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A xanthone and a phenylanthraquinone from the roots of *Bulbine frutescens*, and the revision of six *seco*-anthraquinones into xanthones

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ABSTRACT

Phytochemical investigation of the dichloromethane/methanol (1:1) extract of the roots of *Bulbine frutescens* led to the isolation of a new xanthone, 8-hydroxy-6-methylxanthone-1-carboxylic acid (**1**) and a new phenylanthraquinone, 6′,8-*O*-dimethylknipholone (**2**) along with six known compounds. The structures were elucidated on the basis of NMR and MS spectral data analyses. The structure of compound **1** was confirmed through X-ray crystallography which was then used as a reference to propose the revision of the structures of six *seco*-anthraquinones into xanthones. The isolated compounds were evaluated for cytotoxicity against human cervix carcinoma KB-3-1 cells with the phenylanthraquinone knipholone being the most active (IC_{50} = 0.43 μM). Two semi-synthetic knipholone derivatives, knipholone Mannich base and knipholone-1,3-oxazine, were prepared and tested for cytotoxic activity; both showed moderate activities (IC_{50} value of 1.89 and 2.50 μM, respectively).

1. Introduction

Bulbine frutescens (family Asphodelaceae; sub-family Asphodeloideae) is known to occur in Southern and Eastern part of Africa (Van Wyk et al., 1995). Although the plant is widely recognized for its ornamental value owing to colorful (yellow) flowers and succulent leaves, is also known for its use in traditional medicinal practice. The leaf exudate of *B. frutescens* is used for the treatment of various ailments, particularly for wound healing and esthetic purposes (Rabe and van Staden, 1997; Abegaz et al., 2002; Pather et al., 2011; Pather and Kramer, 2012). The gel extract has recently been patented as a promoter of wound healing (Lusunzi and Karuso, 2001). *B. frutescens* produces a variety of polyketide derivatives including anthraquinones (Van Wyk et al., 1995), isofuranonaphthoquinones (Bringmann et al., 2008), and phenylanthraquinones (Abegaz et al., 2002; Mutanyatta et al., 2005; Bezabih et al., 1997; Bringmann et al., 2008). Among these, phenylanthraquinones, which comprise an anthraquinone and an acetylphloroglucinol moiety, have gained considerable attention due to their antiplasmodial (Abegaz et al., 2002; Bringmann et al., 1999) and anticancer (Reid, 1993; Habtemariam, 2010; Huyssteen et al., 2011) activities. As part of our on-going program in search for new bioactive natural compounds from Eastern African plants of the Asphodelaceae family (Abdissa et al., 2013; Bringmann et al., 2008), now we report the isolation of two new natural products (**1** and **2**) and six known compounds. The cytotoxicity of knipholone (**3**) and two semi-synthetic knipholone derivatives (**4** and **5**) is also reported.

2. Results and discussion

Compound **1** was obtained as a yellow crystalline solid. Its molecular formula was established as $C_{15}H_{10}O_5$ on the basis of its HR-EIMS (*m*/*z* 270.0520; M^+, calcd. 270.0528, eleven degrees of unsaturation). The UV–vis absorption at λ_{max} 225, 254, 362, and

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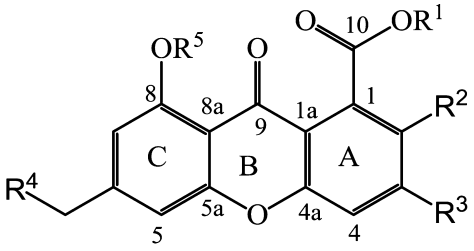
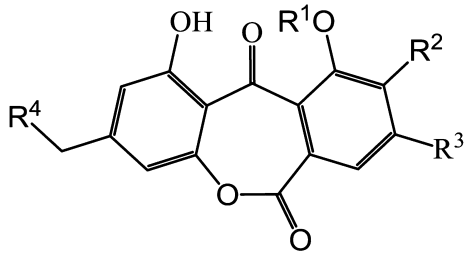
386 nm and the NMR spectra (Tables 1 and 2) suggested a xanthone skeleton (Yang et al., 2001; Shao et al., 2008). The presence of methyl (δ_H 2.45, δ_C 22.6), chelated hydroxyl (12.25, *s*) and carboxylic acid (δ_C 169.8 for C=O, ν_{max} 3142–3486 cm^{-1} for OH) substituents were also evident. The ^1H NMR spectrum showed three mutually coupled aromatic protons with AMX spin multiplicity pattern centered at δ_H 7.68 (*dd*, *J* = 8.5, 1.1 Hz, H-2), 7.94 (*dd*, *J* = 8.5, 7.3 Hz, H-3) and 7.45 (*dd*, *J* = 7.3, 1.1 Hz, H-4) of a monosubstituted (at C-1) ring-A. The substituent at C-1 was established to be the carboxylic acid from a $^3J_{C,H}$ HMBC correlation of H-2 (δ_H 7.68) with the acid carbonyl carbon at δ_C 169.8 (C-10). In ring-C two *meta*-coupled aromatic protons that resonated at δ_H 6.89 (*br d*, *J* = 1.5 Hz, H-5) and 6.66 (*br d*, *J* = 1.5 Hz, H-7) were also observed; these protons showed long-range correlation (in the ^1H–^1H COSY spectrum) and NOE (in the NOESY spectrum) interaction with methyl protons at δ_H 2.45 (δ_C 22.6) placing the methyl group at C-6 and the chelated hydroxyl at C-8. Thus, based on the above spectroscopic evidence the compound was characterized as 8-hydroxy-6-methylxanthone-1-carboxylic acid (**1**; Fig. 1).

