

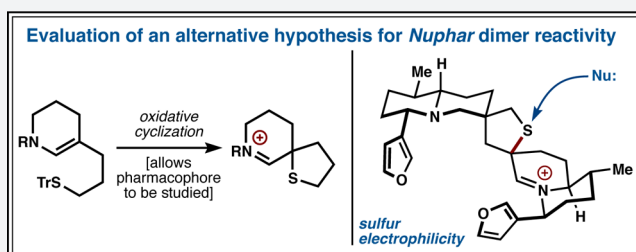
Synthesis and Sulfur Electrophilicity of the *Nuphar* Thiaspirane Pharmacophore

Norihiro Tada,[‡] Daniel J. Jansen,^{†,‡} Matthew P. Mower, Megan M. Blewett, Jeffrey C. Umotoy, Benjamin F. Cravatt, Dennis W. Wolan, and Ryan A. Shenvi*

Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, United States

S Supporting Information

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 pK_a and exhibit half-lives less than 5 h at a 5 mM concentration of thiol. A simple thiophane 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 apoptosis^{7–10} 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 IC_{50} of 29 nM and inhibits mouse lung tumor formation at 90% versus control over 10 days.¹¹ Semipurified mixtures of the dimers down-regulate NF κ B 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 μ M, whereas 6'-OH-TNL (2c) is completely inactive at 10 μ M.⁸ 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 In contrast, the electrophilic promiscuity of the unstabilized iminium/hemiaminal of 6'-hydroxy dimers is thought to prevent access to a specific

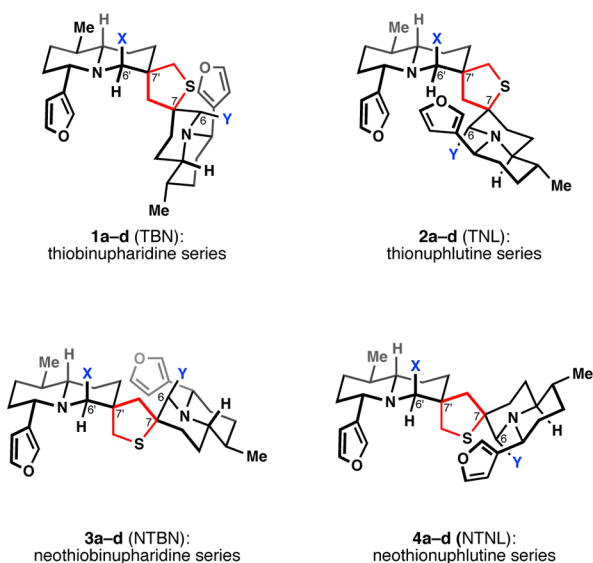
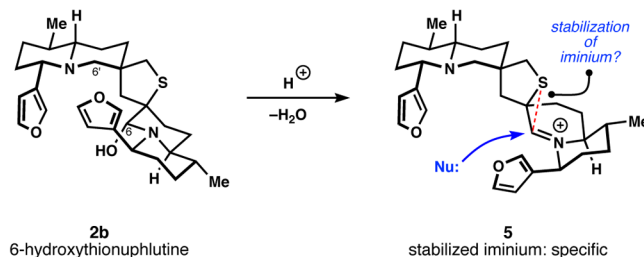
target.² Dihydroxy-thiaspirane dimers would then be expected to not show activity or exhibit the low activity of 6'-hydroxydimers since they also give rise to an unstabilized iminium. Yet 6,6'-dihydroxy-1 (1a) and 6-hydroxy-1 (1b) inhibit invasion of collagen-matrix by B-16 melanoma cells¹¹ and promote apoptosis^{7,8} 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,⁸ 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 pK_a . 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

Received: April 18, 2016

Published: June 13, 2016

a. The stereoisomeric nuphar thiaspirane alkaloids

b. LaLonde hypothesis: bioactivity involves C=N⁺ as electrophilic agent

c. Alternative hypothesis: bioactivity involves electrophilic S

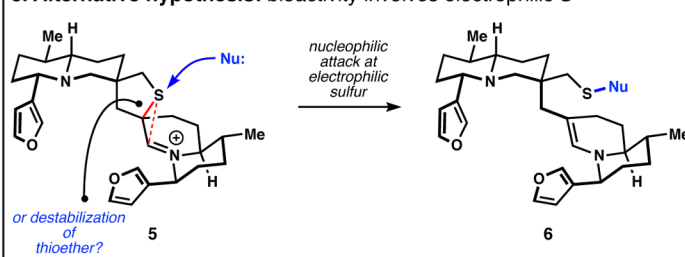
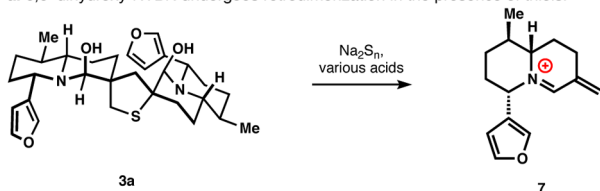


