New Metabolites with Nematicidal and Antimicrobial Activities from the Ascomycete *Lachnum papyraceum* (Karst.) Karst

VIII. Isolation, Structure Determination and Biological Activities of Minor Metabolites Structurally Related to Mycorrhizin A

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(Received for publication October 24, 1995)

Five new minor metabolites, papyracon D (**6**), 6-*O*-methylpapyracon B (**9**), 6-*O*-methylpapyracon C (**10**), lachnumfuran A (**11**) and lachnumlactone A (**12a**), together with the known chloromycorrhizinol A (**4**), have been isolated from extracts of the culture fluids of the ascomycete *Lachnum papyraceum*. The compounds, which structures were determined by spectroscopic methods, are structurally related to the nematicidal and antibiotic mycorrhizin A (**1**), which also is produced by the fungus. The nematicidal, antibiotic and cytotoxic activities of the new compounds are weaker compared to those of mycorrhizin A (**1**). Papyracon D (**6**) possesses the highest antibiotic activities while lachnumlactone A (**12a**) is the most nematicidal and cytotoxic.

A considerable number of biologically active metabolites have been isolated from the ascomycete *Lachnum papyraceum* (for a review see reference 1). Its secondary metabolism appears to start with the formation of 3-methyl-3,4-dihydroisocoumarins which may be halogenated at C-4[2]. These are decarboxylated and in some cases halogenated a second time[3], and isoprenylated to eventually form for example dechloromycorrhizin A (**3**), mycorrhizin A (**1**) and chloromycorrhizin A (**2**)[4]. In an effort to characterise new metabolites from *L. papyraceum* which may shed further light on the mechanism by which these compounds exert their biological activities as well as the biosynthetic pathways leading to them, 5 new metabolites were isolated from the extracts of the culture fluids obtained after the fermentation of *L. papyraceum* in a 100 liter scale.

Materials and Methods

General

The fractionation of the extract was performed by chromatography on silica gel columns eluted with mixtures of heptane and EtOAc. TLC experiments were performed on Merck silica gel 60 F_{254} precoated plates. HPLC chromatography (analytical and preparative) was performed on reverse phase columns with water/MeOH gradients as mobile phases. UV spectra were obtained with a Perkin Elmer λ 16, and IR spectra with a Bruker IFS 48. The optical rotation was measured with a Perkin Elmer 1541 polarimeter with a cell path of 10 cm. EI-MS and HREI-MS spectra (direct inlet, EI at 70 eV) were recorded with a Jeol JMS-SX102 spectrometer. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX 500 spectrometer with an inverse 5 mm probe equipped with a shielded gradient coil. COSY, HMQC and HMBC experiments were performed with gradient enhancements using sine shaped gradient pulses, and for the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}=145$ Hz and $^2J_{CH}=10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001).

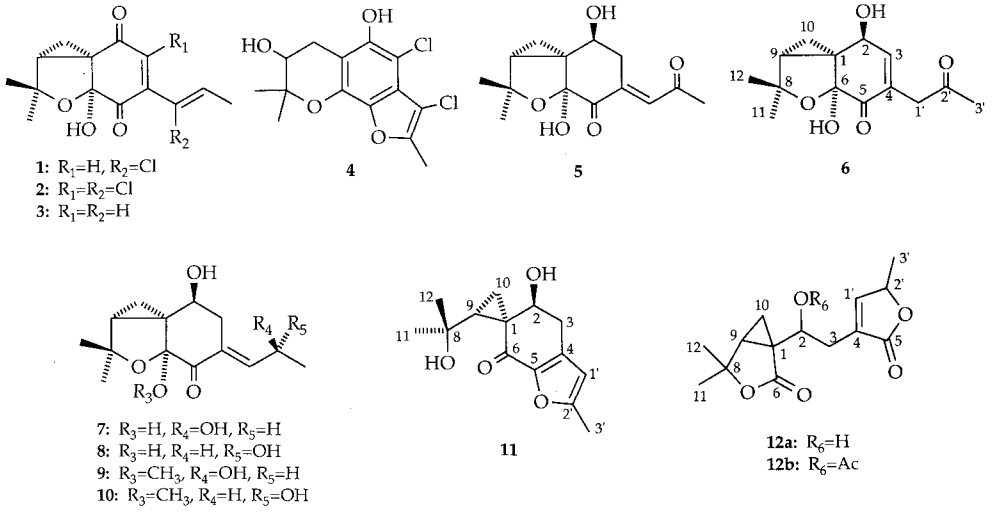
Taxonomy of Producer

The origin of the producing strain, *Lachnum papyraceum* A 48-88, its maintainance and growth conditions have been described before[4].

