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Full Papers

Kyanomycin, a Complex of Unusual Anthracycline−Phospholipid Hybrids from *Nonomuria* Species

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A blue secondary metabolite complex, named kyanomycin (**2**), in addition to ϵ-rhodomycinone (**1**), was detected in the mycelium extract of *Nonomuria* sp. NN 22303 by HPLC-diode array and HPLC-electrospray mass spectrometry screening. The chemical structures of the novel compounds were determined to be unusual anthracycline−phosphatidylethanolamine hybrids by spectroscopic and chemical degradation experiments.

In the course of ongoing research efforts aimed at exploring the biosynthetic potential of rare actinomycetes, the secondary metabolite profile of a *Nonomuria* species NN 22303 was subjected to a closer scrutiny.[2] HPLC coupled with diode array detection and electrospray mass spectrometry (ESMS) revealed a pattern of anthracycline-derived metabolites, of which the major compound was identified as ϵ-rhodomycinone (**1**).[3] A minor constituent was detected in the mycelium extract, attracting attention due to its intense blue color. The present report describes the structural characterization of this blue compound complex, named kyanomycin (**2**), by spectroscopic methods, including NMR and MS corroborated by chemical methods.

Results and Discussion

Actinomycete strain NN 22303, obtained from the Novo Nordisk culture collection, was isolated from a soil collected in India in 1991. Taxonomic studies based on chemotaxonomical and 16S rDNA analyses have revealed that this isolate belongs to the genus *Nonomuria*, which has been created recently.[4]

Batch fermentations of *Nonomuria* sp. NN 22303 were carried out in a 3-L airlift fermenter using a complex medium. Production of **1** and **2** commenced after about 100 hours of fermentation time, when biomass reached a maximal level of 13 mg/L dry weight. After incubation for about 180 h, **1** and **2** reached concentrations of 26 mg/L and 2.8 mg/L, respectively.

Both compounds were isolated from the biomass by extraction with MeOH. After concentration of the MeOH extract, **1** was extracted from the aqueous residue using ethyl acetate. The aqueous layer that contained **2** was applied to an Amberlite XAD-16 column. Compound **2** was extracted from the aqueous resin eluate at pH 9 with *n*-butanol and purified by Si gel adsorption chromatography. Finally, pure **2** was obtained after Sephadex LH-20 chromatography using DMSO as eluent, resulting in a blue solid after lyophilization. Although apparently homogeneous, ESMS analysis (negative mode) of **2** exhibited a cluster of molecular ion peaks spaced by 14 mass units [*m*/*z*

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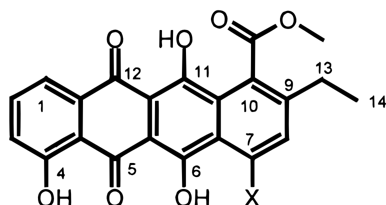


Figure 1. NMR-derived substructure of kyanomycin (**2**).

1097/1111/1125/1139 (1:5:8:3)], indicating a mixture of congeners. The NMR spectra of the complex were in several solvents characterized by broad, unresolved resonances revealing only limited structural information. However, in a 2:1 mixture of methanol-d_4 and chloroform-*d*, reasonably resolved spectra, including COSY, TOCSY, ROESY, HSQC, and HMBC data, were obtainable.

The aromatic region of the ^{1}H NMR spectrum exhibited signals characteristic of anthracycline D-ring protons, and resonances corresponding to a methyl ester and an isolated ethyl-group implied that **2** constitutes an addition to the ε-rhodomycinone (and not the daunomycinone) series of the anthracyclines. The presence of an additional one-proton singlet signal in the aromatic region and the absence of resonances corresponding to aliphatic A-ring protons, combined with the characteristic deep blue color, implied extension of the ε-rhodomycinone chromophore by aromatization of the A ring (Figure 1). Striking similarities between the ^{1}H NMR and UV-absorption data recorded for the blue complex and those reported for anhydromaggiemycin (**3**), a metabolic product of an unspecified *Streptomyces* strain,[5] further supported the presence of the proposed substructure.

