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Cervimycin A–D: A Polyketide Glycoside Complex from a Cave Bacterium Can Defeat Vancomycin Resistance

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Abstract: Cervimycins A–D are novel polyketide glycosides with significant activity against multi-drug-resistant staphylococci and vancomycin-resistant enterococci. They are produced by a strain of *Streptomyces tendae*, isolated from an ancient cave. The structures of the cervimycins were determined by performing extensive NMR and chemical degradation studies. All cervimycins have a common tetracyclic polyketide core that is substituted with unusual di- and tetrasaccharide chains, composed exclusively of trideoxysugars; however, they differ in the acetyl and carbamoyl ring substituent and in the highly unusual terminal methylmalonyl and dimethylmalonyl residues.

Keywords: antibiotics · glycosides · natural products · polyketides · streptomyces

Introduction

Gram-positive bacteria, such as staphylococci, are the most common cause of severe nosocomial infections. The resistance of these pathogens to many commonly used antibiotics, such as penicillins, cephalosporins, methicillins, and tetracyclines, has increased dramatically.[1] Until recently, the glycopeptide antibiotic vancomycin has been considered as the last resort against serious infections caused by multi-drug-resistant, Gram-positive bacteria. During the past decade, vancomycin-resistant enterococci[2–4] and, more recently, staphylococci,[5–7] have spread worldwide with unanticipated rapidity.[8] The occurrence of life-threatening infections and the lack of new powerful antibiotics to combat such pathogens cause serious concerns among the medical community. However, natural products continue to play a major role in drug discovery and represent an important source of novel antibacterial therapies.[3,9]

In a program to search for new antiinfective agents, we studied the biosynthetic capabilities of rare microorganisms and the inhabitants of unusual environments that displayed the potential for bioactive metabolite production. The *Grotta dei Cervi*, Italy, which is not accessible to the public, harbors a rich microbial flora that has remained undisturbed since ancient times.[10] In the course of these studies, we isolated a bacterial strain from a rock wall that was covered with 5000-year-old neolithical wall paintings in red ochre or black (Figure 1). Here, we report on the isolation, structural elucidation, and characterization of a novel antibiotic complex produced by this cave bacterium, which may provide a new lead in the fight against multi-drug-resistant pathogens.

Figure 1. Origin of the cervimycin producer. Neolithic paintings made of bat dung in the *Grotta dei Cervi*, Italy, about 3000 years b.c. (*lat.* cervus = deer).

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Results and Discussion

The cave isolate, strain HKI 0179, was classified under the genus *Streptomyces*, based on morphological and chemotaxonomic characteristics, such as the presence of LL-diaminopimelic acid (LL-A_2pm),[11,12] major menaquinones consisting of MK-9(H_6), MK-9(H_8), and MK-9(H_4),[13] and the predominating fatty acids (MIDI-System, Agilent). Comparison of the 16S rRNA gene sequence revealed that strain HKI 0179 was most closely related to *Streptomyces tendae*[14] (16S rRNA gene sequence similarity 99.5 %). Morphological and physiological characteristics,[15,16] and the comparison of the fatty acid profiles (Sherlock database of the MIDI-System) supported this affiliation. According to Locci,[17] *Streptomyces tendae*[14] is a subjective synonym of *Streptomyces rochei*.[18]

The crude extract of the yellow-pigmented culture broth of the *S. tendae* strain HKI 0179 showed a very good activity against Gram-positive pathogens, especially against multi-drug-resistant *Staphylococcus aureus* and, most remarkably, against vancomycin-resistant *Enterococcus faecalis* strains. To obtain sufficient material of the active principle for structural elucidation and the establishment of the bioactivity profile, strain and fermentation procedures were optimized. The organic extract (30 g), obtained by a large-scale fermentation (180 L), was subjected to purification on two successive sephadex LH20 columns of different size. Bioactivity-guided fractionation by repeated RP-HPLC yielded four main components of the complex, named cervimycin A (**1**), B (**2**), C (**3**), and D (**4**) (Figure 2).

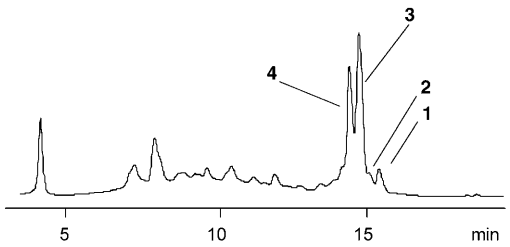

Figure 2. Chromatographic profile of the crude extract obtained from cultures of *S. tendae* strain HKI 0179.

All four compounds showed remarkably strong antimicrobial effects against standard test strains in agar diffusion assays. Most importantly, they proved to be active against multi-drug-resistant *Staphylococcus aureus* and even against vancomycin-resistant *Enterococcus faecalis* (Table 1).

The structures of cervimycin A–D were fully resolved by conducting UV/Vis, HRMS, MS^n, IR, and extensive ^1H and ^{13}C NMR investigations, as well as chemical degradation studies. The main component **3** was obtained as an orange powder with a melting point of 167–168 °C and $[\alpha]_D^{22} = +26.5$ ($c = 0.51$ in MeOH). The molecular formula ($C_{61}H_{81}NaNO_{25}$) was established from the results of HRESI-MS analysis ($m/z = 1250.5002$ $[M+Na]^+$) and ^{13}C NMR data (61 carbon atoms). ESI-MS^n experiments revealed pseudodaughter ions

Table 1. MIC values ($\mu g\,mL^{-1}$) for cervimycins A–D compared to tetracycline and vancomycin (in vitro). Test strains: ATCC 6633: *Bacillus subtilis* ATCC 6633; SG511: *Staphylococcus aureus* SG511; MRSA: *S. aureus* 134/93; VRE: vancomycin-resistant *Enterococcus faecalis* 1528; EfS4: efflux-resistant *S. aureus* EfS4.

