Structural and Biosynthetic Investigations of the Rubromycins

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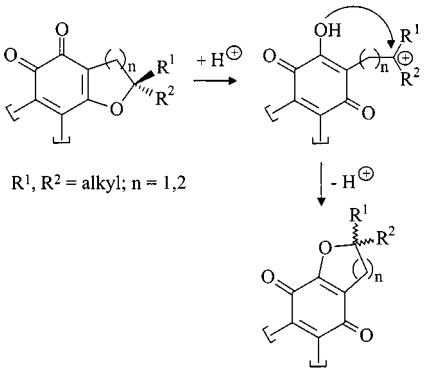
The structure of the known secondary metabolite β-rubromycin was corrected, based on spectroscopic and chemical investigations, from *o*-quinone **1** to *p*-quinone **6**. By feeding [U-^{13}C3]malonic acid to the rubromycin-producing strain, *Streptomyces* sp. A1, the polyketide origin of the skeleton was verified, but the identity of the starter unit and the folding mechanism of the polyketide chain are still unclear. From the culture broth of the strain A1, in addition to **6**, the co-metabolites γ-rubromycin (**3**), δ-rubromycin (**4**) and 3′-hydroxy-β-rubromycin (**7**) were isolated. Their structures were determined or confirmed by detailed spectroscopic analysis. The rubromycins inhibit HIV-1 reverse transcriptase (RT) and are cytostatically active against different tumor cell lines.

The red, antibacterially active β-rubromycin was first isolated from cultures of a *Streptomyces* species by H. Brockmann et al., and its structure was established by chemical transformation and degradation reactions.[1−3] It was suggested that the structure of β-rubromycin contains an *o*-quinone moiety, this was based on a condensation reaction with *o*-phenylenediamine and the relatively positive redox potential of di-*O*-acetyl-β-rubromycin. β-Rubromycin can be quickly transformed into γ-rubromycin (**3**) by an acid-catalyzed reaction. For this purpose a mechanism similar to the β-lapachone/α-lapachone rearrangement was proposed (Scheme 1).[4]



Scheme 1. β-Lapachone/α-lapachone rearrangement[4]

The investigations we made by modern spectroscopic methods appeared to contradict previous findings, especially with regard to the structure of β-rubromycin. The two spiroketals, β-rubromycin and γ-rubromycin, both show significant features in their CD spectra. Surprisingly, in the case of γ-rubromycin (**3**), it was inconsequential whether **3** was obtained from biological material or by acid-catalyzed transformation of β-rubromycin. This fact is not compatible with the postulated mechanism for the transformation of **1** to **3**[3] because in this case γ-rubromycin (**3**) should have been formed as a racemate. Further problems arose with the assignment of the ^{13}C-NMR data of β-rubromycin for the biosynthetic studies. The 2D-NMR analysis (HMQC and HMBC experiments) of this molecule remains incomplete because of the unfavourable ratio of quaternary carbon to hydrogen-binding carbon atoms. This fact, together with some overlapping ^{13}C-NMR shifts, led to the result that only 19 of 27 ^{13}C-NMR signals could definitely be assigned.

On the basis of these findings, we have resumed the structure elucidation of β-rubromycin and we will now go on to report the necessary correction of the chromophore moiety. For the revision of the structure of this antibiotic, some derivatives of β-rubromycin and their spectroscopic characterization were very helpful. Further valuable information resulted from several feeding experiments with [^{13}C]-labeled precursors during the course of biosynthetic investigations. As a result of the renewed fermentations of available rubromycin-producing strains, we discovered, under altered fermentation conditions, besides the known γ-rubromycin (**3**), small amounts of the co-metabolites δ-rubromycin (**4**) and 3′-hydroxy-β-rubromycin (**7**). The structure elucidation of these two metabolites is described. Furthermore, we compile the known data about the biological activities of the rubromycins and supplement them with new facts.

In addition to the described rubromycin-producing strains[2][5] and large amounts of β-rubromycin from H. Brockmann et al., we used a new strain. This strain was isolated from enrichment cultures prepared from orange grove soil samples from Israel.[6] The inhibition of reverse transcriptase by β- and γ-rubromycin (**3**), however, was published for the first time by M. E. Goldman et al. in 1990.[7]

Chemical Investigations

A first indication that the structure of β-rubromycin does not contain an *o*-quinone moiety resulted from the attempt, to prepare a Diels−Alder product of β-rubromycin with

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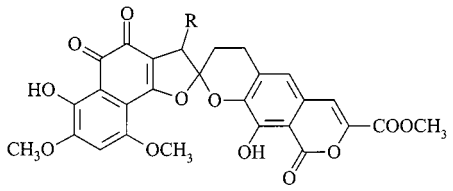
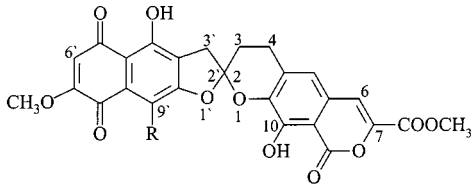


1: R = H, **2**: R = OH

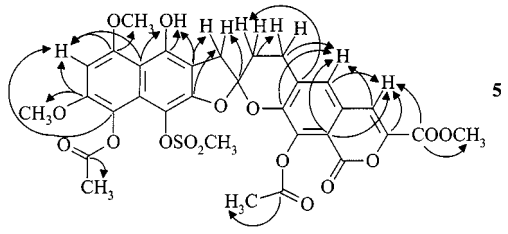


3: R = OH, **4**: R = H

cis-stilbene.[8][9] In spite of variation of the solvent and the temperature, we were not able to achieve any reaction.

