Biofilm blocking sesquiterpenes from *Teucrium polium*

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Introduction

*Teucrium polium* is a member of the Lamiaceae family with the genus including more than 300 species. Plant species in this genus are distributed ubiquitous around the world, with a concentration in the Mediterranean basin. Chemical investigations have shown that members of this genus are rich in monoterpenes, sesquiterpenes, sterols, saponins, iridoids, flavonoids, polyphenolic compounds, fatty acids, alkaloids and essential oils (Perez et al., 2005; Perez et al., 1993; Kamel and Sandra, 1994; Piozzi et al., 1998; Ulubelen et al., 2003). Several plants of this genus are used in folk medicine for treatment of fungal infections and abscesses (Batanouny, 2005). In addition, these plants are used for digestive disorders, inflammation, hypertension, fever, diabetes, rheumatism, parasitic diseases, such as amoebiasis, and as tonics, stimulants and antiseptics (Moustapha et al., 2011). Previous studies have demonstrated the therapeutic efficacy of *Teucrium* species as antibacterial (Belmekki et al., 2013), antipyretic (Autore et al., 1984), anti-inflammatory (Menichini et al., 2009), antioxidant (Sharififar et al., 2009), hypoglycemic (Afifi et al., 2005; Esmaeili and Yazdanparast, 2004), anticancer (Nematollahi-Mahani et al., 2007), and anti-nociceptive agents (Abdollahi et al., 2003). *T. polium* contains phenylpropanoid glycosides, iridoid glycosides, flavonoids (De Marino et al., 2012), diterpenes (Fiorentino et al., 2010), and monoterpenes (Wassel and Ahmed, 1974), as well as sesquiterpenes (Cozzani et al., 2005). The plant is widely used in folk medicine for abdominal colic, headache, and kidney stones, as well as vermifuge, anti-inflammatory, and antipyretic (Aburjai et al., 2006). In Egypt, *T. polium* is used for wound healing, as well as an appetite, expectorant, and hypoglycemic (Kamel, 1995). In addition, an extract showed activity against both yeast and carrageenin pyrexia in rats. The interest in this study was in antibiotic activity of the crude extract, as well as with four of the isolated metabolites, was observed with *Staphylococcus aureus* anti-biofilm activity in the low μM range. Diverse sesquiterpene-skeleton structure and corresponding comprehensive enzyme capacity is discussed.

**Keywords:**
Teucrium polium 
Lamiaceae 
Sesquiterpenes 
Biofilm blocking

**A B S T R A C T**

The chemical composition and antibacterial activity of *Teucrium polium* L. (Lamiaceae) were assessed; sixteen compounds were isolated from a CH2Cl2/MeOH extract of the aerial parts of the plant including four sesquiterpenes 4\(\beta\)-\(\delta\)-epoxy-7\(\alpha\)-H-gemmar-10(14)-en-6\(\gamma\)-ol-1-one, 4\(\beta\)-\(\delta\)-epoxy-7\(\alpha\)-H-gemmar-10(14)-en-1\(\beta\)-hydroperoxy-6\(\gamma\)-ol, 4\(\beta\)-\(\delta\)-epoxy-7\(\alpha\)-H-gemmar-10(14)-en-1\(\beta\)-hydroperoxy-6\(\gamma\)-ol, together with seven known sesquiterpenes, one known iridoid glycoside, two known flavonoids, and one known phenylpropanoid glycoside. Structures were elucidated on the basis of spectroscopic (UV, 1H and 13C NMR) data, as well as two-dimensional NMR (\(^1\)H-\(^1\)H COSY, HMQC, NOESY and HMBC), and ESI-MS analysis. The relative stereochemistry of the ketone was established by X-ray crystallography, while its absolute configuration was attained by a modified Mosher’s method. Antibacterial activity of the crude extract, as well as with four of the isolated metabolites, was observed with *Staphylococcus aureus* anti-biofilm activity in the low μM range. Diverse sesquiterpene-skeleton structure and corresponding comprehensive enzyme capacity is discussed.
toxicity of antibiotics. Blocking or retarding formation of biofilms improves the efficacy of antibiotics (Götz, 2002). Staphylococcus aureus is frequently the causal agent in biofilm-associated infections (Otto, 2008) and treatment is becoming increasing less effective due to antibiotic prevalent resistance (Begun et al., 2007; Darabpour and Hossein, 2010). The focus of this study is to mine for T. polium metabolites with antibiotic activity.