Fermentation

Lachnum papyraceum was grown in MGP medium (maltose 2%, glucose 1%, soy peptone 0.1%, yeast extract 0.1%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, $CaCl_2\cdot 2H_2O$ 10 mM, $CaBr_2$ 50 mM, $FeCl_3$ 6 μM, $ZnSO_4\cdot 7H_2O$ 6 μM) in a 100 liter fermentor (Braun Biostat U) at 24°C with an aeration rate of 15 liters/minute and agitation (120 rpm). The oxygen saturation of the culture broth

Fig. 1. Metabolites isolated from *Lachnum papyraceum*.



was measured using a Braun Oxygen electrode, and the pH value was determined on line using an Ingold pH electrode. The pH was adjusted to 5.5 before sterilisation. The inoculum was 15 liters of a seven days old 20 liter scale culture, which was grown as previously described[4]. The course of the fermentation in this scale was very similar to that observed previously in a 20 liter scale[4], and no separate fermentation diagram is given here. Aliquots of the culture fluid were assayed daily for nematicidal and antimicrobial activities (with *Caenorhabditis elegans*, *Nematospora coryli* and *Bacillus brevis* as test organisms). The fermentation was harvested 36 hours after the biological activities of the culture fluid had reached their maximum.

Isolation

The culture fluid was filtered through a Buechner funnel to remove the mycelium. The culture filtrate (91 liters) was applied onto a Mitsubishi DIAION HP21 column (containing 2 liters of resin). The column was rinsed with 10 liters of water and thereafter eluted with 8 liters of acetone. The acetone was evaporated *in vacuo* at 40°C and the remaining aqueous residue (500 ml) was extracted five times with 500 ml of ethyl acetate. Evaporation of the combined organic phases yielded an extract (45 g) which was fractionated by chromatography on silica gel in an EtOAc-heptane gradient followed by HPLC on reversed phase material with H_2O-MeOH mixtures. The Rf values of the compounds in two different TLC systems are given in Table 1. Besides the compounds that already have been reported from similar fermentations of this fungus[4,5], 25 mg chloromycorrhizinol A (**4**), 10 mg papyracon D (**6**), 15 mg 6-*O*-methylpapyracon B (**9**), 10 mg 6-*O*-methylpapyracon C (**10**), 50 mg lachnumfuran A (**11**) and 30 mg lachnumlactone A (**12a**) were obtained. The methoxy groups of compounds **9** and **10** were not introduced by the solvent during the reversed phase separations, as both compounds were present in the fractions obtained from the silica gel separations.

2-*O*-Acetylpapylactone A (**12b**)

Compound **12b** was obtained as colourless crystals (m.p. 100~102°C) as the only product of acetylation of lachnumlactone A (**12a**) (10 mg) with acetic anhydride (0.5 ml) in pyridine (1 ml). $[\alpha]_D^{22}$ −52° (*c* 0.7 in $CHCl_3$). UV (MeOH) λ_{max} (ε): 210 (5300). IR (KBr): 3330, 2985, 1755, 1635, 1375, 1325, 1235, 1100 and 1025 cm^{-1}. ^1H NMR (500 MHz in $CDCl_3$), δ, multiplicity, *J* (Hz): 7.19, dm, $J_{1'-2'}=1.3$, 1'-H; 4.99, dq, $J_{1'-2'}=1.3$, $J_{2'-3'}=6.6$, 2'-H; 4.95, dd, $J_{2-3a}=9.5$, $J_{2-3b}=5.0$, 2-H; 3.23, dd, $J_{2-3a}=9.5$, $J_{3a-3b}=14.8$, 3-Ha; 2.98, dd, $J_{2-3b}=5.0$, $J_{3a-3b}=14.8$, 3-Hb; 2.13, dd, $J_{9-10a}=7.9$, $J_{9-10b}=5.0$, 9-H; 2.03, s, Ac; 1.39 and 1.32, s, 11-H_3 and 12-H_3; 1.37, d, $J_{2'-3'}=6.6$, 3'-H_3; 1.22, dd, $J_{9-10a}=7.9$, $J_{10a-10b}=5.0$, 10-Ha; 1.06, dd, $J_{9-10b}=5.0$, $J_{10a-10b}=5.0$, 10-Hb. ^{13}C NMR (125 MHz in $CDCl_3$), δ: 173.5 C-6; 173.1 C-5; 170.1 Ac; 152.3 C-1'; 129.7 C-4; 81.4 C-8; 77.6 C-2'; 70.7 C-2; 32.6 C-9; 32.3 C-1; 29.1 and 23.7 C-11 and C-12; 28.1 C-3; 20.8 Ac; 18.9 C-3'; 15.8 C-10. MS (EI, 70 eV), *m/z*: 293 (2%, M−CH_3), 248.1055 (M−CH_3COOH, 24%, $C_{14}H_{16}O_4$ requires 248.1048), 230 (19%), 187 (32%), 155 (88%), 137 (56%), 112 (100%), 109 (49%). MS ($CINH_3$) *m/z*: 326 (M+NH_4, 100%), 309 (M+H, 11%), 249 (M−CH_3COOH+H, 25%).