The fact, that phenols react, after being transferred into their methanesulfonic acid esters by reduction with H_2/Pd in protic solvents, to the corresponding aromatic compounds,[10] could be used to incorporate further protons in the chromophore of β-rubromycin and thereby obtain more data for the analysis of the 2D-NMR experiments.

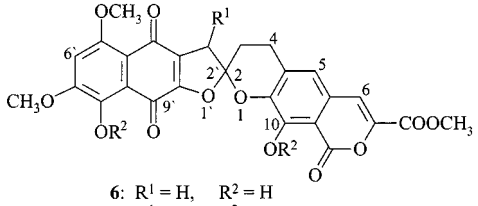
For this purpose it was intended to synthesize di-*O*-acetyl-di-*O*-mesyl-dihydro-β-rubromycin by the reaction of di-*O*-acetyl-β-rubromycin[2] with palladium/pyridine/methanesulfonic anhydride under H_2 in THF. The following hydrogenation in the presence of Pd/C in methanol should yield a pentasubstituted naphthalene derivative, after reductive removal of the *O*-mesylate protecting groups. The first step led, in 72% yield, to 8′,10-di-*O*-acetyl-9′-*O*-mesyl-dihydro-β-rubromycin (**5**) (Figure 1), the structure of which could already be determined by means of two-dimensional NMR-spectroscopy.



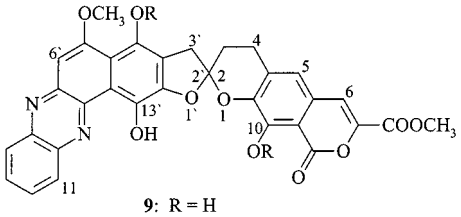
5

Figure 1. $^{n}J_{C,H}$ long-range couplings observed in the β-rubromycin derivative **5** by HMBC pulse sequences at 500 MHz

Of particular importance to derivative **5** is the fact that the phenolic proton shows correlations, in the HMBC as well as in the COLOC spectrum, with three carbon atoms which were characterized by chemical shifts at δ_C = 107.0, 107.7, and 149.9. The values of these ^{13}C-NMR resonances verify that no other oxygen-binding carbon atom occurs in *ortho* position relative to the phenolic carbon atom (δ_C = 149.9). The $^{2}J_{C,H}$ and $^{3}J_{C,H}$ correlations of **5** indicate that the chromophore could only have been derived from a *para*-quinone. It is not possible to obtain a mesylate with an equivalent substitution pattern from structure **1**. From the new proposal of structure **6** as the structure of β-rubromycin, it follows that for its transformation to γ-rubromycin (**3**) only the hydrolysis of a *peri*-positioned methoxy group is required. The given spirochirality in β-rubromycin (**6**) can be preserved, which is confirmed by the CD spectra of **3** and **6**.

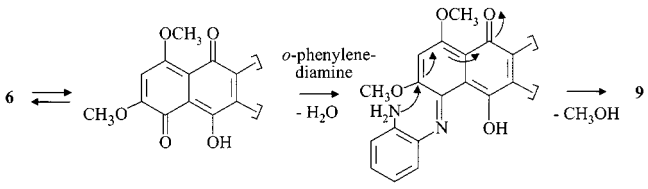


6: R^1 = H, R^2 = H
7: R^1 = OH, R^2 = H
8: R^1 = H, R^2 = Ac



9: R = H
10: R = Ac

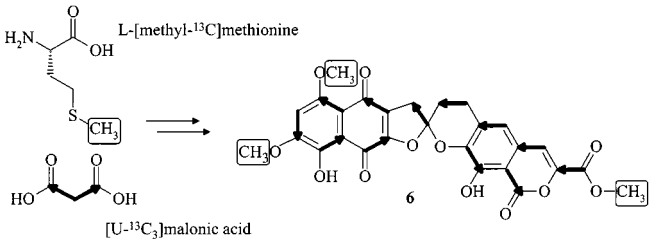
H. Brockmann et al. obtained from the reaction of β-rubromycin with *o*-phenylenediamine in concentrated acetic acid at 60°C under the elimination of two molecules H_2O and hydrolysis of one methoxy group a condensation product (in 42% yield) which does not show any kind of quinone carbonyl bands in its IR spectrum. It was assumed that only an *o*-quinone can be transformed into a quinoxaline derivative. Due to the fact that this derivative was hardly soluble in organic solvents and water it was acetylated with acetic anhydride/sodium acetate to its more lipophilic diacetate.[2] The evaluation of the one- and two-dimensional NMR spectra of this derivative led to structure **10**, from which structure **9**, the structure of the product of the transformation with *o*-phenylenediamine, could be derived. The established structures **9** and **10** harmonize with the fact that the CD spectrum of **10** shows minima at 266 and 312 nm, and maxima at 278 and 348 nm. The formation of the quinoxaline derivative by opening the spiroketal system can therefore be excluded. We assume that the reaction starts with a tautomerization of the hydroxy-1,4-naphthoquinone to its corresponding hydroxy-1,5-naphthoquinone. After the reaction of the sterically less-hindered quinone carbonyl group to an azomethine, the elimination of the neighboring methoxy group at C-7′ results in the formation of the quinoxaline (Scheme 2). Such a condensation reaction makes it clear that the formation of quinoxalines under special conditions is not a secure proof of an *o*-quinone moiety.[11]



