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Scabrosin esters and derivatives: chemical derivatization studies and biological evaluation

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Abstract—Several derivatives of the natural scabrosin esters were synthesized in order to elucidate the structural features present, which are responsible for the biological activities. The studies demonstrate that full anti-proliferative activities of the scabrosin esters, *both* the carboskeleton core as well as the ability to form the dithiol and/or the disulfide linkage of the epidithiopiperazine-2,5-dione are required. The presence of the epoxide rings on the scabrosin esters do not contribute to the observed biological activities.

1. Introduction

In 1999, we unambiguously established the structure of the scabrosin esters (**1**) and confirmed that they belong to the epidithiopiperazine-2,5-dione (ETP) class of compounds.[1] A large number of ETP natural products show potent biological activities,[2] which has been attributed to the disulfide linkage that spans the α-carbon centres of the piperazine-2,5-dione ring.[3] It should however be noted such activities are not limited to ETP natural products as a number of other piperazine-2,5-dione natural products are also biologically active.[4]

In previous studies we demonstrated that scabrosin diacetate (**1a**) and scabrosin acetate butanoate (**1c**) have comparable IC_{50} values (IC_{50} 0.56 and 0.5 μM) against P815 mastocytoma cancer cell line, which have activities much lower than the well known ETP natural product, gliotoxin (**2**) (IC_{50} 2.9 μM).[1] Although the biological mechanisms for the scabrosin esters have not been established, studies have shown that the mitochondrial ATP synthase of P388D1 cells is an early target.[5] Interestingly, the prolyl ETP compound (**3**), a simple model of the scabrosin esters, which lacked the outer ring systems, was shown to be inactive within the concentration range studied.[6] This prompted a biological evaluation of several derivatives of scabrosin esters, in order to elucidate, which structural features were responsible for the biological activities. The present work describes the chemistry and biology of the scabrosin esters and derivatives, and unequivocally demonstrates that the epoxide rings present in the natural product do not contribute to the biological activities.

2. Results and discussion

The scabrosin esters were isolated from *Xanthoparmelia scabrosa*, collected from the summit of a coastal cliff face south east of Bateman's Bay, New South Wales, Australia, using the procedure outlined previously.[1] Due to the paucity of the scabrosin esters, the derivatization studies described here utilizes both the dibutanoate ester (**1b**) and the acetate butanoate ester (**1c**). Our targets for a structure–activity relationship study included derivatives where the sulfur functionality is modified and derivatives where the reactive functionalities in the outer ring system are transformed or removed.

2.1. Derivatization of scabrosin esters

It is recognized that epoxides often act as alkylating agents in biological systems.[7] As such our initial objective was to ring open the epoxide rings of the scabrosin esters in order to access derivatives suitable for

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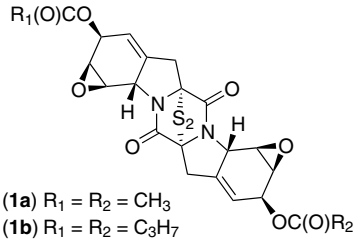
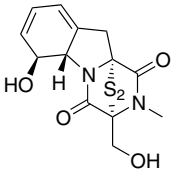
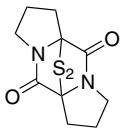


(**1a**) $R_1 = R_2 = CH_3$
(**1b**) $R_1 = R_2 = C_3H_7$
(**1c**) $R_1 = CH_3$, $R_2 = C_3H_7$
(**1d**) $R_1 = CH_3$, $R_2 = C_5H_{11}$
(**1f**) $R_1 = C_3H_7$, $R_2 = C_5H_{11}$



(**2**)



(**3**)

biological study. In this manner, the importance of the contribution of the epoxide ring to the anti-proliferative properties of the scabrosin esters could be ascertained. In our studies treatment of the scabrosin esters **1b** and **1c** with various acid catalysts (e.g., silica gel, Amberlyst-H^+ catalyst, trifluoroacetic acid) in the presence of water or methanol failed to yield any ring opened products. The stability of the epoxide rings in the scabrosin esters towards ring opening reactions was surprising and cannot be easily rationalized.

