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EPOXIDATION OF 6,7- AND 10,11-OXIDOSQUALENES BY THE SQUALENE EPOXIDASE PRESENT IN RAT LIVER MICROSOMES

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Abstract: Incubation of 6,7-oxidosqualene (**2**) or 10,11-oxidosqualene (**3**) with rat liver microsomes led to the formation of mixtures of the corresponding dioxidosqualenes (**4** and **5**, or **6** and **7**, respectively), resulting from the epoxidation of **2** and **3** at their terminal double bonds. The epoxidation requires the presence of both NADPH and FAD. In addition, the HPLC analysis of the Mosher esters resulting from the controlled hydrolysis of dioxide **5** to give the corresponding epoxydiols **9** followed by derivatization with (*R*)-MTPA, showed that the epoxidation had been stereoselective. These facts support the hypothesis that these dioxidosqualenes had been generated by the squalene epoxidase present in the incubation medium.

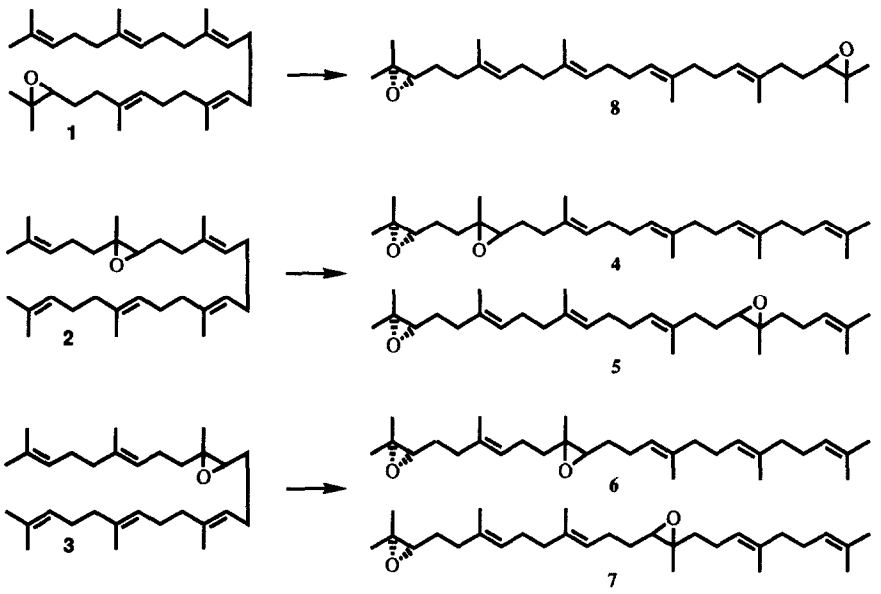
Keywords: dioxidosqualene; oxidosqualene; squalene epoxidase (EC 1.14.99.7); epoxidation.

We have recently reported the synthesis, characterization and inhibitory activity elicited by several dioxidosqualenes on the oxidosqualene-lanosterol cyclase (OSLC, EC 5.4.99.7) present in rat liver microsomes [1]. In particular, high activities were found for two of these derivatives, i.e., 2,3:18,19-dioxidosqualene (**5**, IC_{50} = 0.11 μM, Scheme I) and 2,3:10,11-dioxidosqualene (**6**, IC_{50} = 13.0 μM), being the former comparable to those exhibited by the most potent OSLC inhibitors described to date [2]. Therefore, the occurrence of these dioxido derivatives in living organisms could be of physiological significance for the regulation of sterol biosynthesis.

Concerning the detection of dioxidosqualenes in tissues, only references related to 2,3;22,23-dioxidosqualene (**8**, Scheme I) have been reported. Thus, the accumulation of 2,3-oxidosqualene (**1**) and dioxide **8** was observed in animal cells [3] or liver homogenates treated with OSLC inhibitors [4]. Moreover, the detection of **8** in incubations of **1** with partially purified squalene epoxidase (SE, EC 1.14.99.7) from pig liver has been recently described [5]. In this context, the fact that oxidosqualenes **2** and **3** have been found in nature [6] makes feasible that dioxides **5** or **6** could be formed by the action of SE on their respective terminal double bonds. The present communication reports our results on the formation of dioxidosqualenes **4-7** by incubation of oxides **2** and **3** (as racemates) with rat liver microsomes under conditions for eliciting squalene epoxidase activity, i.e. in the presence of cytosol, NADPH and FAD [7,8].

