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Identification of Inhibitors of Inducible Nitric Oxide Synthase from Microbial Extracts

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A new member of the angucycline family, vineomycin C (**3**), together with four known metabolites saquayamycin A1 (**1**), A-7884 (**2**), rabelomycin (**5**) and xanthomegnin (**6**) were isolated from microbial extracts. The structures were determined by 1D and 2D NMR techniques and chemical degradation. Compounds **1**~**3** and **5** were isolated from a fermentation of *Streptomyces* sp., while **6** was isolated from a fungal fermentation extract. All five compounds have shown potent inhibitory activity in the inducible nitric oxide synthase (iNOS) assay.

Nitric oxide (NO) is a reactive, gaseous, lipophilic molecule that functions at high concentrations as a defensive cytotoxin against tumor cells and pathogens, and at low concentrations as a signal in many diverse physiological processes including blood flow regulation, neurotransmission, learning and memory[1,2]. Nitric oxide synthases (NOS), expressed as cytokine-inducible (iNOS), endothelial (eNOS), and neuronal (nNOS) isozymes, catalyze the NADPH and oxygen-dependent oxidation of L-arginine to citrulline and nitric oxide, thereby controlling NO distribution and concentration in higher eukaryotes. The iNOS enzyme is critical for the immune response, but under conditions of excessive formation, may cause neurotoxicity[3], focal ischemia[4], Alzheimer's and Huntington's disease[5]. Hence, the regulation of nitric oxide synthesis has received a great deal of interest for potential therapeutic applications, as well as to advance our understanding of basic physiology.

As a part of our efforts to identify small molecules with iNOS inhibitory properties, we have characterized a new molecule, vineomycin C (**3**), and four known compounds including saquayamycin A1 (**1**), A-7884 (**2**), rabelomycin (**5**) and xanthomegnin (**6**). Compounds **1**~**3**, and **5** were identified from an actinomycete fermentation extract, while **6** was isolated from a fungal (family Eurotiaceae, possibly an *Anixiopsis* sp.) fermentation extract. The structures were deduced from 2D NMR data and by comparing spectroscopic properties with those of other known related compounds. All five compounds demonstrated inhibition of iNOS activity.

Bioactivity-guided fractionation of the EtOAc extract of the culture broth of a *Streptomyces* sp. yielded four compounds as the active components of the extract. Compounds **1**~**3**, and **5** have a benz[a]anthraquinone skeleton related to saquayamycin[6] or vineomycin[7]. Our standard dereplication protocol[8] was used to establish the structures of the known compounds.

Materials and Methods/Experimental

Spectral Analysis

^{1}H and ^{13}C NMR spectra were recorded at 300 K on a Bruker AC 300 spectrometer operating at 300 MHz and 75 MHz, respectively. The samples for NMR characterization were dissolved in chloroform-d at a concentration of approximately 5~10 mg/ml. Mass and MS/MS spectra were obtained on a PE Sciex API III triple-quadruple mass spectrometer interfaced with a Sciex Ion-Spray probe. Exact mass measurements were performed on a VG 70SEQ

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spectrometer at high resolution (HRFAB) using PEG300/thiogly as an internal standard. UV spectra were run on a Perkin-Elmer Lambda 6 spectrometer. IR spectra were recorded on a Perkin-Elmer FTIR 1600 spectrometer. Optical rotations were measured with a Perkin-Elmer 243B polarimeter in $CHCl_3$.

Microorganism

Both the Streptomycete strain #AM1699 and the fungal strain #AM1675 (family Eurotiaceae, possibly an *Anixiopsis* sp.) were isolated from soil samples collected in Queensland, Australia.

Fermentation of the *Streptomyces* Strain #AM1699

The microorganism was cultivated in 250 ml shake flasks containing 30 ml of medium using a two-stage fermentation protocol. The seed stage consisted of 2 days fermentation at 28°C. The seed medium was composed of Pharmamedia (Traders) 15 g/liter, glucose 20 g/liter, ammonium sulfate (anhydrous) 3 g/liter, zinc sulfate heptahydrate 30 mg/liter, calcium carbonate 4 g/liter, and yeast extract 5 g/liter. One milliliter of the seed broth was transferred to the production medium, which was composed of mannitol 60 g/liter, soybean meal (ADM) 12.5 g/liter, citric acid (free acid, anhydrous) 2.5 g/liter, and yeast extract 0.5 g/liter. Concentrated KOH was added into the medium to adjust its pH to 7.0 before autoclaving. The microorganism was fermented at 28°C for additional 5 days before harvesting.

