Comparative Antimalarial Activities and ADME Profiles of Ozonides (1,2,4-trioxolanes) OZ277, OZ439, and Their 1,2-Dioxolane, 1,2,4-Trioxane, and 1,2,4,5-Tetraoxane Isosteres

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Comparative Antimalarial Activities and ADME Profiles of Ozonides (1,2,4-trioxolanes) OZ277, OZ439, and Their 1,2-Dioxolane, 1,2,4-Trioxane, and 1,2,4,5-Tetraoxane Isosteres

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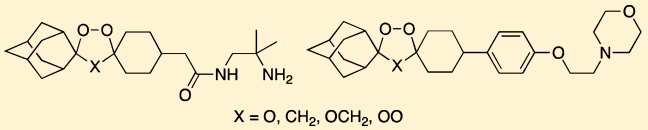
ABSTRACT: To ascertain the structure–activity relationship of the core 1,2,4-trioxolane substructure of dispiro ozonides OZ277 and OZ439, we compared the antimalarial activities and ADME profiles of the 1,2-dioxolane, 1,2,4-trioxane, and 1,2,4,5-tetraoxane isosteres. Consistent with previous data, both dioxolanes had very weak antimalarial properties. For the OZ277 series, the trioxane isostere had the best ADME profile, but its overall antimalarial efficacy was not superior to that of the trioxolane or tetraoxane isosteres. For the OZ439 series, there was a good correlation between the antimalarial efficacy and ADME profiles in the rank order trioxolane > trioxane > tetraoxane. As we have previously observed for OZ439 versus OZ277, the OZ439 series peroxides had superior exposure and efficacy in mice compared to the corresponding OZ277 series peroxides.



The discovery of artemisinin from *Artemisia annua* in the early 1970s gave rise to the semisynthetic artemisinins[1] and, more recently, to structurally diverse synthetic peroxide[2] antimalarials. Although the precise mechanism of action of these drugs is still not fully understood, it is hypothesized that the pharmacophoric peroxide bond[3] undergoes reductive activation by heme released during parasite hemoglobin digestion[4] to produce carbon-centered radicals that alkylate heme and parasite proteins.[2a,5] The first synthetic peroxide drug development candidates were fenozan B07[6] and arteflene,[7] of which the latter progressed to a phase II clinical trial before further development was discontinued[8] (Figure 1).

In 2012, ozonide OZ277 (**1a**),[9] also known as arterolane maleate,[10] was approved for the Indian market as a combination product with piperaquine phosphate (Synriam) for the treatment of malaria. More recently, the next-generation ozonide OZ439 (**1b**)[11] has completed phase IIa clinical trials (Figure 2). In addition to these ozonides (1,2,4-trioxolanes), 1,2,4-trioxane PA1103/SAR116242[12] and 1,2,4,5-tetraoxane RKA 182[13] were identified as synthetic peroxide drug development candidates.[8] To ascertain the structure–activity relationship of the core 1,2,4-trioxolane substructure in dispiro 8′-alkyl (**1a**) and 8′-aryl (**1b**) 1,2,4-trioxolanes, we now describe the synthesis and comparative antimalarial activities and ADME profiles of the 1,2-dioxolane (**2**), 1,2,4-trioxane (**3**), and 1,2,4,5-tetraoxane (**4**) isosteres (Figure 2). This study is the first to provide a head-to-head comparison of these four peroxide heterocycles within a common structural framework.

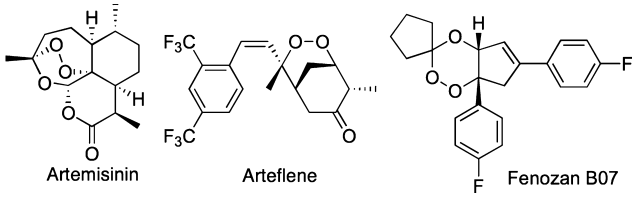


Figure 1. Artemisinin and first generation synthetic peroxides.

CHEMISTRY

Ozonides **1a** and **1b** were obtained via Griesbaum coozonolysis[14] as previously described.[2c,9,11a] The key step in the synthesis of 1,2-dioxolanes **2a** and **2b** was fragmentation of triethylsilylperoxy ketals **7** and **8** with $SnCl_4$[15] to form the corresponding peroxycarbenium ions that underwent annulation[16] with 2-methyleneadamantane[17] to form dioxolane esters **9** and **10** (Scheme 1). Subsequent hydrolysis followed by either amide or ether bond formation afforded **2a** and **2b**, respectively. Dioxolanes **2a** and **2b** were obtained as single diastereomers and assigned a cis configuration based on the stereochemistry observed in similar reactions.[16]

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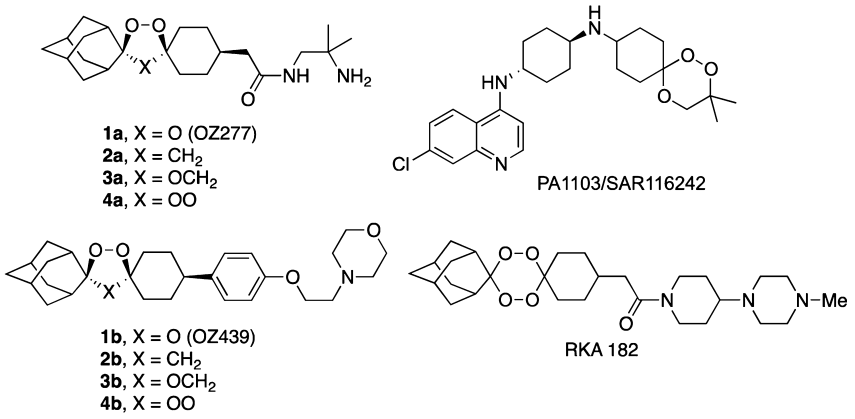
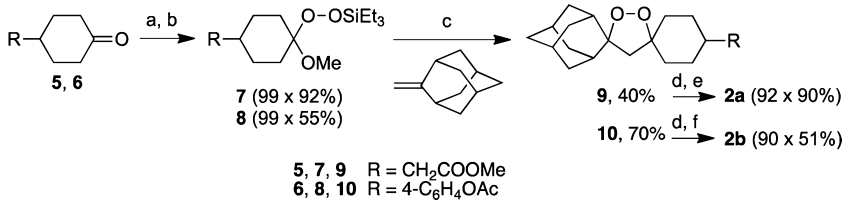


Figure 2. Next generation synthetic peroxides and OZ277 and OZ439 isosteres.

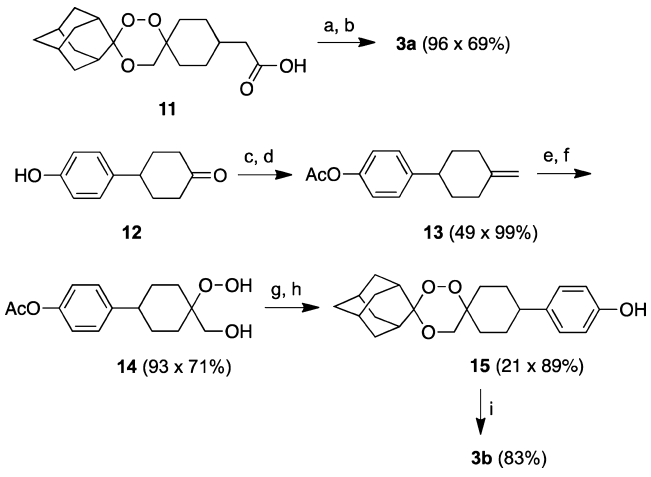
Scheme 1[a]



[a]Reagents and conditions: (a) 30% H_2O_2, I_2, MeOH, rt, 24 h; (b) Et_3SiOTf, Et_3N, DMF, 0 °C to rt, 24 h; (c) 2-methyleneadamantane, 1 N $SnCl_4$ in CH_2Cl_2, −78 to −30 °C, 12 h; (d) 15% aq KOH, EtOH, 60 °C, 20 h, then AcOH; (e) (1) HOSu, EDCI, DMF, rt, 24 h, (2) 1,2-diamino-2-methylpropane, $CHCl_3$, rt, 4 h, (3) *p*-TsOH, ether; (f) powdered NaOH, $(Bu)_4NHSO_4$, *N*-(2-chloroethyl)morpholine HCl, CH_3CN, rt to 60 °C, 12 h, then MSA, CH_2Cl_2/ether, rt.

