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1α,10-EPOXYFURANOEREMOPHILANE—A NEW INHIBITOR OF NADH-DEHYDROGENASE FROM *SENECIO LEPTOLOBUS*

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Key Word Index—*Senecio leptolobus*; Asteraceae; sesquiterpenes; furanoeremophilanes; respiratory chain inhibitors; cytotoxicity.

Abstract—The aerial parts of the Brazilian species *Senecio leptolobus* afforded the known sesquiterpene 1α,10-epoxyfuranoeremophilane (0.05% dry wt). This compound was shown to inhibit NADH-dehydrogenase, the first complex of the respiratory chain. The I_{50} is 2.4×10^{-4} M and the inhibition is uncompetitive. The growth inhibiting effect on V 79 Chinese hamster fibroblasts was investigated using a proliferation assay.

INTRODUCTION

It is known that crude extracts from several plants contain compounds which inhibit the activity of the respiratory chain [1]. A well known example is rotenone from the East Asian *Derris elliptica* (Papilionaceae). The compounds isolated so far are of different chemical natures and act at various sites of the respiratory chain, but a certain hydrophobicity is a common feature.

As part of our investigation on Brazilian *Senecio* spp. and their relevance in diseases of ruminants, we isolated the pyrrolizidine alkaloids neosenkirkine, florosenine, and integerrimine [2, 3], the latter being very common in the genus *Senecio*. These alkaloids are accepted to be the active principle of *Senecio leptolobus* DC. We now present our results on the isolation and physiological effects of the sesquiterpene 1α,10-epoxyfuranoeremophilane (**1**).

RESULTS AND DISCUSSION

The structure of compound **1** was elucidated by ^{1}H and ^{13}C NMR and the data obtained indicated that it is identical with the compound described by Schild [4] and by Bohlmann *et al.* [5]. According to Pinder [6], the furan ring can be opened under mild oxidising conditions to give a butenolactone which may act as a Michael acceptor. As the epoxide may be attacked by nucleophilic groups the compound may be considered to be bifunctional.

We are not aware of any literature data dealing with the biological activity of **1** or related compounds, except for a paper by Jennings *et al.* [7] who investigated *Tetradymia glabrata* (Asteraceae) for its active principle. They isolated tetradymol, i.e. 10β-hydroxyfuranoeremophilane, and showed it to be an effective decoupler of the respiratory chain. Due to the obvious structural similarity of tetradymol, we followed this information and investigated the effect of **1** on intact mitochondria as well as ETPs (i.e. electron transporting particles) from beef heart.

By means of a Clark-type electrode, we showed that the respiratory rate of a mitochondrial suspension was decreased (data not shown) when **1** was added to the suspension in the μM range. The choice of an appropriate substrate (i.e. glutamate/malate) indicated that the site of inhibition is located in complex I of the respiratory chain. Complex II remained unaffected when succinate served as the substrate.

In order to quantify the effect, we determined the I_{50} of **1** in an ETP preparation (Fig. 1) and showed it to be 2.4×10^{-4} M. This shows that **1** is more than twice as effective as the common inhibitor of NADH-DH, amytal [1]. The mechanism of the inhibition was investigated by using beef heart ETPs. A Michaelis–Menten plot indicated both, a decrease of the apparent K'_M as well as v_{max} with increasing inhibitor concentrations. The Lineweaver–Burk plot of the same data (Fig. 2) established that the inhibition was indeed uncompetitive.

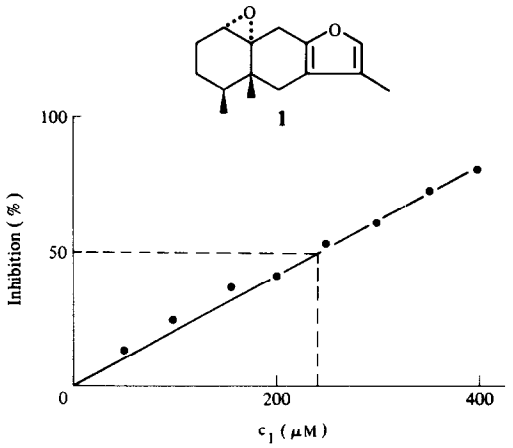

Fig. 1. Titration of inhibition of NADH oxidation by **1**. Samples of ETP (0.5 mg protein) were added to 2.5 ml buffer. In the presence of 0.1 mM NADH increasing amounts of **1** ($=c_I$), dissolved in *n*-PrOH, the total volume of which did not exceed 3%, were added. Oxygen consumption was monitored polarographically with a Clarke type electrode

