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Diketopiperazines derivatives isolated from *Bacillus thuringiensis* and *Bacillus endophyticus*, establishment of their configuration by X-ray and their synthesis

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ABSTRACT

Four known diketopiperazines have been isolated from *Bacillus thuringiensis* strain and two of them were isolated from *Bacillus endophyticus* also. Their structure was elucidated by a complete spectroscopy and the configuration of three of them was established by X-ray analysis. The dicetopiperazine *cyclo*-(L-Proline-L-Tyrosine), isolated from both strains, was polymorphic showing different physical properties. The synthesis has been successfully realized in two simple steps. Their biological activities were probed against some Gram-positive and Gram-negative bacteria and six fungi. The results indicated that these compounds had no antibacterial activity but they had antifungal activity.

Diketopiperazines are relatively simple cyclodipeptides (Fig. 1) which consist of rings obtained by the condensation of two α-amino acids that are produced by fungi, bacteria, the plant kingdom, and mammals.[1–3] Diketopiperazines possess diverse biological activities such as plant-growth promoters,[4] antitumor,[5] antifungal,[6] and antibacterial.[7] They are not only a class of naturally occurring privileged structures that have the ability to bind to a wide range of receptors but they also have a structure that confers high stability and resistance to human digestion that make them attractive scaffolds for drug discovery. In addition, the compounds show a common scaffold, easily obtained by conventional procedures, that favors structural diversity as a function of substituent side chains particularly orientated. Therefore, diketopiperazines are attractive structures for the discovery of new lead compounds for the rational development of a new therapeutic agent.

The genus *Bacillus* consists of a large number of diverse, rod-shaped and spore forming Gram-positive bacteria that have antagonistic activity against fungal and some bacterial pathogens.[8] *Bacillus thuringiensis* is a well-studied bacterium which is biotechnologically employed due to its bioinsecticidal property.[9] This bacteria can produce different kinds of antibiotics[10] or other natural products with different biological activities.[11] *Bacillus endophyticus*

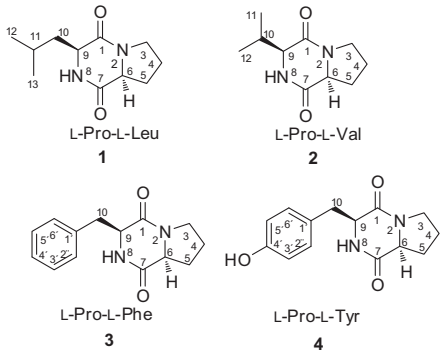

Figure 1. The structures of diketopiperazines.

was first isolated from the inner tissues of cotton plants which genome sequence was recently reported.[12,13] Multiple *Bacillus* species have been identified as plant growth-promoting rhizobacteria (PGPR) that promote growth by producing antibiotics, inhibiting plant ethylene synthesis, and inducing plant systemic resistance to pathogens.[14]

Recently we have reported the potential as antibiotic of *Bacillus thuringiensis* strain against Gram negative and Gram positive bacteria, maybe due to the secondary metabolites secreted by this bacterium. This bacterium is naturally a melanin producer, which is a

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natural photo-protective agent.[11] We extracted a brown pigment corresponding to melanin, that was compared with a commercial melanin.

We report herein the isolation of four diketopiperazines from *Bacillus thuringiensis* and *Bacillus endophyticus*, their structure elucidation, the establishment of their configuration by X-ray and biological activities of these compounds.

The bacteria were isolated from soil and were characterized as *Bacillus thuringiensis*[11] and *Bacillus endophyticus*. These strains were cultured at 29 °C on a rotary shaker at 175 rpm. The fermentation broths (1 L) were centrifuged to eliminate the cells and the sobrenadants were extracted with EtOAc (3:1) for three times to yield 400 mg of brown oily extract. The EtOAc extracts were then subjected to silica gel column chromatography with EtOAc and acetone as a system of eluents to yield four diketopiperazines *cyclo*-(L-Pro-L-Leu) **1**, *cyclo*-(L-Pro-L-Val) **2**, *cyclo*-(L-Pro-L-Phe) **3** and *cyclo*-(L-Pro-L-Tyr) **4** from *Bacillus thuringiensis* and *cyclo*-(L-Pro-L-Val) **2**, and *cyclo*-(L-Pro-L-Tyr) **4** from *Bacillus endophyticus*. The structures of these compounds were completely elucidated by using extensive spectroscopic methods.