Fermentation of the Fungal Strain #AM1675

The microorganism was cultivated in 250 ml shake flasks containing 30 ml of medium using a two-stage fermentation protocol. The seed stage consisted of 2 days of fermentation at 28°C. The seed medium was the same as described above. The production medium was composed of α-lactose 30 g/liter, glucose 5 g/liter, Pharmamedia (Traders) 20 g/liter, yeast extract 3 g/liter, sodium nitrate 1 g/liter, potassium phosphate, dibasic (anhydrous) 0.5 g/liter, potassium chloride 0.7 g/liter, histidine 1 g/liter, and magnesium sulfate, heptahydrate 14 mg/liter; pH 7.0.

Inducible Nitric Oxide Synthase Assay

The iNOS enzyme was partially purified from the macrophage cell line RAW 264.7 according to the procedure described by STUEHR *et al.*[9] The activity was measured by using a batch resin extraction procedure for separating ^{3}H citrulline from ^{3}H arginine. Briefly, 20 μl of iNOS assay mix containing 50 nM ^{3}H arginine; 20.0 μM arginine, 1.0 mM NADPH, 2.0 μM FAD, 2.0 μM THB, and 10 μl of test sample was added to respective wells of a 96 well plate. The reaction was started by the addition of 20 μl of partially purified iNOS enzyme for a total assay volume of 50 μl. After 30 minutes incubation at 37°C, the reaction was terminated by the addition of 50 μl of a 50% slurry of AG50W-X8 (Biorad) resin, 100~200 mesh, sodium form. Scintillation fluid (100 μl) was added to each well. The plates were sealed with adhesive film, vortexed and read in a Wallac Microbeta plate reader. Calculation of IC_{50} values were made from curves using non-linear regression employing GraphPad Prism software.

Extraction and Isolation

The pooled fermentation mixtures obtained from *Streptomyces* strain #AM1699 (6 liters) and the fungal strain #AM1675 (6 liters) were extracted and fractionated using our standard protocol[10].