Initially, incubation of epoxide **1** with rat liver microsomes for 14 h at room temperature [8b] afforded a crude mixture from which dioxidosqualene **8** was isolated. The identification of this compound was carried out by comparing its analytical (HPLC, GC) and spectral (MS) features with those of an authentic standard [1b]. The amount of dioxide **8** formed was 16 nmol (HPLC, external standard method).

Scheme I



Incubation of epoxide **2** under the same conditions led to the formation of dioxidosqualenes **4** and **5**. The GC and TLC traces of the crude incubation mixtures showed that compound **4** was present in higher amount. However, its instability in front of chromatographic procedures [1b] forced the use of a rapid purification process through silica gel impregnated with Et_3N to minimize its decomposition. This procedure did not allow the complete separation of both dioxides (approximately 10-15% of cross contamination), although a pure sample of **5** could be isolated by a further purification. Nevertheless, the fact that these dioxidosqualenes can be analyzed by HPLC and GC/MS/CI permitted their unambiguous identification and quantification. Thus, dioxide **4** appeared as a single peak by HPLC, which could be clearly differentiated from that corresponding to dioxide **5** (R_T = 11.3 and 9.2 min, respectively, under our analysis conditions [8b]). The GC/MS/CI of the fraction containing dioxide **4** as major component showed a profile superimposable to that exhibited by the corresponding synthetic standard [1b]. Thus, the peak assigned to compound **4** exhibited a mass spectrum with the fragmentation pattern common to dioxidosqualenes [1b], i.e., a molecular peak (M+1) at m/z 443, a base peak at m/z 425 (M+1-H_2O) and a peak at m/z 407 (M+1-2 H_2O). In addition, the GC/MS trace revealed the presence of a second peak with a lower retention time. Its mass spectrum showed a base peak at m/z 443 and the absence of fragments resulting from the loss of water, which suggests that this compound could be a ketone generated by thermal rearrangement of the parent dioxide under the analysis conditions. Dioxide **4** contents were estimated as 15 nmol (HPLC), although this amount does not account for all the dioxide formed due to the partial loss of compound occurred during the purification procedure.

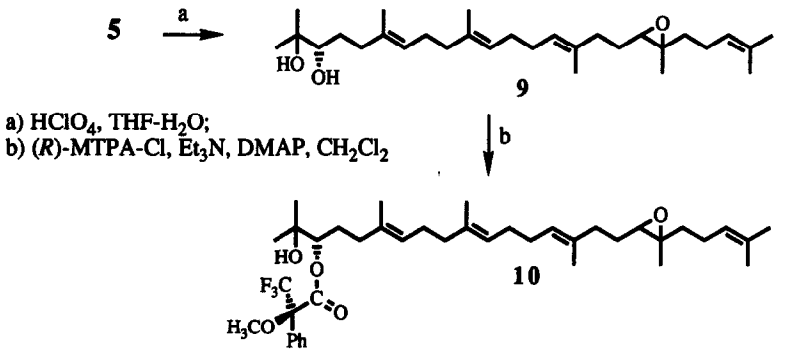
Likewise, the other dioxidosqualene generated during the incubation of epoxide **2** with rat liver microsomes was identified as dioxide **5** (GC and HPLC, coelution with an authentic standard). Confirmation of the chemical structure was carried out by GC/MS/CI. The mass spectrum of this metabolite showed the presence of the three representative fragments of dioxide **5** (m/z 443, 425 and 407), with the same relative

abundances (55%, 100% and 25%, respectively). The amount of this dioxide formed in the incubation was 15 nmol, which indicates that although the enzyme does not epoxidize regioselectively one of the terminal double bonds present in **2**, it exhibits some preference for the one contiguous to the original epoxide moiety. In this respect, Prestwich and coworkers have shown a similar behaviour in the formation of 26-hydroxy-2,3-oxidosqualene and its corresponding 29-hydroxy regioisomer (3:1 isomeric ratio) by incubation of tritiated 26-hydroxysqualene with partially purified SE from pig liver [9].