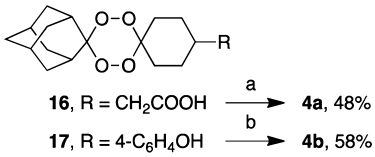
1,2,4-Trioxane **3a** was obtained by conversion of trioxane acid **11**[18] into the corresponding HOSu active ester followed by amide bond formation (Scheme 2). The synthesis of trioxane **3b** (Scheme 2) began with conversion of ketophenol **12** into alkylidene acetate **13** followed by epoxidation and regioselective perhydrolysis[19] to form *β*-hydroperoxy alcohol **14**, predominately as its trans isomer.[18] Acid-catalyzed condensation of **14** with 2-adamantanone followed by acetate hydrolysis (Scheme 2) yielded trioxane phenol **15**, which was alkylated with *N*-(2-chloroethyl)morpholine to afford **3b**. 1,2,4,5-Tetraoxanes **4a** and **4b** (Scheme 3) were obtained by condensation of

Scheme 2[a]



[a]Reagents and conditions: (a) HOSu, EDCI, DMF, rt, 24 h; (b) 1,2-diamino-2-methylpropane, $CHCl_3$, rt, 5 h, then *p*-TsOH, EtOAc; (c) $Ph_3P{=}CH_2$, 4-(4-hydroxyphenyl)cyclohexanone, rt for 1 h, then reflux for 12 h; (d) Ac_2O, pyridine, CH_2Cl_2, 0 °C to rt, 12 h; (e) peracetic acid, $CHCl_3$, NaOAc, 0 °C to rt, 12 h; (f) 50% H_2O_2, $MoO_2(acac)_2$, Et_2O, $MgSO_4$, rt, 12 h; (g) 2-adamantanone, CSA, CH_2Cl_2, rt, 12 h; (h) 15% aq KOH, EtOH/THF, 50 °C, 4 h, then AcOH; (i) see (f) for Scheme 1.

Scheme 3[a]



[a]Reagents and conditions: (a) HOSu, EDCI, 1,2-diamino-2-methylpropane, CH_3CN/CH_2Cl_2, rt, 16 h, then MSA, EtOAc; (b) see (f) for Scheme 1.

tetraoxane acid **16**[13] with the corresponding diamine and *O*-alkylation of tetraoxane phenol **17**[18] with *N*-(2-chloroethyl)-morpholine, respectively. Peroxides **1a**−**3a** were isolated and tested as their tosylate salts; the remaining peroxides were isolated and tested as their mesylate salts.

ANTIMALARIAL ACTIVITY

The activity data shown in Table 1 reveals several SAR trends. Consistent with our previous data[17] demonstrating that 1,2-dioxolanes react with iron(II) primarily by two-electron reduction to form inactive diol reaction products rather than one-electron reduction to form carbon-centered radicals, **2a** and **2b** were 2 orders of magnitude less potent than the corresponding ozonides **1a** and **1b**. Erhardt et al.[20] demon-

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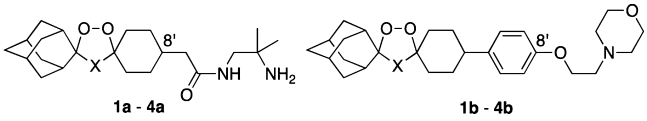
Table 1. Activity of Dispiro Peroxides against *P. falciparum* in Vitro and *P. berghei* in Vivo (1 × 30 mg/kg po)



compd	X	IC_{50} (nM)[a] K1/NF54	in vivo act. (%)[b]	survival (days)	cures[c]
control	–	–	0	6–7	–
1a[d]	O	1.8/1.6	99.9	11	0/5
2a	CH_2	170/440	ND[d]	ND	ND
3a	OCH_2	19/29	99.1	15	0/3
4a	OO	0.90/1.7	99.5	9	0/3
1b[d]	O	2.8/3.4	>99.9	>30	5/5
2b	CH_2	370/1,200	ND	ND	ND
3b	OCH_2	2.2/2.4	99.2	27	3/5
4b	OO	2.9/4.0	99.3	15	0/3
AS[d]	–	3.4/4.2	92	9	0/5
CQ[d]	–	120/9.9	99.9	10	0/5
MF[d]	–	7.2/14	99.6	22	0/5

[a]Mean from n = 2–3. Individual measurements differed by less than 50%. [b]Individual measurements differed by less than 10%. [c]No detectable parasites at 30 days postinfection. [d]Artesunate (AS), chloroquine (CQ), and mefloquine (MF) data from Vennerstrom et al.[9] and Charman et al.[11a] [d]ND = not determined.

strated that β-scission reactions that form carbon-centered radicals are accelerated by the adjacent oxygen atom present in ozonides (1,2,4-trioxolanes), but absent in 1,2-dioxolanes. Although the trioxane (**3a**) and tetraoxane (**4a**) isosteres of OZ277 (**1a**) had good potency against *Plasmodium falciparum* in vitro, neither compound was curative in the *P. berghei* model. Similarly, trioxane **3b** and tetraoxane **4b** were no less potent than OZ439 (**1b**) in vitro, but of these, only **3b** was partially curative in the *Plasmodium berghei* model.

PHYSICOCHEMICAL AND ADME PROPERTIES

Additional studies were conducted to explain why the ozonide heterocycle in this particular dispiro structural framework (**1a** and **1b**) appears to have the best overall antimalarial profile.

The predicted physicochemical parameters (Table 2) indicated that OZ277 and its isosteres were more basic and less lipophilic than OZ439 and its isosteres. While the OZ277 series would be expected to have better aqueous solubility at physiological pH, the OZ439 series would likely have better permeability properties due to a higher log D and lower polar surface area (PSA). However, within the two peroxide series, the differences in these calculated physicochemical properties provided little insight to account for the divergent antimalarial activities and ADME properties.

Table 2. Calculated Physicochemical Properties of Dispiro Peroxides

compd	log P/log $D_{pH\ 7.4}$	pK_a	PSA (Å^2)	H-bond donor/acceptor
1a	2.07/0.69	9.05	82.8	3/6
2a	3.21/1.83	9.05	73.6	3/5
3a	2.44/1.05	9.05	82.8	3/6
4a	2.26/0.87	9.05	92.0	3/7
1b	4.63/4.53	6.81	49.4	0/6
2b	5.66/5.56	6.82	40.2	0/5
3b	4.88/4.78	6.82	49.4	0/6
4b	4.81/4.71	6.81	58.6	0/7

Metabolic stability studies in mouse liver microsomes indicated that with the exception of trioxane **3a**, which was quite stable, the remaining OZ277 isosteres (**1a**, **2a**, and **4a**) exhibited similar metabolic stabilities (Table 3). For OZ439 and its isosteres, trioxolane **1b** was marginally more metabolically stable than the remaining isosteres (**2b**, **3b**, and **4b**), and similar to that of OZ277 (**1a**).

