Tetrahedron Letters 58 (2017) 257–261

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Inducing new secondary metabolites through co-cultivation of the fungus *Pestalotiopsis* sp. with the bacterium *Bacillus subtilis*

Shuai Liu [a], Haofu Dai [b], Christian Heering [c], Christoph Janiak [c], Wenhan Lin [d], Zhen Liu [a,*], Peter Proksch [a,*]

[a] Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany
[b] Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, China
[c] Institute of Inorganic and Structural Chemistry, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany
[d] State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100191, China

ARTICLE INFO

Article history:
Received 8 November 2016
Revised 6 December 2016
Accepted 7 December 2016
Available online 9 December 2016

Keywords:
Pestalotiopsis sp.
Bacillus subtilis
Co-cultivation

ABSTRACT

Two new lactones, pestalotiolactones A (**1**) and B (**2**), together with three known compounds (**3**–**5**) were isolated from the axenic culture of the endophytic fungus *Pestalotiopsis* sp., obtained from fruits of *Drepanocarpus lunatus* (Fabaceae). Co-culture of this fungus with *Bacillus subtilis* afforded two new sesquiterpenoids pestabacillins A (**6**) and B (**7**) as well as eight known compounds (**8**–**15**). Their structures were elucidated by extensive analysis of the NMR and MS data as well as by comparison with literature data. All isolated compounds were evaluated for their cytotoxic and antimicrobial activities but proved to be inactive.

Introduction

Fungi and bacteria co-exist in many environments, such as in the oral cavity, on cheese surface, in cyanolichens, on medical devices, on mural paintings as well as in agricultural and forest environments.[1] The long evolutionary coexistence produced different scenarios of fungal-bacterial interactions. For example, *Penicillium* molds can produce β-lactam antibiotics (penicillin G) to inhibit the growth of *Staphylococcus* species,[2] while bacteria can produce lipopeptides (surfactin) to impair the fungal cell membranes.[3] Auxofuran produced by *Streptomyces* sp. can promote the extension of fungal mycelium.[4] On cheese surfaces, the lactate metabolism and alkaline production of the yeasts can create less acidic environment for the growth of some bacteria, which are very important for cheese ripening.[5] The fungus *Cryptococcus neoformans* can use the bacterial melanin precursor homogentisic acid to synthesize melanin to protect itself from UV and other environmental stress.[6]

In previous studies of our group, co-cultivation of fungi with bacteria was shown to induce the accumulation of new secondary fungal metabolites.[7–9] This paper reports the isolation and structure elucidation of metabolites from the mangrove-derived fungus *Pestalotiopsis* sp. in axenic culture and in co-culture with *Bacillus subtilis*. Two new lactones (**1**–**2**) and three known compounds (**3**–**5**) were isolated from the axenic culture of *Pestalotiopsis* sp., while two new sesquiterpenoids (**6**–**7**) and eight known compounds (**8**–**15**) were obtained from co-cultures of *Pestalotiopsis* sp. and *B. subtilis* (Fig. 1).

Results and discussion

Compound **1** was isolated as colorless oil. The molecular formula $C_{10}H_{16}O_3$ was determined on the basis of the HR-ESIMS data, indicating three degrees of unsaturation. The ^{13}C NMR spectrum (Table 1) displayed ten signals including a carbonyl carbon at δ_C 181.6 (C-1), two oxygenated carbons at δ_C 79.5 (C-5) and 81.3 (C-7), three aliphatic methine carbons at δ_C 51.2 (C-3), 45.1 (C-4) and 36.1 (C-2), one aliphatic methylene carbon at δ_C 47.8 (C-6) and three methyl carbons at δ_C 25.6 (C-10), 18.8 (C-8) and 8.3 (C-9). The ^1H NMR spectrum of **1** (Table 1) exhibited signals of four methine groups at δ_H 4.96 (t, H-7), 2.97 (qd, H-2), 2.61 (ddd, H-3) and 1.86 (dq, H-4), signals of a methylene at δ_H 2.15 (d, H-6a) and 1.88 (dd, H-6b) as well as three methyls at δ_H 1.26 (d, Me-8), 1.25 (s, Me-10) and 1.05 (s, Me-9). These data suggested a bicyclic skeleton for **1**. The COSY correlations between Me-9/H-4, H-4/H-3, H-3/H-7 and H-7/H-6b along with key HMBC correlations from Me-10 to C-4, C-5 and C-6 established a cyclopentane ring with a hydroxy group at the C-5 position and two methyl groups

