Low-Abundance Kutznerides from *Kutzneria* sp. 744

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Five new (**5**−**9**) and four known (**1**−**4**) kutznerides were isolated from the actinomycete *Kutzneria* sp. 744. Compounds **1**−**9** all consisted of a cyclohexadepsipeptide core with the general structure 2-(1-methylcyclopropyl)-D-glycine−(2*S*,3a*R*,8a*S*)-6,7-dichloro-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid−3-hydroxy-D-glutamic acid−*O*-methyl-L-serine−L-piperazic acid−(*S*)-2-hydroxy-3,3-dimethylbutyric acid. Compounds **5**, **6**, and **8** contained *erythro*-3-hydroxy-D-glutamic acid, whereas **7** and **9** contained the *threo* isomer and the hydroxy acid was present as (*S*)-2-hydroxy-3-methylbutyric acid in **5** and **7**. The piperazic acid was C-5−N unsaturated and 4-hydroxylated in **6**, 4-chlorinated in **8**, and C-5−N unsaturated in **9**. Minimal inhibitory concentrations for bacteria were found down to 6 μM (*Staphylococcus aureus* and *Erwinia carotovora*) and for fungi down to 70 μM (*Fusarium culmorum*). The trichlorinated **2** and **8** showed the highest antimicrobial activity, whereas **6**, with a hydroxylated piperazic acid unit, did not show any inhibition of the pathogens at 230 μM.

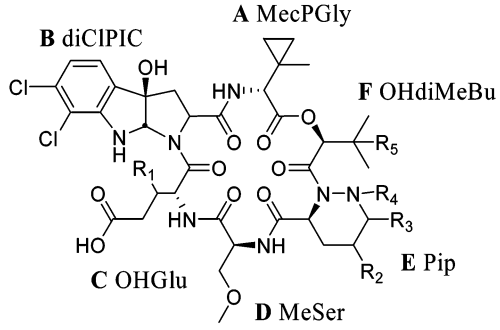
The actinomycete *Kutzneria* sp. 744 was previously found to inhibit growth of the root pathogens *Pythium undulatum*, *Ceratobasidium bicorne*, and *Fusarium avenaceum* in dual culture agar plates. Chemical investigation revealed that the isolate produced a spectrum of cyclic hexadepsipeptides, named kutznerides 1−4 (**1**−**4**), which were moderately active against several common root rotting fungi.[1] The compounds all contained several unusual amino acids: 2-(1-methylcyclopropyl)-D-glycine (MecPGly), 3-hydroxy-D-glutamic acid (OHGlu), and *O*-methyl-L-serine (MeSer), and the original amino acid (2*S*,3a*R*,8a*S*)-6,7-dichloro-3a-hydroxypyrrolidino[2,3-*b*]indole-2-carboxylic acid (diClPIC), together with (*S*)-2-hydroxy-3,3-dimethylbutanoic acid (OHdiMeBu). Kutznerides 1 and 3 also contained L-piperazic acid (Pip), whereas the unreported (*R*)-4-chloro-L-piperazic acid (ClPip) was found in kutzneride 2, and the C-5−N unsaturated L-piperazic acid analogue (Pip*) was found in kutzneride 4.

In this study the focus was set on isolation of additional low-yield kutznerides and a broadened investigation of their antimicrobial properties. Improved growth conditions made it possible to isolate five additional kutznerides, **5**−**9**, which differed in the structure of the piperazic acid and the hydroxy acid moiety together with the relative configuration of the 3-hydroxyglutamic acid.

Results and Discussion

The liquid culture of *Kutzneria* sp. 744 was fractionated with SPE and preparative gradient HPLC (Figure 1). All fractions with retention times similar to the previously isolated kutznerides were screened with NMR (^{1}H and ^{1}H−^{1}H COSY experiments) and direct injection ESIMS. This revealed the presence of several minor kutznerides as well as the previously isolated **1**−**4**. The pure compounds **1**−**9** were obtained after an additional chromatographic step. The kutznerides **5**−**9** all consisted of a basic hexadepsipeptide skeleton containing MecPGly, diClPIC, OHGlu, MeSer, Pip or a derivative thereof, and OHdiMeBu or its monomethylated analogue 2-hydroxy-3-methylbutanoic acid (OHMeBu).

Compound **5** displayed a molecular ion in ESIMS at *m*/*z* 838.1 [M − H]$^-$, which pointed to a molecular mass of 839. The isotope distribution pattern indicated the presence of two Cl atoms, which was confirmed by HRFABMS (*m*/*z* 840.2792 [M + H]$^+$), establishing the molecular formula as $C_{36}H_{47}O_{12}N_7Cl_2$. This suggested that **5** was one methyl group short in comparison with kutznerides **1** and **3**. The compound was subjected to NMR analysis in $CDCl_3$, which made it possible to identify the subunits and their primary sequence.



	R_1	R_2	R_3	R_4	R_5
Kutzneride 1	HO (dashed)	H	H	H	CH_3
Kutzneride 2	HO (dashed)	Cl	H	H	CH_3
Kutzneride 3	HO (wedge)	H	H	H	CH_3
Kutzneride 4	HO (wedge)	H	π-bond		CH_3
Kutzneride 5	HO (wedge)	H	H	H	H
Kutzneride 6	HO (wedge)	OH	π-bond		CH_3
Kutzneride 7	HO (dashed)	H	H	H	H
Kutzneride 8	HO (wedge)	Cl	H	H	CH_3
Kutzneride 9	HO (dashed)	H	π-bond		CH_3

Subunit A (MecPGly) displayed an isolated upfield CH_2−CH_2 spin system in COSY at δ_H 1.06/0.68 (H-4a/H-4b) and 0.66/0.40 (H-5a/H-5b), indicating a cyclopropyl ring attached to a quarternary carbon. A methyl singlet at δ_H 1.07 showed correlations in HMBC to C-5, C-4, the quarternary C-3 (δ_C 19.2), and C-2 (δ_C 60.6). In COSY, the H-2 doublet at δ_H 4.15 coupled to a signal at δ_H 7.81 (N*H*) and in HMBC to a carbonyl at δ_C 173.8. All NMR data of