Diketopiperazine **1** was achieved as white crystals and its molecular formula was assigned as $C_{11}H_{18}N_2O_2$ by high resolution FAB-HRMS (*m*/*z* 211.1444 $[M+H]^+$, Calcd for 211.1447. The molecular formula allowed assigning the number of insaturations that was four. In the ^1H NMR spectrum, one broad signal at 5.78 ppm was observed indicating the presence of an amino group and two double signals at 1.01 and 0.96 ppm indicating an isopropyl group. The ^{13}C NMR spectrum showed 11 carbon signals, attributable to two carbonyl carbons at 170.1 and 166.1 ppm, four methylene carbons (22.7, 28.1, 38.6 and 45.5 ppm), three methine carbons (24.7, 53.3 and 59.0 ppm), and two methyl carbons (21.2 and 23.2 ppm). A detailed analysis of the ^1H–^1H correlation spectroscopy (COSY) spectrum showed connectivity for two proton spin systems, H_3–H_4–H_5–H_6 and NH–H_9–H_{10}–H_{11}–H_{12}–H_{13}. These data defined the presence of proline moiety and isopropyl moiety in **1** and allowed to assign unequivocally all the signals of the ^1H and ^{13}C NMR spectra.[15] The compound **1** was crystallized confirming the proposed structure and the absolute configuration was determined as 6*S*,9*S*.[16] This compound **1** was identified as *cyclo*-(L-Pro-L-Leu) and was achieved only from *Bacillus thuringiensis* strain.

Diketopiperazine **2** was achieved as needle crystals and its molecular formula was assigned as $C_{10}H_{16}N_2O_2$ by high resolution EI-HRMS (*m*/*z* 196.1192, Calcd for 196.1212. The molecular formula allowed assigning the number of insaturations that was four. The IR absorptions of 3212, 1669, and 1426 cm^{-1} showed the presence of the amide group. In the ^1H NMR spectrum, one broad signal at 5.89 ppm was observed indicating the presence of an amino group and two double signals at 1.1 and 0.9 ppm indicating an isopropyl group. The ^{13}C NMR spectrum showed 10 carbon signals, attributable to two carbonyl carbons at 169.9 and 164.8 ppm, three methylene carbons (22.3, 28.5 and 45.1 ppm), three methine carbons (28.5, 58.7 and 60.1 ppm), and two methyl carbons (19.1 and 16.0 ppm). All recovered data indicated that compound **2** contained two rings in the molecule and an isopropyl group. A detailed analysis of the ^1H–^1H correlation spectroscopy (COSY) spectrum showed connectivity for two proton spin systems, H_3–H_4–H_5–H_6 and NH–H_9–H_{10}–H_{11}–H_{12}. The heteronuclear multiple bond correlations (HMQC) of H-3 to C-6 and H-9 to C-12, were observed. These data defined the presence of a proline moiety and isopropyl moiety in **2** and allowed to assign unequivocally all the signals of the ^1H and ^{13}C NMR spectra.[17] The compound **2** was crystallized confirming the proposed structure and the absolute configuration was determined as 6*S*,9*S*, as shown in Figure 3.[19,20] This compound **2** was identified as *cyclo*-(L-Pro-L-Val). This compound was obtained both with *Bacillus thuringiensis* and *Bacillus endophyticus* strains.

Diketopiperazine **3** was achieved as a white solid and its molecular formula was assigned as $C_{14}H_{16}N_2O_2$ by high resolution EI-HRMS (*m*/*z* 244.1177, Calcd for 244.1212. The molecular formula allowed assigning the number of insaturations that was eight. In the ^1H NMR spectrum, a multiple signal at 7.29 ppm indicating the presence of a phenyl group and one broad signal at 5.60 ppm, indicating the presence of an amino group, were observed. The ^{13}C NMR spectrum showed 14 carbon signals, attributable to two carbonyl carbons at 169.3 and 165.0 ppm, four methylene carbons (22.5, 28.3, 36.7 and 45.4 ppm), two methine carbons (56.1 and 59.1 ppm), and six aromatic carbons. All recovered data indicated that compound **3** contained two rings in the molecule. A detailed analysis of the ^1H–^1H correlation spectroscopy (COSY) spectrum showed connectivity for two proton spin systems, H_3–H_4–H_5–H_6 and NH–H_9–H_{10}–Ph′. The heteronuclear multiple bond correlations (HMQC) of H-3 to C-6, H-6 to C-7, H-5 to C-7 and H-2′ to C-6′, were observed. These data defined the presence of a proline moiety and phenyl moiety in **3**.[22] This compound **3** was identified as *cyclo*-(L-Pro-L-Phe) and was achieved only from *Bacillus thuringiensis* strain.

