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RESEARCH ARTICLE

Erymildbraedin A and B, two novel cytotoxic dimethylpyrano-isoflavones from the stem bark of *Erythrina mildbraedii*: evaluation of their activity toward endocrine cancer cells

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Abstract

Two new dimethylpyrano-isoflavones, named erymildbraedin A (**4**) and B (**5**), were isolated from the stem bark of the Cameroonian medicinal plant *Erythrina mildbraedii*, along with four known ones, the linear congeners, scandenone (**1**), erysenegalinsein M (**2**), 5,4′-dihydroxy-2′-methoxy-8-(3,3-dimethylallyl)-2″,2″-dimethylpyrano[5,6:6,7]isoflavone (**3**), and the angular isoflavone eryvarin B (**6**), and two other compounds, fraxidin and scoparone. Their structures were elucidated by the usual spectroscopic methods and isoflavone effects on the growth of human breast, prostate, and endometrial adenocarcinoma cells were determined. Isoflavones **1**, **3**, and **6** strongly inhibited the growth of all three cell lines, supporting the notion that a non-oxidized isoprenyl group at C-8 is requisite for cytotoxic activity.

Keywords: Erythrina mildbraedii *(Fabaceae); prenyl-dimethylpyrano-isoflavones; endocrine cancer; estrogen receptor*

Introduction

The genus *Erythrina* (Fabaceae) is known for its use in traditional medicine practice, especially for the treatment of microbial infections[1]. Various parts of these plants (roots, barks, leaves, and wood) are commonly used in African folk medicine[2]. Earlier work on the seeds of *Erythrina* species revealed the presence of many physiologically active alkaloids[3]. *Erythrina mildbraedii* is a tree growing up to 30 m in height, endemic in West Africa[4], and its seeds were reported to contain erythrinan alkaloids that could be related to its potential physiological activity[5]. A number of reports on this species have revealed several biologically active constituents: three pterocarpans, erybraedin A, B, and C, isolated from the roots of *Erythrina mildbraedii* were reported to be active against *Staphylococcus aureus* ATCC 13709 and *Mycobacterium smegmatis* ATCC 607[6]. The pterocarpene erycristagallin isolated from the same species was found to exhibit anti-inflammatory activity[7]. Flavonoids with a prenylated B ring from the same species showed protein tyrosine phosphatase-1B inhibitory activity[8]. Flavanones and 2-arylbenzofurans with isoprenoid groups are known to be moderately cytotoxic against cancerous human cells[9]. Recently we reported that a 3-formyl-2-arylbenzofuran isolated from *Onobrychis ebenoides* exhibits strong cytotoxic activity toward a large variety of human cancer cells, which is most likely associated with its C-5 isoprenyl group[10,11].

As a contribution to the study of the neutral components of the *Erythrina* genus, we hereby report the isolation and structural elucidation of two novel prenylated

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dimethylpyrano-isoflavones along with four known ones and two known coumarins from the stem bark of *E. mildbraedii*. We also report on the growth inhibitory effects that the new and known isoflavones exert on human breast, endometrial, and prostate cancer cells and on the structural determinants associated with high cytotoxicity in this dimethylpyrano-isoflavone series.

Materials and methods

Phytochemistry

Plant material

E. mildbraedii Harms, stem bark, was collected at Mbalmayo, south Cameroon, in November 2004. A herbarium specimen documenting the collection was deposited at the National Herbarium, Yaoundé (Cameroon) (No: 50452/HNC).

Extraction, isolation, and structure determination

Air-dried and pulverized stem bark of the collected sample (1.3 kg) was extracted at room temperature with cyclohexane for 5 days. The residue of extraction was then macerated with CH_2Cl_2 (12 L) for 72 h; the extract was concentrated under vacuum to afford a viscous residue (15 g) of CH_2Cl_2 extract.