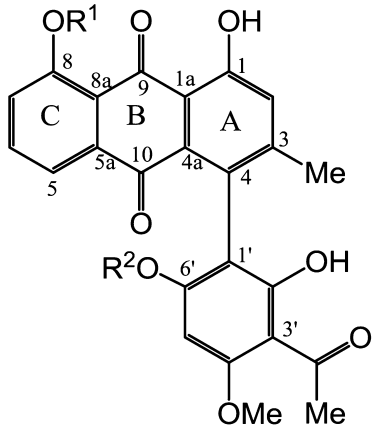
An alternative structure, an aromatic lactone (**6**) a *seco*-anthraquinone, as in janthinone (**7**) (Marinho et al., 2005) was also considered. This is because the NMR data of **1** is comparable with what has been reported for janthinone except for the presence of chelated OH in **1** rather than methoxyl in janthinone (**7**). Interestingly, Shao et al. (2008) isolated a compound with the same spectral data as those reported for janthinone (Marinho et al., 2005) and proposed the compound to be a xanthone, methyl-8-hydroxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate (**7a**), instead of the *seco*-anthraquinone **7**. The structure of the xanthone was confirmed by single-crystal X-ray crystallography, and it was further suggested that the structure of janthinone (**7**) be revised (Shao et al., 2008) to **7a**. With such conflicting literature information, we also performed a single-crystal X-ray structure analysis (Fig. 2) for compound **1** and confirmed that the compound is indeed the



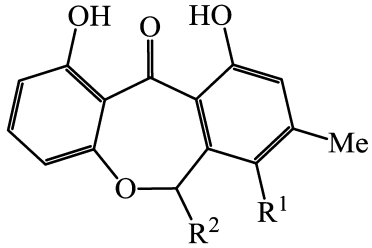
1 $R^1 = R^2 = R^3 = R^4 = R^5 = H$
1a $R^1 = R^5 = Me$, $R^2 = R^3 = R^4 = H$
7a $R^1 = Me$, $R^2 = R^3 = R^4 = R^5 = H$
8a $R^1 = Me$, $R^2 = R^4 = R^5 = H$, $R^3 = OH$
9a $R^1 = Me$, $R^3 = OMe$, $R^2 = R^4 = R^5 = H$
10a $R^1 = R^2 = R^4 = R^5 = H$, $R^3 = OMe$
11a $R^1 = R^2 = R^5 = H$, $R^3 = OMe$, $R^4 = OH$
12a $R^1 = Me$, $R^2 = R^3 = R^5 = H$, $R^4 = OH$
13a $R^1 = Me$, $R^3 = R^5 = H$, $R^2 = R^4 = OH$



6 $R^1 = R^2 = R^3 = R^4 = H$
7 $R^1 = Me$, $R^2 = R^3 = R^4 = H$
8 $R^1 = R^2 = R^4 = H$, $R^3 = OMe$
9 $R^1 = Me$, $R^3 = OMe$, $R^2 = R^4 = H$
10 $R^1 = Me$, $R^2 = R^4 = H$, $R^3 = OH$
11 $R^1 = R^2 = H$, $R^3 = OMe$, $R^4 = OH$
12 $R^1 = Me$, $R^2 = R^3 = H$, $R^4 = OH$
13 $R^1 = Me$, $R^3 = H$, $R^2 = R^4 = OH$



2 $R^1 = R^2 = Me$
3 $R^1 = R^2 = H$



14 $R^1 = OH$, $R^2 = H$
15 $R^1 = H$, $R^2 = OH$

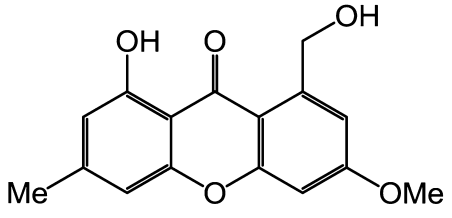


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Fig. 1. Structure of the compounds.

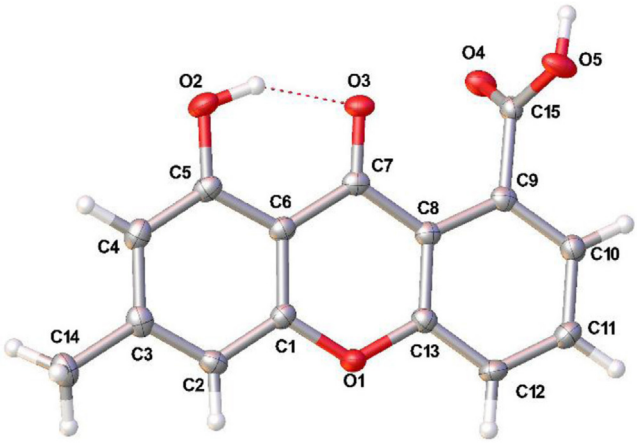


Fig. 2. ORTEP-like view of the molecular structure of 8-hydroxy-6-methylxanthone-1-carboxylic acid (**1**).

xanthone, 8-hydroxy-6-methylxanthone-1-carboxylic acid rather than *seco*-anthraquinone (**6**). The single-crystal X-ray analysis of **1** is comparable to the previously reported crystal structure for 8-hydroxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylic acid methyl ester (Macias et al., 2001) with the acid group being above the tricyclic moiety with torsional angle of 113.5°. This is the first report on the occurrence of compound **1** in nature having previously been reported as a synthetic intermediate (Kudav et al., 1976). It is worth mentioning that this is the first report on the isolation of a xanthone from the family Asphodelaceae.