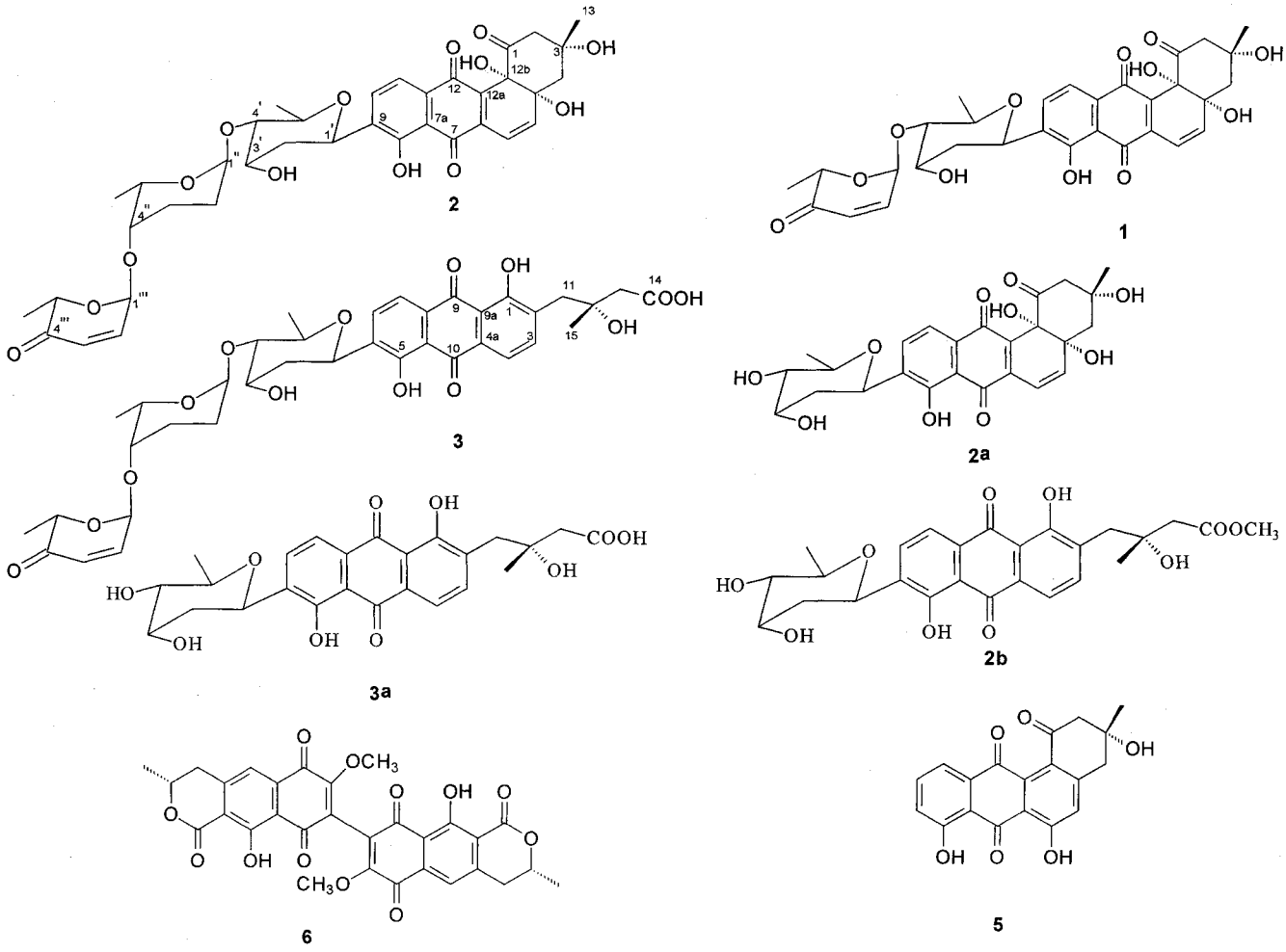
In the case of strain #AM1699 the activity was concentrated into two centrifugal partition chromatography (CPC) peaks eluting at 45~63 (fractions 16~23) and 69~84 minutes (fractions 24~28). The active fractions were pooled and evaporated under reduced pressure to dryness. Final purification was achieved using semi-preparative HPLC. The chromatography system consisted of a Waters HPLC equipped with a Waters 600 system controller and a Waters 996 photodiode array detector. Separation was achieved on two semi-preparative C_{18}-cartridges connected in series (25×100 mm, Nova-pak 6 micron). The mobile phase was pumped as a binary system at a rate of 10 ml/minute and consisted of a 35 minutes linear gradient, starting with 50% H_2O, 50% CH_3CN and ending with 100% CH_3CN. Saquayamycin A1, A-7884 and vineomycin C were isolated from the CPC fractions 16~23 and rabelomycin was isolated from fractions 24~28.

The activity of the fungal extract was concentrated into one CPC peak eluting at 72~99 minutes (fractions 25~34). The active fractions were pooled and evaporated under reduced pressure to dryness which afforded xanthomegnin. Final purification was achieved on semi-preparative HPLC as described above.

Saquayamycin A1 (**1**): Dark yellow solid (30 mg); mp 167~169°C; $[\alpha]_D$ +120 (*c* 1.0, $CHCl_3$); *m/z* 597 $(M+H)^+$; UV λ_{max} (MeOH): 218, 255, 302, 425 nm; ^{1}H and ^{13}C NMR data were identical to the reported data[6].

A-7884 (**2**): Dark yellow solid (40 mg); mp 167~169°C; $[\alpha]_D$ +120 (*c* 1.0, $CHCl_3$); UV λ_{max} (MeOH): 218, 254, 298, 425 nm; IR ν_{max}: 3420, 1720, 1660, 1640, 1526 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.15 (dd, J=13.41, 2.99 Hz, H-2), 2.49 (d, J=13.41 Hz, H-2), 2.25 (dd, J=15.31, 2.81 Hz, H-4), 1.83 (d, J=15.30 Hz, H-4), 6.41 (d, J=10.00 Hz, H-5), 6.89 (d, J=10.00 Hz, H-6), 7.85 (d, J=7.79 Hz, H-10), 7.58

Scheme 1.



