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N-Substituted acridone alkaloids from *Toddaliopsis bremekampii* (Rutaceae: Toddalioideae) of south-central Africa

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Abstract

Toddaliopsins A–D, four novel 1,2,3-trioxygenated acridone alkaloids, have been isolated from the leaves of *Toddaliopsis bremekampii*. Toddaliopsins B–D are the first reported acridone alkaloids with substituted N-methyl groups, in the light of which the chemotaxonomic relationship of *Toddaliopsis* and *Vepris* is discussed. Toddaliopsin C possesses moderate anti-inflammatory activity, which may be related to the hydroxy group present at C-1.

Keywords: *Toddaliopsis bremekampii*; Rutaceae; *Vepris*; Toddaliinae; Acridone alkaloids; Toddaliopsins A–D; 1,2,3-Trimethoxyacridone; 1,2,3-Trimethoxy-10-acetoxymethylacridone; 1-Hydroxy-2,3-dimethoxy-10-acetoxymethylacridone; 1,2,3-Trimethoxy-10-methoxymethylacridone; Anti-inflammatory activity

1. Introduction

The African genus *Toddaliopsis* Engl. of the Rutaceae was assigned by Engler (1931) to the subtribe Toddaliinae of the subfamily Toddalioideae, along with 12 other genera, including *Vepris* Comm. ex A.Juss. A total of five Toddaliinae genera occur in Africa. *Toddaliopsis* comprises only two species, *Toddaliopsis sansibarensis* (Engl.) Engl. of East Africa (Kokwaro, 1982) and *Toddaliopsis bremekampii* I.Verd. of South-central Africa. *T. bremekampii* (wild mandarin or wart-berry) is a multi-stemmed shrub or small tree (to 6 m) of sand and dune forests in the hot lowlands of Swaziland, Mozambique and Zimbabwe, extending as far south as Maputaland in South Africa (Pooley, 1993). Although fairly widespread, it has only once been documented as an ethnomedicinal subject, by Williams et al. (2001), who identified its bark in inter-provincial trade under the Zulu name *intane*. As with most other family members, the crushed leaves are aromatic, in this case lemon-scented (Schmidt et al., 2002). This genus was separated from *Vepris* on account of its lack of endosperm, and possession of strongly warty fruits (Verdoorn, 1926). However, some authors (Kokwaro, 1982; Lebrun and Stork, 1992) have questioned the validity of this generic distinction. Neither *Toddaliopsis* species has previously been investigated phytochemically; accordingly biochemical systematic data have not previously been available to support either position.

2. Results and discussion

Four novel acridone alkaloids, toddaliopsins A–D **1**–**4** were isolated from the dichloromethane extract of the leaves of *T. bremekampii*.