	ATCC 6633	SG511	MRSA	EfS4	VRE
cervimycin					
A	0.8	3.1	6.3	3.1	3.1
B	1.6	25.0	12.5	6.3	12.5
C	0.4	12.5	25.0	3.1	3.1
D	0.1	3.1	6.3	6.3	1.6
tetracycline	0.2	0.4	25.0	>100	100
vancomycin	0.2	0.8	1.6	1.6	>100
ciprofloxacin	<0.05	0.2	12.5	100	0.8

with $m/z = 1022$ $[M+N-2\times114]^+$, $m/z = 680$ $[M+Na-5\times114]^+$, $m/z = 593$ $[5\times114+Na]^+$, and $m/z = 452$ $[M+Na-7\times114]^+$, which account for the loss of fragments with $M = 114$, for example, trideoxyhexosyl units.

The UV spectrum showed absorption maxima at 242 nm, with an overlaying shoulder at about 280 nm, and 434 nm, which indicated a conjugated aromatic system. Addition of 1 N NaOH resulted in a bathochromic shift from 434 nm to 563 nm with concomitant change of the complementary color from yellow to violet, a typical property of naphthoquinoid systems that form phenolate salts under alkaline conditions. The IR absorption peaks correspond to valence oscillation of the aromatic −C=C− (1567/1593/1626 cm^{-1}), carbonyl groups (1727 cm^{-1}), aliphatic −C−H_2− (2854/2922/2953 cm^{-1}), and hydroxy groups (3351 cm^{-1}). Data obtained from ^{13}C, DEPT, and HSQC NMR experiments revealed 44 sp^3 carbon atoms, represented by 8 methyl, 1 methoxy, 13 methylene, 20 methine, and 2 quaternary carbon residues. In addition, **3** contains 17 sp^2 carbon atoms, comprising 2 methine and 15 quaternary carbon atoms. The ^1H NMR data for [D_5]pyridine showed 78 signals with two deep-field shifted singlets at $\delta = 10.23$ and 10.48 ppm, which proved to be signals of the carbamoyl NH_2 protons. Two additional signals of chelated protons were detected as a sharp singlet at $\delta = 13.35$ ppm (OH-11) and broad singlets at $\delta = 17.98$ (OH-3) and 12.71 ppm (COOH) in [D_6]DMSO and $CDCl_3$, respectively. The HSQC experiment established all single-bond ^1H–^{13}C connectivities.

Due to the limited number of proximate protons in the aromatic system, only a single spin system appeared in the COSY spectrum that permitted the analysis of a substructure. However, COSY data and diagnostic ^1H–^{13}C long-range couplings in the aglycone fully established the framework and substitution pattern of the tetracyclic ring system (Figure 3), which is reminiscent of, but not identical to, the structure of tetracyclines. COSY data and diagnostic ^1H–^{13}C long-range couplings, that is, of H-4, H-4a, H-5, H-6, and H-9 to the adjacent carbon residues (C-4 to C-12a), revealed the composition of rings B, C, and D. These results were supported by the HMBC couplings of OH-11 and OCH_3-8. The exact HMBC classification (to $^2J_{CH}$, $^3J_{CH}$, or $^4J_{CH}$) was successfully deduced from the intensity of the couplings.

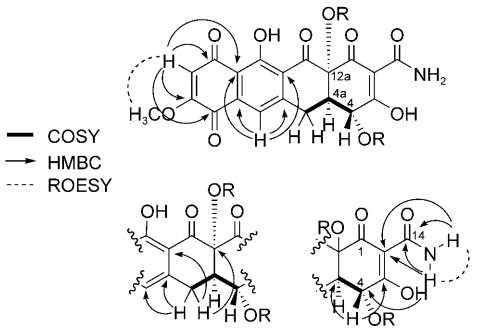

Figure 3. H,H COSY and selected HMBC and ROESY correlations of the cervimycin C aglycone.

The elucidation of ring A was more challenging, due to the lack of protons. ^{13}C NMR shifts $\delta = 194.93$, 192.03, 101.04, and 174.74 ppm in [D_5]pyridine, and the downfield-shifted OH-group ($\delta = 17.98$ ppm in $CDCl_3$) disclosed an enol–keto architecture at C-1, C-2, C-3, and C-14, as in the tetracyclines. Furthermore, ^{1}H-(NH_2-14)-^{13}C long-range couplings provided the strongest evidence for a carbamoyl substituent at C-2 in ring A (Figure 3).

The composition and relative configuration of the monosaccharide substituents was fully resolved by performing NMR spectroscopy and chemical degradation studies. The presence of 6 methine (99–104 ppm), 6 methyl (17–19 ppm), 12 methylene (22–32 ppm), and 12 methine (65–80 ppm) carbon atoms suggested the presence of six constitutionally similar trideoxyhexose residues, which supports the results obtained from the MS^n experiments. The anomeric protons (H-1B, H-1C, H-1D) of monosaccharides B, C, and D appeared as singlets ($J_{B,C,D} < 1$ Hz), which corroborates an α-configuration. Conversely, monosaccharides A, E, and F are clearly connected as β-glycosides, because the anomeric protons (H-1A, H-1E, H-1F) appeared as doublets ($J_A = 9.1$ Hz, $J_E = 8.6$ Hz, $J_F = 9.4$ Hz). The H-5 of sugar residues B, C, and D appeared as quartets with a very small coupling constant between H-4 and H-5 ($J_{B,C,D} < 1$ Hz), indicating an equatorial (H-4)–axial (H-5) orientation. The coupling constant between H-4 and H-5 in sugar E ($J = 6.1$ Hz) indicates an axial–axial configuration. The coupling constant ($J_{4,5}$) of A and F could not be defined, due to the overlying signals of H-5 and a multiplet (ddd) of H-4. However, such a multiplet in the 4-position should only be observed in the case of an axial–axial orientation of H-4, H-5 and H-3, H-4, which indicated the presence of amicetose units. In contrast, the H-4 of sugar residues B, C, and D showed broad singlets in $CDCl_3$ without any splitting, which indicated the presence of rhodinose moieties. COSY, HMBC, and ROESY experiments provided further strong evidence that the saccharide units A, E, and F are identical with β-amicetose, whereas B, C, and D consist of α-rhodinose (Figure 4).

