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2,3:18,19-DIOXIDOSQUALENE: SYNTHESIS AND ACTIVITY AS A POTENT INHIBITOR OF 2,3-OXIDOSQUALENE-LANOSTEROL CYCLASE IN RAT LIVER MICROSOMES

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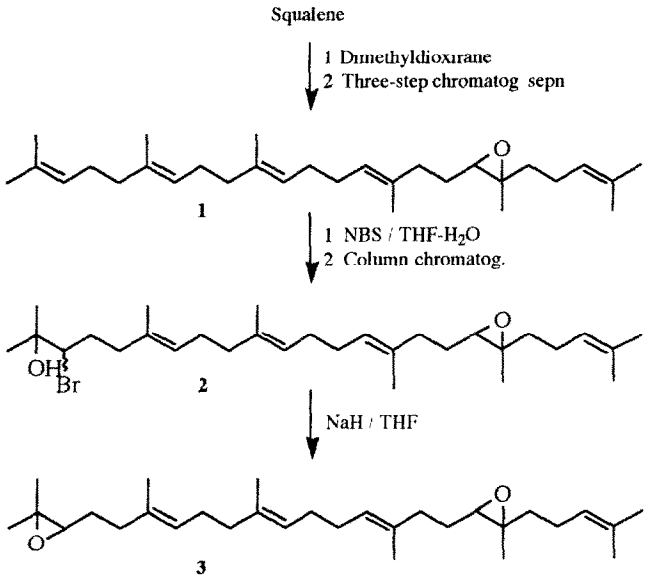
Abstract: 2,3:18,19-Dioxidosqualene (**3**) was synthesized and isolated as a diastereomeric mixture. Incubation of 2,3-oxidosqualene with rat liver microsomes in the presence of dioxide **3** caused a high inhibition of lanosterol production ($K_i = 0.11$ μM). The kinetic data showed that this inhibition was non-competitive.

Keywords : 2,3:18,19-dioxidosqualene; inhibition; 2,3-oxidosqualene-lanosterol cyclase (EC 5.4.99.7).

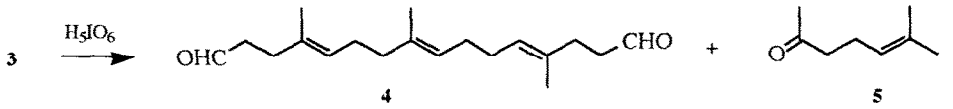
The inhibition of oxidosqualene-lanosterol cyclase (OSLC, EC 5.4.99.7), the enzyme that catalyzes the cyclization of (3*S*)-2,3-oxidosqualene to lanosterol in mammals and fungi has constituted an attractive goal for the development of potential hypocholesteremic agents. An advantage of this strategy is that the above enzymatic step is located in the cholesterol biosynthetic pathway beyond the bifurcations for the synthesis of other important terpenoid biomolecules such as dolichol and ubiquinones. The OSLC inhibitors described so far include substrate mimics, product mimics, transition state analogues and irreversible inactivators [1]. Although some of these molecules are structurally related to squalene or to its 2,3-epoxy derivative, it appears that the different squalene dioxides had not been previously considered as potential modulators of the above enzyme. In fact, up to our knowledge the only squalene dioxide that has been characterized so far is 2,3:22,23-dioxidosqualene (**6**) [2]; this compound exhibited a moderate angiotoxic activity in rabbits [2c]. In the context of a an ongoing study on the occurrence, characterization and biological activity of the different possible squalene dioxides, the present communication reports on the synthesis of 2,3;18,19-dioxidosqualene (**3**) and its inhibitory activity on rat liver microsomal OSLC.

Synthesis of compounds. The preparation of compound **3** is shown in Scheme I. Thus, epoxidation of squalene with equimolecular amounts of dimethyldioxirane [3] afforded a mixture of mono and diepoxy derivatives [4]. The corresponding 6,7-epoxy derivative **1** [5] was isolated after three sequential chromatographic steps which included a final column chromatography on silicagel impregnated with $AgNO_3$ to separate compound **1** from its positional isomer at C10,C11. Reaction of **1** with *N*-bromosuccinimide in THF/H_2O led to the formation of the diastereomeric bromohydrins **2**, which were purified by column chromatography and further converted into bisepoxide **3** by treatment with sodium hydride in anhydrous THF. Compound **3** was isolated as a mixture of diastereomers and characterized by its spectral features [6].



Scheme I . Synthesis of 2,3:18,19-dioxidosqualene (**3**).

A complementary identification of **3** was carried out by GC/MS analysis (chemical ionization with methane) of the crude resulting from its reaction with periodic acid. As shown below, the presence of 6-methylhept-5-en-2-one (**5**, identified by comparison with an authentic standard) and of a compound with a fragmentation pattern in agreement with the structure of dialdehyde **4** [peaks at m/z 329 (M + 29), 291 (M+1), 273 (M-H_2O+1), 111 (base peak)] confirmed the structural assignation for **3** [7]:



A second squalene bisepoxy derivative, i.e. 2,3:22,23-dioxidosqualene (**6**) was synthesized for comparison purposes. In this case, treatment of squalene with excess NBS, followed by column chromatography purification of the corresponding bisbromohydrin and epoxide formation by reaction with sodium hydride in THF afforded compound **6** also as a diastereomeric mixture [6].