Results and discussion

The CH₃Cl₂-MeOH extract of T. polium aerial plant material was partitioned with a gradient of n-hexane, dichloromethane, and methanol. Compounds from the eluted fractions were purified using a combination of Sephadex, and silica gel CC, as well as, by RP-HILIC. Four new compounds 1-4, along with 12 known 5-16 (Fig. 1) were isolated and characterized.

Compound 1 was isolated as a colorless crystals with a specific rotation of [α]D = −8.3 (c 0.9, CHCl₃). The mass spectrum of 1 exhibited a HRESI-MS molecular ion peak [2M+Na⁺] at m/z 527.3339 (calc. 527.3348), and [M⁺] at m/z 253.1794 (calc. 253.1798), suggesting a molecular formula C₁₁H₂₀O₅. Its IR spectrum showed absorptions due to a hydroxyl group (3412 cm⁻¹), one terminal exocyclic double bond, and a carbonyl group (2954, 1669 and 928 cm⁻¹) (Barreroa et al., 1999). On the basis of a DEPT experiment, the fifteen ¹³C signals corresponded to one exocyclic double bond, three methyls, four methylenes, and four methines (two of which are oxygenated), as well as two quaternary carbon atoms one of which is a ketone. The ¹H and ¹³C NMR spectra (Tables 1 and 2, respectively) established the following fragments: one tertiary methyl signal at δH 1.25, isopropyl methyl signals appearing at δH 0.88 and 0.99 (δD 6.8 Hz) respectively, and a hydroxyl-bearing methine at δH 3.43 (dd, δJ = 7.7), and an exocyclic methylene δH 5.88 and 6.22 (s). The degree of saturation indicated one tertiary methyl signal at δH 1.25, isopropyl methyl signals at δH 0.88 and 0.99, allowed for the assignment of C-11, C-12 and C-13, respectively.

Based on the relative up-field proton chemical shifts and clear doublet signals for H-12 and H-13 identified the presence of an isopropyl group. Moreover, HMBC between H-7 and C-11, as well as C-12, established a C-7 linkage of the isopropyl unit. COSY coupling of H₂-8 to δH signals as 2.16 and 2.79 allowed for the assignment of C-9, which was supported by the HMBC between H-7, C-8 and C-9. A COSY correlation between H₉-9 (δH 2.79 brd.) and H-14 (δH 5.88, d, 1.8), as well as a HMBC between H-9, C-10 and C-14, confirmed the connectivity of C-9 and C-10.

The relative stereochemistry of the chiral centers in 1 was resolved by a combination of X-ray crystallography (Fig. 4) and 2-D NOESY data, as well as analysis of the coupling constants and was supported by data from literature (Barreroa et al., 1999) (Fig. 3). The cross peaks observed in the NOESY spectrum between H-6 and H-15, as well as between H-5 and H-11, implied that H-15, H-6, and H-7 were on the same molecular face of germacrone ring; thus H-5 was on the opposite side to H-15, H-6 and H-7 which was confirmed by X-ray crystallography of 1 (Fig. 4). The multiplicities of the carbon signals were deduced from DEPT experiments, whereas the assignment of all proton resonances and their connectivities to adjacent proton and carbon signals were established from 2-D HMBC and 2-D ¹H-¹H COSY experiments. A modified Mosher’s method was performed to determine the absolute configuration of the secondary alcohols at C-6 (Ohtani et al., 1991). Treatment of two aliquots of 11 with (S)- and (R)-MTPA chloride in dry pyridine gave the corresponding esters 1a and 1b, respectively, with a molecular ion peak at m/z 469.2201 consistent with derivatized products. The pattern of A(δS-δR) values (Fig. 5) allowed for the absolute configuration at C-6 to be R and the complete stereochemistry of 1 based on NMR, X-ray crystallography and Mosher data was assigned to 45S, 55S, 6R, and 7S as shown in Fig. 1 with the name 4α,5z-epoxy-7αH-germacar-10(14)-en-6β-ol-1-one.