Figure 1. (a) The *Nuphar* family of thiaspirane diastereomers and congeners (a: X, Y = OH; b: X = H, Y = OH; c: X = OH, Y = H; d: X, Y = H). (b) Activity was proposed to involve an electrophilic carbon. (c) Here we propose an electrophilic sulfur as a basis for bioactivity.

a. 6,6'-dihydroxy-NTBN undergoes retrodimerization in the presence of thiols.



b. An electrophilic sulfur likely underlies the epimerization of 1b in hot chloroform.

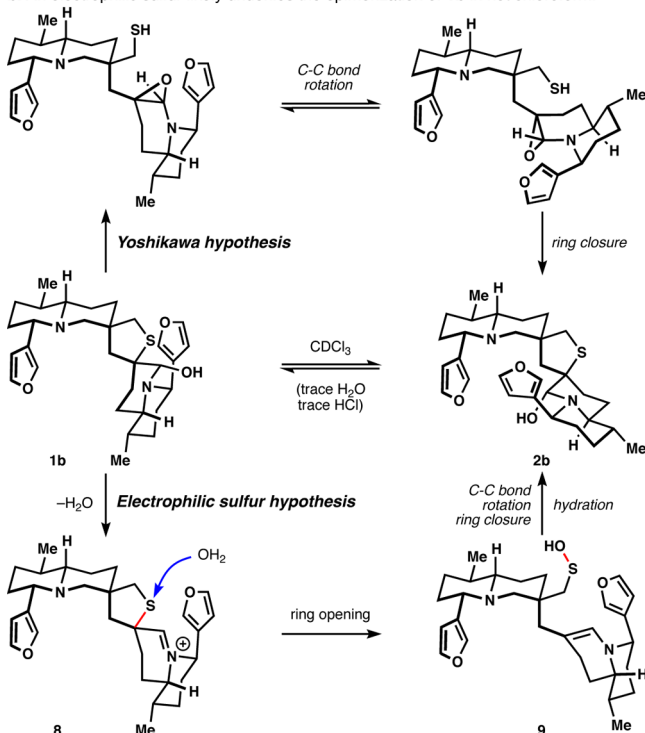


Figure 2. (a) Retrodimerization of 3a could be effected by nucleophilic addition to sulfur. (b) An electrophilic sulfur probably also underlies the epimerization of 1b to 2b.

this pharmacophore and its Trojan horse-capacity suggest a future role as a component of biological tools or therapeutics.¹⁸

■ 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'-dihydroxynothiobinupharidine (3a).¹⁹ 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.^{5,20–37} 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).²⁰ 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).³⁸ A sulfur nucleophile would not require the same activation energy to cleave the C–S bond as water or chloride,³⁹ but it is unlikely that dihydroxydimers would have been subjected to acids and thiols simultaneously during the isolation process.

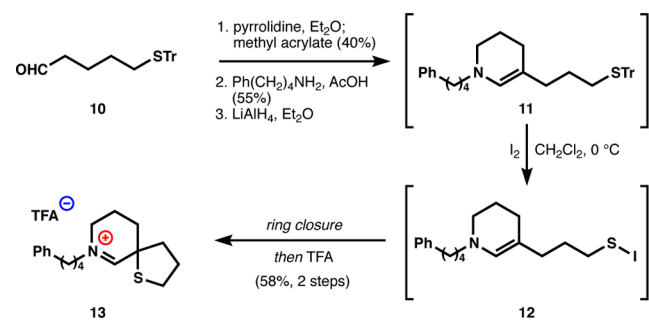
This invocation of an electrophilic thioether during retrodimerization or epimerization relies on circumstantial evidence rather than direct observation of an electrophile-nucleophile adduct.⁴⁰ Therefore, we sought a simple monomeric system that would allow us to fully characterize a thiol adduct and study its formation in more detail.^{41–43} 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**).¹⁹ 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 5-(tritylthio)-

