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The Iromycins, a New Family of Pyridone Metabolites from *Streptomyces* sp. I. Structure, NOS Inhibitory Activity, and Biosynthesis

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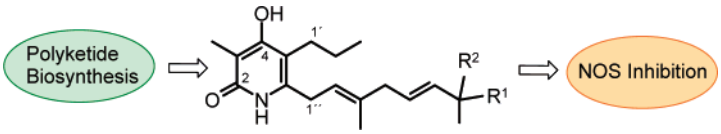

The iromycins A−D are members of a new family of rare α-pyridone metabolites. The isolation and structure elucidation of these microbial secondary metabolites from *Streptomyces* sp. Dra 17 revealed a *N*-heterocyclic core structure with two unusual side chains. Iromycins act as inhibitors of nitric oxide synthases (NOS), a protein family, which produces the crucial second messenger nitric oxide (NO). Importantly, these compounds inhibit selectively endothelial NOS rather than neuronal NOS and thus set prospects for both medical therapy and basic research. Feeding experiments with ^{13}C- and ^{15}N -labeled precursors indicated an uncommon type of polyketide biosynthesis and clearly ruled out an isoprenoid origin. A detoxification pathway of a particular secondary metabolite in the host strain is a rare observation and here we demonstrate it with the iromycin family.

Introduction

Polyketides are extraordinarily diverse natural products which exhibit various biological activities and are successfully used as biochemical tools as well as therapeutical compounds in clinical application.[1] Even though pyridone ring containing natural products from bacteria or funghi are unusual, they are fairly common among alkaloids from plants.[2] The known examples of microbial pyridone metabolites as well as some of their pyrone and pyridine analogues exhibit rather different yet important biological activities, which include selective antimicrobial properties,[3] inhibition of the mitochondrial electron transport chain complex I,[4,5] and neuritogenic properties.[6] Therefore, they are used as powerful biochemical tools and allow for the detailed investigation of certain subcellular molecular pathways.

Nitric oxide (NO) is a gaseous radical with diverse physiological functions essential for neurotransmission, host defense, and the cardiovascular system in mammals. However, NO overproduction leads to harmful pathological processes like septic/cytokine-induced circulatory shock and chronic inflammatory diseases.[7] Three isoforms of NO synthases (neuronal (nNOS), inducible (iNOS), and endothelial (eNOS)) catalyze the conversion of arginine to citrulline, thereby producing NO.

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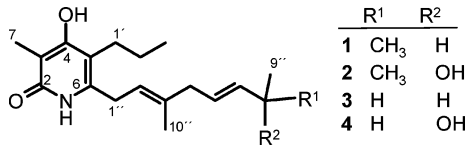
In the past decade it has become clear that their activity is very tightly controlled, yet the contribution of the single NOS isoforms to physiological and pathophysiological processes is far from being fully understood.[7,8] Thus, isoform-specific inhibitors of NO production represent ideal tools to study the NO synthase network and, additionally, would be widely appreciated for therapeutic intervention.[9] Herein, we report the isolation, structure elucidation, and unsual polyketide biosynthesis of the new metabolite family of the iromycins A (**1**), B (**2**), C (**3**), and D (**4**) which are isoform-specific inhibitors of NO synthases.

Results and Discussion

In our chemical screening program for new metabolites, the strain *Streptomyces bottropensis* sp. Gö Dra 17 was isolated from a soil sample collected on lime neglected grassland close to Göttingen, Germany.[10] Liquid cultures contained traces of a new compound, which could be stained blue-violet with anisaldehyde reagent.[11] Fermentations of the producer strain were performed in a 1 L fermentor or 300 mL shaking flasks for 72 h at 28 °C, using the complex medium S that contained starch, glucose, glycerol, peptone, and yeast extract.[12] In total, four new metabolites were isolated by extraction with EtOAc followed by chromatography on silica gel, size exclusion chromatography (Sephadex LH-20, MeOH), and reverse phase HPLC.[12] A particular high yield of up to 18 mgL^{-1} of **1** was efficiently achieved with the OSMAC method ("*O*ne-strain-*ma*ny-*c*ompounds"),[13] whereas the amounts of the purified derivatives where significantly lower (12 mg/L for **2**, 0.5 mg/L for **3**, and 0.25 mg/L for **4**).



	R1	R2
1	CH_3	H
2	CH_3	OH
3	H	H
4	H	OH

From high-resolution mass spectrometry the empirical formulas were determined as $C_{19}H_{29}NO_2$ for **1**, $C_{19}H_{29}NO_3$ for **2**, $C_{18}H_{27}NO_2$ for **3**, and $C_{18}H_{27}NO_3$ for the minor component **4**.

