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Isolation and structure revision of the actin-binding macrolide rhizopodin from *Myxococcus stipitatus* (Myxobacteria)

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ABSTRACT

Rhizopodin was isolated as cytostatic and weakly antifungal macrolide (**1**) and later characterized as potent actin-depolymerizing agent. It is produced by the myxobacterium *Myxococcus stipitatus*, which enables a fermentative supply of the drug for biological studies. We here report a revised structure that characterizes rhizopodin (**2**) as the first known dimeric bis-lactone exhibiting side chains that terminate in *N*-methyl-vinylformamide groups, which are otherwise found in smaller marine toxins also targeting the actin cytoskeleton. Compound **2** might function as bivalent inhibitor forming ternary complexes with actin which would explain its high efficacy.

Myxobacteria are not only a rich source for antifungal or antibacterial antibiotics,[1–3] but also for compounds targeting the cytoskeleton of mammalian cells, such as the tubulin polymerizing epothilones (Ixempra®)[4] or the depolymerizing tubulysins,[5] which are currently used or developed as anticancer drugs. Further, myxobacterial compounds interfere with the actin microfilaments of the cytoskeleton, such as chondramides,[6] showing cytotoxic actin stabilization.[7] By contrast, a metabolite from *Myxococcus stipitatus*, strain Mx f164, has been initially described in 1993[8] as inhibitor of the propagation of mammalian cell cultures without killing the cells. The development of branching and reticular cell extensions resembling the rhizopodia of protozoa led to the name rhizopodin for the compound that was described as the oxazole-containing macrolide **1**. Rhizopodin (**1**) is chemically closely related to marine oxazole macrolides, for example, halichondramides and sphinxolides.[9] Since these macrolides depolymerize actin microfilaments, a study comparing the effects of rhizopodin and latrunculin B showed that the dramatic morphological changes of the cells are caused by inhibition of actin polymerization.[10] This mechanism of action makes rhizopodin a valuable molecular probe to elucidate the interaction of macrolides with actin and the biological function of actin in greater detail.

Recently, it was demonstrated that chondramide and rhizopodin, as examples of compounds acting on the dynamics of the actin skeleton of macrophages reduced the phagocyto efficiency for yeast cells.[11]

The current report describes the isolation and NMR spectroscopic data of rhizopodin (**2**) for the first time and provides a reassignment of the planar chemical structure. It was initiated by a parallel X-ray study of the actin–rhizopodin complex[12] which did not match the original structural assignment. This comprehensive reinvestigation unambiguously revealed the symmetrical dilactone structure **2** for rhizopodin rather than the previously proposed monomeric lactone **1**.[8]

For production of rhizopodin (**2**), a 200 L fermentation batch of *M. stipitatus*, strain Mxf 164, was cultivated in presence of 2 L of Amberlite XAD-16. At the end of the fermentation, the resin was recovered by sieving [200 mesh] and washed with water to remove adherent cells. The more polar fraction of absorbed material was removed from the XAD by washing with two bed volumes of 50% aqueous methanol, before the rhizopodin-containing fraction was eluted with 8 L of methanol. The fraction was concentrated in vacuo to give approximately 1 L of an oil/water mixture, which was extracted with three portions of ethyl acetate. The combined organic layer was evaporated to give 7.2 g of an oily residue. The

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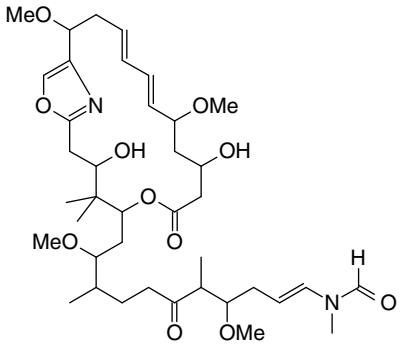
semisolid raw material was passed through a column of Sephadex LH 20 in methanol (6.2 × 63 cm). The rhizopodin (**2**) containing fraction was collected according to HPLC or TLC analyses. Evaporation of the solvent provided 0.37 g of a residue which was separated batch-wise by preparative RP-HPLC on Nucleosil 100 C-18 using 80% aqueous methanol. The rhizopodin-containing fraction was concentrated in vacuo and finally freeze-dried to give a total yield of 130 mg of **2**, a yield of 0.65 mg/L fermentation broth.

