Synthesis and Sulfur Electrophilicity of the Nuphar Thiaspirane Pharmacophore

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ABSTRACT: We describe a general method to synthesize the iminium tetrahydrothiophene embedded in the dimeric Nuphar alkaloids. In contrast to prior studies, the sulfur atom of the thiaspirane pharmacophore is shown to be electrophilic. This α-thioether reacts with thiophenol or glutathione at ambient temperature to cleave the C=S bond and form a disulfide. Rates of conversion are proportional to the corresponding ammonium ion pKₐ and exhibit half-lives less than 5 h at a 5 mM concentration of thiol. A simple thioether analogue of the Nuphar dimers causes apoptosis at single-digit micromolar concentration and labels reactive cysteines at similar levels as the unsaturated iminium “warhead”. Our experiments combined with prior observations suggest the sulfur of the Nuphar dimers can react as an electrophile in cellular environments and that sulfur-triggered retrodimerization can occur in the cell.

BACKGROUND
The Nuphar dimers (1−4, Figure 1) are a small family of bioactive terpene alkaloids defined by the presence of a nonsymmetric spiro-tetrahydrothiophene (thiaspirane) linker.1,2 Members of the class exhibit weak antimicrobial activity,3−5 but strong immunosuppressive activity.6 Some members also promote apoptosis7−10 and inhibit metastasis,11,12 both in vitro and in a murine model. The most potent member, 6-hydroxythiobinupharidine (1b), exhibits antimetastatic activity with an in vitro IC₅₀ of 29 nM and inhibits mouse lung tumor formation at 90% versus control over 10 days.11 Sempurified mixtures of the dimers down-regulate NFκB at low concentrations, although the active constituents in this mixture were not definitively proven and therefore potency values could not be defined.13

The structural basis for much of this bioactivity is not well explained. Cytotoxicity assays indicate that a hemiaminal adjacent to the tert-alkyl thioether (C6) is necessary for induction of apoptosis, whereas a thioether adjacent to a quaternary center (C6′) displays low potency.7,8 For example, 6-OH-TNL (2b, Figure 1a) is strongly apoptotic below 1 μM, whereas 6′-OH-TNL (2c) is completely inactive at 10 μM.9 Simplified analogues that replace the thioether with a hydroxyl are also reported to lose all activity in fungicidal experiments.14

One explanation put forth for these structural requirements is the enhanced stability of the iminium adjacent to a thioether in 6-hydroxydimers (Figure 1b).15−17 which may allow this otherwise strong carbon electrophile to penetrate the cell and reach a specific target.2 Dihydroxy-thiaspirane dimers would then be expected to not show activity or exhibit the low activity of 6′-hydroxydimers since they also give rise to an unstabilized iminium. Yet 6,6′-dihydroxy-1 (1a) and 6-hydroxy-1 (1b) inhibit invasion of collagen-matrix by B-16 melanoma cells11 and promote apoptosis7,8 at nearly identical levels. Therefore, the promiscuity of an unstabilized iminium and the preservation of a stabilized iminium cannot be the basis for the different activity of C6-hydroxy versus C6′-hydroxy dimers. A recent report by MacMillan, Eastman, and Wu demonstrates that all diastereomers and enantiomers of dihydroxydimers 1a, 2a, 3a, 4a have similar apoptotic activity,7 suggesting a common mechanism of action between the stereoisomers, at least for the dihydroxy-dimers.

We hypothesize that a dormant electrophilic sulfur atom embedded in the Nuphar dimers might better account for these unexplained observations. Such reactivity also explains the stereochemical equilibration observed in 6-hydroxy dimers (see Figure 2 below). Here we report an oxidative cyclization to synthesize the thiaspirane-iminium pharmacophore and demonstrate proof-of-principle selective thiol capture at the electrophilic sulfur. Rates of thiol capture can be tuned with the electronics of the imine and correlated to ammonium pKₐ.

