or glycogen synthase kinase 3β.

As already mentioned above, strain HB383 produces not only the nocapyrones but also three molecules belonging to the diketopiperazine compound class. As far as these compounds are concerned, only one (2E/5Z)-2-[(4-methoxyphenyl)methylene]-5-(2-methylpropylylen)-3,6-piperazinedione (5) (Figure 3)—could be sufficiently purified for structure elucidation. The molecular formula C16H18N2O3 of 5 was established by HRESIMS (m/z 309.1219 [M + Na]+, calc 309.1210), and the structure was elucidated based
Therefore, isomerization of the to H-11 was observed in one of the isomers, but not in the other. Contrary to that, a NOESY correlation from H-1 under the applied conditions.

were observed. As the two double bonds in the molecule, the formation of four different isomers is possible, yet only two signals in the NMR spectra. With two double bonds in the molecule, rapidly reverted to a mixture again, which led to doubled sets of though they seemed to be separable by HPLC, the single compounds.

were determined by 1D and 2D NMR experiments (1H, 13C, 1H-13C-HSQC, and 1H-13C-HMBC). The data matched those of a synthetic compound perfectly,\textsuperscript{1,2} but it was isolated from a natural source for the first time.

Compound 5 was isolated as a mixture of two isomers. Even though they seemed to be separable by HPLC, the single compounds rapidly reverted to a mixture again, which led to doubled sets of signals in the NMR spectra. With two double bonds in the molecule, the configuration could not be deduced from the coupling constants of connected protons. Therefore, a NOESY spectrum was measured in order to clarify the configurations of the double bonds. Therefore, isomerization of the double bond readily occurs under the applied conditions.

Compound 5 exhibited weak cytotoxic activity. The treatment of NIH-3T3 and HepG2 cells with 50 \( \mu \text{M} \) 5 resulted in a reduction of the metabolic activities by 64\% and 44\%, respectively. Although 5 is described for the first time as a natural product, \( \delta \)-configured in \( \alpha \)-methylated derivative. For these two compounds molecular weights of 320.34 and 334.37, respectively, have been reported, which fit in well with the peaks detected in the extract of strain HB383.

### Experimental Section

#### General Experimental Procedures.

The optical rotation was measured on a Perkin-Elmer model 241 polarimeter. UV spectra were obtained on a NanoVue (GE Healthcare). NMR spectra were recorded on a Bruker DRX500 spectrometer (500 and 125 MHz for 1H and 13C NMR, respectively), using the signals of the residual solvent protons as internal references (\( \delta \text{H} \) 3.31 and \( \delta \text{C} \) 49.0 ppm for methanol-\( \text{d}_4 \); \( \delta \text{H} \) 2.50 and \( \delta \text{C} \) 49.31 ppm for DMSO-\( \text{d}_6 \)). High-resolution mass spectra were acquired on a Bruker Daltonics with positive electrospray ionization. Analytical reversed-phase HPLC/UV/MS experiments were performed with a C\text{18} column (Phenomenex, Bruker Daltonics) with positive electrospray ionization. Analytical reversed-phase HPLC/UV/MS experiments were performed on a Bruker DRX500 spectrometer (500 and 125 MHz for 1H and 13C NMR, respectively), using the signals of the residual solvent protons and the solvent carbons as internal references (\( \delta \text{H} \) 3.31 and \( \delta \text{C} \) 49.0 ppm for methanol-\( \text{d}_4 \); \( \delta \text{H} \) 2.50 and \( \delta \text{C} \) 49.31 ppm for DMSO-\( \text{d}_6 \)).

#### Isolation and Identification of Nocardiopsis sp. HB383

Strain HB383 was obtained from the marine sponge \textit{Halichondria panicea} collected from the Baltic Sea (Germany). DNA extraction, 16S rDNA amplification, and 16S rRNA gene sequencing as well as the comparison of this sequence (1395 nucleotides; GenBank/EMBL/DDBJ acc. no GQ863933) with sequences in the EMBL nucleotide database available online at the European Bioinformatics Institute homepage using the Basic Local Alignment Search Tool (nucleotide BLAST) and of RDP-II Project homepage were done as described in Schneemann et al.\textsuperscript{5} The sequence analysis revealed that the strain was affiliated with the genus \textit{Nocardiopsis}. The most closely related type strain was \textit{Nocardiopsis alba} DSM 43377\textsuperscript{7} (GenBank/EMBL/DDBJ acc. no. X978883) with a sequence identity of 99.8\%.

#### Cultivation.

For chemical analyses \textit{Nocardiopsis} sp. HB383 was grown in GYMCaCl\textsubscript{2} medium (0.4 g glucose, 0.4 g yeast extract, 0.4 g malt extract, 0.2 g CaCl\textsubscript{2}, 1 L H\textsubscript{2}O, pH 7.2) for 18 days at 28 °C and 120 rpm in 12 × 2000 mL Erlenmeyer flasks in the dark. Each flask was inoculated with 10 mL of a 3-day-old GYM4 preculture (0.4 g glucose, 0.4 g yeast extract, 0.4 g malt extract, 0.2 g CaCO\textsubscript{3}, 100 mL H\textsubscript{2}O, pH 7.2; 120 rpm, 28 °C). These precultures were inoculated with cryoconserved cultures of strain HB383.