In further attempts to derivatize the scabrosin esters, we attempted to hydrogenate the alkene moiety of scabrosin acetate butanoate (**1c**) using hydrogen in the presence of a catalytic amount of Pd/C. This, however, failed to yield any of the desired saturated compound presumably due to the poisoning of catalyst in the presence of the sulfur moiety. When a modified Wilkinson's catalyst [$RhBr(PPh_3)_3$] was used, only unreacted starting material was recovered. Longer reaction times as well as an increase in the catalyst to substrate ratio failed to facilitate the hydrogenation of the ester **1c**. In latter cases, when large excesses of the 'catalyst' were used, decomposition of the ester **1c** was observed.

We then sought to modify the disulfide functionality present in the scabrosin esters in order to assess the importance of the disulfide linkage. The scabrosin dibutanoate (**1b**) was readily converted to the monosulfide **4** using 1 equiv of triphenylphosphine.[8] In the ^1H NMR spectrum of the product, the AB doublets due to the methylene protons of the fused five-membered ring are shifted downfield to 2.65 and 3.40 ppm, respectively. Similarly, the methine protons on this ring system are also shifted downfield to 4.22 ppm. It is also clear from the ^1H NMR spectrum of the monosulfide **4** that the epoxide rings remained intact. This further demonstrates the stability of the epoxides in the presence of nucleophiles. In addition, the electron impact mass spectrum of the product showed a molecular ion peak at *m*/*z* 528, diagnostic of the monosulfide **4**.

As observed above, in spite of the presence of reactive functional groups in the scabrosin esters, the disulfide functionality of scabrosin ester **1b-c** can be manipulated chemoselectively. Scabrosin ester **1b** was quantitatively reduced to the dithiol **5a** by treatment with sodium borohydride in methanol/dichloromethane. As the dithiol is susceptible to air oxidation, conversion of the dithiol to the dithioether was achieved using a 'one-pot' protocol in which excess methyl iodide was added to the reaction mixture after 30 min. The desired dithioether **5b** was isolated in 63% yield.

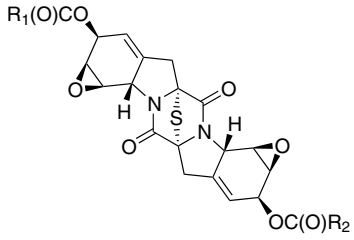
Using a similar method the scabrosin acetate butanoate **1c** was reduced with sodium borohydride and subsequent in situ *S*-acylation with acetic anhydride afforded the dithioacetate **5c** in 37% yield. The singlet observed at 2.29 ppm in the ^1H NMR spectrum of the product is diagnostic of the methyl protons of the dithioacetate **5c**, with the corresponding carbonyl resonance observed at 191.0 ppm in the ^{13}C NMR spectrum.

Our attempts to desulfurize the scabrosin esters **1b** and **1c** using W2 Ra Ni in refluxing ethanol or by using Al amalgam failed to yield any isolable products. The reagents and conditions for desulfurization were optimized for a model ETP compound (*N*,*N*-diethyl ETP) but were not applicable to the scabrosin esters. In spite of clean conversions of the *N*,*N*-diethyl ETP to the *N*,*N*-diethylpiperazine-2,5-dione under the conditions utilized, all attempts to desulfurize the scabrosin esters gave complex mixtures of inseparable products.