Biological assays. OSLC assays were carried out by using rat liver microsomes and the amount of lanosterol formed was quantified by GC chromatography [8]. *N*,*N*-dimethyldodecylamine-*N*-oxide was used as a reference inhibitor [9b]. The IC_{50} values determined for monoepoxide **1**, dioxide **6** and dioxide **3** were 83.5, 142 and 0.11 μM, respectively. Therefore, compound **3** appeared to be a potent inhibitor of the OSLC, thus being 800-fold more active than its putative precursor, i.e. monoepoxide **1**, and over three orders of magnitude more potent when compared to the inhibition caused by the structurally related dioxide **6**. These results demonstrate the importance of the epoxide moiety at C18-C19 for eliciting a high inhibitory activity. On the other hand, the K_i value for compound **3** was determined from replots of slopes and intercepts of Lineweaver-Burk double-reciprocal plot (Figure 1) and it resulted to be 0.11 μM. In addition, either Dixon or Cornish-

Bowden plots of these kinetic data showed that the inhibition elicited by compound **3** was essentially non-competitive. All these results showed that dioxide **3** elicited an activity comparable to the most potent inhibitors described so far [1].

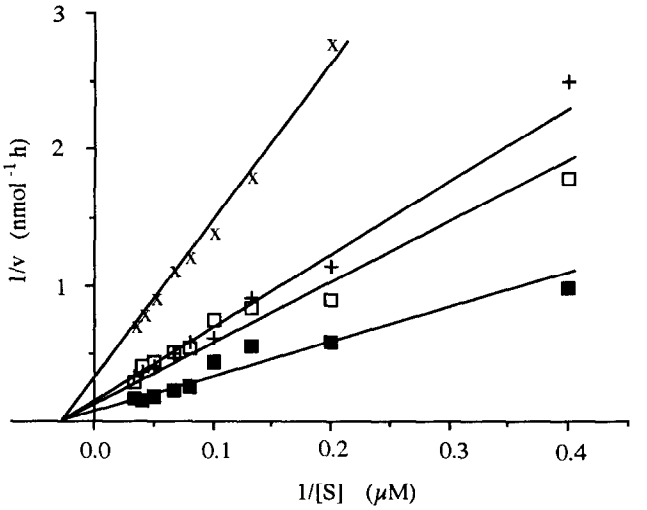


Figure 1. Inhibition of rat liver 2,3-oxidosqualene-lanosterol cyclase by 2,3:18,19-dioxidosqualene (**3**). The concentrations of **3** are: (-■-) none, (-□-) 0.1 μM, (-+-) 0.2 μM, (-x-) 0.4 μM. Protein concentration: 1.57 mg microsomal protein/ml.

Two final remarks. First, it should be noted that dioxide **3** was assayed as a mixture of four diastereomers. However, it is reasonable to expect that the observed inhibitory activity could be due to either one or both stereoisomers with 3*S* configuration, whereby its or their potency could be even higher than that herein reported. In this respect, the stereoselective synthesis of these diastereomers will be needed to confirm our hypothesis. Finally, the occurrence of epoxide **1** in nature [5c] brings up the possibility of formation of dioxide **3** under physiological conditions, which would give a particular relevance to the OSLC inhibitory activity found for this compound. Work on these lines is now in progress in our laboratory.

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6. **1**: ^{1}H NMR (300 MHz, $CDCl_3$): δ, 5.3-5.0 (5H), 2.71 (t, 1H, J = 6 Hz), 2.2-1.9 (16H), 1.68 (s, 6H), 1.62 (d, 3H, J = 1 Hz), 1.60 (br. s, 12H), 1.8-1.3 (4H) and 1.25 (s, 3H) ppm. ^{13}C NMR (75 MHz): δ, 135.2 (C), 134.8 (C), 134.1 (C), 131.7 (C), 131.2 (C), 124.8 (CH), 124.3 (CH), 124.2 (CH), 124.1 (CH), 123.7 (CH), 63.4 (CH), 60.7 (C), 39.7 (CH_2), 38.8 (CH_2), 36.2 (CH_2), 28.2 (CH_2), 28.1 (CH_2), 27.3 (CH_2), 26.7 (CH_2), 26.6 (CH_2), 25.7 (CH_3), 23.8 (CH_2), 17.6 (CH_3), 17.6 (CH_3), 16.5 (CH_3), 16.0 (CH_3), 16.0 (CH_3) and 16.0 (CH_3) ppm (cf. ref. 5b). MS (CI, CH_4), m/z: 455 (M+29), 427 (base peak, M+1).