Tests for Biological Activities

The tests for nematicidal[1,4], antimicrobial[5] and cytotoxic[6] activities were carried out as reported before.

Results and Discussion

Structure Determination

The physico-chemical properties of the new compounds are presented in Table 1, while the NMR data are given in Table 2 (^{1}H NMR) and Table 3 (^{13}C NMR). The structure determinations are based on 2D NMR spectroscopy (including HMQC, HMBC, COSY and NOESY experiments), although only pertinent results are given here. The spectroscopic data of chloromycorrhizinol A (**4**) isolated in this investigation were found to be identical with those previously reported[7].

High resolution EI-MS measurements of papyracon D (**6**) indicated that its molecular composition is $C_{14}H_{18}O_5$. The NMR data closely resemble those of isomer papyracon A (**5**)[5], although the lack of a ^{1}H-^{1}H coupling between allylic methylene protons and 2-H suggested that the double bond had migrated. The structure of papyracon D (**6**) was determined by the HMBC correlations between 1'-H_2 and C-3, C-4, C-5 and C-2', between 3'-H_3 and C-2', and and between 3-H and C-1 as well as C-5. The relative stereostructure was suggested to be the same as in papyracon A (**5**)[5], by the NOESY correlations observed between for example 10-Hβ and 9-H as well as 2-H, and between 10-H_α and 11-H_3.

Neither compound **9** nor compound **10** gave a molecular ion (*m/z* 282) during EIMS experiments, instead the fragments *m/z* 267 (M$-CH_3$) and 250 (M$-CH_3OH$) were observed. Both compounds gave similar CI (NH_3) mass spectra, with *m/z* 300 (M$+NH_4$), 268 (M$-CH_3+$

Table 1. Physico-chemical properties of compounds **6**, **9**, **10**, **11** and **12a**.

	6	9	10	11	12a
Appearance	Yellowish oil	Yellowish oil	Yellowish oil	Yellowish oil	Yellowish oil
$[\alpha]_D^{22}$	+86° (*c* 0.2 in $CHCl_3$)	−2° (*c* 0.3 in $CHCl_3$)	+25° (*c* 0.2 in $CHCl_3$)	+126° (*c* 1.1 in $CHCl_3$)	−17° (*c* 0.6 in $CHCl_3$)
Molecular formula	$C_{14}H_{18}O_5$	$C_{15}H_{22}O_5$	$C_{15}H_{22}O_5$	$C_{14}H_{18}O_4$	$C_{14}H_{18}O_5$
HREI-MS (*m/z*)					
Observed	266.1139 M^+	267.1241 M^+-CH_3	267.1257 M^+-CH_3	250.1205 M^+	248.1043 M^+-H_2O
Calculated	266.1154 for $C_{14}H_{18}O_5$	267.1232 for $C_{14}H_{19}O_5$	267.1232 for $C_{14}H_{19}O_5$	250.1205 for $C_{14}H_{18}O_4$	248.1048 for $C_{14}H_{16}O_4$
EI-MS	266 (9%), 251 (7%), 230 (41%), 202 (78%), 187 (100%), 179 (73%), 161 (58%), 123 (58%),	267 (13%), 223 (34%), 187 (38%), 161 (35%), 137 (40%), 109 (67%), 84 (78%), 43 (100%)	267 (9%), 223 (26%), 187 (33%), 161 (29%), 137 (42%), 109 (83%), 84 (88%), 43 (100%)	250 (3%), 235 (100%), 217 (7%), 215 (6%), 179 (88%), 173 (28%), 161 (41%), 145 (11%)	266 (1%), 251 (3%), 248 (6%), 155 (90%), 137 (61%), 112 (100%) 109 (74%), 67 (31%)
UV (MeOH)					
λ_{max} nm (ε)	229 (4900)	244 (8100)	242 (7700)	232 (1000), 293 (6700)	210 (6100)
IR (KBr) cm^{-1}	3425, 2970, 1710, 1675, 1360, 1175, 1110, 1065 and 1020	3420, 2970, 2930, 1710, 1635, 1375, 1100, 1085 and 1055	3400, 2975, 2930, 1715, 1635, 1370, 1105, 1080 and 1055	3390, 2975, 1635, 1530, 1460, 1430, 1205, 1065 and 1030	3420, 2935, 1750, 1615, 1375, 1320, 1105, 1025 and 920
TLC (Rf)	0.20[a], 0.70[b]	0.22[a], 0.72[b]	0.26[a], 0.68[b]	0.37[a], 0.82[b]	0.38[a], 0.87[b]