In addition to janthinone (**7**) (Marinho et al., 2005), some related structures having the same *seco*-anthraquinone skeleton (Fig. 1) *vis* wentiquinones A (**8**) and B (**9**) (Sun et al., 2013), 1,8-dihydroxy-10-methoxy-3-methyldibenzo[b,e]oxepine-6,11-dione (**10**) (Pan et al., 2010), and wentiquinone C (**11**) (Li et al., 2014), along with **12** and **13** (Carvalho et al., 2001) have been reported. In line with the above discussion, the NMR data reported for these compounds were compared with those of compound **1** (Tables 1 and 2) and related xanthones (Shao et al., 2008; Song et al., 2013). From this comparison we suggest that the *seco*-anthraquinone structures **8**, **9**, **10**, **11**, **12** and **13** should be revised to the xanthones **8a**, **9a**, **10a**, **11a**, **12a** and **13a**, respectively. The NMR assignments for the revised structures are shown in Tables 1 and 2.

Besides comparison of the NMR data of these compounds with xanthones with proven structures (**1** and **7a**) through X-ray crystallography, the principal argument in this revision is that the chemical shift value δ_C 52–53 ppm assigned for aromatic methoxyl in the *seco*-anthraquinone structures **8**, **9**, **12** and **13** correspond to methyl ester [as the related xanthones reported in Shao et al. (2008) and Song et al. (2013)] rather than methoxyl group attached to aromatic ring (typical value *ca*55–56 ppm). In the case of **13** the

Table 1
^{1}H NMR spectral data (δ_H, m, *J* in Hz) of xanthones.

Position	**1**	**1a**	**8a**	**9a**	**10a**	**11a**	**12a**	**13a**
2	7.68 (dd, 8.5, 1.1)	7.47 (dd, 8.5, 1.1)	7.18 (d, 2.2)	6.89 (d, 2.5)	7.18 (d, 2.4)	7.16 (d, 2.0)	7.45 (dd, 7.3, 1.1)	
3	7.94 (dd, 8.5, 7.3)	7.66 (dd, 8.5, 7.3)					7.93 (dd, 8.3, 7.3)	7.48 (d, 8.0)
4	7.45 (dd, 7.3, 1.1)	7.26 (dd, 7.3, 1.1)	6.98 (d, 2.2)	6.87 (d, 2.5)	6.98 (d, 2.4)	6.93 (d, 2.0)	7.77 (dd, 8.3, 1.1)	7.62 (d, 8.0)
5	6.89 (br. d, 1.5)	6.87 (d, 1.5)	6.86 (br. d, 1.0)	6.69 (br. d, 1.1)	6.87 (br. d, 1.2)	6.95, br. s	7.00 (d, 1.1)	7.00 (d, 1.1)
7	6.66 (br. d, 1.5)	6.61 (d, 1.5)	6.66 (br. d, 1.1)	6.60 (br. d, 1.1)	6.67 (br. d, 1.2)	6.75, br. s	6.80 (d, 1.1)	6.78 (d, 1.1)
Me/CH_2OH	2.45 (d, 0.6)	2.47 (s)	2.40 (s)	2.41 (s)	2.41 (s)	4.59 (s)	4.60 (d, 5.4)	4.60 (br. s)
10-COMe	4.05 (s)	3.95 (s)	4.01 (s)				3.90 (s)	3.89 (s)
8-OMe		3.99 (s)						
3-OMe				3.97 (s)	3.96 (s)	3.94 (s)		
8-OH	12.25 (s)		12.32 (br. s)	12.27 (br. s)	12.32 (s)	12.41 (s)	12.06 (br. s)	12.25 (br. s)
2-OH								10.5 (br. s)
6-OH							5.40 (t, 5.4)	5.55 (br. s)

1 in acetone-d_6; **1a**, **9a** in $CDCl_3$; **8a**, **10a**, **11a**, **12a**, **13a** in DMSO-d_6. For compounds **8a**–**13a** NMR data was extracted from literature and reassigned on the revised structure: **8a** and **9a** (Sun et al., 2013), **10a** (Pan et al., 2010), **11a** (Li et al., 2014), **12a** and **13a** (Carvalho et al., 2001).

Table 2
^{13}C NMR spectral data (δ_C) of xanthones.

Position	**1**	**1a**	**8a**	**9a**	**10a**	**11a**	**12a**	**13a**
1	135.8	134.2	136.6	135.1	137.1	137.0	133.6	117.4
1a	117.8	120.1	105.8	106.7	106.4	106.5	116.6	117.4
2	120.1	118.8	101.1	101.5	101.7	101.0	122.9	149.2
3	136.6	133.5	164.8	164.7	165.4	164.8	136.0	125.7
4	123.8	122.4	111.7	112.1	112.3	111.7	119.7	120.5
4a	157.1	154.9	157.6	158.1	155.7	157.7	155.5	151.1
5	108.3	109.9	107.1	107.1	107.7	103.8	104.2	104.2
5a	156.8	157.6	155.1	155.8	155.7	155.3	155.5	155.8
6	150.7	146.9	148.7	148.6	149.3	153.6	154.5	154.4
7	112.2	107.0	111.2	111.7	111.8	107.6	107.0	106.9
8	162.2	160.5	160.5	161.5	161.0	160.6	160.5	160.8
8a	107.7	110.2	109.7	111.4	110.2	109.7	107.7	107.5
9	181.6	174.9	179.2	179.7	179.8	179.3	180.1	180.1
10	169.8	170.3	168.8	169.2	169.4	168.9	168.6	167.2
Me/CH_2OH	22.6	22.5	21.8	22.5	22.4	62.3	62.3	62.7
10-COMe		53.1	52.5	53.1			52.7	52.6
8-OMe		56.5						
3-OMe				56.1	57.1	56.5		

1 in acetone-d_6; **1a**, **9a** in $CDCl_3$; **8a**, **10a**, **11a**, **12a**, **13a** in DMSO-d_6.
For compounds **8a**-**13a** NMR data was extracted from literature and reassigned on the revised structure: **8a** and **9a** (Sun et al., 2013), **10a** (Pan et al., 2010), **11a** (Li et al., 2014), **12a** and **13a** (Carvalho et al., 2001).

