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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and reactive oxygen species scavenging activity of halogenated alkaloids from boldine

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Abstract Six halogenated alkaloids have been semi-synthesized from natural boldine as starting material. Their antioxidant activity toward reactive oxygen species (ROS) generation by neutrophils stimulated with *N*-formyl-methionyl-leucyl-phenylalanine and in the hypoxanthine–xanthine oxidase system was evaluated. Most of the alkaloids synthesized inhibited ROS generation in both systems in a concentration-dependent manner. The alkaloids with phenolic substituents displayed more powerful anti-oxidative activity than those containing methoxylated groups. None of the alkaloids assayed had any effect on xanthine oxidase activity. Therefore, halogenated phenanthrene alkaloids can become in promising candidates for the development of novel and potent anti-inflammatory drugs.

Keywords Halogenated phenanthrene alkaloids · Halogenated aporphine alkaloids · Semi-synthesis · Antioxidant activity

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Introduction

The increasing recognition of the participation of free radical-mediated oxidative events in the initiation and/or progression of cardiovascular, tumoural, inflammatory, and neurodegenerative disorders (Bauerova and Bezek, 1999; Jaeschke, 1995; Zhao, 2009; Dinkova-Kostova, 2008; Griendling *et al*., 2000), has given rise to the search for new antioxidant molecules either natural or synthetic. In fact, the decreased activity of a large number of enzymes involved in reactive oxygen species (ROS) defence, particularly with aging process, can explain the main etiological factor of acute or chronic disorders. In this regard, boldine and other related aporphine alkaloids have been shown to behave as potent antioxidants in a number of experimental models (Speisky *et al*., 1991; Martínez *et al*., 1992; Cederbaum *et al*., 1992; Jang *et al*., 2000; Youn *et al*., 2002; Rancan *et al*., 2002; Milián *et al*., 2004; Yu *et al*., 2009).

Some years ago our group isolated two phenanthrene alkaloids from the roots of *Dennettia tripetala*, uvariopsine, and stephenanthrine (López-Martín *et al*., 2002). These alkaloids were able to inhibit Ang-II-induced leukocyte-endothelial cell interactions in vivo (Estellés *et al*., 2003). This effect was partly mediated through inhibition of the generation of ROS, down-regulation of P-selectin expression on the endothelial cell and blockade of PAF-induced responses by interacting with its receptor. Based on their structural features, we semi-synthesized four phenanthrene and one aporphine alkaloid from natural boldine and evaluated their inhibitory effect on ROS generation (Milián *et al*., 2004). The reasons for this semi-synthetic process were two: first to increase the number of aporphine and phenanthrene alkaloids to enhance their ROS scavenging properties and second, to obtain enough

quantities of the compounds to test their pharmacological activity in different in vivo and in vitro models.

In that study, boldine, secoboldine, and boldine methine displayed more powerful ROS scavenger activity than other semi-synthesized alkaloids or even uvariopsine and stephenanthrine and the classic antioxidant ascorbic acid, used as control in the same assays (Milián *et al*., 2004). Furthermore, boldine and the semi-synthesized secoboldine and boldine methine could inhibit Ang-II-induced leukocyte-endothelial cell interactions in vivo.

Secoboldine and boldine methine were also capable of reducing neutrophil infiltration into the peritoneal cavity of the rat, CXC chemokine and PAF release elicited by Ang-II in vivo (Estellés *et al*., 2005). The effects displayed by these two alkaloids were partly mediated through inhibition of the endothelial generation of ROS and interleukin-8 release after Ang-II stimulation. The inhibition of ROS generation seems to be the key mechanism by which these alkaloids inhibits Ang-II-induced responses since P-selectin up-regulation and inhibition of CXC chemokine synthesis are events linked to this capability (Riaz *et al*., 2003; Gaboury *et al*., 1994; Johnston *et al*., 1996). By contrast, the aporphine alkaloid boldine although endowed with antioxidant properties did not markedly affect the inflammatory activity elicited by Ang-II (Estellés *et al*., 2005). In this way, the use of a phenanthrene alkaloid such as boldine methine could constitute an alternative therapy for the control of Ang-II-induced neutrophilic inflammation in cardiovascular disease states where Ang-II plays a critical role.