   2 (diastereomeric mixture): ^{1}H NMR ($CDCl_3$): δ, 5.3-5.0 (4H), 3.98 (dd, 1H, J_1 = 11.5, J_2 = 2 Hz), 2.71 (t, 1H, J = 6 Hz), 2.4-1.9 (14H), 1.68 (d, 3H, J = 1 Hz), 1.62 (br. s, 3H), 1.60 (br. s, 9H), 1.58 (s, 3H), 1.9-1.1 (6H), 1.34 (s, 3H), 1.33 (s, 3H) and 1.25 (s, 3H) ppm.

   3 (diastereomeric mixture): ^{1}H NMR ($CDCl_3$): δ, 5.3-5.0 (4H), 2.71 (t, 2H, J = 6 Hz), 2.3-1.9 (14H), 1.68 (d, 3H, J_1 = 1 Hz), 1.62 (br s, 9H), 1.60 (s, 3H), 1.8-1.2 (6H), 1 30 (s, 3H), 1.26 (s, 3H) and 1.25 (s, 3H) ppm. ^{13}C NMR: δ, 135.1 (C), 134.2 (C), 134.0 (C), 131.8 (C), 124.9 (CH), 124.8 (CH), 124.2 (CH), 123.7 (CH), 64.2 (CH), 63.3 (CH), 60.8 (C), 58.3 (C), 39.6 (CH_2), 38.8 (CH_2), 36.3 (CH_2), 28.3 (CH_2), 28.2 (CH_2), 27.5 (CH_2), 27.3 (CH_2), 26.7 (CH_2), 25.7 (CH_3), 24.9 (CH_3), 23.9 (CH_2), 18.7 (CH_3), 17.6 (CH_3), 16.5 (CH_3), 16.0 (CH_3) and 16.0 (CH_3) ppm. MS (CI, CH_4), m/z: 443 (M+1), 425 (base peak, M-18+1).

   6 (diastereomeric mixture) [2a]: ^{1}H NMR ($CDCl_3$); 5.2-5.1 (4H), 2.70 (t, 2H, J = 6 Hz), 2.2-1.9 (16H), 1,62 (d, 6H, J = 0.5 Hz), 1,60 (d, 6H, J = 1 Hz), 1.7-1.4 (4H), 1.31 (s, 6H), 1.26 (s, 6H). ^{13}C NMR; 134.9 (C), 133.9 (C), 124.8 (CH), 124.3 (CH), 64.1 (CH), 58.3 (C), 39.6 (CH_2), 36.3 (CH_2), 28.2 (CH_2), 27.4 (CH_2), 26.6 (CH_2), 24.9 (CH_3), 18.7 (CH_3), 16.0 (CH_3), 16.0 (CH_3).

7. Full details of a study carried out on the identification of the different squalene dioxides by GC/MS will be presented elsewhere (Abad, J.L. et al., manuscript in preparation).

8. *Assay method for OSLC.* Isopropyl alcohol solutions of the substrate and inhibitors were added to the test tubes (the alcohol contents did not exceed 1% of the overall test mixture), followed by addition of 0.1 M phosphate buffer (pH 7.4), EDTA (final concn. 0.1 mM), Tween-80 (final concn. 0.15% w/v), 100 μl of rat liver microsomal suspension and 200 μl cytosolic fraction. Microsomes (from Sprague-Dawley males) were prepared as described in ref. 9a. For determinations of IC_{50} values final concentration of substrate (*R,S*)-2,3-oxidosqualene was 40 μM and OSLC activity was 3.1 ± 0.4 nmol/hr/mg protein (N = 8) The substrate concentrations used for the calculation of the kinetic parameters were half of that of (*R,S*)-2,3-oxidosqualene given to the incubation medium. The mixture (final volume 1 ml) was flushed with nitrogen and incubated anaerobically for 60 minutes at 37 °C. The enzymatic reaction was quenched by addition of 1 ml of 6% KOH in methanol and incubated for 60 minutes at 37 °C. Then, 24,25-dihydrolanosterol was added as internal standard and the mixture was extracted 3 times with an equivalent volume of hexane. The combined extracts were evaporated to dryness, redissolved in a 10:1 hexane: *tert*-butyl methyl ether mixture (3 x 40 μl), loaded onto a column fitted with silicagel (0.75 g, SDS, 40-60 μm) and eluted with the same solvent mixture. After discarding the first 7 ml, lanosterol and internal standard were collected in a 6 ml fraction. The eluates were evaporated to dryness, redissolved in hexane and injected onto a GC system (Hewlett-Packard model 5890). Conditions of analysis were: SPB-5 capillary column (15 m, 0.32 mm i.d.), gradient temperature from 240 (1 min) up to 300 °C at 2 °C/min.. Under these conditions, retention times for 24,25-dihydrolanosterol and lanosterol were 13.86 and 14.72 min., respectively. Incubations were performed by duplicate and a minimum of two experiments per point were carried out

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