We show analogous reactivity under cell-relevant conditions in a synthetic dimer by observing retrodimerization to highly electrophilic monomers. This simple dimeric thiaspirane analogue exhibits sub-micromolar cytotoxicity and causes rapid cell death. The thiol-triggered activation exhibited by...
The stereoisomeric nuphar thiaspirane alkaloids

The Nuphar family of thiaspirane diastereomers and congeners (a: X, Y = OH; b: X = H, Y = OH; c: X = OH, Y = H; d: X, Y = H).

Activity was proposed to involve an electrophilic carbon.

Here we propose an electrophilic sulfur as a basis for bioactivity.

INITIAL OBSERVATIONS OF S-ELECTROPHILICITY

We recently reported the first chemical synthesis of a Nuphar dimer, (−)-neothiobinupharidine (3d), via the intermediacy of its probable biosynthetic precursor 6,6′-dihydroxyneothiobinupharidine (3a). This latter compound proved difficult to isolate because the sulfurous reagents used for its synthesis caused retrodimerization to 7 upon acidification, concentration, and addition to silica (Figure 2a). These observations indicated that the final ring closure of the tetrahydrothiophene could be easily reversed via cleavage of the C−S bond, which surprised us since retrodimerization had not been previously reported in the extensive isolation literature. However, this same tert-alkyl center undergoes epimerization upon heating in chloroform, which was rationalized by a stereoretentive epoxide formation and reopening by a pendant thiol (Figure 2, top pathway). More likely, the presence of trace hydrochloric acid at high temperature induces iminium ion formation (8), whereby water or chloride anion attacks the thioether to form a sulfenic acid/enamine pair (9) that collapses to 2b after σ-bond rotation (bottom pathway). A sulfur nucleophile would not require the same activation energy to cleave the C−S bond as water or chloride but it is unlikely that dihydroxydimers would have been subjected to acids and thiols simultaneously during the isolation process.

This invocation of an electrophilic thioether during retrodimerization or epimerization relies on circumstantial evidence rather than direct observation of an electrophile-nucleophile adduct. Therefore, we sought a simple monomeric system that would allow us to fully characterize a thiol adduct and study its formation in more detail. So, we devised a new method to easily generate a monomeric Nuphar thiaspirane, which would allow us to study its reactivity.
Oxidative Spirocyclization

During our work on the syntheses of 3a−d, we observed that triphenylmethanethiol (TrSH) could mediate formation of acyclic dimers from monomeric iminium ions. Addition of an oxidant completed the tetrahydrothiophene—a stepwise variant of the dimerization we disclosed (i.e., 7 to 3a). Using this information, we designed a simple oxidative cyclization method to forge the thiaspirane.

As shown in Scheme 1, tetrahydropyridine 11 is readily available in three steps.44 Michael reaction of S-(tritylthio)-pentanal (10) with methyl acrylate is effected through the intermediacy of a pyrrolidine enamine. Subsequent amidation with 4-phenyl-butylamine occurred smoothly, as did lactam reduction with lithium aluminum hydride. While enamines similar to 11 were isolated in prior work,26 we used crude solutions due to its sensitivity to water and oxygen. After some experimentation, we found that treatment of solutions of dichloromethane at 0 °C resulted in gradual formation of a single thiophenol adduct 7.

However, addition of thiophenol to a solution of tetrahydrothiophene 13 resulted in oxidative cyclization of the iminium-thiaspiranes 20 proved too unstable to study extensively. Instead we turned to benzyl substituents, since para-substitution on benzylamines modulate their pKa.35 While 16 did undergo oxidative cyclization, the iminium-thiaspiranes 20 were isolated in prior work,25 we used crude reaction components by evaporation over several hours; otherwise, disulfide exchange would occur in the presence of excess thiophenol to yield diphenyldisulfide. However, only a trace amount of the free thiol derived from 15 was observed in any given experiment.

