Organic & Biomolecular Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Journal Name

RSCPublishing

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

Antibacterial epipolythiodioxopiperazine and unprecedented sesquiterpene from *Pseudallescheria boydii*, a beetle (Coleoptera)-associated fungus

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Pseudallescheria boydii residing in the gut of coleopteran (*Holotrichia parallela*) larva produces four new epipolythiodioxopiperazines (ETPs) boydines A−D (**3**−**6**) and two novel sesquiterpene boydenes A (**7**) and B (**10**), in addition to bisdethiobis(methylthio)-deacetylaranotin (**1**), bisdethiodi(methylthio)-deacetylapoaranotin (**2**), AM6898 A (**8**) and ovalicin (**9**). The structure elucidation was accomplished by a combination of spectral methods with quantum chemical calculations of optical rotations and electronic circular dichroism (ECD) spectra. Boydine B (**4**) was shown to be active against the clinical strains *Bifidobacterium* sp., *Veillonella parvula*, *Anaerostreptococcus* sp., *Bacteroides vulgates* and *Peptostreptococcus* sp. with an MIC range of 0.2−0.8 μM, and the pharmacophore 3-hydroxy-2,4,6-trimethyl-5-oxooct-6-enoyl chain of 4 was determined to have (2*R*,3*S*,4*S*)-configurations. Boydene A (**7**) possessed an unprecedented carbon skeleton, suggesting an unusual biochemistry that permits an intramolecular Aldol addition in the fungus. Collectively, the finding may inspire the discovery of new antibacterial agents and the understanding on biosyntheses of polythiodioxopiperazine and sesquiterpene metabolites.

Introduction

Some insect-associated fungi such as *Paecilomyces tenuipes* and *Cordyceps sinensis* are folk remedies capable of treating diseases due to the presence of biologically active molecules produced as a result of their long-time co-evolution.[1−3] The cross-kingdom interactions between fungus and insect promoted the generation of promising compounds valuable for further development of antimalarial, immunosuppressive and antimicrobial drugs.[4−7] The fungus *Pseudallescheria boydii* of Phylum Ascomycota has a broad host range, and its secondary metabolite profiles seem residence-dependent. As noted, the fungus has been found to be an opportunistic fungal pathogen that causes fatal invasive infections in both immunocompromised patients and immunocompetent individuals.[8, 9] It produces an array of biomolecules such as glucosylceramides, glycoconjugates, and polysaccharides, which are involved in cell differentiation, cell recognition and virulence.[10, 11] Concerning its generation of bioactive secondary metabolites, the antifungal tyroscherin and N-methyltyroscherin have been characterized from a culture of *P. boydii* isolated from a termite *Nasutitermes* sp.;[12] the *P. boydii* strain residing in the littoral plant *Sesuvium portulacastrum* is able to produce cytotoxic polyketide boydone B;[13] another *P. boydii* strain isolated from afflicted patients generates cyclic peptides pseudacyclins A−E.[14] Few attempts, however, focused on the small molecule compounds biosynthesized by any *P. boydii* strain associated with insects. In the light of the speedier gene modification of symbionts,[15, 16] the intricate interaction of *P. boydii* with its insect host was presumed to facilitate the production of a uniquely structured compound library, from which new bioactive molecules may be screened. As hypothesized, the *P. boydii* BC-f4 isolated from the gut of *Holotrichia parallela* larva has been shown here to produce a diverse array of secondary metabolites including four new

epipolythiodioxopiperazines (ETPs) we have named boydines A−D (**3**−**6**), and two novel sesquiterpenes boydenes A (**7**) and B (**10**) with the former representing an unprecedented sesquiterpene skeleton (Fig. 1). Boydine B (**4**) was assessed to be active against a panel of clinical anaerobic bacteria with an MIC range of 0.2−0.8 μM. The unusual framework of boydene A (**7**) suggests an interesting metabolomic branch point where the skeletal diversification starts to generate novel shunt products through the ovalicin-related biosynthetic pathway.

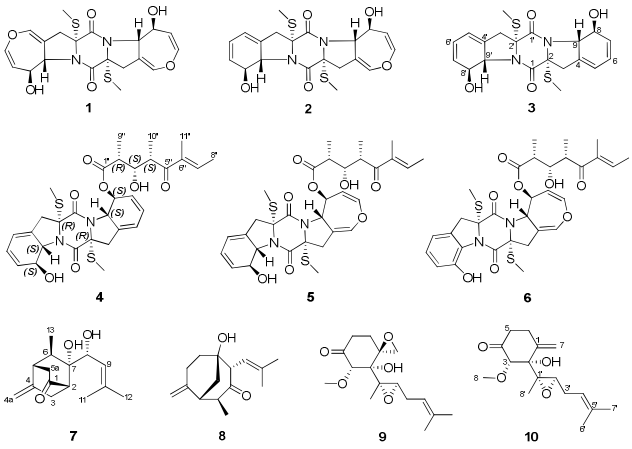

Fig. 1 Structures of **1**−**10**.

Results and discussion

Structural elucidation of boydines A−D and boydenes A−B

Compounds **1** and **2** were spectrally identified as bisdethiobis(methylthio)-deacetylaranotin and bisdethiobis(methylthio)-deacetylapoaranotin, respectively.[17, 18] The molecular formula of boydine A (**3**) was indicated to be $C_{20}H_{22}N_2O_4S_2$ by the protonated molecular ion at *m/z* 419.1099 $[M+H]^+$ in its high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) ($C_{20}H_{23}N_2O_4S_2$ requires 419.1094). Its intense IR absorption bands at 3360 and 1642 cm^{-1} suggested the presence of hydroxyl and amide groups. The ^1H and ^{13}C NMR spectra of **3** suggested that it was a symmetrical molecule (Table 1). In particular, its ^{13}C NMR spectrum gave only ten resonance lines consisting of a methyl (δ_C 14.9), a methylene (δ_C 38.6), five methine (an oxygenated one at δ_C 74.5) and three quaternary carbon signals (δ_C 167.4, 131.2 and 73.0). This hypothesis was confirmed by its 2D NMR results (^1H-^1H COSY, ROESY and HMBC) which allowed the exact assignment of all ^1H and ^{13}C NMR signals. In the HMBC spectrum of **3**, both pairs of diastereotopic protons (H-3/3') correlated with C-5(5') and C-9(9'). These spectral data corroborated collectively that compound **3** was a diketopiperazine condensed from two molecules of 7-hydroxy-2-(methylthio)-2,3,7,7a-tetrahydro-1H-indole-2-carboxylic acid. The formulated relative configuration of **3** was supported by its ROESY spectrum, and its absolute stereochemistry was elucidated to be 2*R*,2'*R*,8*S*,8'*S*,9*S*,9'*S* by comparing its experimental CD curve with the CD spectra calculated for all options (Fig. S8). Compound **3** was hypothesized to be the precursor of **1** and **2** in *Aspergillus terreus*, which was later confirmed by the presence of diketopiperazine in all three compounds within a single batch culture of the title fungus .[17]