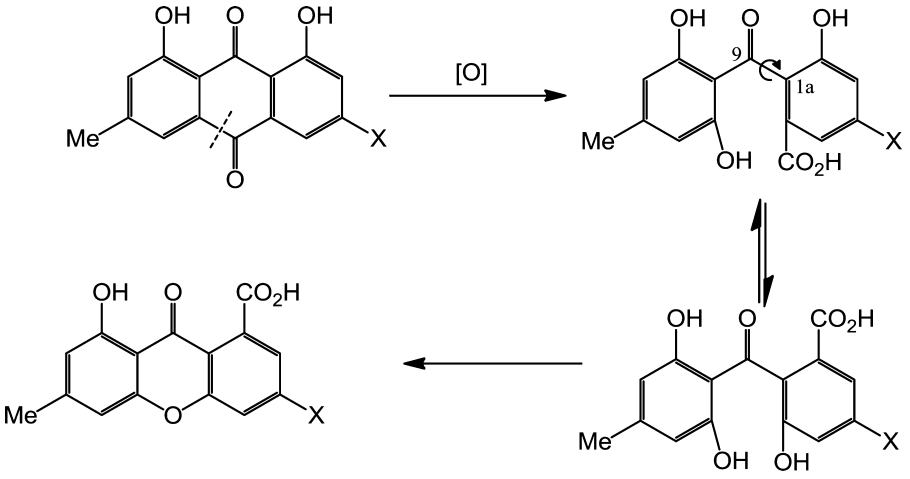
methoxyl signal should even be more down field shifted (above 59 ppm) since it would be di-*ortho*-substituted and hence could only be the methyl ester **13a**. Methylation of **1** gave **1a** whose NMR spectral data (Tables 1 and 2) showed typical methoxyl (δ_H 3.99; δ_C 56.5) and methyl ester (δ_H 4.05;δ_C 53.1) signals, which not only confirmed the structure of **1** as 8-methoxy-6-methylxanthone-1-carboxylic acid methyl ester, but also put the basis for the revision of the *seco*-anthraquinone structures **8**, **9**, **12** and **13** into xanthone methyl esters (**8a**, **9a**, **12a** and **13a**). In the case of structure **11**, if this was the correct structure it should have showed two chelated hydroxyl groups as in leptosphaerin D (**14**) (Lin et al., 2010) and arugosin F (**15**) (Hein et al., 1998) (only one is reported for **11**, Table 1) in the ^{1}H NMR spectrum, and this is only possible in the xanthone structure **11a**. The C-9 carbonyl in all these compounds (except for **1a** which lacks chelation and C-9 appear at δ_C 174.9 ppm) appeared in the narrow range of 179.2–181.6 ppm (Table 2) and is consistent with xanthone structure. If the *seco*-anthraqunone structures **8**–**13** were correct the C-9 carbonyl should have been as de-shilded as in leptosphaerin D (**14**, δ_C 198.5) (Lin et al., 2010) and arugosin F (**15**, δ_C 197.2) (Hein et al., 1998).

In the case of compound **10**, two xanthones, having hydroxymethylene at C-1 have been reported along with it (Pan et al., 2010). The structure of one of these xanthones (**16**), which appears to be derived from **10a** through reduction of the ester carbonyl, has been confirmed by X-ray diffraction analysis; it is then reasonable to propose the revision of **10** to the xanthone **10a**, whose NMR data is very similar to the other xanthones (Tables 1 and 2). The methoxyl group which appears at δ_C 57.1 in this compound is in agreement with its placement at C-3 as in **9a**. In line with the above discussion, we also question the correctness of two *seco*-anthraquinone reported by Huang et al. (2010).

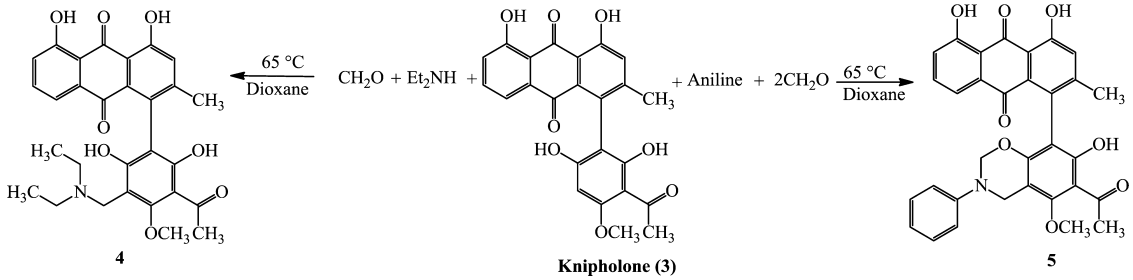
Furthermore, that compounds **8a**–**13a** are xanthones appear to be agreeable from biogenetic point of view as these compounds co-occur with the corresponding anthraquinone precursors. Oxidative cleavage of ring B of anthraquinoid precursor(s) forms a benzophenone intermediate(s); rotation of ring A along C_{1a}–C_9 axis of the benzophenone(s) and cyclization of ring B produces the xanthones as shown in Scheme 1. The occurrence of the benzophenone intermediates, moniliphenone (Song et al., 2013), 3,4-dihydro-3,9-dihydroxy-6,8-dimethoxy-3-methylanthracen-1(2*H*)-one and 2-(2,6-dihydroxy-4-methylbenzoyl)-3,5-dimethoxybenzoic acid (Sun et al., 2013) along with the corresponding anthraquinones and xanthones in *Aspergillus* species supports this biogenetic proposal. The role of moniliphenone as a biosynthetic intermediate between anthraquinones and xanthones in culture filtrate of *Monilinia fructicola* has already been reported (Kachi and Sasa, 1986).

