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Graphical Abstract

Secondary Metabolites from the Endophytic Fungus *Pestalotiopsis virgatula* Isolated from the Mangrove Plant *Sonneratia caseolaris*

David Rönsberg[a], Abdessamad Debbab[a], Attila Mándi[b], Victor Wray[c], Haofu Dai[d], Tibor Kurtán[b], Peter Proksch[a], Amal H. Aly[a,*]

[a]Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität, Universitätsstrasse 1, 40225 Düsseldorf, Germany

[b]Department of Organic Chemistry, University of Debrecen, POB 20, 4010 Debrecen, Hungary

[c]Helmholtz Centre for Infection Research, Inhoffenstraße 7, 38124 Braunschweig, Germany

[d]Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, Hainan, P. R. China

[*] Corresponding author. Tel.: +49-211-81-14173; fax: +49-211-81-11923; e-mail: amal.hassan@uni-duesseldorf.de

Abstract

Chromatographic analysis of an ethyl acetate extract of *Pestalotiopsis virgatula* rice cultures yielded four new -pyrone derivatives, pestalotiopyrones I-L, and the new (6*S*,1'*S*,2'*S*)-hydroxypestalotin, in addition to three known compounds. The planar structures of the new compounds were elucidated on the basis of extensive NMR spectroscopic and mass spectrometric analyses. The absolute configurations of the new compounds were determined on the basis of biosynthetic considerations, coupling constants and for pestalotiopyrones L by TDDFT ECD calculations of solution conformers.

1. Introduction

The search for new drugs from natural sources is nowadays becoming more indispensable than ever, due to a comprehensive knowledge on the mode of action in terms of specificity and efficiency, possible side effects and the growing risk of developing resistance. Accordingly, natural products and drugs derived from natural sources increasingly represent a significant proportion among newly developed drugs.[1-3]

Bioactive secondary metabolites from endophytic fungi, isolated from higher plants, are a major focus of natural product research. Of particular interest are plant sources of endophytic fungi, which are able to survive in extreme conditions such as drought, salinity, heat or cold, and with a variety of competing organisms, which live in their natural environments. Examples of such natural habitats where all organisms have to adapt morphologically and physiologically to withstand the existing evolutionary pressure are mangrove forests. Chemical variations are required, inter alia, for these

organisms to obtain evolutionary benefits. These facts make endophytes from such environments very promising targets in the search for new bioactive secondary metabolites.[4-8]

Members of the genus *Pestalotiopsis* have attracted considerable attention in recent years. Many important secondary metabolites that are potential leads for the treatment of human diseases and control of plant diseases have been identified from this genus.[9,10] A prominent example is the discovery of paclitaxel as an endophytic secondary metabolite from the fungal strain *P. microspora*.[9] The genus is characterized by its extensive distribution and the wide genetic and biological variability of its members, which make it a vast reservoir of bioactive natural products still to be explored.[11]