* Corresponding authors.
E-mail addresses: zhenfeizi0@sina.com (Z. Liu), proksch@uni-duesseldorf.de (P. Proksch).

http://dx.doi.org/10.1016/j.tetlet.2016.12.026
0040-4039/

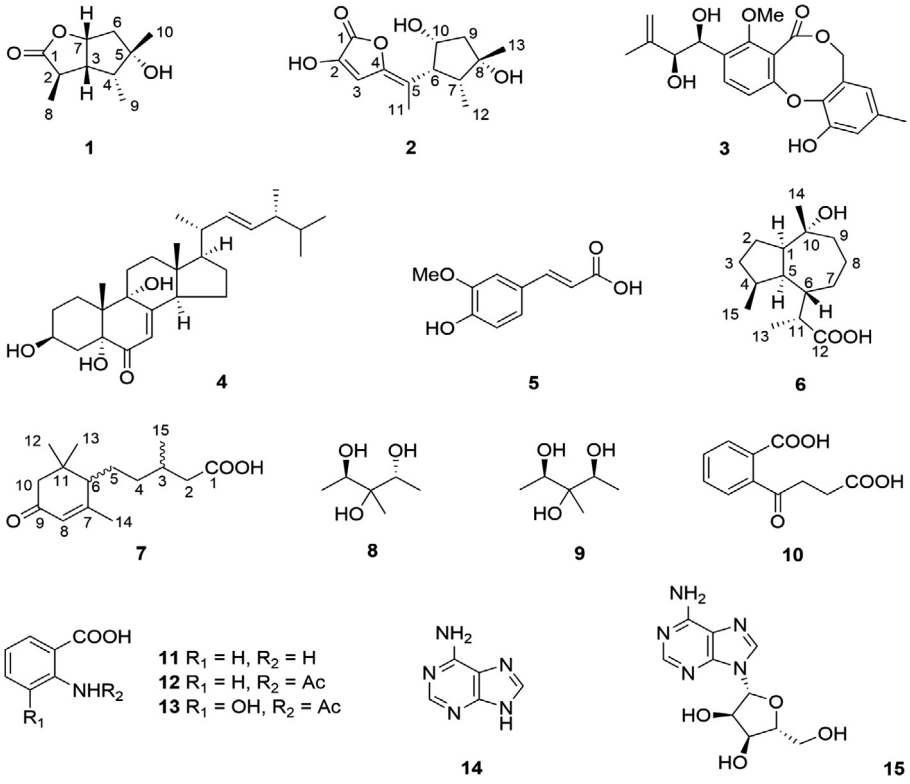

Fig. 1. Structures of isolated compounds.

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of **1** and **2**.

Position	**1**[a]		**2**[b]	
	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type
1		181.6, C		168.0, C
2	2.97, qd (7.6, 5.1)	36.1, CH		145.4, C
3	2.61, ddd (8.2, 7.8, 5.1)	51.2, CH	6.72, s	110.1, CH
4	1.86, dq (8.2, 7.0)	45.1, CH		145.5, C
5		79.5, C		121.8, C
6	2.15, d (15.3)	47.8, CH_2	3.71, dd (9.6, 6.6)	48.9, CH
	1.88, dd (15.3, 7.0)			
7	4.96, ddd (7.8, 7.0)	81.3, CH	2.16, dq (9.6, 7.5)	48.0, CH
8	1.26, d (7.6)	18.8, CH_3		79.1, C
9	1.05, d (7.0)	8.3, CH_3	2.09, dd (13.7, 6.6)	50.8, CH_2
			2.03, dd (13.7, 6.6)	
10	1.25, s	25.6, CH_3	4.34, q (6.6)	75.2, CH
11			2.08, s	17.0, CH_3
12			0.89, d (7.5)	10.8, CH_3
13			1.30, s	29.6, CH_3