The CH_2Cl_2 extract was subjected to silica gel column chromatography using mixtures of CH_2Cl_2/MeOH of increasing solvent polarity as eluent, to give 45 fractions. These were collected and combined on the basis of thin layer chromatography (TLC) analysis leading to six main series A–F. The pure compounds were obtained either by direct crystallization or after further purification by column chromatography. Workup of series C (2 g) by repeated column chromatography on silica gel with CH_2Cl_2/MeOH and increasing concentrations of MeOH yielded **1**, **2**, fraxidin, scoparone, and **4**. Series B (250 mg) were separated by column chromatography over silica gel using CH_2Cl_2/MeOH (98:1; 98:2) to yield **3**. Series F (1.3 g) were separated by successive column chromatography on silica gel with CH_2Cl_2/EtOAc (99:1) and increasing concentrations of EtOAc to yield **5** and eryvarin B (**6**).

*Erymildbraedin A (**4**)* White solid; [α] + 40 (c 0.2174, CH_2Cl_2), UV λ nm (logε): 284.5 (4.18), IR ν (cm^{-1}): 3374, 2995, 1653, 1614, 1515, 1436, 1227, 1174, 1084, and 838; ^1H and ^{13}C NMR ($CDCl_3$, 300 and 75 MHz) see Table 1; EI-MS: *m/z* 450 (38) M^+; 435 (38); 379 (100); 361 (22); 349 (55).

*Erymildbraedin B (**5**)* Pale yellow solid; [α] + 50 (c 0.9543, MeOH), UV λ nm (logε): 275.4 (4.69), IR ν (cm^{-1}): 2918, 1652, 1516, 1435, and 1215; ^1H and ^{13}C NMR ($CDCl_3$, 300 and 75 MHz) see Table 1; EI-MS: *m/z* 418 (63) M^+; 389 (52); 375(100); 361(42); 349 (31); 333 (28); 321 (93).

Cell culture and assessment of cell proliferation

The effects of dimethylpyrano-isoflavones on the growth of MCF-7, LNCaP, and Ishikawa cells were assessed in 96-well microculture plates using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as already described[10,11]. Ishikawa and MCF-7 cells were plated in full growth medium (containing physiologically relevant levels of estradiol as well as fetal bovine serum, FBS) at a density of 2000 and 5000 cells/well, respectively, in phenol red-free minimal essential medium (MEM) containing 5% FBS, 0.1 nM 17β-estradiol, and 1 μg/mL insulin. LNCaP cells were plated at a density of 5000 cells/well in phenol red-free RPMI 1640 containing 5% FBS and 1 nM 17β-estradiol. The cells were exposed 72 h later to increasing concentrations of test compound, ICI 182,780 (1 μM), or vehicle only (0.1% dimethylsulfoxide, DMSO), and cell growth after 4 days of treatment was assayed photometrically at 550 (MTT) or 565 (sulforhodamine B, SRB) nm.

The binding affinities of **1–6** relative to that of 17β-estradiol (relative binding affinity, RBA; the RBA of estradiol was set equal to 100) for the human estrogen receptor (ER) were determined using fluorescence polarization (FP) as already described[12,13]. Briefly, the concentration of 17β-estradiol and **1–6** that inhibited the binding of the fluorescent estrogen ES2 to ER by 50% (IC_{50}) were determined using an FP reader, and RBA was then deduced by:

$$[RBA = (IC_{50\,estradiol}/IC_{50\,isoflavone}) \times 100]$$

Results and discussion

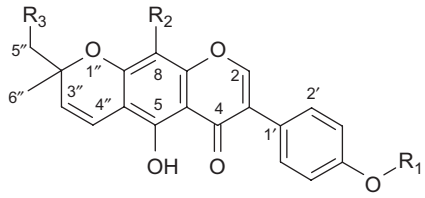
Phytochemistry

The CH_2Cl_2 extract of the stem bark of *E. milbraedii* was fractionated by silica gel column chromatography to obtain two new linear dimethylpyrano-isoflavones, named erymildbraedin A (**4**) and B (**5**), along with three linear congeners (**1–3**), one angular isoflavone (**6**), and two known coumarins (fraxidin[14] and scoparone[15]). The isoflavones **1–3** and **6** were identified as scandenone (**1**)[16], erysenegalensein M (**2**)[17], 5,4′-dihydroxy-8-(3,3-dimethylallyl)-2″-hydroxymethyl-2″-methylpyrano[5,6:6,7]isoflavone (**3**)[18], and eryvarin B (**6**)[19] (Figure 1).