Compound 2 was isolated as a colorless syrup, with a specific rotation of [α]D = +12.5 (c 0.6, CHCl₃). Its mass spectrum exhibited a HRESI-MS molecular ion peak [M⁺] at m/z 271.1896 (calc. 271.1903) indicating a molecular formula of C₁₀H₁₆O₄. The IR spectrum showed absorptions due to a hydroxyl group (3356 cm⁻¹), (C=O) and one terminal exocyclic double bond (2955, 2928, 2870, 2360, 1701, 1558, 1008, 906, 818 and 668 cm⁻¹). The structure of 2 is similar to 1, based on NMR spectroscopic data, except for the replacement of the ketone group in 1 with a methine-bearing hydroperoxide in 2 (Fig. 1). The hydroperoxide at C-1 followed from a downfield chemical shift for C-1 to δC 85.9 and a new proton signal at δH 4.44 corresponding to H-1'. EIMS was consistent with the presence of a hydroperoxide. The HMBC between H₂-14 (δH 5.11, 5.14) and δC signal at 85.9 confirmed the C-1 position (Fig. 2). The presence of a peroxy group was positive employing commercially available Baker Testrips (Thomas Scientific). The relative stereochemistry of the chiral centers for 2 was resolved by...
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* Solvent: methanol d₅.

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* Solvent d₅ MeOH.

NOESY (Fig. 3) with cross-peaks observed between H-1, H-15, H-6 and H-7, indicating that these protons were on the same molecular face of the germacrane ring. Analysis of the vicinal couplings between H-5 and H-6 were found to be J₅,₆ (8.24 Hz) pointing to an anti-coplanar arrangement. Assuming the same stereochemistry for both 1 and 2, C-1 was assigned an S configuration and the structure of 2 was established as 4,β,5α-epoxy-7,4β-hydropyranyloxy-10(14)-en,1β-hydropyranyloxy,6β-ol, also a new natural product.

Compound 3 was isolated as a colorless syrup with a specific rotation of [α]D²⁵ = +74° (c 0.40, CHCl₃), its mass spectrum exhibited a HRESI-MS molecular ion peak [M⁺] at m/z 271.1895 (calc. 271.1903) indicating a molecular formula C₁₅H₂₀O₄. Its IR spectrum showed absorptions due to a hydroxyl group (3386 cm⁻¹), (C=O) and one terminal exocyclic double bond (2957, 2870, 2707, 1670 and 925 cm⁻¹). The structure of 3 was similar to 2, based on NMR spectroscopic data, except for an inverted stereo-center at C-5, based on NOE data, in which a correlation was observed between C-5 and C-1, C-4, C-6, and C-7 which was not observed in 2 (Fig. 3). Structure 3 was established as 4/5β-epoxy-7/4H-germacr-10(14)-en,1β-hydropyranyloxy,6β-ol, also a new natural product.

Compound 4 was isolated as a colorless syrup with a specific rotation of [α]D²⁵ = +37.5° (c 0.11, CHCl₃). Its mass spectrum exhibited a HRESI-MS molecular ion peak [M⁺] at m/z 271.1897 (calc. 271.1903) indicating a molecular formula C₁₅H₂₀O₄. Its IR spectrum showed absorptions due to a hydroxyl group (3405 cm⁻¹), (C=O) and one terminal exocyclic double bond (2957, 2871, 2710, 1670, 1018, 910, and 736 cm⁻¹). The structure of 4 was similar to 3, based on NMR spectroscopic data except for the stereochemistry at C-4 and C-6 (Fig. 3). Based on NOE data, a correlation was observed between C-4 and C-6, these being in the opposite configuration to C-1, C-5, and C-7. The absence of NOE coupling between H-6 and H-7, as well as between H-1 and H₂-15, confirmed these configuration assignments. So 4 was assigned the name 4x,5β-epoxy-7xH-germacr-10(14)-en,1β-hydropyranyloxy,6x-ol, also a new natural product.

In addition, 12 known compounds consisting of seven sesquiterpenes: 10x,1β/4/5x-diepoxy-7xH-germacr-6-ol (5) (Sanz and Alberto, 1991), teucadiol (6) (Bruno et al., 1993), 4/6x-dihydroxy-1z,5zβ(H)-guai-9-ene (7) (Mahmond, 1997), opl-ophanan (8) (Dastlilk et al., 1989), oxyphylidendiol A (9) (Muraoka et al., 2001), eudesm-3-ene-1β-diol (10) (Mahmond, 1997), rel-1β,3β,6β-trihydroxyedem-4-ene (11) (Stavri et al., 2004), artecliniet (12) (Khagay et al., 1983), and two flavonoids including 7,4α-0-dimethylcinyllactone-1(5b,6b)-6,7-dimethoxyfla- one (14) and salvigenin (15) (Okuda et al., 1975; Seshadri and Sharma, 1973) as well as two glycosides: teucardoside (13) (Ruhdorfer and Rimpler, 1981) and poliumoside (16) (De Marino et al., 2012) were isolated and identified by direct comparison of their spectroscopic data with those reported in the literature.