A full assignment of the absolute configuration was possible following sequential hydrolysis and comparison with reference compounds. The total hydrolysis of **3** gave a colorless syrup of a mixture of monosaccharides, which was subsequently derivatized with 2,4-dinitrophenylhydrazine. After prepurification of the reaction mixture (see Experimental Section), a single hydrazone fraction was obtained, which could be separated by semipreparative RP-HPLC, yielding two fractions with m/z 312. The optical rotation of the derivatives revealed the identities of the 2,4-dinitrophenylhydrazones to be D-amicetose ($[\alpha]_D^{22} = -10.7°$, $c = 0.15$ in pyridine)[19] and L-rhodinose ($[\alpha]_D^{22} = -21.4°$, $c = 0.14$ in pyridine),[20] respectively, in good agreement with published data. By considering these results together, the absolute configuration of the monosaccharide units B, C, and D was established as α-L-rhodinose (α-2,3,6-trideoxy-*threo*-hexopyranose), and that of units A, E, and F as β-D-amicetose (β-2,3,6-trideoxy-*erythro*-hexopyranose). HMBC long-range couplings unequivocally revealed a 1,4-O-glycosidic linkage between all monosaccharide moieties, and the connection of the H-4 to the C-1A carbon of the aglycone and from the H-1E to the C-12a carbon (Figures 4 and 5). The relative configuration of the protons H-4 and H-4a was determined

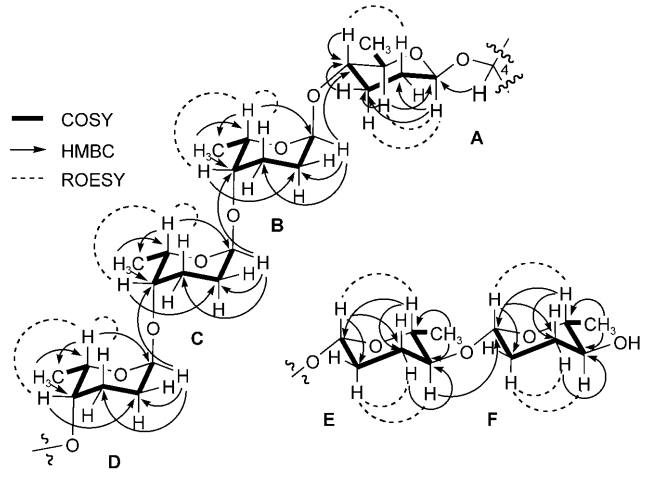

Figure 4. The H,H COSY, HMBC, and ROESY correlations of the amicetose and rhodinose side-chains of cervimycin C (**3**).

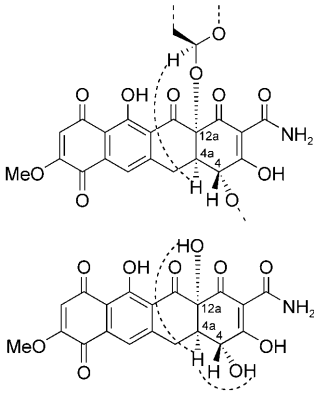

Figure 5. ROESY correlations of cervimycin C (**3**) between H-1E and H-4a, and of the aglycone between OH-12a, OH-4, H-4, and H-4a.

as *trans*-diaxial, by means of their vicinal coupling constant of $^{3}J_{4,4a} = 11.8$ Hz, which is in accordance with related structures, that is, tetracyclines.[21,22] The absence of any ROESY contacts between H-4 and H-4a in cervimycin C confirmed this observation. Furthermore, the ROESY correlation of H-4a and H-1E of the substituent at C-12a in **3**, and the ROESY couplings between OH-4, H-4a, and OH-12a in the aglycone of **3**, correspond to a *syn*-position of these moieties (Figure 5).

Surprisingly, despite its mass being identical to that of the trideoxysugars, the sixth substituent at the C-4-saccharide terminus does not represent a saccharide residue. The presence of two downfield-shifted carbonyl carbon atoms C-1′ and C-3′ in the ^{13}C NMR spectrum, and a ^{1}H–^{13}C long-range correlation from H-4D to an ester carbonyl carbon, indicated the connection between the tetrasaccharide side-chain and an acyl residue. In addition, HMBC connections of two diastereotopic methyl carbon atoms, CH_3-5′ and CH_3-4′, to the quaternary C-2′ established the structure of an unprecedented dimethylmalonyl substituent (Figure 6).

The physicochemical and spectroscopic properties of compound **1** ($m/z = 1226$) were strikingly similar to those of **3**, except for a mass difference of one unit. The molecular formula $C_{62}H_{82}O_{25}$ deduced from HRMS and ESI-MS^n experiments, which revealed odd-numbered daughter ions with $m/z = 1021$ $[M+\mathrm{Na}-2\times114]^+$, 679 $[M+\mathrm{Na}-5\times114]^+$, 593 $[5\times114+\mathrm{Na}]^+$, and 451 $[M+\mathrm{Na}-7\times114]^+$, indicated the loss of nitrogen in the aglycone. Comparison of the NMR spectroscopic data of **1** and **3** (Table 2) revealed an additional acetyl methyl carbon in **1** ($\delta = 29.03$ ppm, C-15), in lieu of the carbamoyl group in **3**. In addition, the NMR data of the aglycone of **1** were very similar to the published data of polyketomycin (**5**)[23,24] and dutomycin (**6**, Figure 6).[25]

Compounds **2** ($m/z = 1212$, $C_{61}H_{80}O_{25}$) and **4** ($m/z = 1213$, $C_{60}H_{79}NO_{25}$) differ from **1** and **3**, respectively, by the presence of a monomethylmalonyl unit in place of the dimethylmalonyl unit (Figure 6). ^{1}H and ^{13}C NMR spectra confirmed the loss of one sp^3 methyl carbon and an additional sp^3 methine group. This was supported by two-dimensional-NMR correlations, which showed the connectivity between CH_3-4′ and CH-2′, and their protons to carbonyl carbon atoms C-1′ and C-3′.