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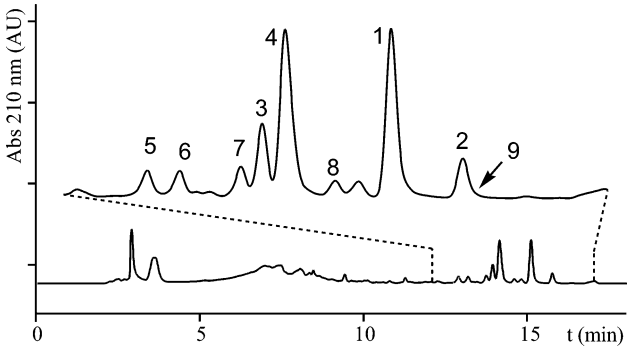

Figure 1. Chromatogram of preparative gradient HPLC run of the 95% CH_3CN SPE fraction from *Kutzneria* sp. 744 (10 to 100% CH_3CN in 10 min with a hold at 100% CH_3CN for 8 min). The numbers indicate elution of the individual kutznerides.

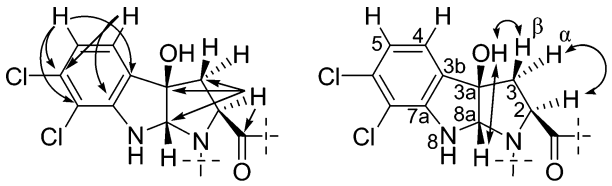

Figure 2. Left: HMBC correlations in the diClPIC subunit. Right: ROESY correlations for determination of the relative configuration in diClPIC.

subunit A were thus in favor of a MecPGly unit. In ROESY, the N*H* displayed internal coupling to H-2 and CH_3, but also to a resonance at δ_H 5.33 (H-2 of subunit B).

Subunit B (diClPIC) displayed three isolated spin systems in COSY: H-2 (δ_H 5.33) coupled to H-3α and H-3β (δ_H 2.16/2.79); H-4 (δ_H 7.14) and H-5 (δ_H 6.95) constituted an isolated spin system on the dichlorinated aromatic ring; and H-8a (δ_H 5.40) and N*H* (δ_H 6.37) correlated in COSY. In addition, an exchangeable singlet was present at δ_H 5.88, representing a hydroxy proton. The tricyclic system, including the five quarternary carbons, could be confirmed in HMBC with the help of correlations from H-2, H-4, and H-5 (Figure 2). The N*H* of subunit B correlated with H-2 of subunit C in ROESY.

Subunit C (OHGlu) displayed a single spin system where N*H* (δ_H 6.75) coupled to H-2 (δ_H 5.23), which in turn coupled to a methine at δ_H 4.74 (H-3) with a terminal correlation to a methylene at δ_H 2.70 (H-4). The shift of C-3 (δ_C 70.0) indicated that the carbon was hydroxylated. In HMBC, H-2 displayed coupling to C-1 (δ_C 171.0, amide carbonyl) and H-4 with C-5 (δ_C 174.3, free carboxylic acid). The data for subunit C were thus indicative of an OHGlu unit. In ROESY, the N*H* displayed coupling to H-2 (δ_H 4.56) of subunit D.

Subunit D (MeSer) also consisted of a single spin system comprised of N*H* (δ_H 7.62), H-2 (δ_H 4.56), and a terminal methylene at δ_H 3.87/3.31 (H-3a/H-3b). The H-3b resonance showed correlations in HMBC to a methoxy carbon at δ_C 60.5 (*C*H_3 with protons at δ_H 3.22) as well as to C-2 (δ_C 73.8) and a carbonyl at δ_C 172.0 (C-1). In ROESY, the N*H* displayed coupling to H-2 (δ_H 5.06) of subunit E.

Subunit E (Pip) consisted of a single spin system starting with H-2 at δ_H 5.06, then three methylenes at δ_H 2.35/1.69 (H-3a/H-3b), 2.12/1.58 (H-4a/H-4b), and 3.16/2.78 (H-5a/H-5b), with a terminal N*H* at δ_H 5.09. In HMBC, H-2 correlated with a carbonyl at δ_C 174.8. This was in agreement with the presence of a piperazic acid unit. In ROESY, the H-5b correlated with H-3 (δ_H 2.22) of subunit F.

Subunit F displayed one spin system with a methine at δ_H 5.72 (H-2) coupling to a second methine at δ_H 2.22 (H-3), which in turn correlated with two methyl groups at δ_H 1.14 (H-4a) and 1.10 (H-4b), respectively. H-2 showed a coupling to a carbonyl in HMBC at δ_C 172.9 (C-1 amide), and the C-2 shift at δ_C 78.2 suggested that the carbon was attached to an oxygen. The subunit F was then determined as a 2-hydroxy-3-methylbutanoic acid (OHMeBu). In ROESY, a coupling was present between H-2 of subunit F and CH_3 (δ_H 1.07) of subunit A.

The absolute configuration of the subunits A, C, D, E, and F of **5** was determined by a previously described enantiomeric resolution method. A part of **5** was subjected to acidic hydrolysis, catalytic hydrogenation, esterification with 2-(*S*)-BuOH, derivatization with pentafluoropropionic acid anhydride, and subsequently GC-MS analysis. During hydrolysis or esterification, diastereomers of all derivatives were formed at low levels (<10%). The peaks of the diastereomers were used as internal references, as the elution order of the D- and L-derivatives was known from the previous work.[1] For OHMeBu, which was not present in kutznerides **1**−**4**, standards were derivatized and analyzed in the same way.

Subunit A was identified as 2-(1-methylcyclopropyl)-D-glycine (detected as D-*tert*-leucine formed in the hydrogenation step),[2] subunit C as *erythro*-3-hydroxy-D-glutamic acid, subunit D as *O*-methyl-L-serine (detected as L-serine formed in the acidic hydrolysis), subunit E as L-piperazic acid (detected as L-ornithine formed in the hydrogenation step),[3] and subunit F as (*S*)-2-hydroxy-3-methylbutanoic acid. The absolute configuration for the subunits was in line with the previously investigated **1**−**4**.