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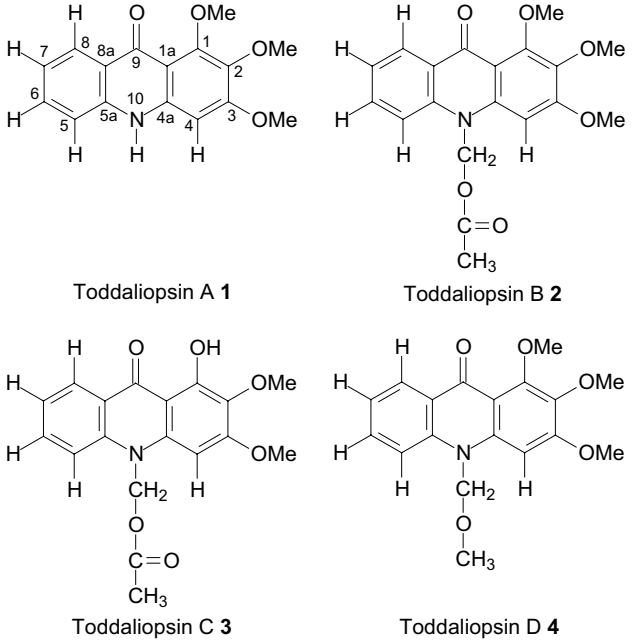
A positive Cl HRMS of toddaliopsin A **1** showed an $[M + H]^+$ peak at *m/z* 286.1081, corresponding to the molecular formula $C_{16}H_{16}NO_4$, and that of $C_{16}H_{15}NO_4$ for the alkaloid itself. Inspection of the IR, ^1H and ^{13}C NMR spectra of **1** showed it to possess a carbonyl carbon (δ_C 177.0 (*s*); 1633 cm^{-1}, C=O stretch), an aromatic N–H group (δ_H 10.39 (*br s*), 1H; 3275 cm^{-1}, N–H stretch), three methoxy groups (δ_H 4.00 (*s*), 3.84 (*s*), 3.74 (*s*), each 3H), and five aromatic proton signals, and suggested that it was an acridone alkaloid. A correlation in the HMBC spectrum between the C-9 carbonyl resonance and a 1H doublet at δ_H 8.41 (J = 8.1 Hz) established this as H-8, with a correlation in the COSY spectrum then permitting the assignment of a 1H multiplet resonance at δ_H 7.17 to H-7. This signal, in turn, displays a correlation in the COSY spectrum to two superimposed 1H multiplet resonances at δ_H 7.53, assigned to both H-6 and, by virtue of a correlation in the NOESY spectrum to the N–H signal at δ_H 10.39, to H-5. All three methoxy substituents in **1** are thus attached to the second aromatic ring. A further correlation in the NOESY spectrum between the N–H resonance and the remaining 1H singlet aromatic signal at δ_H 6.75 established this as H-4, as expected from both its upfield position, relative to that of H-8, and correlation in the NOESY spectrum to only one methoxy group resonance. Toddaliopsin A **1** is thus the novel 1,2,3-trimethoxyacridone.

The positive CI HRMS of toddaliopsin B **2** showed a $[M + H]^+$ peak at *m/z* 358.1291, corresponding to $C_{19}H_{20}NO_6$, and thus a molecular formula, for the alkaloid itself, of $C_{19}H_{19}NO_6$. The IR, ^1H and ^{13}C NMR spectra of **2** were similar to those of **1**, with those of **2** again displaying the carbonyl carbon (δ_C 176.8 (*s*); 1644 cm^{-1}), four COSY-coupled aromatic 1H proton resonances (δ_H 8.43 (*d*, J = 8.1 Hz, H-8, by HMBC correlation, as before, to C-9); 7.30 (*dd*, J = 8.1, 7.0 Hz, H-7); 7.65 (*dd*, J = 8.6, 7.0 Hz, H-6); 7.50 (*d*, J = 8.6 Hz, H-5)), a fifth 1H singlet aromatic resonance, and three methoxy group proton resonances (δ_H 4.01 (*s*), 4.01 (*s*), 3.99 (*s*), each 3H). The difference, relative to **1**, of $C_3H_4O_2$ is accounted for by additional resonances attributable to an acetate group (δ_H 2.20 (*s*), 3H; δ_C 21.0 (*q*), 170.6 (*s*)) and an oxymethylene linkage (δ_H 6.29 (*br s*), 2H, δ_C 71.5 (*t*)), which was assigned, as the IR and ^1H NMR signals ascribed to the amino proton in **1** were missing, to N-10. This placement was confirmed by a correlation in the NOESY spectrum between the H-5 and N–CH_2 proton signals, while a further NOESY correlation between the latter resonance and the remaining 1H singlet aromatic signal at δ_H 6.88 established this, as in **1**, as H-4. Toddaliopsin B **2** is thus the novel 1,2,3-trimethoxy-10-acetoxymethylacridone.

