Antibiotics from Gliding Bacteria, LXXVIII[◇]

Ripostatin A, B, and C: Isolation and Structure Elucidation of Novel Metabolites from *Sorangium cellulosum*

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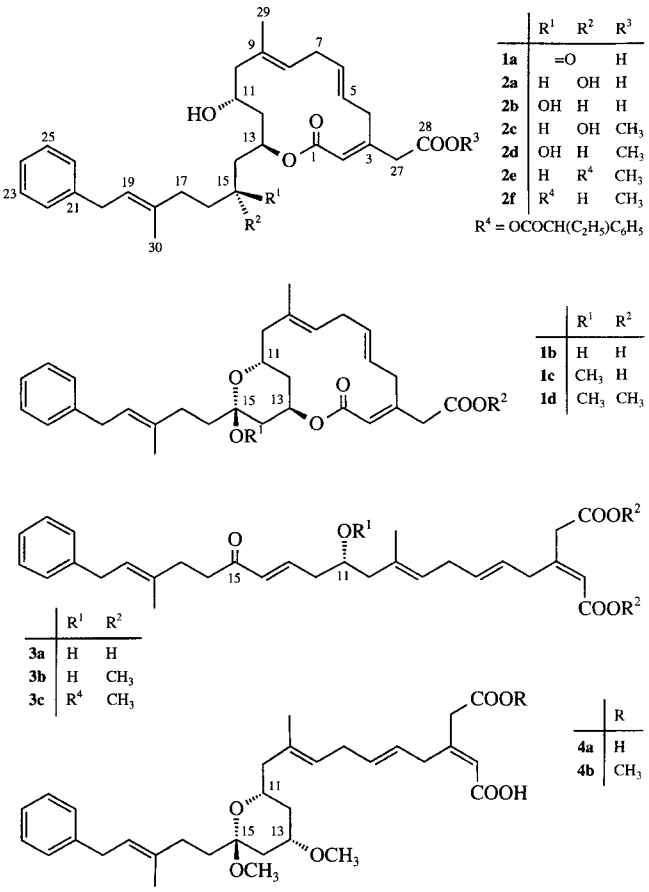
Three closely related new metabolites named ripostatins were isolated from the myxobacterium *Sorangium cellulosum* and their structures elucidated by spectroscopic methods. Two of them, ripostatin A (**1a**, **b**) and B (**2a**), are 14-membered macrolides with an acetic acid and a phenylalkyl side chain, whereas the third metabolite ripostatin C (**3a**) is an acyclic derivative of ripostatin A. By application of the method of Helmchen the absolute stereochemistry could be determined as (11*R*,13*R*) for ripostatin A, 11*R*,13*S*,15*R* for ripostatin B and 11*S* for ripostatin C. The polyketide origin of A was revealed by feeding experiments with ^{13}C-labeled precursors demonstrating the incorporation of one molecule of phenylacetic acid derived from phenylalanine, one propionate unit, and ten acetate units.

In the course of our screening program for new antibiotics from gliding bacteria we isolated from the myxobacterium *Sorangium cellulosum*, strain So ce 377, a group of three new metabolites. Two of them, ripostatin A (**1a**, **b**) and B (**2a**), are active against Gram-positive bacteria, the third, ripostatin C (**3a**), is biologically inactive. A detailed description of the producing organism, fermentation, and biological properties was published recently[2]. In this paper we report on the isolation, structure elucidation, chemical properties, and the biosynthesis.

During the growth phase of the organism the ripostatins were excreted into the culture medium, from which they were adsorbed on added Amberlite XAD 1180. After harvesting the resin, it was filled into a chromatography column and eluted with mixtures of 0.05 M NaH_2PO_4 buffer and methanol. As shown in Scheme 1, starting with 50% methanol, the methanol content was increased by 10-% steps after two bed volumes of solvent had passed the resin. The fractions were monitored by HPLC. In this way, a crude fraction I, containing all three ripostatins, and a crude fraction II, containing the bulk of ripostatin A, were obtained. Fraction I required two further separation steps by chromatography on silica gel RP 8 followed by chromatography on Sephadex LH 20 to yield pure compounds, whereas fraction II required only chromatography on silica gel RP 8 to give pure ripostatin A. The ripostatins are colorless oils of high viscosity.

The antibiotic spectrum is narrow, the MIC value of some strains of *Staphylococcus aureus* and a mutant of *Escherichia coli* with an altered outer membrane is about 1 μg/ml for both A and B. It should be noted that the synthetic derivative 15-epi-ripostatin B displays the same bio-