#### Isolation of Nocapyrones A—D and 5.

The culture broth (12 L) was homogenized with an Ultra Turrax T25 basic (IKA-Werke GmbH.
and Co., Staufen, Germany) at 16,000 rpm for 30 s and extracted with 6 L of EtOAc. The solution was dried to yield 804 mg of an organic extract. An aliquot was analyzed with HPLC-UV/MS. Nocapyrones A (1) and B (2) were detected at 3.2 and 4.5 min, respectively.

Subsequently, the extract was subjected to preparative HPLC (eluents: H₂O (A); MeCN (B); gradient 0 min 5% B, 16 min 70% B, 16.5 min 100% B; flow 18 mL/min). Nocapyrones A (3.7 mg) and B (0.3 mg) eluted at 6.5 and 9.1 min, respectively.

Nocapyrones C (3) and D (4) were isolated from out of combined extracts that were fractionated on a Sephadex LH 20 column with MeOH as eluent. One fraction (approximately 20 mg) yielded 4.8 mg of crude 3 and 2.6 mg of crude 4 by HPLC separation (45% ACN to 55% ACN in 13 min). Compounds 3 and 4 eluted at 4.8 and 5.5 min, respectively. The compounds were purified further on a semipreparative normal-phase column. Applying a gradient from 2-propanol/hexane (25:75) to 2-propanol/hexane (20:80) in 16 min and a flow of 5 mL/min, the derivatives eluted at 7.2 min (3) (0.9 mg of a mixture of two isomers) and 9.1 min (4) (0.2 mg), respectively.

Compound 5 precipitated in the MeOH extracts as a colorless solid. Part of the precipitate was washed with H₂O several times. Subsequently MeOH (4 x 1 mL) was added. The supernatant was subjected to preparative HPLC, and separation was carried out by a H₂O/ACN gradient of 45% ACN to 90% ACN in 13 min. Compound 5 eluted at 5.0 min (0.6 mg of a mixture of two isomers).

**Biogenic Studies.** For the labeling experiments 1-13C-sodium acetate, 1-13C-sodium propionate, and methyl-13C-methionine were added in the form of sterile filtered solutions (dissolved in H₂O). The isolation of nocapyrone A was carried out according to the isolation procedure described above. The yields of labeled nocapyrone A were approximately 1 mg in each batch.

**Nocapyrone A (1):** colorless, amorphous solid; UV (MeOH) \( \lambda_{\text{max}} \) (log e) 253 (4.06), 204 (4.00) nm; for 1D and 2D NMR data see Table 1 and SI; HRESIMS m/z 269.1847 [(M + H)]⁺ (calcd for C₁₅H₂₅O₃, 269.1847).

**Nocapyrone B (2):** colorless, amorphous solid; UV (MeOH) \( \lambda_{\text{max}} \) (log e) 253 (4.02), 209 (4.07) nm; for 1D and 2D NMR data see Table 2 and SI; HRESIMS m/z 253.1798 [(M + H)]⁺ (calcd for C₁₅H₂₅O₄, 253.1798).

**Nocapyrone C (3):** colorless, amorphous solid; [α]_D^20 +11 (c 0.07, MeOH); UV (MeOH) \( \lambda_{\text{max}} \) (log e) 253 (4.06), 204 (4.00) nm; for 1D and 2D NMR data see Table 2 and SI; HRESIMS m/z 283.1903 [(M + H)]⁺ (calcd for C₁₆H₂₆O₄, 283.1903).

**Nocapyrone D (4):** colorless, amorphous solid; [α]_D^20 +120 (c 0.005, MeOH); UV (MeOH) \( \lambda_{\text{max}} \) (log e) 253 (4.06), 205 (4.10) nm; for 1D and 2D NMR data, see Table 2 and SI; HRESIMS m/z 281.1749 [(M + H)]⁺ (calcd for C₁₆H₂₆O₄, 281.1747).

**Antibacterial Activities.** Antimicrobial assays of compounds 1, 2, and 5 were performed using Bacillus subtilis (DSM 347), Erwinia amylovora (DSM 50001), 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), Staphylococcus epidermidis (DSM 20044), Staphylococcus lenta (DSM 6672), Xanthomonas campestris (DSM 2405), and the yeast Candida glabrata (DSM 6425). The assays were performed according to Schneemann et al.**

**Cytotoxicity Assays.** The sensitivity of the cell lines NIH-3T3, HepG2, and HT29 to the isolated compounds (1, 2, and 5) was evaluated by monitoring the metabolic activity according to Schneemann et al.

**Enzyme Inhibitory Activity.** See the Supporting Information.

**Acknowledgment.** The authors gratefully thank Dr. I. Kajahn for fruitful discussions, K. Schumann for cultivation experiments, A. Erhard for bioactivity assays, and G. Kohlmeyer-Yilmaz, M. Höftmann, and Prof. Dr. F. Sönnichsen (University of Kiel, Germany) for running and processing NMR experiments. This study is from the Kieler Wirkstoff-Zentrum KiWiZ at the Leibniz Institute of Marine Sciences IFM-GEOMAR and was supported by the Ministry of Science, Economic Affairs and Transport of the State of Schleswig-Holstein (Germany) in the frame of the “Future Program for Economy”, which is co-financed by the European Union (EFRE).

**Supporting Information Available:** Additional information such as 1H NMR spectra of 1–5, 13C NMR spectra of 1, NMR spectra of labeling experiments and spectroscopic data of 2–5, as well as the description of enzyme inhibitory activity are available free of charge via the Internet at http://pubs.acs.org.

**References and Notes**