Table 3. ADME Properties of Dispiro Peroxides

compd	in vitro CL_{int} (μL/min/mg)/EH[a]	in vitro blood $T_{1/2}$ (h)[b]	in vivo AUC_{0-24h} (nM·h)[c]
1a	17/0.43	0.93	5130[d]
2a	12/0.35	1.0	ND[e]
3a	<7/<0.2	2.3	22377
4a	14/0.37	0.91	13511
1b	14/0.38	10.4	52726
2b	28/0.55	*f*	ND
3b	38/0.63	8.6	34419
4b	31/0.58	2.6	18448

[a]Mouse liver microsomes. [b]Mouse blood. [c]30 mg/kg oral dose to mice. [d]AUC to 8 h only as concentrations were below the limit of quantitation at 24 h. [e]ND = not determined. [f]No measurable degradation.

For the OZ277 series, the stability in mouse blood showed the same trend as seen with the microsomes, with trioxane **3a** being about 2-fold more stable than **1a**, **2a**, and **4a** (Table 3). Consistent with previous results in human and rat blood,[11a] OZ439 (**1b**) was about 10-fold more stable in mouse blood compared to OZ277 (**1a**). No degradation was detected over the 4 h incubation period for dioxolane **2b**, whereas **3b** exhibited similar stability to **1b**, and **4b** was about 4-fold less stable compared to **1b**.

In vivo, **3a** had the highest area under the curve (AUC) of the OZ277 isosteres, consistent with its better metabolic stability and marginally better blood stability; this trioxane also produced the longest average survival time for the OZ277 series, despite having somewhat weaker activity against *P. falciparum* in vitro (Table 3). As shown in Figure 3, panel A, **3a** was also the only compound from the OZ277 series that had plasma concentrations above about 10 ng/mL for more than 24 h. In contrast, concentrations of both **1a** and **4a** dropped below 1 ng/mL by the 24 h time point. Interestingly, the 2-fold increase in the AUC of **4a** vs **1a** did not translate into markedly superior in vivo antimalarial efficacy of the former. For the OZ439 series, the rank ordering of AUC values mirrored the in vitro blood stability data with **1b** and **3b** having similar AUC values, whereas **4b** had an AUC that was approximately 2- to 3-fold lower. The rank order of exposure (i.e., **1b** > **3b** > **4b**) was also consistent with the in vivo efficacy results where **1b** resulted in the longest survival (>30 days), **3b** resulted in an average survival of 27 days, and animals treated with **4b** survived for an average of only 15 days. Each of the compounds from the OZ439 series maintained plasma concentrations above about 30 ng/mL for more than 24 h, with concentrations of **1b** and **3b** being above about 200 ng/mL at the 24 h time point (Figure 3, panel B).

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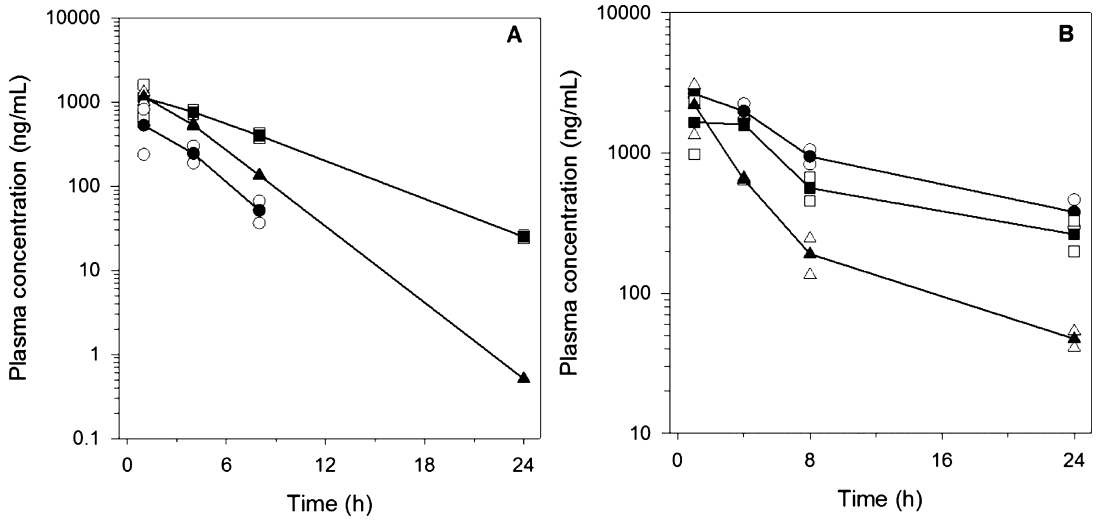

Figure 3. Plasma concentration versus time profiles for (A) OZ277 isosteres and (B) OZ439 isosteres following oral administration of 30 mg/kg to noninfected Swiss outbred mice. Open symbols represent individual concentrations for $n = 2$ mice per time point, and filled symbols represent the average concentrations. Symbols: **1a** and **1b** (circles), **3a** and **3b** (squares), and **4a** and **4b** (triangles). For **1a**, concentrations were below the limit of quantitation of 0.5 ng/mL by the 24 h time point.

SUMMARY AND CONCLUSIONS

In summary, this work confirms that dioxolanes such as **2a** and **2b** have very weak antimalarial properties, which we attribute to their reaction with ferrous iron to form inactive diol reaction products.[17] In the OZ277 series, trioxane **3a** had the best ADME profile, but this was compensated by a somewhat lower antimalarial potency, which may be due in part to subtle differences in the iron(II) and heme reaction profiles of trioxanes, trioxolanes, and tetraoxanes.[19b,21] For the OZ439 series, there was a good correlation between antimalarial efficacy and in vivo exposure (AUC) with the rank order being trioxolane **1b** > trioxane **3b** > tetraoxane **4b**. As we have noted before,[11a] the superior exposure profile of 8′-aryl (OZ439) vs 8′-alkyl (OZ277) ozonides translates into superior antimalarial efficacy (as evidenced by increased survival time) of the former. Our data suggest that this same trend also holds true for trioxanes (e.g., **3b** versus **3a**) and tetraoxanes (e.g., **4b** versus **4a**), which is consistent with recent data[22] indicating that aryl substituted tetraoxanes may have superior antimalarial efficacies compared to alkyl substituted tetraoxanes such as RKA182.[13] Finally, we suggest that the inferior blood stability and PK profiles of tetraoxanes **4a** and **4b** versus the corresponding trioxanes **3a** and **3b** results from the second peroxide bond of the former. For example, each of the degenerate conformers of the tetraoxanes has an equatorial peroxide bond with a more accessible LUMO[4e,19b,21c] that would be expected to react more readily with iron(II) than the corresponding trioxanes which have only one conformer with an equatorial peroxide bond.

EXPERIMENTAL SECTION

General. Melting points are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a 500 MHz spectrometer. All chemical shifts are reported in parts per million (ppm) and are relative to internal $(CH_3)_4Si$ (0 ppm) for ^{1}H and $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.7 ppm) for ^{13}C NMR. Combustion and HPLC analysis confirmed that all target compounds possessed purities ≥95%. As indicated below, starting materials were commercially available or were prepared according to known procedures.