Scheme 1. General Strategy for Synthesis of Monomeric Iminium Thiaspiranes

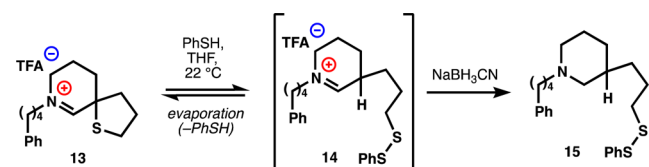


pentanal (**10**) with methyl acrylate is effected through the intermediacy of a pyrrolidine enamine. Subsequent amidation with 4-phenyl-butylamine occurred smoothly, as did lactam reduction with lithium aluminum hydride. While enamines similar to **11** were isolated in prior work,²⁵ we used crude solutions due to its sensitivity to water and oxygen. After some experimentation, we found that treatment of **11** with I_2 in a solution of dichloromethane at 0 °C effected tandem trityl removal^{45,46} and oxidative cyclization to directly form iminium tetrahydrothiophene **13**. Trifluoroacetic acid was added prior to purification to exchange the iodide counteranion, which was sensitive to autoxidation. This simple protocol could yield significant quantities of the isolated pharmacophore and could be adapted to many analogues (see **Scheme 3**).

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^{1,8}—it can be formed in methanol and can be characterized by NMR in methanol- d_4 . No hemiaminal is observed in equilibrium even at -50 °C (see **Supporting Information**), reflecting the stability of the *Nuphar* thiaspirane noted previously (see below).^{15–17} However, addition of thiophenol to a solution of **13** (**Scheme 2**) resulted in gradual formation of a single thiophenol adduct stable enough to be observed by liquid chromatography—mass

Scheme 2. Demonstration of S-Electrophilicity of Thiaspiranes



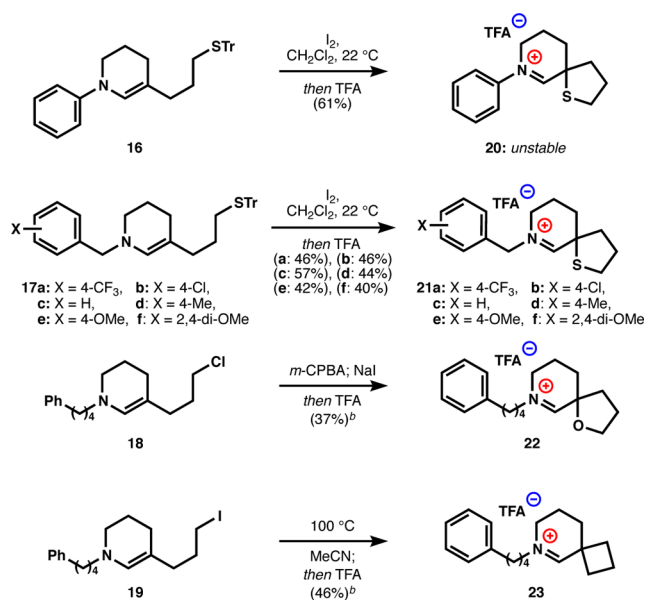
spectrometry (LCMS). Complete removal of the volatile reaction components by evaporation over several hours delivered very pure starting material (**13**), indicating that if formation of disulfide **14** had occurred, it could also be rapidly reversed. Nevertheless, none of this information proved whether bond formation had occurred at the iminium carbon or the thiophane 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 thiophane **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.

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 **20** proved too unstable to study extensively. Instead we turned to benzyl substituents, since *para*-substitution on benzylamines modulate their pK_a by 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 S_N2 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- d_4 (see **Supporting Information**). Like *N*-4-phenylbutyl-iminium **13**, *N*-benzyl-thiophanes **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- d_4 , either reflecting decreased donation of the ether *n*-electrons into the C=N antibonding orbital compared to the thiophane¹ 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

Scheme 3. Synthesis of a Small Library of Spirocyclic Iminiums^a

^aYields from enamide (two steps) based on ¹H NMR internal standard, unless noted. ^bYield after HPLC.

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.