[a] Merck, Kieselgel 60 F_{254}: Toluene-aceton (7:3).
[b] Merck, Kieselgel 60 F_{254}: Methylene chloride-methanol (10:1).

Table 2. ^{1}H (500 MHz) NMR data (δ; multiplicity; *J*) for papyracon D (**6**), 6-*O*-methylpapyracon B (**9**), 6-*O*-methylpapyracon C (**10**), lachnumfuran A (**11**), and lachnumlactone A (**12a**), in $CDCl_3$ (compounds **6**, **9**, **11** and **12a**) or $CDCl_3$ with 5% CD_3OD (compound **10**) with the $CHCl_3$ signal (7.26 ppm) as reference. The coupling constants *J* are given in Hz.

H	6	9	10	11	12a
2	4.87; br s	3.90; dd; 4, 4	3.87; dd; 3.0, 6.8	3.55; dd; 4, 4	4.10; ddd; 5, 5, 7
3α	6.92; ddd; 0.8, 0.8, 2.0	2.98; m	2.98; ddd; 2, 3, 15	3.04; dd; 3.8, 17.0	2.71; d; 6.7
3β	—	2.98; m	2.70; ddd; 2, 6.8, 15.0	2.87; dd; 4.0, 17.0	2.71; d; 6.7
9	1.86; dd; 4.4, 8.1	1.71; dd; 5.1, 8.3	1.68; dd; 4.9, 8.2	1.59; dd; 8.1, 9.3	2.08; dd; 4.6, 7.8
10α	1.09; dd; 4.4, 5.0	1.37; dd; 5, 5	1.16; dd; 5, 5	1.68; dd; 4.5, 8.1	1.00; dd; 5, 5
10β	0.95; dd; 5.0, 8.1	0.85; dd; 5.0, 8.3	0.82; dd; 4.9, 8.2	1.27; dd; 4.5, 9.3	1.34; dd; 5.1, 7.8
11	1.26; s	1.29; s	1.22; s	1.37[a]; s	1.37; s
12	1.16; s	1.25; s	1.17; s	1.33[a]; s	1.47; s
1'a	3.48; d; 16.6	6.79; br d; 8.3	6.62; ddd; 2, 2, 8.0	6.12; s	7.27; m
1'b	3.27; d; 16.6	—	—	—	—
2'	—	4.62; dd; 6.4, 8.3	4.52; dd; 6.5, 8.0	—	5.08; dq; 1.2, 6.9
3'	2.25; s	1.34; d; 6.4	1.26; d; 6.5	2.39; s	1.43; d; 6.9
OCH_3	—	3.16; s	3.12; s	—	—
Ac	—	—	—	—	—
2-OH	—	—	—	—	3.23; d; 5.1
8-OH	—	—	—	5.31; s	—

[a] Interchangable.

Table 3. ^{13}C (125 MHz) NMR data (δ; multiplicity) for papyracon D (**6**), 6-*O*-methylpapyracon B (**9**), 6-*O*-methylpapyracon C (**10**), lachnumfuran A (**11**), and lachnumlactone A (**12a**), in $CDCl_3$ (compounds **6**, **9**, **11** and **12a**) or $CDCl_3$ with 5% CD_3OD (compound **10**) with the $CDCl_3$ signal (77.0 ppm) as reference.