Scheme 2. Reaction of β-rubromycin (**6**) with *o*-phenylenediamine

Biosynthesis

By feeding [U-$^{13}C_3$]glycerol to the cultures of the strain *Streptomyces* sp. A1, which were grown on oat-bran medium, we obtained the first information about the polyketide origin of β-rubromycin (**6**). For every chemical shift of the 24 skeleton carbon atoms in the ^{13}C-NMR spectrum of **6** there appeared, besides the natural ^{13}C-NMR signals, one doublet caused by a $^1J_{C,C}$ coupling. The observed incorporation of intact C_2 units can be explained by the metabolic transformation of glycerol via glyceraldehyde 3-phosphate and phosphoenol pyruvate to acetyl-CoA. Whereas the tolerance of the strain A1 against glycerol was quite good, the addition of the direct precursor acetate caused great difficulties. Feeding 10 mmol/L sodium acetate or malonic acid during the product formation phase, in shake flasks or in the Biostat M fermentor, led to a complete suppression of the rubromycin biosynthesis. In the case of the addition of malonic acid, it was possible to neutralize the suppression by the improvement of the fermentation process (installation of a pH barrier, addition of vitamin B_{12} to the culture broth) and in this way establish a basis for further incorporation experiments. By feeding [2-^{13}C]malonic acid, [U-$^{13}C_3$]malonic acid and L-[methyl-^{13}C]methionine, we succeeded in elucidating the biogenesis sources of β-rubromycin (**6**), and determining the incorporation direction of the acetate units. Moreover, we were able to confirm the new proposal for the structure of β-rubromycin by means of the $^1J_{C,C}$ couplings, and finally to exclude structure **1** established by H. Brockmann et al.[3] The carbon skeleton of the rubromycins is composed of twelve acetate units, the methoxy groups derive from L-methionine. The labeling pattern of the rubromycin carbon skeleton seems to be identical with that of the aglycon of heliquinomycin,[12] which was published recently.



Scheme 3. Labeling pattern of **6** derived from [2-^{13}C]malonic acid, [U-$^{13}C_3$]malonic acid, and L-[methyl-^{13}C]methionine

The structure of **6** and the labeling pattern represented in Scheme 3 exclude the creation of the rubromycin carbon skeleton by direct folding and enzymatically controlled aldol condensation of a dodecaketide precursor. For the biosynthesis of **6**, the following alternatives can be put forward:

Table 1. ^{13}C-NMR resonances [ppm] of **6** and **7**, $^1J_{C,C}$ coupling constants [Hz], and specific incorporations[14] after feeding with [U-$^{13}C_3$]malonic acid or [U-$^{13}C_3$]glycerol (I), [2-^{13}C]malonic acid (II) and L-[methyl-^{13}C]methionine (III)

C atom	δ_C[a]	I **6**	II **6**	III **6**	δ_C[b]	I **7**	II **7**
C-2	111.1	43	9.9	1.2	112.8	48	9.4
C-3	29.6	32	0.2	0.4	22.9	32	0.4
C-4	22.2	32	7.5	0.3	21.0	32	11.2
C-4a	131.6	60	0.1	0.4	132.0	59	1.3
C-5	118.2	60	8.1	0.2	118.8	59	11.7
C-5a	127.45	54	1.3	1.0	127.5	54	0
C-6	113.6	54	6.6	0.1	113.3	54	9.9
C-7	141.25	93	1.7	0.6	140.4	93	0.5
C-9	164.9	72	0.7	0.6	163.3	73	0.6
C-9a	106.6	72	8.4	0.7	106.4	73	7.6
C-10	150.6	80	0.3	0.8	148.9	79	1.6
C-10a	141.23	80	6.4	0.8	140.6	79	10.7
7-*C*$O_2$$CH_3$	160.5	93	9.2	0.2	159.9	93	10.0
7-CO_2*C*H_3	52.9	–	0	51.5	52.6	–	1.2
C-3′	40.1	43	0.3	0.5	75.2	48	0.6
C-3′a	127.43	61	8.0	0.9	127.7	61	8.9
C-4′	179.2	61	0.4	0.4	177.7	61	0
C-4′a	110.6	73	6.9	0.3	109.6	73	12.4
C-5′	155.8	73	0.5	0.3	155.4	73	0.2
C-6′	103.7	72	7.4	0	105.6	71	10.9
C-7′	155.4	72	0.5	0.2	154.7	71	0.7
C-8′	149.6	68	9.0	0.1	148.6	68	18.3
C-8′a	114.8	68	0.2	0.7	114.5	68	0.4
C-9′	181.9	64	7.7	0.8	182.1	62	10.2
C-9′a	154.9	64	0.5	0.8	155.6	62	0.6
5′-OCH_3[c]	56.4	–	0.1	60.3	56.5	–	0.8
7′-OCH_3[c]	57.1	–	0.1	66.8	56.8	–	0.9

[a] 125.7 MHz, $CDCl_3$. – [b] 125.7 MHz, [D_6]DMSO. – [c] Assignment exchangeable.

a) Two-chain hypothesis: The carbon skeleton is formed by the connection of two polyketide chains. It is conceivable that a decaketide is connected with a diketide, or a heptaketide is connected with a pentaketide.

b) One-chain hypothesis: The carbon skeleton is derived from a dodecaketide, which undergoes an oxidative C−C bond cleavage in the course of biosynthesis.