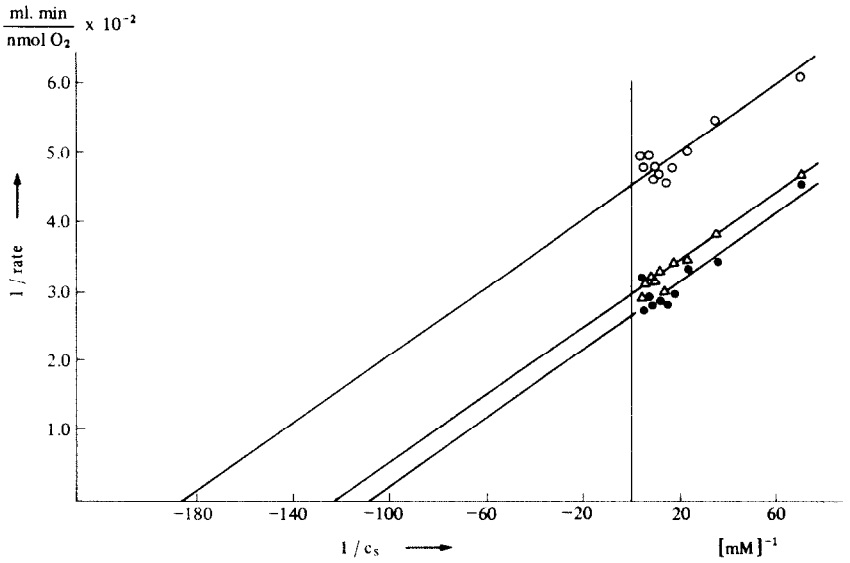

Fig. 2. Lineweaver–Burk plots for two concentrations of **1**. ●—●, no inhibitor present; △—△, 60 μM **1**; ○—○, 240 μM **1**. ETP protein content set at 0.2 mg/ml.

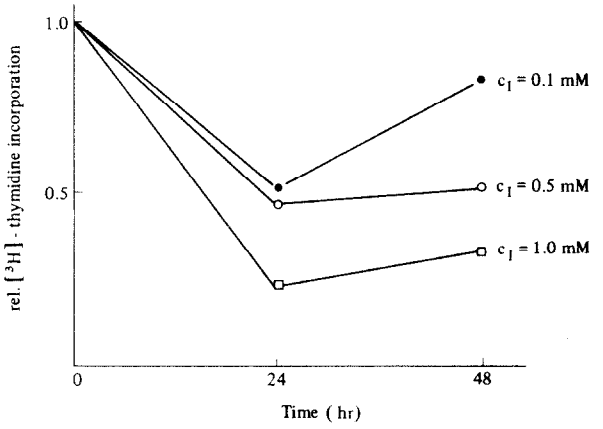

Fig. 3. Uptake of ^{3}H-thymidine by V 79 Chinese hamster fibroblasts subcultured in microwell tissue culture plates. Compound **1** was added in concentrations of 1, 0.5 and 0.1 mM and the incubations were terminated after 24 and 48 hr. The incorporation of ^{3}H-thymidine was measured over the last 8 hr of each incubation period. Controls contained a corresponding volume of solvent (MéCN). Means are given and standard deviation of means was lower than 15%.

We assumed that the marked inhibition of energy metabolism by **1** should have an effect on intact cells, providing **1** could reach its site of action. To test this hypothesis we used V 79 Chinese hamster fibroblasts, thymidine subcultured in microwell tissue culture plates. The uptake of ^{3}H-thymidine was measured over an 8 hr period in cells which had been grown for 16 and 40 hr, respectively, in the presence of increasing amounts of **1** (Fig. 3).

Depending on the concentration, the uptake of thymidine was reduced in the first 24 hr after which time the cells recovered. The fact that the inhibition was reversed after 48 hr may be explained by decomposition or detoxification of **1**. In each case more cells than in the control cultures were pycnotic and non-adherent. These cells could be stained with Trypan Blue indicating the cytotoxic effect of **1**. Each adherent fibroblast showed a number (5–10) of vesicles which appeared as highly refractive spherical objects under the microscope. After fixation with 50% ethanol the cells were treated with a Sudan III solution. The spheres then appeared red which can be interpreted as incorporation of the dye into lipid vesicles.

These findings indicated that the cytotoxic effect is accompanied by intracellular changes in lipid metabolism. This takes the form of an accumulation of substrates because NADH cannot be oxidized by the respiratory chain. Therefore our results are a strong hint that the observed cytotoxic effect is correlated with the inhibition described above.

At the present time, we cannot prove that **1** remains

unmetabolized on its passage through the cell membrane, cytoplasma and mitochondrial membranes to the location of complex I.

EXPERIMENTAL

Extraction of the plant material was carried out in a Soxhlet apparatus using petrol as solvent. The resulting petrol extract (4.3% dry wt) was filtered through a column of silica gel with petrol to separate chlorophylls. From this enriched mixture, **1** was isolated by prep TLC on silica gel using the solvent system EtOAc–petrol (3:97). This procedure yielded 0.05% of **1** (dry wt). The compound was detected by Ehrlich's reagent (*N,N*-dimethylaminobenzaldehyde) and its purity was monitored by GC.

