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Characterization of the two major CYP450 metabolites of ozonide (1,2,4-trioxolane) OZ277

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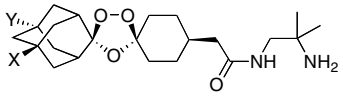
Abstract—The antimalarial synthetic ozonide OZ277 (RBx11160) was hydroxylated by human liver microsomes at the distal bridgehead carbon atoms of the spiroadamantane substructure to form two carbinol metabolites devoid of antimalarial activity.

The antimalarial sesquiterpene lactone artemisinin contains a peroxide bond in the form of a 1,2,4-trioxane heterocycle.[1] The peroxide bond in artemisinin, semisynthetic artemisinins, and synthetic peroxides is essential, but not sufficient, for high antimalarial efficacy.[1,2] A synthetic peroxide antimalarial drug has yet to be identified,[3,4] although a synthetic ozonide (OZ277 or RBx11160)[5] is now in Phase II clinical trials. Understanding the stability of the pharmacophoric peroxide bond in OZ277 (**1**)[6] to cytochrome P450 (CYP450) metabolism is key to elucidating its pharmacokinetics and pharmacodynamics. To this end, we studied the reaction profile of **1** with human liver microsomes, and we now report the structural identification, synthesis,[7] and antimalarial activity of two major OZ277 hydroxylated metabolites **2** (OZ397) and **3** (OZ381) (Fig. 1).



1 (OZ277) X, Y = H
2 (OZ397) X = OH, Y = H
3 (OZ381) X = H, Y = OH

Figure 1. Ozonide **1** (OZ277) and its two hydroxylated metabolites **2** (OZ397) and **3** (OZ381).

Incubation of **1** (Fig. 2A) with human liver microsomes in vitro produced three new peaks on LC/MS (Fig. 2B), each with an increase in molecular weight of 16 suggesting the presence of hydroxylated metabolites.[8] The hydroxylated metabolite peaks were further characterized by MS/MS (Fig. 2D and E). The MS/MS fragmentation of spiroadamantane ozonides (such as **1**) in ESI–MS is characterized by peroxide bond scission and subsequent rearrangement resulting in the elimination of an adamantane lactone fragment with the loss of 166 mass units (Fig. 2C). Peaks b and c exhibited a loss of 182 mass units in the MS/MS spectra indicating incorporation of an oxygen atom in the adamantane substructure (Fig. 2D). The minor hydroxylated metabolite (peak d) showed a loss of 166 mass units indicating that the adamantane moiety had not been modified and that incorporation of the oxygen atom had occurred at the cyclohexyl side of the molecule (Fig. 2E). Although the sites of hydroxylation could not be deduced from the MS/MS fragmentation spectra alone, it was speculated that the sites of hydroxylation of the two major metabolites (peaks b and c) were at the two distal bridgehead positions. This is consistent with the observed preference for bridgehead oxidation in

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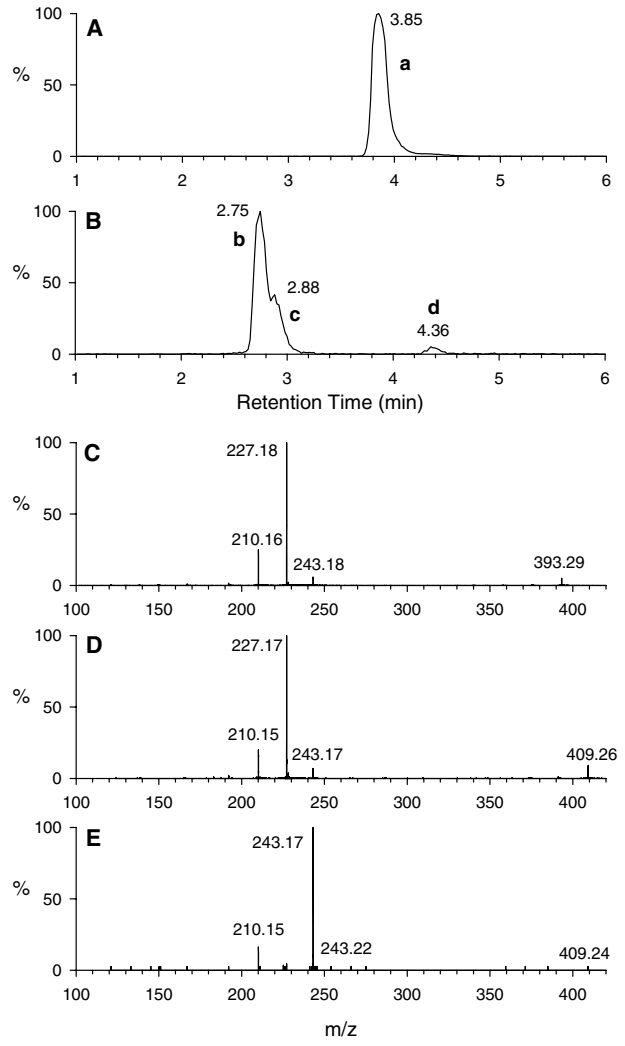