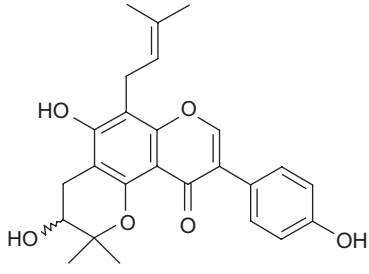
Compound **4** was obtained as a white solid. Its electron ionization-mass spectrum (EI-MS) exhibited a molecular ion at *m/z* 450, corresponding to a $C_{26}H_{26}O_7$ formula, appropriate for 14 degrees of unsaturation. The infrared (IR) spectrum showed absorption bands at 3374 (hydroxyl group), 1653 (chelated C=O), and 1614 cm^{-1} (double bond C=C). The ^1H nuclear magenetic resonance (NMR) spectrum of **4** (Table 1) displayed a singlet signal at δ_H 7.88, assigned to H-2, characteristic of the isoflavone skeleton. A downfield signal at δ_H 13.40 was assigned to a hydroxyl group at C-5. It also showed the typical 2,2-dimethylpyran ring resonances at δ_H 5.67 (1H, d, J = 10.11 Hz, H-3″), 6.76 (1H, d, J = 10.11 Hz, H-4″), and 1.50 (6H, s), two *para*-substituted ring B doublets at δ_H 7.40 (H-2′/H-6′) and 6.91 (H-3′/H-5′) (J = 8.70 Hz), and one methoxyl singlet at δ_H 3.40. The ^1H NMR spectrum also indicated the presence of a 3,3-dimethyloxiranylhydroxymethyl (oxyprenyl) group due to a set of signals resonating as two methyl singlets at δ 1.21 (CH_3-4‴) and 1.30 (CH_3-5‴) and two oxymethine doublets at 3.72 (H-2‴, J = 7.42 Hz) and 4.71 (H-1‴, J = 7.42 Hz). The oxyprenyl group was confirmed by

the ^{13}C NMR spectrum (Table 1), which displayed carbon resonances at δ_C 65.9 (C-1‴), 75.3 (C-2‴), 77.8 (C-3‴), 19.9 (C-4‴), and 25.3 (C-5‴). The oxidation of the C-1 position of the isoprenyl moiety, leading to the hydroxyl group, is rare in the literature; to the best of our knowledge, only derivatives of two coumarins[20,21], one flavanone[22], and one xanthone[23] were found to carry the same oxyprenyl group. The locations of the three groups, the methoxyl, the 2,2-dimethylpyran ring, and the 3,3-dimethyloxiranylhydroxymethyl, on the isoflavone skeleton were based on heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) experiments. The HMBC spectrum showed correlations between H-1‴ (δ_H 4.71) and carbons C-8 (δ_C 106.5), C-7 (δ_C 157.4), and C-8a (δ_C 156.7), suggesting that the oxyprenyl moiety is at the C-8 position. It also showed correlations from proton H-4″ (δ_H 6.76) to carbons C-5 (δ_C 157.4) and C-7 (δ_C 157.4), and from the proton H-3″ (δ_H 5.67) to carbon C-6 (δ_C 104.2), confirming that the fusion of the 2,2-dimethylpyran ring was linear. Finally, correlations from proton H-2′ (δ_H 7.40) and the methoxyl protons (δ_H 3.40) to carbon C-4′ (δ_C 156.7) confirmed the location of the methoxyl group at the C-4′ position. Consequently, the structure of **4** was established, as 5-hydroxy-4′-methoxy-8-(3,3-dimethyloxiranylhydroxymethyl)-2″,2″-dimethyl-pyrano[5,6:6,7]isoflavone, and named erymildbraedin A.