Subunit B in kutznerides **1**−**4** was reported to have the (2*S*,3a*R*,8a*S*) configuration.[1] This means that H-8a and the hydroxy group at 3a are *cis*, and both of them *trans* to H-2. This was deduced by ROESY experiments in combination with molecular modeling. Strong ROE correlations were observed between H-8a and O*H*, O*H*, and H-3β, as well as between H-2 and H-3α. Substantially weaker ROEs were found between H-2 and H-3β and between O*H* and H-3α.[1] No ROESY correlation was found between H-2 and H-8a, which has been reported for the cyclic peptide kapakahine D, where O*H*-3a, H-8a, and H-2 were determined to be on the same side of the ring system.[4] Determination of the absolute configuration of the subunit in kutznerides **1**−**4** was done by comparison between experimental ROEs and proton distances between subunits A and B obtained by molecular modeling.[1] The presence of a correlation between the diClPIC N*H* and H-2 of subunit A indicated that the PIC subunit was present as the (2*S*,3a*R*,8a*S*) isomer. For compound **5**, all ROEs were in line with the data that have been reported for kutznerides **1**−**4** (Figure 2), and subunit B was identified as (2*S*,3a*R*,8a*S*)-6,7-dichloro-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid (Figure 2). Compound **5** was thus identical to kutzneride **3** with the exception of the incorporation of (*S*)-2-hydroxy-3-methylbutanoic acid (OHMeBu) instead of (*S*)-2-hydroxy-3,3-dimethylbutanoic acid.

The structure elucidation of **6**−**9** was done in an analogous manner, and only the differences compared with **1**−**5** will be highlighted, as the major part of the data is similar for all kutznerides. In **6**−**9**, the subunits A, B, and D were all found to be identical to those found in **5**, and for kutzneride **7**, also subunits E and F.

The molecular ion of compound **6** in ESIMS at *m*/*z* 866.1 $[M - H]^-$ gave a molecular mass of 867, and HRFABMS data at *m*/*z* 868.2681 $[M + H]^+$ provided a best-fit molecular formula of $C_{37}H_{47}O_{13}N_7Cl_2$. In NMR, chemical shifts and coupling patterns for subunits A−D were essentially identical with those of **5**. The spin system of subunit E contained only five hydrogens. H-2 (δ_H 5.27) coupled to a methylene at δ_H 2.60/1.63 (H-3a/3b), which was followed by two sequential methines at δ_H 4.68 (H-4) and 7.09 (H-5). The downfield H-5 resonance showed an HSQC correlation to the carbon at δ_C 149.3, and since no N*H* signal was found for subunit E, this indicated the presence of a double bond between C-5 and the neighboring nitrogen, as previously found in **4**. C-4 had a shift at δ_C 60.4, which suggested that the carbon was substituted with a hydroxy group.

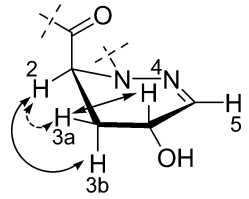

Figure 3. ROESY correlations for determination of the relative configuration in the piperazic acid subunit of **6**.

Analysis of **6** by ESIMS with CH_3OH-d_4 as solvent shifted the monoisotopic ion eight mass units, indicating eight exchangeable protons, which is in accord with a hydroxy group at C-4 in the Pip subunit. The subunit was thus determined to be 5,6-didehydro-4-hydroxypiperazic acid (OHPip*). Hydroxylated Pip units have been reported from polyoxypeptins,[5] while a C-5−N unsaturated 3-hydroxylated analogue is present in the luzopeptins.[6,7] The experimental NMR data for subunit E in **6** were in good agreement with the combined reported literature values. ROESY correlations were found to be useful as indicators of the relative configuration of the subunit. Strong ROEs were found between H-2 and H-3b and between H-4 and H-3a, while a weaker correlation was present between H-2 and H-3a and none between H-4 and H-3b. Apparently, H-2 and H-3b seemed to have a *cis* relation and were *trans* to both H-3a and H-4 (Figure 3). Presupposing that the OHPip* residue had the L-configuration (as found for Pip in **1**, **3**, **5**, and **7**), the subunit E was proposed to be present as (4*R*)-5,6-didehydro-4-hydroxy-L-piperazic acid.

For subunit F, a diagnostic singlet at δ_H 1.11 in ^{1}H NMR (9H, H-4a/4b/4c) indicated that the hydroxy acid moiety was present as OHdiMeBu. The subunit was determined to have an *S*-configuration. Kutzneride **6** was thus identical to **4** with the addition of a hydroxyl group at C-4 of the Pip* residue. The OHPip* subunit has not been described before.

Compound **7** had a molecular ion in ESIMS at 838.1 $[M - H]^-$, and HRFABMS of the proton adduct ion yielded a pseudomolecular mass of 840.2782. The molecular formula was deduced as $C_{36}H_{47}O_{12}N_7Cl_2$, i.e. identical to **5**. NMR data differed substantially only for subunit C, where H-2 resonated as a doublet (J = 10.8 Hz) instead of a triplet, and the N*H* signal had a chemical shift of δ_H 7.15, i.e., 0.4 ppm downfield compared to **5**. Determination of absolute configuration revealed that OHGlu in **7** was present as *threo*-D-OHGlu as opposed to the *erythro* isomer found in **5**.

The molecular formula of **8** was established as $C_{37}H_{48}O_{12}N_7Cl_3$ by HRFABMS, i.e., identical to kutzneride **2**. Differences in NMR between **8** and **2** were found for subunit C, in the same manner as between **5** and **7**. This suggested that **8** contained *erythro*-D-OHGlu, which was verified by absolute configuration determination. Compound **9**, with a molecular formula of $C_{37}H_{48}O_{12}N_7Cl_2$ derived from HRFABMS, was identical to kutzneride **4** with the exception of the presence of *threo*-D-OHGlu instead of *erythro*-D-OHGlu.

The primary sequence of **5**−**9** was deduced from ROESY experiments (Figure 4), except for **7**, where the expected correlations between subunit A and F were obscured by overlapping resonances. However, the molecular formula of **7** suggested by HRFABMS had an unsaturation index of 16, which could be achieved if the peptide backbone was present as a macrocycle. In addition, all NMR shifts of subunits A and F were analogous with the ones reported for the macrocyclic kutznerides with identical units.