Compound **2** was isolated as yellow amorphous solid. HR-EIMS showed a molecular ion peak at *m*/*z* 462.1314 corresponding to molecular formula of $C_{26}H_{22}O_8$. The UV spectrum (λ_{max} 220, 256, 286, and 415 nm) is similar to that of knipholone (**3**) suggesting a phenylanthraquinone chromophore (Dagne and Steglich, 1984). Similar to **3**, the ^{1}H NMR spectrum displayed the presence of three adjacent and mutually coupled aromatic protons at δ_H 7.74 (*dd*, *J* = 7.8, 1.1 Hz, H-5), 7.64 (*t*, *J* = 7.8 Hz, H-6) and 7.29 (*dd*, *J* = 7.8, 1.1 Hz, H-7) with ABX spin system for ring-C protons. In ring-A, a singlet at δ_H 7.25 was assigned to H-2 with the biosynthetically expected aromatic methyl (δ_H 2.10; δ_C 20.7) being at C-3 and the acetylphloroglucinol moiety (whose presence was evident from the downfield shifted singlet at δ_H 13.91 due to the chelated hydroxyl proton, the singlet aromatic proton shifted upfield to δ_H 6.14 for H-5′, an acetyl proton, δ_H 2.69 at C-3′ and a methoxy group, δ_H 4.02; δ_C55.4) at C-4. The only difference between **2** and knipholone (Dagne and Steglich, 1984) is the presence of two additional methoxyl signals at δ_H 4.08 (δ_C 56.6) and 3.79 (δ_C 55.7). Consequently, compound **2** is a dimethyl ether derivative of knipholone. The positions of methoxyl groups were established on the bases of HMBC and NOE experiments (Section 3.3.3). Thus, the methoxyl groups resonating at δ_H 4.02 (δ_C 55.4) and 3.79 (δ_C 55.7) were placed at C-4′ and C-6′ respectively based on the NOE interaction between these methoxyl protons and H-5′; whereas the other methoxyl group, δ_H 4.08 (δ_C 56.6), was placed at C-8 based on its NOE correlation with H-7. Therefore, compound **2** was characterized and named as 6′,8-*O*-dimethylknipholone (**2**). In the CD spectrum of **2**, no significant Cotton effect was observed, indicating that the compound occurs as a scalemic or racemic mixture. In agreement with this, HPLC analysis on a chiral stationary phase column (Chiralcel OD-H) revealed that the compound is nearly 1:1 mixture (52:48) of atropisomers. This is in line with the previous assertion that phenylanthraquinones are found as scalemic or nearly racemic mixtures in *B. frutescens* (Mutanyatta et al., 2005).

Interestingly we have also isolated the 'southern' part of compound **2**, 2-hydroxy-4,6-dimethoxyacetophenone, previously reported from the leaves of *Peperomia glabella* (Soares et al., 2006). 6′-*O*-Methylknipholone, having the same 2-hydroxy-4,6-dimethoxyacetophenone moiety, has been reported earlier from *Bulbine capitata* (Bezabih et al., 1997). It is then possible that the oxidative coupling leading to the phenylanthraquinones **2** and 6′-*O*-methylknipholone could have occurred between chrysophanol (or chrysophanol-8-methyl ether in the case of **2**) and 2-hydroxy-4,6-dimethoxyacetophenone. It has already been shown that 2,4-dihydroxy-6-methoxyacetophenone (but



Scheme 1. Proposed biogenesis of xanthones from anthraquinones (X = H, OH, or OMe).



Scheme 2. Partial synthesis of knipholone analogs, **4** and **5**.

not 2,4,6-trihydroxyacetophenone) is the substrate toward the formation of knipholone and related phenylanthraquinone derivatives (Bringmann et al., 2007).

The other known compounds isolated in this study were identified as 4-*O*-methyleleutherol (Bringmann et al., 2008), 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (Krupa et al., 1989), chrysophanol (Bezabih et al., 1997), knipholone (Dagne and Steglich, 1984) and isoknipholone (Yenesew et al., 1994). Whereas the anthraquinones of this plant including the phenylanthraquinones are chrysophanol-based, where the octaketide chain has folded in the common way; the octaketide chain in 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid appears to have folded in an unusual way as in aloesaponarin II (Dagne et al., 1994). 3,8-Dihydroxy-1-methylanthraquinone-2-carboxylic acid has previously been reported from *Streptomyces* species (Krupa et al., 1989), however this is the first report on the isolation of such anthraquinone from the genus *Bulbine*.

In view of the current interest on the biological activities of phenylanthraquinones (Reid, 1993; Habtemariam, 2010), the new compounds (**1** and **2**) and the major compound of this plant, knipholone (**3**), were tested for cytotoxicity on the human cervix carcinoma KB-3-1 cell line. The phenylanthraquinone, knipholone (**3**) showed good activity with IC_{50} value of 0.43 μM, while compounds **1** and **2** did not show significant activities.