In order to clarify whether there is more than one precursor unit, the strain A1 was fed with *S*-[1,3-$^{13}C_2$]acetoacetyl-*N*-acetylcysteamine[11] in the presence of the β-oxidation inhibitor 3-(tetradecylthio)propanoic acid[13] and vitamin B_{12}. The evaluation of the ^{13}C-NMR spectrum indicated that [1,3-$^{13}C_2$]acetoacetic acid was not incorporated into **6**. Currently, this negative result does not allow to differentiate between the mentioned alternatives. Further experiments with other rubromycin-producing strains are in progress.

Variation of the Cultivation Conditions

Besides β-rubromycin (**6**) the strain A1 produces the equally known γ-rubromycin (**3**)[2] and the structurally new secondary metabolites δ-rubromycin (**4**) and 3′-hydroxy-β-rubromycin (**7**). The yields of the single compounds as shown in Table 2 are highly dependent of the type of the medium which was chosen.

Table 2. Isolated yields of the rubromycins [mg/L], obtained from fermentations in 250-mL Erlenmeyer flasks by using different media

Medium	**3**	**4**	**6**	**7**
Oat bran	3.3	traces	40	traces
Millet	0.6	−	53	2.0
Quinoa	1.0	1.7	43	1.2
Oat bran + Celite	3.2	2.5	173	8.2

The structure of the red γ-rubromycin (**3**),[3] which was identified by EI-MS, resisted the close examination given to the structure of β-rubromycin. Its ^{13}C-NMR data were assigned completely by using a coupled ^{13}C-NMR spectrum and a HMBC experiment.

The yellow-orange compound was named δ-rubromycin and revealed in its HREI mass spectrum a molecular ion peak at m/z = 506 with the formula $C_{26}H_{18}O_{11}$. The NMR spectra are very similar to those of γ-rubromycin (**3**) and indicate that δ-rubromycin, like **3**, contains the spiroketal system with the isocoumarin moiety. The difference between the colors of δ- and γ-rubromycin is reflected by the long-waved absorption bands in the UV spectra (λ_{max} = 424 nm, compared with λ_{max} = 513 nm for **3**), corresponding to the change of the absorption bands from juglone (yellow) to naphthazarine (red).[15] Consequently, the naphthoquinone chromophore of δ-rubromycin has, in comparison with **3**, one hydroxy group less, and in its place one aromatic proton (δ_H = 7.14) more. The chemical shift of 6′-H (δ_H = 6.16 or 6.17, respectively) remained fairly unchanged. Among the possible constitution isomers, the given isomer was chosen by consideration of comparable chemical shifts,[16] HMBC correlations of the chromophore, and biosynthetic considerations. All structural data are in agreement, based on the assumption that δ-rubromycin (**4**) is the direct precursor of γ-rubromycin (**3**), which implies that 9′-H derives from the methyl group of an acetate in the polyketide chain. In the case of γ-rubromycin (**3**) the oxygen atom is incorporated in position C-9′ by an oxygenase late in the biosynthesis.

The ^{1}H-NMR spectrum of the most polar compound of the four isolated dyes does not differ greatly from that of β-rubromycin (**6**), except that the chemical shifts for 3′-H_2 at δ_H = 3.29 and 3.62 (AB system),[11] which appeared in the spectrum of **6**, is missing. Instead of the AB system in $CDCl_3/CD_3OD$, a singlet at δ_H = 5.42 was detected. The mass which was derived from DCI-MS (m/z = 552) is 16 units higher than the mass of **6**, which was to be expected in the case of a rubromycin hydroxylated in 3′-position. As expected, the upward-orientated signal of C-3′ in the APT spectrum has a chemical shift of δ_C = 75.2 (in [D_6]DMSO), similar to the ^{13}C-NMR data of 3,4-didesoxygriseorhodin C.[17] Structure **7**, which is analogous to structure **6**, could be proved (Table 1) by obtaining [^{13}C]-labeled 3′-hydroxy-β-rubromycin from feeding experiments. The ^{13}C-NMR data of this compound could be completely assigned. One compound which matches **7** in its spectroscopic data is the object of a patent.[18] Structure **2**, which was specified for the patented secondary metabolite BQ180B, should be corrected to **7**.

The four described rubromycins are chiral, but their configurations have not yet been examined. The determination of the absolute configuration of the spiro center by CD methods by comparison to the known stereochemistry of heliquinomycin[19] is in progress.

According to literature there are only a few natural products (purpuromycin,[20−24] heliquinomycin,[12,19,25,26] the griseorhodins[17,27−34] and the DK-7814 compounds[35]) which are structurally related to the rubromycins. These compounds differ slightly from γ-rubromycin (**3**). Positions C-3, C-4, and C-3′ are partially occupied by *O*-substituents, and the griseorhodins have a methyl group instead of a methoxycarbonyl group at position C-7. These structural differences are presumably due to differences in their late biosynthesis. The common carbon skeleton, similarities in their spectra of activity against prokaryotic and eukaryotic cells, as well as the finding that the rubromycins, purpuromycin, heliquinomycin, the griseorhodins, and the DK-7814 compounds were isolated exclusively from *actinomycetes*, makes it seem appropriate, to group these colored secondary metabolites in the so-called "rubromycin-group of antibiotics".

Biological Activities

β-Rubromycin (**6**), γ-rubromycin (**3**), 3′-hydroxy-β-rubromycin (**7**) and 8′,10-di-*O*-acetyl-β-rubromycin (**8**) show a marked growth inhibition against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*. Furthermore, **3** and **6**

are active against other Gram-positive germs.[2] But an inhibition of the rubromycins against *Candida albicans* was not detectable.

The described inhibition of HIV-1 RT by **3** and **6** could be confirmed,[7] but the observed potency of **6** was relative low. The established inhibition of this enzyme by **7** (see Table 3) was unknown up to now.

