Isolation and selective glycosylation of antisalmonellal anthraquinones from the stem bark of Morinda lucida Benth. (Rubiaceae)

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Abstract

In this work we report the isolation, identification and antibacterial activity of two anthraquinones, 2-hydroxy-1-methoxyanthraquinone (1) and 2,5-dihydroxy-1-methoxy-6-methoxymethylanthraquinone (2), from the stem bark of *Morinda lucida*. These two natural products were selectively converted into two new glycosylated derivatives, 2-hydroxy-1-methoxyanthraquinone-4’-O-methyl-2-O-β-D-glucopyranoside (3) and 2,5-dihydroxy-1-methoxy-6-methoxymethylanthraquinone-4’-O-methyl-2-O-β-D-glucopyranoside (4) by the filamentous fungus *Beauveria bassiana* ATCC 7159. Structure elucidation was accomplished based on the 1D and 2D NMR, IR and mass spectra. The glycosylated compounds 3 and 4 showed higher *in vitro* antibacterial activity against *S. enterica* subsp. *enterica* sérovars Typhimurim (MIC of 8 µg/mL each) than the corresponding aglycons 1 and 2 (MIC of 16 µg/mL and 32 µg/mL, respectively). These results indicated that microbial glycosylation is an effective approach to modify natural products for enhanced biological activities.

**Keywords:** *Morinda lucida*, anthraquinones, *Beauveria bassiana*, microbial biotransformation, 4’-O-methylglycosylation, antibacterial activity
1. Introduction

*Morinda lucida* Benth. (Rubiaceae) is an evergreen medium-sized tree bearing a dense crown of slender crooked branches (Adeleye et al., 2018). It grows in Central and West Africa, where all the parts (leaves, stem bark and roots) of this plant are used independently to treat malaria and other tropical diseases (Idowu et al., 2010), even though toxicity has been reported (Idowu et al., 2010). This medicinal plant was reported to produce substantial amounts of anthraquinones (Koumaglo et al., 1992). Anthraquinones are well known for their diverse biological activities such as antimalarial (Koumaglo et al., 1992), anticancer (Huang et al., 2005), antibacterial, antifungal and antiviral (Zhan and Gunatilaka, 2006a). Despite wide therapeutic applications, some anthraquinones only show weak bioactivities and others were reported to have certain toxicity (Huang et al., 2005). Therefore, it is desirable to obtain new derivatives with improved physical, chemical and biological properties (Huang et al., 2005). Such derivatives can be prepared through chemical or biological transformations. Filamentous fungi have shown promise in structural modification of natural products. One such fungal biocatalyst is *Beauveria bassiana* ATCC 7159, which has been reported to catalyze a variety of reactions such as hydroxylation, reduction, oxidation, conjugation, hydrolysis, and heteroatom dealkylation (Holland et al., 1999). In addition, this strain has also been used for O-methylation (Zhan and Gunatilaka, 2005) and glycosylation (Zeng et al., 2010; Zhan and Gunatilaka, 2006a). Glycosylation represents an effective method to enhance the water solubility/bioavailability (Zeng et al., 2010), stability (Flores-Fernandez and Griebenow, 2012) and biological activity (Bowles et al., 2006; Ross et al., 2001) of natural products. For example, Ruefer et al. found that daidzein glucoside had better bioavailability than daidzein through oral administration (Ruefer et al., 2008). Another example is curcumin-β-
diglucoside which showed greater antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* than curcumin (Parvathy et al., 2009).