Kutznerides **1**−**4** were previously tested against several root rotting fungi and were found to be moderately active.[1] In order to further characterize their biological properties, compounds **1**−**9** were tested against several other bacterial and fungal human and agricultural pathogens (Table 1). *Candida albicans*, *Pseudomonas sevastanoi* pv. *sevastanoi*, and *Aspergillus fumigatus* were not inhibited by **1**−**4** at concentrations up to 590 μM. The most potent antimicrobials were the trichlorinated **2** and **8**, with inhibition of

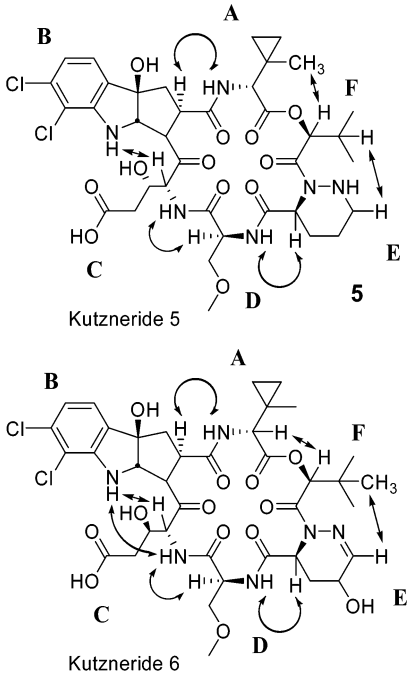

Figure 4. ROESY correlations between subunits used for determination of the primary sequence of the kutznerides, exemplified for **5** and **6**.

Table 1. Minimal Inhibitory Concentrations of Kutznerides **1**−**9** (μM)

	pathogen			
compound	*Drechslera sorokiniana*[a]	*Erwinia carotovora*[b]	*Fusarium culmorum*[a]	*Staphylococcus aureus*[c]
1	230	60	210	12
2	110	6	70	6
3	260	12	260	9
4	>590[d]	120	>590[d]	140
5	>240[d]	120	>240[d]	120
6	>400[d]	>230[d]	>230[d]	>230[d]
7	>420[d]	180	>420[d]	120
8	230	6	110	6
9	>230[d]	230	>230[d]	60
standard				
A[e]	4		40	
F[f]	0.3		17	
S[g]				17

[a] Fungal agricultural pathogen. [b]Bacterial agricultural pathogen. [c]Human bacterial pathogen. [d]No inhibition at the indicated concentration. Depending on the amount of material available, the highest concentration applied for the individual compounds ranged from 230 to 590 μM. [e] Amphotericin B. [f]Fungazil A. [g]Streptomycin.

Erwinia carotovora and *Staphylococcus aureus* at 6 μM. Compound **2** inhibited *Drechslera sorokiniana* at 110 μM and *Fusarium culmorum* at 70 μM, while **8** was slightly less active against these pathogens. The relative configuration of the OHGlu subunit does not seem to have a large influence on the antimicrobial activity, as the compound pairs where this is the only difference, i.e., **1** and **3**, **2** and **8**, **4** and **9**, and **5** and **7**, do inhibit the pathogens at similar levels. On the other hand, the presence of a monomethylated hydroxy acid, as in **5** and **7**, weakens activity in comparison with their dimethylated counterparts **3** and **1**, respectively. Inclusion of a hydroxylated Pip residue, as in **6**, seems to be detrimental for the investigated antimicrobial activity.

Other cyclic peptides that share the PIC subunit with the kutznerides have been reported to have diverse biological activities. Nonchlorinated PIC moieties are found in the cytotoxic compounds himastatin,[8] kapakahine C,[4] phakellistatin 3,[9] and the nematocidal omphalotins B, C, and D.[10] These all have the same *cis* relation

between OH at C-3a and H-8a and *trans* to H-2, as found in the kutznerides. However, kapakahine D[4] and isophakellistatin 3,[9] which instead have an all-*cis* relation of the PIC subunit, are reported as noncytotoxic. The isolation of both *cis* and *trans* forms from the same organisms as for the kapakahines and phakellistatines has called for the proposal that an existing PIC element is not inserted during peptide biosynthesis, but rather derived from oxidation of a tryptophan-containing peptide.[4]

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR data were acquired on a Bruker DRX600 MHz NMR spectrometer equipped with a 5 mm QNP probe (^{1}H/^{13}C/^{31}P/^{15}N) or a 2.5 mm SEI microprobe (^{1}H/^{13}C). All NMR experiments were recorded at 30 °C. For complete structure elucidation, 1D ^{1}H NMR, COSY, TOCSY, ROESY, DEPT-HSQC, and HMBC experiments were applied. Pulse sequences were applied as provided by the manufacturer. Chemical shifts were determined relative to internal $CHCl_3$ (δ_C 77.23; δ_H 7.27). HRFABMS was performed on a four-sector tandem mass spectrometer (Jeol SX/SX102A) with glycerol as matrix and PEG as internal standard. Positive and negative ion mode ESI mass spectra were obtained on a Bruker Esquire ion-trap MS with CH_3OH or CH_3OH-d_4 as solvent, and GC-MS on a HP5890/5970 GC-MS (Hewlett-Packard). SPE was performed with 1 g or 10 g prepacked columns or columns packed in-house with bulk C_{18} material [all Isolute C18 (EC), International Sorbent Technology, Hengoed, UK]. Preparative HPLC was run on a Gilson system at a flow rate of 10 mL/min with UV monitoring at 210 or 254 nm. Fractions were collected in polypropylene, 2 mL square well plates. For the mobile phase CH_3CN of HPLC isocratic grade and deionized filtered H_2O were used.

Isolate Origin and Identity. The actinomycete *Kutzneria* sp. 744 was isolated from mycorrhizal root tips of bare root cultivated spruce [*Picea abies* (L.) H. Karst (Pinaceae)] seedlings in a forest nursery in Lithuania.[11] It was assigned to the genus *Kutzneria* on the basis of 16S rDNA sequence data (GenBank accession number DQ181633) and has been deposited at the culture collection of the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala.

Production of Bacterial Cultures. Cultures of the isolate 744 were grown in 500 mL Erlenmeyer flasks filled with 300 mL of liquid modified Melin Norkrans medium (MMN).[12] The cultures were started by adding three colonized MMN agar plugs (3 mm diameter) to each Erlenmeyer flask, and the cultures were then incubated for 14 days at 21 ± 2 °C on a rotary shaker (120 rpm). Cells were removed by filtration (50 μm) under reduced pressure, and cell-free supernatants were immediately fractionated by SPE or stored at +6 °C for a maximum of 24 h before being processed.