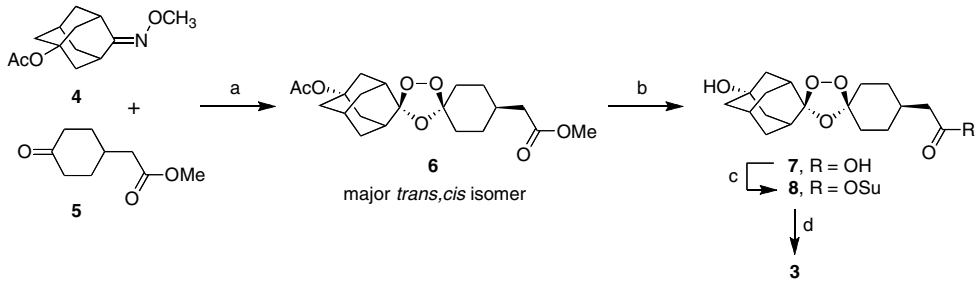
Figure 2. Mass chromatograms of (A) ozonide **1** (OZ277) and (B) three hydroxylated metabolites formed upon incubation with human liver microsomes. Peak a, ozonide **1** (OZ277); peak b, adamantane hydroxylated metabolite **2** (OZ397); peak c, adamantane hydroxylated metabolite **3** (OZ381) and peak d, cyclohexyl hydroxylated metabolite. MS/MS spectra of (C) ozonide **1** (OZ277), (D) adamantane hydroxylated metabolites **2** (OZ397) and **3** (OZ381), and (E) the cyclohexyl hydroxylated metabolite.

adamantane-containing structures.[9] We also reasoned that hydroxylation at the two proximal bridgehead carbon atoms was less likely to occur due to an inductive effect of the ozonide heterocycle. Based on this hypothesis, we synthesized putative hydroxylated metabolites **2** and **3** (vide infra) and found that they had identical chromatographic retention characteristics and MS fragmentation patterns to the two major in vitro metabolites formed in human liver microsomes. A repeat of the in vitro metabolism studies quantitating metabolite formation indicated that **2** was the major, and **3** was the minor, metabolite, in an approximate 4:1 ratio.

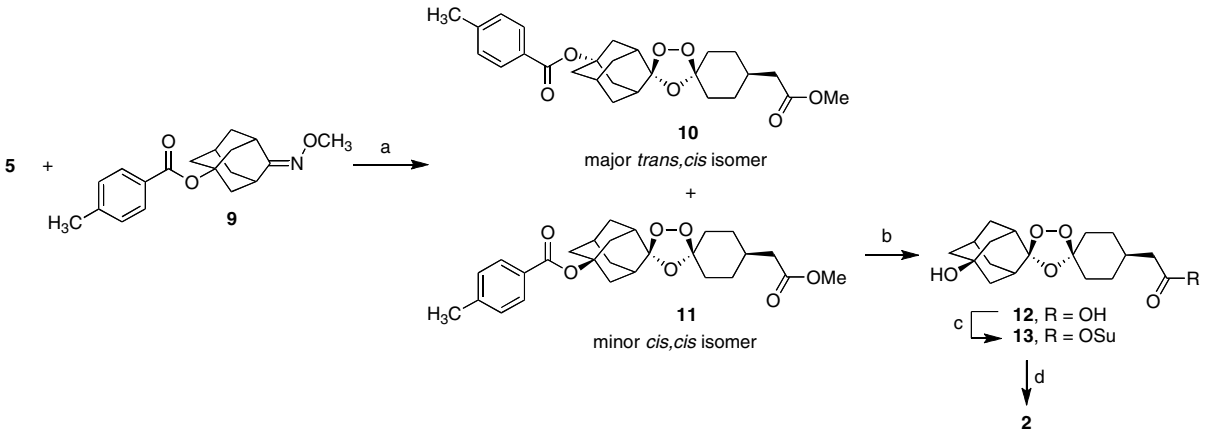
Minor metabolite **3**[10] was obtained in a four-step sequence (Scheme 1) starting with a Griesbaum coozonolysis[11,12] reaction between 5-acetoxy-2-adamantanone *O*-methyl oxime (**4**) and keto ester **5**. Oxime ether **4** was obtained by treatment of 5-hydroxy-2-adamantanone with methoxylamine HCl and pyridine (97%) followed by acetylation with Ac_2O, pyridine, and DMAP (cat.) (91%). Ozonolysis of unsymmetrical oxime ethers such as **4** produce enantiomeric carbonyl oxide intermediates, which in cycloaddition reactions with 4-substituted cyclohexanones (such as **5**) could form four isomeric ozonides.[13] Based on our previous data,[14,15] we expected to observe the formation of two major ozonide isomers with substituent and peroxo groups at the equatorial and axial positions in the cyclohexane ring. Indeed, proton and carbon NMR of the purified (sg, 10% ether in hexanes) reaction mixture (30%) indicated formation of two predominant ozonide diester isomers[16] from which we were able to crystallize the major isomer **6** in 17% yield. Conversion of **6** to minor metabolite **3** was relatively straightforward beginning with ester hydrolysis (96%) to hydroxy acid **7** followed by conversion to active ester **8** (89%). Amide bond (80%) and salt formation with TsOH (94%) completed the reaction sequence. The *trans*,*cis* configuration of **6**, and therefore of **3**, was assigned based on the conversion of *trans*,*cis* ozonide diester **10** (vide infra) to **7** (86%).