On the other hand, both boldine and semi-synthetic derivatives (Sobarzo-Sánchez *et al*., 2002) have attracted the attention by the wide range of pharmacological activities. Thus, it has recently been reported that boldine displays anti-proliferative activity against glioma cells (Gerhardt *et al*., 2009) opening the study of the potential anticancer activities of boldine and its derivatives. Methiodides of boldine and derivatives showed neuronal nicotinic acetylcholine receptor blockers (Iturriaga-Vásquez *et al*., 2007) or more recently boldine and derivatives can be envisaged as new therapeutic candidates with novel mechanisms of action for treating resistant forms of tuberculosis (Guzman *et al*., 2010) or new phenanthrene alkaloids semi-synthesized from boldine for the treatment of *S. pneumoniae* infections resistant to other antibiotics (García *et al*., 2011). Based on these results, in this study we have synthesized novel antioxidant agents. The alkaloids synthesized have been halogenated in order to investigate and perform further studies regarding its structure–activity relationships searching for more powerful compounds than those previously investigated. Therefore, they may become in the future promising candidates for the development of novel and potent anti-inflammatory drugs.

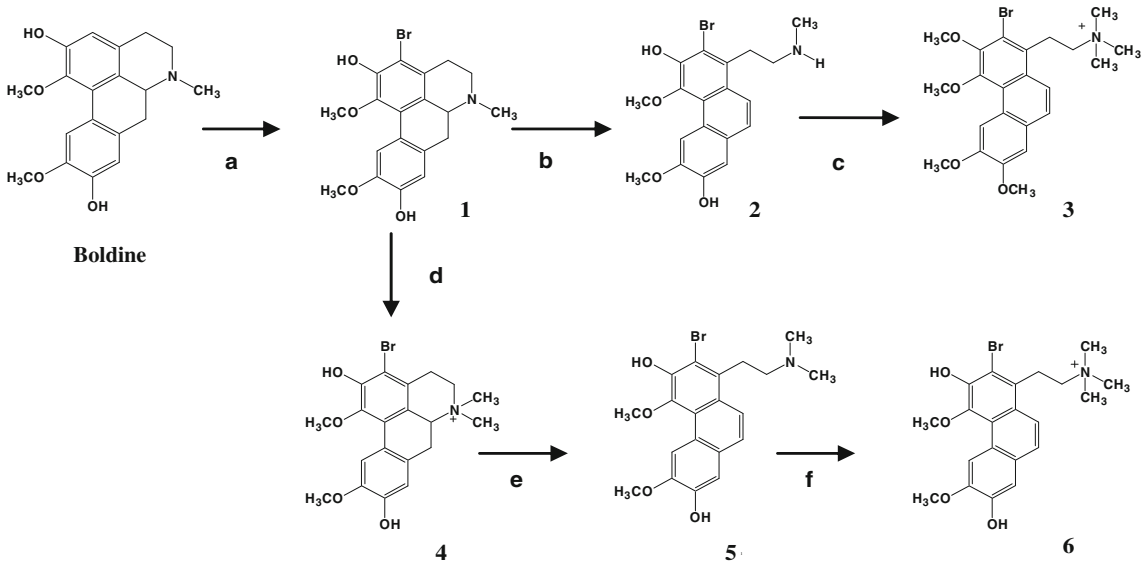
Results and discussion

The synthesis of halogenated aporphinods (compounds **1**–**6**) using boldine as starting material is shown in Scheme 1. Several strategies have been employed for the synthesis of halogenated compounds. Among the different methods available for the formation of halogenated aporphine, the treatment with the appropriate *N*-halosuccinimide in trifluoroacetic acid has been proved to be useful, notably in the synthesis of *S*(+)-3-bromoboldine, *S*(+)-3,8-dibromoboldine, and *S*(+)-3-chloroboldine (Martínez *et al*., 1999).