In this study four new α-pyrone derivatives, pestalotiopyrones I-L (**1-4**), a new hydroxypestalotin diastereomer (**5**), as well as three known compounds, hydroxypestalotin, pestalotin and pestalopyrone, were isolated from the mangrove-derived endophytic fungus *P. virgatula* by chromatographic methods. The structure elucidation of the new compounds (**1-5**) (Figure 1) is described in detail.
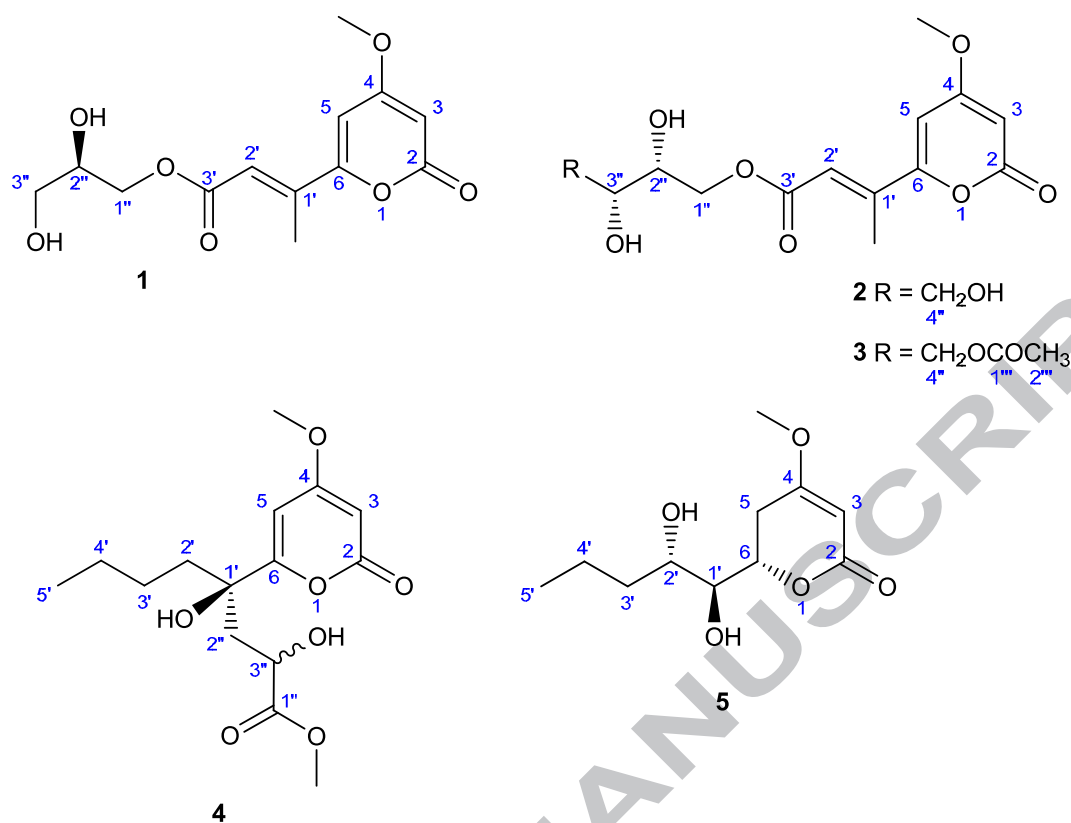
Figure 1. Chemical structures of compounds **1**-**5**.

2. Results and Discussion

The ethyl acetate extract was fractionated by column chromatography initially using silica gel and then Sephadex LH-20 or Diaion HP20 as stationary phases. Each substance was purified by subsequent semi-preparative HPLC.

Compound **1** was found to have the molecular formula $C_{13}H_{16}O_7$ based on the prominent signal at m/z 285.0968 $[M+H]^+$ in the HRESIMS. The ^{1}H NMR spectrum (Table 1) indicated the presence of one olefinic methyl group at δ(H) 2.42 ppm (1'-CH$_3$), one methoxy group at δ(H) 3.94 ppm (4-OCH$_3$), two oxygenated methylene groups at δ(H) 3.63 (CH$_2$-3'') and 4.21/4.30 ppm (CH$_2$-1''), an oxygenated aliphatic proton at δ(H) 3.92 ppm (H-2''), two *meta*-coupled aromatic protons at δ(H) 5.78 (H-3) and 6.6 ppm (H-5), and one olefinic proton singlet at δ(H) 6.74 ppm (H-2'). In total,

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thirteen signals were detected in the ^{13}C NMR spectrum (Table 1) including signals for two ester carbonyl groups, six olefinic and three oxygenated aliphatic carbons, one methoxy and one methyl group. The unsaturated δ-lactone part found in **1** was confirmed by the HMBC correlations of H-3 to C-4 and C-5, and of H-5 to C-3, C-4 and C-6 (Table S1). The position of the methoxy function was proven by its correlation to C-4. Through two-dimensional NMR analyses, it was furthermore shown that the α-pyrone core structure of **1** was attached to a side chain at C-6, which consists of isocrotonic acid esterified with glycerol. The presence of the glycerol moiety was indicated by the COSY correlations establishing the downfield shifted spin system $CH_2(1'')CH(2'')CH_2(3'')$. HMBC correlations of CH_2-1'' to C-2'', of H-2'' to C-1'' and C-3'', and of CH_2-3'' to C-2'' offered further evidence (Table S1). The isocrotonic acid moiety was confirmed by the HMBC correlations of H-2' to C-1', 1'-CH_3 and C-6, and of 1'-CH_3 to C-1', C-2' and C-6 (Table S1). The HMBC correlation of H-5 to the sp^2 carbon C-1' of the side chain confirmed the linkage of C-6 to the β-position of the carboxylic acid. Moreover, esterification with the glyceryl moiety was shown by the correlation of H-1'' to the carboxylate ester atom C-3'. The (*E*) geometry of the isocrotonic acid part was determined by the observed correlation from H-5 to H-2' in the ROESY spectrum. Thus, the structure of **1** was therefore unambiguously determined as 2,3-dihydroxypropyl (2*E*)-3-(4-methoxy-2-oxo-2H-pyran-6-yl)but-2-enoate for which we suggest the name pestalotiopyrone I. Comparison of the $[\alpha]_D$ value of **1** (−13.1, MeOH) with reported values for structurally related compounds as (2*S*)-1-O-*p*-coumaroylglycerol (+12.8, MeOH),[12] (2*R*)-glycerol monoacetate (−9.2, pyridine) and (2*R*)-glycerol monobenzoate (−13.3, pyridine)[13] suggested that the C-2'' chirality center of **1** may have (*R*) absolute configuration.

