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RESOLUTION AND BIOLOGICAL ACTIVITIES OF OPTICAL ISOMERS OF 1,4-DIETHYL-3,6-EPIDITHIOPIPERAZINE-2,5-DIONE

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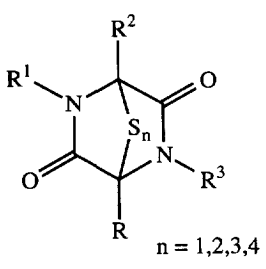
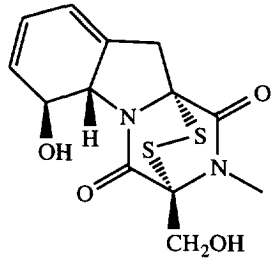
Abstract. Epipolythiopiperazine-2,5-diones (ETPs) are an important class of biologically active fungal metabolites. We have successfully synthesised and resolved racemic 1,4-diethyl-3,6-epidithiopiperazine-2,5-dione using chiral HPLC. The biological activities of the enantiomers were investigated to determine the correlation between biological activity and chirality at the bridgehead carbons. © 1997 Elsevier Science Ltd.

Epipolythiopiperazine-2,5-diones constitute an important class of biologically active compounds, characterised by a bridged polysulfide piperazine ring (**1**).[1-3] In particular, considerable interest is centred on gliotoxin (**2**) because of the diverse range of biological activities it displays, which include antiviral, antiphagocytic, immunosuppressive properties, and its important role in the etiology of diseases such as invasive aspergillosis.[4] The crucial element responsible for the observed biological properties has been shown to be the sulfide linkage.[2,5,6]

Many instances have been reported where compounds that differ in chirality display varying biological properties.[7] The ETP compounds observed in nature are found to be enantiomerically pure. Gliotoxin and sporidesmin have a *R,R* configuration at the sulfide bridgehead carbons and display antiviral properties.[1,8,9] In contrast, chaetocin has a *S,S* configuration and exhibits no antiviral activity.[10] For synthetic ETP compounds, a complete study of the correlation between biological activity and bridgehead chirality has not yet been investigated. In this paper we report our biological studies with the enantiomers of 1,4-diethyl-3,6-epidithiopiperazine-2,5-dione. In addition, this is the first reported resolution of an ETP compound using chiral chromatographic methods.

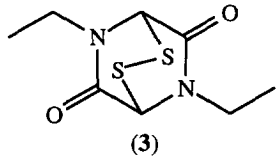


(**1**)



(**2**)

Resolution. The synthesis of 1,4-diethyl-3,6-epidithiopiperazine-2,5-dione (**3**)[11] was achieved in 65% yield via a modification of the Trown procedure[12,13] starting from 1,4-diethylpiperazine-2,5-dione.[14] The analytically pure racemic 1,4-diethyl-3,6-epidithiopiperazine-2,5-dione was chromatographed using HPLC,[15] employing the CHIRALPAK-AS column (amylose carbamate, 250 x 4.6 mm, Daicel Chemical Industries LTD). Using an isocratic solvent system of 50% hexane/isopropyl alcohol, two peaks with retention times of 7.1 and 11.8 min were observed (Figure 1).



(**3**)

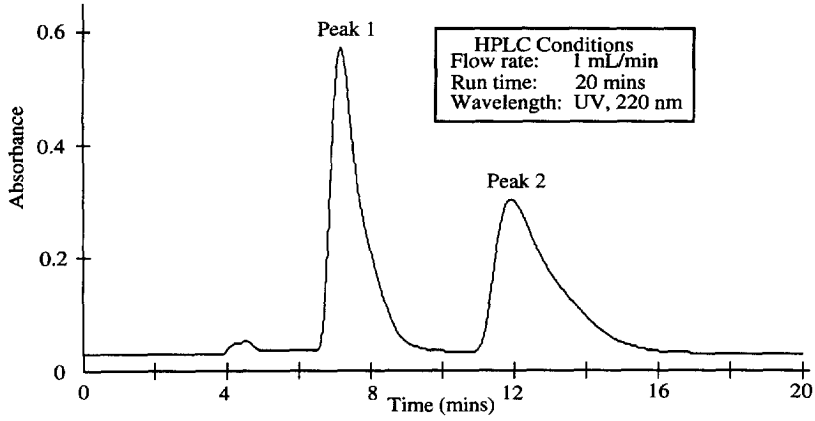


Figure 1: HPLC trace of 1,4-Diethyl-3,6-epidithiopiperazine-2,5-dione.