***cis*-Adamantane-2-spiro-3′-8′-[[[(2′-amino-2′-methylpropyl)amino]carbonyl]methyl]-1′,2′-dioxaspiro[4.5]-decane *p*-Tosylate (2a). Step 1.** To a solution of I_2 (0.254 g, 1.0 mmol) and 50% H_2O_2 (4.5 mL, 40 mmol) in MeOH[15b] (50 mL) was added methyl 2-(4-oxocyclohexyl)acetate (**5**) (1.70 g, 10 mmol). The mixture was stirred at rt for 24 h and concentrated to a residue that was partitioned between CH_2Cl_2 (30 mL) and water (30 mL). The aqueous layer was extracted with CH_2Cl_2 (2 × 30 mL). The combined extracts were washed with water and brine, dried over $MgSO_4$, filtered, and concentrated to afford methyl 2-(4-hydroperoxy-4-methoxycyclohexyl)acetate as a 1:1 mixture of diastereomers (2.15 g, 99%), which was used immediately in the next step: ^{1}H NMR ($CDCl_3$) δ 0.92−2.46 (m, 11H), 3.30 (s, 1.5H), 3.34 (s, 1.5H), 3.70 (s, 3H), 7.42 (s, 0.5H), 7.52 (s, 0.5H). **Step 2.** The unpurified methyl 2-(4-hydroperoxy-4-methoxycyclohexyl)acetate (2.15 g, 9.86 mmol) in DMF (100 mL) was treated with Et_3N (4.5 mL, 32 mmol) and Et_3SiOTf (2.54 mL, 12 mmol) at 0 °C. The reaction mixture was stirred at rt for 24 h and then diluted with ice-cold hexane (100 mL) and ice−water (100 mL). The organic layer was separated, and the aqueous layer was extracted with hexane (3 × 100 mL). The extracts were combined, dried over $MgSO_4$, and concentrated to afford methyl 2-[4-methoxy-4-(triethylsilylperoxy)cyclohexyl]acetate (**7**) as a 1:1 mixture of diastereomers (3.02 g, 92%), which was used immediately in the next step: ^{1}H NMR ($CDCl_3$) δ 0.68−0.80 (m, 6H), 0.94−1.08 (m, 9H), 0.84−2.44 (m, 11H), 3.26 (s, 1.5H), 3.29 (s, 1.5H), 3.67 (s, 3H). **Step 3.** To a −78 °C solution of **7** (3.02 g, 9.10 mmol) in CH_2Cl_2 (50 mL) was added 2-methyleneadamantane (0.67 g, 4.53 mmol) followed by 1 M $SnCl_4$ in CH_2Cl_2 (10 mL, 10 mmol). The resulting mixture was stirred at −78 °C for 30 min and then kept at −30 °C overnight. The reaction mixture was allowed to warm to −3 °C and quenched with ice−water (50 mL). After separation of the organic layer, the aqueous layer was extracted with CH_2Cl_2 (2 × 50 mL). The combined extracts were washed with water (50 mL) and brine (50 mL), dried over $MgSO_4$, filtered, and concentrated. Purification by silica gel (sg) chromatography (0−10% ether in hexane) afforded *cis*-adamantane-2-spiro-3′-8′-[(methoxycarbonyl)-methyl]-1′,2′-dioxaspiro[4.5]decane (**9**) as a colorless solid (0.60 g, 40%): mp 119−120 °C; ^{1}H NMR ($CDCl_3$) δ 1.24−1.36 (m, 2H), 1.44−1.96 (m, 17H), 1.95−2.02 (m, 2H), 2.06−2.14 (m, 2H), 2.13 (s, 2H), 2.20 (d, J = 7.5 Hz, 2H), 3.66 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 26.44, 26.99, 29.07, 33.45, 34.93, 35.64, 36.24, 37.21, 41.17, 51.42, 55.47, 84.02, 88.71, 173.47. **Step 4.** To a solution of **9** (0.45 g, 1.35 mmol) in EtOH (20 mL) was added 15% aq KOH solution (2 mL). The resulting mixture was stirred at 60 °C for 20 h, concentrated to ∼5 mL, then diluted with water (10 mL), and acidified with acetic acid (5 mL). The precipitate was collected by filtration, washed with cold water, and dried in vacuo at 40 °C to afford *cis*-adamantane-2-spiro-3′-

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8′-(carboxymethyl)-1′,2′-dioxaspiro[4.5]decane as a colorless solid (0.40 g, 93%): mp 184−185 °C; ^{1}H NMR ($CDCl_3$) δ 1.27−1.40 (m, 2H), 1.42−1.96 (m, 17H), 1.95−2.04 (m, 2H), 2.06−2.14 (m, 2H), 2.13 (s, 2H), 2.24 (d, J = 7.5 Hz, 2H), 11.14 (brs, 1H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 26.44, 26.99, 29.00, 33.26, 33.45, 34.90, 35.64, 36.24, 37.20, 41.04, 55.46, 83.98, 88.75, 178.74. **Step 5.** A solution of *cis*-adamantane-2-spiro-3′-8′-(carboxymethyl)-1′,2′-dioxaspiro[4.5]-decane (0.22 g, 0.69 mmol), HOSu (0.09 g, 0.78 mmol), and EDCI (0.15 g, 0.78 mmol) in DMF (10 mL) was stirred at rt for 24 h. Under ice cooling, the reaction was quenched with water (30 mL). The precipitate was collected by filtration, washed with cold water, and dried in a vacuum oven at 40 °C to afford the dioxolane active ester as a colorless solid (0.28 g, 99%): mp 188−189 °C; ^{1}H NMR ($CDCl_3$) δ 1.33−1.98 (m, 19H), 1.98−2.06 (m, 2H), 2.06−2.14 (m, 2H), 2.13 (s, 2H), 2.27 (d, J = 7.0 Hz, 2H), 2.96 (s, 4H). **Step 6.** To a solution of 1,2-diamino-2-methylpropane (0.30 g, 3.41 mmol) in $CHCl_3$ (10 mL) was added dropwise to the solution of the dioxolane active ester (0.28 g, 0.68 mmol) in $CHCl_3$ (10 mL). The resulting mixture was stirred at rt for 4 h and then quenched with water (10 mL). After separation of the organic layer, the aqueous layer was extracted with $CHCl_3$ (2 × 20 mL). The combined extracts were washed with water (2 × 10 mL) and brine (10 mL), dried over $MgSO_4$, filtered, and concentrated. The residue was dissolved in ether (10 mL), and then the solution of *p*-toluenesulfonic acid monohydrate (0.14 g) in ether (10 mL) was added. The precipitate was collected by filtration to afford **2a** as a colorless solid (0.35 g, 90%): mp 224−225 °C; ^{1}H NMR ($CDCl_3$) δ 0.96−1.08 (m, 2H), 1.33 (s, 6H), 1.24−1.94 (m, 21H), 2.06 (s, 2H), 2.02−2.16 (m, 2H), 2.39 (s, 3H), 3.39 (d, J = 6.5 Hz, 2H), 7.25 (d, J = 9.0 Hz, 2H), 7.30 (brs, 3H), 7.66 (d, J = 9.0 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 21.35, 23.98, 26.47, 27.02, 28.92, 32.08, 33.21, 33.48, 34.93, 35.65, 36.24, 37.24, 42.97, 46.23, 55.52, 56.07, 84.00, 88.49, 125.53, 129.44, 140.34, 141.54, 173.26. Anal. Calcd for $C_{30}H_{46}N_2O_6S$: C, 64.03; H, 8.24; N, 4.98. Found: C, 64.08; H, 7.99; N, 4.71.