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Table 1. ^{1}H and ^{13}C NMR data of **1-3** (MeOH-d_4, δ in ppm, J in Hz)

Nr.	1		2		3	
	$\delta_H{}^a$	$\delta_C{}^b$	$\delta_H{}^c$	$\delta_C{}^d$	$\delta_H{}^a$	$\delta_C{}^b$
2		165.6		165.7		165.6
3	5.78, d (1.9)	91.3	5.73, d (2.0)	91.4	5.78, d (2.0)	91.3
4		172.9		173.1		173.0
5	6.6, d (1.9)	104.0	6.55, d (2.0)	104.2	6.6, d (2.1)	104.0
6		160.2		160.3		160.2
1'		144.1		144.1		144.1
2'	6.74, bs	121.0	6.70, d (1.1)	121.3	6.76, d (1.2)	121.1
3'		167.3		167.6		167.4
4'		-		-		-
5'		-		-		-
1"	4.30, dd (4.3, 11.5)	66.7	4.40, dd (2.8, 11.6)	67.5	4.46, dd (2.4, 11.4)	67.1
	4.21, dd (6.3, 11.5)		4.22, dd (6.5, 11.6)		4.28, dd (5.5, 11.5)	
2"	3.92, m	71.1	3.79, m	71.3	3.82, m	71.0
3"	3.63, dd (1.7, 5.4)	64.1	3.60, m	73.6	3.82, m	71.0
4"	-	-	3.59, dd (6.0, 14.4)	64.7	4.37, dd (2.8, 11.6)	67.1
			3.76, dd (3.4, 14.4)		4.20, dd (5.5, 11.5)	
1'''		-		-		172.9
2'''	-	-	-	-	2.12, s	20.8
1'-CH$_3$	2.42, d (0.8)	13.7	2.37, d (1.2)	13.8	2.43, d (1.2)	13.7
1"-OCH$_3$		-		-		-
4-OCH$_3$	3.94, s	57.2	3.89, s	57.4	3.94, s	57.2

Compound **2** had the molecular formula $C_{14}H_{18}O_8$ as indicated by the HRESIMS signal at *m/z* 315.1072 [M+H]$^+$. The ^{1}H and ^{13}C NMR data of **2** (Table 1) resembled those of **1**, except for the existence of one additional hydroxy methine group resonating at δ(H) 3.60 ppm (δ(C) 73.6 ppm, CH-3''), which was in accordance with the increase in molecular weight of **2** by 30 amu compared to **1**. Further confirmation was achieved by the observed COSY correlations of CH$_2$-1'' to H-2'', of H-2'' to H-3'', and of H-3'' to CH$_2$-4'' (Table S1). Accordingly, the side chain of **2** is composed of isocrotonic acid esterified with a butane-1,2,3,4-tetrol unit. Thus, **2** was identified as the new natural product 2,3,4-trihydroxybutyl (2*E*)-3-(4-methoxy-2-oxo-2H-pyran-6-yl)but-2-enoate and given the name pestalotiopyrone J.