Incubation of epoxide **3** under the same conditions described above led to the formation of dioxidosqualenes **6** and **7**. In this case, all efforts to resolve the two peaks appearing in the HPLC profile were unsuccessful. However, these dioxides are stable enough to allow their complete separation by TLC with good recovery. The identification of these dioxides was carried out by comparison of their mass spectra and chromatographic features with those of the corresponding synthetic standards [1b]. On the other hand, a production of 20 nmol for each dioxide was determined by HPLC, which suggests that the internal oxirane is far enough from the reaction site for exerting an influence in the regioselectivity of the epoxidation.

Different assays were carried out to support that the epoxidation reactions observed during the incubation of oxidosqualenes **1**, **2** and **3** were due to the SE action. Thus, we confirmed that the formation of dioxidosqualenes required, in addition to NADPH, the presence of FAD in the incubation medium, which discarded the intervention of a cytochrome P-450 monooxygenase in the epoxidation process. An additional proof of the enzymatic mediation was obtained from the HPLC analysis of the Mosher esters **10** obtained from the purified sample of dioxide **5** formed during the incubation of epoxide **2** (Scheme II).

Scheme II



For this purpose, the required standards were prepared as described [10]. Thus, synthetic dioxide **5** (mixture of diastereomers) was converted into the corresponding mixture of epoxydiols **9** resulting from the regioselective opening of the terminal epoxide ring. The formation of the epoxydiols was preferred to simplify the further derivatization process; to this aim, the conditions of this hydrolysis were carefully controlled to minimize the formation of tetrol derivatives. Reaction of the purified epoxydiol mixture with the (*R*) enantiomer of Mosher acid (MTPA) led to the formation of the corresponding MTPA esters [10], which appeared as two peaks in reversed-phase HPLC. These peaks were attributed to the diastereomers of (3*R*) and (3*S*) configurations in the starting substrate [11]. Conversely, when dioxide **5** coming from the incubation of epoxide

2 was subjected to the above sequence, the HPLC analysis of the corresponding (*R*)-MTPA esters revealed the presence of a single peak (the one with the lower retention time). This result indicated that only one stereoisomer at C-3 was formed during the epoxidation, presumably that with (3*S*) configuration. The assignation of this configuration was confirmed by the HPLC analysis of the (*R*)-MTPA esters obtained from the hydrolysis and further derivatization of the 2,3-oxidosqualene formed by incubation of squalene under the same conditions. Once again, and in contrast to which occurred with the standards from synthetic origin, the presence of the peak with the lower retention time was only detected.

The confirmation that epoxides **2** and **3** were substrates for the SE present in rat liver microsomes raised the question about their potential inhibitory effect on the activity of this enzyme for the epoxidation of squalene, its natural substrate. The IC_{50} values found for epoxides **2** and **3** (31.8 and 50.9 μM, respectively [8a]) are three to five fold higher than the corresponding substrate concentration used, which indicates a rather low inhibitory activity for both compounds.

Finally, the availability of these compounds and the corresponding dioxido derivatives as pure enantiomers will permit to obtain more information about the stereochemical requirements involved in the biological activities of these intermediates on both SE and OSLC enzymatic systems. Work along this line is in progress in our laboratory.

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8. *Assay method for SE.* a) Sprague-Dawley male rats were sacrificed during the dark period and microsomes were prepared as described by Satoh at al. (Satoh, T.; Hidaka, Y.; Kamei, T.*J. Lipid Res.* **1990**, *31*, 2095-2101). Oxido- and dioxidosqualenes used as substrates and standards were prepared