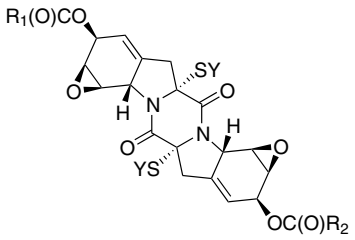
2.2. Biological studies

To assess and compare the biological responses of the prolyl ETP (**3**), scabrosin esters **1b** and **1c** and derivatives **4**–**6** on cell proliferation, the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay[9] was used. The anti-proliferative assays conducted in this work involved incubation of the murine P388 macrophage cancer cell line with the test compounds for a period of 3 days. The EC_{50} values for each compound in the MTT assay, as summarised in Table 1, were determined from a semi-logarithmic plot of concentration against absorbance at 590 nm ($A_{590\,nm}$). A nonlinear regression analysis was used to fit the sigmoidal concentration-response curve through data points obtained from quadruplicate sets of data.

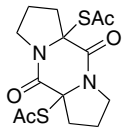
From these studies, it is apparent that the monosulfide **4** and the dithioether derivative **5b** of scabrosin ester show greatly reduced biological activity indicating that the epoxide rings contribute little or not at all to the biological activity of the scabrosin esters. Interestingly the dithioacetate **5c** shows activity comparable to that of the natural ETP compound. In these assays, the prolyl



(**4**) $R_1 = R_2 = C_3H_7$



(**5a**) $R_1 = R_2 = C_3H_7$, Y=H
(**5b**) $R_1 = R_2 = C_3H_7$, Y=Me
(**5c**) $R_1 = CH_3$, $R_2 = C_3H_7$, Y=Ac



(**6**)

Table 1. EC_{50} values of scabrosin esters and derivatives tested against murine P388 cell line

Compound	EC_{50} value (μM)
Scabrosin dibutanoate (**1b**)	0.03
Scabrosin acetate butanoate (**1c**)	0.015
Scabrosin monosulfide derivative (**4**)	0.15
Scabrosin dithioether derivative (**5b**)	>10
Scabrosin dithioacetate derivative (**5c**)	0.02
L-Prolyl-L-proline ETP (**3**)	1.0
L-Prolyl-L-proline dithioacetate (**6**)	3.4

ETP **3** and the corresponding dithioacetate **6** displays a large decrease in anti-proliferative activity as compared to the scabrosin esters.

The comparable biological activities of the dithioacetates and the parent ETP compounds are most likely to be due to hydrolysis of the thioacetyl groups upon uptake into the cells to yield the dithiol, which could in turn be oxidized to the ETP compound.[10] The observed lower activities for the epimonothiopiperazinedione **4** as compared to the epidithiopiperazinedione **1b** have been reported in other epipolythiopiperazinedione systems, for example, in sirodesmin[11] and acetylaranotin.[12] The marked difference in activity between the cyclic monosulfide **4** and the dithioether **5b** suggests that the activity of the former may be due to the lability of the strained cyclic monosulfide. Thus although the anti-proliferative activity of scabrosin esters is *mainly* attributed to the ability to form the dithiol and/or the disulfide linkage of the ETP compound, the S–S linkage (or the ability to form the S–S linkage) is not the sole requirement for biological activity. The large difference in the anti-proliferative properties of scabrosin esters and the prolyl ETP compounds further attest to this. As the dithioether derivative **5b** and the monosulfide **4**, which have the other reactive functionalities intact in the outer rings, have greatly reduced activity, this implies that the structural requirement for full activity requires *both* the carboskeleton core of the scabrosin esters *and* the S–S linkage (or a 'pro-drug' to the disulfide e.g., the dithioacetate). Interestingly the X-ray crystallographic data of the dibutanoate ester **1b**,[1] the CSSC dihedral bond angle of 14.4° is slightly larger than that for the prolyl ETP compound[13] at 8.5° and that for gliotoxin, at 12.3°.[14] These ETP compounds show significant strain across the disulfide bridgehead of the ETP carboskeleton although it is unclear as to how this correlates with biological activities. One can, however, envisage that the ease of reduction of the S–S bond to the dithiol and the converse oxidation of the dithiol to the disulfide is reflected in the equilibrium constant of the redox process. Previous studies with gliotoxin and prolyl ETP suggest that the difference in biological activities of the two ETP compounds may be due to this.[6] As the equilibrium constant of the scabrosin esters is not known, further conclusions on the physicochemical requirements for biological activity cannot be made at this time.