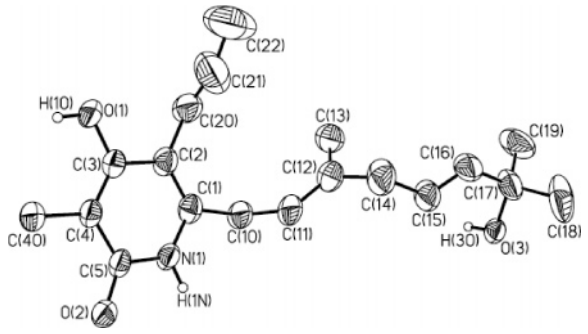


FIGURE 1. Crystal structure of iromycin B (**2**) with atomic labeling scheme (CCDC 614260).

^{1}H NMR and ^{13}C NMR data indicated identical carbon backbones for **1** and **2**. The characteristic IR absorption peaks of **1** suggested hydroxyl (3398 cm^{-1}) and carbonyl groups (1636 cm^{-1}).[14] The UV spectra showed absorption maxima at 210 and 292 nm which significantly shifted upon addition of HCl (208, 275 nm) and NaOH (220, 279 nm). The ^{1}H NMR and ^{13}C NMR spectra (CD_3OD) in accordance with COSY and HSQC experiments of **1** exhibited signals of an isopropyl group (δ_H = 0.98, 2.26 ppm), three methyl groups (δ_H = 0.97, 1.73, 1.98 ppm), three olefinic methine groups (δ_H = 5.17, 5.32, 5.42 ppm), and four methylene units. The six quaternary carbon atoms comprise carbonyl (δ_C = 165.9 ppm), hydroxy (δ_C = 166.1 ppm), and olefinic (δ_C = 106.0, 113.7, 143.0, 138.8 ppm) moieties. All 2D-NMR data from COSY, HSQC, HMBC, and NOESY experiments as well as the fragmentation patterns of EI-MS analyses were consistent with both tetramic acid or pyridone skeletons with a C_{10} side chain with nonconjugated double bonds.[12] The semisynthetic products after methylation (CH_3I/K_2CO_3 at rt) and acetylation (Ac_2O/pyridine at rt) of **1** confirmed the presence of the assigned OH and NH groups.[12] Fortunately, X-ray structure analysis of a single crystal of **2** gave final evidence for the pyridone structure defining the new metabolite family of iromycins including iromycins A (**1**), B (**2**), C (**3**), and D (**4**).[12] Detailed analysis of the bond lengths showed a shortened bond length for the single bond of C(3)/O(1) and a longer one for C(3)/C(4) (Figure 1) indicating the partial presence of the keto−enol tautomer (3-CH_2/4-C=O) of **1**, but did not support any lactim character. Both characteristic and unusual features of compounds **1**−**4** are a fully substituted α-pyridone ring together with a propyl moiety and the unique unsaturated side chain. The latter does not resemble common geranyl moieties but instead bears a rare 1,4-pentadiene motif and additionally in the case of derivatives **1** and **2** shows a terminal branching, while **3** and **4** lack one carbon atom at this position. These uncommon structural features aroused our interest in the biosynthetic pathway and structure−activity relationships.

Database searches resulted in one match for the structure of **1**, which refers to the metabolite NK26588 in the patent literature.[15] This metabolite was isolated by extraction and high-performance chromatography from cultures of a *Streptomyces* strain (strain no. 26588), while the derivatives **2**−**4** were previously unknown. Therefore, the members of this new family

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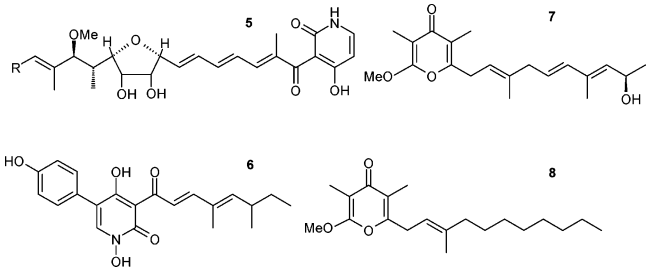
of pyridone metabolites were named iromycins. The iromycins share the pyridone structure with a few other active natural products, for example kirromycin (**5**),[3] an inhibitor of the elongation factor TU, or militarinones and tenellin (**6**),[6,16] which are produced by insect pathogenic fungi *Beauveria* sp. and *Paecilomycies* sp. Similarities to the exceptional side chains are rare and can only be found in some bioactive heterocyclic metabolites like actinopyrone, kalkipyrone (**7**), or piericidin as well as the just recently identified verticipyrone (**8**).[4,5,17,18]