Boydine B (**4**) displayed a molecular ion at *m/z* 637.2016 $[M+Na]^+$ in its HR-ESI-MS spectrum, suggesting a molecular formula of $C_{31}H_{38}N_2O_7S_2$ (calcd. for $C_{31}H_{38}N_2O_7S_2Na$, 637.2013). Though comparable to those of **3**, the ^1H and ^{13}C NMR spectra of **4** were typical of a nonsymmetric bis-(methylthio)-diketopiperazine (Table 1). The ^1H NMR spectrum of **4,** however, displayed an extra set of four methine signals at δ_H 2.58 (qd, *J*=7.0, 5.0 Hz, H-2"), 4.22 (dd, *J*=7.0, 5.0 Hz, H-3"), 3.51 (dq, *J*=7.5, 7.0, H-4") and 6.94 (qq, *J*=7.0, 1.0 Hz, H-7"), and four methyl resonances at δ_H 1.89 (dd, *J*=7.0, 1.0 Hz, H-8"), 1.14 (d, *J*=7.0 Hz, H-9"), 1.16 (d, *J*=6.5 Hz, H-10"), 1.76 (t, *J*=1.0 Hz, H-11"). This suggests that boydine B (**4**) might have a 3-hydroxy-2,4,6-trimethyl-5-oxooct-6-enoyl group,[18] which resonated in its ^{13}C NMR spectrum at δ_C 174.7 (C-1"), 43.8 (C-2"), 73.7 (C-3"), 43.1 (C-4"), 205.1 (C-5"), 138.1 (C-6"), 138.6 (C-7"), 15.0 (C-8"), 11.3 (C-9"), 14.9 (C-10") and 11.2 (C-11") (Table 1). This assumption was confirmed by its 2D NMR results (^1H-^1H COSY, HSQC, ROESY and HMBC), which led to the unequivocal assignment of all ^1H and ^{13}C NMR signals. The location of the acyl chain on C-8 is supported by the magnitude of the H-8 signal at δ_H 6.02, moved downfield by >1.0 ppm from that of **3**, and the ROESY cross-peaks of H-8" with H-7" and H-11", but no correlation between H-7" and H-11", indicating a *trans* configuration of the 6",7"-double bond. The relative configuration of the diketopiperazine motif of **4** was assigned by its ROESY spectrum. Biogenetic consideration suggested that **4** might be identical with **3** in the absolute configuration of the diketopiperazine motif. This proposal was confirmed by the liberation of **3** upon the alkaline hydrolysis of **4** as detailed in the Experimental Section.

The acyl residue of **4** has been found to be a motif of diketopiperazine derivatives, but its stereochemistry remains unverified.[18] Furthermore, the acyl moiety seems to be the pharmacophoric moiety since compound **4** is substantially antibacterial whereas its unacylated analogue **3** is inactive. The close association of the acyl unit with bioactivity motivated us to clarify the configuration of the releasable acid which remained a new chemical entity. Thus, the acid (**11**) liberated from **4** was purified and analyzed to have a molecular formula of $C_{11}H_{18}O_4$, which was supported by the molecular ion at *m/z* 237.1090 $[M+Na]^+$ ($C_{11}H_{18}O_4Na$ requires 237.1091). The ^1H and ^{13}C NMR spectral data of **11** (Table S1) were assigned unequivocally by its ^1H-^1H COSY, HSQC, ROESY and HMBC spectra. Compound **11** was demonstrated to be 3-hydroxy-2,4,6-trimethyl-5-oxooct-6-enoic acid, which, to our knowledge, was not described before. To understand its stereochemistry, the ROESY spectra of **4**−**6** (see below) and **11** were correlatively scrutinized to guarantee the recognition of all key cross-peaks. In particular, the eight possible configurations of acid **11** were reduced to two options (*RSS* and *SRR* for C-2~C-4), which might have permitted the ROESY correlations of H-3 with H-2 (but not H-4), and of H-10 with H-3 and 3-OH as illustrated in the Newman projection formulae **A**/**A*** and **B**/**B*** (Fig. 2). Subsequently, optical rotations ($[\alpha]_D$) of this enantiomer pair of **11** were calculated and compared with the experimental value, which is a believable approach for

Table 1 ^{1}H and ^{13}C NMR data of **3**−**6** (*J* in Hz).