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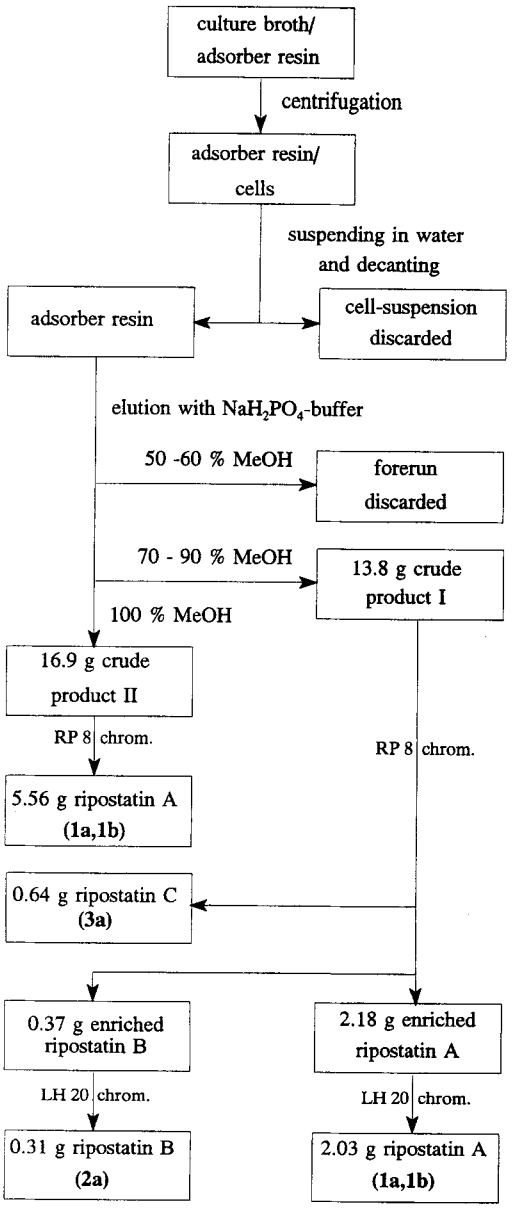
Scheme 1. Isolation of ripostatins



logic activity, whereas all other derivatives are inactive. As the primary target is the inhibition of eubacterial **ribo**nucleic acid **po**lymerase, the new compounds were named **ripo**statins.

A first inspection of the ^{13}C-NMR spectra revealed that the signals of the main component ripostatin A (**1a**, **b**) appear doubled in a constant ratio of 4:3 suggesting an equilibrium mixture of two isomers. Therefore, structure elucidation was first performed with ripostatin B (**2a**). The (−)-FAB-MS exhibits a molecular ion [M − H] at *m/z* 495 and [M − COOH] at *m/z* 451. From (−)-FAB HRMS the molecular formula $C_{30}H_{40}O_6$ was deduced. The ^{13}C-DEPT-NMR spectrum (Table 1) disclosed 2 $-CH_3$, 9 $-CH_2-$, 3 $-CH(O)-$, 10 $=CH-$, 4 $=C<$, and 2 $O=C(O)$ groups. The interpretation of ^{1}H- (Table 2), ^{1}H,^{1}H-COSY-, ^{1}H,^{13}C-COSY-, and the HMBC-NMR spectra led to the establishment of a 14-membered lactone ring bearing a carboxymethyl group and a side chain with a phenyl ring. The geometry of the four double bonds was determined by NOE difference spectra as (2*Z*, 5*E*, 8*E*, 18*E*). Consistent with the structure are the UV and IR spectra. The UV spectrum having a maximum at 207 nm (ε = 19 500) is the sum of the absorptions of an α,β-unsaturated ester and a phenyl group. The presence of these two functional groups was further corroborated by strong IR bands at 1717, 1646, 742, and 698 cm^{-1}. The chiral centers at C-11, C-13, and C-15 remained to be elucidated (see below).

Table 1. ^{13}C-NMR chemical shifts of ripostatin A (**1a**, **1b**), ripostatin B (**2a**), and ripostatin C (**3a**) in [D_4]methanol (δ values)

C atom	1a	1b	2a	3a
1	166.48	168.63	166.76	169.14
2	120.79	120.20	120.97	120.45
3	155.15	155.20	154.87	154.85
4	35.40	35.43	35.00	36.05
5	126.33	128.41	126.14	127.08
6	129.36	129.32	129.19	132.84
7	31.90	31.37	31.82	31.94
8	126.61	126.33	126.58	126.27
9	135.35	133.82	135.51	133.96
10	50.85	44.49	51.09	48.57
11	66.33	67.17	66.16	69.74
12	37.28	33.24	37.14	40.87
13	69.63	69.27	71.60	146.38
14	45.84	37.00	40.53	133.08
15	210.29	101.65	69.31	202.83
16	42.62	36.86	36.60	35.13
17	34.30	34.59	36.63	39.38
18	135.96	137.04	136.98	135.99
19	124.96	124.84	124.41	125.04
20	35.16	35.31	35.00	35.02
21	142.71	142.61	142.90	142.67
22,26	130.15	129.55	130.09	129.24
23,25	129.26	129.45	129.23	129.31
24	126.73	126.92	126.58	126.70
27	45.70	48.43	45.66	43.36
28	173.51	176.72	173.51	173.72
29	16.77	18.46	16.68	16.38
30	16.29	16.50	16.22	16.32

The UV spectrum of ripostatin A is identical with that of ripostatin B, also the IR spectrum except for the most intense band, which is shifted from 1717 to 1720 cm^{-1}. The (−)-FAB mass spectrum of ripostatin A reveals a molecular ion [M − H] at *m/z* 493 and [M − COOH] at *m/z* 449. From (−)-FAB-HRMS a molecular formula $C_{30}H_{38}O_6$ was deduced, 2H atoms less than that of ripostatin B. In the ^{13}C-NMR spectrum (Table 1) the signal of one of the CH(O) groups is replaced by two signals at δ = 101.65 and 210.29, characteristic of an acetal and a ketone, respectively. With regard to the doubling of other signals, ripostatin A exists as an equilibrium mixture of a ketone **1a** and hemiacetal form **1b**. In methanolic solutions used for the NMR measurements the equilibrium mixture consists of about 55% ketone and 45% hemiacetal. When a solution of ripostatin A in aqueous methanol was concentrated until all methanol had been removed, ripostatin A precipitated from the aqueous phase. After the dried precipitate had been dissolved in methanol it turned out to be the pure keto form,