Rhizopodin (**2**) was obtained as white amorphous solid; UV (MeOH): λ_{max} ($\log\varepsilon$) = 231 (4.65), 241 sh (4.49); IR ($CHCl_3$): ν = 3475, 3011, 2937, 2828, 1725, 1693, 1658, 1080 cm^{-1}; $[\alpha]_D^{20}$ −53.4 (*c* 1, MeOH). The unpublished spectroscopic structure assignment as a monomeric lactone **1** was based on NMR and misleading MS data.[8] Recently, we repeated the HPLC–MS analysis of **2** using ESI-MS spectrometry: in the positive mode strong ions at *m*/*z* 1469, 1486, and 1491 were detected for $(M+H)^+$, $(M+NH_4)^+$, and $(M+Na)^+$. In the negative mode a low abundant *m*/*z* 1467 was observed for $(M-H)^-$ accompanied by a cluster with acetic acid $(M-H+AcOH)^-$ at *m*/*z* 1527. These observations now clearly indicate the double molecular mass for **2**. HR (+) ESI MS of the sodiated molecular ion *m*/*z* 1491.8618 [calcd. for $C_{78}H_{124}N_4O_{22}$ ^{23}Na $(M+Na)^+$ 1491.8605] provided the elemental composition $C_{78}H_{124}N_4O_{22}$ for **2** which includes 19 double bond equivalents.

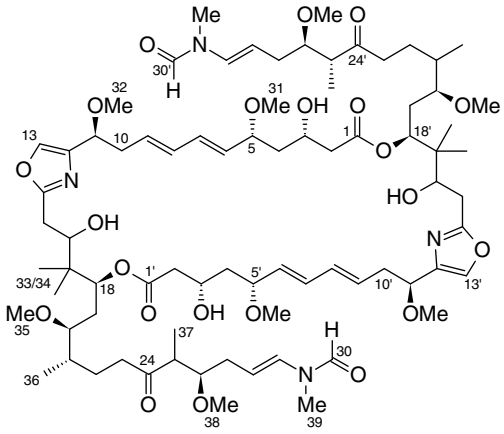
Table 1
NMR data of rhizopodin (**2**) in CD_3OD[a]

H	δ_H[b]	M	*J* (Hz)	ROESY[c]	C	δ_C[d]	m	HMBC[c]
—	—	—	—	—	1	174.01	s	2a, 2b, 18
2a	2.57	dd	14.7, 4.8[f]	4a/b > 5	2	44.13	t	4a
2b	2.50	dd	14.7, 8.4	4a/b > 5				
3	4.07	dddd	8.4, 8.7, 4.8, 4.1	5, 4b, 2a/b > 6, 7, 20	3	67.35	d	2b, 4a > 5, 4b
4a	1.83	ddd	13.8, 8.7, 6.3	2a, 6	4	43.97	t	2a/b, 5, 6
4b	1.65	ddd	13.8, 7.3, 4.1	2a, 6				
5	3.82	ddd	8.2, 7.3, 6.3	31, 7, 3 > 4a/b > 2a/b	5	81.44	d	31 > 4ab, 7, 6
6	5.40	dd	15.2, 8.2	8, 31 > 3, 4b > 4a	6	132.62	d	4a/b, 8
7	6.20	dd	15.2, 10.4	9, 5 > 31, 10	7	135.13	d	5, 9 > 8
8	6.10	m	-	6, 19 >31 >11	8	133.47	d	6, 10
9	5.64	dt	14.9, 7.4	7, 11, 10 >13	9	131.55	d	7, 11, 10
10ab	2.62	m	(2H)	8, 13	10	38.69	t	11, 8, 9
11	4.22	t	6.6	10, 9, 13 >8	11	76.94	d	32 > 10, 9
—	—	—	—	—	12	140.98	s	10, 13 >11
13	7.70	s	—	11, 32	13	138.03	d	11 ($J_{H,C}$ 208 Hz)
—	—	—	—	—	14	166.49	s	15a/b, 13, 16
15a	2.96	dd	15.1, 2.7	18, 33/34 >	15	32.17	t	18, 16, 22b[g]
15b	2.84	dd	15.1, 10.4	33/34 >18				
16	3.94	dd	10.4, 2.7	15a, 18, 33/34 > 19, 15b > 2a/b	16	74.49	d	33,34 > 15b > 15a,18
—	—	—	—	—	17	42.79	s	33 + 34 ≫ 18, 16
18	5.30	dd	9.4, 2.4	16, 20, 33/34, 35 > 2b	18	76.65	s	33,34 > 19, 26
19ab	1.60	m	(2H)	16, 33/34 > 22	19	32.24	t	18, 16, 22b[g]
20	3.05	dt	9.1, 3.1 br	35, 18, 21/22a, 19 > 23, 3	20	83.27	d	35, 36 > 19, 18
21	1.79	m	[e]	35[e]	21	35.65	d	36 > 23, 22a/b > 19
22a	1.77	m	(2H)[e]	36[e]	22	26.04	t	36 > 23 > 20
22b	1.25	m		36				
23	2.58	m	[f]	25, 21/22a > 36, 37	23	42.04	d	22b, 21/22a
—				—	24	216.29	s	37, 23, 25 > 22, 26
25	2.81	dq	8.7, 7.0	23 > 28, 27b	25	50.41	d	37, 26, 27a > b
25Z	2.79	dq		—				
26	3.48	m		27a/b, 37, 23 > 28	26	84.11	d	37, 38 > 25, 27b, 27a
27a	2.49	m	[h]	>29 > 37	27	31.34	t	25, 29, 28
27b	2.21	m	[h]	>29 > 37				
28	5.19	ddd	14.3, 7.9, 6.6	39 ≫ 38 > 26, 27a/b	28	107.43	d	27b, 27a, 26 > 29
29	6.73	d	13.9	30 ≫ 39, 27b > 27a	29	132.08	d	39, 27a/b, 28 > 30
30	8.32	s		29 ≫ 39	30	164.79	d	29, 39 ($J_{H,C}$ 200 Hz)
31	3.22	s	(3H)	5, 6 >7, 8	31	56.46	q	5
32	3.27	s	(3H)	11 > 13	32	56.91	q	11
33	0.93	s	(3H)	18, 16, 15 > 19	33	19.46	q	18, 33/34
34	0.93	s	(3H)	18, 16, 15 > 19	34	19.05	q	18, 16, 33/34
35	3.33	s	(3H)	20, 18, 22a/21	35	58.37	q	20
36	0.85	d	7 (3H)	23, 19 > 20	36	16.10	q	22b, 21/22a
37	1.00	d	7 (3H)	26, 27b > 23	37	13.20	q	25, 26
37Z	1.00	d	7					
38	3.31	s	(3H)	26 > 28	38	58.04	q	26
39	3.02	S	(3H)	28 >	39	27.81	q	39, 30
Z isomer (minor component)								
28Z	5.27	M			28Z	109.55		27Zb, 29Z > 26, 27Za
29Z	7.13	d	14.7 (28%)	—	29Z	127.38		39Z, 30Z, 28Z, 27Zb
30Z	8.08	s	(29%)	39Z >	30Z	163.39	d	39Z
39Z	3.11	S		30Z, 28Z	39Z	33.65		29Z, 30Z