(d, J=7.79 Hz, H-11), 1.25 (s, H-13), 12.26 (s, OH), 4.84 (d, J=11.18 Hz, H-1′), 2.45 (ddd, J=10.32, 4.71, 1.00Hz, H-2′), 1.34 (m, H-2′), 3.77 (bdd, J=8.29, 3.57 Hz, H-3′), 3.03 (t, J=8.6 Hz, H-4′), 3.51 (dq, J=12.05, 4.91 Hz, H-5′), 1.37 (d, J=4.94 Hz, H-6′), 4.96 (bs, H-1″), 2.08 (m, H-2″), 1.46 (m, H-3″), 3.69 (m, H-4″), 4.20 (m, H-5″), 1.33 (d, J=6.73 Hz, H-6″), 5.23 (d, J=3.32 Hz, H-1‴), 6.85 (dd, J=10.54, 3.32 Hz, H-2‴), 6.06 (d, J=10.54 Hz, H-3‴), 4.53 (q, J=6.74 Hz, H-5‴), 1.35 (d, J=6.4 Hz, H-6‴); ^{13}C NMR ($CDCl_3$): δ 205.4 (C-1), 50.6 (C-2), 76.5 (C-3), 43.6 (C-4), 80.9 (C-4a), 144.6 (C-5), 117.9 (C-6), 139.1 (C-6a), 188.3 (C-7), 114.3 (C-7a), 158.5 (C-8), 138.5 (C-9), 134.1 (C-10), 120.2 (C-11), 130.6 (C-11a), 182.5 (C-12), 138.5 (C-12a), 77.8 (C-12b), 30.6 (C-13), 71.4 (C-1′), 39.1 (C-2′), 71.8 (C-3′), 89.3 (C-4′), 74.9 (C-5′), 18.4 (C-6′), 99.9 (C-1″), 25.5 (C-2″), 24.7 (C-3″), 74.9 (C-4″), 68.2 (C-5″), 17.4 (C-6″), 95.7 (C-1‴), 143.2 (C-2‴), 127.9 (C-3‴), 197.0 (C-4‴), 71.1 (C-5″), 15.6 (C-6‴); API-MS (positive): m/z 711 $(M+H)^+$.

Conversion of A-7884 (**2**) to aquamycin (**2a**): 10 mg of **2** dissolved in 0.05 N HCl-MeOH (10 ml) were stirred at room temperature for an hour. The reaction mixture was extracted with EtOAc, washed with water, and concentrated to dryness. The residue was applied onto a silica gel column. The column was developed with $CHCl_3$-MeOH (9 : 1), affording one main orange band. The band was concentrated to dryness, which afforded **2a** as orange compound (6.5 mg). $[\alpha]_D$ +141 (c 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.23 (s, 3H), 1.37 (d, J=5.9 Hz, 3H), 2.02 (q, J=15.12 Hz, 2H), 2.74 (q, J=12.94 Hz, 2H), 6.28 (d, J=10.00 Hz, 1H), 6.84 (d, J=10.00 Hz, 1H), 7.50 (d, J=8.11 Hz, 1H),7.79 (d, J=10.00 Hz, 1H), 12.31 (s, 1H). API-MS (positive): m/z 487 $(M+H)^+$.

Conversion of A-7884 (**2**) to **2b**: 10 mg of **2** in 5% HCl-

MeOH (10 ml) was heated at 90°C for 20 hours and concentrated to dryness under reduced pressure. The residue was applied onto a silica gel column. The column was developed with $CHCl_3$ - MeOH (9 : 1) affording one main yellow band. The band was concentrated to dryness, which afforded **2b** as yellow compound (7 mg). ^{1}H NMR ($CDCl_3$): δ 1.20 (s, 3H), 1.34 (d, J=5.00 Hz, 3H), 2.52 (m, 2H), 2.61 (m, 2H), 2.84 (d, J=12.94 Hz, 1H), 3.31 (d, J=12.46 Hz, 1H), 3.58 (s, 3H), 4.83 (d, J=11.27 Hz, 1H), 7.46 (d, J=8.00 Hz, 1H), 7.56 (d, J=8.00 Hz, 1H), 7.63 (d, J=8.11 Hz, 1H), 7.79 (d, J=8.00 Hz, 1H), 12.81 (s, 1H). API-MS (positive): m/z 501 $(M+H)^+$.