Table 2. ^{1}H-NMR data of ripostatin A (**1a**, **1b**), ripostatin B (**2a**), and ripostatin C (**3a**) in [D_4]methanol

	1a		**1b**		**2a**		**3a**	
H atom	δ	J [Hz]	δ	J [Hz]	δ	J [Hz]	δ	J [Hz]
2	5.71 d	1.3	5.87 s		5.74 s		5.83 s	
4a	2.55 dd	3.5,13.1	2.65 dd	6.4,13.4	2.50 m	13.2	3.45 d	7.2
4b	4.02 dd	8.5,13.1	3.98 dd	7.1,13.4	4.01 dd	8.8,13,2	-	
5	5.30 m	3.5,8.5,15.4	5.25 ddd	6.4,7.1,15.0	5.23 m	8.8,15.0	5.45 m	15.0,7.2
6	5.43 m	6.8,15.4	5.43 m	15.0	5.37 m	15.0	5.57 dt	15.0,7.2
7a	2.43 m	13.0	2.60 m	7.8,14.6	2.47 m	8.0,14.2	2.77 dd	6.8,7.2
7b	2.51 dt	13.0,6.8	2.68 m	7.8,14.6	2.59 m	8.0,14.2	-	
8	5.36 m	6.8	5.19 t	7.8	5.26 t	8.0	5.27 t	6.8
10a	1.99 t	11.8	1.89 dd	11.3,12.2	2.01 dd	11.0,12.0	2.17 dd	9.1,13.4
10b	2.21 dd	2.9,11.8	2.35 dd	3.0,12.2	2.20 dd	4.4,12.0	2.23 dd	3.8,13.4
11	3.76 m	2.9,9.0,11.8	3.75 ddt	1.0,3.0,11.3	3.86 m	4.4,8.7,11.0	3.91 m	3.8,9.1
12a	1.40 ddd	2.8.9.0,15.7	1.27 ddd	2.7,11.3,14.0	1.50 ddd	2.5,8.7,15.4	2.32 m	7.2
12b	2.22 m	15.7	2.29 dt	14.0,1.0	2.16 m	15.4	2.43 m	7.2
13	5.41 m	2.8,7.2	5.01 m	1.0,2.7,4.7	5.14 m	2.5,7.2	7.02 dt	18.0,7.2
14a	2.98 d	7.2	1.82 dd	4.7,15.1	1.88 dt	14.2,7.2	6.19 d	18.0
14b	-		2.01 dd	1.5,15.1	2.07 m	14.2	-	
15	-		-		3.80 m	6.8	-	
16a	2.64 t	7.8	1.61 ddd	5.7,11.8,14.2	1.61 dt	6.8,8.2	2.78 t	6.8
16b	-		1.90 ddd	4.5,12.8,14.2	-		-	
17a	2.34 t	7.8	2.06 ddd	4.5,11.8,14.1	2.08 m	8.2,14.5	2.43 m	6.8
17b	-		2.12 ddd	5.7,12.8,14.1	2.15 m	8.2,14.5	-	
19	5.40 m	8.3	5.41 t	7.2	5.37 t	7.2	5.39 t	7.6
20	3.37 d	8.3	3.38 d	7.2	3.33 d	7.2	3.37 d	7.6
22.26	7.15 d	7.1	7.16 d	7.3	7.15 d	7.8	7.17 d	8.1
23,25	7.27 t	7.1	7.20 t	7.2	7.26 t	7.5	7.27 t	8.1
24	7.19 t	7.1	7.17 t	7.2	7.16 t	7.5	7.18 t	8.1
27a	3.14 d	15.1	3.08 d	14.7	3.12 d	15.1	3.15 s	
27b	3.22 dd	1.3,15.1	3.13 d	14.7	3.18 d	15.1	-	
29	1.54 s		1.64 s		1.48 s		1.68 s	
30	1.77 s		1.79 s		1.70 s		1.74 s	

but after one day the aforementioned equilibrium had reestablished. From the ^{1}H,^{1}H−COSY−NMR spectrum it follows that the vicinal coupling along the backbone is interrupted at C-15. Therefore, it is this carbon atom, which is oxidized to a carbonyl group in ripostatin A. This relationship between ripostatin A and ripostatin B was confirmed by $NaBH_4$ reduction of ripostatin A to a mixture of C-15 epimeric alcohols, one of them being identical in all respects with ripostatin B.

The ^{13}C-NMR spectrum of the third component ripostatin C (**3a**) differs from that of ripostatin B in three positions. Two signals of the three −CH(O)− groups and one $-CH_2-$ group are missing. Instead, the signals of two new =CH− groups and a >C=O group are observed. The chemical shifts of the new olefinic protons at δ = 7.02 and 6.19 with a coupling constant of 18 Hz indicate that the new double bond is conjugated to a carbonyl group and has the configuration *E*. The signal of the proton of the remaining −CH(O)− group at δ = 3.91 indicates a secondary alcohol and excludes an ester or lactone function. Furthermore, the AB multiplets of 4-H_2 (δ = 2.50, 4.01) and 7-H_2 (δ = 2.47, 2.59), typical of the lactone ring in ripostatin A and B, collapse to A_2 multiplets at δ = 3.45 (4-H_2) and 2.77 (7-H_2) for ripostatin C. From these results it follows that the lactone ring of ripostatin A is opened by β-elimination leading to structure **3a** for ripostatin C. This could be verified by heating of ripostatin A in a buffer solution (pH 8) at 40°C. Thus, after 1 hour 36% of ripostatin A had been converted to ripostatin C. Considering the mild conditions for this conversion, ripostatin C, unambiguously found in the culture broth, may be formed in the same way. Consistent with the structure **3a** are the (−)-FAB-MS results indicating the molecular formula $C_{30}H_{38}O_6$. The UV spectrum as compared to that of ripostatin B has a larger ε value of 33 000 at 205 nm and a shoulder at 232 nm (ε = 17 000) due to the additional chromophore of an α,β-unsaturated ketone moiety.