In summary, cervimycin A–D represent unusual bisglycosylated polyketides that are ring-substituted with either a carbamoyl or an acetyl moiety, and bear either a dimethylmalonyl or a monomethylmalonyl residue attached to the longer sugar side-chain (Figure 6). Although the structures of cervimycins A and B are novel, database searches suggest that the cervimycins C and D may have the same constitution as compounds A2121–3 and A2121–2, which have been reported in the Japanese patent literature only.[26] Unfortunately, the data set submitted with the patent is not complete, and one-dimensional-NMR data ([D$_6$]DMSO) were not assigned with the structures. Furthermore, no studies revealing the stereochemistry of the compounds, in particular the characterization of the saccharide units, have been reported, and, as the submitted data set of ^{13}C-shifts is incomplete, it is not yet possible to establish unequivocally the nature of the aglycone. Thus, we were not able to prove if cervimycin C and D are in fact identical with A2121–3 and A2121–2. Furthermore, it is remarkable that compounds A2121–3 and A2121–2 have been reported only in the context of proline hydroxylase inhibition and hepatoprotection, and no antibacterial activity has been disclosed.

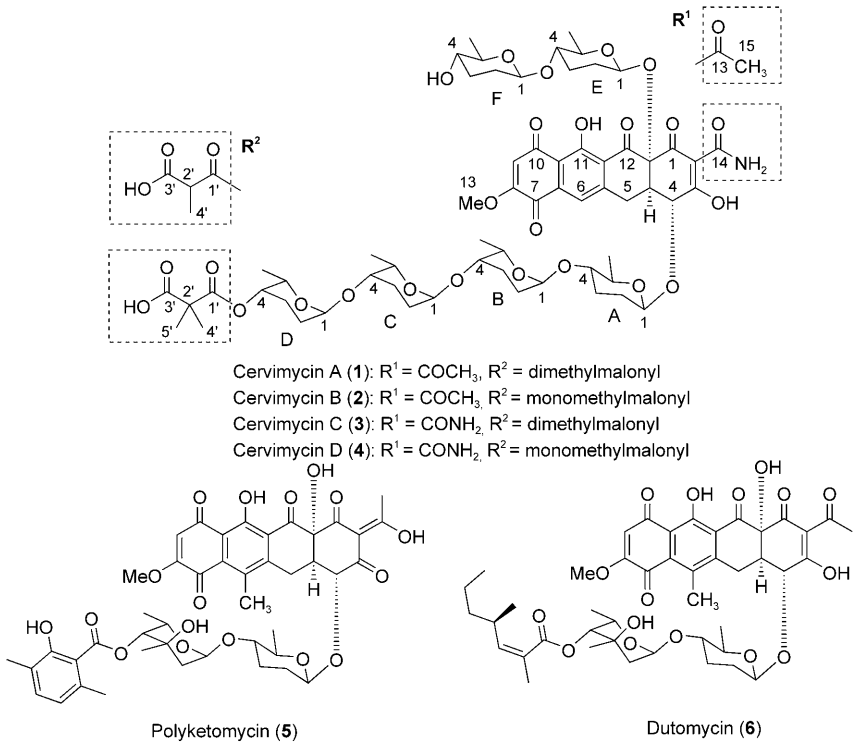

Figure 6. Structures of cervimycins A–D (**1**–**4**), polyketomycin (**5**), and dutomycin (**6**). (Relative configuration; for simplicity, numbering is not in accordance with IUPAC.)

The cervimycins, as well as the compounds of the A2121 complex, represent members of a small and yet relatively unexplored class of chromoglycosides that share a quinoid system on ring D of the naphthacene core, which is inverted relative to classical tetracyclines. The only known structural relatives are the antibacterial polyketomycin[23,24] and the antitumoral dutomycin,[25] which are also produced by *Streptomyces* species. To date, the cervimycins are the only known

Table 2. ^{13}C NMR data of the cervimycins **1**–**4**.

	δ [ppm] for B, C, D			
Position	**1**	**2**	**3**	**4**
1	193.6	193.3	194.9	195.0
2	113.8	113.3	101.0	101.1
3	190.8	190.5	192.0	192.1
4	74.2	73.8	74.1	74.2
4a	42.8	42.4	43.5	43.5
5	28.6	28.2	28.6	28.6
5a	153.2	152.8	153.3	153.3
6	120.2	119.8	120.3	120.3
6a	134.0	133.6	134.0	134.1
7	179.0	178.6	179.1	179.1
8	161.2	160.8	161.3	161.3
9	110.4	110.0	110.5	110.5
10	190.6	190.2	190.7	190.7
10a	113.0	112.6	113.0	113.0
11	163.3	162.9	163.3	163.4
11a	125.3	124.9	125.7	125.7
12	189.4	188.9	189.2	189.2
12a	86.3	85.9	86.2	86.2
13	56.8	56.3	56.8	56.8
14	202.8	202.4	174.7	174.8
15	29.0	28.5	–	–
1A	102.6	102.1	102.7	102.7
2A	30.8	30.5	30.9	31.0
3A	29.8	29.5	29.9	29.9
4A	79.0	78.6	79.1	79.1
5A	74.9	74.5	74.9	75.0
6A	18.3	17.9	18.4	18.4
1B	99.3	98.9	99.4	99.4
2B	25.2	24.8	25.3	25.3
3B	24.9	24.5	25.0	25.0
4B	74.9	74.5	74.9	75.0
5B	67.0	66.6	67.1	67.1
6B	17.3	17.0	17.5	17.5
1C	99.6	99.2	99.6	99.7
2C	25.1	24.7	25.2	25.2
3C	24.7	24.4	24.9	24.9
4C	75.0	74.7	75.1	75.2
5C	67.2	66.8	67.3	67.3
6C	17.3	17.0	17.5	17.5
1D	99.2	98.9	99.3	99.4
2D	24.5	24.2	24.7	24.7
3D	23.1	22.8	23.2	23.4
4D	70.5	70.2/70.4[a]	70.5	70.7/70.9[a]
5D	66.0	65.5/65.6[a]	66.1	66.0/66.1[a]
6D	17.2	16.9/16.9[a]	17.3	17.3/17.4[a]
1E	99.9	99.5	100.1	100.1
2E	31.6	31.3	31.8	31.8
3E	30.8	30.5	30.9	31.0
4E	79.6	79.2	79.7	79.7
5E	75.4	75.0	75.5	75.5
6E	18.5	18.2	18.7	18.7
1F	103.3	103.0	103.5	103.5
2F	32.0	31.7	32.2	32.2
3F	31.6	31.3	31.8	31.8
4F	71.1	70.7	71.2	71.2
5F	76.7	76.3	76.8	76.8
6F	18.8	18.4	18.9	18.9
1′	173.4	170.6/170.7[a]	173.5	171.1/171.2[a]
2′	50.6	46.7/46.9[a]	50.7	47.2/47.4[a]
3′	175.5	172.4/172.5[a]	175.5	172.9/173.0[a]
4′	23.3	13.9/13.9[a]	23.4	14.4/14.4[a]
5′	23.3	–	23.4	–

