The First Low Molecular Weight Antibiotic from Lactic Acid Bacteria: Reutericyclin, a New Tetramic Acid

Alexandra Höltzel, Michael G. Gänzle, Graeme J. Nicholson, Walter P. Hammes, and Günther Jung*

Based on a screening of lactic acid bacteria from cereal fermentations, an inhibitory effect of *Lactobacillus reuteri* LTH2584 against Gram-positive bacteria has been demonstrated.[1] This strain had been isolated as one of the strains dominating the flora of a sourdough which is prepared for the production of a commercial baking aid.[2] Here, we describe for the first time a low molecular weight antibiotic, reutericyclin (**1**), which is produced by lactic acid bacteria.

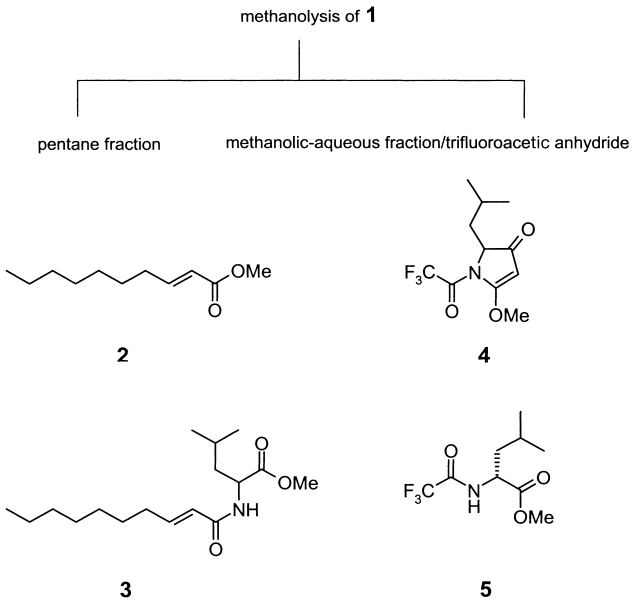
Compound **1** was purified as a yellow-brown oil from cell extracts and culture filtrate of *L. reuteri* LTH2584 with a yield of approximately 1 mg L^{-1}.[3] The elemental formula of **1** was determined by high-resolution ESI-FT-ICR mass spectrometry (ICR = ion cyclotron resonance; $[M + H^+]$ for $C_{20}H_{31}NO_4$: calculated *m*/*z* 350.23257, experimental *m*/*z* 350.23210). The UV/Vis spectrum (acetonitrile) showed absorption maxima at 238 and 286 nm. An intense absorption at 1726 cm^{-1}, as well as several strong bands between 1657 cm^{-1} and 1617 cm^{-1}, in the IR spectrum (film) pointed to the presence of several carbonyl groups in the molecule. The structure of **1** was elucidated by NMR spectroscopy and GC-MS analysis of methanolysis products.

The HSQC spectrum showed that **1** consists of four methyl and seven methylene groups as well as two aliphatic and two olefinic methine groups. Signals for five quarternary carbons were identified in the ^{13}C NMR spectrum, two of which were classified as carbonyl carbons of acid, ester, or amide functionalities, and two as ketone carbons (Table 1). TOCSY spectra (acquired with short and long mixing times) exhibited signals for two distinct spin systems, the first one including 5-H to 9-H and the second one 13-H to 21-H. The *E* configuration of the double bond between C-13 and C-14 was reflected by the vicinal coupling constant ^3J(13-H, 14-H) = 15.4 Hz. Connectivities observed in the HMBC spectrum (Table 1) confirmed the assignments within the spin systems and enabled the addition of the quarternary carbons C-4 and C-12 to the respective structural fragments. A third fragment was established from the HMBC connectivities of the methyl protons 11-H to the quarternary carbons C-3 and C-10; the ^1H NMR signal for 11-H appeared at $\delta = 2.51$, suggesting the vicinal connectivity of methyl group and keto function. HMBC connectivities were not observed between the three structural fragments established so far or to the carbonyl carbon C-2.

Table 1. ^1H and ^{13}C NMR chemical shifts for compound **1**, as well as observed HMBC correlations ([D_3]acetonitrile, 298 K). Due to keto–enol tautomerism two signal sets were detected for several nuclei (**1a** to 60%, **1b/1c** up to 40%).