***cis*-Adamantane-2-spiro-3′-8′-[4′-[2′-(4′-morpholinyl)-ethoxy]phenyl]-1′,2′-dioxaspiro[4.5]decane Mesylate (2b). Step 1.** To a solution of I_2 (0.254 g, 1.0 mmol) and 30% H_2O_2 (4.5 mL, 40 mmol) in MeOH[15b] (50 mL) was added 4-(4-acetoxyphenyl)-cyclohexanone (**6**) (2.32 g, 10 mmol). The mixture was stirred at rt for 24 h and concentrated to a residue that was partitioned between CH_2Cl_2 (30 mL) and water (30 mL). The aqueous layer was extracted with CH_2Cl_2 (2 × 30 mL). The combined extracts were washed with water and brine, dried over $MgSO_4$, filtered, and concentrated to afford 4-(4-hydroperoxy-4-methoxycyclohexyl)phenyl acetate as a 3:2 mixture of diastereomers (2.8 g, 100%), which was used immediately in the next step: ^{1}H NMR ($CDCl_3$) δ 1.52−2.00 (m, 6H), 2.17−2.34 (m, 5H), 2.48−2.62 (m, 1H), 3.34 (s, 1.2H), 3.36 (s, 1.8H), 6.97−7.04 (m, 2H), 7.20−7.26 (m, 2H), 7.58 (s, 0.4H), 7.62 (s, 0.6H); ^{13}C NMR ($CDCl_3$) δ 21.12, 30.06, 30.30, 30.78, 31.30, 33.96, 41.28, 43.01, 48.31, 48.56, 104.90, 105.12, 121.31, 121.33, 127.75, 127.76, 143.65, 143.75, 148.86, 169.73. **Step 2.** The unpurified 4-(4-hydroperoxy-4-methoxycyclohexyl)phenyl acetate (2.8 g, 10 mmol) in DMF (100 mL) was treated with Et_3N (4.5 mL, 32 mmol) and Et_3SiOTf (2.54 mL, 12 mmol) at 0 °C. The reaction mixture was stirred at rt for 24 h and then diluted with ice-cold hexane (100 mL) and ice−water (100 mL). The organic layer was separated, and the aqueous layer was extracted with hexane (3 × 100 mL). The extracts were combined, dried over $MgSO_4$, and concentrated. Purification by sg chromatography (0−10% ether in hexane) afforded 4-[4-methoxy-4-(triethylsilylperoxy)cyclohexyl]phenyl acetate (**8**) as a 2:3 mixture of diastereomers (2.18 g, 55%): ^{1}H NMR ($CDCl_3$) δ 0.68−0.82 (m, 6H), 0.94−1.07 (m, 9H), 1.42−1.84 (m, 6H), 2.18−2.25 (m, 1.2H), 2.29 (s, 1.8H), 2.29 (s, 1.2H), 2.31−2.38 (m, 0.8H), 2.50−2.59 (m, 1H), 3.32 (s, 1.8H), 3.34 (s, 1.2H), 6.97−7.03 (m, 2H), 7.17−7.25 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 3.79, 3.80, 5.79, 6.57, 6.75, 6.79, 21.13, 30.21, 30.51, 31.32, 31.71, 43.01, 43.26, 48.15, 48.40, 104.20, 104.35, 121.26, 121.32, 127.68, 127.79, 143.99, 144.17, 148.82, 169.68. **Step 3.** To a −78 °C solution of **8** (8.0 g, 20 mmol) in CH_2Cl_2 (200 mL) was added 2-methyleneadamantane (3.2 g, 21.6 mmol) followed by 1 M $SnCl_4$ in CH_2Cl_2 (30 mL, 30 mmol). The resulting mixture was stirred at −78 °C for 30 min and then kept at −30 °C overnight. The reaction mixture was allowed to warm up to −3 °C and quenched with ice−water (100 mL). After separation of the organic layer, the aqueous layer was extracted with CH_2Cl_2 (2 × 100 mL). The combined extracts were washed with water (100 mL) and brine (100 mL), dried over $MgSO_4$, filtered, and concentrated. Crystallization from EtOH afforded *cis*-adamantane-2-spiro-3′-8′-(4′-acetoxyphenyl)-1′,2′-dioxaspiro[4.5]-decane (**10**) as a colorless solid (5.60 g, 70%): mp 149−150 °C; ^{1}H NMR ($CDCl_3$) δ 1.52−1.88 (m, 16H), 1.93−1.98 (m, 2H), 2.07−2.16 (m, 4H), 2.18 (s, 2H), 2.28 (s, 3H), 2.45−2.53 (m, 1H), 6.98 (d, J = 8.5 Hz, 2H), 7.19 (d, J = 8.5 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 21.14, 26.46, 27.02, 30.52, 33.47, 35.63, 35.67, 36.28, 37.22, 42.83, 55.61, 83.89, 88.79, 121.25, 127.75, 144.49, 148.70, 169.73. **Step 4.** To a solution of **10** (5.60 g, 14.0 mmol) in THF (80 mL) and EtOH (160 mL) was added 15% aq KOH solution (5 mL) The resulting mixture was stirred at 60 °C for 20 h. The solution was concentrated to ∼5 mL, and the residue was diluted with water (10 mL) and acidified with acetic acid (5 mL). The precipitate was collected by filtration, washed with cold water, and dried in a vacuum oven at 40 °C to afford *cis*-adamantane-2-spiro-3′-8′-(4′-hydroxyphenyl)-1′,2′-dioxaspiro[4.5]-decane as a colorless solid (4.50 g, 90%): mp 210−211 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.52−1.90 (m, 16H), 1.93−2.00 (m, 2H), 2.06−2.17 (m, 4H), 2.18 (s, 2H), 2.38−2.48 (m, 1H), 4.72 (brs, 1H), 6.75 (d, J = 8.5 Hz, 2H), 7.07 (d, J = 8.5 Hz, 2H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 26.45, 27.01, 30.71, 33.48, 35.67, 35.72, 36.28, 37.22, 42.47, 55.63, 84.03, 88.83, 115.10, 127.83, 139.25, 153.71. **Step 5.** To a solution of *cis*-adamantane-2-spiro-3′-8′-(4′-hydroxyphenyl)-1′,2′-dioxaspiro[4.5]decane (0.50 g, 1.40 mmol) in dry acetonitrile (80 mL) were added powered NaOH (0.30 g, 7.50 mmol) and tetrabutylammonium hydrogen sulfate (0.06 g, 0.20 mmol). The mixture was stirred at rt for 30 min before 3-chloropropylamine hydrochloride (0.70 g, 3.75 mmol) was added. The reaction mixture was stirred at 60 °C overnight, cooled to rt, filtered, and washed with CH_2Cl_2. After the filtrate was concentrated, the residue was dissolved in CH_2Cl_2, washed with water and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was dissolved in CH_2Cl_2 (5 mL), and then a solution of methanesulfonic acid (MSA) (0.11 g) in ether (20 mL) was added. The precipitate was collected by filtration to afford **2b** as a colorless solid (0.41 g, 51%): mp 175−176 °C; ^{1}H NMR ($CDCl_3$) δ 1.52−1.87 (m, 16H), 1.92−1.97 (m, 2H), 2.07−2.16 (m, 4H), 2.18 (s, 2H), 2.40−2.50 (m, 1H), 2.83 (s, 3H), 3.02−3.12 (m, 2H), 3.50−3.57 (m, 2H), 3.62−3.70 (m, 2H), 3.97−4.04 (m, 2H), 4.06−4.16 (m, 2H), 4.44−4.51 (m, 2H), 6.82 (d, J = 8.5 Hz, 2H), 7.14 (d, J = 8.5 Hz, 2H), 11.84 (brs, 1H); ^{13}C NMR ($CDCl_3$) δ 26.45, 27.00, 30.63, 33.47, 35.63, 35.66, 36.28, 37.20, 39.36, 42.45, 52.88, 55.59, 56.79, 62.84, 63.86, 83.90, 88.81, 114.36, 128.02, 140.85, 155.20. Anal. Calcd for $C_{30}H_{45}NO_7S$: C, 63.92; H, 8.05; N, 2.48. Found: C, 63.69; H, 7.89; N, 2.52.