Diketopiperazine **4** was achieved as white square crystals from *Bacillus endophyticus* and as a white solid from *Bacillus thuringiensis* and its molecular formula was assigned as $C_{14}H_{16}N_2O_3$ by high resolution EI-HRMS (*m*/*z* 260.1160, Calcd for 260.1161. The molecular formula allowed assigning the number of insaturations that was eight. The IR absorptions of 3292, 1632, and 1514 cm^{-1} showed the presence of the amide group. In the ^1H NMR spectrum of the compound, two double signals at 7.07 and 6.80 ppm indicating the presence of *para*-substituted phenyl group and two broad sig-

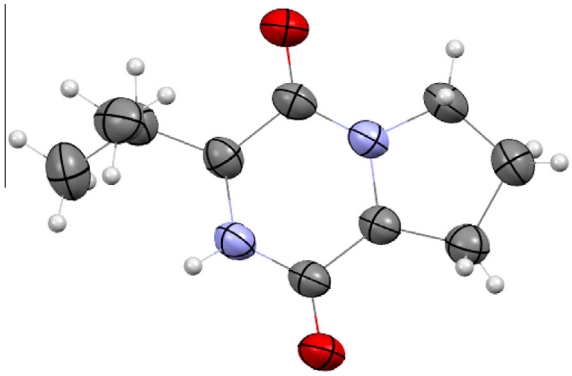


Figure 2. ORTEP representation of compound **2**.

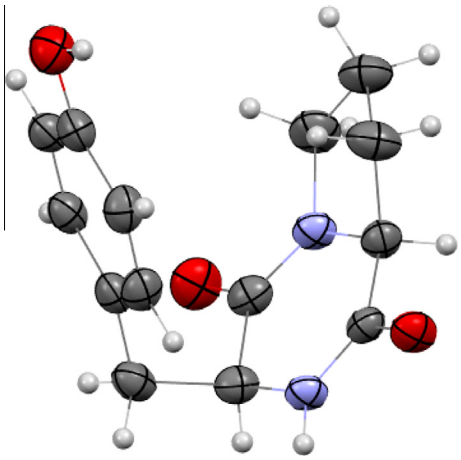
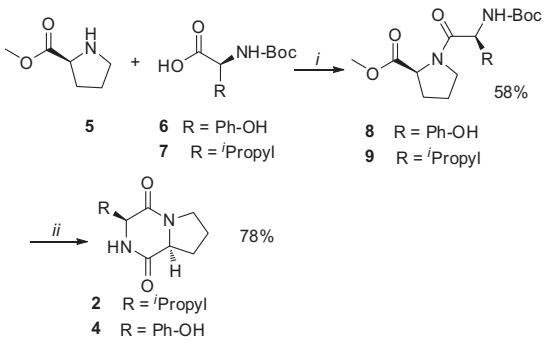


Figure 3. ORTEP representation of compound **4**.

nals at 6.36 and 5.79 ppm were observed indicating the presence of hydroxyl and amino groups respectively. An important difference was noted in NMR ^{1}H spectra of this compound from both strains; the signal at 6.36 ppm of hydroxyl group corresponded to the compound **4** achieved from *Bacillus endophyticus*; however, in NMR ^{1}H spectra of the compound **4** achieved from *Bacillus thuringiensis* this signal corresponding to hydroxyl group appeared at 3.57 ppm overlapped with the signals of H_3 and H_{10}. This could be deduced by the integration of this signal which corresponded for four hydrogens instead of three. The ^{13}C NMR spectrum showed 14 carbon signals, attributable to two carbonyl carbons at 169.7 and 165.1 ppm, four methylene carbons (22.5, 28.2, 35.9 and 45.3 ppm), two methine carbons (56.1 and 59.0 ppm), and six aromatic carbons. All recovered data indicated that compound **4** contained two rings in the molecule. A detailed analysis of the ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum showed connectivity for two proton spin systems, H_3–H_4–H_5–H_6 and NH–H_9–H_{10}–Ph. The heteronuclear multiple bond correlations (HMQC) of H-3 to C-6, H-6 to C-7, H-5 to C-7 and H-2′ to C-6′, were observed. These data defined the presence of proline moiety and phenol moiety in **4**.[23] The compound **4**, extracted from *Bacillus endophyticus*, was crystallized confirming unequivocally the proposed structure and the absolute configuration was determined as 6*S*,9*S*, as shown in Figure 2.[24] This compound **4** was identified as *cyclo*-(L-Pro-L-Tyr) and was obtained both with *Bacillus thuringiensis* and *Bacillus endophyticus* strains as white solid and square crystal respectively, showing a difference in the displacement of hydroxyl group in NMR ^{1}H spectrum.