Following the good activity observed for knipholone (**3**), two new knipholone derivatives (**4** and **5**) were prepared (Scheme 2) using catalyst free Mannich type reaction (Zhao et al., 2013). The reaction involves a C–C bond formation where knipholone as an active hydrogen atom containing molecule reacts with formaldehyde and an amine derivative with elimination of water. The use of the secondary amine, diethylamine allowed the modification of acetylphloroglucinol part of knipholone at C-5′ (Scheme 2) into knipholone Mannich base (**4**). In another reaction, the presence of the primary amine aniline, the Mannich base initially formed condensed, involving the vicinal hydroxyl group at C-6′ with a second formaldehyde molecule through imidazole cyclization reaction, forming knipholone-1,3-oxazine (**5**, Scheme 2). All the spectroscopic data (refer to Section 3) of these compounds were in agreement with the proposed structures. When tested against human cervix carcinoma KB-3-1 cells, these knipholone derivatives (**4** and **5**) showed moderate activity with IC_{50} value of 1.89 and 2.50 μM, respectively.

3. Materials and methods

3.1. General

Melting points were measured on Butch melting point B-540, Switzerland. Column chromatography and flash chromatography were carried out on silica gel (0.06–0.2 mm, Merck) impregnated with 3% aq. oxalic acid. Analytical TLC was performed on Merck pre-coated silica gel 60 F_{254} plates. Gel filtration was performed on Sephadex LH-20. UV spectra were recorded on a Specord S600, Analytik Jena AG, Germany. HREI-MS was done on Micromass GC-TOFmicro mass spectrometer (Micromass, Wythenshawe, Waters Inc., UK). CD spectra were measured on JASCO J-810 spectropolarimeter. IR spectra were recorded on a JASCO Fourier transform IR-460 spectrometer. ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, and NOESY spectra were recorded on a Bruker Avance 500 spectrometer using the residual solvent peaks as reference. Enantiomeric analyses were determined by Chiralcel OD-H HPLC.

3.2. Plant material

The roots of *B. frutescens* were collected from University of Nairobi, Chiromo campus garden in May 2012. The plant material was identified at University of Nairobi, School of Biological Science Herbarium where a voucher specimen (voucher number SGM-AYT-2004–27) has been deposited.

3.3. Extraction and isolation

The air-dried roots (940 g) of *B. frutescens* were extracted using dichloromethane/methanol (1:1) three times for 24 h by cold percolation. The extract was then concentrated under vacuum using a rotary evaporator to yield a dark brown residue (56 g, 5.96%). A 50 g portion of the extract was subjected to flash column chromatography (column size: 80 cm length and 90 mm diameter) on oxalic acid impregnated silica gel (600 g) eluting as follows: CH_2Cl_2 (frs 1–8), CH_2Cl_2–EtOAc (1:1) (frs 9–16) and EtOAc–MeOH (9:1) (frs 17–22) each of *ca.* 250 mL. Fractions 3–6 were combined based on their TLC profile and then loaded on Sephadex LH-20 column (60 cm length and 3 cm diameter; eluent: dichloromethane/methanol, 1:1, *ca.* 50 mL each) followed by purification on prep. TLC (silica gel; petroleum ether/EtOAc, 4:1) to give chrysophanol (12.3 mg, ≥98% purity), 4-*O*-methyleleutherol (5.4 mg, ≥96% purity) and 6′,8-*O*-dimethylknipholone (**2**, 4.5 mg, ≥97% purity). Fractions 9–15 were combined and subsequently subjected to column chromatography (column size: 60 cm length and 40 mm diameter) on silica gel (300 g) impregnated with oxalic acid (eluent: increasing gradient of EtOAc in petroleum ether; 0:10, 1:9, 1:4, 2:3, 1:1, 3:2, 10:), *ca.* 700 mL each) followed by further purification of the fractions on Sephadex LH-20 (60 cm length and 3 cm diameter; eluent: dichloromethane/methanol, 1:1, *ca.* 50 mL each) yielding 8-hydroxy-6-methylxanthone-1-carboxylic acid (**1**, 12.2 mg, ≥99% purity), 2-hydroxy-4,6-dimethoxyacetophenone (7.4 mg, ≥96% purity), isoknipholone (6.5 mg, ≥98% purity), and knipholone (**3**, 124.2 mg, ≥99% purity). Similar treatment of fractions 17–20 resulted in the isolation of 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (6.8 mg, ≥97% purity).

3.3.1. 8-Hydroxy-6-methylxanthone-1-carboxylic acid (*1*)

Yellow crystalline solid, m.p. 267–268 °C. UV λ_{max} (acetonitrile): 225, 254, 362, 386 nm. IR (KBr) ν_{max} cm^{-1}: 3486–3142, 1650, 1612, 1589, 1476, 1450. ^{1}H (500 MHz, Table 1). ^{13}C NMR (125 MHz, Table 2). EIMS (70 eV, rel. int.): m/z = 270 (100 [M]$^+$), 252 (75), 226

(62), 223 (25), 197 (17), 130 (18). HR-EIMS m/z = 270.0520 $[M]^+$ (calculated for $C_{15}H_{10}O_5$, 270.0528). X-ray single crystal analysis of **1**: Single crystals of **1** were coated with a layer of hydrocarbon oil and attached to a MicroMount™. A suitable crystal was selected and mounted on a SuperNova, Dual Atlas diffractometer. The crystal was kept at 100(1) K during data collection. The structure was solved using OLEX2 (Sheldrick, 2008) and refined with SHELX-97 (Dolomanov et al., 2009). Crystal Data: monoclinic, a = 15.6907(12) Å, b = 4.9175(4) Å, c = 15.3304(11) Å, β = 92.895(6)°, V = 1181.36(16) Å^3, space group $P2_1/c$ (no. 14), Z = 4, μ(Cu Kα) = 0.973, 3854 reflections measured, 2053 unique (R_{int} = 0.0242) which were used in all calculations. The final wR_2 was 0.0984 (all data) and R_1 was 0.0365 ($I > 2\sigma(I)$). Further details can be obtained *via* CCDC. CCDC 968696 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