Position	**3** ($CDCl_3$)[a]		**4** (acetone-d_6)[a]		**5** (acetone-d_6)[a]		**6** (acetone-d_6)[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		167.4		169.2		165.1		164.7
2		73.0		74.7		71.2		71.4
3	(α) 2.93, d (16.0)	38.6	3.04, br d (15.5)	39.6	3.18, dt (16.0, 1.5)	40.2	3.32, br d (15.6)	40.2
	(β) 3.08, d (16.0)		3.14, br d (15.5)		3.22, dt (16.0, 2.0)		3.42, br d (15.6)	
4		131.2		135.6		112.1		111.8
5	5.97, br s	120.6	6.02-6.09, m	120.4	6.76, br s	138.4	6.81, br t (1.5)	138.4
6	5.90, br d (9.5)	122.9	6.02-6.09, m	125.9	6.40, dd (8.3, 2.3)	140.6	6.42, dd (8.4, 2.4)	140.6
7	5.78, br d (10.0)	130.7	5.61, br d (10.5)	128.7	4.73, dd (8.5, 2.0)	107.0	4.76, dd (8.4, 1.8)	107.0
8	4.94, br d (13.0)	74.5	6.02-6.09, m	76.9	5.74, dt (8.0, 2.1)	72.9	5.78, dt (7.8, 2.4)	73.0
9	4.88, br d (13.5)	68.8	5.19, br d (10.0)	64.5	5.12, br d (8.0)	61.0	5.21, br d (8.4)	61.1
1'		167.4		165.8		168.8		166.4
2'		73.0		74.4		74.5		74.6
3'	(α) 2.93, d (16.0)	38.6	3.02, (2H, br s)	39.2	3.05, br d (15.5)	38.9	3.46, br d (16.8)	40.0
	(β) 3.08, d (16.0)				3.09, br d (15.5)		3.72, br d (16.8)	
4'		131.2		134.3		134.3		133.6
5'	5.97, br s	120.6	5.98, br s	120.1	5.99, br s	119.9	6.91, br d (7.8)	117.2
6'	5.90, br d (9.5)	122.9	5.90, ddd (9.5, 4.5, 2.5)	123.9	5.92, ddd (9.5, 4.5, 2.5)	123.9	7.18, t (7.8)	129.7
7'	5.78, br d (10.0)	130.7	5.66, br d (10.0)	131.1	5.66, br d (10.0)	131.4	6.83, br d (7.8)	118.4
8'	4.94, br d (13.0)	74.5	4.76, ddd (13.5, 5.5, 3.0)	75.1	4.78, br d (13.5)	75.0		147.5
9'	4.88, br d (13.5)	68.8	4.82, br d (13.5)	69.7	4.88, br d (13.5)	69.9		147.7
1"				174.7		174.7		174.7
2"			2.58, qd (7.0, 5.0)	43.8	2.52, qd (7.0, 5.5)	43.6	2.55, qd (7.2, 5.2)	43.6
3"			4.22, dd (7.0, 5.0)	73.7	4.16, dd (6.5, 5.5)	73.7	4.20, dd (6.4, 5.2)	73.7
4"			3.51, dq (7.5, 7.0)	43.1	3.45, dq (6.8,6.9)	43.2	3.47, br d (6.6)	43.2
5"				205.1		204.9		204.9
6"				138.1		138.1		138.1
7"			6.94, qq (7.0, 1.0)	138.6	6.88, qq (6.8, 1.5)	138.4	6.91, m	138.5
8"			1.89, dd (7.0, 1.0)	15.0	1.89, dd (7.0, 1.0)	14.4	1.89, dd (6.6, 1.2)	14.4
9"			1.14, d (7.0)	11.3	1.14, d (7.5)	11.8	1.15, d (7.2)	11.7
10"			1.16, d (6.5)	14.9	1.16, d (7.0)	14.8	1.18, d (6.6)	15.0
11"			1.76, t (1.0)	11.2	1.75, t (1.3)	11.3	1.76, t (1.2)	11.3
2-SCH_3	2.26, s	14.9	2.30, s	14.8	2.29, s	14.9	2.37, s	15.0
2'-SCH_3	2.26, s	14.9	2.18, s	14.5	2.24, s	14.9	2.23, s	15.0
8'-OH	5.36, s		5.46, s		5.29, s			
3"-OH			4.15, d (5.5)		3.97, d (5.5)		4.00, d (5.4)	

[a] Data record at ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR [b] Data record at ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR

determining theoretically absolute configurations of organic chiral molecules.[19-21] The computed $[\alpha]_D$ value for 2*R*,3*S*,4*S* is +16.2, which has only 1.8 deviation from that of **11**, but strikingly differs from those computed for other possible steroisomers (Table 2).

Table 2 Calculated optical rotations $[\alpha]_D$ (calc.) for all possible configurations of **11** and their comparisons with experimental value $[\alpha]_D$ (expt.) .

Enantiomer pairs[a]	AC[b]	$[\alpha]_D$ (calc) [c_e]	Δ[f]	AC[b]	$[\alpha]_D$ (calc) [c_e]	Δ[f]
RSS-SRR	*2R*,3*S*,4*S*	16.2	–1.8	*2S*,3*R*,4*R*	–16.2	34.2
RSR-SRS	*2R*,3*S*,4*R*	66.3	–84.3	*2S*,3*R*,4*S*	66.3	48.3
RRS-SSR	*2R*,3*R*,4*S*	158.6	140.6	*2S*,3*S*,4*R*	–158.6	176.6
RRR-SSS	*2R*,3*R*,4*R*	77.7	59.7	*2S*,3*S*,4*S*	–77.7	95.7

[a] Chirality of C-2~C-4, e.g., RSS means "*2R*,3*S*,4*S*"; [b] Absolute configuration; [c] $[\alpha]_D$ in deg·[dm·g/cm^3]$^{-1}$; [d] B3LYP/6-31G(d,p) within polarizable continuum model (PCM, dielectric constant for methanol solvent is 32.63); [e] The conformations with Boltzmann population and optical rotations are given in Table S2-S5 and Fig.S28; [f] Δ = $[\alpha]_D$ (calc.) – $[\alpha]_D$ (expt.), and $[\alpha]_D$ (expt.) is 18.0 deg·[dm·g/cm^3]$^{-1}$.

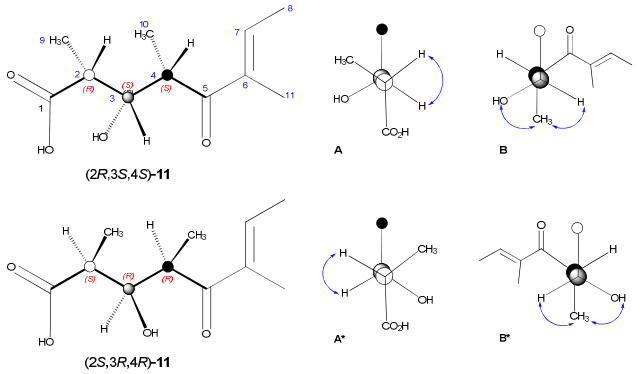

Fig. 2 Conformational analyses of two optional enantiomers of **11**.