The earlier reported *Streptomyces* metabolite NK26588 (**1**) was the first bacterial pyridone for which an inhibitory effect on NO synthesis could be observed by using J774.1 macrophage cells.[15] Herein, we performed a detailed investigation using several members of the iromycin family (**1**−**3**) and addressed substrate specificity regarding eNOS and nNOS. Chinese hamster ovary (CHO) cells expressing human eNOS or nNOS, respectively, were treated with Ca^{2+}-ionophore (A23187) to stimulate NOS activity in the absence or presence of the potential inhibitors **1**−**3**.[19] Regarding eNOS, iromycin A (**1**) showed a strong inhibitory effect on NOS activity, measured by the formation of citrullin. As controls, the ratios of citrullin production upon stimulation in the presence or absence of the known standard NOS inhibitor L-N^{ω}-nitroarginine (L-NNA) were used. **1** turned out to be more potent than both iromycin C (**3**) and the hydroxylated **2**, which showed only little inhibitory capacity. In contrast, neither of the iromycins revealed an inhibitory effect toward nNOS. These experiments proved that the iromycins, comparable with the known synthetic, nonselective inhibitors like L-NNA,[7] act as NOS inhibitors in micromolar ranges. Furthermore it is of great interest that **1**−**3** act with a different inhibitory capacity on each of the tested NOS isoforms. While nNOS is not significantly affected the eNOS isoform is strongly inhibited by **1.** We want to point out that **1** bears a terminal *i*Pr-moiety, whereas the oxygenated **2** and the derivative **3** are less effective regarding NOS inhibition (Figure 2). This apparent structure−activity relationship lays the basis for the design of derivatives with refined biological properties, which certainly is an attractive scientific aim.

Regarding the biosynthesis of the iromycins, the structures initially suggested several options, e.g., isoprenoid,[12,20] hybrid

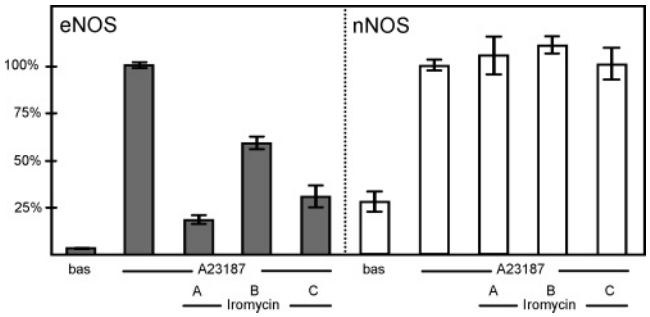


FIGURE 2. Effect of iromycins A−C (**1**−**3**) on NOS activity. Iromycins (100 μM) were added 30 min prior to stimulation with A23187 (3 μM) and eNOS and nNOS activity were determined by arginine to citrulline conversion assay,[19] calculated as the difference for citrulline production in the presence of the NOS inhibitor L-NNA (100 μM) and expressed as relative values. Similar results were obtained by using iromycins with 200 μM (bas: basal activity).

polyketide/nonribosomal peptide,[21] or polyketide based pathways.[22] Hitherto, even though limited in number the microbial pyridone metabolites use a variety of biosynthetic sources and routes: For example, feeding experiments and the identification of the biosynthetic gene cluster suggest that the pyridone ring of kirromycin (**5**) derives from β-alanine as the final extender unit in the biosynthesis.[23] In contrast, phenylalanine and a conjugated polyketide chain create the structures of tenellin (**6**) and the militarinones.[6,16] Generally accepted, the pyrone analogues actinopyrone and kalkipyrone (**7**) and the 2-methoxy pyridine piericidin (**8**) derive from single polyketide chains.[12]

To tackle the question of the microbial production of the iromycins, biosynthetic investigations were carried out by performing feeding experiments with stable isotope-labeled precursors. [1-^{13}C]Acetate and [1-^{13}C]propionate were fed to growing cultures of strain *Streptomyces* sp. Dra 17 (12 h of continuous feeding starting 24 h after inoculation). ^{13}C-Enrichments of **1** and **2** were analyzed by ^{13}C NMR spectroscopy. High incorporation rates of the ^{13}C at positions C-4, C-6, and C-4″ of the carbon skeleton upon feeding with [1-^{13}C]-acetate as well as at positions C-2, C-1′, and C-2″ upon feeding with [1-^{13}C]propionate clearly indicates a polyketide biosynthetic pathway (Scheme 1, Table 1).[12] Furthermore, a feeding experiment with [1,2-$^{13}C_2$]acetate proved the intact incorporation of acetate units by strong spin−spin couplings and gave evidence for the direction of the polyketide chain. The iromycins **1**−**4** are the first pyridone metabolites bearing two alkyl side chains and therefore require an uncommon step in the biosynthetic machinery.[12] Presumably, propionyl-CoA and acetyl-CoA are condensed and further processed giving rise to a propylmalonyl-CoA precursor that forms the pentanoate (valerate) extender unit (C-3′/C-2′/C-1′/C-4/C-3) which is incorporated into the single PKS chain of the iromycins. An alternative biosynthetic pathway would comprise only a propionate and acetate based polyketide chain, and the C-1′/C-2′/C-3′ alkyl chain would be attached to C-5 as an external propionate unit. This "two-chain-biosynthesis" is considered unlikely, since such condensations are uncommon; furthermore, incorporation rates of this propionate unit are comparable to the remaining ones in the iromycin