Sample Workup and Isolation Procedures. Cell-free supernatant (8 L) was fractionated on a 180 g SPE column. The column was packed and activated with 500 mL of CH_3CN and equilibrated with 500 mL of H_2O before sample loading. Hydrophilic components were washed out with 750 mL of aqueous 5% CH_3CN before the lipophilic fraction was eluted with 750 mL of aqueous 95% CH_3CN. The lipophilic eluate was evaporated under reduced pressure and fractionated by gradient preparative HPLC (10% to 100% CH_3CN in H_2O in 10 min with an 8 min hold at 100%). Fraction collection was started after the void volume at five fractions per minute. The fractions 53 to 72 were screened with ^{1}H NMR, ^{1}H−^{1}H-COSY, and ESIMS, which detected kutznerides **1**−**9**. To obtain pure compounds, a second HPLC step was performed. Preparative chromatography was run at 50% CH_3CN in H_2O with 0.1% TFA for 15 min and then changed to 60% CH_3CN over 5 min, ending with a hold at 60% for 20 min, to yield compounds **3**−**7**. Compounds **1**, **2**, **8**, and **9** were isolated by 60% CH_3CN in H_2O with 0.1% TFA for 15 min and then changed to 70% CH_3CN over 5 min, ending with a hold for 10 min at 70%. The column for gradient and isocratic HPLC was a Reprosil-pur C_{18} (100 × 20 mm with guard column 30 × 20 mm, 5 μm.).

MIC Determination. Determination of MIC values was performed using a previously developed bioassay, based on inhibition of spore germination or cell growth in microtiter plates.[13−15]

The pathogen isolates of *Aspergillus fumigatus* Fresen. (Trichocomaceae) and *Staphylococcus aureus* Rosenbach (Staphylococcaceae) were provided by the Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden, and the isolates of *Erwinia carotovora* (Jones) Bergey et al. (Enterobacteriaceae), *Fusarium culmorum* (W. G. Sm.) Sacc. (Nectriaceae), and *Drechslera sorokiniana* (Sacc.) Subram.& B. L. Jain (Pleosporaceae) by the Plant Pathology and Biocontrol Unit, Swedish University of Agricultural Sciences, Uppsala, Sweden. The isolate of *Candida albicans* (C. P. Robin) Berkhout (Saccharomycetaceae) was provided by the Laboratory of Clinical Microbiology, Centre of Laboratory Medicine, Uppsala University Hospital, Uppsala, Sweden. The isolate of *P sevastanoi* pv. *sevastanoi* CCM 3580 (Pseudomonadaceae) was purchased from the Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic. All isolates were maintained as described by Levenfors et al.[13] The strains are deposited at the bacterial and fungal culture collection at MASE Laboratories, Uppsala, Sweden.

Pure compounds dissolved in MeOH were transferred to 96-well microtiter plates, from which the solvents were evaporated in a fume hood. To each well was added 100 μL of spore suspension at 10^4 spores/mL or cell suspension at 10^4 cells/mL in appropriate culture media. Spore/cell suspensions were used as positive controls, and sterile medium was used as negative control. For both detection of antimicrobial fractions and MIC determinations, bioassay plates were incubated in darkness at 27−28 °C (*Erwinia carotovora* and *P. sevastanoi* pv. *sevastanoi*, both 24 h, *D. sorokiniana* 48 h, *F. culmorum* 72 h, and *A. fumigatus* 96 h) or at 37 °C for 24 h (*C. albicans* and *S. aureus*). Results were evaluated visually according to the following scale: 3, full inhibition of growth comparable to negative control; 2, intermediate inhibition of growth; 1, detectable inhibition of growth; 0, no detectable inhibition of growth, comparable to positive control. All MIC tests were performed in triplicate and repeated once. MIC values for the standards amphotericin B, fungazil A, and streptomycin were determined using identical bioassay conditions and pathogen strains, except for the exclusion of *Erwinia carotovora* and *P. sevastanoi* pv. *sevastanoi*.[16]

Compound 5: 0.7 mg; white powder; ^{1}H NMR ($CDCl_3$, 600 MHz) δ MecPGly 7.81 (1H, d, J = 9.4 Hz, N*H*), 4.15 (1H, d, J = 9.7 Hz, H-2), 1.07 (3H, s, CH_3), 1.06 (1H, m, H-4a), 0.68 (1H, m, H-4b), 0.66 (1H, m, H-5a), 0.40 (1H, m, H-5b); diClPIC 7.14 (1H, d, J = 7.7 Hz, H-4), 6.95 (1H, d, J = 7.7 Hz, H-5), 6.37 (1H, d, J = 4.9 Hz, N*H*), 5.88 (1H, s, O*H*), 5.40 (1H, d, J = 5.1 Hz, H-8a), 5.33 (1H, d, J = 7.9 Hz, H-2), 2.79 (1H, d, J = 14.7 Hz, H-3β), 2.16 (1H, dd, J = 8.0, 14.5 Hz, H-3α); OHGlu 6.75 (1H, d, J = 10.8 Hz, N*H*), 5.23 (1H, t, J = 9.7 Hz, H-2), 4.74 (1H, dt, J = 4.4, 9.4 Hz, H-3), 3.26 (1H, s, O*H*), 2.70 (2H, m, H-4); MeSer 7.62 (1H, br d, J = 8.5 Hz, N*H*), 4.56 (1H, br d, J = 8.4 Hz, H-2), 3.87 (1H, d, J = 9.0 Hz, H-3a), 3.31 (1H, dd, J = 9.0, 3.1 Hz, H-3b), 3.22 (3H, s, CH_3); Pip 5.09 (1H, br d, J = 11.7 Hz, N*H*), 5.06 (1H, d, J = 5.6 Hz, H-2), 3.16 (1H, br d, J = 13.7 Hz, H-5a), 2.78 (1H, d, J = 14.3 Hz, H-5b), 2.35 (1H, m, H-3a), 2.12 (1H, m, H-4a), 1.69 (1H, m, H-3b), 1.58 (1H, m, H-4a); OHMeBu 5.72 (1H, d, J = 5.1 Hz, H-2), 2.22 (1H, m, H-3), 1.14 (3H, d, J = 7.1 Hz, H-4a), 1.10 (3H, d, J = 7.1 Hz, H-4b); ^{13}C NMR ($CDCl_3$, 150 MHz) δ MecPGly 173.8 (CO, C-1), 60.6 (CH, C-2), 19.6 (CH_3, *C*H_3), 19.2 (C, C-3), 15.4 (CH_2, C-4), 12.7 (CH_2, C-5); diClPIC 172.5 (CO, C-1), 146.8 (C, C-7a), 133.3 (C, C-6), 131.0 (C, C-3b), 122.5 (CH, C-5), 122.4 (CH, C-4), 115.5 (C, C-7), 91.8 (C, C-3a), 85.1 (CH, C-8a), 61.5 (CH, C-2), 39.8 (CH_2, C-3); OHGlu 174.3 (CO, C-5), 171.0 (CO, C-1), 70.0 (CH, C-3), 55.7 (CH, C-2), 39.5 (CH_2, C-4); MeSer 172.0 (CO, C-1), 73.8 (CH_2, C-3), 60.5 (CH_3, *C*H_3), 56.2 (CH, C-2); Pip 174.8 (CO, C-1), 51.7 (CH, C-2), 40.0 (CH_2, C-5), 24.0 (CH_2, C-3), 21.3 (CH_2, C-4); OHMeBu 172.9 (CO, C-1). 78.2 (CH, C-2), 31.0 (CH, C-3), 20.4 (CH_3, C-4a), 18.4 (CH_3, C-4b); HRFABMS *m*/*z* 840.2792 $(M + H)^+$ (calcd for $C_{36}H_{48}O_{12}N_7Cl_2$ 840.2738).