The molecular formula of boydine C (**5**) was supported by the molecular ion at *m/z* 653.1961 $[M+Na]^+$ in its HR-ESI-MS spectrum ($C_{31}H_{38}N_2O_8S_2Na$ requires 653.1962). The ^1H and ^{13}C NMR data of **5** (Table 1) were similar to those of bisdethiobis(methylthio)-deacetylapoaranotin (**2**).[17, 18] As in **4**, the spectra gave a whole set of NMR signals ascribable for the hydroxy-2,4,6-trimethyl-5-oxooct-6-enoyl group on C-8, supported by the H-8 signal of **5** signified at δ_H 5.74, shifted downfield by 0.97 ppm from that of **2** (δ_H 4.71).[18] The absolute configuration of **5** should be identical to that of **4** since their CD spectra were quite similar (Fig. S38). The HR-ESI-MS spectrum of boydine D (**6**) displayed a molecular ion at *m/z* 651.1803 $[M+Na]^+$ (calcd. for $C_{31}H_{36}N_2O_8S_2Na$: 651.1805). The ^1H and ^{13}C NMR, and DEPT spectra of **6** suggested that it could be a dehydrogenated and aromatized derivative of **5**. The ^1H-^1H COSY, HSQC, ROESY and HMBC spectra of **6** reinforced the hypothesis, and allowed the unambiguous assignment of all ^1H and ^{13}C NMR data (Table 1). In particular, the five proton coupling sequence from H-5' through H-9' exhibited in the ^1H NMR spectrum of **5** was missing, and replaced by sequentially coupled (J = 7.8 Hz) aromatic proton signals at 6.91, 7.18 and 6.83 ppm in that of **6**. As to the absolute configuration of **6**, the experimental CD data and specific rotation suggested that the diketopiperazine moiety possessed (2*R*,2'*R*,8*S*,9*S*)-configurations (Fig. S38). The ^1H and ^{13}C NMR signals due to the hydroxy-2,4,6-trimethyl-5-oxooct-6-enoyl group of **5** and **6** were almost identical with those of **4** (Table 1), suggesting that the three compounds carried the same acyl chain. This assumption was confirmed by the alkaline hydrolysis of **5** and **6**, which afforded the same (2*R*,3*S*,4*S*,*E*)-3-hydroxy-2,4,6-trimethyl-5-oxooct-6-enoic acid as liberated from **4** (see above).

Boydene A (**7**) displayed, in its HR-ESI-MS spectrum, a molecular ion at *m/z* 273.1467 $[M+Na]^+$ (calcd. for $C_{15}H_{22}O_3Na$: 273.1461). In correlation with the 20-proton integration in the ^1H NMR spectrum of **7**, this molecular formula suggested the presence of two hydroxyl groups. The ^{13}C NMR and DEPT data of **7** suggested a sesquiterpene carbon skeleton composed of three methyl, three methylene (an sp^2-hybridized C at δ_C 107.2), five methine (an oxygenated C at δ_C 70.0 and an sp^2-hybridized C at δ_C 122.1) and four quaternary carbon signals (an oxygenated C at δ_C 76.3, two sp^2-hybridized ones at δ_C 147.5 and 138.5, and a ketone at δ_C 213.2). This hypothesis was confirmed by the ^1H-^1H COSY, ROESY, HMQC and HMBC spectra of **7**, which resulted in the exact assignment of all ^1H and ^{13}C NMR signals (Table 3). The ^1H-^1H COSY spectrum of **7** exhibited the coupling sequences arising from 1-substituted 3-methylbut-2-en-1-ol (C-8 through C-10), 2,5-disubstituted 4-methylenecyclohexanone (C-1 through C-5, C-4a and C-5a) and a 1,1-disubstituted ethyl (C-6 and C-13) motifs. These moieties, along with the oxygenated quaternary carbon, were pieced together into an unprecedented carbon skeleton by the key HMBC correlations of C-7 with H-3, H-5 and H-13, and of C-8 with H-2 and H-6. The ROESY spectrum of **7** reinforced the elucidation by the cross-peak of H-8 with H-2 and H-6. Furthermore, the relative configuration of **7** was assigned from the ROESY correlations of H-5a (δ_H 2.63) with H-8 and H-11. With this assignment, sesquiterpene **7** was demonstrated to have (2*S*,5*S*,6*R*,7*S*,8*R*)-configurations by comparing its CD spectrum with its calculated ECD curves of all possible diastereomers (Fig. 3).

Table 3 Assignments of ^1H, ^{13}C and HMBC NMR spectra of **7** ($CDCl_3$).

position	δ_C	δ_H (mult. *J*)	HMBC
1	213.2		2, 5a
2	53.1	2.80 t (2.8)	8
3	27.8	2.45 ddd (18.5, 5.3, 2.3) 2.69 dd (18.5, 2.0)	2, 4a
4	147.5		2, 4a, 5a
4a	107.2	4.74 d (1.0) 4.90 br s	
5	46.1	2.38 dd (4.5, 2.5)	4a, 6, 13
5a	38.7	(a) 2.13 ddd (18.5, 3.3, 1.8) (b) 2.63 dd (19.0, 2.0)	6
6	37.8	1.69 qt (7.5, 1.5)	2, 3
7	76.3		13
8	70.0	4.29 d (9.5)	6
9	122.1	5.42 dt (9.5, 1.3)	8, 11, 12
10	138.5		8, 12
11	18.5	1.74 d (1.5)	
12	26.1	1.79 d (1.5)	11
13	12.9	1.02 d (7.5)	

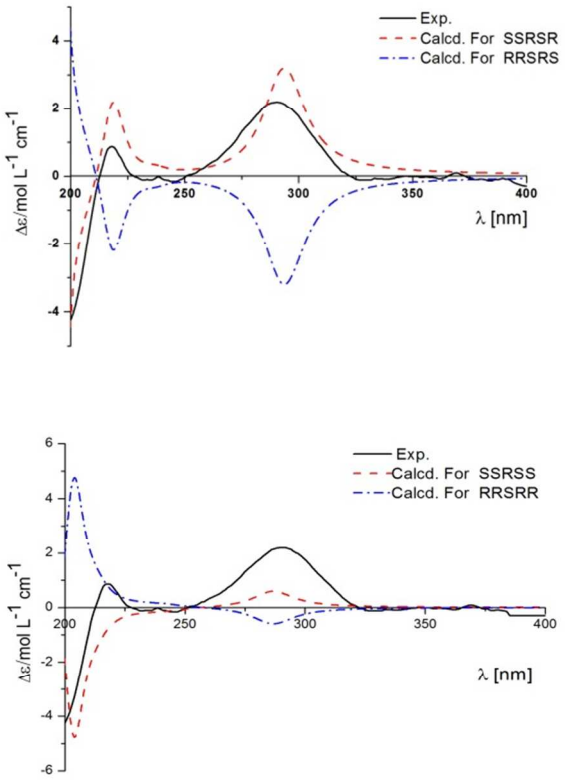

Fig. 3 Comparison of the ECD spectrum of **7** with those calculated for its optional stereoisomers [(2*S*, 5*S*, 6*R*, 7*S*, 8*R*)-, (2*R*, 5*R*, 6*S*, 7*R*, 8*S*)-, (2*S*, 5*S*, 6*R*, 7*S*, 8*S*)- and (2*R*, 5*R*, 6*S*, 7*R*, 8*R*)-**7**].