Table 3. Inhibition of HIV-1 RT by the natural rubromycins

Compound	Residual activity[a] [%]
3	10.8 ± 1.1
4	47.3 ± 10.5
6	60.3 ± 3.1
7	6.8 ± 1.2

[a] The residual enzymatic activity was measured in the presence of 10 μmol/L inhibitor. Each Figure represents the average activity of at least three independent experiments ± standard errors.

Tests with four tumor cell lines [HMO2 (stomach adeno carcinoma), Kato III (colon carcinoma), HEP G2 (liver carcinoma), and MCF 7 (mamma carcinoma)] led to the result that all four natural rubromycins and the two derivatives of β-rubromycin, **5** and **8**, possess a marked cytostatic activity, whereas the diacetate **8** shows slightly increased activity compared with the parent compound **6**. The GI_{50} and *TGI* values of the rubromycins are more likely to be unfavourable than those of doxorubicine, with the exception of the doxorubicine-resistant cell line Kato III (Table 4). The mode of action of the rubromycins is still unknown.

Table 4. Cytostatic activity against different tumor cell lines, GI_{50} and *TGI* values in [μmol/L][36]

	HMO2		Kato III		HEP G2		MCF7	
Compound	GI_{50}	*TGI*	GI_{50}[a]	*TGI*[b]	GI_{50}	*TGI*	GI_{50}	*TGI*
3	0.76	5.0	1.2	5.7	n. d.	n. d.	< 0.1	6.5
4	0.1	50	0.6	2.5	0.1	9.0	5.5	7.8
5	1.0	14	0.75	7.5	n. d.	n. d.	< 0.1	5.0
6	0.5	1.8	0.84	7.5	n. d.	n. d.	< 0.1	2.6
7	< 0.1	5.0	0.5	1.0	< 0.1	1.4	6.0	8.0
8	0.62	1.8	0.5	1.5	n. d.	n. d.	< 0.1	1.2
doxorubicine	< 0.1	0.14	0.4	> 50	0.25	1.0	< 0.1	0.2

[a] GI_{50} = concentration, which results in a 50% inhibition of the cell growth. − [b] TGI = concentration, which results in a complete inhibition of the cell growth.

Experimental Section

Melting points: Reichert hot-stage microscope, uncorrected values. − IR: Perkin−Elmer FT IR-1600 spectrometer (KBr pellets). − UV: Kontron Uvikon 860 spectrophotometer. − CD: Jasco J 500 A spectrometer. − Optical rotation values: Perkin−Elmer 343 − EI-MS: Finnigan MAT 95, 70 eV, high resolution with perfluorokerosine as internal standard. − FAB-MS: Finnigan MAT 95 (matrix: 3-nitrobenzyl alcohol, glycerol). − DCI-MS: Finnigan MAT 95, 200 eV (reactant gas: NH_3). − Elemental analysis: Mikroanalytisches Labor der Universität Göttingen − ^1H and ^{13}C NMR: Varian Unity 300, Bruker AMX 300, Varian Inova 500; chemical shifts are expressed in δ values with solvents as internal standard. Assignments which are insecure are indicated by an asterisk (*). − TLC: Silica gel 60 F_{254} (Merck, 0.25 mm); R_f values were determined on 20 × 20 cm plates, the evaluation length was 10 cm. − Column chromatography: Silica gel < 0.08 mm (Macherey & Nagel), glutaric acid silica gel,[2] Sephadex LH-20 (Pharmacia). − Fermentor: Biostat M (Braun-Diessel, 1 L) − Labeled precursors: all 99% ^{13}C, purchased from Chemotrade, Deutero GmbH and Cambridge Isotope Laboratories.

Media: Oat-bran medium: 30 g/L oat bran, pH = 7.8 prior to sterilization. − Oat bran + Celite medium: 30 g/L oat bran + 30 g/L Celite, pH = 7.8 prior to sterilization. − Quinoa medium: 30 g/L ground quinoa, pH = 8.4 prior to sterilization. − Millet medium: 30 g/L ground millet, pH = 7.8 prior to sterilization. − S/M Medium: 20 g/L degreased soybean meal + 20 g/L mannitol, pH = 7.0 prior to sterilization.

Fermentation: *Streptomyces* sp. A1 was maintained as a stock culture on agar slants containing S/M-agar (S/M medium + 20 g/L agar) stored at 6°C. Fermentations were carried out in 250-mL Erlenmeyer flasks with three baffles. Caps of foamed plastic material were used as closures. Each flask was filled with medium (50 mL), sterilized 30 min at 121°C and then inoculated with a 4-cm^2 piece of agar from 5 d old cultures. The submerged cultures were cultivated on a rotary shaker (250 rpm) at 28°C for 92−96 h. The number of flasks for the different attempts varied between 40−80 (2−4 L).

Isolation and Purification of the Rubromycins: The following description of the workup procedure of a fermentation (on a 4.0 L scale) in quinoa medium serves as an example of the isolation of the rubromycins. The harvested culture broth (pH ≈ 5.5) was separated into mycelium and culture filtrate by filtration, after the addition of 80 g celite. The mycelium was extracted five times with 500 mL of CH_2Cl_2/MeOH (1:1) and the combined organic layers were concentrated under reduced pressure. The resulting crude product was stirred in 100 mL of cyclohexane, filtered, and the obtained precipitate washed with 50 mL of cyclohexane. The precipitate was extracted with 300 mL of CH_2Cl_2/MeOH (95:5), filtered again, and the extract evaporated to dryness. The amorphous residue (312 mg) was purified by chromatography on glutaric acid silica gel (CH_2Cl_2/acetone gradient, 98:2 → 94:6; column 25 × 4 cm) yielding 7 mg of δ-rubromycin (**4**), 4 mg of γ-rubromycin (**3**), 174 mg of β-rubromycin (**6**) and 5 mg of 3′-hydroxy-β-rubromycin (**7**). The rubromycins on TLC plates were detected immediately because of their intensive color.