Typhoid fever has been a marked public health problem in developing countries in general, and is endemic in Sub-Saharan Africa in particular (Gatsing et al., 2006). Typhoid fever is caused by *Salmonella enterica* subsp. *enterica* sérovars Typhi, whereas paratyphoid fevers are caused by *S. enterica* subsp. *enterica* sérovars Paratyphi A and B (Gatsing and Adoga, 2007). The greater prevalence of resistance (Gatsing and Adoga, 2007) to all first-line antimicrobials (ampicillin, chloramphenicol and cotrimoxazole) has prompted the continuous search for new and effective anti-typhoid agents. In our ongoing effort to discover bioactive natural products to combat tropical infectious diseases such as typhoid fever, we isolated two anthraquinones, 2-hydroxy-1-methoxyanthraquinone (1, Fig. 1) and 2,5-dihydroxy-1-methoxy-6-methoxymethylanthraquinone (2, Fig. 1), from the methylene chloride extract of the stem bark of *M. lucida*. These two anthraquinones were converted into two new compounds by *B. bassiana* ATCC 7159 (Mei et al., 2014; Zeng et al., 2010; Zhan and Gunatilaka, 2006a, b), which were characterized as 2-hydroxy-1-methoxyanthraquinone-4’-O-methyl-2-O-β-D-glucopyranoside (3, Fig. 1) and 2,5-dihydroxy-1-methoxy-6-methoxymethylanthraquinone-4’-O-methyl-2-O-β-D-glucopyranoside (4, Fig. 1), respectively. All the compounds were tested against a panel of *Salmonella* strains, which showed that the two new glycosylated anthraquinones have higher *in vitro* antibacterial activity against *S. enterica* subsp. *enterica* sérovars Typhimurim than their aglycons.

**Fig. 1**

**2. Results and discussion**

The methylene chloride extract of the stem bark (62 g) of *M. lucida* Benth. was subjected to vacuum liquid chromatography. Two anthraquinones, 1 and 2 (Fig. S1), were obtained as
crystals from the fractions, which were filtered and recrystallized from hexane. The purified compounds were analyzed on LC-MS (Figs. S2 and S3), and their molecular weights were determined to be 254 and 314 Da, respectively.

Compound 1 was obtained as yellow crystals. Its $^{13}$C NMR spectrum portrayed significant peaks at $\delta_C$ 182.7, 182.1, 155.6 and 146.6, allowing us to propose an anthraquinone skeleton with two oxygenated aromatic carbons. From its $^1$H NMR spectrum, an AB system was visible between the protons at $\delta_H$ 7.32 ($J = 8.5$ Hz) and 8.14 ($J = 8.5$ Hz), and an Ar-OCH$_3$ signal was observed at $\delta_H$ 4.05. These data were in perfect agreement with those reported by Wu et al. (2009) for 2-hydroxy-1-methoxyanthraquinone, indicating that they are the same compound. This was further supported by its melting point of 177-178°C. Compound 1 was previously isolated from Morinda officinalis, which was found to have weak antiosteoporotic activity on osteoblasts and osteoclasts (Wu et al., 2009). This plant is commonly used in China to strengthen bones and kidneys (Wu et al., 2009).

Compound 2 was also obtained as yellow crystals. Its $^{13}$C NMR spectrum was similar to that of 1 with some exceptions such as the signals at $\delta_C$ 187.9 and 68.1, which suggested an anthraquinone with a different substitution pattern. Its $^1$H NMR spectrum revealed two AB systems between the protons at $\delta_H$ 7.32 ($J = 8.6$ Hz) and 7.95 ($J = 8.6$ Hz) as well as $\delta_H$ 7.63 ($J = 7.8$ Hz) and 7.75 ($J = 7.8$ Hz). Two methoxy group signals were observed, including an Ar-OCH$_3$ at $\delta_H$ 3.89 and an alkyl-OCH$_3$ at $\delta_H$ 3.40. A sharp proton signal at $\delta_H$ 13.01 indicated a phenolic OH hydrogen bonded to one of the carbonyls while a broad singlet at $\delta_H$ 10.98 suggested a free phenolic OH group. According to these data, structure 2 was proposed for this compound, which was strongly supported by literature (Lin et al., 2007).
These two compounds (1 and 2, 20 mg each) were then incubated with the fungus B. bassiana ATCC 7159 (100 mL of potato dextrose broth) for 14 days. Extraction with ethyl acetate/methanol/acetic acid in the ratio 89:10:1 and HPLC purification afforded 10 mg of 3 and 4 mg of 4 (Fig. S1).