Detailed analysis of the 2D NMR data furnished evidence for the presence of glycerol and hydroxyethyl residues. The remaining resonances were attributable to branched aliphatic ester groups, the presence of which was manifested by high-intensity aliphatic and carbonyl absorption bands in the 2850−2950 cm^{-1} range and at 1740 cm^{-1}, respectively, in the IR spectrum. Severe signal overlap precluded in-depth NMR analysis of the unhomogeneous aliphatic portion, and recourse was taken to chemical degradation for further characterization of the structure.

The MS and NMR data implied that the unhomogeneity was related to the presence of homologous and isomeric long-chain aliphatic acyl groups, which prompted us to attempt deacylation of the complex. Treatment with NaOMe in MeOH followed by solvent partition against heptane afforded a colorless heptane extract comprising a mixture of fatty acid methyl esters and a blue methanolic phase, dominated by one major component exhibiting UV-absorption properties similar to those of the parent compound.

Chromatographic separation afforded a homogeneous specimen of the blue deacylation product. Inspection of its ^{1}H NMR spectrum confirmed that the acyl groups had been efficiently cleaved off, and that the anthracycline, hydroxyethyl, and glycerol units were intact. The methine proton and one set of methylene protons in the glycerol moiety of the deacylation product exhibited marked upfield shifts compared to the corresponding resonances of the parent molecule (δ 5.32→3.77 and δ 4.44/4.25→3.61/3.54, respectively), convincingly identifying the sites of deacylation. HRFABMS analysis supplemented by NMR-derived constraints on the elementary composition established the molecular formula as $C_{27}H_{28}NO_{13}P$, based on which the structure **4** is suggested for the deacylation product.

Further evidence for the proposed structure became available by incidental decomposition of **4** to a less polar component (see Experimental Section), which by NMR and MS analysis was characterized as **5** and proved to be identical with anthracyclinone-blue B.[6]

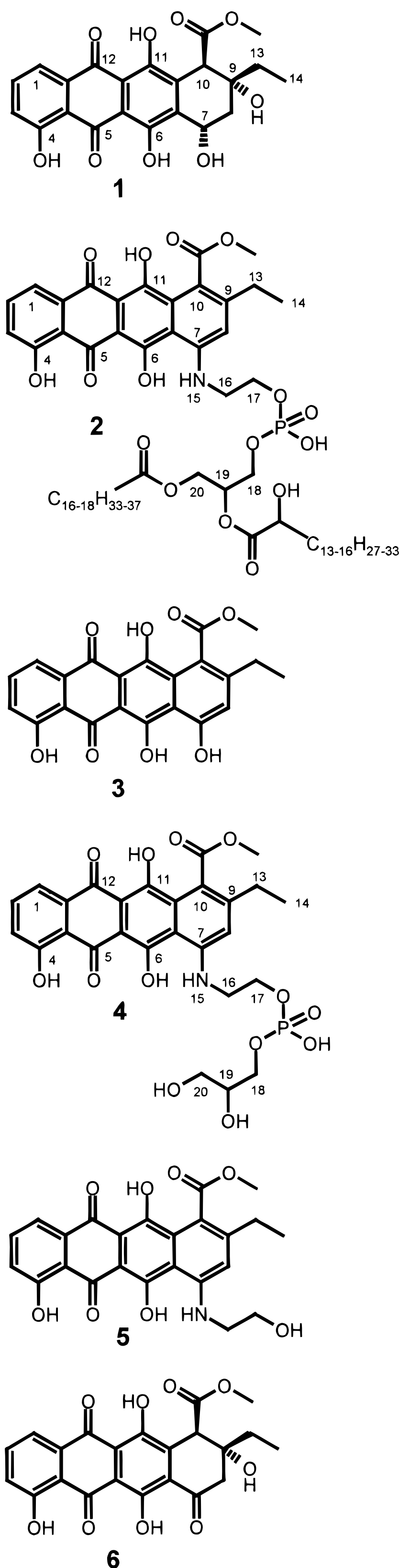
The heptane-extractable fraction from the deacylation reaction was separated by normal-phase HPLC into a polar and a nonpolar fraction, comprising a mixture of 2-hydroxy-substituted and unsubstituted fatty acid methyl esters, respectively. The constituents of the polar fraction were identified by GC analysis as methyl esters of 2-hydroxy-13-methyl-tetradecanoic acid (*iso*-15:0-2OH, 0.6%), 2-hydroxy-pentadecanoic acid (15:0-2OH, 1.6%), 2-hydroxy-14-methyl-pentadecanoic acid (*iso*-16:0-2OH, 36.0%), 2-hydroxy-hexadecanoic acid (16:0-2OH, 21.8%), hydroxy-15-methyl-hexadecanoic acid (*iso*-17:0-2OH, 12.7%), 2-hydroxy-14-methyl-hexadecanoic acid (*anteiso*-17:0-2OH, 9.8%), 2-hydroxy-heptadecanoic acid (17:0-2OH, 16.5%), and 2-hydroxy-octadecanoic acid (18:0-2OH, 1.0%). Similarly, the constituents of the nonpolar fraction were identified as methyl esters of 10-methyl-hexadecanoic acid (10-Me-16:0, 18%), 10-methyl-heptadecanoic acid (10-Me-17:0, 68%) and 10-methyl-octadecanoic acid (10-Me-18:0, 14%).