As a consequence of the fact that ripostatin A could be transformed into B as well as into C, the stereochemistry at C-11 must be the same for A, B, and C, that at C-13 the same for A and B. To determine their absolute configuration the method of Helmchen[3] was applied. First, Ripostatin C containing only one chiral center was converted into the 11-acyl derivatives **3c** both with (*RS*)-2-phenylbutyric anhydride and (*R*)-2-phenylbutyric anhydride. As shown in Table 3, in the (*R*)-acyl derivative the signals of 13-H and 14-H are shifted upfield, whereas in the (*S*)-acyl derivative the CH_3-29 signal is shifted upfield. According to the rules described by Helmchen, C-11 is assigned an (*S*) configuration for ripostatin C and the configuration (*R*) for ripostatin A and B. In the hemiacetal form of ripostatin A (**1b**) C-11 and C-13 are part of a six-membered ring. Provided

the methyl acetal of ripostatin A (**1c**) could be prepared, the configuration at C-13 could be correlated with C-11 on the basis of coupling constants and NOE data. Initial experiments with acid catalysis failed to give the methyl acetal, but the compound was smoothly formed in good yield by heating of ripostatin A in methanol without acid catalysis. To ensure a higher stability the methyl ester **1d** was prepared for measurements in [D_6]benzene by subsequent treatment with diazomethane. The proton 12ax-H (δ = 1.12) (Table 4) shows two strong couplings (11.0, 13.9 Hz) with 11-H and 12eq-H and a weak one (2.4 Hz) with 13-H, the proton 14ax-H (δ = 1.31) exhibits a strong coupling (15.0 Hz) with 14eq-H and a weak one (4.7 Hz) with 13-H. No individual couplings are recognizable for 13-H which gives rise to a broad signal at δ = 5.05 with a half width of 9–10 Hz. Consequently, 13-H must have an equatorial position, which was further supported by NOE measurements. Irridiation of 13-H caused an enhancement of the axial as well of the equatorial signals of 12-H and 14-H, respectively. Additional NOEs are observed between 12ax-H and 14ax-H as well as 11-H and 15-OCH_3. From the absolute configuration at C-11 for ripostatin A the configuration (13*R*) and for ripostatin B the configuration (13*S*) were deducted.

Table 3. Selected ^{1}H-NMR data of **1d** in [D_2]dichloromethane

H atom	δ	*J* [Hz]
11	3.91	11,12a = 11.2; 11,12e = 3.5
12a	1.12	12a,12e = 14.0; 12a,13 = 2.5
12e	2.48	12e,13 = 1.8
13	5.04	13,14a = 4.8; 13,14e = 1.7
14a	1.31	14a,14e = 15.1
14e	1.95	

Table 4. ^{1}H-NMR data of 2-phenylbutyrates in [D_4]methanol

H atom	*(2'R)*-11-acyl-**3c**	*(2'S)*-11-acyl-**3c**	$\delta_R - \delta_S$
13	6.44	6.65	-0.21
14	5.85	6.03	-0.18
29	1.59	1.46	+0.13
	2e	**2f**	$\delta_{2e} - \delta_{2f}$
14a	1.91	1.78	+0.13
14b	2.05	2.04	+0.01
16a	1.56	1.66	-0.10
16b	1.66	1.66	±0.00
17	2.19	2.22	-0.03

Finally, the absolute configuration of C-15 of ripostatin B (**2a**) had to be determined. Treatment of a solution of ripostatin B in dichloromethane with (*R*)-2-phenylbutyric anhydride/pyridine failed to produce an acylated product. However, a modification of the reaction conditions employed by Zeeck et al.[4] using (*R*)-2-phenylbutyric acid/trifluoracetic anhydride gave the 11-*O*-(trifuloroacetyl)-15-[(2*R*)-2-phenylbutyryl] derivative, which on mild hydrolysis provided the 15-monoacylated ripostatin B (**2e**). In the same way the corresponding derivative of 15-*epi*-ripostatin B **2f** was prepared and the ^{1}H-NMR spectra compared (Table 4). 14-H_a of ripostatin B gives rise to an upfield shift of 0.13 ppm, whereas downfield shifts of 0.10 ppm for 16-H_a and 0.04 ppm for 17-H are observed relative to the signals of 15-*epi*-ripostatin B. On the basis of these results and application of the rules given by Helmchen[3] the configuration (15*R*) is assigned to ripostatin B and the configuration (15*S*) to 15-*epi*-ripostatin B.