Compound 3 was obtained as a yellow solid. Its molecular weight was determined to be 430 Da based on the [M+Na]+ ion peak at m/z 453 in the ESI-MS spectrum (Fig. S4A), which is 176 mass units larger than the substrate, suggesting that a 4'-O'-methylglucose moiety has been introduced to 1. Further high resolution ESI-MS analysis (Fig. S4B) revealed that this compound has a molecular formula of C_{22}H_{22}O_{9}, based on the [M+H]+ ion peak at m/z 431.1332 (calcd. for C_{22}H_{23}O_{9}: 431.1348). Its IR spectrum showed OH band at 3410 cm\(^{-1}\) and a carbonyl band at 1674 cm\(^{-1}\). A comparison of its \(^1\)H NMR spectrum with that of 1 revealed prominent signals at \(\delta_H 5.15\) (1H, d, \(J = 7.7\) Hz) attributed to H-1', \(\delta_H 3.48\) (3H, s) assigned to 4'-OCH\(_3\), as well as signals at \(\delta_H 3.66\) and 3.54, 3.50, 3.46, 3.39, and 3.10 attributed respectively to H-2', H-5', H-3', H-2' and H-4', respectively (Fig. S5). These data are in agreement with the sugar moiety reported by Zhan & Gunatilaka (Zhan and Gunatilaka, 2006a). Its \(^{13}\)C NMR spectrum showed seven additional signals including those at \(\delta_C 100.2, 79.3\) and 60.2 attributed to C-1', C-4' and 4'-OCH\(_3\) (Fig. S6), further confirming that a 4'-O-methylglucose moiety has been attached to 1. The great shift of the H-3 signal from 7.32 ppm (for compound 1) to 7.64 ppm showed the effect of 4'-O-methylglycosylation on the nearby carbon atom, indicating that the sugar moiety is attached to 2-OH. HMBC spectrum showed the HMBC correlation of H-1'to C-2, which confirmed that the connection of the sugar moiety to the aglycone at C-2, as shown in Fig. 2. Other HMBC, HSQC and \(^1\)H-\(^1\)H COSY correlations (Figs. S7-S9) further supported the structure and its assignments.
Compound 4 was obtained as a yellow solid with a melting point of 251-252°C. Its molecular weight was determined to be 490 Da according to the [M+H]^+ peak at m/z 491.1 in the ESI-MS spectrum (Fig. S10A). Similarly, it is 176 mass units larger than 2, suggesting that a 4'-O-methylglucose moiety has also been introduced to the substrate by B. bassiana ATCC 7159. This was supported by the high resolution ESI-MS spectrum (Fig. S10B), which showed a [M+H]^+ at m/z 491.1543 (cacld. for C_{24}H_{27}O_{11}: 491.1559). Its IR spectrum revealed a free OH band at 3395 cm\(^{-1}\) and a hydrogen-bonded OH at 3320 cm\(^{-1}\), a free carbonyl stretching band at 1674 cm\(^{-1}\) and a hydrogen bonded carbonyl at 1634 cm\(^{-1}\). The \(^1\)H NMR spectrum of 4 (Fig. S11) showed a peak at \(\delta_H\) 13.01, indicating that the 5-OH was not glycosylated in the biotransformation. By contrast, the proton signal for 1-OH of 2 at \(\delta_H\) 10.98 disappeared. A chemical shift change was equally observed for H-3 from \(\delta_H\) 7.32 in 2 to \(\delta_H\) 7.65 in 4, indicating that the 4'-O-methylglycosylation occurred at a nearby position, C-2. This was further supported by HMBC correlation of H-1' to C-2 (Fig. 2). The \(^13\)C NMR spectrum of 4 (Fig. S12) revealed seven more carbon atoms than the substrate 2. Based on the 1D and 2D (Figs. S13 and S14) NMR spectra, 4 was characterized as 2,5-dihydroxy-1-methoxy-6-methoxymethylantraquinone-4'-O-methyl-2-O-\(\beta\)-D-glucopyranoside.

Compounds 3 and 4 are two new compounds. Compound 1 only has one free hydroxyl group, so it is not surprising that this position was taken by the glycosyltransferase in B. bassiana ATCC 7159 as the glycosylation site. By contrast, 2 has two free phenolic hydroxyl groups (C-2 and C-5, respectively) on two separate aromatic rings. The glycosyltransferase only transfers the 4'-O-methylglucose moiety to the 2-OH instead of 5-OH of 2, indicating that this enzyme is highly regio-selective.