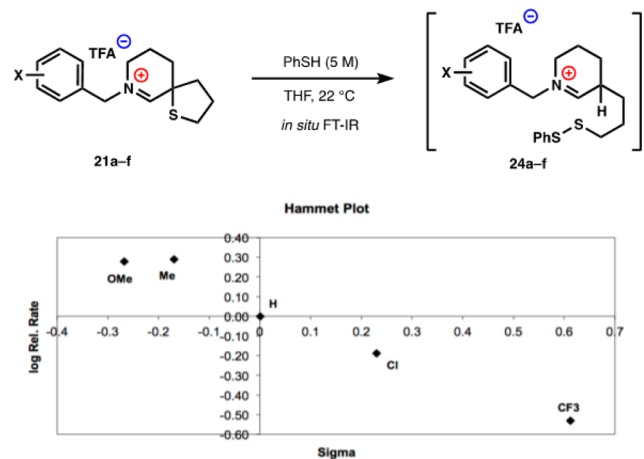


Figure 3. Relative rates of benzyl analogues plotted against sigma values.

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 ¹H NMR. At ambient temperature, peak broadening prevented interpretation, but at –40 °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.

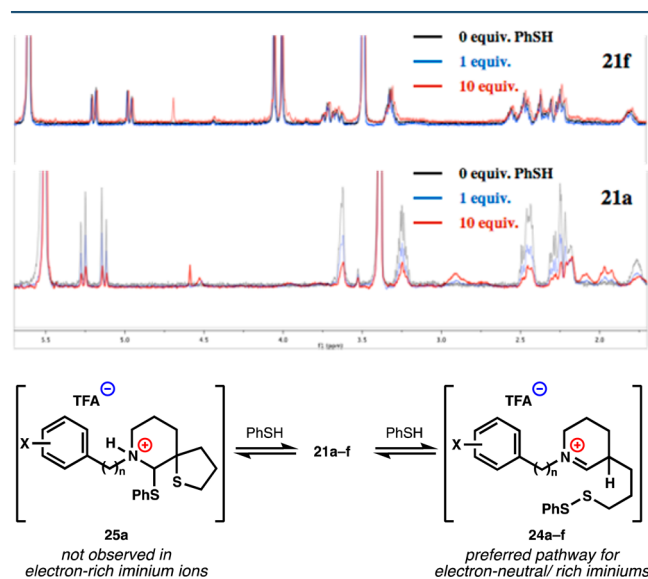


Figure 4. Titration of PhSH to electron-rich or -deficient iminiums. Measured by ¹H NMR in methanol-*d*₄ at –40 °C.

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,⁵⁵ 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 *Nuphar* monomers and the similar reactions of chalcones⁴² 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-

Scheme 4. Dimerization of Simple Iminium 26 to an Unnatural Dimer 27

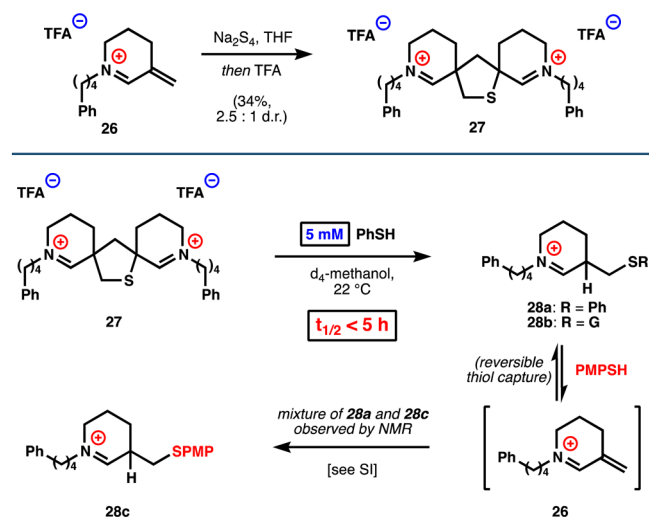


Figure 5. Dimeric thiaspiranes retrodimerize quickly at 5 mM thiol.

tion reaction also occurs with 5 mM aqueous glutathione but more slowly because the dimers are poorly water-soluble. The resulting unsaturated iminium 26 captures thiols at the β -position to form a stable adduct (28a) in solution. The disulfide itself apparently funnels to 28a (as determined by ^1H NMR), probably due to the lower pK_a of persulfides relative to thiols and the instability of persulfides.⁵⁷ Release of H_2S ,⁵⁸ a common degradation product of persulfides, was identified by odor and confirmed by reaction with AgNO_3 to yield Ag_2S . However, β -thioether 28 itself acts as a latent Michael acceptor and will exchange with other thiols, like 4-methoxythiophenol, to give mixtures of the two adducts (^1H NMR). The identities of adducts 28a–c were confirmed by independent synthesis from iminium ion 26, which rapidly captures thiols at the β -carbon exclusively (not the iminium carbon).