Biofilm inhibition was observed in the bacterial line S. aureus strain AH133-GFP via confocal laser scanning microscopy (CLSM) which showed that the depth of biofilm formed in DMSO containing-disk was greater than when treated with 3, 10, or 15 (Fig. 6). At the 0.18 and 0.67 μMol doses, 3 and 15, respectively were as
effective in inhibiting biofilm formation as 0.02 μMol of a commercially used broad-spectrum inhibitor of biofilm formation for bacteria, gentamicin, which is potent antibiotic standard, while partial inhibition was observed with 0.79 μMol of 10. The rest of the compounds produced variable levels of inhibition (data not shown).

Concluding remarks

Three distinct sesquiterpene skeletons have been reported from *T. polium* collected in Tunisia (Ghiglione et al., 1976), Saudi Arabia (Hassan et al., 1979), and Serbia (Kovacevic and Lakusic, 2001), eudesmol, guaiol, and germacrene, respectively. Plants collected from Egypt contain all three of these sesquiterpene skeletons. This establishes that the enzymes that convert the common precursor FPP (farnesyl diphosphate) to the germacrane skeleton, germacrone A synthase, as well as germacrone cyclase that generates the guaiane and eudesmane skeletons, (Piet et al., 1995, 1996) are functional in the Egyptian ecotype of *T. polium*.

**Experimental**

**General experimental procedures**

Optical rotations were measured in CHCl₃ on an Autopal IV automatic polarimeter (from Rudolph Research Analytical) equipped with a sodium lamp (λmax = 589 nm) and a 10 cm microcell. IR (KBr) spectra were recorded on a ThermoNicolet model IR 100 spectrophotometer. High-resolution ESI mass spectrometry (HRESI-MS) was carried out on a Micromass QTOF spectrometer and electrospray ionization mass spectrometry (ESI–MS) experiments were performed on an API 2000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). High-performance liquid chromatography (HPLC) separations in the isocratic mode were achieved on Agilent 1100 apparatus equipped with a Rheodyne injector, refractive index, and with UV detectors, using an Agilent Prep-C18 column (21.2 × 250 mm, 10 μm). NMR spectra were obtained on a Varian (Palo Alto, CA) Unity Inova 500 NMR spectrometer (1H at 500 MHz and 13C at 125 MHz) equipped with V NMR 6.1C software and Sun hardware, δ (ppm), J in Hz, spectra relative to CD3OH (δH = 3.31) and as internal standard. Chemical shifts are referenced to the residual solvent signal (CDCl₃: δH 7.26, δC 77.0). The multiplicities of 13C NMR resonances were determined by DEPT experiments. One-bond heteronuclear 1H–13C connectivities were determined with the HMQC experiment. Two- and three-bond 1H–13C connectivities were determined by HMBC experiments. Nuclear Overhauser Effect (NOE) measurements were obtained from 2D NOESY experiments. Column chromatography (CC) was carried out using EMD silica gel 60 (70–230 mesh). Analytical TLC was performed on Merk silica gel 60 F₂₅₄ sheets 0.25 mm thick.

**Plant material**

Air-dried aerial parts of *T. polium* were collected in June 2010, from North Sinai, Egypt. A voucher specimen SK-105 has been deposited in the Herbarium of St. Katherine protectorate, Egypt.