Using the general strategy of Scheme 1, we were then able to assemble a small library of spirocyclic iminium ions (20−23) to study their stability and reactivity. Initially, we explored aniline enamines (e.g., 16), which might have formed the basis of a straightforward Hammett analysis.47 Although substrates of type 16 did undergo oxidative cyclization, the iminium-thiaspiranes proved too unstable to study extensively. Instead we turned to benzyl substituents, since para-substitution on benzylamines modulate their pKa, about 1 unit—an observable but not destabilizing electronic effect.48−50 These benzyl substituted enamines (17a−f) cyclized cleanly and could be purified by high-performance liquid chromatography (HPLC) as stable iminium salts 21a−f. As benchmark iminiums for these and future studies, we were also able to adapt this route to the synthesis of a spirocyclic tetrahydrofuran iminium (18 → 22) and a cyclobutane iminium (19 → 23) as shown in Scheme 3. In the first case, peracid-mediated epoxidation/ring-opening of 18 delivers an alpha-hydroxy iminium, which undergoes S2 displacement to tetrahydrofuran 22. In the second case, direct 4-exo-tet cyclization of the enamine onto a pendant organoiodide furnishes cyclobutane 23.

The solvolytic stabilities of the iminiums 21−23 were probed by measuring their NMR spectra in methanol-d4 (see Supporting Information). Like N-4-phenylbutyl-iminium 13, N-benzyl-thiophenes 21a−f exist exclusively as the iminium ions on the NMR time scale at temperatures as low as −60 °C even in the case of electron-deficient 4-trifluoromethylbenzyl-iminium 21f. Interestingly, spiro-tetrahydrofuran 22 exists mostly as hemiaminal diastereomers in MeOH-d4, either reflecting decreased donation of the ether π-electrons into the C=N antibonding orbital compared to the thiophene or increased inductive withdraw from the oxygen. Supporting this latter idea, spiro-cyclobutane 23 is observed as the iminium ion in methanol, not the hemiaminal (see Supporting Information).

Observation of Disulfide Formation

The ability to form the pharmacophore in isolation from other functional groups and not embedded in a dimeric structure allowed us to study its reactivity with nucleophiles. Obviously, the tetrahydrothiophene is stable to alcoholic nucleophiles— it can be formed in methanol and can be characterized by NMR in methanol-d4. No hemiaminal is observed in equilibrium even in the case of electron-decific 4-trifluoromethylbenzyl-iminium 21f. Interestingly, spiro-tetrahydrofuran 22 exists mostly as hemiaminal diastereomers in MeOH-d4, either reflecting decreased donation of the ether π-electrons into the C=N antibonding orbital compared to the thiophene or increased inductive withdraw from the oxygen. Supporting this latter idea, spiro-cyclobutane 23 is observed as the iminium ion in methanol, not the hemiaminal (see Supporting Information).

Rates of Thiol Addition to Sulfur

Access to 21a−f allowed us to study the effect of N-substituent electronics on the electrophilicity at sulfur. In order to reach rates that would allow us to easily study relative reactivity, we increased thiophenol concentration to 5 M. Not surprisingly, the tetrahydrofuran 22 did not react under these conditions, but neither did the strained cyclobutane 23. The large amounts of thiophenol frustrated our attempts to follow the reactions in mass spectrometry (LCMS). Complete removal of the volatile reaction components by evaporation over several hours delivered very pure starting material (13), indicating that if formation of disulfide 14 had occurred, it could also be rapidly reversed. Nevertheless, none of this information proved whether bond formation had occurred at the iminium carbon or the thiophene sulfur. Unfortunately, reduction of the reaction mixture with sodium borohydride did not deliver a thiophenol adduct, but a fully reduced piperidine-thiol. Eventually, we discovered that addition of sodium cyanoborohydride to the reaction cleanly delivered disulfide 15, demonstrating for the first time that the Nuphar thiaspirane pharmacophore can function as a sulfur electrophile and that it is selective for thiol nucleophiles over water or alcohols. The disulfide in 15 must be lower in electrophilicity than thiophene 13; otherwise, disulfide exchange would occur in the presence of excess thiophenol to yield diphenyldisulfide. However, only a trace amount of the free thiol derived from 15 was observed in any given experiment.