3.3.2. Methylation of 8-hydroxy-6-methylxanthone-1-carboxylic acid (**1**)

To a solution of **1** (6.0 mg, 11.8 mmol), in acetone (20 mL), Na_2CO_3 (150 mg) and dimethylsulfate (0.3 mL) were added. The mixture was stirred for 6 h at room temperature. TLC was done using 20% ethyl acetate in petroleum ether to monitor the completion of the reaction. The mixture was dried using a rotary evaporator and then partitioned between ethyl acetate and water. The organic layer was then concentrated and purified on silica gel (eluent: increasing gradient of ethyl acetate in *n*-hexane) to afford 8-methoxy-6-methylxanthone-1-carboxylic acid methyl ester (**1a**, 5.3 mg, 88.3% yield). Yellow amorphous solid. ^{1}H NMR (Table 1). ^{13}C NMR (Table 2).

3.3.3. 6′,8-O-Dimethylknipholone (**2**)

Yellow amorphous solid, m.p. 263–264 °C. UV λ_{max} (acetonitrile): 220, 256, 286, 415 nm. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 13.91 (s, 1H, 2′-OH), 13.39 (s, 1H, 1-OH), 7.74 (*dd*, *J* = 7.8, 1.1 Hz, 1H, H-5), 7.64 (*t*, *J* = 7.8 Hz, 1H, H-6), 7.29 (*dd*, *J* = 7.8, 1.1 Hz, 1H, H-7), 7.25 (*s*, 1H, H-2), 6.14 (*s*, 1H, H-5′), 4.08 (*s*, 3H, 8-OCH_3), 4.02 (*s*, 3H, 4′-OCH_3), 3.79 (*s*, 3H, 6′-OCH_3), 2.69 (*s*, 3H, $COCH_3$), 2.10 (*s*, 3H, 3-CH_3). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 203.5 (CO), 189.0 (C-9), 183.5 (C-10), 162.9 (C-4′), 162.6 (C-6′), 162.4 (C-2′), 162.3 (C-1), 160.2 (C-8), 149.4 (C-3), 136.9 (C-5a), 135.3 (C-6), 130.9 (C-4a), 126.6 (C-4), 125.3 (C-2), 120.6 (C-8a), 120.0 (C-5), 117.2 (C-7), 116.2 (C-1a), 109.3 (C-1′), 106.2 (C-3′), 86.2 (C-5′), 56.6 (8-OCH_3), 55.7 (6′-OCH_3), 55.4 (4′-OCH_3), 33.3 ($COCH_3$), 20.7 (3-CH_3). HMBC: H-5 (C-7, C-8a, C-10), H-6 (C-5a, C-8), H-7 (C-5, C-8, C-8a), H-2 (C-1, C-1a, C-4, 3-CH_3), H-5′ (C-1′, C-3′, C-6′), 8-OMe (C-8), 4′-OMe (C-4′), 6′-OMe (C-6′), COMe (C-3′, CO), 3-Me (C-2, C-3, C-4), 2′-OH (C-1′, C-2′, C-3′), 1-OH (C-1, C-1a, C-2). NOE: H-7 (8-OCH_3), H-2 (CH_3), H-5′ (4′-OCH_3, 6′-OCH_3). EIMS (70 eV, rel. int.): m/z = 462 (15 $[M]^+$), 447 (14), 431 (9), 357 (17), 342 (24), 105 (35), 100 (100), 72 (37). HR-EIMS m/z = 462.13142 $[M]^+$ (calculated for $C_{26}H_{22}O_8$, 462.13147).

3.4. Preparation of knipholone Mannich base (**4**) and knipholone-1,3-oxazine (**5**)

In a 250 mL round bottom flask, 11.5 mg (0.027 mmol) of knipholone (**3**) was dissolved in 50 mL of dioxane and 2.0 mL of diethyl amine and 0.8 mL of formaldehyde (aq.) were added. The mixture was then stirred for 48 h at 65 °C and the reaction progress was monitored by TLC using 20% EtOAc in petroleum ether. The solvent was evaporated and the reaction mixture was purified by column chromatography (60 cm length and 4 cm diameter) on oxalic acid impregnated silica gel (increasing gradient of EtOAc in petroleum ether as eluting solvent) to give **4** (6.3 mg, 54.7% yield, ≥98% purity). Compound **5** was also prepared following the same procedure where knipholone (16.0 mg; 0.037 mmol), formaldehyde (2.2 mL) and aniline (2.7 mL) were dissolved in 50 mL of dioxane. The mixture was refluxed at 65 °C for 24 h, and then purified by column chromatography (60 cm length and 4 cm diameter) on oxalic acid impregnated silica gel (increasing gradient of EtOAc in petroleum ether as eluting solvent) to give 7.6 mg (47.5% yield, ≥97% purity) of **5**.