Similarly, major metabolite **2**[17] was obtained in a four-step sequence (Scheme 2) starting with a Griesbaum coozonolysis[11,12] reaction between 5-(4-methylbenzoxy)-2-adamantanone *O*-methyl oxime (**9**) and keto ester **5** to afford, after chromatography (sg, 0–20% ether in hexanes), a mixture of four ozonide diester diastereomers (54%).[13,16] Oxime ether **9** was obtained by acylation of 5-hydroxy-2-adamantanone with 4-methylbenzoyl chloride in pyridine (90%) followed by treatment with methoxylamine HCl and pyridine (94%). The 4-methylbenzoate (rather than acetate) was chosen to increase molecular weight to allow for more convenient fractional crystallization of the minor ozonide diester isomers, and to provide convenient benzylic singlet proton NMR signals to distinguish between isomers. Repeated chromatography (sg, 8% ether in hexanes) gave four fractions (isomers A + B), isomer B, isomers B + C, and isomers C + D. Crystallization of isomer B from acetone gave **10** as colorless crystals, established as the *trans*,*cis* diastereomer by X-ray crystallographic analysis (Fig. 3). Repeated chromatography (sg, 8% ether in hexanes) of isomers B + C gave isomer C which was crystallized from acetone to give **11** as colorless crystals, established as the *cis*,*cis* diastereomer by X-ray crystallographic analysis (Fig. 3). Diester ozonide **11** was converted to major metabolite **2** following a similar sequence to that described for minor metabolite **3**: ester hydrolysis to hydroxy acid **12** (81%), conversion to active ester **13** (83%) followed by amide bond formation and conversion to the tosylate salt (71% combined yield).

The crystallization conditions described above delivered suitable crystals for X-ray analysis of **10** and **11**.[18] In both cases, single crystals were mounted in a loop and data were collected on a STOE Imaging Plate Diffraction System (STOE, Darmstadt) with Mo-radiation (0.71 Å) at room temperature. Data were processed with STOE IPDS-software and the crystal structures were



Scheme 1. Reagents and conditions: (a) O_3, cyclohexane:CH_2Cl_2 3:1, 0 °C; (b) 1.25 M aq NaOH:EtOH:THF 0.8:1:1, 50 °C, 4 h, then acidification to pH 3 with 1 M HCl; (c) HOSu, EDCI, DMF, rt, 24 h; (d) 1,2-diamino-2-methylpropane, $CHCl_3$, rt, 1 h, then PTSA, EtOH, rt.



Scheme 2. Reagents and conditions: (a) O_3, cyclohexane:CH_2Cl_2 4:1, 0 °C; (b) 1 M aq NaOH:EtOH:THF 1:1:1, 50 °C, 18 h, then acidification to pH 4 with 0.2 M HCl; (c) HOSu, EDCI, DMF, rt, 24 h; (d) 1,2-diamino-2-methylpropane, $CHCl_3$, rt, 3 h, then PTSA, ether:CH_2Cl_2 5:1, rt.