Fig. 2
The compounds were tested against some strains of *Salmonella* and the results are shown in Table 1 below. It was observed that the glycosylated compounds 3 and 4 showed higher *in vitro* antibacterial activity (MIC of 8 µg/mL each) than the substrates 1 and 2 (MIC of 16 µg/mL and 32 µg/mL respectively) against *S. enterica* subsp. *enterica* sérovars Typhimurim. All the compounds showed varying activities against over different *Salmonella* strains. According to established cut off points (Kuete, 2010), the antibacterial activity of phytochemicals is considered significant when MICs are lower than 10 µg/mL, moderate when MICs are between 10 µg/mL and 100 µg/mL and low when MICs are higher than 100 µg/mL. On the basis of this scale, compounds 3 and 4 showed significant antibacterial activity against *S. typhimurium* (MICs of 8 µg/mL). Elsewhere, moderate antibacterial activities were observed for all the compounds against ST566 (resistant isolate of *S. enterica* subsp. *enterica* sérovars Typhi) and STS (*S. enterica* subsp. *enterica* sérovars Typhi ATCC 6539) with MICs ranging from 16 to 64 µg/mL. Antimicrobial compounds are considered as bactericidal when the ratio MBC/MIC < 4 and bacteriostatic when the ratio MBC/MIC > 4 (Gatsing et al., 2006). Accordingly, compound 1 and its glycosylated counterpart 3 are both bactericidal against *S. enterica* subsp. *enterica* sérovars Typhi (ST) and *S. enteritidis* (STE) with MBC/MIC < 4. Also both compounds are bacteriostatic against *S. typhimurium* (STM) with MBC/MIC > 4. The results showed that the different *Salmonella* strains had different tolerance to the compounds. Compounds 3 and 4 showed better activity against *S. enterica* subsp. *enterica* sérovars Typhimurim than 1 and 2, suggesting that introduction of the sugar moiety to the substrates makes them more inhibitory to this strain.

**Table 1**

3. Conclusion

Phytochemical investigation of *M. lucida* led to the isolation and characterization of two anthraquinones, 2-hydroxy-1-methoxyanthraquinone (1) and 2,5-dihydroxy-1-methoxy-6-
methoxymethylanthraquinone (2). Structural modification of these two compounds were performed with B. bassiana ATCC 7159, leading to the production of two new glycosylated derivatives, which were structurally characterized as 2-hydroxy-1-methoxyanthraquinone-4'-O-methyl-2-O-β-D-glucopyranoside (3) and 2,5-dihydroxy-1-methoxy-6-methoxymethylanthraquinone-4'-O-methyl-2-O-β-D-glucopyranoside (4). The glycosyltransferase in B. bassiana ATCC 7159 selectively transferred a 4'-O-methylglucose moiety to the C-2 position of the two anthraquinone substrates. Glycosylation enhanced the in vitro activities of 1 and 2 against S. typhimurium, and thus is a useful tool for structural modification of natural products for bioactive agents. The in vivo activity of these new glycosylated anthraquinones can be further tested in future studies. Additional anthraquinones and other bioactive aromatics may be used to prepare new derivatives with this fungal strain.

4. Experimental

4.1. General experimental procedures

Melting points (mp) were determined on a Meltemp II apparatus and are uncorrected. 1H (500 MHz), 13C (125 MHz) and 2D (1H-1H COSY, HMQC and HMBC) NMR spectra were recorded on a Bruker Avance III HD Ascend-500 NMR spectrometer. The 1H and 13C NMR chemical shifts are expressed in ppm with the solvent signals of DMSO (δH 2.50/δC 39.5-40.5) and CDCl3 (δH 7.25/δC 77.0-77.5) as reference. IR spectra were recorded for KBr pellets on a Shimadzu FTIR-8400. Thin-layer chromatography (TLC) was performed on Merck silica gel plates. TLC plates were visualized with a UV-lamp (UVGL-58) at 254 or 366 nm and later exposed to iodine. Column chromatography was performed with glass column (60 cm length, 11 cm external diameter) using silica gel 60 (63 to 200 µm). An Agilent 1200 HPLC equipped with an Agilent Eclipse XDB-C18 column (5 µm, 250 mm × 4.6 mm) was used for compound analysis and purification.
resolution ESI-MS spectra were obtained on an Agilent 6130 single quadrupole LC-MS. The fungus, Beauveria bassiana ATCC 7159 was obtained from the American Type Culture Collection (ATCC) and was grown in potato dextrose broth (PDB, Difco, Plymouth, MN, USA) medium. The test microorganisms including S. enterica subsp. enterica sérovars Typhi (ST), resistant isolate of S. enterica subsp. enterica sérovars Typhi (ST566), S. enteritidis (STE), and S. enterica subsp. enterica sérovars Typhimurim (STM), were obtained from the Medica Bacteriology Laboratory of the “Centre Pasteur”, Yaoundé, Cameroon. S. enterica subsp. enterica sérovars Typhi ATCC 6539 was obtained from ATCC. The culture media used in this study were Salmonella Shigella Agar (Italy Liofilchem) for activation and maintenance of Salmonella strains/isolates, and Mueller Hinton Broth (MHB) for the determination of the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs).