The reaction of boldine in TFA with *N*-bromosuccinimide was carried out at room temperature. Under these conditions, two reaction products were obtained, 3-bromoboldine and 3,8-dibromoboldine, which were characterized through spectroscopic data. The crude was extracted with dichloromethane and the organic phase subjected to silica gel column using EtOAc as mobile phase to afford 3-bromoboldine (**1**) as a single product. First attempts to obtain 2-bromosecoboldine led to the synthesis of the protonated form. It was revealed when synthesis of product **6** from **2** failed but it was possible to obtain 2-bromo-3,7-dimethoxy-*N*,*N*-dimethylsecobodine iodide (**3**) when **2** was treated at room temperature with an excess of both methyl iodide and K_2CO_3. Mass spectrometry of **2** was done revealing the presence of an extra proton. To obtain 2-bromosecoboldine in its basic form, basification of the crude of reaction was needed. The treatment of **1** with aqueous NH_4OAc and EtOH under reflux during 48 h, led to the opening of the piperidinium ring to give after crystallization compound **2** in 51% yield. Compound **4** was simply prepared by methylation overnight at room temperature of compound **1** with an excess of methyl iodide (100% yield) in a mixture of CH_3CN–MeOH (7.5:5).

Compound **5** was obtained in a more efficient synthesis by treating compound **4** with aqueous ammonium as described above for compound **2**. The crude was concentrated and extracted with dichloromethane, then 2-bromo-*N*-methylsecoboldine was obtained in 79% yield. Finally, the treatment of compound **5** with methyl iodide at room temperature gave compound **6** (29% yield).

The effect of the halogenated aporphine and phenanthrene alkaloids on free radical generation was investigated by two different methods: measurement of ROS generation in stimulated human PMNs (Schudt *et al*., 1991), and measurement of ROS scavenging activity in a enzymatic system (Sekiguchi and Nagamine, 1994). In the first assay, all the alkaloids tested except compound **3** inhibited in a concentration-dependent manner *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP)-induced ROS generation in human PMNs although with different potency. The order of potency, based on the calculated inhibitory concentration 50% (IC_{50}) values (Table 1) showed that the phenanthrene alkaloid with phenolic groups at C-3, C-7 (compound **2**, **5**,



Scheme 1 Synthesis of compounds **1**–**6**. Reagents and conditions: (*a*) NBS; (*b*) NH_4AcO, EtOH; (*c*) MeOH, CH_3CN, K_2CO_3, CH_3I; (*d*) MeOH, CH_3CN, CH_3I; (*e*) NH_4AcO, EtOH; (*f*) MeOH, CH_3CN, CH_3I

and **6**) were more potent than their corresponding equivalents with aporphine skeleton. Phenanthrene alkaloids without phenolic group displayed lower activity than even those with aporphine skeleton (**3**, Fig. 1). All the alkaloids assayed were more potent than ascorbic acid (IC_{50} 99.6 μM), the reference drug previously employed (Milián *et al*., 2004). In general, it seems that halogenation of the alkaloid does not affect its antioxidant activity in this system since most of the alkaloids assayed presented similar IC_{50} values than their corresponding non-halogenated alkaloids (Milián *et al*., 2004).

Since most of these compounds may exert this action through their free radical scavenging activity, they were also assayed on a cell-free ROS generation system. All the alkaloids tested were capable of scavenging ROS generated by the hypoxanthine–xanthine oxidase system in a concentration-dependent manner (Fig. 2).

The order of potency based on the calculated IC_{50} values (Table 1) revealed that all the synthesised compounds were more active than the classic antioxidant ascorbic acid (IC_{50} 167.8 μM). In this assay, a similar profile of structure–activity relationship to that encountered in ROS generated in f-MLP-stimulated neutrophils was observed. Indeed, phenanthrene alkaloids with phenolic substituents were the most active, followed by the group of the aporphine alkaloids with phenolic groups. Only, compound **6**, 2-bromo-