according to ref. 1b. The procedure used for the SE assays was based on that reported by Tai and Bloch (see ref. 7a) with minor modifications. Thus, isopropyl alcohol solutions of the substrate (endogen plus exogen squalene, 10 μM, containing 50.000 dpm of [4,8,12,13,17,21-^{3}H]-squalene, 15.5 Ci/mmol, NEN) and inhibitors were added to the test tubes (the alcohol contents did not exceed 1% of the overall test mixture), followed by addition of Tween-80 (0.075% w/v), 1 mM EDTA, 80 μM *N,N*-dimethyldodecylamine *N*-oxide (an inhibitor of OSLC, Ceruti, M.; Delprino, L.; Cattel, L.; Bouvier-Navé, P.; Duriatti, A.; Schuber, F.; Benveniste, P. *J. Chem. Soc. Chem. Comm.* **1985**, 1054-1055.), rat liver microsomal protein (2.3 mg/mL), cytosolic protein (8.0 mg/mL), 0.1 mM FAD and 1 mM NADPH in a total volume of 1 mL 0.1 M Tris-HCl buffer (pH 7.5). The incubation mixture was shaked for 1 h at 37 °C and quenched by treatment with 1 mL of 10% KOH in methanol for 1 h at 37 °C. Then, the mixture was extracted 3 times with an equivalent volume of 3:2 hexane:AcOEt. The combined extracts were evaporated to dryness, redissolved in CH_2Cl_2 (3 x 60 μl), spotted onto a silica gel TLC plate and eluted with a 10:1 hexane:methyl *tert*-butyl ether mixture. Bands corresponding to squalene and 2,3-oxidosqualene were detected with an RITA TLC-radioscanner (Isomess), cutted off and radioactivity was counted with a liquid scintillation counter (LKB 1217 Rackbeta). The endogen squalene (1.4 nmol/mL incubation mixture) was determined by treatment of 1 mL of the assay suspension with 10% KOH in methanol (1 h at 37 °C), followed by addition of dotriacontane as internal standard, extraction with 3:2 hexane:AcOEt, purification by column chromatography and capillary GC analysis of the eluates. Incubations were performed by duplicate and a minimum of two experiments per point were carried out. SE activity was 1.28 ± 0.08 nmol/h.mg protein (N = 6). The IC_{50} values for each inhibitor were determined by interpolation from the respective plot of percent inhibition vs. log of inhibitor concentration (5-80 μM). Protein contents were determined according to Bradford (Bradford, M M. *Anal. Biochem.* **1976**, *72*, 248-254).

b) In the experiments carried out to identify the produced dioxidosqualenes, isopropyl alcohol solutions of the corresponding oxidosqualene (**1**, **2** or **3**, 75 μM, as racemates) were added to the test tubes, followed by addition of Tween-80 (0.075% w/v), 1 mM EDTA, 150 μM *N,N*-dimethyldodecylamine *N*-oxide, rat liver microsomal protein (2.9 mg/mL), cytosolic protein (9.8 mg/mL), 0.1 mM FAD and 1 mM NADPH in a total volume of 12 mL 0.1 M Tris-HCl buffer (pH 7.5). The mixture was incubated for 14 h at room temperature, and then quenched and extracted as indicated above. Dioxidosqualenes were purified by column chromatography on silica gel impregnated with Et_3N, eluting with a hexane:methyl *tert*-butyl ether gradient mixture, and identified by comparison with authentic standards from synthetic origin [1b] (GC, reversed phase HPLC, GC/MS/CI in the positive mode using methane as ionization gas). Quantification of formed dioxidosqualenes was performed by HPLC (Spherisorb ODS-2, 85:15 CH_3CN:H_2O at 1 mL/min, λ = 210 nm). A calibration curve was established with known injected amounts of the corresponding dioxidosqualene ranging from 10-100 pmol.

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10. Dioxide **5** (10 mg) [1b] was treated with 5 mL of a 3:2 THF:H_2O solution containing 10 μL of $HClO_4$ for 2 h at room temp.. The crude reaction mixture was treated with $NaHCO_3$ satd. soln. (100 μL) and solvents were evaporated under vacuum. The residue was extracted with a 1:1 hexane: methyl *tert*-butyl ether mixture, dried with $MgSO_4$ and purified by column chromatography on silica gel (4:1 hexane: methyl *tert*-butyl ether) to give epoxydiols **9** (as a diastereomer mixture) in 70% yield. 2,3-Dihydro-2,3-dihydroxy-

18,19-oxidosqualene (**9**): ^{1}H NMR (300 MHz, $CDCl_3$): δ, 5.28–5.02 (4 H), 3.35 (br.d, 1 H, J = 9 Hz), 2.72 (t, 1 H, J = 6.5 Hz), 2.43-2.30 (1 H, OH), 2.30-1.90 (14 H), 1.85-1.70 (1 H, OH), 1.68 (s, 3 H), 1.62 (s, 9 H), 1.60 (s, 3 H), 1.70-1.00 (6 H), 1.26 (s, 3 H), 1.20 (s, 3 H), 1.16 (s, 3 H). ^{13}C NMR (50 MHz): δ, 135.1 (C), 134.9 (C), 134.2 (C), 131.8 (C), 125.0 (CH), 124.8 (CH), 124.3 (CH), 123.7 (CH), 78.3 (CH), 72.9 (C), 63.4 (CH), 60.9 (C), 39.6 (CH_2), 38.8 (CH_2), 36.8 (CH_2), 36.3 (CH_2), 29.6 (CH_2), 28.2 (CH_2), 28.1 (CH_2), 27.3 (CH_2), 26.56 (CH_2), 26.4 (CH_3), 25.7 (CH_3), 23.9 (CH_2), 23.2 (CH_3), 17.7 (CH_3), 16.5 (CH_3), 16.0 (CH_3), 16.0 (CH_3), 15.9 (CH_3).