The carboxy group of ripostatin A can be methylated with diazomethane, the 11-OH group can be formylated with formic acid/acetic anhydride/pyridine and, as metnioned before, the ketone can be transformed into a methyl acetal or reduced to a mixture of epimeric alcohols. A characteristic feature of ripostatin A however is the behavior of the lactone. Under mild alkaline conditions the lactone ring is opened by β-elimination to yield ripostatin C; no trace of a 13-hydroxy acid from a regular hydrolysis could be detected. If ripostatin A was heated in methanol (45 °C, 30 min) with various acid catalysts, a complex mixture of compounds was formed, from which the main components **4a** and **4b** were separated by TLC. As deduced from the NMR spectra, the lactone ring is opened in both compounds. However, instead of the expected enone a tetrahydropyrane ring with two CH_3O substituents at C-13 and C-15 has formed. An additional CH_3O signal at δ = 3.79 for compound **4b** is indicative of a C-28 methyl ester. A quartet at δ = 1.15 (*J* = 11.8 Hz) for 12a-H and a triplet at δ = 1.28 (*J* = 11.7 Hz) for 14a-H indicate *trans*-diaxial coupling between 13-H, 12a-H, and 14a-H. Therefore, the compounds **4a** and **4b** must have formed from an intermediate like ripostatin C (**3a**) by addition of methanol to the double bond of the enone. In contrast, ripostatin B (**2a**) is more stable. Heating of ripostatin B in methanol with acidic catalysis gave the methyl ester **2c** without opening of the lactone ring. Only under more basic conditions (pH = 12) is the lactone ring opened hydrolytically.

Ripostatin is a further member of secondary metabolites with a monosubstituted phenyl ring frequently found in the group of myxobacteria such as crocacin[5], nannochelin[6], phenalamide[7], phenoxan[8], socein[9], soraphen[10], and thiangazole[11]. In this series ripostatin is the first example of a 14-membered macrolide. The well-known macrolides of this size, e.g. erythromycin[12], oleandomycin[12], and megalomycin[12], are all glycosylated and have no phenyl substituent. Regarding partial structures a large portion of molecule from C-1 to C-15 shows a similarity to latrunculin B, a marine toxine isolated from the Red Sea sponge *Latrunculia magnifica*[13].

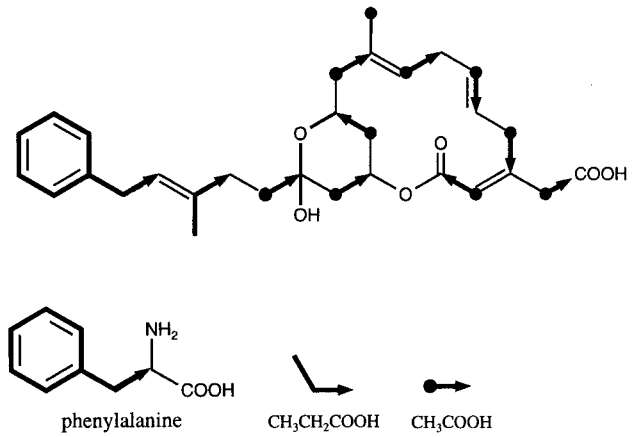
From the substitution pattern the ripostatins seem to be polyketides. To check this assumption feeding experiments with sodium [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate, [1-^{13}C]propionate, [1-^{13}C]phenylacetate as well as with [$^{13}CH_3$]methionine and [2-^{13}C]phenylalanine were undertaken. The incorporation of the precursors was determined by ^{13}C-NMR spectroscopy. As can be seen from Table 5 and Figure 1, the starter unit, corresponding to the C-19–C-26 segment, is derived from phenylalanine, which is incorporated at a high rate (66%). Phenylalanine could not be replaced by sodium phenylacetate, as was shown by feeding of the [u-^{14}C]-labeled compound or its methyl ester. No trace of in-

corporation was detected. The next building block corresponding the C-17–C-18 and C-30, originates from propionate, which is also incorporated at a high rate (51%). The remaining part of the molecule was constructed of ten acetate units, eight of them being used for the elongation of the chain from C-16 to C-1, the other two for the pendant substituents the carboxymethylene (C-27–C-28) and the methyl group (C-29). [$^{13}CH_3$]Methionin was not incorporated. The origin of C-29 was deduced from the incorporation of [1,2-$^{13}C_2$]acetate. Thus, in the ^{1}H-NMR spectrum the methyl group gives rise to a singlet in contrast to the other signals appearing as doublets. The carboxy group, initially adjacent to C-29 in the precursor molecule, had been lost during biosynthesis similarly to the biosynthesis of myxopyronin[13]. The rate of incorporation was about five times lower for the acetate units than that for phenylalanine and propionate. This agrees well with the results found with other macrolides of *Sorangium* species.

Table 5. ^{13}C abundance in ripostatin A (**1a**) relative to C-2 = 1.0 obtained from a feeding experiment with Na[1-^{13}C]acetate

C atom	δ	^{13}C content	C atom	δ	^{13}C content
1	166.48	8.3	15	210.29	8.6
2	120.79	1.0	16	42.62	1.1
3	155.15	6.6	17	34.30	2.0
4	35.40	1.4	18	135.96	0.9
5	126.33	11.1	19	124.96	1.1
6	129.36	1.4	20	35.16	1.2
7	31.90	11.9	21	142.71	0.9
8	126.61	1.1	22,26	130.15	1.1
9	135.35	9.7	23,25	129.26	1.2
10	50.85	1.0	24	126.73	1.2
11	66.33	11.1	27	45.70	0.9
12	37.28	1.0	28	173.51	5.3
13	69.63	11.1	29	16.77	1.1
14	45.84	1.1	30	16.29	1.2

Figure 1. Incorporation of ^{13}C-labelled precursors into ripostatin A



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Experimental

Analytical TLC: aluminum sheets silica gel Si 60 F_{254}, (Merck); detection: UV absorption at 254 nm, spray reagent vanillin/sulfuric acid and heating to 120°C. – Analytical HPLC: HD-Sil C18, 5 μ (250 mm × 4 mm). – UV: Shimadzu UV, 2102 PC, methanol as solvent. – IR: Nicolet FTIR 20 DXB. – NMR: Bruker AM 300 and AM 600. – MS: Finnigan MAT 95, matrix: 3-NBA. – Optical rotation: Perkin-Elmer polarimeter 241. – Elemental analyses: Labor I. Beetz, D-96 301 Kronach.