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The HRESIMS of **3** exhibited a prominent peak at *m/z* 357.1180 [M+H]+ which is in accordance with the molecular formula $C_{16}H_{20}O_9$. The NMR spectra of **3** showed the same signals detected for **2** as well as signals corresponding to an additional acetyl group (Table 1). The methyl group of the latter was detected at δ(H) 2.12 ppm (δ(C) 20.8 ppm, CH_3-2''') and the ester carbonyl carbon at δ(C) 172.9 ppm (C-1'''). This was further confirmed by the downfield chemical shift of CH_2-4'' (δ(H) 4.20/4.37 and δ(C) 67.1 ppm) and the HMBC correlation observed for CH_2-4'' to the carboxylate ester carbon C-1'''. Compound **3** was thus identified as the new natural product pestalotiopyrone K (4-(acetyloxy)-2,3-dihydroxybutyl (2*E*)-3-(4-methoxy-2-oxo-2H-pyran-6-yl)but- 2-enoate).

In order to determine the relative configuration in combination with the NMR data, conformational analysis of the diastereomeric (*R,R*)- and (*R,S*)-**2** were carried out. Their initial MMFF search provided 700 and 806 conformers, respectively, which were optimized at B3LYP/6-31G(d) with PCM solvent model for chloroform to produce 11 and 18 conformers, respectively (Figures S1 and S2). Since the tetrol moiety showed large conformational flexibility for both diastereomers with versatile dihedral angles and hydrogen bondings, the correlation of the computed conformers with the $^3J_{H,H}$ data did not allow the determination of the relative configuration of **2**. However, biosynthetic considerations indicate that the butane-1,2,3,4-tetrol unit in **2** and **3** may arise from either one of the two natural tetrols, also known as sugar alcohols, threitol [(2*R*,3*R*)-butane-1,2,3,4-tetrol] and erythritol [(2*R*,3*S*)-butane-1,2,3,4-tetraol]. Erythritol is optically inactive due to the presence of a plane of symmetry in its structure (*meso* form), whereas threitol is optically active.[14,15] The [α]$_D$ values of **2** and **3**, measured in MeOH, were −22.5 and −20.1, respectively, suggesting threitol (−14.0 in EtOH)[16] as the tetrol unit of **2** and **3**　provided that the acylation of the tetrol unit did not involve an asymmetric desymmetrization. Although **2** showed a

distinct ECD spectrum determined mainly by the C-2'' chirality center, ECD calculations were not successful as it was not possible to distinguish between the (2''*R*,3''*R*)- and (2''*R*,3''*S*)-epimers.

The molecular formula of pestalotiopyrone L (**4**) was determined to be $C_{15}H_{22}O_7$ by HRESIMS (*m/z* 315.1433, [M+H]$^+$). The ^{1}H NMR data of **4** (Table 2) revealed the presence of two *meta*-coupled aromatic protons (δ(H) 5.59, δ(C) 88.2 ppm, H-3, and δ(H) 6.40, δ(C) 101.3 ppm, H-5), two methoxy groups (δ(H) 3.92, δ(C) 57.0 ppm, 4-OCH$_3$, and δ(H) 3.72, δ(C) 52.6 ppm, 1''-OCH$_3$), an AMX system including a deshielded methine (δ(H) 4.21 ppm, H-2'') and two methylene protons (δ(H) 2.02/2.39 ppm, CH$_2$-3''), and a butyl group (CH$_2$-2'' to CH$_3$-5'). The above observations indicated an α-pyrone core structure for **4**. This was further confirmed by COSY correlations between H-3 and H-5, and HMBC correlations of H-3 to C-2, C-4 and C-5, and of H-5 to C-3, C-4 and C-6 (Table S1). The position of the methoxy group at C-4 was confirmed by its HMBC correlation to C-4 (δ(C) 173.7 ppm) and its ROESY correlations to H-3 and H-5. The 1-hydroxypentyl (C-1' to CH$_3$-5') side chain was disclosed by COSY correlations establishing the butyl spin system CH$_2$(2')CH$_2$(3')CH$_2$(4')CH$_3$(5'), which was confirmed by HMBC correlations of the respective protons, and correlations of CH$_2$-2' to the deshielded quaternary C-1' (δ(C) 76.5 ppm) (Table S1). HMBC correlations of 1''-OCH$_3$, H-2'' and CH$_2$-3'' to the ester carbonyl C-1'' (δ(C) 175.4 ppm) established the 2-hydroxypropanoic acid methyl ester moiety, and those of H-2'' and CH$_2$-3'' to C-1' confirmed its attachment to the side chain at C-1', which was further corroborated by the correlations observed for CH$_2$-2' and CH$_2$-3'' to C-3'' and C-2', respectively (Table S1). Correlations of both CH$_2$-2' and CH$_2$-3'' to C-6 confirmed that the side chain was linked to the α-pyrone at this position. Comparison of the measured NMR data with values reported in the literature showed a close correlation with the known compound PC-2,[17] which lacks the 2-hydroxypropanoic acid methyl ester moiety.