[a] Signal doubling possibly due to diastereomeric forms of monomethylmalonyl-substituted cervimycins.

bis-glycosylated polyketides of this kind. Various secondary metabolites[27] bear the 2,3,6-trideoxypyranose rhodinose,[28,29] for example, urdamycin,[30] and amicetose;[31,32] however, no examples of the cumulation of the trideoxysugars in the disaccharide and tetrasaccharide side-chains have yet been reported.

Besides the oligo-trideoxyhexose residues, one of the most remarkable structural features of the cervimycins is the presence of the terminal mono- and dimethylmalonyl substituents. Although malonate esters are components of a variety of natural products, such as in the macrolides malonylniphimycin[33] and brasilinolide A,[34] the occurrence of C-2 monoalkylated malonic acid derivatives, for example, monobutylmalonyl, is much rarer.[35] A methylmalonic acid monoester comparable to cervimycins B and D has been described only for angucycline BA-12100.[36] The dimethylmalonic acid substituent is unique to cervimycins A and C, as well as to compounds A2121–3 and A2121–6. Labeling studies in our laboratory revealed that this novel dimethylmalonyl unit is derived from valine, and not from a malonate precursor, as one might assume.[37]

The results of antimicrobial testing showed that all cervimycins A–D proved to be highly active against various Gram-positive bacteria, such as *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* SG511, and multi-drug-resistant *S. aureus* 134/93 (MRSA), and displayed potencies comparable to those of tetracycline, vancomycin, and ciprofloxacin (Table 1). More importantly, they are also highly active against efflux-resistant *S. aureus* EfS4 and vancomycin-resistant *Enterococcus faecalis* 1528 (VRE) within a MIC range of 1.6–12.5 $\mu g\,mL^{-1}$, in contrast to tetracycline, vancomycin, or ciprofloxacin, which are ineffective against VRE and EfS4, respectively.

Conclusion

We have isolated a novel *Streptomyces tendae* strain from an ancient cave, *Grotta dei Cervi*, and have purified a remarkably active antibiotic complex, cervimycins A–D, by a scaled-up fermentation process. The structures of the cervimycins were fully resolved by conducting extensive one- and two-dimensional-NMR studies, as well as by chemical degradation studies. Cervimycins A–D have a common tetracyclic polyketide core that is substituted with unusual di- and tetrasaccharide chains composed exclusively of trideoxysugars; however, they differ in the acetyl and carbamoyl ring substituent and in the highly unusual terminal methylmalonyl and dimethylmalonyl residues. The most important result is that the cervimycins are highly active against multi-drug-resistant bacterial pathogens, and also defeat vancomycin resistance. Further studies are in progress to improve the bioactivity profile of the cervimycins and to investigate the as yet unknown biological target of these novel antibiotics.

Experimental Section

General: NMR spectra were measured by using Bruker Avance DRX 500 and DPX 300 instruments. ESI-MS was performed by using a VG Quattro Fisons Instrument, and HRESI-MS was conducted by using a Finnigan MAT 95XL sector field mass spectrometer equipped with a compatible ion source. ESI-CID-MSn was performed by using a Finnigan LCQ benchtop mass spectrometer equipped with an electrospray ion source and ion-trap mass analyzer. IR spectra were measured by using an FTIR spectrometer Satellite FTIR Mattson (Chicago, USA). Melting points were determined by using a Polytherm A (Wagner & Munz, Munich, Germany). Optical rotation was measured in MeOH or pyridine by using a 0.5-dm cuvette in a Propol Polarimeter (Dr. Kernchen, Seelze, Germany). UV spectra were measured by using a Specord 200 photometer (Analytik Jena AG, Germany).

Strain isolation and taxonomic characterization: Strain HKI 0179 was isolated from a rock wall covered with 5000-year-old (neolithic) red paintings, inside the cave *Grotta dei Cervi* in southern Italy, near Porto Badis-

Table 3. ^{1}H NMR data of the cervimycins **1**–**4** ([D_5]pyridine); (s) singlet, (brs) broad singlet, (d) doublet, (wd) wide doublet, (t) triplet, (m) multiplet.