The molecular formula of toddaliopsin C **3** was established by positive CI HRMS to be $C_{18}H_{17}NO_6$ and thus, relative to **2**, to be missing a CH_2 group. Inspection of the IR, ^1H and ^{13}C NMR spectra of **3** showed it to have, in common with **2**, the C-9 carbonyl group (δ_C 181.6 (*s*); 1643 cm^{-1}), N-10 acetoxymethyl group (δ_H 2.19 (*s*), 3H, δ_C 20.9 (*q*), 170.4 (*s*), 1747 cm^{-1}; δ_H 6.33 (*br s*), 2H, δ_C 70.3 (*t*)), four COSY-coupled aromatic 1H proton resonances (δ_H 8.41 (*d*, J = 8.1 Hz, H-8, by HMBC correlation, as before, to C-9); 7.34 (*dd*, J = 8.1, 7.1 Hz, H-7); 7.73 (*dd*, J = 8.6, 7.1 Hz, H-6); 7.58 (*d*, J = 8.6 Hz, H-5)), and a fifth 1H singlet aromatic resonance (δ_H 6.59 (*s*), H-4, by correlation in the NOESY spectrum to N–CH_2). However, only two methoxy group proton resonances (δ_H 3.99 (*s*), 3.90 (*s*), each 3H) were visible in the ^1H NMR spectrum, while the IR spectrum displayed an O-H stretching band at 3455 cm^{-1}. The methoxy groups were established as 3-OCH_3 and 2-OCH_3, respectively, by stepwise correlation, in the NOESY spectrum, from H-4; the positioning of the hydroxy group at C-1 was confirmed by the shift of C-9, C-1a, C-1 and C-2 from δ_C 176.8 (*s*), 112.1 (*s*), 154.7 (*s*) and 138.8 (*s*) in **2** to δ_C 181.6 (*s*), 105.5 (*s*), 156.1 (*s*) and 131.0 (*s*) in **3**, which is attributed to chelation between the C-9 carbonyl and C-1 hydroxy groups (Adinolfi et al., 1986). Toddaliopsin C **3** is thus the novel 1-hydroxy-2,3-dimethoxy-10-acetoxymethylacridone.



Toddaliopsin D **4** was assigned the molecular formula $C_{18}H_{19}NO_5$ on the basis of positive CI HRMS data, and is therefore missing, relative to **2**, a carbon and an oxygen atom. The disappearance, in the NMR and IR spectra, of the signals attributable to the acetoxy group, and their replacement by an additional upfield methoxy group proton resonance (δ_H 3.58 (*s*), 3H; δ_C

55.9 (*q*)), suggested that **4** was the N-methoxymethyl analogue of **2**. This was confirmed by a correlation in the NOESY spectrum between this methoxy group proton signal and a 2H broad singlet at δ_H 5.52, attributed (its upfield shift from δ_H 6.29 in **2** notwithstanding) to the N–CH_2 resonance by correlations in the NOESY spectrum to both H-5 (δ_H 7.49 (*d*, $J = 8.6$ Hz) and H-4 (δ_H 6.80 (*s*)); the latter resonances were assigned both by direct comparison with the corresponding values in **2**, and by analysis of correlations in the COSY and NOESY spectra as detailed in the structural elucidation of **2** and **3**. Toddaliopsin D **4** is thus the novel 1,2,3-trimethoxy-10-methoxymethylacridone.

As only a single acridone alkaloid has previously been reported with anti-inflammatory properties (Dictionary of Natural Products, 2004), toddaliopsins A–D **1**–**4** were tested for their anti-inflammatory activity in the chemiluminescence assay. They displayed moderate activity, with mean IC_{50} values of 27.3, 48.3, 4.21 and 79.1 $\mu g.mL^{-1}$, respectively. Of these, toddaliopsin C **3** had the greatest activity, suggesting that the presence of a hydroxy group at C-1 may enhance the anti-inflammatory properties of these compounds.

To our knowledge, toddaliopsins B–D **2**–**4** are the first reported acridone alkaloids with substituted N-methyl groups, although a number of 4- and 5-quinolinones possessing an N-acetoxymethyl substituent have been reported from the Rutaceous genera *Boronia* SM. (Rutoideae; Boroniae) (Duffield and Jeffries, 1963; Ahsan et al., 1993) and *Zanthoxylum* L. (Rutoideae; Zanthoxylaceae) (Stermitz and Sharifi, 1977; Brader et al., 1993). This unusual feature at once distinguishes these acridones from those previously reported from the closely related genus *Vepris*, of which at least eight species have been chemically characterised to date (Dagne et al., 1988; Dictionary of Natural Products, 2004). Of perhaps greater significance in the current investigation is the absence of furoquinoline alkaloids, a class consistently found in *Vepris* species (Dagne et al., 1988; Dictionary of Natural Products, 2004), although this may be at only trace levels (Waterman et al., 1978). To a lesser extent pyranoquinolone alkaloids are also known from *Vepris* (Dagne et al., 1988; Dictionary of Natural Products, 2004); again these were not detected in the leaves of *Toddaliopsis*.