3. Experimental

Proton (^1H) and carbon (^{13}C) NMR spectra were recorded on a Varian Gemini II NMR spectrometer, operating at 300 MHz for proton and 75.4 MHz for carbon spectroscopy. Deuterochloroform ($CDCl_3$) was used as the solvent unless otherwise indicated. Low and high resolution mass spectra were recorded on a VG Micromas 7070F double focussing mass spectrometer using positive ion electron impact techniques. Melting points are uncorrected and were recorded on a Leica Galen III microscope.

The foliose lichen *Xanthoparmelia scabrosa* (Taylor) Hale, was collected from the head of a coastal cliff face at Lilli Pilli, south east of Bateman's Bay, New South Wales, Australia and the scabrosin esters were isolated as reported previously.[1] The spectroscopic data of the scabrosin acetate butanoate **1c** and scabrosin dibutanoate **1b** isolated were identical to that reported.[1]

3,6-Epidithio-L-prolyl-L-proline anhydride was synthesized following modifications of the literature procedures reported by Schmidt and co-workers.[15]

3.1. Derivatization studies

3.1.1. 3,6-Dithioacetyl-L-prolyl-L-proline anhydride (6). Sodium borohydride (21 mg, 0.554 mmol) was added to a stirred solution of 3,6-epidithio-L-prolyl-L-proline anhydride (71 mg, 0.277 mmol) in dichloromethane (10 mL) and methanol (2 mL) at 0 °C. The reaction mixture was stirred under nitrogen for 30 min before the addition of acetic anhydride (2 mL, excess). The resultant solution was then stirred for 16 h after which time

the reaction mixture was washed with 10% hydrochloric acid (5 mL). The aqueous phase was extracted with dichloromethane (3 × 5 mL) and the organic phases were combined, dried ($MgSO_4$) and the solvent removed in vacuo. The title compound was obtained as a colourless solid (63 mg, 66%) with physical and spectroscopic properties consistent with those previously reported. The compound was purified via column chromatography (ethyl acetate, R_f: 0.45). Mp 158–160 °C (lit.: 156–161 °C).[15] ^{1}H NMR: δ 2.00–2.40 (m, 6H, 2 × NCH_2CH_2-CH_aH_b); 2.30 (s, 6H, 2 × $SC(O)CH_3$); 3.09 (dd, 2H, $^2J_{AB}$ = 15 Hz, ^3J = 6 Hz, 2 × $NCH_2CH_2CH_aH_b$); 3.62–3.76 (m, 4H, 2 × $NCH_2CH_2CH_aH_b$). ^{13}C NMR: δ 20.5 ($NCH_2CH_2CH_2$); 31.3 ($SC(O)CH_3$); 36.0 (NCH_2CH_2-CH_2); 46.4 ($NCH_2CH_2CH_2$); 163.5 (ring *C*(O)); 191.9 ($SC(O)CH_3$).