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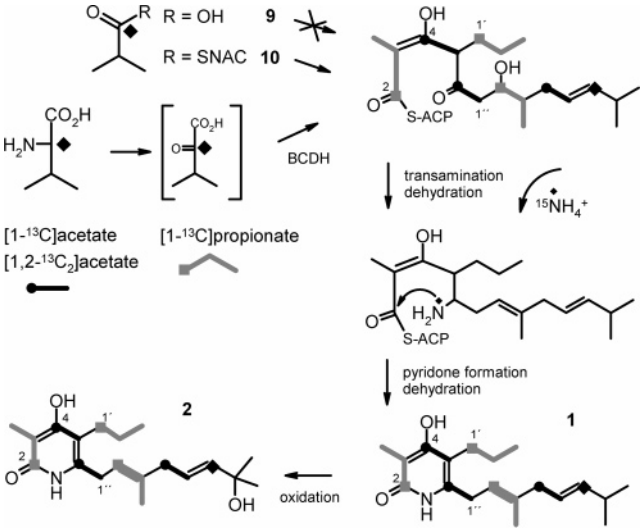
SCHEME 1. Labeling Pattern of 1 and 2 from Feeding Experiments with ^{13}C- and ^{15}N-Labeled Precursors and Hypothesis for Biosynthetic Pathway[a]



[a] BCDH: enzymatic action of a putative branced chain dehydrogenase.

skeleton and no unsubstituted iromycins were detected.[24] As yet, a pentanoate extender unit was only proposed for the polyketide biosynthesis of the immunosuppressant FK506 (tacrolimus) of *Streptomyces* sp. on the basis of sequence data. The respective acetyl transferase (AT 4) domain for FK506 shows conserved motives of methylmalonyl-CoA specific ATs, thus substrate availability or other AT motives determine the longer chain specificity. The second example is given from the metabolite pattern of *Streptomyces hygroscopicus*, which comprises the metabolite FK520 (containing a butyrate unit), and further FK520 propionate and pentanoate analogues suggesting a broad specificity of the butyrate-specific AT domain.[25] However, in the case of the iromycin producer strain Gö Dra 17 no C-5 analogues have been detected via HPLC-MS-DAD analysis so far.

Since branched chain carboxylic acids are the starter unit of several bacterial polyketides like bafilomycin, avermectin, tautomycin, and myxothiazol[26,27] we investigated its role as primer for the polyketide chain of the iromycins. [1-^{13}C]-Isobutyrate (IB) (**9**) was efficiently synthesized via Grignard reaction with $Ba^{13}CO_3$[12] and used in feeding experiments; however, no isotope enrichment for **1** and **2** could be detected. Lack of appropriate activation might be the reason for this failure since one can assume sufficient cell permeability for IB. Similar results were obtained for myxothiazol, but not in the case of the bafilomycins and avermectins.[26,28] The [1-^{13}C]-labeled isobutyrate-*N*-acetylcysteamine (SNAC) thioester (**10**) was prepared as a cell-permeable and activated CoA analogue.[12,29] The isolated iromycins from the feeding experiment with **10** gave ^{13}C NMR spectra which showed strong signal enrichments of C-6″ for **1** and **2** and therefore pointed to IB as the direct primer unit. Previous work showed that short branched-chain carboxylic acids as the starter units for fatty acids and polyketides usually derive from the amino acids valine, leucine, or isoleucine via deamination and decarboxylation.[27,28] A branched-chain α-keto acid dehydrogenase reaction was found to provide the activated α-branched-chain acyl-CoA precursors.[28] To verify the origin of the IB starter unit [2-^{13}C]-labeled valine was used, which gave strong signal enhancement for C-6″ of isolated **1** and **2** (Scheme 1, Table 1).[12]

These results shed light on the biosynthesis of the hexaketide carbon framework (Scheme 1), which proceeds from the IB starter and includes a pentanoate, two acetates, and two propionate units, presumably condensed by a type I modular polyketide synthase. In contrast, **3** and **4** are synthesized from a propionate starter, which points to a relaxed substrate specificity of the respective AT or CoA-ligase of the loading domain. This exchange of a branched with a linear starter unit is very uncommon; usually different branched starter units prime the biosynthesis of polyketides as in the case of avermectin.[30] The obtained labeling patterns for **1** and **2** clearly rule out the isoprenoid origin, neither mevalonate nor non-mevalonate pathway, which would give substantially different results.[12]