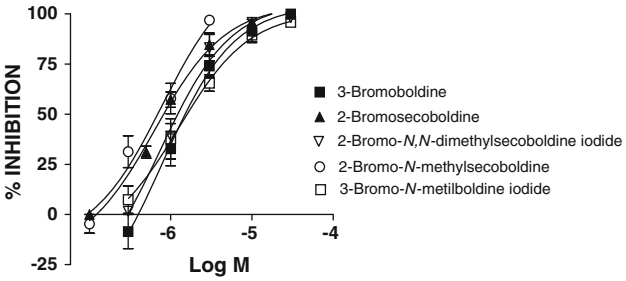


Fig. 1 Effect of alkaloids on ROS generation in f-MLP-stimulated PMNs

Table 1 IC_{50} of the alkaloids tested for ROS generation by f-MLP-stimulated human neutrophils or hypoxanthine–xanthine oxidase system

Alkaloid	f-MLP-stimulated human neutrophils IC50 (μM)	Hypoxanthine–xanthine oxidase system IC50 (μM)
3-Bromoboldine (**1**)	0.81 ± 0.03**	0.55 ± 0.10**
2-Bromosecoboldine (**2**)	0.64 ± 0.03**	0.16 ± 0.10**
2-Bromo-3,7-dimethoxy-*N*,*N*-dimethylsecoboldine iodide (**3**)	A 100 μM 62% inhibition	3.01 ± 0.05**
3-Bromo-*N*-methylboldine iodide (**4**)	1.27 ± 0.02**	2.00 ± 0.06**
2-Bromo-*N*-methylsecoboldine (**5**)	0.78 ± 0.12**	0.19 ± 0.03**
2-Bromo-*N*,*N*-dimethylsecoboldine iodide (**6**)	0.74 ± 0.07**	2.11 ± 0.08**

Data are means ± SEM; statistical significance ** $P < 0.01$

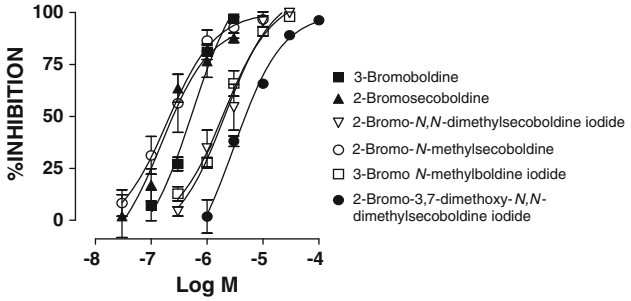


Fig. 2 Effect of alkaloids on ROS generation by hypoxanthine–xanthine oxidase system

N,*N*-dimethylsecoboldine iodide, was slightly less potent than its corresponding alkaloid with aporphine skeleton (compound **4**). Similarly, the phenanthrene alkaloid without phenolic groups showed less potency than any of the alkaloids tested in this system regardless its phenanthrene or aporphine skeleton. When they were compared with their corresponding non-halogenated alkaloids (Milián *et al*., 2004), the halogenated alkaloids were either more potent than the non-halogenated group or exerted similar potency.

In order to confirm that the ROS scavenging activity elicited by aporphine and phenanthrene alkaloids was not due to xanthine oxidase inhibition, another set of experiments was carried out to investigate such possibility. None of the alkaloids assayed affected xanthine oxidase activity (Table 2). Therefore, we have synthesized a potent new class of halogenated phenanthrene alkaloids with ROS scavenger activity.

The analysis of the structure–activity relationship showed that the presence of free phenolic groups is essential for a potent antioxidant activity. In general, the alkaloids with phenanthrene skeleton resulted in more active compounds than their respective alkaloids with aporphine skeleton. The higher activity reported for phenolic-phenanthrenic relative to phenolic-aporphinic molecules may reside on the existence of a third benzylic ring which may confer a greater capacity to delocalize the phenoxy-free radical to the former.