Adamantane-2-spiro-3′-9′-[[[(2′-amino-2′-methylpropyl)-amino]carbonyl]methyl]-1′,2′,4′-trioxaspiro[5.5]undecane *p*-Tosylate (3a). Step 1. A solution of adamantane-2-spiro-3′-9′-(carboxymethyl)-1′,2′,5′-trioxaspiro[5.5]undecane (**11**)[18] (0.20 g, 0.60 mmol), HOSu (0.08 g, 0.70 mmol), and EDCI (0.14 g, 0.73 mmol) in DMF (10 mL) was stirred at rt for 24 h. The reaction was quenched with water (30 mL) at 0 °C, and the precipitate was collected by filtration, washed with cold water, and dried in vacuo at 40 °C to give the trioxane active ester (0.24 g, 96%) as a colorless solid, which was used directly in the next step: ^{1}H NMR ($CDCl_3$) δ 1.00−2.10 (m, 21H), 2.48−2.64 (m, 2H), 2.76−2.90 (m, 4H), 2.95 (brs, 1H), 3.18 (brs, 1H), 3.76 (s, 2H). **Step 2.** To a solution of 1,2-diamino-2-methylpropane (0.20 g, 2.30 mmol) in $CHCl_3$ (5 mL) was added dropwise to the solution of the trioxane active ester (0.24 g, 0.58 mmol) in $CHCl_3$ (15 mL). The resulting mixture was stirred at rt for 5 h and then quenched with water (20 mL). After separation of the organic layer, the aqueous layer was extracted with $CHCl_3$ (2 × 20 mL). The combined extracts were washed with water (2 × 20 mL) and brine (20 mL), dried over $MgSO_4$, filtered, and concentrated to afford the free base of **3a** (0.18 g) as a colorless solid. The free base of **3a** (0.18 g) was dissolved in EtOAc (20 mL) to which was added a solution of *p*-toluenesulfonic acid monohydrate (0.09 g) in EtOAc (10 mL). The solid was collected and dried in vacuo at 40 °C to afford **3a**

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as a colorless solid (0.24 g, 69%): mp 166−167 °C; ^{1}H NMR ($CDCl_3$) δ 1.34 (s, 6H), 0.50−2.15 (m, 23H), 2.41 (s, 3H), 2.41 (brs, 1H), 2.90 (brs, 1H), 3.40 (s, 2H), 3.54 (s, 2H), 7.25 (d, J = 8.0 Hz, 2H), 7.33 (t, J = 7.0 Hz, 1H), 7.65 (d, J = 8.0 Hz, 2H), 7.76 (brs, 3H); ^{13}C NMR ($CDCl_3$): δ 21.39, 23.90, 27.12, 28.51 (br), 31.44 (br), 33.33, 33.58, 36.05 (br), 37.14, 41.97, 46.15, 56.27, 62.45, 77.58, 104.23, 125.50, 129.41, 140.34, 141.78, 172.99. Anal. Calcd for $C_{30}H_{46}N_2O_7S$: C, 62.26; H, 8.01; N, 4.84. Found: C, 62.49; H, 7.86; N, 4.77.