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situ using standard techniques (NMR, HPLC) due to high background signals and peak broadening. Therefore, we turned to transmission IR spectroscopy to effectively screen out the signal from the solvent and thiophenol. An isolated IR signal from the iminium ion was observed at 1778 cm\(^{-1}\), and by following the changes in absorbance at this wavenumber, we could compare initial rates of reaction for each substrate.

We had expected to observe a positive Hammett or negative \(pK_a\) correlation reflecting increased reactivity of a C–S bond adjacent to an electron-deficient iminium due to loss of positive charge in the transition state.\(^{51}\) In fact, we observed the opposite: more electron-rich rings reacted more quickly. We hypothesized that this increased rate of electron-rich iminiums reflects either a decreased susceptibility to nucleophilic addition at carbon, i.e., a higher concentration of iminium reactant,\(^{52}\) or a more rapid protonation of electron-rich intermediate enamines. To probe these possibilities, we added thiophenol in low concentration to electron-deficient substrate 21a and measured its \(^1\)H NMR. At ambient temperature, peak broadening prevented interpretation, but at \(-40^\circ\text{C}\), we observed a decrease of iminium peaks and an appearance of a small diagnostic signal at 4.5 ppm, which increased at higher concentrations of thiophenol. On the basis of the NMR data of isolated thiophenol hemiaminals,\(^{53,54}\) this peak appears attributable to the thioaminal methine (25a). This adduct is unstable and cannot be observed by LCMS, unlike a disulfide adduct (for additional spectra, see Supporting Information).

Electron-rich substrate 21f, on the other hand, does not display these new peaks at ambient or low temperature even in the presence of 10 equiv of PhSH. Therefore, correlation of initial rates of reaction with \(pK_a\) of the ammonium/iminium ion appears to reflect varying concentrations of iminium ion in solution (Figure 4). Addition of Brønsted acids did not affect rates, ruling out enamine protonation as the rate-determining step.

Although these small electronic changes held a profound influence on the reactivity of the iminium, neither thiohemiaminal 25a nor disulfides 24a–f formed at concentrations of nucleophile relevant to a cellular environment (1–5 M). Whereas monomeric thiaspiranes were useful for characterizing nucleophile–electrophile adducts, we hypothesized that because 21a–f can undergo intramolecular resulfenylation of the nascent disulfide,\(^{56}\) these compounds show appreciable reaction with thiols only at high concentrations. So, we generated a simple analogue of the dimers (analogous to 1–4a), in which a retro-Mannich reaction would compete with resulfenylation, and tested its reactivity with thiols. Dimer 27 can be synthesized from 26 in analogy to our biomimetic dimerization of \(N\)uphar monomers and the similar reactions of chalcones\(^{42}\) reported by Reinecke (Scheme 4).

In contrast to the monomers, dimer 27 retrodimerizes to 28a with a half-life of less than 5 h at a PhSH concentration of only 5 mM (Figure 5)—a relevant concentration to cellular environments since the integrated, intracellular concentration of glutathione lies between 1 and 10 mM.\(^{56}\) This retrodimeriza-

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**Figure 3.** Relative rates of benzyl analogues plotted against sigma values.

**Figure 4.** Titration of PhSH to electron-rich or -deficient iminiums. Measured by \(^1\)H NMR in methanol-\(d_4\) at \(-40^\circ\text{C}\).

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\(^{44}\)Yields from enamide (two steps) based on \(^1\)H NMR internal standard, unless noted. \(^{45}\)Yield after HPLC.
Scheme 4. Dimerization of Simple Iminium 26 to an Unnatural Dimer 27

Figure 5. Dimeric thiaspiranes retrodimerize quickly at 5 mM thiol.

Figure 6. Extent of retrodimerization after 5 h at 2.5 mM dimer and 5 mM thiophen depends on iminium electronics.
to poor cellular stability or low membrane permeability. Together, the foregoing data do not indicate whether retrodimerization occurs in vivo.