Vineomycin C (**3**): Dark yellow solid (12 mg); mp 123~162°C; $[\alpha]_D$ +110 (c 1.0, $CHCl_3$); UV λ_{max} (MeOH): 231, 258, 290, 442 nm; IR ν_{max}: 3420, 3000~2500, 1730, 1660, 1625, 1526 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 7.49 (d, J=7.66 Hz, H-3), 7.56 (d, J=7.66 Hz, H-4), 7.83 (d, J=7.90 Hz, H-7), 7.68 (d, J=7.90 Hz, H-8), 3.12 and 2.81 (d, J=13.46 Hz, H-11), 2.62 (m, H-13), 1.22 (s, H-15), 12.88 (s, OH), 12.99 (CO*OH*), 4.83 (d, J=11.37 Hz, H-1′), 2.61 (m, H-2′), 1.46 (m, H-2′), 3.88 (bdd, J=8.29, 3.57 Hz, H-3′), 3.09 (m, H-4′), 3.54 (dq, J=12.05, 4.91 Hz, H-5′), 1.36 (d, J=4.94 Hz, H-6′), 4.96 (bs, H-1″), 2.08 (m, H-2″), 1.46 (m, H-3″), 3.69 (m, H-4″), 4.20 (m, H-5″), 1.33 (d, J=6.73 Hz, H-6″), 5.25 (d, J=3.38 Hz, H-1‴), 6.85 (dd, J=10.19, 3.38 Hz, H-2‴), 6.10 (d, J=10.10 Hz, H-3‴), 4.53 (q, J=6.74 Hz, H-5‴), 1.35 (d, J=6.4 Hz, H-6‴); ^{13}C NMR ($CDCl_3$): δ 161.5 (C-1), 134.8 (C-2), 139.7 (C-3), 119.2 (C-4), 138.8 (C-4a), 187.9 (C-10), 115.5 (C-10a), 159.3 (C-5), 138.8 (C-6), 133.6 (C-7), 119.9 (C-8), 131.8 (C-8a), 188.2 (C-9), 115.8 (C-9a), 40.8 (C-11), 77.0 (C-12), 45.7 (C-13), 175.5 (C-14), 30.1 (C-15), 71.4 (C-1′), 38.1 (C-2′), 72.0 (C-3′), 89.0 (C-4′), 74.9 (C-5′), 18.9 (C-6′), 99.9 (C-1″), 25.5 (C-2″), 24.7 (C-3″), 76.0 (C-4″), 68.3 (C-5″), 17.4 (C-6″), 95.7 (C-1‴), 143.2 (C-2‴), 127.9 (C-3‴), 197.1 (C-4‴), 71.1 (C-5″), 15.6 (C-6‴); API-MS (positive): m/z 711 $(M+H)^+$; HRFAB-MS calcd. for $C_{37}H_{17}O_{14}$ (M^++H) 711.2369; found 711.2370.

Hydrolysis of vineomycin C (**3**) to **3a**: 10 mg of **3** was hydrolyzed by 0.05 N HCl-MeOH using same conditions as described above. API-MS (positive): m/z 487 $(M+H)^+$.

Rabelomycin (**5**): Yellowish solid (5 mg); mp 190~192°C; UV λ_{max} (MeOH): 231, 258, 442 nm; API-MS (positive): m/z 339 $(M+H)^+$. ^{1}H and ^{13}C NMR data were identical to the reported data[12].

Xanthomegnin (**6**): Orange powder (45 mg). mp 256~258°C; UV λ_{max} (MeOH): 228, 290, 394 nm; API-MS (positive): m/z 475 $(M+H)^+$. ^{1}H and ^{13}C NMR data were identical to the reported data[13].