Adamantane-2-spiro-3′-9′-[4′-[2′-(4′-morpholinyl)ethoxy]-phenyl]-1′,2′,4′-trioxaspiro[5.5]undecane Mesylate (3b). **Step 1.** To a solution of *n*-BuLi (2.5 M in hexane, 6.3 mL, 15.8 mmol) in dry ether (50 mL) was added methyltriphenylphosphonium bromide (5.7 g, 16.0 mmol). The resulting mixture was stirred at rt for 1 h, and then a solution of 4-(4-hydroxyphenyl)cyclohexanone (**12**) (1.5 g, 7.9 mmol) in dry ether (30 mL) was added dropwise. The mixture was then refluxed overnight. After removal of ether, the residue was partitioned between ether (30 mL) and water (30 mL). The aqueous layer was extracted with ether (2 × 30 mL). The combined extracts were washed with water and brine, dried over $MgSO_4$, filtered, and concentrated. Purification by sg chromatography (0−10% ether in hexane) afforded 4-(4-methylenecyclohexyl)phenol as a colorless solid (0.92 g, 49%): mp 89−90 °C; ^{1}H NMR ($CDCl_3$) δ 1.43−1.55 (m, 2H), 1.91−1.99 (m, 2H), 2.12−2.22 (m, 2H), 2.37−2.44 (m, 2H), 2.56−2.66 (m, 1H), 4.63 (s, 1H), 4.67 (m, 2H), 6.73−6.78 (m, 2H), 7.03−7.10 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 35.15, 35.75, 43.24, 107.28, 115.10, 127.87, 139.28, 148.91, 153.61. **Step 2.** To a solution of 4-(4-methylenecyclohexyl)phenol (0.92 g, 4.65 mmol) in CH_2Cl_2 (10 mL) at 0 °C was added dry pyridine (5 mL) followed by acetic anhydride (2 mL). The resulting mixture was stirred at rt overnight. After removal of the solvents, the residue was partitioned between CH_2Cl_2 (30 mL) and water (30 mL). The aqueous layer was extracted with CH_2Cl_2 (2 × 30 mL). The combined extracts were washed with 1 M HCl (2 × 10 mL) and water (10 mL), dried over $MgSO_4$, filtered, and concentrated to afford 4-(4-methylenecyclohexyl)phenyl acetate (**13**) as a colorless solid (1.06 g, 99%): ^{1}H NMR ($CDCl_3$) δ 1.43−1.60 (m, 2H), 1.91−1.99 (m, 2H), 2.12−2.22 (m, 2H), 2.29 (s, 3H), 2.37−2.44 (m, 2H), 2.56−2.66 (m, 1H), 4.68 (m, 2H), 6.99 (d, J = 8.5 Hz, 2H), 7.20 (d, J = 8.5 Hz, 2H). **Step 3.** Peracetic acid (5.7 g, 32 wt % in aq acetic acid) was mixed with $CHCl_3$ (25 mL), the aqueous layer was discarded, and the organic layer was dried over $MgSO_4$ to yield an anhydrous peracetic acid solution, which was added dropwise to an ice cold solution of **13** (1.80 g, 7.83 mmol) in $CHCl_3$ (15 mL) containing sodium acetate (0.5 g). The reaction mixture was then warmed to rt and stirred overnight. The reaction mixture was washed with water (3 × 20 mL) and saturated $NaHCO_3$ (3 × 20 mL), dried over $MgSO_4$, filtered, and concentrated to afford 4-(1-oxaspiro[2.5]octan-6-yl)-phenyl acetate as a pale yellow oil (1.80 g, 93%): ^{1}H NMR ($CDCl_3$) δ 1.32−1.50 (m, 2H), 1.76−2.00 (m, 4H), 2.00−2.16 (m, 2H), 2.30 (s, 3H), 2.56−2.66 (m, 1H), 2.70 (s, 2H), 7.01 (d, J = 8.5 Hz, 2H), 7.24 (d, J = 8.5 Hz, 2H). **Step 4.** Anhydrous $MgSO_4$ (10 g) was added to a mixture of 50% H_2O_2 (10.0 g, 114 mmol) and ether (150 mL) at 0 °C. After stirring for 20 min, the mixture was filtered and the filtrate was added to a mixture of 4-(1-oxaspiro[2.5]octan-6-yl)phenyl acetate (1.80 g, 7.32 mmol) and molybdenyl acetylacetonate (228 mg, 0.7 mmol) in ether (10 mL). After the reaction mixture was stirred overnight at rt, it was washed with water (100 mL) and brine (100 mL), dried over $MgSO_4$, filtered, and concentrated to afford 4-[(4-hydroperoxy-4-(hydroxymethyl)cyclohexyl)]phenyl acetate (**14**) as a pale yellow oil (1.46 g, 71%): ^{1}H NMR ($CDCl_3$) δ 1.35−2.20 (m, 8H), 2.30 (s, 3H), 2.46−2.56 (m, 1H), 3.91 (s, 2H), 7.01 (d, J = 8.5 Hz, 2H), 7.21 (d, J = 8.5 Hz, 2H), 7.63 (brs, 1H). **Step 5.** 10-Camphorsulfonic acid (CSA) (0.20 g, 0.88 mmol) was added to a mixture of **14** (1.46 g, 5.20 mmol) and 2-adamantanone (1.20 g, 8.00 mmol) in CH_2Cl_2 (50 mL). The reaction mixture was stirred overnight at rt, washed with saturated $NaHCO_3$ (20 mL), water (20 mL), and brine (20 mL), dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by crystallization from 5:1 EtOH:H_2O to afford adamantane-2-spiro-3′-9′-(4′-acetoxyphenyl)-1′,2′,4′-trioxaspiro[5.5]undecane as a colorless solid (0.46 g, 21%): mp 155−156 °C; ^{1}H NMR ($CDCl_3$) δ 1.30−2.10 (m, 20H), 2.28 (s, 3H), 2.56−2.66 (m, 1H), 2.61 (brs, 2H), 3.87 (brs, 2H), 7.00 (d, J = 8.0 Hz, 2H), 7.18 (d, J = 8.0 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 21.13, 27.12, 28.60 (br), 29.66 (br), 30.55 (br), 33.18 (br), 33.36, 36.00 (br), 37.14, 39.23, 42.96, 62.28, 77.65, 104.40, 121.40, 127.63, 143.37, 148.87, 169.70. **Step 6.** To a solution of adamantane-2-spiro-3′-9′-(4′-acetoxyphenyl)-1′,2′,4′-trioxaspiro[5.5]undecane (0.30 g, 0.73 mmol) in THF (10 mL) and EtOH (20 mL) was added 15% aq KOH (1 mL). The resulting mixture was stirred at 50 °C for 4 h, concentrated to ∼5 mL, diluted with water (30 mL), and acidified with acetic acid (2 mL). The precipitate was collected by filtration, washed with cold water, and dried in vacuo at 40 °C to afford adamantane-2-spiro-3′-9′-(4′-hydroxyphenyl)-1′,2′,4′-trioxaspiro[5.5]undecane (**15**) as a colorless solid (0.24 g, 89%): mp 178−179 °C; ^{1}H NMR ($CDCl_3$) δ 1.30−2.20 (m, 20H), 2.51−2.60 (m, 1H), 2.82 (brs, 1H), 2.98 (brs, 1H), 3.89 (brs, 2H), 6.77 (d, J = 8.5 Hz, 2H), 7.06 (d, J = 8.5 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 27.13, 30.69 (br), 32.26 (br), 33.18, 33.36, 34.18 (br), 37.15, 42.67, 62.30, 77.75, 104.40, 115.17, 127.74, 138.19, 153.78. **Step 7.** To a solution of **15** (0.10 g, 0.27 mmol) in dry acetonitrile (20 mL) were added powered NaOH (0.20 g, 5.0 mmol) and tetrabutylammonium hydrogen sulfate (0.01 g, 0.03 mmol). The mixture was stirred at rt for 30 min before *N*-(2-chloroethyl)-morpholine hydrochloride (0.14 g, 0.75 mmol) was added. The reaction mixture was stirred at 60 °C overnight, cooled to rt, filtered, and washed with CH_2Cl_2. After the filtrate was concentrated, the residue was dissolved in CH_2Cl_2, washed with water and brine, dried over $MgSO_4$, filtered, and concentrated. The residue (0.12 g) was dissolved in EtOAc (10 mL) to which was added a solution of MSA (0.02 g) in ether (20 mL). The precipitate was collected by filtration to afford **3b** as a colorless solid (0.13 g, 83%): mp 188−189 °C; ^{1}H NMR ($CDCl_3$) δ 1.30−2.14 (m, 20H), 2.53−2.62 (m, 1H), 2.83 (s, 3H), 2.74−2.90 (m, 1H), 2.96 (brs, 1H), 3.02−3.13 (m, 2H), 3.51−3.58 (m, 2H), 3.64−3.70 (m, 2H), 3.89 (brs, 2H), 3.98−4.05 (m, 2H), 4.06−4.16 (m, 2H), 4.47−4.53 (m, 2H), 6.84 (d, J = 8.5 Hz, 2H), 7.13 (d, J = 8.5 Hz, 2H), 11.81 (brs, 1H); ^{13}C NMR ($CDCl_3$) δ 27.12, 28.46 (br), 29.68 (br), 30.62 (br), 33.36, 34.77 (br), 36.01 (br), 37.13, 39.36, 42.66, 52.94, 56.86, 62.28, 62.88, 63.83, 77.65, 104.40, 114.45, 127.92, 139.70, 155.35. Anal. Calcd for $C_{30}H_{45}NO_8S·0.5H_2O$: C, 61.20; H, 7.88; N, 2.38. Found: C, 61.15; H, 7.95; N, 2.36.

Adamantane-2-spiro-3′-9′-[[[(2′-amino-2′-methylpropyl)-amino]carbonyl]methyl]-1′,2′,4′,5′-tetraoxaspiro[5.5]-undecane Mesylate (4a). To a solution of adamantane-2-spiro-3′-9′-(carboxymethyl)-1′,2′,4′,5′-tetraoxaspiro[5.5]undecane (**16**)[13] (339 mg, 1 mmol) in CH_3CN (10 mL) and CH_2Cl_2 (10 mL) was added HOSu (127 mg, 1.1 mmol) followed by EDCI (211 mg, 1.1 mmol). After the mixture was stirred at rt for 16 h, 1,2-diamino-2-methylpropane (264 mg, 3 mmol) was added. After stirring for 2 h, the mixture was concentrated, diluted with water (50 mL), and extracted with CH_2Cl_2 (2 × 20 mL). The combined organic layers were washed with water (30 mL) and brine (20 mL), dried over $MgSO_4$, and concentrated. To a solution of the residue in EtOAc (5 mL) was added a solution of MSA (96 mg, 1 mmol) in EtOAc (5 mL). The precipitate was collected by filtration and purified by crystallization from CH_3CN to give **4a** as a colorless solid (243 mg, 48%): mp 150 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.18−1.37 (m, 2H), 1.36 (s, 6H), 1.42−2.08 (m, 19H), 2.17 (d, J = 7.3 Hz, 2H), 3.00−3.26 (m, 2H), 11.15 (brs, 1H); ^{13}C NMR ($CDCl_3$) δ 23.93, 26.97, 26.99, 27.75 (br), 28.36 (br), 28.78 (br), 30.04 (br), 31.07 (br), 33.07, 33.11, 33.44, 34.24 (br), 36.87, 39.86, 42.63, 46.25, 56.14, 107.62, 110.37, 172.80. Anal. Calcd for $C_{23}H_{40}N_2O_8S$: C, 54.74; H, 7.99; N, 5.55. Found: C, 54.79; H, 8.01; N, 5.42.