Three possibilities for the arrangement of the fatty acids exist. The 2-hydroxy fatty acid residues may either reside (a) exclusively on the secondary hydroxy function (C-19), (b) exclusively on the primary hydroxy function (C-20), or (c) on either position. The mass spectra exhibit no signals corresponding to species containing none or two hydroxy fatty-acid residues, excluding a random distribution, and speaking in favor of an ordered substitution of the acyl-substituents as in (a) or (b). In attempts to clarify this issue, selective enzymatic deacylation of kyanomycin by different classes of phospholipases was attempted. However, no hydrolysis or formation of complex mixtures was observed. The rate of formation, as determined by NMR spectroscopy (see Experimental Section), for unsubstituted fatty acid ester during the chemical deacylation reaction was found to be much higher compared to that of 2-hydroxy-ester. This is, however, ambiguous evidence, inasmuch as the relative contribution from steric and electronic factors to the observed overall reaction rate is not known. We tentatively assign the structure **2** to kyanomycin, but note that the acyl substituents might have to be interchanged. Due to the complex composition, the stereochemical identity of the fatty acids remains unsettled, as does the configuration of the stereogenic center in the glycerol unit.

The antimicrobial spectrum of kyanomycin (**2**) was examined using an agar-plate diffusion assay. The compound exhibited only a weak activity against *Agrobacterium tumefaciens* DSM 30205, reaching an inhibition zone of 8 mm at a concentration of 1 mg/mL. No growth-inhibitory activities were observed against other Gram-positive bacteria, such as *Bacillus brevis* DSM 30, *Bacillus subitilis* DSM 10, *Clostridium pasteurianum* DSM 525, *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* DSM 20231, and *Streptomyces viridochromogenes* Tü 57, nor against Gram-negative bacteria, yeasts, or filamentous fungi.

Anthracyclines comprise a large group of tetracyclic polyketide metabolites, which have attracted considerable attention due to their impact on cancer chemotherapy.[7] A common feature for the majority of biologically active anthracyclines is the attachment of aminosugar-containing oligosaccharides to C-7 via a glycosidic linkage. The *Nonomuria* metabolite kyanomycin (**2**) described here is formally derived by dehydration of the condensation product formed by reaction between the amino group of a diacylphosphatidylethanolamine and the 7-keto group of maggiemycin (**6**).[5]

Anthracyclines carrying an ethanolamino residue at C-7, such as anthracyclinone-blue B (**5**), have been described as metabolic products of blocked *Streptomyces galilaeus* and *Streptomyces griseus* mutants,[6] but members of the kyanomycin complex are, to our knowledge, the first example of phospholipid–anthracycline hybrids.