[a] Recorded in $CDCl_3$.
[b] Recorded in CD_3OD.

at C-4 and C-5 (Fig. 2). The presence of an additional five-membered lactone ring was confirmed by the COSY correlations between H-3/H-2 and H-2/Me-8 in addition to key HMBC correlations from H-7 and Me-8 to C-1. The relative configuration of **1** was determined by ROESY data. The NOE relationships between H-7/H-6b, H-7/H-3, H-3/Me-8, H-3/H-4, H-4/Me-10, Me-10/H-6b suggested that these protons were β-oriented, while the NOE correlation between H-2 and Me-9 indicated their α-orientation (Fig. 2). Thus, the structure of compound **1** was elucidated as shown, for which the name pestalotiolactone A is proposed.

Pestalotiolactone B (**2**) possessed the molecular formula $C_{13}H_{18}O_5$ as determined by the HR-ESIMS data, with five degrees of unsaturation. In the ^{13}C NMR spectrum of **2** (Table 1), thirteen signals including a carbonyl carbon at δ_C 168.0 (C-1), four olefinic carbons at δ_C 145.5 (C-4), 145.4 (C-2), 121.8 (C-5) and 110.1 (C-3), two oxygenated aliphatic carbons at δ_C 79.1 (C-8) and 75.2 (C-10), two aliphatic methine carbons at δ_C 48.9 (C-6) and 48.0 (C-7), one aliphatic methylene carbon at δ_C 50.8 (C-9) and three methyl carbons at δ_C 29.6 (C-13), 17.0 (C-11) and 10.8 (C-12) were observed. The ^{1}H NMR spectrum of **2** (Table 1) showed one olefinic proton at δ_H 6.72 (t, H-3), one proton attached to an oxygenated carbon at δ_H 4.34 (q, H-10) and three methyls at δ_H 2.08 (s, Me-11), 1.30 (s, Me-13) and 0.89 (d, Me-12). The COSY correlation between H-9a (δ_H 2.09, dd)/H-10, H-9b (δ_H 2.03, dd)/H-10, H-10/H-6 (δ_H 3.71, dd), H-6/H-7 (δ_H 2.16, dd) and H-7/Me-12, together with the HMBC correlations from Me-13 to C-7, C-8 and C-9

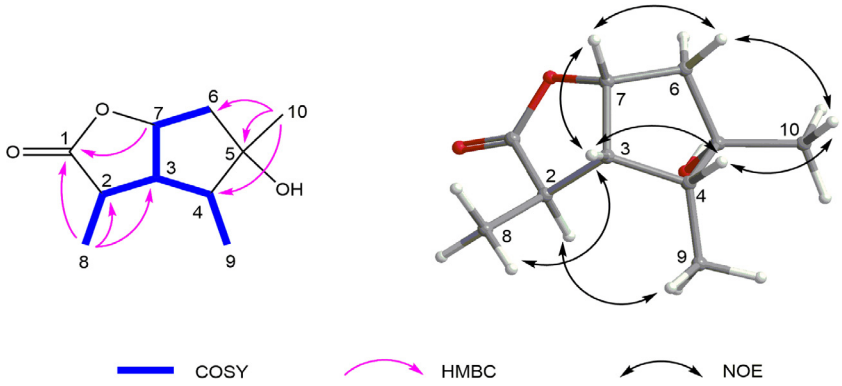

Fig. 2. COSY, key HMBC and NOE correlations of compound **1**.