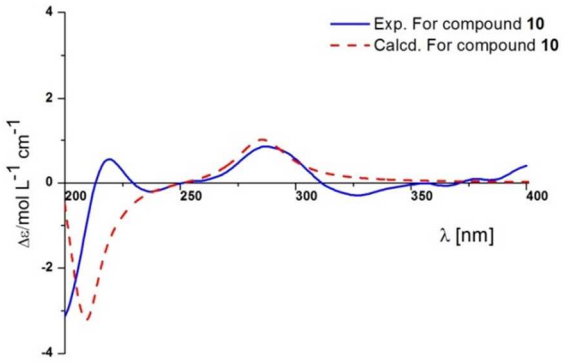

Fig. 4 Assignment of the absolute configuration of boydene B (**10**) by comparing experimental and calculated CD spectra.

Sesquiterpenes **8** and **9** were identified as AM6898 A and ovalicin, respectively, by comparing the spectral data with those reported.[22, 23] Compound **10** was shown to have a molecular formula of $C_{16}H_{24}O_4$ molecular ion at *m/z* 303.1555 $[M+Na]^+$ in its HR-ESI-MS ($C_{16}H_{24}O_4Na$ requires 303.1560). The ^1H and ^{13}C NMR spectra were close to those of ovalicin (**9**).[23] However, the oxygenated methylene signal due to the spiro-epoxide in the ^1H NMR spectrum of **9** was replaced by the exomethylene-derived singlets at δ_H 5.12 (s) and 5.18 (s) in that of **10** (Table 4). The ^1H-^1H COSY, ROESY, HMQC and HMBC spectra of **10** confirmed the assumption, and indicated that it was 1,8-deoxygenated derivative of ovalicin (**9**). The absolute stereochemistry was determined to be 2*S*,3*S*,1'*S*,2'*R* by comparing its experimental CD spectrum with those calculated for all possible diastereoisomers (Fig. 4). We have named compound **10** boydene B after boydene A (**7**).

Table 4 Assignments of ^1H, ^{13}C and HMBC NMR spectra of **10** ($CDCl_3$).

position	δ_C	δ_H (mult. *J*)	HMBC
1	144.7		3, 5
2	79.2		6, 8, 1', 8'
3	86.5	3.83 s	7
4	206.5		6
5	38.5	2.36-2.44 m	
		2.47-2.56 m	
6	30.9	2.47-2.56 m	5, 8
		2.75 m	
7	58.9	3.51 s	
8	113.5	5.12 s	
		5.18 s	
1'	61.4		
2'	57.6	2.99 t (6.3)	8'
3'	27.4	2.23 dt (15.0, 7.6)	2'
		2.36-2.44 (m)	
4'	118.2	5.20 br t (6.3)	2'
5'	134.8		3'
6'	25.7	1.73 s	
7'	18.0	1.65 s	6'
8'	14.1	1.42 s	

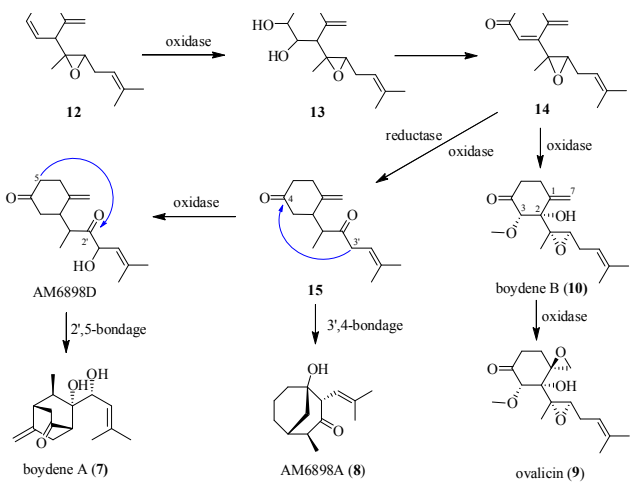

Fig. 5 Proposed biosynthesis pathway of **7** and **10**.

Biosynthesis of boydene A

Owing to the structural and biological significance, the biosynthetic pathway of ovalicin and its related analogues are receiving intense attention.[24] The present co-isolation of boydenes A (**7**) and B (**10**), AM6898A (**8**) and ovalicin (**9**) indicates the shunt product diversity of this family of sesquiterpenes (Fig. 5). As a key intermediate,[24] β-*trans*-bergamotene (**12**) is most probably subjected to sequential oxidation and dehydration to produce proposed precursors **13** and **14**. Subsequently, 2,3-epoxidation and 3-O-methylation of

14 would form **10**, which might produce ovalicin (**9**) after 1,7-epoxidation. Reduction of **14**, followed by oxidation, would supposedly yield compound **15**, which could be the common precursor of AM6898A (**8**) and AM6898D, two immunosuppressants against IgE production.[25, 26] The unprecedented carbon skeleton of boydene A (**7**) forms most probably from AM6898D by an intramolecualr Aldol condensation (Fig. 5).[26]

Biological activity

All isolated fungal metabolites were assayed for *in vitro* antibacterial activity against pathogenic anaerobes isolated from clinical specimens. Boydine B (**4**) showed strong inhibition against *Bifidobacterium* sp., *Veillonella parvula*., *Anaerostreptococcus* sp. and *Peptostreptococcus* sp. with minimum inhibitory concentrations (MICs) at 0.2 μM. It was also active against *Bacteroides vulgates* with an MIC of 0.8 μM. These MIC values are lower than those of the positive control tinidazole (Table 5), an antibacterial drug prescribed mainly to treat bacterial infections.[27, 28] All other compounds had no significant effect on bacterial inhibition.

Table 5 *In vivo* antibacterial activity of **4** (MICs in μM).

Strains	**4**	tinidazole
Bifidobacterium sp.	0.2	404.9[29]
Veillonella parvula.	0.2	0.5
Anaerostreptococcus sp.	0.2	1.0
Bacteroides vulgates	0.8	2.0
Peptostreptococcus sp.	0.2	2.0

Experimental Section

General procedures

Silica gel (SiO_2, 200-300 mesh) for column chromatography (CC) and silica GF_{254} (10~20 μm) for TLC were purchased from Qingdao Marine Chemical Factory, Qingdao, China. Sephadex LH-20 was obtained from Pharmacia Biotech, Uppsala, Sweden. Optical rotations were measured on a Rudolph Research Analytical Autopol IV automatic polarimeter. IR spectra were acquired on a Nexus 870 FT-IR spectrometer. NMR spectra were recorded on a Bruker DRX-500 NMR spectrometer in $CDCl_3$ or acetone-d_6 with ^1H and ^{13}C nuclei observed at 500 and 125 MHz, respectively. HPLC separation was carried out on a Hitachi L-7110 pump, and UV detector L-7400 equipped with an Apollo C18 column (5 μm, 250 × 4.6 mm; Alltech Associates, Inc. Chicago, USA). HR-ESI-MS spectra were measured on an Agilent 6210 TOF LC/MS machine with an ESI probe operating in the positive-ion mode. All chemicals used herein were of analytical grade.