β-Rubromycin (6): R_f = 0.60 (TLC; $CHCl_3$/MeOH, 9:1). − CD ($CHCl_3$): λ_{extr} ([Θ]) = 274 nm (+2.69 × 10^3), 283 (+2.82 × 10^3), 296 (−1.65 × 10^3), 323 (+3.10 × 10^4). − ^{13}C NMR (125.7 MHz, $CDCl_3$): See Table 1. − Additional spectroscopic data are given in ref.[2]

γ-Rubromycin (3): R_f = 0.62 (TLC, $CHCl_3$/MeOH, 9:1). − CD ($CHCl_3$): λ_{extr} ([Θ]) = 258 nm (−2.86 × 10^4), 299 (−1.37 × 10^4), 327 (+1.06 × 10^4). − ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 22.3 (t, C-4), 29.7 (t, C-3), 39.5 (t, C-3′), 52.9 (q, 7-CO_2CH_3), 56.7 (q, 7′-OCH_3), 106.5 (s, C-4′a), 106.6 (s, C-9a), 110.1 (d, C-6′), 111.7 (s, C-2), 113.0 (s, C-8′a), 113.5 (d, C-6), 118.2 (d, C-5), 122.9 (s, C-3′a), 127.4 (s, C-5a), 131.5 (s, C-4a), 141.2 (s, C-7), 141.3 (s, C-10a), 150.1 (s, C-9′), 150.6 (s, C-10), 153.5 (s, C-9′a), 158.9 (s, C-4′), 160.0 (s, C-7′), 160.5 (s, 7-CO_2CH_3), 164.9 (s, C-9), 179.0 (s, C-8′), 183.7 (s, C-5′). − Additional spectroscopic data are given in ref.[2]

3′-Hydroxy-β-rubromycin (7): M.p. > 300°C. − R_f = 0.44 (TLC; $CHCl_3$/MeOH, 9:1). − IR (KBr): $\tilde{\nu}$ = 3441 cm^{-1} (br.), 1732, 1690, 1623, 1446, 1273, 1235. − UV ($CHCl_3$): λ_{max} (lg ε) = 316 nm

(4.25), 349 (3.99), 363 (3.95), 516 (3.70). − CD ($CHCl_3$): λ_{extr} ([Θ]) = 280 nm (+3.84 × 10^3), 295 (−2.46 × 10^3), 321 (+4.27 × 10^4). − ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ ≈ 9:1): δ = 2.20 (ddd, *J* = 13.5, 13.5, 6.0 Hz, 1 H, 3-H_a), 2.53 (ddd, *J* = 13.5, 6.0, 2.0 Hz, 1 H, 3-H_b), 2.98 (ddd, *J* = 17.0, 6.0, 2.0 Hz, 1 H, 4-H_a), 3.29 (ddd, *J* = 17.0, 13.5, 6.0 Hz, 1 H, 4-H_b), 3.91 (s, 3 H, 7-CO_2CH_3), 3.96 and 3.97 (s, 6 H, 5′-OCH_3, 7′-OCH_3), 5.42 (s, 1 H, 3′-H), 6.74 (s, 1 H, 6′-H), 6.91 (s, 1 H, 5-H), 7.41 (s, 1 H, 6-H). − ^{13}C NMR (125.7 MHz, [D_6]DMSO): See Table 1. − DCI-MS for $C_{27}H_{20}O_{13}$ (552.45); *m/z*: 570 (96) [M + NH_4^+], 553 (100) [M + H^+].

δ-Rubromycin (4): M.p. > 280 °C. − R_f = 0.65 ($CHCl_3$/MeOH, 9:1). − IR (KBr): $\tilde{\nu}$ = 3432 cm^{-1} (br), 2918, 1734, 1687, 1636, 1447, 1317, 1230. − UV ($CHCl_3$): λ_{max} (lg ε) = 312 nm (4.28), 351 (4.03), 365 (4.01), 424 (3.57). − $[\alpha]_D^{22}$ = − 30 (*c* = 0.03 in $CHCl_3$). − CD ($CHCl_3$): λ_{extr} ([Θ]) = 264 nm (−6.71 × 10^4), 295 (−7.11 × 10^3), 318 (+1.81 × 10^3), 366 (+5.24 × 10^3). − ^{1}H NMR (500 MHz, $CDCl_3/CF_3COOD$ ≈ 9:1): δ = 2.33 (ddd, *J* = 13.3, 13.3, 6.0 Hz, 1 H, 3-H_a), 2.46 (ddd, *J* = 13.3, 6.0, 1.7 Hz, 1 H, 3-H_b), 3.00 (ddd, *J* = 17.5, 6.0, 1.7 Hz, 1 H, 4-H_a), 3.37 (ddd, *J* = 17.5, 13.5, 6.0 Hz, 1 H, 4-H_b), 3.38 (d, *J* = 18.0 Hz, 1 H, 3′-H_a), 3.70 (d, *J* = 18.0 Hz, 1 H, 3′-H_b), 3.89 (s, 3 H, 7′-OCH_3), 3.97 (s, 3 H, 7-CO_2CH_3), 6.17 (s, 1 H, 6′-H), 6.98 (s, 1 H, 5-H), 7.14 (s, 1 H, 9′-H), 7.50 (s, 1 H, 6-H). − ^{13}C NMR (125.7 MHz, $CDCl_3$/CF_3COOD ≈ 9:1): δ = 22.4 (t, C-4), 29.4 (t, C-3), 38.5 (t, C-3′), 53.7 (q, 7-CO_2CH_3), 57.0 (q, 7′-OCH_3), 104.4 (d, C-9′), 106.4 (s, C-9a), 109.2 (d, C-6′), 110.1 (s, C-4′a), 111.2 (s, C-2), 114.7 (d, C-6), 119.1 (d, C-5), 119.4 (s, C-3′a), 127.2 (s, C-5a), 132.3 (s, C-4a), 132.8 (s, C-8′a), 140.6 (s, C-7), 141.8 (s, C-10a), 150.0 (s, C-10), 159.0 (s, C-4′), 160.9 (s, C-7′), 161.6 (s, 7-CO_2CH_3), 163.5 (s, C-9′a), 165.5 (s, C-9), 180.2 (s, C-8′), 190.7 (s, C-5′). − EI-MS; *m/z* (%): = 506 (35) [M^+] (high resolution calcd. for $C_{26}H_{18}O_{11}$ 506.0849, found 506.0849), 257 (100).