Compound 6: 1.2 mg; white powder; ^{1}H NMR ($CDCl_3$, 600 MHz) δ MecPGly 7.82 (1H, d, J = 9.2 Hz, N*H*), 4.10 (1H, d, J = 9.2 Hz, H-2), 1.08 (3H, s, CH_3), 1.05 (1H, m, H-4a), 0.72 (1H, m, H-4b), 0.69 (1H, m, H-5a), 0.41 (1H, m, H-5b); diClPIC 7.16 (1H, d, J = 7.7 Hz, H-4), 6.98 (1H, d, J = 7.7 Hz, H-5), 6.25 (1H, d, J = 3.2 Hz, N*H*), 5.84 (1H, s, O*H*), 5.30 (1H, d, J = 7.7 Hz, H-2), 5.25 (1H, d, J = 3.3 Hz, H-8a), 2.80 (1H, d, J = 14.7 Hz, H-3β), 2.17 (1H, dd, J = 14.7, 7.7 Hz, H-3α); OHGlu 6.78 (1H, d, J = 10.0 Hz, N*H*), 5.14 (1H, dd, J = 10.1, 8.2 Hz, H-2), 4.45 (1H, br quart, J = 6.8 Hz, H-3), 2.60 (2H, m, H-4a/4b/4c); MeSer 8.17 (1H, br s, N*H*), 4.36 (1H, br d, J = 3.8 Hz, H-2), 3.81 (1H, d, J = 8.8 Hz, H-3a), 3.37 (1H, br d, J = 8.8 Hz, H-3b), 3.27 (3H, s, CH_3); Pip 7.09 (CH, br s, C-5), 5.27 (1H, d, J = 3.3 Hz, H-2), 4.68 (1H, m, H-4), 2.60 (1H, m, H-3a), 1.63 (1H, m,

H-3b); OHdiMeBu 5.89 (1H, s, H-2), 1.11 (9H, s, H-4); ^{13}C NMR ($CDCl_3$, 150 MHz) δ MecPGly 173.0 (CO, C-1), 59.9 (CH, C-2), 19.9 (C, C-3), 19.8 (CH_3, *C*H_3), 15.8 (CH_2, C-4), 12.9 (CH_2, C-5); diClPIC 171.5 (CO, C-1), 146.8 (C, C-7a), 133.2 (C, C-6), 131.0 (C, C-3b), 123.0 (CH, C-5), 122.7 (CH, C-4), 115.5 (C, C-7), 91.8 (C, C-3a), 85.9 (CH, C-8a), 61.6 (CH, C-2), 40.2 (CH_2, C-3); OHGlu 176.7 (CO, C-5), 170.6 (CO, C-1), 69.9 (CH, C-3), 54.3 (CH, C-2), 38.7 (CH_2, C-4); MeSer 172.1 (CO, C-1), 72.7 (CH_2, C-3), 60.8 (CH_3, *C*H_3), 56.9 (CH, C-2); Pip 170.5 (CO, C-1), 149.3 (CH, C-5), 60.4 (CH, C-4), 52.1 (CH, C-2), 27.6 (CH_2, C-3); OHdiMeBu 172.6 (CO, C-1). 79.8 (CH, C-2), 34.8 (C, C-3), 27.4 ($3CH_3$, C-4a/4b/4c); HRFABMS *m*/*z* 868.2681 $(M + H)^+$ (calcd for $C_{37}H_{48}O_{13}N_7Cl_2$ 868.2687).