Fungal material

A living *Holotrichia parallela* larva was collected from the Zijin Mountain in the suburb of Nanjing (China) in September 2008. The strain BC-f4 was isolated with the fungal isolation medium spread from the grind gut dilution of the insect after cultivating for two weeks at 28 °C as reported.[31] Morphological scrutiny of hyphae and spores combined with 18S rDNA ITS sequence (GenBank number: JQ668664) led to its identification as *Pseudallescheria boydii*.

Culture, extraction and isolation

Fermentation of the strain was initiated in 1 L sized Erlenmeyer flasks, each preloaded with 400 ml of Modified Czapek's medium containing sucrose 3.0 g, $NaNO_3$ 3.0 g, K_2HPO_4 g, yeast extract 1.0 g, KCl 0.5 g, $MgSO4{\cdot}7H_2O$ 0.5 g and $FeSO_4$ 0.01 g for 1 L volume in tap water. The seed was prepared by inoculating activated fungal cakes from agar Petri dish into 200 ml Modified Czapek's medium preloaded in each of 1 L Erlenmeyer flask, followed by cultivation for 5 days at 28 ºC and 150 rpm. Approximately 50 ml aliquots of the inoculum was then transferred to fermentation medium and further incubated for 13 days at 28 ºC and 120 rpm on rotary shakers. The resulting culture broth was extracted with an equal volume of EtOAc (four times) after removal of fungal cells. The *in vacuo* evaporation of solvent at 35 ºC gave a crude extract (3.5 g), which was subsequently separated by silica gel CC eluted with chloroform/methanol mixtures of a growing polarity (v:v always, 100:0, 100:1, 100:2, 100:4, 100:8, 100:16) to afford four fractions F1−F4. Compound **8** (13.0 mg) was separated from F1 by CC over silica gel eluted petroleum ether/acetone gradient (60:1→1:1). F2 was fractionated by a silica gel CC eluted with petroleum ether/acetone gradient (20:1→1:1) to yield three subfractions F2-1~F2-3. F2-1 (55 mg) was purified through gel filtration over Sephadex LH-20 in MeOH, followed by reversed-phase HPLC separation using MeOH/H_2O (62:38) to furnish **3** (3.0 mg) and **10** (3.3 mg). Boydene A (**7**, 3.8 mg) was obtained from gel filtration of F2-2 over Sephadex LH-20 followed by semipreparative HPLC with MeOH/H_2O (4:1). Compound **9** (22.5 mg) was isolated by Sephadex LH-20 filtration of F2-3 in MeOH, and by semi-preparative HPLC with MeOH/H_2O (7:3). F3 was separated over a silica gel CC eluted with petroleum ether/acetone gradient (30:1→1:1) afforded three subfractions (F3-1~F3-3). F3-1 was separated by the gel filtration over Sephadex LH-20 in MeOH, followed by semipreparative HPLC with MeOH/H_2O (22:3) to furnish **1** (6.0 mg) and **2** (13.5 mg). Further HPLC purification of F3-2 with MeOH/H_2O (7:3) gave boydines C (**5**, 12.2 mg) and D (**6**, 0.4 mg), and compound **4** (4.5 mg) was separated from F3-3 in the same manner.

Antibacterial assay

As described,[32, 33] the *in vitro* antibacterial assay was determined by inhibiting the growth of clinical anaerobic bacteria isolated at Department of Clinical Laboratory of the First Affiliated Hospital of Nanjing Medical University (Nanjing, P. R. China). The MICs were assessed by incubating the susceptible bacteria for 48 h at 35 ℃ in an atmosphere of 80% N_2, 10% CO_2 and 10% H_2. The MIC values of the particular compound were obtained by reading the concentration, at which no turbidity could be detected on the microtiter plates. All assays were repeated three times for the reliability and reproducibility.

Hydrolysis of boydines B−D (4−6)

Each of compounds **4**−**6** was dissolved in 1 mL mixture of tetrahydrofuran/H_2O (67:33), and adjusted to pH 10 by adding 1 M NaOH dropwise. After standing overnight at room temperature, the mixture was dried under vacuum at 20℃, dissolved in 20 ml H_2O and extracted twice with CH_2Cl_2 (20 ml each). By dropwise addition of 0.1 M HCl, the aqueous layer was adjusted to pH=2, followed by extraction with CH_2Cl_2. Evaporation of the solvent from extract, and the residue was dissolved in methanol, followed by the reversed-phase HPLC purification using MeOH/H_2O (67:33, containing 0.1%

trifluoroacetic acid) to give (2*R*,3*S*,4*S*,*E*)-3-hydroxy-2,4,6-trimethyl-5-oxooct-6-enoic acid (**11**).

Boydine A (**3**): light yellow amorphous powder; $[\alpha]^{20}_{D}$= −37 (*c* 0.16, CH_3OH); ^1H and ^{13}C NMR data listed in Table 1; FT-IR (KBr) : ν_{max}/cm^{-1} 3360, 3051, 2923, 2853, 1642; UV/VIS (CH_3OH): λ_{max} (log ε) = 203 (6.6), 224 (6.5), 265 nm (6.0 mol^{-1}dm^{3}cm^{-1}); CD (MeOH, c = 3.8×10^{-4} M): $\Delta\varepsilon_{218}$= −1.4, $\Delta\varepsilon_{283}$= +3.0 cm^{2}mol^{-1}; HR-ESI-MS: *m*/*z*: calcd. for $C_{20}H_{23}N_2O_4S_2$: 419.1094 $[M+H]^+$; found: 419.1099.

Boydine B (**4**): light yellow amorphous powder; $[\alpha]^{20}_{D}$= −26 (*c*= 0.19, CH_3OH); ^1H and ^{13}C NMR data assigned in Table 1; FT-IR (KBr) : ν_{max}/cm^{-1} 3462, 2977, 2939, 1739, 1678, 1640, 1382, 1194; UV/VIS (CH_3OH): λ_{max} (log ε) = 202 (5.9), 269 nm (5.4 mol^{-1}dm^{3}cm^{-1}); CD (MeOH, c = 3.1×10^{-4} M): $\Delta\varepsilon_{219}$ = −5.5, $\Delta\varepsilon_{281}$ = +1.6 cm^{2}mol^{-1}; HR-ESI-MS: *m*/*z*: calcd. for $C_{31}H_{38}N_2O_7S_2$ Na: 637.2013 $[M+Na]^+$; found: 637.2016.