	δ [ppm] (J in Hz)			
Position	**1**	**2**	**3**	**4**
3-OH	18.03 (brs)[c]	n.d.	17.98 (brs)[c]	18.03 (brs)[c]
4	4.70 (d, 12.0)	4.72 (d, 11.9)	4.68 (m)[b]	4.67 (m)[b]
4a	3.54 (m)[b]	3.55 (m)[b]	4.31 (d, 11.8)[b]	3.53 (m)[b]
5	3.90 (wd, 18.7)	3.93 (m)[b]	3.93 (wd, 18.3)	3.94 (wd, 18.3)
	4.33 (dd, 18.7; 5.3)	4.35 (dd, 18.7; 5.0)	4.33 (dd, 18.3; 5.3)	4.33 (dd, 18.3; 5.1)
6	7.68 (s)	7.72 (s)	7.70 (s)	7.71 (s)
9	6.36 (s)	6.36 (s)	6.34 (s)	6.36 (s)
11-OH	13.39 (s)[c]	13.45 (s)[d]	13.35 (s)[c]	n.d.
13	3.76 (s)	3.74 (s)	3.74 (s)	3.74 (s)
14-NH_2	–	–	10.23 (s); 10.48 (s)	10.44 (s); 10.50 (s)
15	2.71 (s)	2.71 (s)	–	–
1A	5.03 (m)[b] [4.64 (d, 9.8)][c]	5.01 (m)[b]	5.01 (m)[b] [4.62 (d, 9.1)][c]	5.03 (d, 9.8)
2A	1.75 (m)[b]/2.25 (m)[b]	1.73 (m)[b]/2.24 (m)[b]	1.69 (m)[b]/2.21 (m)[b]	1.75 (m)[b]/2.25 (m)[b]
3A	1.60 (m)[b]/2.22 (m)[b]	1.58 (m)/2.22 (m)[b]	1.54 (m)[b]/2.20 (m)[b]	1.58 (m)[b]/2.22 (m)[b]
4A	3.27 (m)[b]	3.27 (m)[b]	3.24 (m)[b]	3.27 (m)[b]
5A	3.27 (m)[b]	3.27 (m)[b]	3.24 (m)[b]	3.27 (m)[b]
6A	0.97 (d, 5.0)	1.00 (d, 4.9)	0.96 (d, 5.4)	1.00 (d, 5.3)
1B	4.96 (brs)	4.99 (brs)	4.95 (s)	4.98 (brs)
2B	1.52 (m)[b]/2.12 (m)[b]	1.53 (m)[b]/2.15 (m)[b]	1.52 (m)[b]/2.10 (m)[b]	1.51 (m)[b]/2.15 (m)[b]
3B	1.85 (m)[b]/1.96 (m)[b]	1.89 (m)[b]/1.97 (m)[b]	1.86 (m)[b]/2.19 (m)[b]	1.89 (m)[b]/1.99 (m)[b]
4B	3.52 (m)[b]	3.51 (m)[b]	3.47 (m)[b]	3.52 (m)[b]
5B	3.97 (q, 6.5)	4.00 (q, 6.4)	3.99 (q, 6.6)	3.99 (q, 6.6)
6B	1.21 (d, 6.6)	1.21 (d, 6.4)	1.21 (d, 6.6)	1.21 (d, 6.4)
1C	5.00 (brs)	5.05 (brs)	4.98 (s)	5.00 (brs)
2C	1.62 (m)[b]/2.03 (m)[b]	1.65 (m)[b]/2.02 (m)[b]	1.69 (m)[b]/1.95 (m)[b]	1.72 (m)[b]/1.99 (m)[b]
3C	1.82 (m)[b]/2.21 (m)[b]	1.85 (m)[b]/2.23 (m)[b]	1.86 (m)[b]/2.09 (m)[b]	1.84 (m)[b]/2.22 (m)[b]
4C	3.55 (m)[b]	3.51 (m)[b]	3.46 (m)[b]	3.52 (m)[b]
5C	4.05 (q, 6.6)	4.07 (q, 6.4)	4.04 (q, 6.5)	4.06 (q, 6.4)
6C	1.16 (d, 6.6)	1.18 (d, 6.4)	1.16 (d, 6.5)	1.17 (d, 6.2)
1D	4.89 (brs)	4.95 (brs)	4.89 (s)	4.95 (brs)
2D	1.60 (m)[b]/2.02 (m)[b]	1.63 (m)[b]/2.03 (m)[b]	1.59 (m)[b]/2.01 (m)[b]	1.62 (m)[b]/2.03 (m)[b]
3D	1.93 (m)[b]/2.12 (m)[b]	1.93 (m)[b]/2.14 (m)[b]	1.90 (m)[b]/2.09 (m)[b]	1.93 (m)[b]/2.14 (m)[b]
4D	5.02 (m)[b]	5.08 (m)[b]	5.00 (m)[b]	5.07 (m)[b]
5D	4.21 (q, 6.6)	4.24 (q, 6.4)/4.24 (q, 6.4)[a]	4.21 (q, 6.6)	4.23 (q, 6.4)/4.23 (q, 6.4)[a]
6D	1.25 (d, 6.6)	1.26 (d, 6.4)/1.32 (d, 6.4)[a]	1.25 (d, 6.5)	1.25 (d, 6.5)/1.31 (d, 6.6)[a]
1E	5.17 (m) [4.60 (d, 9.4)][c]	5.17 (d, 8.6)	5.15 (d, 8.6)	5.17 (m) (8.7)
2E	1.80 (m)[b]/2.55 (m)[b]	1.83 (m)[b]/2.56 (m)[b]	1.83 (m)[b]/2.49 (m)[b]	1.86 (m)[b]/2.54 (m)[b]
3E	1.69 (m)[b]/2.45 (m)[b]	1.81 (m)[b]/2.48 (m)[b]	1.79 (m)[b]/2.45 (m)[b]	1.84 (m)[b]/2.50 (m)[b]
4E	3.36 (m)	3.36 (m)	3.33 (m)	3.38 (m)
5E	3.62 (qd, 9.1; 6.1)	3.62[b] (m)	3.68 (qd, 9.1; 5.9)	3.69 (qd, 9.0; 6.1)
6E	1.38 (d, 6.2)	1.39 (d, 6.0)	1.41 (d, 6.0)	1.41 (d, 6.1)
1F	4.63 (dd, 8.9; 1.8)	4.64 (dd, 8.7; 1.8)	4.65[b] (m) [4.42 (dd, 9.4; 1.8)][c]	4.65 (m)[b]
2F	1.64 (m)[b]/2.15 (m)[b]	1.68 (m)[b]/2.14 (m)[b]	1.65 (m)[b]/2.10 (m)[b]	1.69 (m)[b]/2.16 (m)[b]
3F	1.85 (m)[b]/2.12 (m)[b]	1.86 (m)[b]/2.13 (m)[b]	1.85 (m)[b]/2.10 (m)[b]	1.89 (m)[b]/2.13 (m)[b]
4F	3.44 (m)	3.44 (m)	3.42 (m)	3.43 (m)
5F	3.52 (m)[b]	3.51 (m)[b]	3.52 (m)[b]	3.52 (m)[b]
6F	1.46 (d, 5.9)	1.47 (d, 5.8)	1.47 (d, 5.9)	1.48 (d, 6.0)
2′	–	3.91 (q, 7.2), 3.92 (q, 7.2)[a]	–	3.90 (q, 7.3), 3.89 (q, 7.3)[a]
3′-COOH	12.60 (brs)[d]	n.d.	12.71 (brs)[d]	n.d.
4′	1.72 (s)	1.67 (d, 7.3)/1.66 (d, 7.2)[a]	1.74 (s)	1.67 (d, 7.3), 1.65 (d, 7.2)[a]
5′	1.71 (s)	–	1.72 (s)	–