Table 2 Effect of alkaloids on isoxantopterine formation from pterine by measuring enzyme activity (μmol/min)

Control	3.65 ± 0.25	Control	3.25 ± 0.15
1	4.09 ± 0.42	**2**	2.82 ± 0.57
4	3.45 ± 0.07	**3**	3.77 ± 0.52
5	3.98 ± 0.11	**6**	3.60 ± 0.33

Data are means ± SEM; no statistical significance $P < 0.05$ from control was observed

Experimental section

General experimental procedure

Melting points were determined on a Fisher-John apparatus and are uncorrected. Optical rotation measurements were determined on a Perkin-Elmer 241 polarimeter at room temperature in MeOH solution. UV spectra were taken on a Shimadzu 2101 UV/VIS spectrophotometer in MeOH solution. MS were performed using a VG Auto Spec Fisons Spectrometer. NMR measurements (data reported in δ) were run on a Varian Unity-400 instrument. The chemical shift are referenced to solvent (Cl_3CH, Cl_3CD) signals at 7.25 and 77.0 ppm, respectively. Multiplicities of ^{13}C NMR resonances were determined by distortion less enhancement by polarization transfer. Standard pulse sequences were employed for magnitude correlated spectroscopy. HMQC (heteronuclear multiple quantum coherence) and HMBC (heteronuclear multiple bond connectivity) experiments to establish correlations were carried out. Thin-layer chromatography was run on Merck precoated silica gel F_{254} type 60 plates with detection by UV light and Munnier' reagent.

Commercial chemicals

All chemical are of the highest commercially available purity. Boldine, bovine serum albumin (BSA), dimethylsulfoxide (DMSO), f-MLP, gelatine, glucose, hypoxanthine, luminol, microperoxidase, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were purchased from Aldrich-Chemie (Steinheim, Germany).

Chemistry

*3-Bromoboldine (**1**)*

A solution of boldine (2 g, 6.1 mmol) in TFA (15 ml) was treated with NBS (1.086 g, 6.1 mmol) at room temperature. After 3 h stirring the mixture was poured into cold water (50 ml) and the aqueous solution was adjusted to pH 8–9 with aqueous NH_4OH 25%. The filtrate was extracted with CH_2Cl_2. The combined organic phases were dried and evaporated under vacuum, and the resulting residue was purified by silica gel column chromatography (EtOAc) to give **1** as brown solid, yield 35.9%; mp 181–183°C; $[\alpha_D] = +22.02°$; UV (MeOH): λ_{max} (log ε) = 285 (2.10), 311 (2.02) nm; ^1H NMR (DMSO-d_6) δ: 2.27 (dd, 1H, $J = J'' = 13.3$ Hz, H-7b), 2.30 (m, 1H, H-5b), 2.40 (s, 3H, N–CH_3), 2.68 (m, 2H, H-4a,b), 2.80 (dd, 1H, $J = 13.4$, 3.9 Hz, H-6), 2.95 (dd, 1H, $J = 13.4$, 3.9 Hz, H-7a), 2.97 (dd, 1H, $J = 3.9$, 13.9 Hz, H-5a), 3.49 (s, 3H, OCH_3-1),

3.78 (s, 3H, OCH_3-10), 6.74 (s, 1H, H-8), 7.80 (s, 1H, H-11). ^{13}C NMR (DMSO-d_6) δ: 30.1 (C-4), 33.3 (C-7), 43.5 (NCH_3), 52.6 (C-5), 55.7 (OCH_3, C-10), 59.8 (OCH_3, C-1), 62.3 (C-6a), 110.2 (C-3), 111.5 (C-11), 115.2 (C-8), 121.8 (C-11a), 125.4 (C-1b), 127.3 (C-1a), 129.5 (C-7a), 143.1 (C-1), 146.2 (C-9), 146.3 (C-10), 146.6 (C-2). [HMQC and HMBC data were used to assign proton and carbon correlations] HR-EI-MS: $m/z = 405.0552$ calc. mass 405.0576 $C_{19}H_{20}BrNO_4$: EI-MS: m/z (rel. int.) = 407 (63), 406 (46), 405 (85), 404 (36), 390 (16), 326 (5), 307 (100), 289 (50).