**Extraction and isolation**

Air-dried aerial plant tissue (2 kg) was crushed and extracted with CH₃Cl₂–MeOH (1:1) (4 L) at room temperature. After solvent removal, the residue (210 g) was subjected to silica gel column chromatography (CC) and eluted with n-hexanes, CH₃Cl₂ and MeOH in increasing order of polarity up to 100% CH₃Cl₂ and then to 15% MeOH in CH₃Cl₂ (a total solvent volume of 400 L) to afford 398 1-L fractions.
Fractions (1–7) were combined (2.6 g) based on TLC similarities, concentrated in vacuo, re-dissolved in CH2Cl2 and defatted with MeOH. The lower layer was subjected to silica gel CC, eluted with n-hexanes with increasing amounts of EtOAc up to 1:1 n-hexanes:EtOAc to afford 29 fractions. Pooled fractions 2–9 (600 mg) were subjected to reversed phase HPLC (MeOH-H2O, 30:70) to obtain 9 (2.5 mg). Fractions (84–111) were pooled (1.1 g) based on TLC similarities, concentrated in vacuo and subjected to silica gel CC; eluted with n-hexanes with increasing amounts of EtOAc up to 1:1 n-hexanes:EtOAc. Subfractions 8–10 (310 mg) were pooled, evaporated, then subjected to reversed phase HPLC to afford 12 (2 mg) and 8 (7 mg). Pooled fractions 112–221 (1.4 g) were subjected to series of silica columns eluting with gradients of n-hexane–EtOAc up to 100% EtOAc and EtOAc–acetone up to 100% acetone then gradient acetone–MeOH up to acetone–MeOH (50:50) to afford 122 fractions; subfractions 16–17 (30 mg) were pooled and subjected to reversed phase HPLC (MeOH–CH3CN / H2O (50:50), 20–80) to afford 6 (5 mg), and 7 (3 mg). Subfractions 26–31 (220 mg) were pooled and subjected to a reversed phase HPLC (MeOH–CH3CN/H2O (50:50), 25–75) to afford 5 (11 mg), and 1 (4.7 mg). Fractions (294–332) were re-combined (7 g) based on TLC similarities, concentrated in vacuo and subjected to silica gel CC eluting with a gradient of CH2Cl2–MeOH starting with (10:3) up to (7:3); fractions were monitored by TLC eluting with CH2Cl2–MeOH–H2O (7:3:1), to afford 50 fractions. Sub-fractions 21–28 (2 g) were pooled and subjected to Sephadex LH-20 gel eluted with an isotropic system of n-hexane–CH2Cl2–MeOH (7:4:0.5); 42 sub-fractions were obtained and sub-fractions 13–18 (550 mg) were pooled and purified by RP HPLC eluting with an isotropic MeOH–H2O system (17:83) to afford 16 (24 mg) and 13 (30 mg). Pooled fractions 41–83 (12.6 g) were subjected to a series of silica gel CC procedures, eluting with gradients of n-hexane–EtOAc up to 100% EtOAc and EtOAc–acetone up to 100% acetone then gradient acetone–MeOH up to acetone–MeOH (50:50) to afford 40 fractions. Sub-fractions 26–30 (1.3 g) were pooled and subjected to silica gel CC eluting with a gradient system of n-hexane–EtOAc (7:1) up to 100% EtOAc to afford 45 fractions from which 14 (20 mg), and 15 (35 mg) were isolated. Sub-fractions (16–21) (1.8 g) were pooled and passed through a series of silica gel CC eluting with a gradient system of n-hexane–EtOAc (7:1) up to 100% EtOAc to afford 45 fractions, sub-fraction 14 was identified as 10 (8 mg). Sub-fractions 3–6 (1.1 g) were pooled and subjected to silica gel CC eluting with a gradient system of n-hexane–EtOAc (7:1) up to 100% EtOAc giving 85 fractions to afford 2 (6 mg), 3 (10 mg), 4 (6 mg) and 11 (2 mg).

4j,5j-Epoxy-7α-germacr-10(14)-en-6β,7-ol-1-one (1)

Colorless crystals (dichloromethane-methanol); [α]D20 = –8.33° (CHCl3; c 0.9); mp 183–187°C; UVmax 223; IR (νmax cm–1) 3412 (OH), 2954, 2840, 1669 (C=O), 1433, 1384, 1075, 1034, 928, 826, 605; EIMS (probe) 70 eV m/z (rel. int.): 527.3339 [M+Na]+, 253.1794 [M]+ (calc. 527.3348). For 1H and 13C NMR spectroscopic data, see Table 1.

(5j)-MTPA ester of 1 (1a)

1H NMR (CDCl3, 400 MHz): 3.10 (1H, m, H-2a), 2.40 (1H, m, H-2b), 2.16 (1H, m, H-3a), 1.46 (1H, m, H-3b), 2.62 (1H, d, 7.79, H-5), 5.023 (1H, dd, 7.79, H-6), 1.03 (1H, m, H-7), 1.43 (1H, m, H-8a), 1.89 (1H, m, H-8b), 2.13° (1H, m, H-9a), 2.89 (1H, br d, H-9b), 0.90 (3H, d, 6.87, H-12), 0.97 (3H, d, 6.87, H-13), 5.81 (1H, d, H, H-14), 6.05 (1H, s, H-14), 1.344 (3H, s, H-15); 13C NMR (CDCl3, 400 MHz): 203.9 (C-1), 33.8 (C-2), 37.7 (C-3), 58 (C-4), 64.6 (C-5), 76.6 (C-6), 44.2 (C-7), 25 (C-8), 32.6 (C-9), 150 (C-10), 31.3 (C-11), 20.7 (C-12), 21 (C-13), 127.6 (C-14), 16.8 (C-15), 55.4 (OCH3), 127.8, 128.3, 129.6, and 132.5 (benzene ring), 165.8 (C=O); an asterisks (*) indicates overlapping signals. HRESIMS: m/z 469.2201 [M]+ (calc. 469.2202).