Experimental Section

General Experimental Procedures. HPLC-DAD analyses were performed as described by Fiedler.[8] NMR data were acquired at 303 K on a Bruker DRX400 instrument. Chemical shifts (δ values) are in parts per million (ppm), coupling constants (J) in hertz. EIMS, at 70 eV ionization potential, and HRFABMS were performed on a JEOL AX505W instrument. Electrospray mass spectrometry (ESMS) was performed on a Sciex API III instrument.

Producing Organism and Culture Conditions. *Nonomuria* sp. NN 22303 was cultivated in a 3-L airlift fermenter using a medium composed of (per liter) glucose (10 g), soluble starch (20 g), yeast extract (5 g), casein enzymatic hydrolysate (5 g), calcium chloride (1.47 mg), cobalt chloride (0.2 mg), and deionized H_2O, pH 7.3. The fermenter was inoculated with 5 vol % of shaking cultures grown for 72 h in 500-mL Erlenmeyer flasks with one baffle on a rotary shaker at 120 rpm and 27 °C in the same medium. For production of kyanomycin (**2**), the fermentation was carried out at 27 °C for 182 h with an aeration rate of 2.7 v/v/m.

Isolation and Purification. The fermentation broth (3 L) was filtered, and the mycelium cake was extracted three times with the same volume of MeOH. The combined extracts were concentrated in vacuo to an aqueous residue and extracted three times with EtOAc at pH 4 to separate ϵ-rhodomycinone (**1**), leaving kyanomycin (**2**) in the aqueous layer. Chromatography on Amberlite XAD-16 (column 4 × 30 cm) afforded **2** in the aqueous eluate from which it was extracted with 30 vol % *n*-butanol at pH 9 and 4 °C. The organic layer was concentrated to dryness and subjected to Si gel column chromatography (Merck Si gel 60, 40–63 μm; column 2.5 × 30 cm) using cyclohexane–EtOAc (1+1, added 0.1% triethylamine and 0.1% trifluoroacetic acid) and MeOH as eluents. Compound **2** was desorbed by step-gradient elution at a MeOH content of 10%. Sephadex LH-20 chromatography (column 1.5 × 100 cm) using DMSO as eluent afforded **2** as a blue solid: UV (MeOH) λ_{max} (log ϵ): 259 (4.46), 333 (sh, 3.71), 402 (3.46), 535 (sh, 3.88), 575 (4.07) and 600 (sh, 4.06); IR (KBr) ν_{max} 3260 (br), 2935, 2860, 1740, 1595, 1470, 1425, 1285 and 1230 cm^{-1}; ^{1}H NMR (CD_3OD–$CDCl_3$ 2:1) δ 7.82 (1H, d, J = 8 Hz, H-1), 7.54 (1H, t, J = 8 Hz, H-2), 7.02 (1H, d, J = 8 Hz, H-3), 6.78 (1H, s, H-8), 2.64 (2H, m, H-13), 1.28 (3H, t, H-14), 3.44 (2H, m, H-16), 4.33 (2H, m, H-17), 4.11 (2H, m, H-18), 5.32 (1H, m, H-19), 4.44 (1H, m, H-20a), 4.25 (1H, m, H-20b), 3.26 (3H, s, OMe), 4.15 (1H, m, C*H*OHCO); ^{13}C NMR (CD_3OD–$CDCl_3$ 2:1, δ values extracted from 2D HSQC spectrum) δ 115.7 (d, C-1), 132.6 (d, C-2), 118.0 (d, C-3), 115.0 (d, C-8), 27.0 (t, C-13), 15.1 (q, C-14), 49.3 (t, C-16), 64.7 (t, C-17), 64.0 (t, C-18), 71.5 (d, C-19), 62.3 (t, C-20), 53.1 (q, OMe), 71.1 (d, *C*HOHCO); ESMS (negative mode, $[M - H]^-$) m/z 1097/1111/1125/1139 (1:5:8:3).