Boydine C (**5**): light yellow amorphous powder; $[\alpha]^{20}_{D}$= −69 (*c*= 0.09, CH_3OH); ^1H and ^{13}C NMR data tabulated in text (Table 1); FT-IR (KBr) : ν_{max}/cm^{-1} 3415, 2981, 2945, 1731, 1640, 1386, 1191,; UV/VIS (CH_3OH): λ_{max} (log ε) = 202 (5.6), 224 nm (5.7 mol^{-1}dm^{3}cm^{-1}); CD (MeOH, c = 1.4×10^{-4} M): $\Delta\varepsilon_{224}$= −2.5, $\Delta\varepsilon_{280}$ = +0.2 cm^{2}mol^{-1}; HR-ESI-MS: *m*/*z*: calcd. for $C_{31}H_{38}N_2O_8S_2$Na: 653.1962 $[M+Na]^+$; found: 653.1961.

Boydine D (**6**): light yellow amorphous powder; $[\alpha]^{20}_{D}$= −62 (*c*= 0.045, CH_3OH); ^1H and ^{13}C NMR data edited in Table 1; FT-IR (KBr) : ν_{max}/cm^{-1} 3407, 1646, 1476, 1397, 1261, 1192; UV/VIS (CH_3OH): λ_{max} (log ε) = 202 (5.0), 272 nm (4.3 mol^{-1}dm^{3}cm^{-1}); CD (MeOH, c = 7.2×10^{-5} M): $\Delta\varepsilon_{226}$= −2.5, $\Delta\varepsilon_{299}$ = +0.09 cm^{2}mol^{-1}; HR-ESI-MS: *m*/*z*: calcd. for $C_{31}H_{36}N_2O_8S_2$Na: 651.1805 $[M+Na]^+$; found: 651.1803.

Boydene A (**7**): colorless gum; $[\alpha]^{20}_{D}$= +107 (*c*= 0.08, CH_3OH); ^1H and ^{13}C NMR tabulated in Table 3; FT-IR (KBr) : ν_{max}/cm^{-1} 3342, 2975, 2930, 1723, 1675,1654, 1444, 1402, 1384, 1128; UV/VIS (CH_3OH): λ_{max} (log ε) = 202 nm (5.0); CD (MeOH, c = 3.2×10^{-4} M): $\Delta\varepsilon_{219}$= +0.8, $\Delta\varepsilon_{291}$= +2.2 cm^{2}mol^{-1}; HR-ESI-MS: *m*/*z*: calcd. for $C_{15}H_{22}O_3$Na: 273.1461 $[M+Na]^+$; found: 273.1467.

Boydene B (**10**): colorless gum; $[\alpha]^{20}_{D}$= −21 (*c*= 0.13, CH_3OH); ^1H and ^{13}C NMR given in Table 4; FT-IR (KBr) : ν_{max}/cm^{-1} 3444, 2963, 2924, 2852, 1732, 1650, 1449, 1384, 1261, 1106; UV/VIS (CH_3OH): λ_{max} (log ε) = 201 (4.9); 220 (0.5), 287 nm (0.9 mol^{-1}dm^{3}cm^{-1}); CD (MeOH, c = 4.6×10^{-4} M): $\Delta\varepsilon_{220}$ = +0.5, $\Delta\varepsilon_{237}$ = −0.2, $\Delta\varepsilon_{287}$ = +0.9, $\Delta\varepsilon_{326}$ = −0.3 cm^{2}mol^{-1}; HR-ESI-MS: *m*/*z*: calcd. for $C_{16}H_{24}O_4$Na: 303.1560 $[M+Na]^+$; found: 303.1555.

Computational details for absolute configuration

Conformational analysis of the studied systems was carried out in two ways. Firstly, conformational searching was performed by using consisting valence force field (CVFF). All the stable conformations obtained were then reoptimized using density functional theory (DFT) at B3LYP/6–31G(d,p) level. Next, the harmonic vibrational frequencies of each conformation were calculated using B3LYP/6–31G(d,p) to confirm that the conformation was stable. Finally, the relative free energies were calculated and equilibrium room-temperature Boltzmann populations obtained thence. The optimized conformers were used in the electronic circular dichroism (ECD) and optical rotation calculations. The salvation effects on the electronic structures of the systems were evaluated by quantum chemistry methods through the polarized continuum model (PCM, dielectric constant ε for methanol solvent was 32.63). For the ECD calculation, time-dependent DFT (TDDFT) with the same basis set was carried out to calculate the spin-allowed excitation energy and rotatory strength of the lowest 100 excited states. The ECD spectra were generated using the program SpecDis[34] by applying a Gaussian band shape with the width of 0.20 eV, from oscillator strengths and dipole-velocity rotational strengths, respectively. The optical rotations at the sodium D line were obtained by dipole electric field polarizability calculations. All the ECD and optical rotation calculations were performed with the Gaussian 09 program.[35]

Conclusions

Two important classes of fungal metabolites, as represented by boydines A−D (**3**−**6**) and boydenes A (**7**) and B (**10**), have been characterized from the insect-derived *P. boydii*. Compound **7** possesses an unprecedented carbon skeleton and highlights a new skeletal diversification of the ovalicin-related fungal sesquiterpenes through the intramolecular cyclization. Boydine B (**4**) shows a broader antibacterial spectrum against a panel of anaerobic pathogens isolated from clinic specimens. The acyl side chain of this diketopiperazine has been clarified to be likely (2*R*,3*S*,4*S*,*E*)-3-hydroxy-2,4,6-trimethyl-5-oxooct-6-enoic acid by a combined experimental and computational strategy. Attention to the structure-activity relationship of the cyclic dipeptides suggested that the antibacterial activity might be dependent on the combination of the acyl residue with diketopiperazine nucleus condensed from two (2*R*,7*S*,7a*S*)-7-hydroxy-2-(methylthio)-2,3,7,7a-tetrahydro-1H-indole-2-carboxylic acids. The findings shed lights both on the discovery of new antibacterial agents, and on the engineered diversification of ovalicin-related fungal sesquiterpenes, a unique reservior for drug leads or tool molecules.[24, 30]

Acknowledgements

The work was co-financed by the MOST (2013AA092901), NSFC grants (81121062, 21132004 & 21072092), and the Natural Science Foundation of the Higher Education Institutions of Jiangsu Province (No. 11KJB350003).