While alanine and phenylalanine provide the pyridone nitrogen for kirromycin and tenellin/militarinones, respectively, the nitrogen source of the iromycins is still undiscovered.[12] ^{15}N-labeled **1** and **2** were obtained from feeding experiments with $(^{15}NH_4)_2SO_4$ but not with $Na^{15}NO_3$ in standard medium cultures of strain Gö Dra 17. FT-ICR high-resolution mass spectrometry successfully determined the isotope pattern of the ion peaks (**1**: $C_{19}H_{29}{}^{15}NO_2$, 305 [M + H$^+$]; **2**: $C_{19}H_{29}{}^{15}NO_3$, 321 [M + H$^+$]). The $^{13}C/^{12}C$ ratio and $^{15}N/^{14}N$ ratio pointed to 25% ^{15}N-enrichment of **1** and **2**, and ^{13}C NMR confirmed the ^{15}N incorporation with high field shifts of the signals of C-2 and C-6.[12] This proves the insertion of the nitrogen from the NH_4^+-pool or a close precursor, for example, via transamination of a C-6 carbonyl group. For iromycins **1**−**4** the C-2″/C-3‴ double bond is "shifted", i.e., it is not placed between two extender units, which would be the common site for polyketide chains.[22] The putative transamination and pyridone formation mechanisms do not necessarily induce the shift. Since the other bioactive pyrone analogues (**7**−**8**) also show double bonds in the *β*-position to the heterocycle, we speculate that an additional directed post-PKS biosynthetic reaction such as a dehydration occurs in order to create the biologically active natural products.

Iromycin B (**2**) features much lower NOS inhibitory activity than **1**. Thus, we suggested that the hydroxylated **2** represents a catabolic intermediate of a degradation process of the bioactive metabolite **1**. Oxidation processes of xenobiotics are common detoxification pathways. In the case of the phytotoxin destruxin B, a nonactivated isopropyl moiety is biotransformed by hydroxylation.[31] To prove the oxidation as deactivation and degradation path of **1** to **2** in the producer strain, ancymidol, an inhibitor of cytochrome P450 monooxygenases (CYP), was fed in concentrations of 0, 30, or 300 mg/L in three parallel runs to

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TABLE 1. ^{13}C NMR Data of Iromycin A (1) from Feeding Experiments

Carbon atom	δ_C [ppm]	[1-^{13}C] acetate[a]	[1,2-$^{13}C_2$] acetate[b]	[1-^{13}C] propionate[a]	[1-^{13}C] IB (**9**)[a]	(**10**)[a]	[2-^{13}C] valine[a]
C-2	165.9	0.2	-	**3.5**	-0.1	0.1	0.5
C-3	106.0	–0.2	-	0.1	0.0	0.1	0.7
C-4	166.1	**5.9**	60	0.2	0.4	0.4	-0.5
C-5	113.7	0.0	60	-0.1	0.0	-0.4	-0.4
C-6	143.0	**2.9**	48	-0.2	-0.3	0.0	-0.2
C-7	8.7	–0.1	-	-0.3	-0.2	-0.2	-0.1
C-1'	28.0	0.0	-	**3.2**	-0.3	0.3	-0.2
C-2'	24.1	–0.1	-	-0.2	-0.2	-0.4	-0.2
C-3'	14.5	0.0	-	-0.4	-0.3	-0.4	-0.3
C-1"	30.4	–0.2	48	-0.1	-0.4	-0.5	-0.3
C-2"	121.0	0.2	-	**2.5**	-0.5	0.1	-0.3
C-3"	138.8	0.4	-	0.1	0.0	-0.2	-0.6
C-4"	43.7	**3.6**	43	-0.1	-0.3	-0.1	0.3
C-5"	125.7	–0.2	43	-0.1	-0.3	-0.5	-0.4
C-6"	140.8	–0.1	-	0.0	-0.3	**15.1**	**16.7**
C-7"	32.3	–0.1	-	-0.2	-0.2	-0.3	-0.2
C-8", 9"	23.0	0.0	-	-0.3	-0.2	-0.5	-0.4
C-10"	16.5	0.0	-	0.0	0.0	0.0	0.0

[a] ^{13}C specific incorporation (reference atom: C-10″, significant specific incorporations are indicated by shading). [b] Coupling constants (in Hz). Spectra were recorded in CD_3OD (75.5 MHz).

growing cultures of strain Gö Dra 17 and the metabolite pattern was analyzed.[32] TLC and HPLC-ESI-MS analyses of the crude extracts revealed that the ratio of the microbial production of iromycin B (**2**) to **1** is decreased more than 10-fold in the presence of ancymidol,[12] supporting the role of a CYP in the conversion of iromycin A (**1**) to iromycin B (**2**). On the basis of such an interesting natural diversity of the metabolite family of the iromycins it is of high interest to further evaluate analogues such as semisynthetic derivatives or to investigate the potential of total chemical synthesis.[33]