3.4.1. Knipholone mannich base (**4**)

Red amorphous solid, m.p. 192–193 °C. UV λ_{max} (acetonitrile): 219, 252, 284, 423 nm. ^{1}H NMR (500 MHz, acetone-d_6): δ_H 13.80 (s, 1H, 2′-OH), 7.72 (*t*, *J* = 7.8 Hz, 1H, H-6), 7.53 (*dd*, *J* = 7.8, 1.1 Hz, 1H, H-5), 7.31(*s*, 1H, H-2), 7.24 (*dd*, *J* = 7.8, 1.1 Hz, 1H, H-7), 4.01 (*d*, *J* = 1.9 Hz, 2H, N-CH_2), 3.86 (*s*, 3H, H-OCH_3), 2.73 (*m*, 4H, CH_2), 2.69 (*s*, 3H, $COCH_3$), 2.16 (*s*, 3H, 3-CH_3), 1.10 (*t*, *J* = 7.1 Hz, 6H, CH_3). ^{13}C NMR (125 MHz, acetone-d_6): δ_H 203.4 (CO), 194.1 (C-9), 182.7 (C-10), 166.0 (C-6′), 163.2 (C-4′), 163.1 (C-1), 162.6 (C-8), 161.7 (C-2′), 152.6 (C-3), 138.1 (C-6), 135.7 (C-5a), 132.5 (C-4a), 130.2 (C-4), 125.3 (C-2), 124.0 (C-7), 120.0 (C-5), 116.5 (C-8a), 115.6 (C-1a), 112.5 (C-5′), 108.2 (C-1′), 108.0 (C-3′), 63.1 (4′-OCH_3), 50.9 (N-CH_2), 46.6 (C-CH_2), 31.2 ($COCH_3$), 21.0 (3-CH_3), 11.3 (CH_3). EIMS (70 eV) m/z = 519 $[M]^+$.

3.4.2. Knipholone-1,3-oxazine (**5**)

Red amorphous solid, m.p. 197–198 °C. UV λ_{max} (acetonitrile): 217 nm, 242 nm, 290 nm, 419 nm. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 13.35 (*s*, 1H, 2′-OH), 12.58 (*s*, 1H, 8-OH), 12.07 (*s*, 1H, 1-OH), 7.59 (*t*, *J* = 7.9 Hz, 1H, H-6), 7.52 (*dd*, *J* = 7.5, 1.2 Hz, 1H, H-5), 7.30–7.21 (*m*, 3H, H-2/3″/5″), 7.08–7.01 (*m*, 2H, H-2″/6″), 7.01–6.94 (*m*, 1H, H-4″), 5.34 (*d*, *J* = 10.5 Hz, 1H, H-N-CH_2O), 5.25 (*d*, *J* = 10.5 Hz, 1H, N-CH_2O), 4.72 (*s*, 2H, N-CH_2), 3.93 (*s*, 3H, 4-OCH_3), 2.75 (*s*, 3H, $COCH_3$), 1.97 (*s*, 3H, 3-CH_3). ^{13}C NMR (125 MHz, $CDCl_3$): δ_H 203.5 (CO), 192.9 (C-9), 182.3 (C-10), 162.6 (C-1), 161.8 (C-8), 160.4 (C-2′), 159.3 (C-4′), 157.4 (C-6′), 151.2 (C-3), 147.5 (C-1″), 136.8 (C-6), 134.5 (C-5a), 131.4 (C-4a), 129.3 (C-3″/5″), 127.5 (C-4), 125.2 (C-2), 123.6 (C-7), 122.2 (C-4″), 119.7 (C-5), 118.9 (C-2″/6″), 115.6 (C-8a), 114.9 (C-1a), 112.3 (C-5′), 109.3 (C-3′), 106.1 (C-1′), 80.6 (N-CH_2O), 62.0 (4′-OCH_3), 46.6 (N-CH_2), 31.3 ($COCH_3$), 20.9 (3-CH_3). EI-MS (70 eV) m/z = 551 $[M]^+$.

3.5. Cytotoxicity assay

Cytotoxic activity testing of the compounds was done as described in previous report (Sammet et al., 2010). The KB-3-1 cells were cultivated as a monolayer in DMEM (Dulbecco's modified Eagle medium) with glucose (4.5 g/l), L-glutamine, sodium pyruvate and phenol red, supplemented with 10% (KB-3-1) fetal bovine serum (FBS). The cells were maintained at 37 °C and 5.3% CO_2 in humidified air. On the day before the test, the cells (70% confluence) were detached with trypsin-ethylendiamine tetraacetic acid solution (0.05%; 0.02% in DPBS) and placed in sterile 96-well plates in a density of 10,000 cells in 100 μL medium per well. The dilution series of the compounds were prepared from stock solutions in DMSO of concentrations of 100, 50 or 25 mM. The stock solutions were diluted with culture medium (10% FBS [KB-3-1]) down to pM range. The dilution prepared from stock solution was added to the wells. Each concentration was tested in six replicates. Dilution series were prepared by pipetting liquid from well to well. The control contained the same concentration of DMSO as the first dilution. After incubation for 72 h at 37 °C and 5.3% CO_2-humidified air, 30 μL of an aqueous resazurin solution (175 μM) was added to each well. The cells were incubated at the same conditions for 5 h. Subsequently, the fluorescence was recorded at a wavelength of 588 nm (λ_{ex} = 530 nm). The IC_{50} values were calculated as a sigmoidal dose response curve using GRAPHPAD PRISM 4.03. The IC_{50} values equal the drug concentrations, at which vitality is 50%. Cryptophycin-52

(≥98% purity) with IC_{50} = 36 pM (Eissler et al., 2009) was used as a positive control.

Supplementary data

NMR data for the new compounds are given. 1D and 2D spectra of the known compounds are available upon request.

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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.phytol.2014.04.004.

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