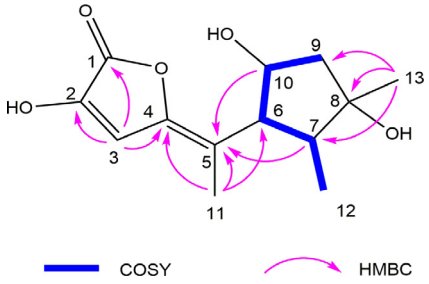

Fig. 3. COSY and key HMBC correlations of compound **2**.

indicated the presence of a cyclopentane ring with a hydroxy group at the C-8 position and two methyl groups at the C-7 and C-8 positions in **2** (Fig. 3). The partial structure from C-4 to C-6 was deduced by the HMBC correlations from Me-11 to C-4, C-5 and C-6 and from H-7 and H-10 to C-5. Combined with its molecular formula, the HMBC correlations from H-3 to C-1, C-2 and C-4 confirmed a lactone substructure from C-1 to C-4 with a hydroxyl group at C-2. The NOE correlation between H-3 and Me-11 was in accordance with a *Z*-configurated C-4/C-5 double bond. The NOE correlations between H-10/H-6, H-6/H-7 and H-7/Me-13 indicated that these protons were oriented on the same face of the ring.

In addition to these new natural products, three known compounds including pestalotiollide B (**3**),[10] 3β,5α,9α-trihydoxyergosta-7,22-diene-6-one (**4**)[11] and (*E*)-ferulic acid (**5**)[12] were also isolated from the axenic culture of the endophytic fungus *Pestalotiopsis* sp.

When the fungus was co-cultivated with *Bacillus subtilis*, the resulting HPLC chromatograms were remarkably different from those of the axenic fungal culture. Ten compounds that were not present in the extract of the axenic fungal culture were isolated, including two new sesquiterpenoids pestabacillins A (**6**) and B (**7**).

Compound **6** was isolated as white amorphous powder. Its molecular formula $C_{15}H_{26}O_3$ was evident from the HRESIMS data. The ^{1}H NMR spectrum (Table 2) indicated the presence of three methyls at δ_H 1.24 (s, Me-14), 0.96 (d, Me-13) and 0.90 (d, Me-15) while the ^{13}C NMR spectrum exhibited fifteen signals including a carbonyl at δ_C 180.1 (C-12), an oxygenated quaternary carbon at δ_C 76.2 (C-10), five methines, five methylenes and three methyls at δ_C 29.6 (C-13), 17.0 (C-11) and 10.8 (C-12). The COSY correlations between Me-15/H-4 (δ_H 2.07, m), H-4/H_2-3, H_2-3/H_2-2, H_2-2/H-1 (δ_H 2.34, q) and H-1/H-5 (δ_H 2.08, m) as well as the HMBC correla-

Table 2
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of **6** and **7** in CD_3OD.

Position	**6**		**7**	
	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type
1	2.34, q (10.0)	52.7, CH		176.6, C
2	1.87, m 1.79, m	28.3, CH_2	2.28, dd (14.9. 6.7) 2.14, ddd (14.9. 7.4, 1.7)	42.2, CH_2
3	1.60, m 1.49, m	33.7, CH_2	1.91, m	31.8, CH
4	2.07, m	37.0, CH	1.51, m 1.32, m	37.1, CH_2
5	2.08, m	48.9, CH	1.81, m 1.48, m	28.6, CH_2
6	2.16, ddd (12.0, 10.0, 2.5)	41.1, CH	1.96, m	52.2, CH
7	1.56, m 1.16, m	31.5, CH_2		169.2, C
8	1.73, m 1.39, m	26.6, CH_2	5.81, s	125.0, CH
9	1.84, m 1.52, m	49.0, CH_2		201.9, C
10		76.2, C	2.45, d (17.4) 1.99, d (17.4)	47.9, CH_2
11	2.59, qd (7.0, 2.5)	43.3, CH		36.9, C
12		180.1, C	1.08, s	27.2, CH_3
13	0.96, d (7.0)	8.8, CH_3	1.02, s	28.8, CH_3
14	1.24, s	23.6, CH_3	2.03, s	24.5, CH_3
15	0.90, d (7.0)	14.7, CH_3	0.98, d (6.7)	19.7, CH_3