	$\delta(^{13}C)$		$\delta(^1H)$		HMBC
	1a	**1b/1c**	**1a**	**1b/1c**	correlations
2	167.3	174.8	–		–
3	106.1	103.6	–		11-H
4	199.2	194.5	–		5-H, 6-H
5	60.2	64.1	4.68	4.41	6-H
6	39.8		1.81	1.76	5-H, 7-H, 8-H, 9-H
7	25.2		1.82		5-H, 6-H, 8-H, 9-H
8	23.8		0.88		9-H
9	22.6		0.93		8-H
10	195.6	189.3	–		11-H
11	22.8	20.2	2.51		–
12	165.9	165.5	–		13-H, 14-H
13	124.2		7.29	7.22	15-H
14	150.7	151.8	7.06	7.10	15-H, 16-H
15	33.3	33.2	2.28	2.29	13-H, 14-H, 16-H
16	28.8		1.50		14-H, 15-H
17	29.8		1.34		15-H, 16-H, 18-H
18	29.8		1.34		16-H, 17-H
19	32.5		1.30		20-H, 21-H
20	23.3		1.32		19-H, 21-H
21	14.3		0.90		20-H

The acid methanolyzate of **1** (formed by treatment with CH_3OH/1.5 N HCl, 110 °C, 15 min) was, after addition of water, extracted with *n*-pentane (Scheme 1). The MeOH/H_2O fraction was derivatized with trifluoroacetic anhydride. The GC-MS of the pentane fraction showed two major peaks. The peak at *m*/*z* 184 was identified as 2-decenoic acid methyl ester (**2**) while the second peak at *m*/*z* 297 was assigned to *N*-(2-decenoyl)leucine methyl ester (**3**), on the basis of character-



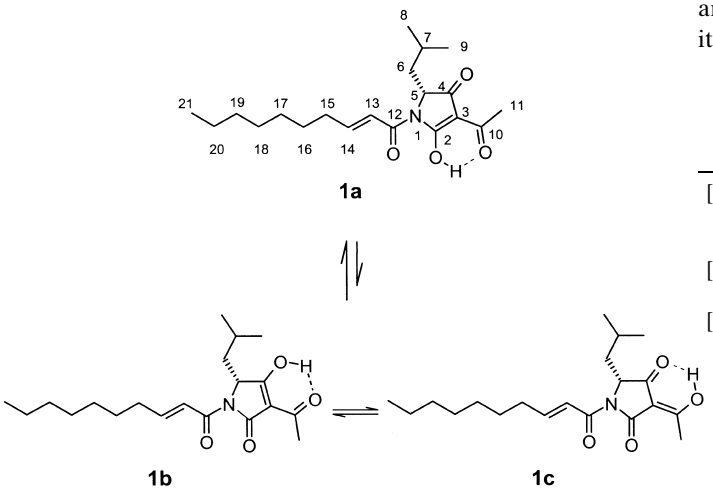
Scheme 1. Methanolysis products of reutericyclin, detected by GC-MS analysis at *m*/*z* 184 (**2**), 297 (**3**), 265 (**4**), and 241 (**5**).

[*] Prof. Dr. G. Jung, Dr. A. Höltzel, G. J. Nicholson
Institut für Organische Chemie der Universität Tübingen
Auf der Morgenstelle 18, 72076 Tübingen (Germany)
Fax: (+49) 7071-29-5560
E-mail: guenther.jung@uni-tuebingen.de
Dr. M. G. Gänzle,[+] Prof. Dr. W. P. Hammes
Institut für Lebensmitteltechnologie
der Universität Hohenheim (Germany)
[+] Current Address:
Lehrstuhl für Technische Mikrobiologie
Institut für Brauereitechnologie und Mikrobiologie
TU München in Freising-Weihenstephan (Germany)

  1433-7851/00/3915-2766 $ 17.50+.50/0 *Angew. Chem. Int. Ed.* **2000**, *39*, No. 15

istic fragmentations. The identification of **3** in the acid methanolyzate of **1** suggested that C-5 and C-12 are connected by N-1 in the native molecule. The observed ^{13}C NMR signals left but one possibility for the arrangement of the third fragment and the carbonyl group at C-2 in the molecule. The formulation of **1** as 3-acyltetramic acid was confirmed by the identification of compound **4** as the main component of the MeOH/H_2O fraction of the acid methanolyzate. The deacylation of 3-acyltetramic acids under acidic conditions has long been known,[4] and similarly the O-alkylation of tetramic acids.[5] The MeOH/H_2O fraction also contained *N*-trifluoroacetylleucine methyl ester **5**. From the absolute configuration of **5**, as determined by GC-MS analysis with a Chirasil-Val column,[6] the (5*R*)-configuration of **1** was concluded. The methanolysis products **3** and **5**, the identification of which was essential for the structure elucidation of **1**, are unusual in that their formation requires the cleavage of a C–C bond. Up to now the release of an amino acid from 3-acyltetramic acids has only been reported upon treatment with oxidative reagents.[4] The structure and the results of the acid methanolysis of **1** were validated by the synthesis of racemic **1**.[7]