Conclusion

Our detailed study in the field of natural product chemistry presents the exciting pyridone metabolite family of iromycins A to D (**1**−**4**), which are the first noticeable natural products that selectively inhibit eNOS but not nNOS. In contrast, most widely used NOS inhibitors resemble derivatives of the substrate arginine,[8] and thus inhibit NO synthases unspecifically. The iromycins are produced by the strain *Streptomyces* Gö Dra 17 via a type I polyketide pathway from a hexaketide chain consisting of acetate, propionate, a rare pentanoate, and isobutyrate building blocks. The unique skeleton of the pyridone ring, the branched unsaturated side chain, and the additional C_3 side chain originate from a unusual set of biosynthetic reactions: Propylmalonyl CoA presumably extends the yet unknown C_{10}-alkenyl tetraketide, giving rise to the branched polyketide chain. The formation of the unusual position of the C-2″/C-3″ double bond is in agreement with a putative dehydration. The nitrogen source of the pyridone is either the ammonia pool or a close precursor like glutamine. By using the enzyme inhibitor ancymidol, the uncommon oxidative degeneration by the producer strain via cytochrome P450 monooxygenase was established and thus linked the biosynthesis of iromycin B (**2**) with **1**, and in analogy **4** with **3**. Detailed questions on the biosynthetic enzyme machinery should be solved by investigation of the biosynthetic gene cluster in the future. Total synthesis of iromycins is a prerequisite for the development of novel derivatives and custom-made analogues for further biological profiling. On the basis of these promising results, work is now in progress to establish the new natural iromycins as valuable tools for studying the important network of NOS isoforms, a challenge important for both basic research and potential therapeutic intervention.

Experimental Section

Cultivation and Isolation. Strain *Streptomyces* sp. Gö Dra 17 was grown on agar plates (glucose 4 g L^{-1}, yeast extract 4 g L^{-1}, malt extract 10 g L^{-1}, calcium carbonate 0.4 g L^{-1}, pH 7.0) at 28 °C until sporulation, and then stored at 4 °C. A 1 cm^2 sample of these agar plates was then used to inoculate 100 mL of soy/glucose/glycerol medium (starch 10 g L^{-1}, glucose 10 g L^{-1}, glycerol 10 g L^{-1}, corn steep powder 2.5 g L^{-1}, caseinpeptone 5 g L^{-1}, yeast extract 2 g L^{-1}, calcium carbonate 3 g L^{-1}, sodium chloride 1 g L^{-1}, pH 7.3) in 300 mL Erlenmeyer flasks. These cultures were incubated on a rotary shaker (250 rpm) at 28 °C for 48 h. A 100 mL portion of this preculture was used to inoculate a 1 L fermentor, medium: 10 g L^{-1} soluble starch, 4 g L^{-1} glycerol, 4 g L^{-1} caseinpeptone, 0.5 g L^{-1} yeast extract, 0.5 g L^{-1} meat extract, 0.5 g L^{-1} liver extract, 1 g L^{-1} NaCl, pH 7.0. The culture was incubated at 28 °C, 1.6 L min^{-1} aeration, 600 rpm and harvested after 72 h. Scale-up fermentations were performed in a 50 L fermenator (28 °C, 1.5 L min^{-1} aeration, 100 rpm, 72 h, workup procedure according to standard protocols).

The culture broth was separated from the mycelium by filtration. The mycelium was extracted with 300 mL of acetone and the culture filtrate was applied to Amberlite XAD-2/methanol (500 mL) to yield the crude extract. The residue was purified by chromatography on silica gel (50 × 2 cm, cyclohexane/ethyl acetate/methanol 5/10/2), size exclusion chromatography (74 × 2.7 cm, Sephadex LH-20, MeOH), and reverse phase HPLC to yield 18 mg/L of **1**, 12 mg/L of **2**, 0.5 mg/L of **3**, and 0.25 mg/L of **4**.

HPLC programs: solution A, H_2O; solution B, acetonitrile

HPLC-columns: A, Nucleodur C-18 100 ec. 5 μm; B, Nucleosil C-8 100 ec. 5 μm.

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(33) Shojaei, H.; Li-Böhmer, Z.; von Zezschwitz, P. *J. Org. Chem.* **2007**, *72*, 5091−5097.

Isolation of 1 and 3: 35% A and 65% B, column A, retention time 18 min for **1** and 13 min for **3**.

Isolation of 2 and 4: 75% A and 25% B, column B, retention time 20 min for **2** and 18 min for **4**.

Iromycin A (1): $C_{19}H_{29}NO_2$ (M_r = 303.45); EI MS *m/z* 303 ([M]$^+$, 31%); HR EI MS 303.2198, found as calculated for $C_{19}H_{29}NO_2$; IR (KBr) $\tilde{\nu}_{max}$ 3398, 2961, 2931, 2871, 1636, 1458, 1381, 1219, 1165 cm^{-1}; UV (MeOH) λ_{max} nm (ϵ) 291 (7065), 206 (32120); ^{1}H NMR (500 MHz, CD_3OD) δ 0.97 (t, J = 7.5 Hz, 3 H, 3′-H_3), 0.98 (d, J = 7.0 Hz, 6 H, 8″-H_3, 9″-H_3), 1.50 (tq, J = 8.0, 7.5 Hz, 2 H, 2′-H_2), 1.73 (s, 3 H, 10″-H_3), 1.98 (s, 3 H, 7-H_3), 2.26 (dqq, J = 7.0, 7.0, 7.0 Hz, 1 H, 7″-H), 2.43 (t, J = 8.0 Hz, 2 H, 1′-H_2), 2.70 (d, J = 6.5 Hz, 2 H, 4″-H_2), 3.32 (d, J = 7.5 Hz, 2 H, 1″-H_2), 5.17 (dt, J = 7.5, 1.5 Hz, 1 H, 2″-H), 5.32 (ddd, J = 15.0, 6.5, 1.0 Hz, 1 H, 5″-H), 5.42 (ddt, J = 15.0, 7.0, 1.0 Hz, 1 H, 6″-H), the signal of the 1″ group is under the methanol signal; ^{13}C NMR, see Table 1.