[a] Internal references CD_3OD at δ_H 3.31 and δ_C 49.15 ppm.
[b] ^1H at 600 MHz. Integral values of two or three protons given in brackets.
[c] The sign > in ROESY and HMBC columns differentiates strong and weak correlations.
[d] ^{13}C at 150 MHz; data with two decimal places enable to distinguish narrow signals. Multiplicity was determined from HMQC spectrum.
[e,f,g] Overlapping signal pairs.
[h] Overlapping with 27a/b of the *Z* isomer.



Formula scheme: the monomeric lactone proposed earlier **(1)**



Formula scheme: rhizopodin **(2)**

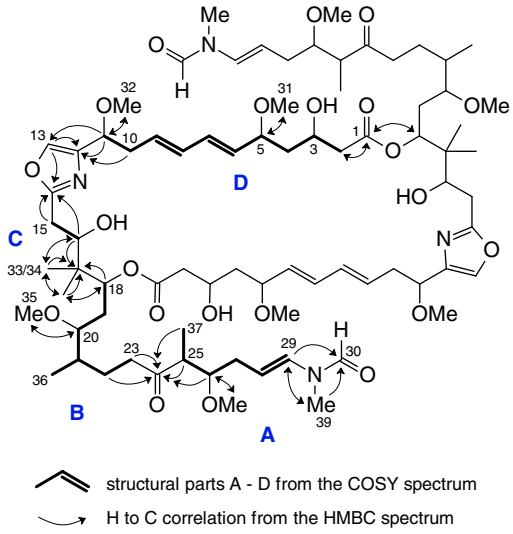


Figure 1a. Structural parts A–D of rhizopodin (**2**) from COSY NMR spectra and selected interconnecting HMBC correlations.

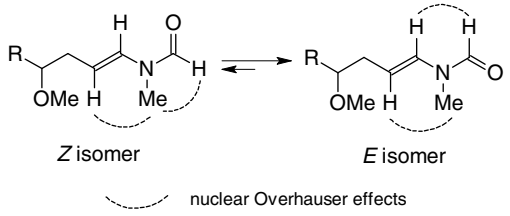


Figure 1b. Geometrical isomers of the *N*-methyl-vinylformamide group in rhizopodin (**2**).

Since only one half of the molecule can be detected in the NMR spectra, rhizopodin (**2**) must have a completely (C2) symmetrical structure. The planar structure of the half-molecule was elucidated by NMR spectroscopy in CD_3OD (Table 1). Although the compound provided only one peak in the HPLC, NMR spectra at ambient temperature partially showed two sets of signals in a ratio of about 2:1. $^1H,^{13}C$ HMQC NMR spectroscopy allowed assigning the direct shift correlations for both sets of signals.