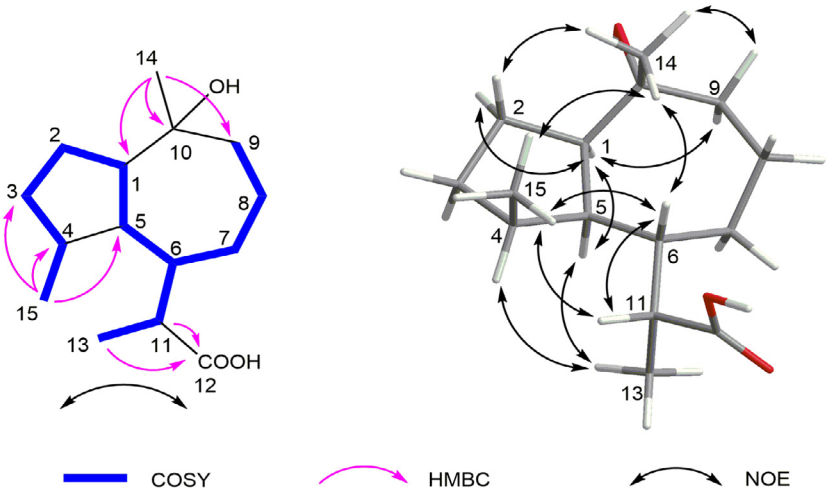

Fig. 4. COSY, key HMBC and NOE correlations of compound **6**.

tions from Me-15 to C-3 (δ_C 33.7), C-4 (δ_C 37.0) and C-5 (δ_C 48.9) revealed the presence of a cyclopentane ring with a methyl substituent attached to C-4 in compound **6** (Fig. 4). An additional seven-membered ring fused at the C-1 and C-5 positions was confirmed by the COSY correlations between H-5/H-6/H_2-7/H_2-8/H_2-9 and the HMBC correlation from Me-14 to C-1 (δ_C 52.7), C-9 (δ_C 49.0) and C-10. Furthermore, the COSY correlations between H-6 (δ_H 2.16, ddd)/H-11 (δ_H 2.59, qd) and H-11/Me-13 and the HMBC correlations from H-11 and Me-13 to C-12 indicated a 1-carboxyethyl group to be attached at the C-6 position. Thus, the planar structure of **6** was elucidated as shown, bearing a zierane-type sesquiterpene skeleton. In the ROESY spectrum of **6**, H-1 showed correlations to H-2a (δ_H 1.87) and H-9b (δ_H 1.52), while Me-14 exhibited correlations to H-2b (δ_H 1.79) and H-9a (δ_H 1.84), suggesting a *trans* orientation of H-1 and Me-14. Assuming H-1 was oriented on the α-face of the ring, Me-14 was accordingly β-oriented (Fig. 4). The NOE correlations between Me-14/H-6, Me-14/Me-15, H-6/Me-15 and H-1/H-5 indicated β-orientation of H-6 and Me-15 while H-4 and H-5 were α-oriented. In addition, the configuration at C-11 was deduced from the NOE correlations from H-11 to H-6 and Me-15 as well as from Me-13 to H-4 and H-5. Furthermore, the relative configuration of **6** was confirmed by X-ray diffraction (Fig. 5).