*2-Bromosecoboldine (**2**)*

A mixture of 3-bromoboldine (200 mg, 0.492 mmol) and 2 M aqueous ammonium acetate and ethanol (1:1) was refluxed in a bath at 103°C during 48 h. After it was cooled at room temperature, the mixture was basified with NH_4OH 25% until pH 9 and extracted with EtOAc to give **2** after crystallization with MeOH/DCM, yield 51%; mp 174–175°C. UV (MeOH): λ_{max} (log ε) = 268 (2.17), 284 (1.21), 320 (0.31) nm. ^{1}H NMR (DMSO-d_6) δ: 2.43 (s, 3H, N–CH_3), 2.76 (m, 2H, $CH_{2\alpha}$), 3.44 (m, 2H, $CH_{2\beta}$), 3.75 (s, 3H, OCH_3-4), 3.94 (s, 3H, OCH_3-6), 7.22 (s, 1H, H-8), 7.47 (d, 1H, $J = 9.2$ Hz, H-9), 7.81 (d, 1H, $J = 9.2$ Hz, H-10), 8.94 (s, 1H, H-5). ^{13}C NMR (DMSO-d_6) δ: 31.82 ($CH_{2\beta}$), 34.74 (NCH_3), 49.89 ($CH_{2\alpha}$), 55.27 (OCH_3, C-6), 59.64 (OCH_3, C-4), 108.19 (C-5), 111.57 (C-8), 120.88 (C-10), 124.45 (C-9). HR-EI-MS: $m/z = 405.0544$ calc. mass 405.0576 $C_{19}H_{20}BrNO_4$: EI-MS: m/z (rel. int.) = 407 (8), 405 (10), 390 (4), 364 (100), 362 (97), 326 (43), 296 (16).

*2-Bromo-3,7-dimethoxy-N,N-dimethylsecoboldine iodide (**3**)*

To a stirred solution of 2-bromosecoboldine (98.58 mg, 0.212 mmol) in acetonitrile (7.5 ml) and methanol (5 ml) was added K_2CO_3 (200 mg, 1.449 mmol) and an excess of CH_3I (0.1 ml, 0.610 mmol) at room temperature during 48 h. The methanol/acetonitrile was evaporated under vacuum and the residue extracted with acetone to give **3** as white solid, yield 34.8%; UV (MeOH): λ_{max} (log ε) = 269 (1.35), 320 (0.13) nm. ^{1}H NMR (DMSO-d_6) δ: 3.30 (s, 9H, N–$(CH_3)_3$), 3.49 (m, 2H, $CH_{2\alpha}$), 3.75 (m, 2H, $CH_{2\beta}$), 3.96 (s, 12H, (4× OCH_3), 7.55 (s, 1H, H-8), 7.88 (d, 1H, $J = 9.2$ Hz, H-9), 7.91 (d, 1H, $J = 9.2$ Hz, H-10), 9.04 (s, 1H, H-5). ^{13}C NMR (DMSO-d_6) δ: 26.70 ($CH_{2\beta}$), 52.30 (N–$(CH_3)_3$), 63.11 ($CH_{2\alpha}$), 55.29, 55.49, 60.28, 60.84 (OCH_3, C-3,4,6,7), 107.93 (C-5), 108.45 (C-8), 127.81 (C-9,10). FAB-MS $m/z = 462.1274$ calc. mass 462.1280 $C_{23}H_{29}BrNO_4$: FAB-MS (M + 1): m/z (rel. int.) = 463 (29), 462 (100), 461 (31), 383(3), 368 (4), 310 (7), 295 (5), 267 (3).