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

1 H. Kikuchi, Y. Miyagawa, K. Nakamura, Y. Sahashi, S. Inatomi, Y. Oshima, *Org. Lett.*, 2004, **6**, 4531−4533.
2 E. J. Buenz, B. A. Bauer, T. W. Osmundson, T. J. Motley, *J. Ethnopharmacol.*, 2005, **96**, 19−29.
3 X. L. Yao, S. Meran, Y. P. Fang, J. Martin, A. Midgley, M. M. Pan, B. C. Liu, S. W. Cui, G. O. Phillips, A. O. Phillips, *Food hydrocoll.*, 2014, **35**, 444−452.
4 Y. L. Zhang, H. M. Ge, W. Zhao, H. Dong, Q. Xu, S. H. Li, J. Li, J. Zhang, Y. C. Song, R. X. Tan, *Angew. Chem. Int. Edit.*, 2008, **47**, 5823−5826.
5 Y. L. Zhang, H. M. Ge, F. Li, Song, Y. C. Tan, R. X. *Chem. Biodivers.*, 2008, **5**, 2402−2407.
6 Z. K. Guo, T. Wang, Y. Guo, Y. C. Song, R. X. Tan, H. M. Ge, *Planta Med.*, 2011, **77**, 2057−2060.
7 I. Molnar, D. M. Gibson, Krasnoff, S. B. *Nat. Prod. Rep.*, **2**010, **27**, 1241−1275.
8 K. J. Cortez, E. Roilides, F. Quiroz-Telles, J. Meletiadis, C. Antachopoulos, T. Knudsen, W. Buchanan, J. Milanovich, D. A. Sutton, A. Fothergill, M. G. Rinaldi, Y. R. Shea, T. Zaoutis, S. Kottilil, T. J. Walsh, *Clin. Microbiol. Rev.*, 2008, **21**, 157−197.
9 M. Maslen, M. Peel, *Mycoses*, 2011, **54**, 442−449.
10 L. C. L. Lopes, M. I. D. da Silva, V. C. B. Bittencourt, R. T. Figueiredo, R. Rollin-Pinheiro, G. L. Sassaki, M. T. Bozza, P. A. J. Gorin, E. Barreto-Bergter, *Mycoses*, 2011, **54**, 28−36.
11 M. R. Pinto, M. L. Rodrigues, L. R. Travassos, R. M. T. Haido, R. Wait, E. Barreto-Bergter, *Glycobiology*, 2002, **12**, 251−260.
12 C. Nirma, V. Eparvier, S D. tien, *J. Nat. Prod*., 2013, **76**, 988−991.
13 Y. C. Chang, T. S. Deng, K. L. Pang, C. J. Hsiao, Y.Y. Chen, S. J. Tang, T. H. Lee, *J. Nat. Prod*., 2013, **76**, 1796−1800.
14 K. Pavlaskova, J. Nedved, M. Kuzma, M. Zabka, M. Sulc, J. Sklenar, P. Novak, O. Benada, O. Kofronova, M. Hajduch, P. J. Derrick, K. Lemr, A. Jegorov and V. Havlicek, *J. Nat. Prod.*, 2010, **73**, 1027−1032.
15 J. P. McCutcheon, N. A. Moran, *Nat. Rev. Microbiol.*, 2012, **10**, 13−26.
16 S. Raffaele, S. Kamoun, *Nat. Rev. Microbiol.*, 2012, **10**, 417−430.
17 C. J. Guo, H. H. Yeh, Y. M. Chiang, J. F. Sanchez, S. L. Chang, K. S. Bruno, C. C. C. Wang, *J. Am. Chem. Soc.*, 2013, **135**, 7205−7213.
18 M. Chinworrungsee, P. Kittakoop, J. Saenboonrueng, P. Kongsaeree, Y. Thebtaranonth, *J. Nat. Prod.*, 2006, **69**, 1404−1410.
19 P. J. Stephens, D. M. McCann, F. J. Devlin, A. B. Smith, III, *J. Nat. Prod.*, 2006, **69**, 1055−1064.
20 P. J. Stephens, J. J. Pan, F. J. Devlin, K. Krohn, T. Kurtán, *J. Org. Chem.*, 2007, **72**, 3521−3556.
21 P. J. Stephens, F. J. Devlin, F. Gasparrini, A. Ciogli, D. Spinelli, B. Cosimelli, *J. Org. Chem.*, 2007, **72**, 4707−4715.
22 Y. Fukuda, Y. Okamoto, *Tetrahedron*, 2002, **58**, 2513−2521.
23 D. E. Cane, D. B. Mcilwaine, *Tetrahedron Lett.*, 1987, **28**, 6545−6548.
24 H. C. Lin, Y. H. Chooi, S. Dhingra, W. Xu, A. M. Calvo, Y. Tang, *J. Am. Chem. Soc.*, 2013, **135**, 4616−4619.
25 Jpn. Pat. 09309859, 1997.
26 Y. Fukuda, M. Sakurai, Y. Okamoto, *Tetrahedron Lett.*, 2000, **41**, 8505−8508.
27 G. Manes, A. Balzano, *Expert. Rev. Anti-Infect. Ther.*, 2004, **2**, 695−705.
28 N. Rani, A. Sharma, G. K. Gupta, R. Singh, *Mini-Rev. Med. Chem.*, 2013, **13**, 1626−1655.
29 K. Ninomiya, S. Kobata, H. Imamura, I. Mochizuki, K.Watanabe, *Jpn. J. Antibiot.*, 1976, **29**, 325−331.
30 C. Kishor, T. Arya, R. Reddi, X. C. Chen, V. Saddanapu, A. K. Marapaka, R. Gumpena, D. W. Ma, J. O. Liu, A. Addlagatta, *J. Med. Chem.*, 2013, **56**, 5295−5305.
31 Z. K. Guo, R. H. Jiao, H. F. Dai, W. L. Mei,, R. X. Tan, H. M. Ge, *Chem. Biodivers.*, 2013, **10**, 296−302.
32 E. Dimodugno, I. Erbetti, L. Ferrari, G. Galassi, S. M. Hammond, L. Xerri , *Antimicrob. Agents Chemother*., 1994, **38**, 2362−2368.
33 D. Rankin, in MIC testing: *Manual of Antimicrobial Susceptibility Testing*, ed. M.B. Coyle, University of Washington, Washington, 2005, pp. 53−62.
34 T. Bruhn, Y. Hemberger, A. Schaumlöffel and G. Bringmann, University of Wüerzburg (Wüerzburg, Germany), 2010.
35 M. J. Frisch, Gaussian 09, Revision A.1, Gaussian, Inc., Wallingford CT, 2009.