[a] Signal doubling possibly due to diastereomeric forms of monomethylmalonyl-substituted cervimycins. [b] Partial overlapping of signals. [c] Signal recorded in $CDCl_3$. [d] Signal recorded in [D_6]DMSO.

co. Isolation was achieved by touching the wall between the paintings with sterile cotton swabs. The adherent bacteria were suspended in sterile 0.15 M sodium phosphate puffer (pH 7) prior to dilution plating on different growth media. Strain HKI 0179 was isolated from peptone yeast extract brain-heart-infusion medium (PY-BHI), following incubation at 28 °C. Laboratory cultivation was conducted by using oatmeal agar plates (ISP medium 3) or agar slant tubes,[15] or in liquid organic medium 79.[38] Stock cultures of strain HKI 0179 in liquid organic medium 79 supplemented with 5% DMSO were maintained in the vapour phase of liquid nitrogen.

Fermentation of *S. tendae* HKI 0179, and purification and characterization of the cervimycins: The cervimycin-producing strain *S. tendae* HKI 179 was grown on oatmeal agar (ISP3 medium containing 1% rolled oates, 1% oatmeal, 1% agar) slants. After 14 d incubation at 28 °C, the spores were harvested. Seed medium (60 mL) consisting of 1.5% glucose, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.5% NaCl, 0.1% $CaCO_3$, and 1 mL L^{-1} trace element solution (R2) in a 500 mL Erlenmeyer flask was inoculated with a spore suspension to yield 2×10^7 mL^{-1}. The seed culture was incubated at 28 °C for 48 h on a rotary shaker at 200 rpm.

Inoculum cultures in 2 L Erlenmeyer flasks with 400 mL liquid medium (2% glucose, 0.05% yeast extract, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.5% NaCl, 0.1% $CaCO_3$, and 1 mL L^{-1} trace element solution) were inoculated with 5% (*v*/*v*) of the seed culture, and incubated at 28 °C for 26–28 h on a rotary shaker at 160 rpm. The large-scale fermentation was performed in a 300 L stirring vessel fermenter (B. Braun Biotech International, Melsungen, Germany). 180 L fermentation medium (2.5% glucose, 0.75% cornsteep, 0.025% KH_2PO_4, 0.5% NaCl) was inoculated with 3 L inoculum cultures and cultivated at 28 °C, 200 mbar pressure, aeration of 50 L min^{-1}, and a stirring rate of 250 rpm. After 48 h, the culture was harvested. Cells and broth were separated by using a separator. The filtrate was adjusted to pH 5.0 and exhaustively extracted with ethyl acetate. The concentrated extract (10 L) was washed with water, dried with Na_2SO_4, and concentrated to dryness under reduced pressure. The residue (30 g) was treated with a small amount of $CHCl_3$, and lipophilic compounds were separated by means of petrol ether precipitation. Prepurification was achieved by performing gel permeation chromatography with a Sephadex LH20 column (50×5.5 cm) and methanol as eluent. The bioactive fraction was subjected to a second purification step by using a Sephadex column (130×6 cm) to yield several active fractions. Final purification by performing preparative HPLC (Shimadzu) using a Prontosil 120–15-C_{18}AQ column (15 μm, 250×20 mm) and a 0.01 M ammonium acetate/acetonitrile gradient (20–99.5% acetonitrile in 25 min) as eluent at a flow rate of 25 mL min^{-1} (UV detection at 247 nm) afforded **1** (375 mg), **2** (33 mg), **3** (1600 mg), and **4** (927 mg).

Cervimycin A (1): Yield: 375 mg. Orange powder; m.p. 156–158 °C; $[\alpha]_D^{22}=+35.2$ ($c=0.96$ in MeOH); IR (ATR-method, solid film): $\tilde{\nu}=1593$, 1626 (aromatic C=C), 1730 (C=O), 2853, 2923, 2954 (aliphatic methins), 3568 cm^{-1} (OH); UV/Vis (MeOH): λ_{max} (ε)=240 (33925), 277 (25830), 434 nm (5683 $mol^{-1}m^3cm^{-1}$); UV/Vis (MeOH+NaOH): λ_{max} (ε)=204 (88296), 251 (27504), 277 (25943), 566 nm (7024 $mol^{-1}m^3cm^{-1}$); MS (ESI^-): *m*/*z* (%): 1225 (100) $[M-H]^-$; MS^n (ESI^+): *m*/*z*: 1021 $[M+Na-2\times114]^+$, 679 $[M+Na-5\times114]^+$, 593 $[5\times114+Na]^+$, 451 $[M+Na-7\times114]^+$; HRMS: *m*/*z*: calcd for $C_{62}H_{82}NaO_{25}$ $[M+Na]^+$: 1249.5043; found: 1249.5046.

Cervimycin B (2): Yield: 33 mg. Orange powder; m.p. 132–134 °C; $[\alpha]_D^{22}=+26.00$ ($c=0.55$ in MeOH); IR (ATR-method, solid film): $\tilde{\nu}=1593$, 1626 (aromatic C=C), 1730 (C=O), 2871, 2929 cm^{-1} (aliphatic methins); UV/Vis (MeOH): λ_{max} (ε)=239 (50121), 280 (36559), 434 nm (5547 $mol^{-1}m^3cm^{-1}$); MS (ESI^-): *m*/*z* (%): 1211 (100) $[M-H]^-$; MS^n (ESI^+): *m*/*z*: 1007 $[M+Na-2\times114]^+$, 679 $[M+Na-4\times114-100]^+$, 579 $[4\times114+100+Na]^+$, 451 $[M+Na-6\times114-100]^+$; HRMS: *m*/*z*: calcd for $C_{61}H_{80}NaO_{25}$ $[M+Na]^+$: 1235.4887; found: 1235.4909.