Compound 7: 1.2 mg; white powder; ^{1}H NMR ($CDCl_3$, 600 MHz) δ MecPGly 7.67 (1H, d, J = 9.4 Hz, N*H*), 4.06 (1H, d, J = 9.4 Hz, H-2), 1.04 (3H, s, CH_3), 1.04 (1H, m, H-4a), 0.69 (1H, m, H-4b), 0.69 (1H, m, H-5a), 0.40 (1H, m, H-5b); diClPIC 7.17 (1H, d, J = 8.1 Hz, H-4), 6.99 (1H, d, J = 8.1 Hz, H-5), 6.40 (1H, d, J = 5.5 Hz, N*H*), 5.86 (1H, br s, O*H*), 5.29 (1H, d, J = 7.7 Hz, H-2), 5.27 (1H, d, J = 5.5 Hz, H-8a), 2.80 (1H, d, J = 14.3 Hz, H-3β), 2.17 (1H, dd, J = 14.3, 7.9 Hz, H-3α); OHGlu 7.15 (1H, d, J = 10.8 Hz, N*H*), 5.31 (1H, d, J = 10.8 Hz, H-2), 4.77 (1H, dd, J = 8.1, 6.1 Hz, H-3), 2.66 (1H, dd, J = 15.6, 8.2 Hz, H-4a), 2.59 (1H, dd, J = 15.6, 5.3 Hz; H-4b); MeSer 7.59 (1H, d, J = 5.8 Hz, N*H*), 4.39 (1H, dt, J = 5.8, 2.5 Hz, H-2), 3.82 (1H, dd, J = 9.4, 2.1 Hz, H-3a), 3.41 (1H, dd, J = 9.4, 3.0 Hz, H-3b), 3.26 (3H, s, CH_3); Pip 5.02 (CH, br d, J = 5.9 Hz, H-2), 4.67 (1H, br d, J = 10.7 Hz, N*H*), 3.15 (1H, br d, J = 13.8 Hz, H-5a), 2.78 (1H, br d, J = 13.8 Hz, H-5b), 2.35 (1H, m, H-3a), 2.08 (1H, m, H-4a), 1.64 (1H, m, H-3b), 1.57 (1H, m, H-4b); OHMeBu 5.69 (1H, s, H-2), 2.23 (1H, m, H-3), 1.17 (3H, d, J = 6.7 Hz, H-4a), 1.03 (3H, d, J = 6.7 Hz, H-4b); ^{13}C NMR ($CDCl_3$, 150 MHz) δ MecPGly 173.0 (CO, C-1), 60.8 (CH, C-2), 19.6 (C, C-3), 19.2 (CH_3, *C*H_3), 14.3 (CH_2, C-4), 12.3 (CH_2, C-5); diClPIC 171.9 (CO, C-1), 146.9 (C, C-7a), 133.2 (C, C-6), 131.0 (C, C-3b), 122.9 (CH, C-5), 122.4 (CH, C-4), 115.7 (C, C-7), 91.8 (C, C-3a), 85.7 (CH, C-8a), 61.5 (CH, C-2), 39.0 (CH_2, C-3); OHGlu 172.3 (CO, C-5), 171.4 (CO, C-1), 68.2 (CH, C-3), 53.2 (CH, C-2), 37.0 (CH_2, C-4); MeSer 172.2 (CO, C-1), 72.2 (CH_2, C-3), 60.2 (CH_3, *C*H_3), 56.4 (CH, C-2); Pip 171.3 (CO, C-1), 51.7 (CH, C-2), 47.1 (CH_2, C-5), 23.7 (CH_2, C-3), 21.1 (CH_2, C-4); OHMeBu 173.4 (CO, C-1). 78.3 (CH, C-2), 30.7 (CH, C-3), 19.4 ($2CH_3$, C-4a/4b/4c); HRFABMS *m*/*z* 840.2782 $(M + H)^+$ (calcd for $C_{36}H_{48}O_{12}N_7Cl_2$ 840.2738).

Compound 8: 0.4 mg; white powder; ^{1}H NMR ($CDCl_3$, 600 MHz) δ MecPGly 7.86 (1H, d, J = 9.5 Hz, N*H*), 4.11 (1H, d, J = 9.4 Hz, H-2), 1.08 (3H, s, CH_3), 1.08 (1H, m, H-4a), 0.71 (1H, m, H-4b), 0.69 (1H, m, H-5a), 0.40 (1H, m, H-5b); diClPIC 7.16 (1H, d, J = 7.9 Hz, H-4), 6.98 (1H, d, J = 7.9 Hz, H-5), 6.33 (1H, d, J = 5.3 Hz, N*H*), 5.67 (1H, br s, O*H*), 5.33 (1H, d, J = 7.9 Hz, H-2), 5.27 (1H, d, J = 5.5 Hz, H-8a), 2.82 (1H, d, J = 13.9 Hz, H-3β), 2.16 (1H, dd, J = 14.1, 7.9 Hz, H-3α); OHGlu 6.76 (1H, d, J = 10.8 Hz, N*H*), 5.24 (1H, t, J = 10.8 Hz, H-2), 4.70 (1H, dt, J = 9.5, 3.7 Hz, H-3), 2.72 (2H, dd, J = 14.9, 3.9 Hz, C-4); MeSer 7.46 (1H, d, J = 7.8 Hz, N*H*), 4.58 (1H, br d, J = 7.7 Hz, H-2), 3.87 (1H, d, J = 9.5 Hz, H-3a), 3.28 (1H, dd, J = 3.2, 9.5 Hz, H-3b), 3.25 (3H, s, CH_3); Pip 5.45 (1H, d, J = 12.6 Hz, N*H*), 5.26 (CH, d, J = 5.1 Hz, H-2), 4.53 (1H, m, H-4), 3.37 (1H, br d, J = 12.6 Hz, H-5b), 2.90 (1H, br d, J = 12.4 Hz, H-5b), 2.85 (1H, br d, J = 12.2 Hz, H-3a), 1.82 (1H, dt, J = 12.2, 5.9 Hz, H-3b); OHdiMeBu 5.78 (1H, s, H-2), 1.15 (9H, s, H-4a/4b/4c); ^{13}C NMR ($CDCl_3$, 150 MHz) δ MecPGly 173.0 (CO, C-1), 60.9 (CH, C-2), 20.0 (C, C-3), 19.7 (CH_3, *C*H_3), 15.4 (CH_2, C-4), 12.0 (CH_2, C-5); diClPIC 172.5 (CO, C-1), 147.2 (C, C-7a), 133.5 (C, C-6), 131.4 (C, C-3b), 123.2 (CH, C-5), 122.9 (CH, C-4), 115.9 (C, C-7), 92.0 (C, C-3a), 85.8 (CH, C-8a), 60.8 (CH, C-2), 40.3 (CH_2, C-3); OHGlu 174.8 (CO, C-5), 171.5 (CO, C-1), 70.3 (CH, C-3), 55.5 (CH, C-2), 39.9 (CH_2, C-4); MeSer 171.8 (CO, C-1), 74.0 (CH_2, C-3), 60.7 (CH_3, *C*H_3), 55.4 (CH, C-2); Pip 173.6 (CO, C-1), 56.4 (CH, C-2), 54.8 (CH_2, C-5), 51.1 (CH, C-4), 35.0 (CH_2, C-3); OHdiMeBu 172.9 (CO, C-1). 78.4 (CH, C-2), 34.3 (C, C-3), 28.2 ($3CH_3$, C-4a/4b/4c); HRFABMS *m*/*z* 888.2568 $(M + H)^+$ (calcd for $C_{37}H_{49}O_{12}N_7Cl_3$ 888.2505).