4.2. Plant material

The stem bark of M. lucida Benth. (Rubiaceae) was collected in Buea, Southwest Region of Cameroon, in June 2017. Identification was done at the National Herbarium in Yaounde, Cameroon, where a voucher specimen (No. 57202/HNC) was deposited.

4.3. Extraction and isolation of compounds from the plant

The stem bark (12 kg) of M. lucida was macerated in methylene chloride for 3 days. Filtration and removal of solvent afforded 62 g of dark brown extract which was absorbed by 62 g of silica gel. Open column chromatography of this extract eluted with gradients of ethyl acetate in hexane (starting from 0 % to 100 % ethyl acetate) afforded 140 fractions (200 mL each), followed by removal of solvent on a rotary evaporator. These fractions were combined into groups on the basis of TLC profiles. Crystallization of group 7 (fractions 71-84 obtained with 15 % (v/v) ethyl acetate-hexane) yielded 405 mg of compound 1 in pure form. Similarly, crystallization of
group 8 (fractions 91-103 obtained with 15% (v/v) ethyl acetate-hexane) gave rise to 62 mg of compound 2. The purity was analyzed by HPLC, eluted with MeOH (containing 0.1% formic acid)/water (containing 0.1% formic acid) [20-90-100% MeOH (0-20-35 min)] at 1 mL/min. The purified compounds were subjected to ESI-MS and NMR analyses.

2-Hydroxy-1-methoxyanthraquinone (1): Yellow crystals, mp 177-178°C. ESI-MS (-): [M-H]⁻ m/z 253.2. ¹H NMR (CDCl₃, 500 MHz): δ4.05 (3H, s, OCH₃), 7.32 (1H, d, J = 8.5 Hz, H-3), 7.86 (2H, m, H-6 and H-7), 8.14 (1H, d, J = 8.5 Hz, H-4), 8.28 (2H, m, H-5 and H-8). ¹³C NMR (CDCl₃, 125 MHz): δ62.3 (OCH₃), 120.3 (C-3), 125.7 (C-14), 125.8 (C-4), 126.8 (C-8), 127.1 (C-5), 127.4 (C-13), 132.9 (C-12), 133.9 (C-6, C-7), 134.4 (C-11), 146.6 (C-1), 155.6 (C-2) 182.1 (C-10), 182.7 (C-9).

2,5-Dihydroxy-1-methoxy-6-methoxymethylanthraquinone (2): Yellow crystals, mp 210-212°C. ESI-MS (-): [M-H]⁻ m/z 313.2. ¹H NMR (DMSO-d₆, 500 MHz): δ3.40 (3H, s, H-16), 3.82 (3H, s, 1-OCH₃), 4.52 (2H, s, H-15), 7.32 (1H, d, J = 8.6 Hz, H-3), 7.63 (1H, d, J = 7.8 Hz, H-8), 7.75 (1H, d, J = 7.8 Hz, H-7), 7.95 (1H, d, J = 8.6 Hz, H-4), 10.98 (1H, brs, 2-OH), 13.01 (1H, s, 5-OH); ¹³C NMR (DMSO-d₆, 125 MHz): δ58.7 (C-16), 61.0 (1-OCH₃), 68.1 (C-15), 115.1 (C-11), 118.7 (C-8), 121.7 (C-3), 125.1 (C-4), 125.5 (C-14), 127.1 (C-13), 133.2 (C-7), 133.9 (C-12), 134.7 (C-6), 148.4 (C-1), 158.8 (C-5), 159.2 (C-2), 181.6 (C-9), 187.9 (C-10).