3.1.2. Scabrosin dibutanoate monosulfide (4). Triphenylphosphine (12 mg, 0.0461 mmol) was added to a solution of scabrosin dibutanoate (23.5 mg, 0.0419 mmol) (**1b**) in dichloromethane (3 mL) at room temperature and the resultant solution was stirred under nitrogen for 16 h. After this time, the solvent was removed in vacuo and the residue was purified via column chromatography (3:2, *n*-hexane/ethyl acetate, R_f: 0.45). The title compound was obtained as a colourless solid (11 mg, 50%). Mp 173–175 °C dec. ^{1}H NMR: δ 0.96 (t, 6H, J = 7.5 Hz, 2 × $C(O)CH_2CH_2CH_3$); 1.68 (sext, 4H, J = 7.5 Hz, 2 × $C(O)CH_2CH_2CH_3$); 2.37 (dt, 4H, ^3J = 7.5 Hz, J = 2.0 Hz, 2 × $C(O)CH_2CH_2CH_3$); 2.65 (d, 2H, J_{AB} = 16.4 Hz, 2 × CH_aH_b); 3.40 (dd, 2H, $^2J_{AB}$ = 16.4 Hz, ^4J = 2.2 Hz, 2 × CH_aH_b); 3.62 (t, 2H, J = 4 Hz, 2 × C*H*); 4.22 (br s, 2H, 2 × C*H*); 4.66 (d, 2H, J = 3.5 Hz, 2 × C*H*); 5.65–5.67 (m, 2H, 2 × C*H*); 5.71–5.74 (m, 2H, 2 × C*H*). EI-MS: *m*/*z* (relative abundance) 528 ($M^{+\cdot}$, 8%); 352 (18%); 318 (66%); 205 (20%); 188 (38%); 160 (68%). Exact mass: $C_{26}H_{28}N_2O_8S$ requires 528.1564. Found 528.1575.

3.1.3. Scabrosin dibutanoate dithiomethyl ether (5b). Sodium borohydride (5 mg, 0.1256 mmol) was added to a solution of scabrosin dibutanoate **1b** (9 mg, 0.0161 mmol) in dichloromethane (3 mL) and methanol (1 mL) at 0 °C. The reaction mixture was stirred under nitrogen for 30 min before the addition of methyl iodide (1 mL, excess). The resultant solution was then stirred for 16 h following, which time the reaction mixture was washed with 10% hydrochloric acid (1 × 2 mL). The aqueous phase was extracted with dichloromethane (3 × 3 mL) and the organic phases were combined, dried ($MgSO_4$) and the solvent removed in vacuo. The title compound was obtained as a pale yellow solid (6 mg, 63%). Mp 180–182 °C. ^{1}H NMR: δ 0.97 (t, 6H, J = 7.4 Hz, 2 × $C(O)CH_2CH_2CH_3$); 1.68 (sext, 4H, J = 7.4 Hz, 2 × $C(O)CH_2CH_2CH_3$); 2.18 (s, 6H, 2 × SCH_3); 2.37 (dt, 4H, ^3J = 7.5 Hz, J = 2.5 Hz, 2 × $C(O)CH_2CH_2CH_3$); 2.97 (br s, 4H, 2 × CH_2); 3.61 (t, 2H, J = 4 Hz, 2 × C*H*); 3.82 (d, 2H, J = 3.4 Hz, 2 × C*H*); 4.46 (br s, 2H, 2 × C*H*); 5.69 (t, 2H, J = 5.4 Hz, 2 × C*H*); 5.89 (m, 2H, 2 × C*H*). EI-MS: *m*/*z* (relative abundance) 590 ($M^{+\cdot}$, 8%); 543 (100%); 408 (20%); 319 (92%); 160 (46%); 132 (34%). Exact mass: $C_{27}H_{31}N_2O_8S$ [$M^{+\cdot}$−SMe] requires 543.1801. Found 543.1821.