*3-Bromo-N-methylboldine iodide (**4**)*

To a solution of **1** (200 mg, 0.492 mmol) in a mixture of methanol (5 ml) and acetonitrile (7.5 ml) was added CH_3I (0.031 ml, 0.492 mmol). The resulting mixture was stirred overnight at room temperature. Compound **4** after crystallization with MeOH/DCM was obtained as yellow needles, yield 100%; mp 185–187°C; $[\alpha_D] = +1.45°$; UV (MeOH): λ_{max} (log ε) = 288 (0.65), 307 (0.7) nm. ^{1}H NMR (DMSO-d_6) δ: 2.95 (s, 3H, N–CH_3), 2.99 (m, 2H, H-4a,b), 3.37 (s, 3H, N–CH_3), 3.53 (s, 3H, OCH_3-1), 3.66 (dd, 1H, $J = 13.4$, 3.5 Hz, H-5b), 3.77 (s, 3H, OCH_3-10), 3.86 (m, 1H, H-5a), 4.61 (m, 1H, H-6), 6.84 (s, 1H, H-8), 7.79 (s, 1H, H-11). ^{13}C NMR (DMSO-d_6) δ: 25.26 (C-4), 27.82 (C-7), 43.08 (NCH_3), 53.05 (NCH_3), 56.79 (OCH_3, C-10), 59.87 (C-5), 60.20 (OCH_3, C-1), 67.74 (C-6a), 111.40 (C-11), 115.30 (C-8). FAB-MS: $m/z = 420.0798$ calc. mass 420.0810 $C_{20}H_{23}BrNO_4$: FAB-MS (M + 1): m/z (rel. int.) = 421 (97), 420 (33), 419 (100), 392 (10), 329 (33), 175 (98).

*2-Bromo-N-methylsecoboldine (**5**)*

A solution of **4** (270 mg, 0.493 mmol) in 2 M aqueous ammonium acetate and ethanol (1:1) was refluxed in a bath a 104°C for 48 h. The ethanol was evaporated under vacuum and the aqueous layer was basified with NH_4OH 25% until pH 8–9 and extracted with DCM. The combined organic extract were dried over anhydrous Na_2SO_4 and compound **5** after crystallization with MeOH/CH_2Cl_2/Hexane mixtures was obtained a brown stars crystal, yield 79.1%; mp 125–128°C; UV (MeOH): λ_{max} (log ε) = 268 (0.59), 284 (0.33), 320 (0.7) nm. ^{1}H NMR (DMSO-d_6) δ: 2.29 (s, 6H, N–$(CH_3)_2$), 2.43 (m, 2H, $CH_{2\alpha}$), 3.37 (m, 2H, $CH_{2\beta}$), 3.74 (s, 3H, OCH_3-4), 3.95 (s, 3H, OCH_3-6), 7.22 (s, 1H, H-8), 7.52 (d, 1H, $J = 9.3$ Hz, H-9), 7.71 (d, 1H, $J = 9.3$ Hz, H-10), 8.93 (s, 1H, H-5). ^{13}C-NMR (DMSO-d_6) δ: 30.41 ($CH_{2\beta}$), 44.95 (N–$(CH_3)_2$), 58.31 ($CH_{2\alpha}$), 55.29 (OCH_3, C-6), 59.77 (OCH_3, C-4), 108.12 (C-5), 111.57 (C-8), 113.92 (C-2), 120.68 (C-10), 121.89 (C-5a), 122.90 (C-10a), 124.16 (C-4a), 124.70 (C-9), 128.08 (C-8a), 132.39 (C-1), 142.69 (C-4), 145.46 (C-3), 146.95 (C-7), 148.10 (C-6). [HMQC and HMBC data were used to assign proton and carbon correlations]. HR-EI-MS: $m/z = 419.3450$ calc. mass 419.3510 $C_{20}H_{22}Br\ NO_4$: EI-MS: m/z (rel. int.) = 421 (85), 420 (35), 419 (100), 418 (17), 339 (27), 281 (5), 253 (6), 238 (60).

*2-Bromo-N,N-dimethylsecoboldine iodide (**6**)*

A solution of methyl iodide was slowly added to a magnetically stirred solution of 2-bromo-*N*-methylsecoboldine (**5**) (155 mg, 0.369 mmol) at room temperature. The