In considering the distribution of alkaloids in the Toddalioideae, Dagne et al. (1988) concluded that no insights into phylogenetic relationships within the group were discernable. The present report of N-substituted acridone alkaloids in *Toddaliopsis* seemingly does not further illuminate proposed intra-subtribal or intra-subfamilial phylogenies (Verdoorn, 1926; Waterman, 1973). Acridone alkaloids are known only from the Rutaceae (Waterman, 1973), in which family they are fairly widespread in the Toddalioideae (Dagne et al., 1988) as well as the Aurantioideae and Rutoideae (Waterman, 1975). These three subfamilies have traditionally accommodated the vast majority (ca. 1800) of rutaceous species (Mabberley, 1997).

The current findings support the retention of *Vepris* and *Toddaliopsis* as distinct genera. We suggest, furthermore, that N-substituted acridone alkaloids may presently be considered as definitive chemotaxonomic markers for the genus *Toddaliopsis* in the Toddalioideae (Rutaceae).

3. Experimental

3.1. General

NMR spectra were recorded at room temperature on a 400-MHz Varian UNITY-INOVA spectrophotometer. Chemical shifts (δ) are expressed in ppm relative to tetramethylsilane (TMS) as internal standard and coupling constants are given in Hz. ^{1}H NMR spectra were referenced against the C<u>H</u>Cl_3 signal at δ_H 7.27, and ^{13}C NMR spectra to the corresponding signal at δ_C 77.0. UV spectra were obtained on a Varian DMS 300 UV–Visible spectrometer with CH_2Cl_2 as solvent. IR spectra were recorded on a Nicolet Impact 400D Fourier-Transform Infrared (FT-IR) spectrometer, using NaCl windows with CH_2Cl_2 as solvent against an air background. HRMS were recorded on a VG 70-SE HRMS instrument, with electron impact and chemical ionisation, by Mr. John Hill at Kent Mass Spectrometry, UK.

3.2. Plant material

T. bremekampii I.Verd. was collected from sandforest near Tembe Elephant Reserve in northern KwaZulu-Natal, South Africa, and a voucher specimen (*N. Crouch 943*, NH) retained for verification purposes.

3.3. Extraction and isolation of compounds

The air-dried, powdered leaf material (43.6 g) was extracted successively for 24 h each in a Soxhlet apparatus with hexane, CH_2Cl_2, ethyl acetate and methanol. ^{1}H NMR spectroscopy showed the presence of fatty acids only in the hexane extract, and sugars only in the ethyl acetate and methanol extracts, and these were not examined further.

Portions of the CH_2Cl_2 extract (12.34 g) of the leaves were loaded onto glass-backed PTLC (Merck 5745) plates and eluted with CH_2Cl_2:ethyl acetate mixtures (9:1–4:1), affording, after further purification on aluminium backed analytical TLC (Merck 5554) plates using the same solvent systems, toddaliopsin A **1** (18.2 mg), toddaliopsin B **2** (20.2 mg), toddaliopsin C **3** (22.4 mg) and toddaliopsin D **4** (10.3 mg).