Iromycin B (2): $C_{19}H_{29}NO_3$ (M_r = 319.45); EI MS *m/z* 319 ([M]$^+$, 31%); HR EI MS 319.2147, found as calculated for $C_{19}H_{29}NO_3$; IR (KBr) $\tilde{\nu}_{max}$ 3366, 2964, 2930, 2871, 1630, 1430, 1376, 1220, 1156 cm^{-1}; UV (MeOH) λ_{max} nm (ϵ) 290 (9540), 207 (41595); ^{1}H NMR (500 MHz, CD_3OD) δ 0.94 (t, J = 7.5 Hz, 3 H, 3′-H_3), 1.24 (s, 6 H, 8″-H_3, 9″-H_3), 1.47 (tq, J = 8.0, 7.5 Hz, 2 H, 2′-H_2), 1.73 (d, J = 1.0 Hz, 3 H, 10″-H_3), 1.95 (s, 3 H, 7-H_3), 2.41 (t, J = 8.0 Hz, 2 H, 1′-H_2), 2.72 (d, J = 6.5 Hz, 2 H, 4″-H_2), 5.18 (dt, J = 7.0, 1.0 Hz, 1 H, 2″-H), 5.57 (dt, J = 15.5, 6.5 Hz, 1 H, 5″-H), 5.62 (d, J = 15.5 Hz, 1 H, 6″-H), the signal of the 1″ group is under the methanol signal; ^{13}C NMR (150.8 MHz, CD_3OD) δ 8.7 (+, C-7), 14.5 (+, C-3′), 16.6 (+, C-10″), 24.1 (−, C-2′), 27.9 (−, C-1′), 29.9 (+, C-8″, C-9″), 30.4 (−, C-1″), 43.3 (−, C-4″), 71.1 (C_{quat}, C-7″), 106.0 (C_{quat}, C-3), 113.7 (C_{quat}, C-5), 121.3 (+, C-2″), 125.3 (+, C-5″), 138.6 (C_{quat}, C-3″), 141.2 (+, C-6″), 142.9 (C_{quat}, C-6), 165.9 (C_{quat}, C-2), 166.0 (C_{quat}, C-4).

Iromycin C (3): $C_{18}H_{27}NO_2$ (M_r = 289.42); EI M: *m/z* 290 [M + H]$^+$; HR EI MS 290.2115, found as calculated for $C_{18}H_{27}NO_2$; IR (KBr) $\tilde{\nu}_{max}$ 3420, 2962, 2932, 2872, 1717, 1636, 1458, 1380, 1220, 1167, 1115, 1054, 970 cm^{-1}; UV (MeOH) λ_{max} nm (ϵ) 293 (1988), 207 (8432); ^{1}H NMR (600 MHz, CD_3OD) δ 0.94 (t, J = 7.5 Hz, 3 H, 3′-H_3), 0.96 (t, J = 7.5 Hz, 3 H, 8″-H_3), 1.47 (tq, J = 8.0, 7.5 Hz, 2 H, 2′-H_2), 1.70 (s, 3 H, 9″-H_3), 1.95 (s, 3 H, 7-H_3), 2.01 (ddq, J = 7.5, 7.5, 1.0 Hz, 2 H, 7″-H_2), 2.40 (t, J = 8.0 Hz, 2 H, 1′-H_2), 2.67 (d, J = 7.0 Hz, 2 H, 4″-H_2), 3.26 (d, J = 7.0 Hz, 2 H, 1″-H_2), 5.15 (dt, J = 7.0, 1.0 Hz, 1 H, 2″-H), 5.37 (dtt, J = 15.5, 7.0, 1.0 Hz, 1 H, 5″-H), 5.50 (dtt, J = 15.5, 7.5, 1.0 Hz, 1 H, 6″-H); ^{13}C NMR (150.8 MHz, CD_3OD) δ 8.7 (+, C-7), 14.3 (+, C-3′), 14.5 (+, C-8″), 16.5 (+, C-9″), 24.1 (−, C-2′), 26.6 (−, C-7″), 28.0 (−, C-1′), 30.4 (−, C-1″), 43.7 (−, C-4″), 106.0 (C_{quat}, C-3), 113.8 (C_{quat}, C-5), 121.0 (+, C-2″), 127.7 (+, C-5″), 135.2 (+, C-6″), 140.4 (C_{quat}, C-6), 140.5 (C_{quat}, C-3″), 165.9 (C_{quat}, C-2), 166.8 (C_{quat}, C-4).