The two fractions were isolated on a preparative scale and characterised. The ^{1}H NMR spectra of each of these isolated fractions were identical to that of the racemic compound. Circular dichroism (CD) and optical rotation measurements showed that the compounds were enantiomeric (% ee > 95%). Thus, this resolution method is simpler, faster, and more efficient in comparison to the chemical resolution method reported previously.[16,17] Hence, chiral chromatography of this kind can be potentially applied to the resolution of other racemic ETP compounds.

The absolute configuration of each of the enantiomers was assigned using CD studies.[18,19] Gliotoxin with a *R,R* configuration at the disulfide bridgehead stereo centres was used as the reference compound. Here a negative and positive Cotton effect at ca. 231 and 268 nm (literature[18] 234 and 272 nm) are observed respectively. The CD spectrum of the second isolated fraction gave similar Cotton effects to gliotoxin (i.e., with a negative and positive Cotton effect at ca. 220 and 256 nm, respectively). By analogy, the absolute configuration for this enantiomer was assigned as *R,R*-(**3**) (Figure 2). In contrast, the CD spectrum of the first

isolated fraction showed reversed Cotton effects to that above. The absolute configuration of this enantiomer was assigned as *S,S*-(**3**) (Figure 2).

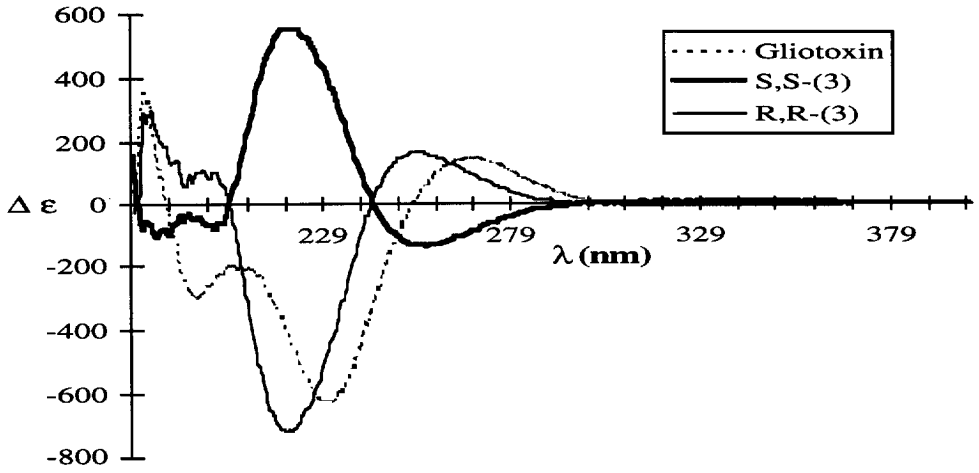


Figure 2: CD Spectra of *R,R* and *S,S*-1,4-Diethyl-3,6-epidithiopiperazine-2,5-dione and Gliotoxin.

Biological Activity. The main interest in ETP compounds centres on the in vitro and in vivo immunosuppressive properties of gliotoxin and their potential clinical applications. Immunosuppressive effects of ETP compounds have been correlated with their antiproliferative capacity and the ability to prevent macrophage adherence.[4] Gliotoxin has also been shown to induce apoptosis in several cell types particularly those of the immune system.[20] In addition, gliotoxin inhibits several proteins with essential thiol groups, such as alcohol dehydrogenase,[21] NF-κB,[22] farnesyl-protein transferase,[23] and creatine kinase.[24] As part of our ongoing studies to evaluate the biological activity of the enantiomers and racemic 1,4-diethyl-3,6-epidithiopiperazine-2,5-diones some of the properties discussed above were investigated.

(1) Thymidine Incorporation. To measure cell proliferation, 100 μL of P815 cells in Eagles Minimum Essential Medium (F15) and 10% Fetal Bovine Serum (FCS) at a final cell concentration of 0.5 x 10^6 per mL were pipetted into 96-well plates containing the serially diluted (1:2 dilution, starting concentration of 200 μM) ETP compounds; *rac*-(**3**), *R,R*-(**3**), *S,S*-(**3**), and gliotoxin (as reference compound). The cells were incubated for 18 h at 37 °C before being pulsed with tritiated thymidine (1 μCi/well) for a further 6 h. Cells were harvested using a Pharmacia Betaplate Liquid Scintillation counter and the results obtained are summarised in Table 1.