Table 1
^{1}H NMR spectral data for Toddaliopsins A–D **1**–**4** ($CDCl_3$, 400 MHz)

Proton	1	2	3	4
1	–	–	–	–
2	–	–	–	–
3	–	–	–	–
4	6.75 (*s*)	6.88 (*s*)	6.59 (*s*)	6.80 (*s*)
5	7.53	7.50 (*d*, 8.6)	7.58 (*d*, 8.6)	7.49 (*d*, 8.6)
6	7.53	7.65 (*dd*, 8.6, 7.0)	7.73 (*dd*, 8.6, 7.1)	7.62 (*dd*, 8.6, 7.1)
7	7.17 (*m*)	7.30 (*dd*, 8.1, 7.0)	7.34 (*dd*, 8.1, 7.1)	7.26 (*dd*, 8.1, 7.1)
8	8.41 (*d*, 8.1)	8.43 (*d*, 8.1)	8.41 (*d*, 8.1)	8.43 (*d*, 8.1)
9	–	–	–	–
NH	10.39 (*br s*)	–	–	–
4a	–	–	–	–
1a	–	–	–	–
8a	–	–	–	–
5a	–	–	–	–
1–OCH_3	4.00 (*s*)	4.01 (*s*)	–	3.90 (*s*)
2–OCH_3	3.84 (*s*)	3.99 (*s*)	3.90 (*s*)	4.01 (*s*)
3–OCH_3	3.74 (*s*)	4.01 (*s*)	3.99 (*s*)	4.00 (*s*)
N–CH_2	–	6.29 (*br s*)	6.33 (*br s*)	5.52 (*s*)
N–CH_2–OCOCH_3	–	2.20 (*s*)	2.19 (*s*)	–
N–CH_2–OCH_3	–	–	–	3.58 (*s*)

Table 2
^{13}C NMR spectral data for Toddaliopsins A–D **1**–**4** ($CDCl_3$, 100 MHz)

Carbon	1	2	3	4
1	153.6 (C)	154.7 (C)	156.1 (C)	154.4 (C)
2	137.8 (C)	138.8 (C)	131.0 (C)	138.6 (C)
3	158.3 (C)	158.2 (C)	159.6 (C)	158.0 (C)
4	94.3 (CH)	93.2 (CH)	87.5 (CH)	93.5 (CH)
5	116.7 (CH)	114.3 (CH)	114.6 (CH)	114.4 (CH)
6	132.7 (CH)	133.3 (CH)	134.4 (CH)	133.0 (CH)
7	121.4 (CH)	122.6 (CH)	122.7 (CH)	122.1 (CH)
8	126.6 (CH)	127.6 (CH)	126.7 (CH)	127.5 (CH)
9	177.0 (C)	176.8 (C)	181.6 (C)	176.9 (C)
4a	140.4 (C)	140.9 (C)	139.7 (C)	141.9 (C)
1a	110.3 (C)	112.1 (C)	105.5 (C)	112.2 (C)
8a	121.9 (C)	124.0 (C)	121.0 (C)	123.8 (C)
5a	140.1 (C)	141.3 (C)	141.7 (C)	141.6 (C)
1-OCH_3	62.0 (CH_3)	61.9 (CH_3)	–	61.9 (CH_3)
2-OCH_3	61.5 (CH_3)	61.4 (CH_3)	60.8 (CH_3)	61.5 (CH_3)
3-OCH_3	55.8 (CH_3)	56.2 (CH_3)	56.2 (CH_3)	56.0 (CH_3)
N–CH_2–	–	71.5 (CH_2)	70.3 (CH_2)	79.6 (CH_2)
N–CH_2–OCOCH$_3$	–	170.6 (C)	170.4 (C)	–
N–CH_2–OCOCH_3	–	21.0 (CH_3)	20.9 (CH_3)	–
N–CH_2–OCH_3	–	–	–	55.9 (CH_3)

*3.3.1. Toddaliopsin A, 1,2,3-trimethoxyacridone (**1**)*

Yellow glass; ν_{max}(NaCl) cm^{-1} 3275 (N–H), 2928, 2843, 1633 (C=O), 1600, 1478, 1312, 1252, 1141, 1102; HRCIMS (70 eV) *m/z* 286.1081 (calc. for [$C_{16}H_{15}NO_4$ + H] 286.1079); CIMS (70 eV) *m/z* (rel. int.) 286 (100), 133 (1), 57 (3); λ_{max}(CH_2Cl_2) nm (logε): 263 (4.34), 297 (3.57), 367 (log e 3.68), 382 (log e 3.69); ^{1}H NMR spectral data (400 MHz, $CDCl_3$) Table 1; ^{13}C NMR spectral data (100 MHz, $CDCl_3$) Table 2.