4.4. Biotransformation of 1 and 2 by B. bassiana ATCC 7159 and isolation of the glycosylation products

Potato dextrose broth (PDB, 2.4 g) was is dissolved in 100 mL of pure water in a shaker flask and autoclaved at 121°C for 15 min, then allowed to cool in a biosafety cabinet. The seed of B. bassiana ATCC 7159 was inoculated into the fresh PDB medium and placed in a 28°C incubator shaker at 250 rpm for three days. Compound 1 (20 mg) was dissolved in DMSO (500 µL) and
added into the fermentation broth of *B. bassiana* ATCC 7159, which was incubated for an additional 14 days. The broth was centrifuged to separate the cells from the liquid and extracted separately with a mixture of ethyl acetate/methanol/acetic acid (89:10:1, v/v/v). Both extracts were combined based on HPLC profiles. On evaporation of the solvents, a yellow precipitate was formed on the walls of the flask which was separated by decantation and purified by HPLC, eluted with MeOH (containing 0.1 % formic acid)/water (0.1 % formic acid) [10-90-100 % MeOH (0-30-45 min)] at 1 mL/min to afford 8 mg of 3. Similarly, 2 was used as a substrate to yield 3 mg of 4 (3 mg). The purified compounds were subjected to ESI-MS and NMR analyses.

2-Hydroxy-1-methoxyanthraquinone-4'-O-methyl-2-O-β-D-glucopyranoside (3): Yellow solid, mp 245-246°C. Low resolution ESI-MS (+): [M+Na]+ m/z 453.0; High resolution ESI-MS (+): [M+H]+ m/z 431.1332; IR (KBr) νmax 3410, 2954, 1674, 1589, 1414 cm⁻¹; ¹H NMR (DMSO-d₆, 500 MHz): δ 3.10 (1H, t, J = 9.1 Hz, H-4'), 3.39 (1H, m, H-2'), 3.46 (1H, m, H-3'), 3.48 (3H, s, 4'-OCH₃), 3.50 (1H, m, H-5'), 3.54 (1H, m, H-6'), 3.66 (1H, m, H-6'), 3.89 (3H, s, 1-OCH₃), 5.15 (1H, d, J = 7.7 Hz, H-1'), 7.64 (IH, d, J = 8.7 Hz, H-3), 7.89 (2H, m, H-6 and H-7), 7.99 (IH, d, J = 8.7 Hz, H-4), 8.15 (2H, m, H-5 and H-8). ¹³C NMR (DMSO-d₆, 125 MHz): δ 60.2 (4'-OCH₃), 60.6 (C-6'), 61.3 (1-OCH₃), 73.8 (C-2'), 76.3 (C-5'), 76.9 (C-3'), 79.3 (C-4'), 100.2 (C-1'), 120.9 (C-3), 124.6 (C-4), 126.7 (C-14), 127.1 (C-8), 127.2, (C-5), 128.2 (C-13), 132.8 (C-12), 134.4 (C-11), 134.8/135.1 (C-6, C-7), 149.6 (C-1), 157.2 (C-2) 182.1 (C-10), 182.2 (C-9).