Compound **5** was obtained as a yellow solid. Its molecular ion peak at *m/z* 418, exhibited by EI-MS, corresponded to $C_{25}H_{22}O_6$ formula, appropriate for 15 degrees of unsaturation. The IR spectrum displayed absorption bands at 3380 (hydroxyl group), 1652 (chelated C=O), and 1612 cm^{-1} (double bond C=C). The ^1H and ^{13}C NMR spectra of **5** (Table 1) exhibited resonances at δ_H 7.93 and δ_C 155.0 (C-2) for the isoflavone type skeleton. The ^1H NMR spectrum of **5** was similar to that of erymildbraedin A (**4**), except for the oxyprenyl moiety, which displayed resonances for a 3-formylbut-2-enyl group at δ_H 3.64 (2H, d, *J* = 7.41 Hz, H-1‴), 6.53 (1H, t, *J* = 7.41 Hz, H-2‴), 9.26 (1H, s, H-4‴), and 1.84 (3H, s, H-5‴), and the ^{13}C NMR spectrum exhibited signals of this group at δ_C 31.3 (C-1‴), 154.8 (C-2‴), 140.7(C-3‴), 198.2 (C-4‴), and 10.5 (C-5‴). The absence of any ring A proton signal



1–5



eryvarin B, **6**

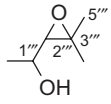
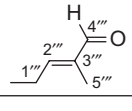
Compound	R_1	R_2	R_3
scandenone, **1**	H		H
erysenegalensein M, **2**	H		H
isoflavone, **3**	H		OH
erymildbraedin A, **4**	CH_3		CH_3
erymildbraedin B, **5**	H		H

Figure 1. *Chemical structures of the new (4, 5) and known (1–3, 6) dimethylpyrano-isoflavones examined in this study.*

Table 1. ^1H and ^{13}C data[a] of erymildbraedin A (**4**) and B (**5**) (300 and 75 MHz).

	4 ($CDCl_3$)		**5** ($CDCl_3$)	
Position	^1H (m, *J* (Hz))	^{13}C	^1H (m, *J* (Hz))	^{13}C
2	7.88 (s)	152.8	7.93 (s)	155.0
3		123.9		123.4
4		181.5		183.0
4a		105.6		107.0
5	13.40 (s, OH)	157.4		158.9
6		104.2		110.4
7		157.4		161.0
8		106.5		107.3
8a		156.6		159.1
1′		122.9		124.7
2′	7.40 (d, 8.70)	130.6	7.27 (d, 8.40)	131.9
3′	6.91 (d, 8.70)	115.9	6.80 (d, 8.40)	117.1
4′		156.7		158.9
5′	6.92 (d, 8.70)	115.9	6.80 (d, 8.40)	117.1
6′	7.40 (d, 8.70)	130.6	7.27 (d, 8.40)	131.9
2″		79.2		80.3
3″	5.67 (d, 10.11)	128.4	5.57 (d, 10.11)	129.0
4″	6.76 (d, 10.11)	116.1	6.65 (d, 10.11)	116.4
5″	1.50 (s)	28.7	1.40 (s)	29.5
6″	1.50 (s)	28.8	1.40 (s)	29.5
1‴	4.71 (d, 7.42) 5.85 (s, OH)	65.9	3.64 (d, 7.41)	31.3
2‴	3.72 (d, 7.42)	75.3	6.53 (t, 7.41)	154.8
3‴		77.8		140.7
4‴	1.21 (s)	19.9	9.26 (s)	198.2
5‴	1.30 (s)	25.1	1.84 (s)	10.49
4′-O-CH_3	3.40 (s)	57.2		

[a]*Data were assigned using HMQC, DEPT, and HMBC experiments.*

suggested that both dimethylchromen and 3-formylbut-2--enyl groups were linked to ring A. The HMBC spectrum exhibited correlations from proton H-1‴ (δ_H 3.64) to both carbons C-7 (δ_C 161.0) and C-8a (δ_C 159.1), indicating that the 3-formylbut-2-enyl group was located at the C-8 location and therefore the dimethylchromen was linear. Correlations observed from proton H-3″ (δ_H 5.57) to carbon C-6 (δ_C 110.4) and from proton H-4″ (δ_H 6.65) to carbon C-5 (δ_C 158.9) also confirmed that the dimethylchromen was linear. Consequently, the structure of compound **5** was determined, as 5,4′-dihydroxy-8-(3-formylbut-2-enyl)-2″, 2″-dimethylpyrano[5,6:6,7]isoflavone, and named erymildbraedin B.