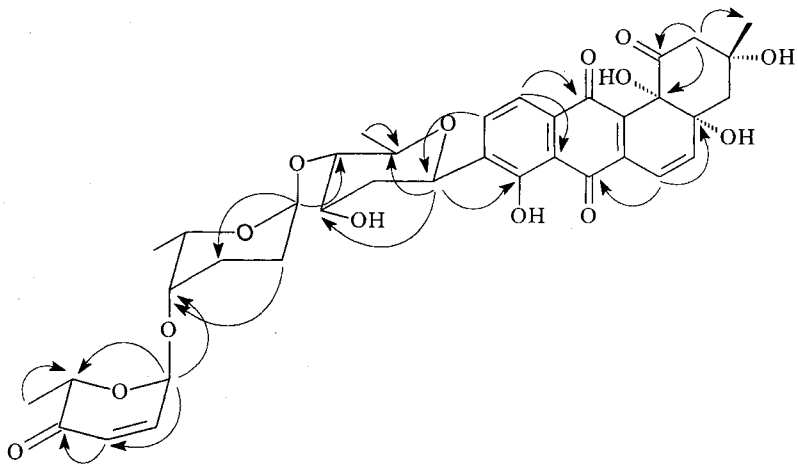
Results and Discussion

Saquayamycin A1 (**1**) was isolated as a dark yellow solid. The molecular weight was determined to be 596 based on atmospheric pressure ionization mass spectrometry (API-MS) data that showed a protonated molecular ion at m/z 597 $(M+H)^+$ in the positive ion mode. The UV-VIS spectrum of **1** suggested that it belonged to the angucycline group of antibiotics. The ^{1}H NMR spectrum of **1** showed characteristic signals at δ 7.85 (d, J=7.81 Hz, H-10), 7.58 (d, J=7.81 Hz, H-11), 6.89 (d, J=9.96 Hz, H-6), 6.42 (d, J=9.96 Hz, H-5) and 12.26 (br s, OH) which were attributable to the chromophore of **1**. It was identified as saquayamycin A1 by comparison of its spectroscopic properties with those reported in the literature[6].

A-7884 (**2**) was also isolated as a dark yellow solid. The molecular weight of m/z 711 $(M+H)^+$ was obtained by API-MS in positive mode, the molecular weight was 114 mu higher than the molecular weight of saquayamycin A1 (**1**). The UV and NMR data of **2** were very similar to **1**; the NMR spectra of **2** showed an additional anomeric signal at δ 4.96 (bs) and 99.9, suggesting the presence of an additional sugar moiety. This third sugar moiety was identified as a 2,3-dideoxysugar as suggested by the ^{1}H-^{1}H COSY spectrum and by comparison of published NMR data for a 2,3-dideoxysugar[6]. A search of natural products databases such as Berdy's BNPD and Chapman & Hall's Dictionary of Natural Products by molecular formula, identified one compound, A-7884[11], an angucycline antibiotic reported in a Japanese patent with similar physical properties as observed for **2**. Since no spectroscopic data for A-7884 were available in the literature, the overall structure was ascertained by extensive 2D NMR studies (*e.g.*, ^{1}H-^{1}H COSY, HMQC and HMBC). The results confirmed the similarities between saquayamycin A1 (**1**) and the aglycone portion of **2**. Like **1** the sugar (olivose) in **2** was connected to C-9 through a *C*-glycosidic linkage which was revealed by the chemical shift of C-1′ at δ 71.4[6] and further confirmed by the HMBC correlation. The remaining connectivities and sequence of the other sugars (rhodinose and aculose) was established by using chemical shift arguments, ^{1}H-^{1}H COSY, and HMBC correlation studies as illustrated in Fig. 1.

Upon acid hydrolysis with 0.05 N HCl-MeOH at room temperature, A-7884 (**2**) gave an orange aglycone **2a**. The optical rotation $[\alpha]_D$ and the ^{1}H NMR spectrum of **2a** were similar to those of saquayamycin[12,13], whose absolute configuration had been determined by the X-ray crystallographic analysis of P-1894B[14] and studies of the

Fig. 1. Some important HMBC correlations of A-7884 (**2**).



absolute configuration of its sugar components[14]. Therefore, it was concluded that compound A-7884 (**2**) would have the same stereochemistry as has been reported for saquayamycin.

Vineomycin C (**3**) was isolated as a yellow powder. API mass spectrometry revealed the molecular ion peak at *m/z* 711 $(M+H)^+$. The molecular formula was established to be $C_{37}H_{17}O_{14}$ on the basis of HRFAB-MS. Comparison of mass and NMR spectra of **3** and **2** suggested a close structural relationship. Like **2**, the NMR spectra indicated the presence of 3-deoxyhexoses (two of them *O*-glycoside, one *C*-glycoside). Especially diagnostic were three methyl doublets at δ 1.33~1.36 and three protons in the typical anomeric region δ 4.83~5.25 of the ^{1}H NMR spectrum and three anomeric carbons δ 71.4, 95.7 and 99.9 of the ^{13}C NMR spectrum. Therefore, it can be concluded that the sugar moieties of **3** are identical to those of **2**. To confirm this, chemical degradations were carried out. Hydrolysis of **3** with 0.05 N HCl-MeOH at room temperature for 1 hour yielded **3a** which as expected had the molecular weight as **2a**. This evidence further supported our initial conclusion that both compounds have the same sugar moieties and their sequences are identical. Thus, the structural difference between **3** and **2** must exist in the aglycone portion.