The $^1H,^1H$ COSY NMR spectrum afforded four basic structural elements A–D (Fig. 1), which were then provided with the remaining moieties using the long-range correlations in the $^1H,^{13}C$ HMBC NMR spectrum. Thus, due to the mutual correlations indicated by double-headed arrows in Figure 1a, structural part A ends at the $\Delta^{28,29}$ trans double bond ($J_{28,29} \sim 14$ Hz) with an *N*-methyl-vinylformamide group, that is known to cause a double set of NMR signals due to a slow conversion of geometrical isomers. Similarly to the observations with sphinxolide,[13] both sets of signals show different NOE correlations in the ROESY NMR spectrum. The main set was identified as the *E*-formyl amide from the strong ROESY correlations between H29 and the formyl H30 on one side and H28 and the *N*-methyl signal on the other side. After rotation around the formyl-*N* bond, the *Z* isomer shows $^1H,^1H$ ROESY correlations of the *N*-methyl signal with both protons, formyl H30 and H28 (Fig. 1b.). The connection between structural parts A and B was indicated by HMBC correlations of the ketone carbon C24 (δ 216.29) with the neighboring protons as indicated by the single-headed arrows in Figure 1a.

The methyl groups C33 and C34, both represented by a common singlet (δ 0.93) in the ^1H NMR spectrum, have to be connected to the quaternary aliphatic C17 (δ 42.79) according to their mutual HMBC correlations and correlations with C17. Further correlations place the structure element with these geminal methyl groups as link between C18 of structural part B and C16 of the small part C.

The existence of a hetero-aromatic ring in **2** was indicated by the direct coupling $^1J_{H,C} = 208$ Hz of the methine C13 group (δ_H 7.70; δ_C 138.03). HMBC correlations of the quaternary carbons C14 (δ 166.49) and C12 (δ 140.98) with H13 then led to the assignment of the oxazole ring. Further correlations of C14 with 15H and 16H on one side and those of C12 with H10 and H11 on the other side connect the oxazole ring with structural elements A and C. The last quaternary carbon C1, a typical carboxyl signal at δ_C 174 in the ^{13}C NMR spectrum, showed a strong HMBC correlation with methylene group C2 of structural element A, and a weaker correlation with oxymethine C18, which also indicated the acylation by a typical low-field shift of its ^1H NMR signal at δ_H 5.30. However, this C18 must, in fact, be C18′ of the second half of the symmetrical molecule. Thus the macro-lactone backbone was established, which then accounts for all 19 double bond equivalents: 6 for two oxazole rings, 4 for two dienes, 4 for the vinyl-formamides, 2 for the ketones, and 2 for the carboxyl groups and the remaining one for the dilactone ring.

Finally, four methoxyl groups, represented by singlets at δ_H 3.2–3.3 and typical shifts of their carbon atoms (δ_C 56–58), were connected to their corresponding oxymethine carbon atoms C5, C11, C20, and C26 according to their mutual $^1H,^{13}C$ long-range correlations. Consequently, the remaining two H/D exchangeable protons, which were not visible in the NMR spectra in CD_3OD, complete the structure of rhizopodin (**2**) with two hydroxyl groups at C3 and C16 (δ_C 67.35 and 74.49).

The all-trans configuration of the dienes is apparent from the coupling constants of 15 Hz for the double bonds and $J_{7,8}$ = 10 Hz for the single bond.

The reinvestigation of the structure of rhizopodin (**2**) unambiguously establishes the new C_2-symmetric dilactone skeleton. With its revised dilactone structure, rhizopodin (**2**) is the only dimeric macrolide possessing side chains terminating in *N*-methyl-vinylformamide groups. Other dimeric macrolides with actin polymerization inhibitory activity include the swinholides, misakinolide A, and bistheonellide B.[14] Although swinholide A features a completely different side chain, it was shown to form a ternary complex with two actin molecules using essentially the same actin-binding site as the monomeric lactones with *N*-methyl-vinylformamide side chains.[15] Analogously, rhizopodin (**2**) may behave as a bivalent inhibitor forming a ternary complex with two actin molecules. The bivalent character of **2** may also be the explanation for the long lasting biological effect in cell culture and for the high efficiency as actin-polymerization inhibitor (5.2 nM) compared to latrunculin (48 nM). Elucidation of the exact nature of the actin–rhizopodin interaction will be of great importance for further studies of the mechanism of action and for chemical studies on the structure–activity relationship. An X-ray study of the absolute configuration of **2** and of its interaction with actin has been accepted.[12]

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