Compound **7** was isolated as a colorless oil. The molecular formula was determined to be $C_{15}H_{24}O_3$ based on the HR-ESIMS spectrum. The ^{1}H NMR spectrum of **7** (Table 2) showed an olefinic

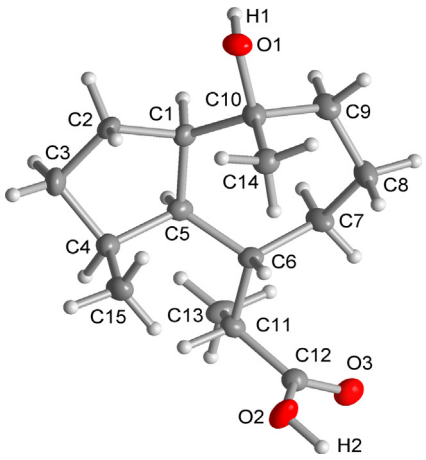

Fig. 5. Molecular structure of **6** from single-crystal X-ray diffractometry.

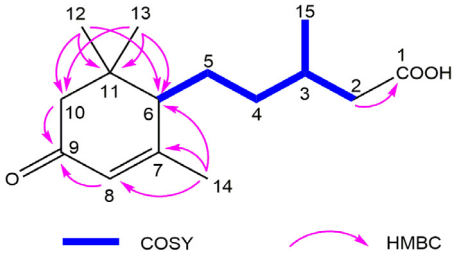

Fig. 6. COSY and key HMBC correlations of compound **7**.

proton at δ_H 5.81 (s, H-8) and four methyl groups at δ_H 2.03 (s, Me-14), 1.08 (s, Me-12), 1.02 (s, Me-13) and 0.98 (d, Me-15), respectively. The HMBC correlations from Me-12 and Me-13 to C-6 (δ_C 52.2), C-10 (δ_C 47.9) and C-11 (δ_C 36.9), from H-8 and H_2-10 (δ_H 2.45 and 1.99) to C-9 (δ_C 201.9) and from Me-14 to C-6, C-7 (δ_C 169.2) and C-8 (δ_C 125.0) established a cyclohexenone substructure with one methyl group at C-7 and two methyls at C-11 (Fig. 6). In addition, a 3-methyl-4-carboxybutyl side chain at the C-6 position was deduced from the COSY correlations between H-6/H_2-5, H_2-5/H_2-4, H_2-4/H-3, H-3/H_2-2 and H-3/Me-15 as well as from the HMBC correlations from H_2-2 to C-1 (δ_C 176.6). Thus, the planar structure of **7** was elucidated as shown. Due to the conformational flexibility of the side-chain, the relative configuration of the two chiral centers at C-3 and C-6 remained unsolved.

The additional known compounds were identified as 3-methyl-2β,3,4α-pentanetriol (**8**),[13] 3-methyl-2β,3,4β-pentanetriol (**9**),[14] *o*-succinylbenzoic acid (**10**),[15] anthranilic acid (**11**),[16] *N*-acetylanthranilic acid (**12**),[17] *N*-acetyl-3-hydroxyanthranilic acid (**13**),[18] adenine (**14**),[19] and adenosine (**15**).[20]

During the co-cultivation experiment with *B. subtilis*, the fungus *Pestalotiopsis* sp. survived but showed a severe growth retardation compared with the axenic fungal control. It took the fungus close to 100 days to cover the surface of the solid rice medium completely, while for the axenic fungal control this time was only 28 days, indicating the severe stress for the fungus during the co-cultivation experiment. When *Pestalotiopsis* sp. was co-cultured with *Streptomyces lividans*,[21] no such retardation of fungal had been observed suggesting that presence of *B. subtilis* causes a serious stress for the fungus when present in the same culture vessel. One reason for this might by the production of known fungicidal compounds such as iturins[22] by *B. subtilis* even though no attempt was made in this study to detect these metabolites in the culture flasks.