■ S-ELECTROPHILICITY IN CELL LYSATE

Therefore, in order to determine if retrodimerization to the unsaturated iminium warhead occurs in a native biological environment, we performed isotopic tandem orthogonal proteolysis-activity-based protein profiling (isoTOP-ABPP).64−66 In this method, cells or cell lysate are treated with an electrophile of interest, then with the general electrophilic probe iodoacetamide alkyne (IA-alkyne). Proteinaceous Cys residues that react with the electrophile will not react with IA-alkyne; this difference can be read out stoichiometrically, and at single amino acid resolution, using mass spectrometry. A primary advantage of the isoTOP-ABPP technology is that the electrophile of interest does not need to be chemically modified for target identification, which is particularly useful when studying low molecular weight electrophiles.

As shown in Figure 8, monomers 13, 22, and 23 react with proteinaceous Cys residues of Jurkat cell lysate only modestly, whereas dimer 27 and warhead 26 display stronger reactivity. These high extents of cysteine modification are consistent with cytotoxicity—there is clear correlation between the cytotoxicity and cysteine adduction. Although these data stop short of definitively proving a detailed mechanism of Nuphar dimer activity in cells, the similar isoTOP-ABPP profiles of dimer 27 and warhead 26, and their deviation from the monomers, support a shared degree and mechanism of reactivity.

It is important to note that not all biological activity exhibited by Nuphar dimers can be ascribed to retrodimerizations and production of an iminium warhead such as 7 or 26. Even though both enantiomers of 1b are apoptotic,8 these 6-monohydroxy dimers cannot retrodimerize. But it also seems unlikely that both enantiomers of the topologically complex dimers are subject to lock-and-key or induced fit. Whether an electrophilic carbon or an electrophilic sulfur underlies the mechanism of 1b or the related monomeric species reported by Wu9 is unknown, but this study opens up the latter possibility to experimentation.

■ CONCLUSION

We have demonstrated that the sulfur atom of the Nuphar thiaspirane iminium pharmacophore reacts as an electrophile with nucleophilic thiols at ambient temperature. The rate of disulfide formation is proportional to the pKₐ of the embedded iminium and may reflect susceptibility to rapidly reversible thiohemiaminal formation in electron deficient substrates. In dihydroxy-dimers (metabolites or their analogues), this reactivity causes rapid retrodimerization to electrophilic, unsaturated iminium ions. In monomeric models of the pharmacophore, the initial disulfide adduct can be isolated and characterized. On the basis of this observed reactivity and rapid induction of apoptosis by 3a and synthetic dimer 27, we propose that some of the observed activity of the dihydroxy Nuphar dimers can be ascribed to retrodimerization; i.e., the dihydroxydimers are prodrugs for a highly reactive covalent binder. Clearly, retrodimerization is not an option for monohydroxydimers, so the mechanism of action for these species is still an open and potentially valuable question; capture of nucleophiles at the thioether is one possible answer.

Figure 7. Dimers promote rapid apoptosis and kill at low concentrations, whereas monomeric iminiums do not.

Figure 8. isoTOP-ABPP of Jurkat cell lysate treated with dimer, warhead, or unsaturated monomers. Jurkat cell lysate was treated with dimer (25 μM), warhead (50 μM), or unsaturated monomers (50 μM) for 1 h at ambient temperature. isoTOP-ABPP was performed as previously described.41,43 Shown on x-axis are top-100 changing Cys-peptides for each tested compound, where change is defined as a high isoTOP-ABPP ratio (DMSO/compound).
We anticipate that these observations of sulfur electrophilicity in the iminium thiispirane could lead to the design of new therapeutics based on the Nuphar pharmacophore and new tools for selective capture of free thiols in a cellular setting. The ability to tune the structure (steric environment) and electronics of the iminium α-thioether could lead to an arsenal of probes with varying activity to profile or inhibit biologically important thiols. In particular, caged iminium thioethers could represent an important class of probes for further exploration.  

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