(2) Macrophage Adherence. Thioglycollate induced mouse peritoneal macrophages at a final cell concentration of 1 x 10^6 per mL were aliquoted into 96-well plates containing the serially diluted (1:2 dilution, starting concentration of 200 μM) ETP compounds; *rac*-(**3**), *R,R*-(**3**), *S,S*-(**3**), and gliotoxin (as reference compound). A measure of phagocytosis was obtained following the neutral red assay[25,26] and the results are summarised in Table 1.

(3) Apoptosis. The extent of apoptotic cell death was determined by evaluating the subdiploid population of propidium iodide (PI) stained cells using flow cytometry.[27] Thymocytes from 10 day old Balb C mice at a final cell concentration of 1 x 10^6 per mL in F15 + 5% FCS media were treated with 3 μM of the ETP compounds; *rac*-(**3**), *R,R*-(**3**), *S,S*-(**3**), and gliotoxin (as reference compound) over a time course of 9 h at 37 °C. At each time point (2, 4, 6, and 9 h) the cells were resuspended in 1 mL PBS and fixed overnight at 4 °C in cold 70% ethanol. The cells were then washed twice in cold PBS and the DNA stained with 1 mL of PBS containing PI (400 μg/mL) and RNase (1 mg/mL) for 30 min in the dark at ambient temperature. Cells were analysed using flow cytometry.[28] The extent of apoptosis after 9 h is summarised in Table 1.

(4) Interaction with Creatine Kinase. The activity of rabbit muscle creatine kinase was determined using a modification of an enzyme coupled assay as described by Tanzer and Gilvarg[29] and more recently by Winterbourn et al.[30] The appropriate ETP compound (50 μM) and creatine kinase (4 μg/mL) were added after 5 min to an assay mixture containing ATP (2.1 mM), phospho(enol)pyruvate (0.5 mM), NADH (0.3 mM), pyruvate kinase (8.96 U/mL), L-lactic dehydrogenase (19.2 U/mL), creatine (13.3 mM) and magnesium sulphate (3.5 mM) in a pH 9 glycine-sodium hydroxide buffer. The reaction was monitored on a CARY UV spectrophotometer at 340 nm for 12 min and the linear rate obtained between 2.5-5.5 min was used for analysis of enzyme activity. The results are summarised in Table 1.

Compound	Thymidine Incorporation (ED_{50} values, μM)	Macrophage Adherence (ED_{50} values, μM)	Creatine Kinase Activity after 6 h (%)	Extent of Apoptosis after 9 h (%)
Control	N/A	N/A	78 ± 1	16 ± 3
Gliotoxin	2 ± 0.1	*0.35 ± 0.1	32 ± 2	59 ± 2
rac-(**3**)	35 ± 3	2.5 ± 0.3	35 ± 1	54 ± 6
R,R-(**3**)	50 ± 8	5 ± 0.5	34 ± 3	53 ± 2
S,S-(**3**)	55 ± 3	8 ± 0.3	34 ± 2	57 ± 2

* Literature value[5] 0.34

Table 1: Biological Activities of the Enantiomers and Racemic 1,4-Diethyl-3,6-epidithiopiperazine-2,5-dione and Gliotoxin.

All four independent assays demonstrate that the enantiomers and racemic 1,4-diethyl-3,6-epidithiopiperazine-2,5-dione are biologically active compounds. In particular, these compounds have been shown to display reduced immunosuppressive properties (macrophage adherence and thymidine incorporation) in comparison to gliotoxin. It should also be noted that in these two assays the biological activities of each enantiomer of (**3**) appear to be less potent than that of the racemic compound. This result is curious and more studies are needed in order to rationalise this interesting behaviour. In contrast, these same compounds are equally effective in inhibiting creatine kinase and inducing apoptosis as gliotoxin. More significantly, each of the enantiomers display equipotent behaviour in every biological assay examined. Our studies therefore show that for this synthetic ETP compound there is no apparent correlation between biological activity and chirality at the

bridgehead carbons. These observations have important implications in the structure activity studies of ETP compounds. Further work examining other synthetic ETP derivatives is in progress.

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11. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.27 (t, 6H, NCH_2CH_3), 3.56 (q, 4H, NCH_2CH_3), 5.35 (s, 4H, 2 x ring CH_2). Microanalysis: Anal. calcd. for $C_8H_{12}N_2O_2S_2$: C 41.36; H 5.21; N 12.06; S 27.60. Found C 41.55; H 5.06; N 11.71; S 27.68.
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