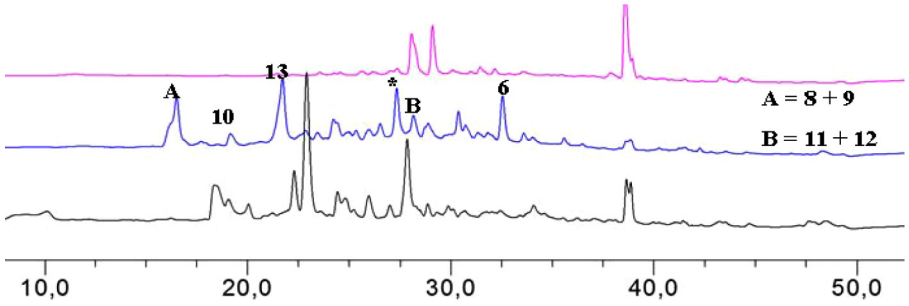

Fig. 7. HPLC chromatograms of the EtOAc extracts from co-culture experiments detected at 235 nm: axenic control of *Pestalotiopsis* sp. (pink), axenic control of *B. subtilis* (brown), co-culture of *Pestalotiopsis* sp. with *B. subtilis* (blue) (*peak disappeared after VLC).

The extract resulting from co-cultivation of *Pestalotiopsis* sp. with *B. subtilis* differed completely from that of axenic fungal control, but also from that of axenically grown *B. subtilis* as shown by HPLC analysis (Fig. 7). Peak A turned out to be a mixture of the two diastereoisomers 3-methyl-2β,3,4α-pentanetriol (**8**) and 3-methyl-2β,3,4β-pentanetriol (**9**) with a ratio of 3:1. Since compound **9** was previously reported from the mangrove-derived bacterium *Bacillus* sp.,[14] the producer of **8** and **9** in the co-culture experiment conducted in this study is likewise suggested to be *B. subtilis*. *o*-Succinylbenzoic acid (**10**), which is a well-known intermediate during the biosynthesis of vitamin K_2 in several gram positive bacteria including *B. subtilis*,[15] is likewise proposed to be produced by *B. subtilis*. The same is suggested for the anthranilic acid derivatives (**11**–**13**) since anthranilic acid (**11**) was repeatedly obtained from co-cultures of different fungi with the same bacterial strain *B. subtilis* as employed in this study.[7,8] Adenine (**14**) and adenosine (**15**) are ubiquituous metabolites, hence no suggestion with regard to the possible producer can be made. Compound **6** is a novel zierane-type sesquiterpene derivative, whose analogues are only known from plants,[23] while compound **7** is a derivative of abscisic acid, which is a plant hormone that has also frequently been isolated from endophytic fungi.[24] Thus compounds **6** and **7** are suggested to be produced by the fungus *Pestalotiopsis* sp.

All isolated new compounds (**1**, **2**, **6** and **7**) were tested for their cytotoxicity against the L5178Y mouse lymphoma cell line. However, none of them showed activity at a dose of 10 μg/mL. In addition, the four new compounds (**1**, **2**, **6** and **7**) were evaluated for their antibacterial activities against *Mycobacterium tuberculosis*, *Staphylococcus aureus* (ATCC25923), *S. aureus* (ATCC700699), *Enterococcus faecalis* (ATCC29212), *E. faecalis* (ATCC51299), *E. faecium* (ATCC35667), *E. faecium* (ATCC700221) and *Acinetobacter baumannii* (ATCCBAA1605). Only compound **6** showed very weak inhibitory effect against *M. tuberculosis* (MIC > 50 μM). Since the fungus grew very slowly during co-cultivation, compounds **6**–**15** were tested for the antifungal activity against *Pestalotiopsis* sp. by agar diffusion test. However, no inhibition was observed when tested at a dose of 50 μg.