Isolation of Ripostatins: A mixture of XAD and cells obtained from a 300-l fermentation was repeatedly stirred with water and decanted until the cells had almost completely been removed. The XAD (2.6 l) was filled into a chromatography column and eluted with a mixture of a 0.05 M NaH_2PO_4 solution and methanol. The methanol content was increased by 10-% steps by starting with 50% of methanol after two bed volumes of solvent had passed the resin. The fractions (500 ml) were monitored by HPLC. Those with a methanol content of 80–90% containing ripostatin A, B, and C were concentrated in a vacuum evaporator until the methanol had been removed. The resulting aqueous suspension was extracted twice with ethyl acetate, the combined extracts were evaporated to dryness yielding 13.78 g of crude product I. The fractions eluted with 100% methanol, containing the bulk of ripostatin A, yielded after evaporation of the solvent 16.95 g of crude product II. The crude product I was dissolved in methanol, the solution applied to a column (75 cm – 6.4 cm, silica gel RP 8, eluent methanol/0.05 M NaH_2PO_4, 7:3). The fractions detected at 227 nm were analyzed by HPLC. Thus, 0.64 g of pure ripostatin C, 0.37 g of enriched ripostatin B, and 2.18 g of enriched ripostatin A were obtained. The enriched fractions were further purified by chromatography on Sephadex LH 20 by using methanol as solvent. 0.31 g of pure ripostatin B and 2.03 g of pure ripostatin A were isolated. The crude product II was treated analogously to yield after silica RP 8 chromatography 5.56 g of pure ripostatin A.

Ripostatin A (**1a, b**): Colorless amorphous oil. – TLC: dichloromethane/methanol (9:1), R_f = 0.61, spraying with vanillin/sulfuric acid and heating at 120°C gave brown-violet spots. – HPLC: Methanol/0.05 M NH_4OAc (7:3), 1.5 ml/min. R_t = 4.57 min. – $[\alpha]_D^{20}$ = +15.1 (*c* = 1 in methanol). – UV (MeOH): λ_{max} (lg ε) = 207 nm (4.29). – IR (KBr): $\tilde{\nu}$ = 3433 cm^{-1} (m), 2924 (s), 1720 (s), 1646 (m), 1382 (m), 1231 (s), 1219 (s), 1179 (s), 1152 (s), 1069 (s), 967 (m), 698 (m). – ^{1}H NMR: Table 2, ^{13}C NMR: Table 1. – (–)-FAB-MS (matrix: 3-NBA), *m/z*: 449 [M – COOH]$^-$; high resolution: calcd. 449.2692, found 449.2681. – $C_{30}H_{38}O_6$ (494.6): calcd. C 72.85, H 7.74, found C 73.00, H 7.63.

Ripostatin B (**2a**): Colorless amorphous solid. – TLC: Dichloromethane/methanol (9:1), R_f = 0.43, spraying with vanillin/sulfuric acid and heating at 120°C gave blue-violet spots. – HPLC: Methanol/0.05 M NH_4OAc (7:3), 1.5 ml/min. R_t = 4.16 min. – $[\alpha]_D^{20}$ = +35.7 (*c* = 1 in methanol). – UV (MeOH): λ_{max} (lg ε) = 207 nm (4.29). – IR (KBr): $\tilde{\nu}$ = 3419 cm^{-1} (m), 2925 (s), 2853 (m), 1717 (s), 1645 (m), 1452 (m), 1231 (s), 1220 (s), 1182 (s), 1152 (s), 1073 (m), 967 (m), 698 (m). – ^{1}H NMR: Table 2, ^{13}C NMR: Table 1. – (–)-FAB-MS (matrix: 3-NBA), *m/z*: 451 [M – COOH]$^-$, high resolution: calcd. 451.2848, found 451.2763. – $C_{30}H_{40}O_6$ (496.6): calcd. C 72.55, H 8.12, found C 72.34, H 8.31.

Ripostatin C (**3a**): Colorless amorphous solid. – HPLC: Methanol/0.05 M NH_4OAc (7:3), 1.5 ml/min. R_t = 3.35 min. – UV (MeOH): λ_{max} (lg ε) 205 nm (4.52), 232 (sh, 4.32). – IR (KBr): $\tilde{\nu}$ = 2927 cm^{-1} (s), 1713 (s), 1646 (s), 1348 (m), 1248 (m), 1177 (s), 972 (m), 699 (m). – ^{1}H NMR: Table 2, ^{13}C NMR: Table 1. – (–)-FAB-MS (matrix: 3-NBA), *m/z*: 493 [M – H]$^-$, high resolution:

calcd. 493.2590, found 493.2653. – $C_{30}H_{38}O_6$ (496.6): calcd. C 72.85, H 7.74, found C 72 58, H 7.78.