Biological activity

We compared the effects of **1–6** on the growth of MCF-7 breast cancer cells, LNCaP prostate cancer cells, and Ishikawa endometrial cancer cells using MTT and/or SRB. Although these cells express the estrogen receptor (ER), only the growth of MCF-7 and LNCaP cells is dependent on estrogen, whereas the growth of Ishikawa cells is estrogen-independent[13,24]. ICI 182,780 (Faslodex®)[25], a non-cytotoxic destabilizer of ER that inhibits the growth of MCF-7 and LNCaP cells, but not that of Ishikawa cells, was also tested for comparison. ICI 182,780 is known to strongly down-regulate ER expression, allowing isoflavone effects on cell growth to be assessed independently of the receptor in cells that do not depend on ER for growth. Notably, we and others have shown that isoflavones can variably affect cell growth, inhibiting it at high concenration, while stimulating it through the ER at lower concentration[14,26]. Since it has been reported that isoflavone levels in the circulation of healthy volunteers given high doses of the compounds are approximately 2–5 μM[27], we exposed the cells to increasing concentrations of **1–6** up to 10 μM at the most. Statistically significant effects (Table 2) on cell growth were considered weak, moderate, or strong depending on whether cell numbers in the presence of **1-6** vs. vehicle were > 66%, ≤ 66–33%, or < 33%, respectively. Table 2 (column 2) shows that the growth of MCF-7 cells was inhibited strongly by **1**, **3**, and **6**, with the effects of **1** and **3** being significantly ($p < 0.05$) higher than that of ICI 182,780, suggesting that **1** and **3** were highly cytotoxic to these cells. Similarly, the growth of LNCaP cells was inhibited strongly by **3** and **6** and moderately by **1** (column 4), the effect of **3** again being significantly higher ($p = 0.05$) than that of ICI 182,780, suggesting that **3** was cytotoxic to these cells as well. Finally, the growth of Ishikawa cells was inhibited strongly by **1** and **3** and moderately by **6**, whereas **2**, **4**, **5**, and ICI 182,780 were ineffective (column 6). When Ishikawa cells were challenged with 10 μM of **1–6** in the presence of 10 μM ICI 182,780, the effects of **1** and **3** remained strong, that of **6** became strong, **2** and **5** became moderately effective, and **4** (the only one with a 4′-methoxy group) remained ineffective (column 8). It is known that interaction of 4′-OH with residues Glu305 and Arg346 of ER is critical for isoflavone binding to the receptor[13,28], and that 17β-estradiol derivatives with long 17α hydrophobic side chains can bind and activate ER[28]. Using fluorescence polarization[29], we determined the binding affinity of **1–6** relative to that of 17β-estradiol for human ER (RBA; mean ± SEM; $n \geq 3$) as being equal to 3.6 ± 1.2, 1.8 ± 0.8, 0.2 ± 0.1, 0.3 ± 0.1, 0.3 ± 0.1, 2.1 ± 0.4, respectively. These data may suggest that in the absence of ICI 182,780, **2**, **3**, **5**, and **6** acted through the ER to variably moderate their inhibitory effects, while **1** appeared to act so as to propagate its inhibitory effect through the receptor. The role of ER in modulating the growth inhibitory effects of **1–6** is currently not clear.

These data show that dimethylpyrano-isoflavones with a non-oxidized isoprenyl group at C-8, namely **1**, **3**, and **6**, whether linear or angular, were growth inhibitory toward all three cell lines tested; that **2**, **4**, and **5**, with an oxidized isoprenyl group, were much less active; and that **4**, in particular, with a 4′-methoxy group, was the least effective of all, indicating that the OH group at C-4′ modulates the growth inhibitory effect of this class of isoflavones possibly through binding to the ER. A comparison with published data on the cytotoxicity of *C*-prenylated flavonoids and their derivatives in general[29,30] shows that the inhibitory activities of **1**, **3**, and **6** are among the highest reported so far and comparable to that of xanthoxumol, a 5′-isoprenyl chalcone known to exhibit strong chemopreventive activity as

Table 2. Cell growth in the presence of **1–6**, ICI 182,780, or vehicle only, as assessed spectrophotometrically using MTT and/or SRB.