Adamantane-2-spiro-3′-9′-[4′-[2′-(4′-morpholinyl)ethoxy]-phenyl]-1′,2′,4′,5′-tetraoxaspiro[5.5]undecane Mesylate (4b). To a solution of adamantane-2-spiro-3′-9′-(4′-hydroxyphenyl)-1′,2′,4′,5′-tetraoxaspiro[5.5]undecane (**17**)[18] (0.5 g, 1.34 mmol) in dry acetonitrile (50 mL) were added powered NaOH (0.30 g, 7.50 mmol) and tetrabutylammonium hydrogen sulfate (0.06 g, 0.20 mmol). The mixture was stirred at rt for 30 min before *N*-(2-chloroethyl) morpholine hydrochloride (0.75 g, 4.03 mmol) was added. The reaction mixture was stirred at 60 °C overnight, cooled to rt, filtered, and washed with CH_2Cl_2. After the filtrate was

concentrated, the residue was dissolved in CH_2Cl_2, washed with water and brine, dried over $MgSO_4$, filtered, and concentrated. The residue (0.60 g) was dissolved in EtOAc (10 mL) to which was added a solution of MSA (0.11 g) in ether (20 mL). The precipitate was collected by filtration to afford **4b** as a colorless solid (0.45 g, 58%): mp 182−183 °C; ^{1}H NMR ($CDCl_3$) δ 1.56−2.10 (m, 20H), 2.53−2.63 (m, 1H), 2.83 (s, 3H), 3.01−3.13 (m, 2H), 3.16−3.36 (m, 2H), 3.48−3.58 (m, 2H), 3.67 (d, J = 12.5 Hz, 2H), 3.95−4.16 (m, 4H), 4.45−4.53 (m, 2H), 6.84 (d, J = 8.5 Hz, 2H), 7.16 (d, J = 7.0 Hz, 2H), 11.83 (brs, 1H); ^{13}C NMR ($CDCl_3$) δ 27.03, 29.84 (br), 31.90 (br), 33.14, 34.28 (br), 36.92, 39.36, 42.76, 52.93, 56.85, 62.87, 63.84, 107.45, 110.52, 114.43, 128.08, 139.74, 155.39. Anal. Calcd for $C_{29}H_{43}NO_9S$: C, 59.88; H, 7.45; N, 2.41. Found: C, 59.97; H, 7.67; N, 2.33.

In Vitro and in Vivo Antimalarial Activity. In vitro and in vivo antimalarial activities[9,11a,23] were measured using the chloroquine-resistant K1 and chloroquine-sensitive NF54 strains of *P. falciparum*, and *P. berghei*-infected mice, respectively. In vivo data were obtained using single 30 mg/kg oral doses (relative to the free base of each compound) of **1**−**4** administered in a nonsolubilizing, standard suspension vehicle (SSV) comprising 0.5% w/v carboxymethyl cellulose, 0.5% v/v benzyl alcohol, 0.4% v/v Tween 80, and 0.9% w/v sodium chloride in water, except for **3a**, which was dosed in 3% v/v ethanol and 7% v/v Tween 80 in water. Activity is defined as percent reduction in parasitemia on day 3 postinfection compared to an untreated control group. For example, a compound with an activity of 99.9% is 10-fold more active than one with an activity of 99.0% and 100-fold more active than one with an activity of 90%. Cures in the *P. berghei* model are defined as having no detectable parasites on day 30 postinfection.

Physicochemical Properties. Physicochemical properties (log $D_{pH\ 7.4}$, pK_a and PSA) were calculated using ACD/Laboratories Release 9.0 software (Advanced Chemistry Laboratories, Toronto).

Metabolic Stability. Metabolic stability was assessed in vitro by incubating compounds (1 μM) at 37 °C in mouse liver microsomes (Xenotech, Lenexa, KS) suspended in 0.1 M phosphate buffer (pH 7.4) at a final protein concentration of 0.4 mg/mL. Metabolic reactions were initiated by the addition of an NADPH-regenerating system (1 mg/mL NADP, 1 mg/mL glucose-6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase) and $MgCl_2$ (0.67 mg/mL) and were quenched at various time points up to 60 min by the addition of ice-cold acetonitrile. Quenched samples were centrifuged, and the relative loss of parent compound was monitored by LC−MS using a Waters/Micromass Xevo TQ triple quadrupole mass spectrometer coupled to a Waters Acquity UPLC (Waters Corporation, Milford, MA). Chromatography was conducted using a Supelco Ascentis Express RP Amide column (50 × 2.1 mm, 2.7 μm particle size, Supelco, Bellefonte, PA) operated at 40 °C and compounds were eluted with a methanol−water gradient buffered with 0.05% formic acid and delivered at a flow rate of 0.4 mL/min. Mass spectrometry was conducted in positive electrospray ionization mode with multiple-reaction monitoring using MS/MS parameters optimized for each compound. Concentration versus time data were fitted to an exponential decay function to determine the first order rate constant for substrate depletion, which was then used to calculate the degradation half-life, an in vitro intrinsic clearance value (mL/min/mg microsomal protein), and subsequently a predicted in vivo intrinsic clearance value.[24] Predicted in vivo CL_{int} values were converted to predicted in vivo hepatic extraction ratios (E_H) using the following equation: $E_H = CL_{int}/Q + CL_{int}$ where Q is liver blood flow, which was assumed to be 90 mL/min/kg for mice.[25]

Blood Stability. Blood stability was assessed by spiking compounds into freshly collected mouse blood and incubating at 37 °C. At various time points over a 4 h period, aliquots of blood were removed and quenched immediately by the addition of 0.4 M potassium dichromate (10% v/v) solution followed by snap freezing. Concentrations were assessed by LC−MS using the conditions described above for the microsomal stability studies. Samples were processed by protein precipitation with acetonitrile using a 2:1 volume ratio, and concentrations were determined by comparison to calibration standards prepared in blank mouse whole blood processed in the same manner.

Mouse Exposure Studies. Animal studies were conducted using established procedures[9,11a] in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the study protocols were reviewed and approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. Exposure following oral administration to noninfected mice was assessed at a dose of 30 mg/kg using an aqueous formulation vehicle containing 0.5% (w/v) hydroxypropylmethylcellulose, 0.5% (v/v) benzyl alcohol, and 0.4% (v/v) Tween 80. Compounds were administered by oral gavage (3 mL/kg) to 6−8 week old male Swiss outbred mice. An abbreviated blood sampling protocol was used to collect samples at 1, 4, 8, and 24 h postdose (two mice at each time point) with blood sampling either by cheek-bleed or terminal cardiac puncture with a maximum of two samples per mouse. Plasma was immediately separated from erythrocytes and frozen at −20 °C prior to analysis by LC−MS. Chromatography, MS, and sample processing were as described for the metabolic stability, and concentrations were determined by comparison to a calibration curve prepared in mouse plasma. Limits of quantitation were between 0.5 and 1 ng/mL for all analytes in plasma. Area under the plasma concentration−time profiles (AUC) were approximated using WinNonLin version 5.2.1 (Pharsight Corporation, Mountain View, CA).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CSA, 10-camphorsulfonic acid; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOSu, *N*-hydroxysuccinimide; MSA, methanesulfonic acid; SSV, standard suspending vehicle

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