following treatment with MeOH/Hexane gave analytically pure **6**, yield 29%; UV (MeOH): λ_{max} (log ε) = 268 (0.54), 284 (0.37), 320 (0.06) nm. ^{1}H NMR (DMSO-d_6) δ: 3.28 (s, 9H, N–$(CH_3)_3$), 3.45 (m, 2H, $CH_{2\alpha}$), 3.72 (m, 2H, $CH_{2\beta}$), 3.76 (s, 3H, OCH_3-4), 3.95 (s, 3H, OCH_3-6), 7.27 (s, 1H, H-8), 7.58 (d, 1H, J = 9.2 Hz, H-9), 7.77 (d, 1H, J = 9.2 Hz, H-10), 8.93 (s, 1H, H-5). ^{13}C NMR (DMSO-d_6) δ: 26.54 ($CH_{2\beta}$), 52.25 (N–$(CH_3)_3$), 55.31 (OCH_3, C-6), 59.82 (OCH_3, C-4), 63.25 ($CH_{2\alpha}$), 108.04 (C-5), 111.65 (C-8), 120.42 (C-10), 125.29 (C-9). FAB$^+$: *m/z* (rel. int.) = 436 (100), 435 (39), 434 (98).

Pharmacology

Measurement of ROS generation from human PMNs

The formation of ROS by human PMNs was assessed by luminol-enhanced chemiluminescence with a modified method (Martinez *et al*., 1999). Assay was carried out in opaque 96-well plates, 10^5 cells per well suspended in an assay volume of 180 µl of Krebs-HEPES buffer containing glucose 5.6 mM, BSA 0.05% (w/v), microperoxidase 2 µM with gelatine 0.1% pH 7.4, alone or in combination with alkaloids (final concentration in 200 µl, 100–0.01 µM) for 30 min at 37°C. All the assays were performed in duplicate. Plates were placed in a Wallac 1420 Victor^2 Multilabel Counter. Then 20 µl of luminol 5 µM, $CaCl_2$ 1 mM and f-MLP 100 nM was added sequentially to each well, except in the blank group. Experiments with the appropriate DMSO concentration were also carried out (1–0.01%). Chemiluminescence was recorded at 4 s intervals over a 100 s period per well and the area under the curve (AUCs) was integrated. Drug-induced reduction was expressed as % inhibition. IC_{50} values were then calculated from the concentration-inhibition curves by non-linear regression analysis.

Measurement of ROS generation by hypoxanthine–xanthine oxidase

The ROS was generated by hypoxanthine–xanthine oxidase system and detected by luminol-enhance chemiluminescence using a modified method (Sekiguchi and Nagamine, 1994). Assays was carried out in opaque 96-well plates. One hundred and eighty microliters of Krebs-HEPES buffer containing hypoxanthine 0.1 mM, glucose 5.6 mM, gelatine 0.1% pH 7.4, alone or in combination with synthesized alkaloids (final concentration in 200 µl, 100–0.01 µM) were added to the wells for 5 min at 37°C. All the assays were performed in duplicate. Plates were placed in a Wallac 1420 Victor^2 Multilabel Counter. Then 20 µl of luminol 5 µM, $CaCl_2$ 1 mM, and xanthine oxidase 0.02 μ/ml was added sequentially to each well, except in the blank group. Experiments with the appropriate DMSO concentration were also carried out (1–0.01%). Chemiluminescence was recorded at 4 s intervals over 100 s period per well and the AUCs was integrated. Drug-induced reduction was expressed as % inhibition. IC_{50} values were then calculated from the concentration-inhibition curves by non-linear regression analysis.

Measurement of the effect on xanthine oxidase activity by isoxantopterine formation from pterine

A direct inhibitory effect on xanthine oxidase activity was tested by measuring isoxantopterine formation from pterine by fluorimetry (excitation wavelength at 345 nm and emission wavelength at 390 nm) following the previously described method (Beckman *et al*., 1989).

Statistical analysis

The IC_{50} values were calculated from non-linear regression by a software of Prisma 3.0 (Graph Pad Software, San Diego, California, USA). All values are shown as mean ± SEM. The difference between two values was determined by use of unpaired Student's t test. The differences were considered statistically significant if the P value was less than 0.05.

Conclusion

The halogenation of some aporphine and phenantrene alkaloids previously synthesized (Milián *et al*., 2004) weakly increases its antioxidant activity in cell free systems. Therefore, halogenated phenanthrene alkaloids may be suitable for being tested in vivo as anti-inflammatory agents since they have shown potent ROS scavenging activity. Thus, they can become promising candidates for the development of novel and potent anti-inflammatory drugs.

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