Table 2. ^{1}H and ^{13}C NMR data of **4** and **5** at 600 (^{1}H) and 150 (^{13}C) MHz (δ in ppm, J in Hz)

Nr.	4		5	
	$\delta_H{}^a$	$\delta_C{}^a$	$\delta_H{}^a$	$\delta_C{}^b$
2		167.0		n.d.[c]
3	5.59, d (2.3)	88.2	5.22, d (1.4)	89.7
4		173.7		173.8
5	6.40, d (2.3)	101.3	3.01, ddd (1.3, 13.3, 17.2)	35.9
			2.28, dd (3.7, 17.2)	
6	-	169.5	4.81, ddd (1.1, 3.7, 13.5)	71.7
1'	-	76.5	3.26, dd (1.1, 9.1)	75.5
2'	1.77, dd (4.7, 11.9)	40.9	3.80, dt (2.4, 9.1)	74.1
	1.86, dd (4.6, 11.9)			
3'	1.09, m	26.3	1.85, m	29.6
			1.44, m	
4'	1.33, m	23.9	1.66, m	18.9
			1.44, m	
5'	0.92, t (7.5)	14.3	1.01, t (7.3)	14.0
1"		175.4		-
2"	4.21, dd (3.6, 9.1)	69.3	-	-
3"	2.02, dd (9.1, 14.5)	43.2	-	-
	2.39, dd (3.7, 14.5)			
1"-OCH$_3$	3.72, s	52.6	-	-
4-OCH$_3$	3.92, s	57.0	3. 85, s	56.2

[a]MeOH-d_4 [b]CHCl$_3$-d

[c]not detected

In contrast to **1**-**3**, the C-1' quaternary chirality center of **4** is adjacent to the 2-oxo-2*H*-pyran chromophore and thus the ECD spectra is governed by this chirality center. Conversely, the chiral center at C-3" will have minor influence as it is separated by a further methylene unit. The ECD spectrum of **4** showed a positive Cotton effect (CE) at 278 nm and negative ones at 233, 205 and 195 nm. In order to determine the absolute configuration of C-1', the solution TDDFT-ECD calculation protocol was pursued. The DFT optimization of the 193 initial MMFF conformers of the arbitrarily chosen (1'*S*,2"*S*)-**4** afforded 11 conformers above 1% population, which differed mostly in the orientation of the butyl side chain (Figure S3). In all the conformers, the oxygen of the 1'-OH was co-planar with the 2-oxo-2*H*-pyran ring and its hydrogen was bonded to the oxygen of the 2"-OH fixing the $\omega_{H\text{-}2",C\text{-}2",C\text{-}3",Ha\text{-}3"}$ and $\omega_{H\text{-}2",C\text{-}2",C\text{-}3",Hb\text{-}3"}$ dihedral angles. These dihedral angles were found