Cervimycin C (3): Yield: 1600 mg. Orange powder; m.p. 167–168 °C; $[\alpha]_D^{22}=+26.5$ ($c=0.51$ in MeOH); IR (ATR-method, solid film): $\tilde{\nu}=1593$, 1626 (aromatic C=C), 1727 (C=O), 2854, 2922, 2953 (aliphatic methins), 3351 cm^{-1} (OH); UV/Vis (MeOH): λ_{max} (ε)=242 (45551), 434 nm (6853 $mol^{-1}m^3cm^{-1}$); UV/Vis (MeOH+NaOH): λ_{max} (ε)=203 (88158), 249 (36490), 273 (29205), 563 nm (8783 $mol^{-1}m^3cm^{-1}$); MS (ESI^-): *m*/*z* (%): 1226 (100) $[M-H]^-$; MS^n (ESI^+): *m*/*z*: 1022 $[M+Na-2\times114]^+$, 680 $[M+Na-5\times114]^+$, 593 $[5\times114+Na]^+$, 452 $[M+Na-7\times114]^+$; HRMS: *m*/*z*: calcd for $C_{61}H_{81}NaNO_{25}$ $[M+Na]^+$: 1250.4995; found: 1250.5002.

Cervimycin D (4): Yield: 927 mg. Orange powder; m.p. 158–160 °C; $[\alpha]_D^{22}=+39.9$ ($c=0.60$ in MeOH); IR (ATR-method, solid film): $\tilde{\nu}=1593$, 1625 (aromatic C=C), 1729 (C=O), 2872, 2932 cm^{-1} (aliphatic methins); UV/Vis (MeOH): λ_{max} (ε)=242 (28451), 434 nm (4109 $mol^{-1}m^3cm^{-1}$); MS (ESI^-): *m*/*z* (%): 1212 (100) $[M-H]^-$; MS^n (ESI^+): *m*/*z*: 1008 $[M+Na-2\times114]^+$, 680 $[M+Na-4\times114-100]^+$, 579 $[4\times114+100+Na]^+$, 452 $[M+Na-6\times114-100]^+$; HRMS: *m*/*z*: calcd for $C_{60}H_{79}NaNO_{25}$ $[M+Na]^+$: 1236.4839; found: 1236.4857.

For NMR data, see Tables 2 and 3.

Total hydrolysis of 3 and hydrazone precipitation: Compound **3** (0.04 mmol) was added to 2 N HCl (10 mL) and the mixture was stirred at 90 °C for 2 d under an argon atmosphere. The reaction was monitored by performing TLC ($CHCl_3$/MeOH, 9:1, stained with anisaldehyde/H_2SO_4). After filtration and neutralization with concentrated NaOH, the solution was evaporated to dryness. The residue was extracted with $CHCl_3$/MeOH (9:1, 100 mL) with stirring for 1 d. The mixture was filtrated and the insoluble residue was washed with $CHCl_3$/MeOH (9:1). The filtrate was concentrated under reduced pressure and used for the hydrazone precipitation.

The solution of the hydrolysate in EtOH (1 mL, 95%) was added to a mixture of 2,4-dinitrophenylhydrazine (0.4 mmol), concentrated H_2SO_4 (0.2 mL), and EtOH (75%, 2 mL), and kept overnight at room temperature. After addition of water (100 mL), the mixture was exhaustively extracted with EtOAc. The combined organic fractions were dried over Na_2SO_4 and evaporated to dryness. The residue (90 mg) was purified on silica with a gradient ($CHCl_3$/MeOH, 15:1 to $CHCl_3$/MeOH, 10:1). A fraction containing substances with *m*/*z*=312 was subjected to preparative TLC ($CHCl_3$/MeOH, 15:1). The mixture (4.9 mg) of the two isomers obtained was separated by using semipreparative HPLC (Nucleosil 100 C18, 5 μm; 125×4.6 mm eluted with ACN/0.1% TFA gradient ranging from (ACN): 1–10 min 0.5%, to 70 min 99.5%, 70–80 min 99.5%, to 90 min 0.5%, at a flow rate of 5 mL min^{-1}), yielding the 2,4-dinitrophenylhydrazones of D-amicetose (1.5 mg) $[\alpha]_D^{22}=-10.7°$ ($c=0.15$ in pyri-

Table 4. ^1H and ^{13}C NMR data of cervimycin F (aglycone of cervimycin C) in [D_6]DMSO.

Position	δ_C [ppm]	δ_H [ppm] (*J* in Hz)
1	195.5	–
2	97.9	–
3	193.9	–
3-OH	–	18. 16 (br s)
4	68.1	3.85 (m)
4-OH	–	5.89 (br s)
4a	44.9	2.63 (ddd, 11.9, 5.0, 1.6)
5	27.0	3.34 (wdd, 18.6, 1.6)
		3.40 (dd, 18.6, 5.0)
5a	150.4	–
6	118.6	7.46 (s)
6a	133.7	–
7	178.5	–
8	160.9	–
9	110.0	6.36 (s)
10	190.2	–
10a	112.8	–
11	161.7	–
11-OH	–	13.35 (s)
11a	124.2	–
12	192.5	–
12a	78.0	–
12a-OH	–	6.85 (br s)
13	57.0	3.89 (s)
14	173.1	–

dine) (literature data: $[\alpha]_D^{25} = -10.0°$ ($c = 0.9$ in pyridine))[19] and L-rhodinose (1.4 mg) $[\alpha]_D^{22} = -21.4°$ ($c = 0.14$ in pyridine) (literature data: $[\alpha]_D^{22} = -17.7°$ ($c = 0.6$ in pyridine)).[20]

Hydrolysis of 3 and isolation of the aglycone: Compound **3** (0.16 mmol) was added to 0.1 N methanolic HCl (50 mL) and stirred overnight at room temperature. The precipitate was filtrated and washed twice with MeOH. The residue afforded 48.3 mg of pure aglycone of **3** (cervimycin F) as an orange solid. M.p. 205–208 °C; $[\alpha]_D^{22} = +86.8°$ ($c = 0.13$ in pyridine); IR (ATR-method, solid film): $\tilde{\nu} = 3370, 3260, 3235, 1669, 1618, 1587\ cm^{-1}$; MS ($ESI^-$): m/z: 428 $[M-H]^-$; HRMS: m/z: calcd for $C_{20}H_{14}O_{10}$ $[M-NH_2+H]^+$: 414.0571; found: 414.0579.

For NMR data, see Table 4.

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