Compound 9: 0.6 mg; white powder; ^{1}H NMR ($CDCl_3$, 600 MHz) δ MecPGly 7.74 (1H, d, J = 9.1 Hz, N*H*), 4.10 (1H, d, J = 9.3 Hz, H-2), 1.06 (3H, s, CH_3), 1.04 (1H, m, H-4a), 0.73 (1H, m, H-4b), 0.71 (1H, m, H-5a), 0.41 (1H, m, H-5b); diClPIC 7.17 (1H, d, J = 7.7 Hz, H-4), 6.99 (1H, d, J = 7.7 Hz, H-5), 6.39 (1H, br s, N*H*), 5.85 (1H, br s, O*H*), 5.29 (1H, obsc, H-2), 5.28 (1H, obsc, H-8a), 2.81 (1H, obsc, H-3β), 2.16 (1H, obsc, H-3α); OHGlu 7.08 (1H, d, J = 10.8 Hz, N*H*), 5.24 (1H, obsc, H-2), 4.67 (1H, dd, J = 9.1, 4.7 Hz, H-3), 2.51 (1H, m, C-4a), 2.46 (1H, m, C-4b); MeSer 7.89 (1H, d, J = 5.8 Hz, N*H*), 4.47 (1H, br d, J = 5.2 Hz, H-2), 3.85 (1H, d, J = 9.0 Hz, H-3a), 3.42 (1H, d, J = 9.2 Hz, H-3b), 3.28 (3H, s, CH_3); Pip 7.11 (1H, br s, H-5), 5.21 (1H, obsc, H-2), 2.84 (1H, m, H-4a), 2.47 (1H, m, H-3a), 2.22 (1H, m, H-4b), 1.59 (1H, m, H-3b); OHdiMeBu 5.91 (1H, s, H-2), 1.11 (9H, s, H-4a/4b/4c); ^{13}C NMR ($CDCl_3$, 150 MHz) δ MecPGly 173.0 (CO, C-1), 60.0 (CH, C-2), 19.9 (C, C-3), 18.5 (CH_3, *C*H_3), 14.5 (CH_2, C-4), 11.7 (CH_2, C-5); diClPIC 171.7 (CO, C-1), 146.7 (C, C-7a), 133.5 (C, C-6), 131.1 (C, C-3b), 122.1 (CH, C-5), 121.7 (CH, C-4), 115.8 (C, C-7), 91.5 (C, C-3a), 85.3 (CH, C-8a), 60.6 (CH, C-2), 39.1 (CH_2, C-3); OHGlu 172.4 (CO, C-5), 171.8 (CO, C-1), 67.0 (CH, C-3), 52.4 (CH, C-2), 36.4 (CH_2, C-4); MeSer 172.4 (CO, C-1), 71.9 (CH_2, C-3), 59.7 (CH_3, *C*H_3), 55.4 (CH, C-2); Pip 171.6 (CO, C-1), 145.4 (CH, C-5), 50.4 (CH, C-2), 20.9 (CH_2, C-4), 17.2 (CH_2, C-3); OHdiMeBu 172.4 (CO, C-1). 78.9 (CH, C-2), 34.7 (C, C-3), 26.4 ($3CH_3$, C-4); HRFABMS *m*/*z* 852.2752 $(M + H)^+$ (calcd for $C_{37}H_{48}O_{12}N_7Cl_2$ 852.2738).

Absolute Configuration of Subunits A, C, D, E, and F. Compounds **5**−**9** (60 μg each) were hydrolyzed at 110 °C in 6 M HCl for 12−16 h in evacuated glass ampules. The solvent was evaporated under a stream of N_2, and the sample was then hydrogenated for 12 h at room temperature under constant stirring (H_2/PtO_2/AcOH). The catalyst was removed by centrifugation, and the supernatant was dried under a stream of N_2. Enantiomeric resolution was based on esterification of the hydrogenated sample with 2*S*-BuOH (200 μL of 2*S*-BuOH/AcCl, 10:1, 100 °C, 40 min in sealed test tubes) followed by evaporation with N_2 and then derivatization with pentafluoropropionic acid anhydride (200 μL, 100 °C, 40 min in sealed test tubes). The samples were dried under N_2 and then dissolved in 100 μL of EtOAc and subjected to GC-MS. The samples were analyzed on a fused silica column (HP-5MS; 0.25 μm, 30 m × 0.25 mm, Agilent Technologies, Palo Alto, CA) using a temperature gradient (60 °C for 5 min, 1 °C/min to 100 °C, 5 °C/min to 150 °C, and 3 °C/min to 240 °C) with the MS detector in SIM mode. The injector was held at 240 °C and the GC-MS interface at 260 °C. Samples (1 μL) were injected in splitless mode, and He was used as a carrier gas at 1 mL/min. Samples of racemic and (*S*)-2-OH-3-methylbutyric acid were derivatized as above, and the elution order of the diastereomers was determined to be (*R*)-OHMeBu before (*S*)-OHMeBu. For all other subunits elution order was previously established,[1] and the minor diastereomers (<10%) formed during hydrolysis or esterification were used as internal references. Retention times in min: 21.03, *R*-OHMeBu; 21.29, *S*-OHMeBu; 23.99, *R*-OHdiMeBu; 24.31, *S*-OHdiMeBu; 38.41, D-*tert*-Leu (from D-MecPGly); 38.90, D-Ser (from D-MeSer); 39.30, L-Ser (from L-MeSer); 39.72, L-*tert*-Leu (from L-MecPGly); 57.65, D-Orn; 57.76, L-Orn; 59.72, *threo*-D-OHGlu; 59.82, *threo*-L-OHGlu; 61.54, *erythro*-D-OHGlu; 61.71, *erythro*-L-OHGlu.

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