It can be noted from the above data that each strain produced different diketopiperazines, secondary metabolite production being strain dependent. Besides, diketopiperazine **4**, *cyclo*-(L-Pro-L-Tyr), was obtained as solid from *Bacillus thuringiensis* strain and as square crystal from *Bacillus endophyticus* strain. All characterization data were the same for this compound except the NMR ^{1}H spectrum which has a marked difference in the OH signal and melting point which was different for the compound extracted from both strains. This difference can be argued by polymorphism of the compound that is the condition by which a solid chemical compound exists in more than one crystalline form differing somewhat in physical and, sometimes, chemical properties.[25,26]

In order to obtain a major quantity of these compounds, their synthesis was carried out following a modified method of Campbell et al.[27] Methyl ester of L-proline **5**, was coupled with *N*-Boc protected amino acids **6** and **7** (L-tyrosine or L-valine) using dicyclohexylcarbodiimide (DCC) mediated conditions to give the compounds **8** and **9** in 58% yield. In contrast with other methods that employed some steps, in only one step we achieved the cleavage of the *N*-Boc group under acidic conditions and intramolecular cyclization with NaOH generating diketopiperazines **2** and **4** in a moderate yield 78% (45% overall yield) for subsequent biological evaluations (Scheme 1). Should be noted that synthetic compound **4** was obtained as a white solid and the NMR ^{1}H was similar as the compound **4** isolated from *Bacillus thuringiensis* confirming the polymorphic property of this compound.



Scheme 1. Reagents and conditions: (i) DCM, Et_3N, 1 h, DMAP, DCC, rt 2 h; (ii) HCl 5 M, AcOEt, 1 h, NaOH 2 M, rt, 30 min.

The compounds **1**–**4** were probed against Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus saprophyticus*, and *Listeria monocytogenes* and Gram-negative bacteria *Escherichia coli*, *Salmonella choleraesuis* and *Vibrio cholera*, by disk diffusion assay[28] resulting in resistance of all probed bacteria to the four compounds at 75 mg/mL. However, when the compounds **1**–**4** were probed by disk diffusion assay[29] against the fungi *Fusarium oxysporum*, *Alternaria* sp., *Rhizopus* sp., *Bipolaris* sp., *Mucor* sp., and *Penicillium* sp. at 75 mg/mL, the compound **2** showed a marked inhibition against *F. oxysporum* and *Penicillium* sp. fungi whereas the compounds **3** and **4** showed a slight inhibition against the same fungi, showing the antifungal activity of these compounds.

In conclusion, we described the isolation of four diketopiperazines *cyclo*-(L-Pro-L-Leu) **1**, *cyclo*-(L-Pro-L-Val) **2**, *cyclo*-(L-Pro-L-Phe) **3** and *cyclo*-(L-Pro-L-Tyr) **4** from *Bacillus thuringiensis* and *cyclo*-(L-Pro-L-Val) **2**, and *cyclo*-(L-Pro-L-Tyr) **4** from *Bacillus endophyticus* probing that biosynthesis of secondary metabolites is dependent of the species. In addition the compound **4** was achieved as a polymorphic form showing similar spectroscopic data but with different physical properties. A complete characterization of the four diketopiperazines is also described and their easy synthesis in only two simple steps. Their biological activities were probed against some Gram-positive and Gram-negative bacteria and six fungi probing their antifungal properties.