8′,10-Di-*O*-acetyl-9′-*O*-mesyl-dihydro-β-rubromycin (5): 269 mg (0.43 mmol) of 8′,10-di-*O*-acetyl-β-rubromycin (**8**),[2] 93 mg of Pd/C (5%), and 1900 mg (10.9 mmol) of methanesulfonic anhydride were suspended in 30 mL of THF and stirred at room temperature under H_2 (1 bar). After 15 min, 0.85 mL (10.5 mmol) of pyridine was added and stirring was continued for 19 h. The reaction mixture was filtered and the residue extracted with 100 mL of CH_2Cl_2. The combined organic layers were washed with H_2O (2 × 30 mL) and concentrated to dryness in vacuo. The crude extract was purified by column chromatography on glutaric acid silica gel (CH_2Cl_2/acetone gradient, 40:1 → 30:1; column 25 × 4 cm) and Sephadex LH-20 ($CHCl_3$, column 90 × 3 cm) yielding 219 mg (72%) of **5**. − M.p. 181 °C. − R_f = 0.63 (TLC; $CHCl_3$/MeOH, 9:1). − IR (KBr): $\tilde{\nu}$ = 3422 cm^{-1} (br), 1739, 1620, 1448, 1369, 1237, 1204, 1020. − UV ($CHCl_3$): λ_{max} (lg ε) = 308 nm (4.33), 336 (4.09). − $[\alpha]_D^{22}$ = − 67 (*c* = 0.29 in $CHCl_3$). − CD ($CHCl_3$): λ_{extr} ([Θ]) = 254 nm (−1.76 × 10^5), 294 (−3.42 × 10^4), 336 (+1.55 × 10^4). − ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.28−2.50 (m, 2 H, 3-H_2), 2.32 and 2.39 (s, 6 H, 2 × $COCH_3$), 2.82 (s br, 3 H, SO_2CH_3), 3.03 (dm, *J* = 17.5 Hz, 1 H, 4-H_a), 3.28−3.42 (m, 1 H, 4-H_b), 3.38 (d, *J* = 17.0 Hz, 1 H, 3′-H_a), 3.62 (d, *J* = 17.0 Hz, 1 H, 3-H_b), 3.88 (s, 3 H, 5′-OCH_3 or 7′-OCH_3), 3.92 (s, 3 H, 7-CO_2CH_3), 4.05 (s, 3 H, 5′-OCH_3 or 7′-OCH_3), 6.55 (s, 1 H, 6′-H), 7.26 (s, 1 H, 5-H), 7.35 (s, 1 H, 6-H), 9.66 (s, 1 H, 4′-OH). − ^{13}C NMR (125.7 MHz, $CDCl_3$, 24 °C): δ = 20.5 and 20.6 (q, 2 × $COCH_3$), 22.1 (t, C-4), 29.2 (t, C-3), 38.7 (q, SO_2CH_3), 38.8 (t, C-3′), 52.7 (q, 7-CO_2CH_3), 56.4 and 56.5 (q, 5′-OCH_3, 7′-OCH_3), 92.6 (d, C-6′), 107.0 (s, C-3′a), 107.7 (s, C-4′a), 111.6 (s, C-2′), 111.6 (d, C-6), 114.7 (s, C-9a), 117.5 (s, C-8′a*), 125.2 (d, C-5), 125.4 (s, C-9′*), 126.3 (s, C-8′), 129.0 (s, C-5a*), 131.4 (s, C-4a), 139.6 (s, C-10*), 141.8 (s, C-7), 146.2 (s, C-10a), 149.6 (s, C-5′ or C-7′), 149.9 (s, C-4′), 149.9 (s, C-9′a), 154.8 (s, C-5′ or C-7′), 156.8 (s, C-9), 160.4 (s, 7-CO_2CH_3), 168.6 and 170.1 (s, 2 × $COCH_3$). − FAB-MS (positive ions); *m/z* (%): 723 (5) [M + Na^+], 701 (40) [M + H^+], 700 (36) [M^+], 659 (39) [M + H^+ − C_2H_2O], 621 (66) [M^+ − CH_3SO_2], 579 (57) [M^+ − C_2H_2O − CH_3SO_2], 537 (100) [M^+ − 2 × C_2H_2O − CH_3SO_2]. − FAB-MS (negative ions); *m/z* (%): 699 (61) [M^- − H], 619 (100) [M^- − 2H − CH_3SO_2], 577 (38) [M^- − 2H − C_2H_2O − CH_3SO_2]. − $C_{32}H_{28}O_{16}S$ (700.62): calcd. C 54.86, H 4.03; found C 54.66, H 4.25.