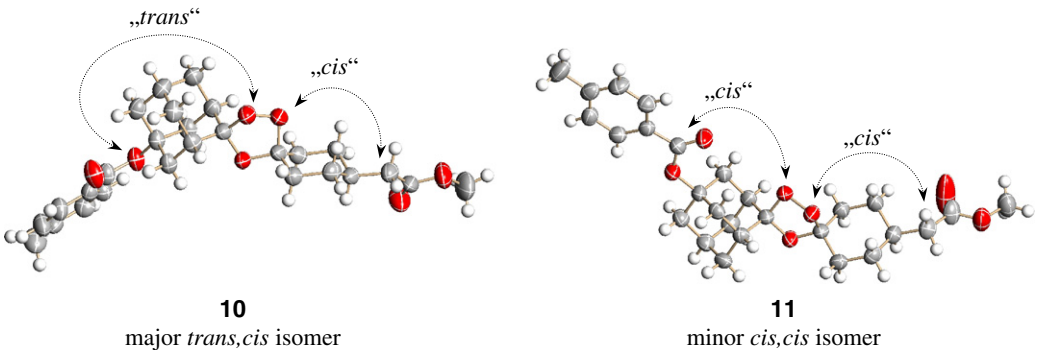


Figure 3. Ellipsoid plots of diester ozonides **10** and **11**; displacement ellipsoids are shown at the 50% probability level. In both structures, the methoxycarbonylmethyl and epoxide substituents are in equatorial positions on the cyclohexane ring, and the peroxide substituent is in the axial position. In **10** and **11**, the 4-methylbenzoate is *trans* and *cis*, respectively, to the peroxide substituent.

solved and refined with ShelXTL (Bruker AXS, Karlsruhe). The relative configuration of **10** was confirmed by an independent X-ray analysis of a second crystal.

Somewhat unexpectedly, we found that both **2** and **3** had IC_{50} values >100 ng/mL against the chloroquine-resistant K1 strain of *Plasmodium falciparum* in vitro; in comparison, **1** has an IC_{50} of 1.0 ng/mL against this same parasite strain.[5] The complete lack of antiplasmodial activity of **2** and **3** demonstrates the essential contribution of an unsubstituted spiroadamantane ring system to the antimalarial properties of **1**. It is conceivable that the steric hindrance provided by the bridgehead carbinols in **2** and **3** prevents efficient alkylation reactions of the spiroadamantane-derived secondary carbon-centered radicals[19] from occurring when the ozonide reacts with iron(II) in the parasite. Consistent with a general SAR trend for this class of antimalarial peroxides,[20] it is also likely that the greater polarity of **2** and **3** (Log *D* 1.8) vs. **1** (Log *D* 3.2) may, in part, account for the inactivity of the former.

Acknowledgment

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8. Ozonide **1** was incubated for 2 h with human liver microsomes (BD Gentest, Discovery Labware Inc., Woburn, MA) at a substrate concentration of 1 μM and a microsomal protein concentration of 0.4 mg/mL as previously described.[5] Loss of parent compound and appearance of metabolites were monitored by LC/MS. LC/MS analysis was conducted on a Waters (Milford, MA) Micromass Q-TOF Micro quadrupole-time-of-flight mass spectrometer coupled to a Waters Alliance 2795 HPLC. Chromatographic separation was achieved using an acetonitrile–water gradient (containing 0.05% formic acid) at a flow rate of 0.4 mL/min and a Phenomenex (Torrance, CA) Luna C8(2) column (50 × 2.1 mm, 5 μm particle size) equipped with a precolumn of the same packing material. Formation of metabolites was monitored using MS full scans and confirmed using collision-induced dissociation experiments.
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16. Two major isomers were formed in an approximate ratio of 3:1; the two minor isomers could not be quantified due to overlapping low intensity signals.
17. *cis,cis*-5-Hydroxyadamantane-2-spiro-3′-8′-[[[(2′-amino-2′-methylpropyl)-amino]carbonyl]methyl]-1′,2′,4′-trioxaspiro[4.5]decane *p*-tosylate (**2**): mp 150–152 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.04–1.20 (m, 2H), 1.16 (s, 6H), 1.44–1.82 (m, 17H), 1.94–2.02 (m, 3H), 2.07 (d, J = 7.3 Hz, 2H), 2.29 (s, 3H), 3.19 (d, J = 5.8 Hz, 2H), 4.47 (br s, 1H), 7.11 (d, J = 8.3 Hz, 2H), 7.47 (d, J = 7.8 Hz, 2H), 7.67 (br s, 3H), 8.05 (t, J = 6.0 Hz, 1H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 20.94, 23.50, 28.61, 29.70, 33.35, 33.43, 37.58, 41.97, 44.50, 46.04, 54.54, 65.09, 108.65, 110.11, 125.66, 128.18, 137.66, 146.06, 172.55. Anal. Calcd for $C_{29}H_{44}N_2O_8S$: C, 59.98; H, 7.64; N, 4.82. Found: C, 59.97; H, 7.40; N, 4.93.
18. Crystallographic data (excluding structural factors) for structures **10** and **11** in this paper have been deposited with the Cambridge Crystallographic Data Centre (CCDC) as supplementary publication numbers CCDC 671176 and 671177. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].
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