Acknowledgments

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15. *cyclo*-(L-Proline-L-Leucine) **1**; extraction yield 2.2%, mp 152–156 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.80 (1H, br s, NH), 4.13 (1H, br t, J = 8.0 Hz, H6), 4.01 (1H, s, J = 10.0, 4.0 Hz, H9), 3.54 (2H, m, $H3_a$, $H3_b$), 2.35 (1H, m, $H5_a$), 2.12 (1H, m, $H5_b$), 2.07 (1H, m, $H10_a$), 2.05 (H, m, $H4_a$), 1.91 (1H, m, $H4_b$), 1.73 (1H, m, H11), 1.55 (1H, m, $H10_b$), 1.00 (3H, d, J = 6.5 Hz, H12), 0.95 (3H, d, J = 6.5 Hz, H13); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 170.1 (C1=O), 166.1 (C7=O), 59.0 (C6) 53.3 (C9), 45.5 (C3), 38.6 (C10), 28.1 (C5), 24.7 (C11), 23.2 (C12), 22.7 (C4), 21.2 (C13). FAB-HRMS: calculated for ($C_{11}H_{19}N_2O_2$), 211.1447; found, 211.1444.
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17. *cyclo*-(L-Proline-L-Valine) **2**; extraction yield 2.4%, mp 162–166 °C; $[\alpha]_D^{25} = -129$ (c 1, MeOH) (lit.,[18] −128 in MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.89 (1H, br s, NH), 4.09 (1H, br t, J = 7 Hz, H6), 3.97 (1H, s, H9), 3.67–3.53 (2H, m, $H3_a$, $H3_b$), 2.64 (1H, sept of d, J = 7, 2.5 Hz, H10), 2.40–2.33 (1H, m, $H5_a$), 2.09–2.01 (2H, m, $H5_b$, $H4_a$), 1.94–1.88 (1H, m, $H4_b$), 1.07 (3H, d, J = 7 Hz, H11), 0.92 (3H, d,

J = 7 Hz, H12); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 169.9 (C1=O), 164.8 (C7=O), 60.1 (C9) 58.7 (C6), 45.0 (C3), 28.5 (C5), 28.3 (C10), 22.3 (C4), 19.1 (C11), 16.0 (C12). $IR_{\upsilon max}$: 3212, 2969, 1669, 1426, 1293, 791 cm^{-1}; EI-HRMS: calculated for ($C_{10}H_{16}N_2O_2$), 196.1212; found, 196.1192.