4′,10-Di-*O*-acetyl-β-rubromycin-quinoxaline (10): R_f = 0.67 (TLC; $CHCl_3$/MeOH, 9:1). − $[\alpha]_D^{22}$ = − 682 (*c* = 0.15 in $CHCl_3$). − CD ($CHCl_3$): λ_{extr} ([Θ]) = = 266 nm (−5.07 × 10^4), 278 (+5.35 × 10^3), 312 (−1.61 × 10^5), 348 (+6.18 × 10^3). − ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 20.5 and 20.6 (q, 2 × $COCH_3$), 22.2 (t, C-4), 29.4 (t, C-3), 40.5 (t, C-3′), 52.8 (q, 7-CO_2CH_3), 56.5 (q, 5′-OCH_3), 100.8 (d, C-6′), 110.5 (s, C-2), 111.9 (d, C-6), 114.9 (s, C-9a), 115.9 and 116.0 (s, C-4′a, C-12′b), 124.1 (s, C-3′a), 125.3 (d, C-5), 127.2 and 128.3 (d, C-8′, C-11′), 129.1 (s, C-5a*), 129.2 and 130.2 (d, C-9′, C-10′), 131.7 (s, C-4a), 134.8 (s, C-4′*), 136.1 (s, C-7′a or C-11′a), 140.0 (s, C-10*), 142.0 (s, C-7), 142.3 (s, C-6′a or C-12′a), 142.3 (s, C-7′a or C-11′a), 143.5 (s, C-13′), 145.3 (s, C-6′a or C-12′a), 145.5 (s, C-13′a), 146.5 (s, C-10a), 156.9 (s, C-9), 159.4 (s, C-5′), 160.6 (s, 7-CO_2CH_3), 169.0 and 169.3 (s, 2 × $COCH_3$). − Additional spectroscopic data are given in ref.[2]

Feeding Experiments: All feeding experiments were carried out in the oat-bran medium by using the Biostat M fermentor. 50 mL of a ca. 48 h old shake culture was used to inoculate a 1-L fermentor containing 600 mL of sterilized medium (Table 5). The stir jar was usually cultivated for 92 h at 1.6 vvm aeration, 400 rpm, and 28 °C operating temperature. At the beginning of every fermentation the

Table 5. Feeding experiments (all fermentations were carried out in a Biostat M fermentor)

Precursor	*c* [mmol/L]	Additive[b]	Feeding time [h]	pH control	pH[c]	Yield [mg/L] **6**	**7**
–	–	–	–	–	4.8	44	4
[U-$^{13}C_3$]glycerol	10.2	–	26−54	5.4 ± 0.3	5.7	40	traces
L-[methyl-^{13}C]-methionine	2.1	–	25−52	5.3 ± 0.3	5.6	26	traces
[2-^{13}C]malonic acid[a]	10.0	A	29−49	5.6 ± 0.6	6.2	48	3
[U-$^{13}C_3$]malonic acid[a]	9.8	A	29−56	5.3 ± 0.5	5.8	31	6
(*S*)-[1,3-$^{13}C_2$]acetoacetyl-*N*-acetyl-cysteamine	2.2	A + B	27−58	5.2 ± 0.5	5.7	11	–

[a] Adjusted to pH = 5.0 with concentrated NaOH. − [b] A: 3.3 mg vitamin B_{12}; B: 100 mg 3-(tetradecylthio)propanoic acid, dissolved in 0.5 mL of DMSO and added in 3 portions after 25, 33, and 42 h. − [c] After 92 h.

pH was adjusted to pH = 7.0 with 0.5 M NaOH. The precursors dissolved in 50 mL of sterile water were added, as soon as first red pigments (direct indication of the start of the rubromycin production) were recognizable in the culture broth. From this time onwards the precursors and additives were pumped continuously into the culture broth by a dosing pump within ca. 24 h. At the beginning of the feeding, a pH barrier adapted to the fermentation process was defined in order to prevent the increase of the pH into the alkaline range; 2 M citric acid was used for the regulation of the pH.

Enzyme: HIV-1 RT is a recombinant enzyme, expressed in *Escherichia coli* and purified from bacterial extracts to homogeneity as described previously.[37] The HIV-1 RT expression plasmid was derived from the BH-10 provirus isolate.[38]

Enzyme Assays: The RT-associated DNA polymerase activity was assayed as described in detail,[39] by monitoring the poly(rA)$_n$·oligo(dT)$_{12-18}$ directed incorporation of [^{3}H]dTTP into TCA-insoluble product. In all inhibition experiments, the enzymes were pre-incubated for 5 min at 30°C in the presence of 10 μmol/L inhibitor. The enzymatic reactions were initiated by adding the substrates followed by incubation at 37°C for 30 min. The enzymatic residual activity was calculated relative to the initial linear reaction rates observed in controls when no drug was added. The rubromycins were dissolved in 100% dimethyl sulfoxide (DMSO) to a final concentration of 10 mmol/L. The final DMSO concentration in the enzymatic assays was 1%, a concentration that did not affect the RT-associated activity.

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