C	6	9	10	11	12a
1	37.2; s	40.9; s	40.4; s	38.0; s	34.8; s
2	63.7; d	67.8; d	65.8; d	75.9; d	67.3; d
3	153.2; d	32.0; t	32.8; t	29.8; t	31.2; t
4	131.6; s	131.8; s	131.9; s	138.1; s	130.6; s
5	194.5; s	194.2; s	195.3; s	146.9; s	174.7; s
6	99.0; s	104.2; s	103.9; s	186.4; s	175.6; s
8	81.3; s	83.4; s	83.1; s	67.0; s	82.1; s
9	31.5; d	32.0; d	31.1; d	42.2; d	31.6; d
10	7.0; t	14.8; t	12.6; t	17.2; t	13.8; t
11	24.6; q	25.0; q	24.7; q	30.5[a]; q	23.8; q
12	29.0; q	29.1; q	29.0; q	30.7[a]; q	29.2; q
1′	43.5; t	143.5; d	143.8; d	109.2; d	152.8; d
2′	205.1; s	63.8; d	64.1; d	160.5; s	78.4; d
3′	30.2; q	22.6; q	22.3; q	14.2; q	18.9; q
OCH_3	—	52.1; q	52.0; q	—	—

[a] Interchangable.

H) and 251 ($M-CH_3OH+H$) as the most important peaks. The NMR data of the two compounds are very similar to each other and to those of papyracon B and C (**7** and **8**)[5]. The structures of compounds **9** and **10** could be determined by ^1H-^{13}C correlation spectroscopy, by which the position of the methoxy group on C-6 was shown by the long-range correlation between the methoxy protons and C-6. The relative configuration of C-1, C-2, C-6 and C-9 of compounds **9** and **10** was established by NOESY correlations between the methoxy protons and 10-Hα as well as 11-H_3, between 9-H and 12-H_3 as well as 10-Hβ, and between 10-Hβ and 2-H. For 1′-H, NOESY correlations were only observed to 2′-H and 3′-H_3, not to 3-H_2, indicating that the double bond is *E* as shown in Figure 1. The relative stereochemistry of C-2′ of papyracon B and C (**7** and **8**) was possible to establish, but due to signal overlap this could not be done for compounds **9** and **10**. However, by comparing the ^{13}C NMR data for the two pairs carbon for carbon, it is evident that the similar difference for each carbon signal observed between papyracon B and C (**7** and **8**)[5] also can be found between compound **9** and **10**, suggesting that compound **9** is 6-*O*-methylpapyracon B and compound **10** is 6-*O*-methylpapyracon C. It is reasonable to assume that compounds **7**/**8** and **9**/**10** are formed by parallel routes, and this is supported by the relative amounts of the two pairs isolated; approximately 50% more of the derivatives **7** and **9** were obtained.

The molecular composition of lachnumfuran A (**11**) was determined by high resolution EI-MS measurements to $C_{14}H_{18}O_4$, and as (according to the NMR data) it contains one carbonyl group and 2 double bonds it should be tricyclic as all the other metabolites related to mycorrhizin A (**1**) isolated from *L. papyraceum*. However, the C-8 hydroxyl proton is observed as a sharp singlet in the ^1H NMR spectrum (long-range ^1H-^{13}C correlations between this and C-8, C-11 as well as C-12 proves its position), showing that the C-6/C-8 ether linkage has been broken. The new ring is by the NMR data shown to be a furan, which is conjugated with the keto function. HMBC correlations are observed between 3′-H_3 and all four furan carbons, although the correlation to C-4 and C-5 are weak, 3-H_2 correlate to C-1, C-2, C-4 and C-5, while the cyclopropane protons 10-H correlate to C-1, C-2, C-6, C-8 and C-9. The small ^1H-^1H coupling constants between 2-H and 3-Hα as well as 3-Hβ (3.8 and 4.0 Hz) suggest that 2-H is equatorial, and the strong NOESY correlation between 1′-H and 3-Hβ (compared to that between 1′-H and 3-Hα) suggest the same for 3-Hβ. The examination of a Dreiding model of lachnumfuran A (**11**) shows that the dihedral angle between 2-H and 3-Hα as well as 3-Hβ in the conformation with the C-2 hydroxyl group axial are approximately equal. 3-Hα, which is on the same side of the ring as 2-H, gives a strong NOESY correlation to 10-Hβ, but not to 9-H, which determines the relative stereostructure of lachnumfuran A (**11**).