The formulation of **1** as (5*R*)-3-acyl-1-(2-decenoyl)-2-hydroxy-5-isobutyl-Δ^2-pyrroline-4-one refers to tautomer **1a** (Scheme 2), which analysis of the ^{13}C NMR signals (Table 1) revealed to be the major tautomer (60%) in acetonitrile



Scheme 2. Reutericyclin is subjected to keto–enol tautomerism in solution. In [D_3]acetonitrile **1a** is present to 60% and **1b** and **1c** in up to 20% each.

solution. The ^{13}C NMR data for the minor signal set indicate equal contributions (20% each) from the internal tautomers **1b** and **1c**. In this respect, **1** differs from all other naturally occurring 3-acyltetramic acids. These prefer almost exclusively the pyrrolidine-2,4-dione form in solution.[8, 9] The exceptional preference for the Δ^2-pyrroline-4-one form in **1** is brought about by the 2-decenoyl substituent at N-1, which not only reduces the hydrogen bridge acceptor ability of the carbonyl group at C-2, but also stabilizes the endocyclic double bond by conjugation.

Among the naturally occurring tetramic acids,[9, 10] which display a remarkable spectrum of biological activity, the structure of **1** is chemically interesting because of the unprecedented N-1 substitution with an α,β-unsaturated fatty acid. For a possible application of **1** as an antibiotic, it is noteworthy that *L. reuteri* is utilized in food fermentations[2] and has been described as a stable constituent of the intestinal microflora of both humans and animals.[11] Investigations on the inhibitory spectrum of **1** and its production by *L. reuteri* LTH2584 support the conclusion that inhibitory concentrations of the amphiphilic compound are present in sourdough fermentations with *L. reuteri*.[1, 12] Lactic acid bacteria produce a multitude of unspecific low molecular weight compounds, mainly side products of carbohydrate metabolism, which contribute to the inhibitory effect of lactic acid.[13] However, all antibiotics from lactic acid bacteria known to date are bacteriocins. The inhibitory effect of bacteriocins is restricted to closely related species and their potential for application to foods is therefore limited.[14] In recent years, a considerable amount of research has focused on the positive effect of lactic acid bacteria on human health (probiotics). In animal tests, a protective effect of antimicrobial substances from lactobacilli against *Helicobacter* and *Salmonella* infections has been demonstrated,[15] but an active substance has not been chemically characterized to date. As **1** differs in its chemical and biological properties from all other active substances produced by lactic acid bacteria, the structure elucidation of this natural compound adds a new dimension to the discussion regarding the application of lactic acid bacteria and their antimicrobial metabolites for food preservation or for positively influencing the human intestinal microflora.

Received: February 11, 2000 [Z14692]

[1] M. G. Gänzle, C. Hertel, W. P. Hammes in *Beijerinck Centennial. Microbial physiology and gene regulation* (Eds.: W. A. Scheffers, J. P. van Dijken), Delft University Press, **1995**, pp. 380–381.

[2] G. Böcker, P. Stolz, W. P. Hammes, *Getreide Mehl Brot* **1995**, *49*, 370–374.

[3] The isolation of **1** from cell extracts involved extraction with a 70/30 mixture of phosphate buffer (50 mM, pH 6.5) and isopropanol, gel filtration on Superdex S30 (Pharmacia, 75/25 mixture of triethylamine buffer (50 mM, pH 7.2) and isopropanol), and medium-pressure reversed-phase chromatography (Pharmacia, 15 μm, eluent A: H_2O/0.1% trifluoroacetic acid (TFA) solution, eluent B: isopropanol/0.1% TFA solution, gradient: from 25% to 50% B in 20 min). The isolation of **1** from culture filtrate followed a slightly modified protocol. In both cases, HPLC (Advanced Separation Technologies, C_{18} column, 5 μm, eluent A: acetonitrile/0.1% TFA solution, eluent B: H_2O/0.1% TFA solution, A/B = 85/15) was used as a final purification step.