Deacylation of 2. Compound **2** (10 mg) was dissolved in 0.1 M NaOMe–MeOH (4.0 mL) and stirred at room temperature. The solution was extracted with portions (4.0 mL) of *n*-heptane after 60, 90, and 120 min of reaction time. After another 30 min, acetic acid (0.2 mL) was added to the methanolic phase and extracted with two more portions (4.0 mL) of *n*-heptane. The combined *n*-heptane extracts and the acidified methanolic phase were separately evaporated to dryness in a vacuum centrifuge. The molar ratios of unsubstituted and 2-hydroxy-substituted fatty acid methyl ester in the heptane extracts from the deacylation reaction were determined to be 14:1 after 60 min, 1.7:1 after 90 min, 1:1.8 after 120 min, and 1:12 after 150 min of reaction time, measured by ^{1}H NMR spectroscopy as the ratio between the intensities of the singlet-signals at δ 3.68 and 3.80 attributable to the methoxy protons in unsubstituted and 2-hydroxy-substituted fatty acid methyl ester, respectively.

Isolation and GC Identification of Fatty Acid Methyl Esters. The fatty acid mixture from the deacylation reaction was separated by normal-phase HPLC (YMC SIL, 5 μm, 250 × 10 mm column, eluted at a flow rate of 5 mL/minute with a linear gradient starting at 5% 2-propanol in *n*-heptane (5 min) and rising to 50% 2-propanol in *n*-heptane over 15 min, and finally, to 100% 2-propanol over a period of 1 min), separated

into polar and nonpolar fractions, comprising a mixture of branched 2-hydroxy-substituted and unsubstituted fatty acid methyl esters, respectively. The constituent fatty acid methyl esters were identified by GC analysis using the MIDI MIS bacterial identification system.[9]

Isolation of 4 and 5. The MeOH-soluble fraction from the deacylation reaction was subjected in three portions to reversed-phase HPLC separation (YMC ODS, 10 μm, 250 × 10 mm column) eluted at a flow rate of 5 mL/min with a linear gradient from water (0.05% TFA) to acetonitrile over a period of 30 min, and employing UV monitoring at 210 and 270 nm. The fractions containing the deacylation product (**4**) were pooled and evaporated in a vacuum centrifuge at ca. 25 °C. In one instance, however, the temperature was accidentally raised to ca. 45 °C, resulting in partial decomposition of **4** to **5**, which was isolated (0.5 mg) by rechromatography utilizing the same system and identified as anthracyclinone-blue B.[6]

Compound 4: obtained (2 mg) as a blue, amorphous solid; ^{1}H NMR (DMSO-d_6) δ 7.95 (1H, d, J = 8 Hz, H-1), 7.69 (1H, t, J = 8 Hz, H-2), 7.19 (1H, d, J = 8 Hz, H-3), 7.11 (1H, s, H-8), 2.66 (2H, m, H-13), 1.29 (3H, t, J = 7 Hz, H-14), 3.68 (2H, m, H-16), 4.21 (2H, m, H-17), 3.94 (2H, m, H-18), 3.77 (1H, m, H-19), 3.61 (1H, dd, J = 11 and 5 Hz, H-20a), 3.54 (1H, dd, J = 11 and 7 Hz, H-20b), 3.96 (3H, s, OMe); ^{13}C NMR (DMSO-d_6, δ values extracted from 2D HSQC spectrum) δ 117.7 (d, C-1), 134.4 (d, C-2), 120.4 (d, C-3), 116.9 (d, C-8), 27.8 (t, C-13), 15.4 (q, C-14), 44.4 (t, C-16), 64.5 (t, C-17), 63.6 (t, C-18), 72.4 (d, C-19), 67.6 (t, C-20) and 52.7 (q, OMe); HRFABMS (negative mode) found 604.1147, calcd for $C_{27}H_{27}NO_{13}P$ 604.1220.

Biological Assays. An agar plate diffusion assay was used to determine the antimicrobial spectrum of **1** and **2**. Samples (10 μL each) were applied to filter disks (6 mm diameter). The test plates were incubated for 24 h at a temperature that permitted an optimal growth of the test organisms. The standard strains for testing the biological activity spectrum were obtained from the stock collection of our laboratory, from DSMZ and ATCC.

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