The structure determination of lachnumlactone A (**12a**) was facilitated by the preparation of its acetate **12b**. The EI-MS spectra of both **12a** and **12b** looked different compared to those of the other metabolites isolated in

this investigation, only small peaks were observed above *m/z* 155. However, in the mass spectra obtained by chemical ionisation (NH_3) the peaks for M+H and M+NH_4 are dominating, and the molecular weight of **12a** is 266 (corresponding to $C_{14}H_{18}O_5$) while that of the monoacetate **12b** is 308 (corresponding to $C_{16}H_{20}O_6$) suggesting that lachnumlactone A (**12a**) is tricyclic. The compound does not react with diazomethane, indicating that the two carbonyls are part of ester or lactone functionalities, and the 2D NMR data unambiguously determine the structure of lachnumlactone A (**12a**). It is interesting to note that the bond between C-5 and C-6 in the mycorrhizin A skeleton has been broken. Although a similar oxidative cleavage has been observed when chloromycorrhizin A (**2**) was treated with sodium periodate[6], this is the first report of a natural product with this skeleton. For obvious reasons it is difficult to establish the relative stereochemistry of lachnumlactone A (**12a**) by spectroscopic methods, although it would be reasonable to assume that the configuration of C-1/C-2 in lachnumlactone A (**12a**) is the same as in all derivatives of this type that have been isolated from *L. papyraceum*. As with papyracon B and C (**7** and **8**), lachnumlactone A (**12a**) was initially obtained as an epimeric mixture (3:2) which was very difficult to separate. Only the major epimer could be obtained as a pure compound, and it is likely that papyracon A (**7**) is the biosynthetic precursor of lachnumlactone A (**12a**).

Table 4. Nematicidal and cytotoxic activities of compounds **4**, **6**, **9**, **10**, **11**, **12a** and **12b** towards *Caenorhabditis elegans* and mammalian cell lines.

Compound	ND_{90} (μg/ml)	IC_{50} (μg/ml)	
	C. elegans	L1210	HL60
1	1	0.05	0.2
4	>100	>100	n.t.
6	100	100	100
9	100	100	100
10	50	100	100
11	100	100	100
12a	50	50	25
12b	100	50	100

n.t.: Not tested.

ND_{90} is the concentration (in μg/ml) making more than 90% of the worms immobile after 18 hours; IC_{50} is the concentration causing lysis of 50% of the cells after 24 hours.

Biological Properties

All compounds isolated in this investigation except chloromycorrhizinol (**4**) exhibit weak nematicidal activity towards *Caenorhabditis elegans* (results presented in Table 4), although they are less potent compared to mycorrhizin A (**1**). In addition, **4** had no antimicrobial or cytotoxic activity and the antimicrobial and cytotoxic activities of the other compounds were rather weak as can be seen in Tables 4 and 5. Only weak activity in the agar diffusion assay towards Gram-positive bacteria and yeasts was noticed, while filamentous fungi and the Gram-negative *Enterobacter dissolvens* were not sensitive. The cytotoxic effects were measured with two mammalian cell lines, and the most active metabolite is lachnumlactone A (**12a**). The acetate **12b** showed slightly weaker activities than its parent compound.

It is obvious that the biological activities are diminished in the mycorrhizin A derivatives in which the C-2 carbonyl group has been reduced, even if they still contain α,β-unsaturated carbonyl functionalities. The possibility that the cyclopropane ring plays a role for the bioactivities of for instance mycorrhizin A (**1**) should not be neglected.

Acknowledgement

Financial support from the Swedish Natural Science Research Council, the Deutsche Forschungsgemeinschaft and the DAAD is gratefully acknowledged.

Table 5. Antimicrobial activities of compounds **4**, **6**, **9**, **10**, **11**, **12a** and **12b** in the agar diffusion assay after 24 hours.

Organism	Compound						
	4	**6**	**9**	**10**	**11**	**12a**	**12b**
Bacillus brevis ATCC 9999	—	14	—	—	11	9	12
Bacillus subtilis ATCC 6633	—	11	9	—	10	11	10
Micrococcus luteus ATCC 381	—	10	9	—	—	8	—
Enterobacter dissolvens LMG 2683	—	—	—	—	—	—	—
Nematospora coryli ATCC 10647	—	15	—	—	—	—	13
Mucor miehei Tu 284	—	10	—	—	—	—	10
Paecilomyces variotii ETH 114646	—	—	—	—	—	—	—
Penicillum notatum	—	—	—	—	—	—	—

—: No zone of inhibition.

100 μg of each compound was applied to the paper disc (diameter 6 mm).

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