[4] C. E. Stickings, *Biochem. J.* **1959**, *72*, 332–340.

[5] a) H.-D. Stachel, K. K. Harigel, H. Poschenrieder, H. Burghard, *J. Heterocycl. Chem.* **1980**, *17*, 1195–1199; b) K. Inami, T. Shiba, *Tetrahedron Lett.* **1984**, *25*, 2009–2012. Both, the 4-methoxy- and the 2-methoxy derivatives have been reported as the methylation products of tetramic acids. The formulation of 2-methoxy-Δ^2-pyrroline-4-one as the methanolysis product of **1** is based on the mass spectrum of **4** in which the base peak at *m*/*z* 209 originates from a McLafferty rearrangement which would require the carbonyl group at position 4.

[6] H. Frank, G. J. Nicholson, E. Bayer, *J. Chromatogr.* **1978**, *167*, 187–196.

[7] U. Marquardt, D. Schmid, G. Jung, *Synlett*, in press.

[8] The naturally occurring 3-acyltetramic acids known to date are—with the exception of magnesidins A and B—either unsubstituted or alkylated at N-1. For these compounds the dominance of the exocyclic pyrrolidine-2,4-dione form, in which the amide carbonyl group acts as

  1433-7851/00/3915-2767 $ 17.50+.50/0

a hydrogen bond acceptor, has been deduced from a comprehensive analysis of the ^{13}C NMR spectra: a) P. S. Steyn, P. L. Wessels, *Tetrahedron Lett.* **1978**, *47*, 4707 – 4710; b) M. J. Nolte, P. S. Steyn, P. L. Wessels, *J. Chem. Soc. Perkin Trans. 1* **1980**, 1057 – 1065.

[9] The major tautomers of magnesidins A and B in solution are the Δ^3-pyrroline-2-one forms: N. Imamura, K. Adachi, H. Sano, *J. Antibiot.* **1994**, *47*, 257 – 261.

[10] B. J. L. Royles, *Chem. Rev.* **1995**, *95*, 1981 – 2001. So far, the naturally occurring *N*-acyltetramic acids known are only 4-methoxy-Δ^3-pyrroline-2-ones (with the exception of magnesidins A and B).

[11] I. A. Casas, W. J. Dobrogosz, *Microecol. Ther.* **1997**, *26*, 221 – 231.

[12] a) The optimal conditions with respect to temperature, pH, and substrate supply correspond to the conditions during sourdough fermentations: M. G. Gänzle, PhD. thesis, Universität Hohenheim, **1998**; b) additionally, it has been shown that reutericyclin-sensitive strains are inhibited in their growth or killed during cofermentation in sourdough containing *L. reuteri* LTH2584: W. P. Hammes, J. Walter, unpublished results; M. G. Gänzle, R. Vogel, unpublished results.

[13] a) P. A. Vandenbergh, *FEMS Microbiol. Rev.* **1993**, *12*, 221 – 337; b) M.-L. Niku-Paavola, A. Laitila, T. Mattila-Sandholm, A. Haikara, *J. Appl. Microbiol.* **1999**, *86*, 29 – 35.

[14] a) R. W. Jack, J. R. Tagg, B. Ray, *Microbiol. Rev.* **1995**, *59*, 171 – 200; b) R. W. Jack, F. Götz, G. Jung in *Biotechnology*, *Vol. 7*, 2nd edition (Eds.: H. Kleinkauf, H. von Döhren), VCH, Weinheim, **1997**, pp. 325 – 368.

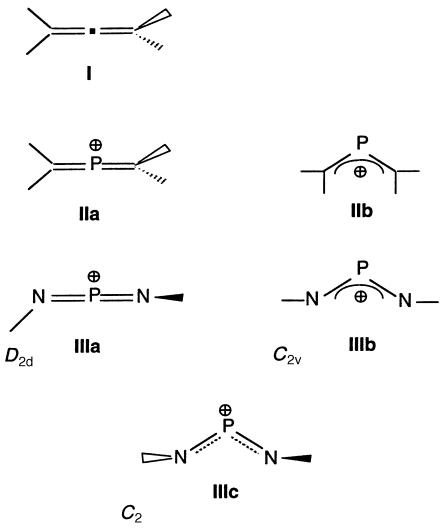
[15] a) M.-H. Coconnier, V. Liévin, E. Hemery, A. L. Servin, *Appl. Environ. Microbiol.* **1998**, *64*, 4573 – 4580; b) M.-F. Bernet-Camard, V. Liévin, D. Brassart, J.-R. Neeser, A. L. Servia, S. Hudault, *Appl. Environ. Microbiol* **1997**, *63*, 2747 – 2753.