Following a general procedure (Ward, D. E.; Rhee, C. K. *Tetrahedron Lett.* **1991**, *32*, 7165-7166), a mixture of oxalyl chloride (10 μL, 0.114 mmol), (*R*)-MTPA (5.6 mg, 0.024 mmol) and DMF (2 mg, 0.026 mmol) in hexane (2 mL) was allowed to react for 1 h at room temp. in an inert atmosphere. The crude reaction mixture was filtered under the same conditions and solvent was evaporated under vacuum. The residue was added to a mixture of epoxydiol **9** (8 mg, 0.017 mmol), triethylamine (8 μL, 0.006 mmol) and few crystals of *N,N*-dimethylaminopyridine in CH_2Cl_2 (2 mL). After stirring 2 h at room temperature, the solvent was evaporated and the residue was treated with $NaHCO_3$ satd. soln., extracted with 9:1 hexane: methyl *tert*-butyl ether mixture, dried with $MgSO_4$ and purified by column chromatography on silica gel (9:1 hexane: methyl *tert*-butyl ether) to give esters **10** (as a diastereomer mixture). **10**: ^{1}H NMR (300 MHz, $CDCl_3$): δ, 7.68–7.57 (2 H), 7.47–7.37 (3 H), 5.25–5.03 (4 H), 4.99 (dd, 1 H, J_1 = 10 Hz, J_2 = 2 Hz), 3.58 (br. 3 H, OCH_3), 2.71 (t, 1 H, J = 6.5 Hz), 2.22-1.70 (14 H), 1.95-1.75 (1 H, OH), 1.68 (s, 3 H), 1.62, 1.61, 1.60 (3 singlets, 9 H), 1.56 (s, 3 H, one diast.), 1.52 (s, 3 H, one diast.),1.70-1.00 (6 H), 1.25 (s, 3 H), 1.23 (s, 3 H, one diast.), 1.17 (s, 3 H, one diast.), 1.16 (s, 3 H, one diast.), 1.13 (s, 3 H, one diast.). ^{13}C NMR (75 MHz): δ, 166.9 (CO), 166.4 (CO), 135.1 (C), 134.2 (C), 133.5 (C), 132.1 (C), 131.8 (C), 129.7 (CH), 129.6 (CH), 128.4 (CH), 127.7 (CH), 127.4 (CH), 125.3 (CH), 125.2 (CH), 124.8 (CH), 124.5 (q, J = 275 Hz, CF_3), 124.2 (CH), 123.7 (CH), 82.7 (CH), 82.4 (CH), 72.7 (C), 72.4 (C), 63.4 (CH), 60.8 (C), 55.5 (OCH_3), 39.6 (CH_2), 38.8 (CH_2), 36.3 (CH_2), 36.1 (CH_2), 35.8 (CH_2), 28.7 (CH_2), 28.2 (CH_2), 28.2 (CH_2), 27.3 (CH_2), 27.0 (CH_2), 26.6 (CH_2), 26.3 (CH_3), 25.7 (CH_3), 24.5 (CH_3), 23.9 (CH_2), 23.8 (CH_2), 17.6 (CH_3), 16.5 (CH_3), 16.0 (CH_3), 16.0 (CH_3), 15.9 (CH_3), 15.9 (CH_3). ^{19}F NMR (275 MHz): δ, 4.90, 4.87 (CF_3COOH used as internal reference).

11. In our opinion, the asymmetric carbon atoms of the epoxide moiety are too far from the site of derivatization to cause further resolution in the HPLC profile of the mixture of standards. Likewise, the ^{19}F NMR spectrum of this mixture also showed only two sharp singlets attributed to the diastereomers with (3*R*) and (3*S*) configuration (cf. note 10).