In summary, compounds **1** and **2** are bicyclic lactones isolated from the EtOAc extract of the axenically grown fungus *Pestalotiopsis* sp. and they share the same 1,2-dimethylcyclopentane subunit. Since **1** is a monoterpenoid and **2** contained three additional carbons compared to **1**, the latter compound is suggested to be formed through condensation of phosphoenolpyruvate (PEP) and a monoterpene moiety. Compounds **6** and **7** are sesquiterpenoids that were obtained from the co-culture of *Pestalotiopsis* sp. and *B. subtilis*. Both are suggested to be produced by the fungus as a consequence of the presence of the bacterium that caused a severe retardation of the fungal growth.

Acknowledgments

S.L. thanks China Scholarship Council for financial support. P.P. wants to thank the Manchot Foundation for support.

A. Supplementary data

Supplementary data (UV, MS and NMR spectra of **1**, **2**, **6** and **7** as well as X-ray data of **6**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.12.026.

References

1. Frey-Klett P, Burlinson P, Deveau A, Barret M, Tarkka M, Sarniguet A. *Microbiol Mol Biol Rev*. 2011;75:583–609.
2. Fleming A. *Br J Exp Pathol*. 1929;10:226–236.
3. Heerklotz H, Seelig J. *Eur Biophys J*. 2007;36:305–314.
4. Riedlinger J, Schrey SD, Tarkka MT, Hampp R, Kapur M, Fiedler HP. *Appl Environ Microbiol*. 2006;72:3550–3557.
5. Corsetti A, Rossi J, Gobbetti M. *Int J Food Microbiol*. 2001;69:1–10.
6. Frases S, Salazar A, Dadachova E, Casadevall A. *Appl Environ Microbiol*. 2007;73:615–621.
7. Ola ARB, Thomy D, Lai D, Brötz-Oesterhelt H, Proksch P. *J Nat Prod*. 2013;76:2094–2099.
8. Chen H, Daletos G, Abdel-Aziz MS, et al. *Phytochem Lett*. 2015;12:35–41.
9. Ebrahim W, El-Neketi M, Lewald LI, et al. *J Nat Prod*. 2016;79:914–922.
10. Xu J, Aly AH, Wray V, Proksch P. *Tetrahedron Lett*. 2011;52:21–25.
11. Kawagishi H, Katsumi R, Sazawa T, Mizuno T, Hagiwara T, Nakamura T. *Phytochemistry*. 1988;27:2777–2779.
12. Xing X, Ho P, Bourquin G, Yeh LA, Cuny GD. *Tetrahedron*. 2003;59:9961–9969.
13. Talontsi FM, Kenla TJN, Dittrich B, Douanla-Meli C, Laatsch H. *Planta Med*. 2012;78:1020–1023.
14. Tang J, Gao H, Hong K, et al. *Chin J Med Chem*. 2008;18:206–209.
15. Plataniappan C, Taber H, Meganathan R. *J Bacteriol*. 1994;176:2648–2653.
16. Regulska E, Samsonowicz M, Świsłocka R, Lewandowski W. *J Mol Struct*. 2009;936:162–170.
17. Giri R, Lam JK, Yu JQ. *J Am Chem Soc*. 2010;132:686–693.
18. Hund HK, De Beyer A, Lingens F. *Biol Chem Hoppe Seyler*. 1990;371:1005–1008.
19. Saladino R, Crestini C, Neri V, Ciciriello F, Costanzo G, Mauro ED. *ChemBioChem*. 2006;7:1707–1714.
20. Patching SG, Baldwin SA, Baldwin AD, et al. *Org Biomol Chem*. 2005;3:462–470.
21. Liu S, Dai H, Makhloufi G, et al. *J Nat Prod*. 2016;79:2332–2340.
22. Gueldner RC, Reilly CC, Pusey PL, et al. *J Agric Food Chem*. 1988;36:366–370.
23. Nakashima K, Oyama M, Ito T, et al. *Chem Biodiversity*. 2012;9:2195–2202.
24. Zeevaart JAD. *Ann Rev Plant Physiol Plant Mol Biol*. 1988;39:439–473.