	MCF-7 cells		LNCaP cells		Ishikawa cells		
Compound	Cell growth[a]	EC_{50}[b] (μM)	Cell growth[a]	EC_{50}[b] (μM)	Cell growth[a]	EC_{50}[b] (μM)	Growth in ICI[c]
Vehicle	100	na	100	na	100	na	97 ± 5
1	4 ± 2#	7.0 ± 0.5	55 ± 5#	6.9 ± 1.0	18 ± 8#	7.4 ± 0.7	30 ± 12#
2	55 ± 5#	7.9 ± 1.5	58 ± 4#	7.6 ± 2.0	95 ± 11	na	50 ± 5#
3	6 ± 3#	6.8 ± 2.3	38 ± 5#	4.1 ± 0.5	26 ± 9#	7.4 ± 0.5	7 ± 3#
4	82 ± 10	na	64 ± 5#	9.1 ± 0.6	94 ± 7	na	79 ± 8
5	96 ± 14	na	56 ± 2#	7.6 ± 0.6	107 ± 2	na	51 ± 6#
6	25 ± 9#	7.1 ± 1.9	42 ± 5#	4.6 ± 0.7	57 ± 2#	7.7 ± 1.7	20 ± 9#
ICI 182,780	25 ± 4#	nd	49 ± 6#	nd	97 ± 5	nd	na

Note. #$p < 0.05$ vs. vehicle (ANOVA); na, not applicable; nd, not determined.

[a]Cell growth (mean ± SEM; $n \geq 3$) in the presence of 10 μM of compound was calculated by: $OD_{compound}$*100/$OD_{vehicle}$. $OD_{vehicle}$ was set equal to 100.

[b]EC_{50} are compound concentrations required to achieve 50% of the cell growth effect at 10 μM, the effect being: ($OD_{vehicle}$ – $OD_{compound}$)*100/$OD_{vehicle}$.

[c]Cell growth (mean ± SEM; $n \geq 3$) in the presence of 10 μM ICI 182,780 alone or in combination with 10 μM **1–6** is expressed relative to vehicle alone.

well as inhibit cancer progression[30]. As for dimethylpyrano-isoflavones, in particular, cytotoxicity data are limited to scandenone and alpinumisoflavone (lacks the C-8 isoprenyl of the former). Scandenone was found to induce apoptosis in HL-60 human leukemia cells[31] and alpinumisoflavone was reported as cytotoxic to KB cells[32], suggesting that both the dimethylpyrano and isoprenyl groups may contribute to cytotoxicity. Notably, 6,8-di-isoprenyl-genistein was found to be ineffective against HL-60 cells[31], supporting that the dimethylpyrano group contributes to the cytotoxicity.

The cytotoxicity of dimethylpyrano-isoflavones may reflect inhibition of cAMP-dependent protein kinase (PKA)[33] and may not be related to their ability to inhibit phospholipase Cγ1[34], since alpinumisoflavone has been reported not to inhibit PLCγ1[34] and yet to be cytotoxic to KB cells[32]. Alternatively, the cytotoxicity may reflect an effect on PTP1B, as reported for prenyl-flavanones[35]. Inhibition of PTP1B can block cadherin-mediated cell adhesion[36], and thus induce cell death by anoikis.

In conclusion, this is the first study to report that dimethylpyrano-isoflavones can strongly inhibit the growth of endocrine cancer cells; that a non-oxidized isoprenyl group at C-8 is requisite for their activity against these cells; and that their ability to bind the ER could modulate their cytotoxic activity. The ER-dependence of the cytotoxicity of these isoflavones may reflect ER cross-talk with PKA and/or PTP1B signaling.

Work to explore these possibilities is in progress. This report also confirms that medicinal plants remain a rich source of bioactive compounds with potentially useful pharmacochemical properties, and that plant-derived isoflavones offer many possibilities for discovering among them new potentially useful bioactive molecules.

Declaration of interest

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