Ripostatin B (**2a**) *and 15-epi-Ripostatin* (**2b**) *from Ripostatin A:* To a solution of 329 mg of **1a**, **b** (0.66 mmol) in 6.5 ml of dry THf 25 mg of $NaBH_4$ (4 equiv.) was added, and the mixture was stirred at room temp. for 20 h. After the solvent had been removed under reduced pressure, the residue was dissolved in water, the solution adjusted to pH 5 with 0.1 N HCl and extracted twice with ethyl acetate. The combined extracts were concentrated under reduced pressure to yield 327 mg of an oily residue, which was separated by chromatography (Eurosil-Bioselect 100–20, C 18 eluent methanol/0.05 M NaH_2PO_4, 65:35). Fraction 1 afforded 82 mg (24%) of **2b**: colorless amorphous solid. – TLC: Dichloromethane/methanol (9:1): R_f = 0.48, spraying with vanillin/sulfuric acid and heating at 120°C gave blue-violet spots. – HPLC: Methanol/0.05 M NH_4OAc (7:3), 1.5 ml/min, R_t = 3.92 min. – $[\alpha]_D^{20}$ = +13.0 (*c* = 1 in methanol). – ^{1}H NMR ([D$_4$]methanol): δ = 3.65 (m, 15-H). – Fraction 2 yielded 126 mg (38%) of **2a**: identical with the fermentation product as revealed by TLC, HPLC, and ^{1}H-NMR analysis.

Ripostatin A 15-Methyl Acetal (**1c**): A solution of 27 mg of ripostatin A in 5 ml of methanol was heated at 50°C for 2 h. The solvent was removed in vacuo and the residue purified by TLC [silicagel, 1 mm (Merck), eluent dichloromethane/methanol, 9:1] Elution of the main band furnished 21 mg (75%) of **1c**. – ^{1}H NMR ($CDCl_3$): δ = 3.18 (OCH_3).

Ripostatin A 15-Methyl Acetal Methyl Ester (**1d**): A solution of 10 mg of **1c** in 1 ml of methanol was cooled to 0°C and treated dropwise with an ethereal solution of diazomethane until the yellow color persisted. The solvent was immediately evaporated to give the quantitative yield of **1d**. TLC (Dichloromethane): R_f = 0.60. – ^{1}H NMR: Table 3. – (+)-FAB-MS, (matrix: 3-NBA), *m/z*: 491 $[M - OCH_3]^+$, high resolution: calcd. 491.2791, found 491.2748.

Ripostatin C (**3a**) *from Ripostatin A:* To a solution of 122 mg (0.25 mmol) of ripostatin A in methanol a solution of 50 mg of $NaHCO_3$ (2.5 equiv.) in 2 ml of water was added, and the mixture was heated at 50°C. An initially formed precipitate dissolved within some min. After 5 h, the reaction was stopped by removal of the methanol under reduced pressure. The aqueous solution was acidified to pH 5 and extracted twice with ethyl acetate. Evaporation of the solvent from the combined extracts afforded a residue (118 mg), which was dissolved in methanol. The solution was applied to a column of Eurosil-Bioselect 100–20, C 18. Elution with methanol/0.05 M NH_4OAc buffer (2:1) yielded 89 mg of **3a** (73%) and 13 mg of recovered ripostatin A (11%).

Ripostatin C Dimethyl Ester (**3b**): A solution of 89 mg of **3a** in 1.5 ml of methanol was treated as described for the preparation of **1d**. The crude material was pure enough for further reactions. – TLC: Dichloromethane/methanol (97:3), R_f = 0.56. – NMR ($CDCl_3$): ^{1}H: δ = 3.65 (s), 3.68 (s), ^{13}C: δ = 51.11 (q), 52.09 (q).

11-O-[(2R)-2-Phenylbutyryl]ripostatin C Dimethyl Ester (**3c**): 20 mg of **3b** (0.04 mmol) was dissolved in 0.5 ml of dichloromethane and 25 mg (2 equiv.) of (*R*)-2-phenylbutyric anhydride in 0.25 ml of dichloromethane and one drop of pyridine. The combined solutions were stirred for about 12 h at room temp. The solvent was evaporated and the residue separated by TLC (silica 60 F_{254}, 1 mm (Merck), eluent dichloromethane/methanol, 98:2). Fraction 1 yielded 8 mg (32%) of (2*Z*)-**3c**, fraction 28 mg (32%) of the (2*E*) isomer of **3c**. – TLC: Dichloromethane/methanol (98:2), R_f = 0.85 [(2*Z*)-**3c**], 0.78 [(2*E*)-**3c**]. – ^{1}H NMR: Table 4. – (+)-FAB-MS (matrix: 3-NBA): $[C_{42}H_{52}O_7 + H]^+$, calcd. 669.3791, found 669.3787.

Analogously, 6 mg of **3b** was treated with (*RS*)-2-phenylbutyric anhydride to furnish a mixture of esters which was subjected to NMR analysis. The signals of the (*S*)-2-phenylbutyric acid ester were assigned after subtracting those of the (*R*)-2-phenylbutyric acid ester (Table 4).

Methyl Esters of Ripostatin B (**2c**) *and 15-epi-Ripostatin B* (**2d**): To cooled (0°C) solutions of 20 mg of **2a** and **2b** in 1 ml of methanol an ethereal solution of diazomethane was each added dropwise until the yellow color persisted. The solvent was immediately evaporated and the residue directly used for further reactions. **2c**: TLC: Dichloromethane/methanol (95:5), R_f = 0.45. – ^{1}H NMR ($CDCl_3$): δ = 3.64 (s, OCH_3). – EI-MS (200°C, 70 eV), *m/z* (%): 510 (2.0) $[M]^+$, 492 (9.9) $[M - H_2O]^+$, 474 (4.0) $[M - 2\,H_2O]^+$, 404 (6.3), 144 (66.6), 91 (100). – **2d**: TLC: Dichloromethane/methanol (95:5), R_f = 0.51. – ^{1}H NMR ($CDCl_3$): δ = 3.65 ppm, s (OCH_3).