around −175° and −58° in all the conformers, which are in accordance with the 9.1 and 3.6 Hz $^3J_{H-2'',H-3''}$ coupling constants. The Boltzmann-averaged ECD spectrum of the conformers of (1'S,2''S)-**4** computed with B3LYP, BH&HLYP and PBE0 functionals and TZVP basis set gave a mirror curve of the solution experimental spectrum (Figure 2), which allowed unambiguous assignment of C-1' as (R). Thus, **4** was identified as the new natural product methyl 2,4-dihydroxy-4-(4-methoxy-2-oxo-2H-pyran-6-yl)octanoate and was named pestalotiopyrone L.
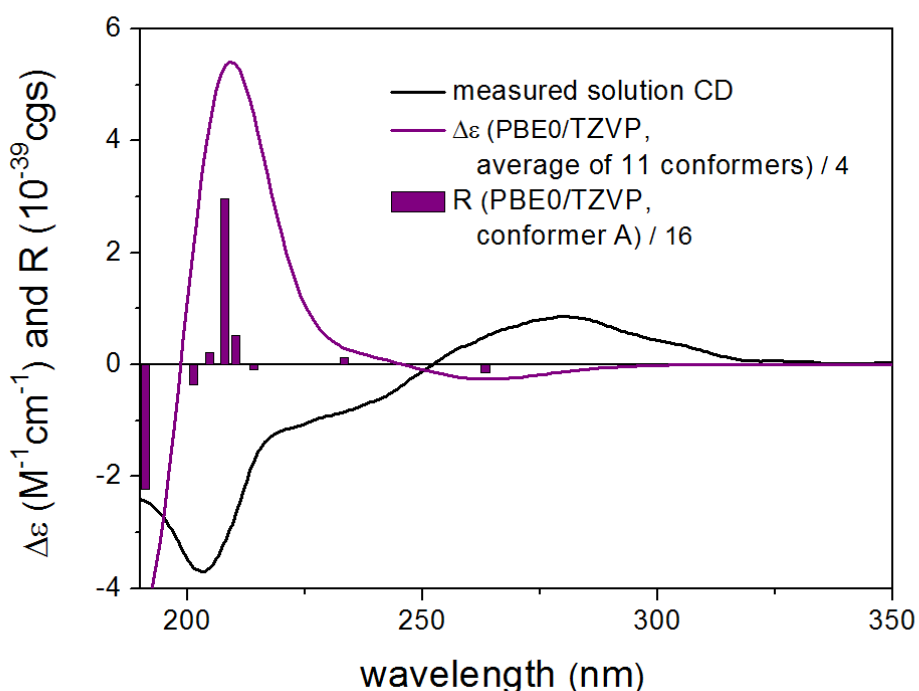
Figure 2. Experimental ECD spectrum of **4** in acetonitrile compared with the Boltzmann-weighted B3LYP/TZVP spectrum calculated for the conformers of (1'S,2''S)-**4** with PCM model for MeCN. Bars represent rotational strength values for the lowest-energy conformer.

The HRESIMS data of **5** and hydroxypestalotin (m/z 231.1227 and 231.1228 [M+H]$^+$, respectively) indicated the same molecular formula of $C_{11}H_{18}O_5$, with three degrees of unsaturation,

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for both compounds. Their UV spectra differed significantly from those recorded for **1**-**4**, which showed UV maxima characteristic for *α*-pyrones,[18] thus indicating that **5** is not an *α*-pyrone derivative. NMR data, however, indicated the presence of a -lactone core structure (Table 2). Hydroxypestalotin [(6*S*,1'*S*,2'*R*)-LL-P880] was identified by comparison of NMR, ECD data and [ ]~D~ value with reported literature data,[19,20] The ECD spectrum of **5** showed a negative CE at 248 nm and a positive one at 218 nm, similar to those of hydroxypestalotin,[20] which proved its (6*S*) absolute configuration. Comparison of the NMR data, including coupling constants, and the optical rotation value of **5** with literature data of (6*S*,1'*S*,2'*R*)-,[19] (6*S*,1'*R*,2'*R*)-,[21] (6*R*,1'*S*,2'*R*)-,[22] (6*R*,1'*R*,2'*R*)-,[21] (6*R*,1'*R*,2'*S*)-,[23] and (6*S*,1'*R*,2'*S*)-LL-P880 [23] showed that ¹H and ¹³C NMR data of **5** were in accordance with those of (6*R*,1'*R*,2'*R*)-LL-P880 , while their optical rotations had opposite signs with similar magnitudes. This led to the conclusion that **5** is the enantiomer of (6*R*,1'*R*,2'*R*)-LL-P880 **and** it was thus assigned as the previously unreported (6*S*,1'*S*,2'*S*)-hydroxypestalotin.

The remaining compounds were identified on the basis of their NMR and mass spectrometric data as well as by comparison with published data as pestalotin[24,25] and pestalopyrone.[26]

All compounds were tested against a panel of selected multidrug-resistant pathogens that included *Klebsiella pneumoniae*, methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However, none of the compounds showed activity against the tested microorganisms (MIC >125 *μ*g/mL). Likewise no cytotoxic activity was detected against murine L5178Y lymphoma cells (cell growth inhibition < 20 % at a concentration of 10 *μ*g/mL). Furthermore, test samples were incorporated into artificial diet and offered to neonate larvae of the polyphagous insect *Spodoptera littoralis* in a nonchoice bioassay. None of the tested samples showed significant larval mortality.

3. Acknowledgement

4. Supplementary data

Supplementary data (figures of calculated low-energy conformers of **2** and **4**, experimental section and compound characterization) associated with this article can be found, in the online version, at http://XXXXXXXXXXXXXX

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