3.1.4. Scabrosin acetate butanoate dithioacetate (5c). Sodium borohydride (3 mg, 0.0793 mmol) was added to a solution of scabrosin acetate butanoate (**1c**) (23 mg, 0.0432 mmol) in dichloromethane (5 mL) and methanol (0.5 mL) at 0 °C. The reaction mixture was stirred under nitrogen for 30 min before the addition of acetic anhydride (2 mL, excess). The resultant solution was then stirred for 16 h following, which time the reaction mixture was washed with 10% hydrochloric acid (5 mL). The aqueous phase was extracted with dichloromethane (3 × 3 mL) and the organic phases were combined, dried ($MgSO_4$) and the solvent removed in vacuo. The residue was purified via column chromatography (1:1, ethyl acetate/petroleum spirit, R_f: 0.26) to afford the title compound (**5c**) as a colourless solid (10 mg, 37%). Mp 111–113 °C. ^{1}H NMR: δ 0.96 (t, 3H, J = 7.4 Hz, $C(O)CH_2CH_2CH_3$); 1.6–1.75 (m, 2H, J = 7.4 Hz, $C(O)CH_2CH_2CH_3$); 2.14 (s, 3H, $C(O)CH_3$); 2.29 (s, 6H, 2 × $SC(O)CH_3$); 2.37 (dt, 2H, ^3J = 7.4 Hz, J = 2.2 Hz, $C(O)CH_2CH_2CH_3$); 3.17 (d, 2H, J_{AB} = 14 Hz, 2 × CH_aH_b); 3.46 (d, 2H, J_{AB} = 14 Hz, 2 × CH_aH_b); 3.60 (apparent t, 2H, 2 × C*H*); 3.99 (apparent t, 2H, 2 × C*H*); 4.47 (br s, 2H, 2 × C*H*); 5.65 (br s, 2H, 2 × C*H*); 5.85 (br s, 2H, 2 × C*H*). ^{13}C NMR: δ 13.6 ($C(O)CH_2CH_2CH_3$); 18.4 ($C(O)CH_2CH_2CH_3$); 20.8 ($C(O)CH_3$); 31.3 (2 × $SC(O)CH_3$); 35.8 ($C(O)CH_2CH_2CH_3$); 42.2 (CH_2); 50.9 (*C*H); 56.2 (*C*H); 56.7 (*C*H); 56.8 (*C*H); 65.0 (*C*H); 65.3 (*C*H); 74.3 (2 × *C*HSAc); 120.1 (quaternary *C*); 120.3 (quaternary *C*); 136.7 (quaternary *C*); 136.8 (quaternary *C*); 163.6 (2 × amide *C*(O)); 170.8 ($C(O)CH_3$); 173.4 ($C(O)CH_2CH_2CH_3$); 191.0 (2 × $SC(O)CH_3$). Exact mass: $C_{26}H_{27}N_2O_9S$ [$M^{+\cdot}$−SAc] requires 543.1437. Found 543.1446.

3.2. Biological experiments

P388 cells were cultured in RPMI 1640 media with 10% Horse Serum, at 37 °C with 5% CO_2. Progression of confluence was monitored by observation using an inverted light microscope. Cell density was determined by use of a haemocytometer, which had a volume of 10^{-4} mL and was transposed with a 5 × 5 grid. The number of cells present in 25 squares of the grid was counted under a light microscope (Olympus, BHB, 20× magnification) and the concentration thereby determined.

Effects on cell proliferation and viability were measured using a colorimetric MTT assay. To wells #2–11 of a 96-well flat bottomed microtitre plate was added 50 μL of fresh media, to well #12 was added 100 μL of fresh media. To well #1 was added 100 μL of ca. 10 μM stock solution of the sample compound (exact concentration in media known, made up from an initial stock solution of the compound, typically 1–5 mM in DMSO). This was serially diluted (1:1) down the plate, discarding after well #10. To wells #1–11 was added 50 μL of cell suspension, so as to achieve a final concentration of 4000 cells per well in 100 μL for the 3 day assay. The cells were then incubated at 37 °C for 3 days. After this time, to each well was added

10 μL of MTT (as a solution in PBS, 5 mg mL^{-1}), before incubation at 37 °C for 5 h. After this time, 100 μL of lysing solution (10% w/v sodium dodecylsulfate in 0.01 M aqueous HCl) was added to each well before incubation overnight at 37 °C. After this time, the absorbance of each well of the plate was read at 590 nm by an ELISA automated spectrophotometric plate reader. Results were expressed as percentages of control (untreated cells) and were analyzed using GraphPad Prism 2.0 software. All determinations were performed in quadruplicate and results were typical of multiple independent experiments.

Acknowledgements

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