X-ray crystallography analysis

X-ray diffraction data for 1 were obtained at room temperature, on a Bruker Smart Apex II CCD diffractometer, using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). Intensity data were collected using o-steps accumulating area detector images spanning at least a hemisphere of reciprocal space. Data were corrected for Lorentz polarization effects, and a multi-scan absorption correction was applied using SADAB (Sheldrick, 2008b). The structure was solved by direct methods and refined by full-matrix least-squares against F2 using SHEXLTL (Sheldrick, 2008a). The OH hydrogen was located from the difference Fourier map and refined isotropically, subject to a distance restraint. All other hydrogen atoms were assigned riding isotropic displacement parameters and constrained to idealized geometries. Due to weak anomalous scattering, the absolute structures could not be determined directly from the data. Crystal Data for 1 are summarized as follow: C31H42O3, colorless platelet, Mw = 525.34, crystal size 0.36 × 0.12 × 0.03 mm3, orthorhombic, space group P212121, a = 10.9799(11) Å, b = 12.924(14) Å, c = 10.160(10) Å, V = 1411(3) Å3, Z = 4, T = 298(2) K, μ = 0.081 mm–1, 4306 reflections collected, unique reflections (Rint) = 0.0240, Σ(θ2) = 2.0422° = 0.0406, wR2 (all data) = 0.0932. CCDG assignment 598590 contains the supplementary crystallographic data for this paper.

Peroxide colorimetric assay

BAKER TESTRIPS for peroxides (Thomas Scientific) is a colorimetric assay for semi-quantitative determination of inorganic...
and organic compounds containing peroxide or a hydroperoxide group. The assay incorporates peroxidase enzyme which transfers oxygen from peroxide to an organic redox indicator converting it to a blue product (Kelly, 1996). The test strip was dipped briefly (ca. 1 s) into the test solution. After 5 s, a blue color indicates the presence of peroxide.

**Preparation of the (R) and (S)-MTPA ester derivatives of 1**

An aliquot of 1 (1.5 mg of each) was dissolved in CH$_2$Cl$_2$ (2 mL) with dry pyridine (0.45 mL) under N$_2$ (g). (S)-or (R)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride (0.1 mL) was added into separate aliquots and the reaction was stirred overnight, quenched with a saturated NaHCO$_3$ solution, and the CH$_2$Cl$_2$ layer was washed twice with distilled H$_2$O. The reaction mixture was dried under reduced pressure to afford the (R) and (S)-MTPA esters.

**Biofilm inhibition assay**

S. aureus strain AH133-GFP, which carries the gene for the green fluorescent protein was grown overnight in Luria Bertani (LB) broth and diluted in LB to ca. 1 x 10$^8$ CFU/ml. The 100 μl culture was spread on an LB agar plate. Sterile cellulose disks were placed on the inoculated plates and three different concentrations from each tested compound were spotted on the disk. The plates were incubated at 37 °C for 16-18 h and the zone of clearing around the disks was measured.

**AH133-GFP biofilm regulation**

Overnight cultures of the tested AH133-GFP were pelleted, washed and re-suspended in fresh LB broth. The cultures were then serially diluted tenfold to obtain inoculums of 1 x 10$^3$ to 1 x 10$^4$ CFU. For each compound, a 6 mm cellulose disks (Becton Dickinson) were placed on the surface of freshly prepared LB agar plates. An aliquot (10 μl) of diluted culture was applied to each disk and plates were incubated at room temperature for 5 min. An aliquot (10 μl) of either DMSO or T. polium test compound (triplicates of each concentration) was added to each disk and plates were incubated at 37 °C for 24 h. Biofilms were quantified by counting CFUs per disk (Hammond et al., 2011). The cellulose disks were removed from the LB plates and placed in an individual 1.5 ml centrifuge tube containing 1 ml of phosphate buffered saline and vortexed to disrupt the biofilm and detach the bacteria. The bacterial suspension was then serially diluted tenfold and 10 μl aliquots of each dilution were spotted on LB agar plates. The number of bacteria per
k = CFU counted × dilution factor × 100.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.03.029.

References