2,5-Diαydroxy-1-methoxy-6-methoxymethylanthaquinone-4'-O-methyl-2-O-β-D-glucopyranoside (4): yellow solid, mp 251-252°C. Low resolution ESI-MS (+): [M+H]+ m/z 491.0, [M+Na]+ m/z 513.1; High resolution ESI-MS (+): [M+H]+ m/z 491.1543. IR (KBr) νmax 3395, 3320, 2954, 1674, 1634, 1589, 1434 cm⁻¹; ¹H NMR (DMSO-d₆, 500 MHz): δ 3.10 (1H, t, J = 9.1 Hz, H-4'), 3.38 (1H, t, H-2'), 3.40 (3H, s, H-16), 3.46 (1H, m, H-3'), 3.48 (3H, s, 4'-OCH₃), 3.50 (1H, m,
H-5'), 3.54 (1H, m, H-6'), 3.66 (1H, m, H-6'), 3.89 (3H, s, 1-OCH₃), 4.56 (2H, s, H-15), 5.15 (1H, d, J = 7.7 Hz, H-1'), 7.65 (1H, d, J = 7.8 Hz, H-3), 7.68 (1H, d, J = 8.8 Hz, H-8), 7.81 (1H, d, J = 7.8 Hz, H-7), 8.04 (1H, d, J = 8.8 Hz, H-4), 13.01 (1H, s, 5-OH); ¹³C NMR (DMSO-d₆, 125 MHz): δ 58.7 (C-16), 60.2 (4'-OCH₃), 60.5 (C-6'), 61.3 (1-OCH₃), 68.1 (C-15), 73.8 (C-2'), 76.3 (C-5'), 76.9 (C-3'), 79.3 (C-4'), 100.1 (C-1'), 115.2 (C-11), 118.9 (C-8), 120.8 (C-3), 124.7 (C-4), 127.3 (C-14), 127.7 (C-13), 133.3 (C-7), 134.2 (C-12), 135.1 (C-6), 149.9 (C-1), 157.8 (C-2), 158.9 (C-5), 181.2 (C-9), 188.1 (C-10).

4.5. Preparation of bacterial inocula

The *Salmonella* strains were grown on Salmonella-Shigella Agar (SSA) at 37 °C for 18 hours. Colonies were collected from the agar plates aseptically with a sterile inoculation loop and suspended in 10 mL of sterile 0.9% saline water to the turbidity of a 0.5 McFarland standard (equivalent to 1.5×10⁸ colony-forming unit/mL (CFU/mL)). These suspensions were diluted 100 times with MHB medium to yield ~1.5×10⁶ CFU/mL bacterial inocula before use.

4.6. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The broth micro-dilution method was used for susceptibility testing of bacteria species. The compounds were tested against five *Salmonella* strains listed on Table 1. The tests were carried out in 96-micro well sterile plates as described by Newton et al. (Newton et al., 2002). Compounds were dissolved in dimethyl sulfoxide (DMSO) and serial diluted to obtain the desired concentrations. The samples were mixed with MHB medium to make a total volume of 100 µL per well. Ciprofloxacin and DMSO (2.5%, w/v) were used as positive and negative controls, respectively. A hundred microliters (100 µL) of each of the 1.5×10⁶ CFU/mL bacterial suspensions was added to the wells containing the test samples or controls, and mixed thoroughly to give the
final concentrations of tested compounds ranging from 0.5 to 1024 µg/mL. These preparations were further incubated at 37°C for 18 hours. The inhibitory concentration of the compounds was detected after addition of 40 µL of 0.2 mg/mL of p-iodonitrotetrazolium chloride (INT) (Sigma Aldrich, South Africa) and incubation at 37°C for 30 min. Viable bacteria change the yellow dye (INT) to pink color. The lowest concentrations at which there were no visible color changes were considered as MIC. The MBC values were determined by adding 50 µL aliquots of the preparations (without INT), which did not show any visible color change after incubation during MIC determination, into 150 µL of fresh broth medium and mixed thoroughly. These preparations were further incubated at 37°C for 48 hours and MBCs were revealed by the addition of INT as above. All compound concentrations to which no color changes were observed were considered as bactericidal concentrations, and the smallest of these concentrations was considered as the MBC. Lower values of MIC or MBC indicate stronger antibacterial activity. The tests were carried out in triplicate.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:
References


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Figure legends

**Fig. 1.** Selective glycosylation of two plant anthraquinones by *B. bassiana* ATCC 7159.

**Fig. 2.** Key HMBC correlations of compounds 3 and 4.
Fig. 1

1 $R_1 = H, R_2 = H$

2 $R_1 = C^{15}H_2OC^{16}H_3, R_2 = OH$

3 $R_1 = H, R_2 = H$

4 $R_1 = C^{15}H_2OC^{16}H_3, R_2 = OH$

Microbial glycosylation

*B. bassiana* ATCC 7159
Fig. 2

3

4