Iromycin D (4): $C_{18}H_{27}NO_3$ (M_r = 305.21); EI MS *m/z* 306 [M + H]$^+$; HR EI MS 306.2063, found as calculated for $C_{18}H_{27}NO_2$; IR (KBr) $\tilde{\nu}_{max}$ 3421, 2962, 2929, 2871, 1700, 1635, 1436, 1376, 1220, 1167, 1114, 1056, 971 cm^{-1}; $[\alpha]^{20}_D$ −150 (*c* 0.1, MeOH); UV (MeOH) λ_{max} nm (ϵ) 289 (911), 206 (3771); ^{1}H NMR (300 MHz, CD_3OD) δ 0.94 (t, J = 7.0 Hz, 3 H, 3′-H_3), 1.19 (d, J = 7.0 Hz, 3 H, 8″-H_3), 1.48 (tq, J = 8.0, 7.0 Hz, 2 H, 2′-H_2), 1.73 (s, 3 H, 10″-H_3), 1.95 (s, 3 H, 7-H_3), 2.41 (dt, J = 8.0, 2.0 Hz, 2 H, 1′-H_2), 2.73 (d, J = 7.0 Hz, 2 H, 4″-H_2), 4.18 (dq, J = 7.0 Hz, 1 H, 7″-H), 5.18 (dt, J = 7.0, 1.0 Hz 1 H, 2″-H), 5.55 (ddt, J = 15.0, 6.5 Hz, 1 H, 5″-H), 5.58 (ddd, J = 15.0, 6.5, 1.0 Hz, 1 H, 6″-H); ^{13}C NMR (75.5 MHz, CD_3OD) δ 8.7 (+, C-7), 14.5 (+, C-3′), 16.6 (+, C-9″), 23.7 (+, C-8″), 24.1 (−, C-2′), 27.9 (−, C-1′), 30.3 (−, C-1″), 43.2 (−, C-4″), 69.1 (+, C-7″), 106.0 (C_{quat}, C-3), 113.8 (C_{quat}, C-5), 121.4 (+, C-2″), 128.4 (+, C-6″), 137.5 (+, C-5″), 138.3 (C_{quat}, C-3″), 142.8 (C_{quat}, C-6), 165.9 (C_{quat}, C-2), 166.4 (C_{quat}, C-4).

Feeding Experiments. Labeled precursors and feeding experiments: Sodium [1-^{13}C]acetate and sodium [1,2-$^{13}C_2$]acetate (0.9 g each), sodium [1-^{13}C]propionate (0.5 g), sodium [1-^{13}C]isobutyrate (0.4 g), sodium [1-^{13}C]isobutyrate-SNAC-ester (0.4 g), [2-^{13}C]valine (0.5 g), [$^{15}N_2$]ammonium sulfate (0.8 g), and [^{15}N]sodium nitrate (1.0 g) were used as sterile aqueous solutions, which were adjusted to pH 7.0, and were added within 12 h after 24 h of incubation to 1 L of growing cultures (valine: 0.5 L). Isolation and purification was performed as described above to yield 6.8 mg of **1** and 12.6 mg of **2** ([1-^{13}C]acetate), 18.5 mg of **1** and 6.8 mg of **2** ([1,2-$^{13}C_2$]-acetate), 4.9 mg of **1** and 11.5 mg of **2** ([1-^{13}C]propionate), 11.8 mg of **1** and 5.8 mg of **2** ([1-^{13}C]isobutyrate), 24.0 mg of **1** and 21.8 mg of **2** ([1-^{13}C]isobutyrate-SNAC-ester), 9.8 mg of **1** and 4.6 mg of **2** ([2-^{13}C]valine), 23.9 mg of **1** and 0.5 mg of **2** [$^{15}N_2$]-ammonium sulfate, and 22.5 mg of **1** and 4.5 mg of **2** [^{15}N]sodium nitrate.

Treatment with ancymidol: Ancymidol (3 and 30 mg, respectively) was added to 100 mL cultures of strain Dra 17 in 300 mL Erlenmeyer flasks after 20 h of inoculation (10% preculture and media as described above). The cultures were harvested after 72 h of cultivation, and cultures were lyophilized and extracted with methanol (2 × 30 mL). After removing the solvent in vacuo 2.5 mL of methanol was added and the ratios of **1** and **2** were determined by coupled HPLC-ESI-MS-DAD analysis.

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Supporting Information Available: General experimental methods, experimental procedures at full length, ^{1}H NMR and ^{13}C NMR spectra of the new metabolites **1**−**4**; a comparison with the structures and biosynthesis of kirromycin (**5**), tenellin (**6**), kalkipyrone (**7**), piericidin A_1, and actinopyrone; hypothetical incorporation pattern for **1** after the [1-^{13}C]acetate feeding experiment in mevalonate and non-mevalonate biosynthetic pathways; and X-ray data for **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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