Synthesis of the Adduct DMAP · BrP(=N-Mes*)$_2$ and of the Salt [(DMAP)$_2$P(=NMes*)$_2$]$^+$Br**

Markus Blättner, Martin Nieger, Alexander Ruban, Wolfgang W. Schoeller, and Edgar Niecke*

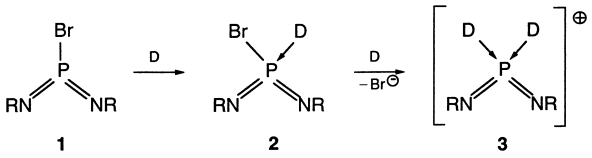
Dedicated to Professor Reinhard Schmutzler on the occasion of his 65th birthday

Allenes **I** are fundamental compounds with a linear structure (D_{2d} symmetry). This implies sp hybridization at the central carbon atom.[1] The hypothetical 2-phosphonio-allenic cation **IIa** is isovalent to **I**, but its central phosphorus atom is not sp hybridized.[2] The s orbital at the phosphorus atom becomes stereochemically active under formation of a "bent" 2-phosphaallyl cation **IIb**.[3] According to ab initio calculations[5] this geometry is favored over that of **IIa**.



Quantum-chemical calculations predict a linear arangement of atoms (D_h) for the cations PO_2^+ and PS_2^+.[6] For these cations a bis-donor adduct has been obtained recently.[7] Here we report on the first synthesis and crystal structure analysis of a bis-donor adduct of the imine analogue of **II** as well as on ab initio calculations on model compounds that support the findings.

Treatment of the bromobis(arylimino)phosphorane **1** (R = Mes* = 2,4,6-tBu$_3$C$_6$H$_2$)[8] with one equivalent of *p*-dimethylaminopyridine (DMAP = D) in CH_2Cl_2 furnished the donor/acceptor adduct **2** in good yield (75 %) in the form of air- and moisture-sensitive light yellow crystals. Subsequent reaction of **2** with a second equivalent of DMAP proceeded by halogen/nucleophile exchange to give the cation **3**, whose bromide was isolated at low temperature as yellow crystals from a little CH_2Cl_2.



Initial information concerning the constitution of **2** and **3** · Br$^-$ was obtained from multinuclear NMR investigations. The ^1H and ^{13}C NMR data clearly indicated the presence of Mes* substituents at the nitrogen atoms as well as the attachment of one (**2**) or two donor molecules (DMAP) (**3**) to the phosphorus atom. As expected the ^{31}P NMR signals appeared at higher fields ($\delta = -93.2$ (**2**), -57.2 (**3**)) than those of bis(imino)phosphoranes,[9] indicating an increase of coordination number at the phosphorus atom.

Crystal structure analyses of **2** and **3** · Br$^-$ gave evidence for the formation as a monoadduct of the bromobis(imino)phosphorane and as a bisadduct of the bis(imino)phosphonium cation (Figures 1 and 2).[10] In both compounds the phosphorus atoms adopt a strongly distorted tetrahedral geometry. The N-P-N bond angle at the phosphorus atom (N1-P1-N2

[*] Prof. Dr. E. Niecke, Dr. M. Blättner, Dr. M. Nieger, Dr. A. Ruban
Anorganisch-Chemisches Institut der Universität
Gerhard-Domagk-Strasse 1, 53121 Bonn (Germany)
Fax: (+49) 228-73-5327
E-mail: e.niecke@uni-bonn.de
Prof. Dr. W. W. Schoeller
Fakultät für Chemie der Universität Bielefeld (Germany)

[**] This work was supported by the Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft (SFB 334). Part of this work was reported at the International Conference on Phosphorus Chemistry, Cincinnati, USA, **1998**. DMAP = *p*-dimethylaminopyridine; Mes* = 2,4,6-tBu$_3$C$_6$H$_2$.

  1433-7851/00/3915-2768 $ 17.50+.50/0