*3.3.2. Toddaliopsin B, 1,2,3-trimethoxy-10-acetoxy-methylacridone (**2**)*

Yellow glass; ν_{max}(NaCl) cm^{-1} 2933, 2858, 1733 (C=O), 1644 (C=O), 1607, 1487, 1291, 1266, 1196, 1135; HRCIMS (70 eV) *m/z* 358.1291 (calc. for [$C_{19}H_{19}NO_6$ + H] 358.1291); CIMS (70 eV) *m/z* (rel. int.) 358 (69), 286 (100), 197 (3), 149 (5), 61 (72); λ_{max}(CH_2Cl_2) nm (log): 263 (4.21), 307 (3.50), 385 (3.53); ^{1}H NMR spectral data (400 MHz, $CDCl_3$) Table 1; ^{13}C NMR spectral data (100 MHz, $CDCl_3$) Table 2.

*3.3.3. Toddaliopsin C, 1-hydroxy-2,3-dimethoxy-10-acetoxymethylacridone (**3**)*

Yellow glass; ν_{max}(NaCl) cm^{-1} 3455 (O–H), 2926, 2860, 1747 (C=O), 1643 (C=O), 1610, 1472, 1202; HRCIMS (70 eV) *m/z* 344.1131 (calc. for $[C_{18}H_{17}NO_6 + H]$ 344.1134); CIMS (70 eV) *m/z* (rel. int.) 344 (100), 286 (64), 198 (1), 149 (2), 61 (84); $\lambda_{max}(CH_2Cl_2)$ nm (log): 223 (3.48), 236 (3.64), 272 (3.64), 320 (3.29), 390 (2.94); ^{1}H NMR spectral data (400 MHz, $CDCl_3$) Table 1; ^{13}C NMR spectral data (100 MHz, $CDCl_3$) Table 2.

*3.3.4. Toddaliopsin D, 1-2,3-trimethoxy-10-methoxy-methylacridone (**4**)*

Yellow glass; ν_{max}(NaCl) cm^{-1} 2923, 2858, 1643 (C=O), 1602, 1467; HRCIMS (70 eV) *m/z* 330.1337 (calc. for $[C_{18}H_{19}NO_5 + H]$ 330.1341); CIMS (70 eV) *m/z* (rel. int.) 330 (100), 314 (11), 298 (15), 286 (25), 270 (4), 242 (1), 197 (2), 149 (1), 125 (1), 97 (2), 71 (3); $\lambda_{max}(CH_2Cl_2)$ nm ($\log\varepsilon$): 264 (3.22), 379 (2.47); ^{1}H NMR spectral data (400 MHz, $CDCl_3$) Table 1; ^{13}C NMR spectral data (100 MHz, $CDCl_3$) Table 2.

3.4. Chemiluminescence assay

Polymorphonuclear leukocytes (PMNs) were prepared from buffycoat residues from healthy volunteers, after centrifugation in Ficoll-Hypaque, according to manufacturer's instructions (Amersham Pharmacia, Uppsala, Sweden). Cells were diluted to 1×10^{-7} PMNs per mL HBSS (Hank's Buffered Salt Solution) and dispensed in white 96-wells flat-bottom microtiter plates in 50 μL amounts. Subsequently, 50 μL of an appropriate dilution range of a test sample and 50 μL of luminol (0.1 mM) were added to each well. The cells were activated with 50 μL (human) serum-treated (opsonized) zymosan (0.8 mg/mL), after which the luminescence of each well was monitored, at 2 min intervals for 30 min, in a Titertek Luminoskan luminometer (TechGen International, Zellik, Belgium). Maximum peak levels were used to calculate the inhibitory activity of the test samples compared with a control, consisting of cells with luminol and buffer (Smit et al., 2000; Van den Worm, 2001).

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