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19. Crystal data for **2**: $C_{10}H_{16}N_2O_2$, M = 196.25, colorless plate, colorless prism, mp = 165.2 °C, 0.444 × 0.128 × 0.085 mm^3, orthorhombic, space group $P2_12_12_1$, cell parameters a = 5.7929(4), b = 10.6294(11), c = 34.938(3) Å, Z = 8, Z' = 2, D_c = 1.212 g cm^{-1}. 11,222 reflections collected on a Xcalibur, Atlas, Gemini diffractometer at rt, with the CuKα radiation (λ = 1.54184 Å) in the range 2θ = 8.696–148.96°, of which 4389 are unique (R_{int} = 0.0275, R_{sigma} = 0.0297). 257 variables refined: R_1 = 0.0604, wR_2 = 0.1681 [$I > 2\sigma(I)$] and R_1 = 0.0906, wR_2 = 0.1988 [all data].[21] The absolute configurations of C6 and C9 were assigned by analysis of anomalous dispersion of data collected. CCDC-1439226 contains the supplementary crystallographic data for this Letter. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK. Fax: +44 1223 336033.
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22. *cyclo*-(L-Proline-L-Phenylalanine) **3**; extraction yield 3.32%, mp 128–131 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.29 (5H, m, Ph), 5.60 (1H, br s, NH), 4.28 (1H, dd, J = 10.5, 3.5 Hz, H9), 4.08 (1H, dd, J = 7.5 Hz, H6), 3.64 (1H, m, $H10_a$), 3.62 (1H, m, $H3_a$), 3.57 (1H, m, $H3_b$), 2.77 (1H, dd, J = 14.5, 10.5 Hz, $H10_b$), 2.34 (1H, m, $H5_a$), 2.03 (1H, m, $H5_b$), 1.99 (1H, m, $H4_a$), 191 (1H, m, $H4_b$); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 169.3 (C1=O), 165.0 (C7=O), 135.8 (C1′), 129.3 (C3′, C5′), 129.0 (C2′,C6′), 127.5 (C4′), 59.1 (C6), 56.1 (C9), 45.4 (C3), 36.7 (C10), 28.3 (C5), 22.5 (C4). EI-HRMS: calculated for ($C_{14}H_{16}N_2O_2$), 244.1212; found, 244.1177.
23. *cyclo*-(L-Proline-L-Tyrosine) **4**; extraction yield 2.9%, $[\alpha]_D^{25}$ = −53 (c 0.7, EtOH); mp = 142–147 °C, ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.07 (2H, d, J = 8.5 Hz, H3′, H5′), 6.80 (2H, d, H2′, H6′), 6.36 (1H, br s, OH), 5.79 (1H, br s, NH), 4.21 (1H, dd, J = 10.5, 3.5 Hz, H9), 4.09 (1H, br t, J = 7.5 Hz, H6), 3.68 (2H, m, $H3_a$, $H3_b$), 3.51 (1H, dd, J = 14.5, 3.5 Hz, $H10_a$), 2.78 (1H, dd, J = 14.5, 10.5 Hz, $H10_b$), 2.34 (1H, m, $H5_a$), 2.05 (1H, m, $H5_b$), 1.90 (2H, m, $H4_a$, $H4_b$); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 169.7 C1=O), 165.1 (C7=O), 155.5 (C4′), 130.2 (C3′, C5′), 127.1 (C1′), 116.1 (C2′,C6′), 59.0 (C9), 56.1 (C6), 45.3 (C3), 35.9 (C10), 28.2 (C5), 22.5 (C4). $IR_{\upsilon max}$: 3292, 1632, 1514, 1433, 1227, 806 cm^{-1}; EI-HRMS: calculated for ($C_{14}H_{16}N_2O_3$), 260.1161; found, 260.1160.
24. Crystal data for **4**: $C_{14}H_{16}N_2O_3$, M = 260.29, colorless plate, colorless prism, mp = 147.2 °C, 0.752 × 0.533 × 0.366 mm^3, orthorhombic, space group P21212, cell parameters a = 11.8825(7), b = 12.0691(7), c = 18.5436(14) Å, Z = 8, Z' = 2, Dc = 1.300 g cm^{-1}. 20,418 reflections collected on a Xcalibur, Atlas, Gemini diffractometer at room temp, with the Cu Kα radiation (λ = 1.54184 Å) in the range 2θ = 8.742–134.146°, of which 4754 are unique (R_{int} = 0.0426, R_{sigma} = 0.0319). 354 variables refined: R_1 = 0.0399, wR_2 = 0.0878 [$I > 2\sigma(I)$] and R_1 = 0.0626, wR_2 = 0.1024 [all data].[21] The absolute configurations of C6 and C9 were assigned by analysis of anomalous dispersion of data collected. CCDC-1439218 contains the supplementary crystallographic data for this Letter. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK. Fax: +44 1223 336033.
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28. 7.5 mg of the compounds were dissolved in 100 μL of distilled sterile water. Sterile filter paper disks containing 10 μL of crude extract (75 mg/mL) were placed on plates of LB and Muller–Hinton agar seeded with 100 μL of suspensions of one day old cultures of bacteria tested. The negative control was a sterile disk impregnated with distilled water and control positive was the antibiotic ampicillin (150 mg/mL) for Gram negative bacteria and vancomycin (50 mg/mL) for Gram positive bacteria. The diameters of the zones of inhibition of growth around the disks were measured after incubation periods of one day at 29 °C.
29. 7.5 mg of the compounds were dissolved in 100 μL of distilled sterile water (75 mg/mL). Fungal spore suspensions were prepared taking a small mycelial fragment of 5 day old PDA plate cultures of each test fungal pathogen and mixing with 6 mL of LB medium. The fraction of inoculated fungus was disintegrated gently with the help of the handle, stirred vigorously for 30 s in a 45° angle and was left at rest in a rack to get the spores separated from hyphae and remaining in the supernatant. The spore suspensions were adjusted to give a concentration of approximately 10^6–10^7 spores mL^{-1}. Sterile filter paper disks, containing 10 μL of the compounds were placed on plates of PDA agar seeded with 100 μL of spore suspensions of the fungal pathogens to be tested. The negative control was a sterile disk impregnated with distilled water and positive control was the antifungal agent miconazole (Neomicol Medix, 20 mg/mL). The diameters of the zones of inhibition of growth around the disks were measured after incubation periods of three days at 29 °C.