15-O-[(2R)-2-Phenylbutyrates] of Ripostatin B Methyl Ester (**2e**) *and 15-epi-Ripostatin B Methyl Ester* (**2f**): To solutions of 10 mg of **2c** and **2d** in 0.4 ml of dichloromethane a solution of 5 mg of (*R*)-2-phenylbutyric acid (1.5 equiv.) in 0.1 ml of dichloromethane and then a solution of 15 mg of trifluoracetic anhydride (3 equiv.) in 0.1 ml of dichloromethane were added, and the mixture was stirred overnight at room temp. The solvent was removed under reduced pressure. and 0.5 ml of a 0.2 N $NaHCO_3$ solution was added to the residue. After stirring of the mixture for 30 min, the aqueous solution was extracted with ethyl acetate, the organic solvent evaporated, and the residue subjected to TLC (silica 60 F, 1 mm, Merck, eluent dichloromethane/methanol, 97:3). – 3.5 mg (27%) of **2e** and 2.4 mg (19%) of **2f** were obtained. TLC: Dichloromethane/methanol (97:3), R_f = 0.39 for **2e** and 0.42 for **2f**. – ^{1}H NMR for **2e** and **2f**: Table 4.

Reaction of Ripostatin A with Hydrochloric Acid in Methanol: A solution of 20 mg of ripostatin A in a mixture of 2 ml of methanol and 2 µl of acetyl chloride was heated at 45°C for 30 min. The resulting solution was concentrated and the residue subjected to PSC chromatography (silica 60 F, 1 mm, Merck, eluent dichloromethane/methanol, 90:10) to afford 5.4 mg (24%) of **4a** and 4.6 mg (20%) of **4b**. TLC: Dichloromethane/methanol (88:12), R_f = 0.52 for **4a** and 0.89 for **4b**. – (–)-FAB-MS (matrix: 3-NBA): *m/z* 495.6 $[M - COOH]^-$ for **4a** and 553.6 $[M - H]^-$ for **4b**. – ^{1}H NMR ($CDCl_3$): δ = 3.47 (s, 3H, 13-OCH_3), 3.21 (s, 3H, 15-OCH_3), 2.33 (dd, *J* = 4.5 and 11.7 Hz, 1H, 14e-H), 2.08 (br d, *J* = 11.8 Hz, 1H, 12e-H), 1.28 (t, *J* = 11.7 Hz, 1H, 14a-H), 1.15 (q, *J* = 11.8 Hz, 1H, 12a-H) for **4a** and **4b**, 3.79 (s, 3H, 28-OCH_3) for **4b**.

Feeding of ^{13}C-Labeled Precursors: The culture volumes for feeding experiments were 500 ml for each precursor. The precursors (100 mg of sodium [1-^{13}C]acetate, 98%; 100 mg of sodium [1,2-$^{13}C_2$]acetate, 98%; 100 mg of [2-^{13}C]phenylalanine, 98%; 250 mg of sodium [1-^{13}C]propionate, 97%; 250 mg of [CH_3-^{13}C]methionine, 98%) were fed in several portions, beginning 3–5 d after the start of cultivation and ending 1 d before harvesting the XAD. The criterion for the harvest was the total consumption of the glucose (concentration at start: 0.3% glucose, 0.3% soluble starch). After work-up as described above about 5–6 mg of ripostatin A was obtained.

[1] G. Höfle, N. Bedorf, H. Steinmetz, D. Schomburg, K. Gerth, H. Reichenbach, *Angew. Chem.* **1996**, *108*, in press; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, in press.
[2] H. Irschik, H. Augustiniak, K. Gerth, G. Höfle, H. Reichenbach, *J. Antibiot.* **1995**, *48*, 787–792.
[3] G. Helmchen, *Tetrahedron* **1974**, *16*, 1527–1530.
[4] E. Egert, M. Noltemeyer, J. Siebers, J. Rohr, A. Zeeck, *J. Antibiot.* **1992**, *45*, 1190–1191.

[5] R. Jansen, unpublished results.
[6] B. Kunze, W. Trowitzsch-Kienast, G. Höfle, H. Reichenbach, *J. Antibiot.* **1992**, *45*, 147–150.
[7] W. Trowitzsch-Kienast, E. Forche, V. Wray, H. Reichenbach, E. Jurkiewicz, G. Hunsmann, *Liebigs Ann. Chem.* **1992**, 659–664.
[8] R. Jansen, B. Kunze, V. Wray, H. Reichenbach, E. Jurkiewicz, G. Hunsmann, G. Höfle, *Liebigs Ann. Chem.* **1991**, 707–708.
[9] N. Bedorf, unpublished results.
[10] N. Bedorf, D. Schomburg, K. Gerth, H. Reichenbach, G. Höfle, *Liebigs Ann. Chem.* **1993**, 1017–1021.
[11] R. Jansen, B. Kunze, H. Reichenbach, E. Jurkiewicz, G. Hunsmann, G. Höfle, *Liebigs Ann. Chem.* **1992**, 357–359.
[12] J. Berdy, *Handbook of Antibiotic Compounds*, vol. II, CRC Press, **1980**, p. 44–72.
[13] D. Blasberger, S. Carmely, M. Cojocaru, I. Spector, N. Shochet, Y. Kashman, *Liebigs Ann. Chem.* **1989**, 1171–1188.

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