1α,10-*Epoxyfuranoeremophilane* (**1**). Colourless oil, decomposing at room temp. and Ar atmosphere within days. Stable in liquid N_2 and in dilute soln. GC: R_t 2.42 min (25 m × 0.27 mm, OV-1 0.2 μm, T_{oven} 200°, T_{inj} 280°, t_{det} 300°, FID, p_{N2} = 0.6 bar); $[\alpha]_D^{20} = -22.6°$ (EtOH; *c* 1) (lit. $[\alpha]_D^{20} = -22.6°$ (EtOH; *c* 1) [4]); IR ν_{max} cm^{-1}: 2950 (C–H), 1640 (furan–C=C), 1565 (furan-C=C), 1245 (epoxide): EIMS (DI, 70 eV, 130°) *m/z* (rel. int.): 232 $[M]^+$ (78), 217 $[M-Me]^+$ (9), 202 (4), 199 $[217-H_2O]^+$ (48), 175 (27), 159 (16), 147 (53), 133 (20), 119 (50), 108 $[C_7H_8O]^+$ (100), 91 (64), 79 (76); ^1H NMR (300 MHz, $CDCl_3$): δ 3.07 (*br s*, H-12), 3.22 and 2.10 (A, B-system, $J_{A,B}$ = 17 Hz, H-9), 3.07 (*m*, H-1), 2.40 and 2.26 (A, B-system, $J_{A,B}$ = 15 Hz, H-6), 1.98 (*m*, H-2), 1.92 (*d*, H-13), 1.79 (*m*, H-4), 1.33 (*m*, H-3), 1.23 (*m*, H-3), 0.92 (*s*, H-14), 0.82 (*d*, H-15); ^{13}C NMR (75 MHz, $CDCl_3$): 146.48 (*s*, C-8), 134.75 (*d*, C-12), 120.01 (*s*, H-7), 116.51 (*s*, C-11), 64.19 (*s*, C-10), 59.12 (*d*, C-1), 36.06 (*s*, C-5), 37.75 (*d*, C-4), 31.33 (*t*, C-6), 31.20 (*t*, C-9), 25.41 (*t*, C-3), 23.04 (*t*, C-2), 15.42 (*q*, C-15), 14.58 (*q*, C-14), 8.06 (*q*, C-13). A 0.04 M solution of **1** in *n*-PrOH was stored under liquid N_2 until used.

Preparation of mitochondria and ETPs. For the preparation of beef heart mitochondria slaughterhouse material was used according to the method described in ref. [8]. The preparation of ETPs followed the procedure of Lee [9], the resulting ETP suspension was adjusted to about 25 mg protein per ml in 0.25 M sucrose. The ETPs thus prepared lost less than 5% of their respiratory activity when they were frozen in aliquots.

Oxygen uptake was determined polarographically at 25° with an oxygen electrode of the Clarke type. The incubation buffer (2.5 ml) contained 0.25 M sucrose, 0.01 M triethanolamine, 0.01 M KCl, 0.005 M $MgCl_2$ and 0.01 M KH_2PO_4, pH 7.2. To this was added 100 μl of mitochondria suspended in the buffer containing glutamate and malate (0.4 mM each). ADP was added at a concentration of 0.2 mM, and when the basal respiratory rate was stable 10 mM of **1** was added. To exclude the formation of an enzyme-inhibitor-complex, the sequence of additions of substrate and inhibitor was reversed. This procedure appeared to have no effect. Another experiment was performed with succinate (0.4 mM) as a substrate.

To determine the I_{50} value of **1**, ETPs were suspended in the buffer at a concentration of 0.2 mg protein per mg. The NADH concentration was 0.1 mM and the concentration of **1** was varied from 0 to 400 μM.

The kinetic measurements were made with inhibitor concentrations of 0, 60 and 240 μM while NADH was varied from 0 to 25 mM. The protein concentration was 0.2 mg/ml. Concentrations of *n*-PrOH up to 5% did not affect the measurements.

Cell culture. V 79 Chinese hamster fibroblasts were grown in RPMI 1640 culture medium + 10% foetal calf serum in culture flasks at 37° in a humid atmosphere containing 5% CO_2. Cells in the exponential growth phase were treated with a trypsin soln (0.25% trypsin and 0.05% EDTA). The split was 1:10 to 1:20, for testing the cells were seeded into microwell tissue plates (500 cells per well, 0.2 ml) [10].

The cells were allowed to become adherent overnight and were incubated with 0.1 mM, 0.5 mM and 1 mM **1** which had to be suspended in MeCN the concentration of which never exceeded 2.5% (not inhibitory itself). After 16 and 42 hr, 10 μl [methyl-^{3}H]thymidine was added (stock soln 20 μCi/ml, 60–90 Ci/mmol) for 8 hr each, then the plates were frozen. The cells were harvested and collected on glass fibre filter mats. All filters were dried at 50° and transferred to scintillation vials. 2 ml of toluene based scintillation fluid was added and radioactivity was measured in a scintillation spectrophotometer [11].

Staining. The cells were seeded into petri dishes and incubated overnight with 100 μM **1** after they had become almost confluent. The medium was decanted after 24 hr, the cells fixed with 50% EtOH and stained with a 1% Sudan III soln in 70% EtOH for 30 min. Other dishes were treated with a 0.5% Trypan Blue soln in 0.9% NaCl in order to test for dye exclusion by vital cells.

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