The UV-visible spectrum of **3** with maxima at λ_{max} 231, 258, 290 and 442 nm suggested the presence of a 1,5- or 1,8-dihydroxy-9,10-anthraquinone skeleton[15]. The infrared absorption of two chelated quinone carbonyl groups at ν_{max} 1625 cm^{-1} and the signals of two hydrogen-bonded quinone carbonyl carbons at δ 187.9 and 188.2 in the ^{13}C NMR spectrum indicated the location of the two hydroxyl groups at the 1 and 5 positions. In addition, two AB-type systems (δ 7.49 and 7.56, J=7.66 Hz and 7.83 and 7.68, J=7.90 Hz) suggested substitutions at the 2- or 4- and 6- or 8-position of the anthraquinone skeleton. Long range correlations from H-1′ to C-5, C-6 and C-7 in the HMBC spectrum revealed that the pyran ring of the olivose moiety was connected to C-6 through a *C*-glycosidic linkage. When the ^{13}C NMR of the aglycones of **2** and **3** were compared, the latter showed three new signals: a nonprotonated sp^2 carbon (δ 134.8), a phenolic carbon (δ 161.5), and a carboxylic acid carbon (δ 175.5). The presence of a carboxylic acid in **3** was also supported by the IR spectrum which showed an absorption at ν_{max} 3000~2500 and 1730 cm^{-1}. Two methylene signals were shifted upfield from δ 50.6 to 45.5 and 43.6 to 40.8. These methylene protons [δ 3.12 (m), 2.81 (d, J=13.46 Hz) and 2.6 (m)] do not couple to each other, whereas those in **2** showed a long range W-type coupling. This suggests that these methylene groups are part of an acyclic structure.

To confirm the above assumption, compound **2** was treated with 8% HCl-MeOH at 90°C under the conditions used for obtaining dehydroaquayamycin (**2b**) from aquayamycin (**2a**)[14]. The resulting product was purified by silica gel column chromatography affording a yellow band. The ^{1}H NMR spectrum of the latter was consistent with the ^{1}H NMR spectrum of the **3a**[14]. Therefore, the structure of the chromophore part and hence the total structure of **3** were determined as shown. The name vineomycin C was proposed for **3** due to its close structural resemblance to vineomycin B[7]. The overall structure was ascertained by detailed 2D NMR studies (^{1}H-^{1}H COSY, HMBC and

Table 1. Inhibition of iNOS activity by purified compounds.

#	Compound	IC_{50} (μM)
1	Saquayamycin A1 (**1**)	101.2
2	A-7884 (**2**)	43.5
3	Vineomycin C (**3**)	25.3
4	Rabelomycin (**5**)	63.9
5	Xanthomegnin (**6**)	5.5
6	L-NMMA	17.0
7	L-NNA	71.0

Purified compounds were tested in duplicate over 12 different concentrations ranging from 100 nM to 10 mM for inhibition of iNOS activity by incubation with the partially purified enzyme from RAW 264.7 cell lysate. Inhibition curves and IC_{50} values were generated by non-linear regression. Data are representative of three different experiments.

HMQC). The stereochemistry of the chiral centers of **3** was proposed by comparing its ^1H and ^{13}C NMR shifts with those reported vineomycin B[7] and saquayamycin[14].

Compound **5** was purified as a yellow powder and on the basis of MS, ^1H NMR, ^{13}C NMR and UV data, identified as rabelomycin, a metabolite previously reported from *Streptomyces olivaceus*[16].

Compound **6** was isolated from the fungal extract as a yellowish powder and identified as the known antibiotic xanthomegnin by comparing its spectroscopic properties with literature data[17].

Biological Properties

All compounds (**1**~**3**, **5**, and **6**) were tested against the iNOS assay (Table 1). Based on the data presented in Table 1, xanthomegnin (**6**) is a potent inhibitor of iNOS while the other compounds exhibited dose-dependent inhibition of iNOS activity with IC_{50} values which were comparable to known standard inhibitors, N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NNA)[18].

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