Using the tetrasulfide mediated dimerization shown in Scheme 4, we prepared a small series of analogues (29–31) that differed in the electronics of the nitrogen substituent (Figure 6). We measured the rate of retrodimerization at 2.5 mM substrate, a lower concentration than Figure 5 to induce

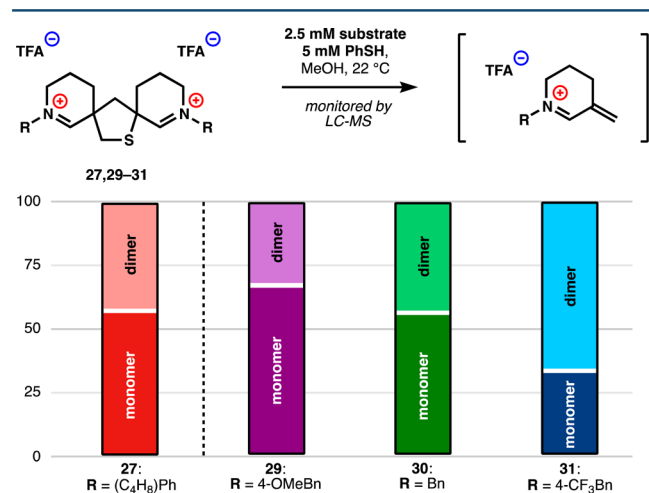


Figure 6. Extent of retrodimerization after 5 h at 2.5 mM dimer and 5 mM thiophenol depends on iminium electronics.

measurable rate differences. Substituent electronics changed the half-lives of retrodimerization of the analogues, which could be roughly correlated to the pK_a of the corresponding amine.^{59,60} This trend matches the same correlation made in the monomeric iminium tetrahydrothiophene series (Figure 3). Whether this reactivity in organic solvent under acidic conditions reflected behavior of these compounds in cellular environments still remained an open question.

RATES OF CELL DEATH

In order to evaluate the cellular relevance of this sulfur electrophilicity and its release of an unsaturated iminium “warhead,” we measured the potency and rate of killing of 3a, 27, and 29–31. Our chosen method for analyzing cell death promotion by *Nuphar* analogues was the CellTiter-Glo luminescence assay that measures depletion of cellular ATP.^{61,62} The dynamic range and normalization of samples for this luminescence-based approach supersedes traditional Western blot-based detection of protein cleavage events that occur during cell death. All cell-based luminescence assays are pragmatically normalized to the number of cells per well, whereas Western blotting normalization is dependent on densitometry analysis of antibody detection of general cellular proteins (i.e., GAPDH and actin). The latter technique can be compromised by sample overloading issues where the densitometry values of antibody binding extends beyond the limited linear range of detection and thus impedes the ability to correlate samples. Importantly, only certain general cellular proteins can be employed as loading controls during apoptosis experiments, as many candidate proteins are subject to degradation during cell death, including actin.⁶³ A recent publication on *Nuphar* analogues employed Western blot-based detection of the apoptosis marker poly(ADP-ribose) polymerase (PARP) and actin as a loading control.^{8,9} Use of actin as a loading control for this publication’s end point assays would likely not be compromised, as the authors assessed PARP cleavage at early stages of cell death (i.e., 2 and 6 h).

We found that simple dimeric analogues 27 and 29–31 kill Jurkat and HT29 cells rapidly at 5 and 10 μM , especially butylphenyl analogue 27, which exhibits rates of killing close to the metabolite 3a. Correlation to bulk-solvent retrodimerization rates is imperfect, which is not surprising given potential differences in cell permeability between the dimers and the different pH encountered by the dimers in the cell versus bulk solvent. However, the simplicity of these dimers make them valuable tools and potential replacements for the complex and difficult to access metabolites 1–4. In contrast, the monomeric iminiums 22–23 did not approach the rates of killing of the dimers, even at 25 μM . The monomeric thiophane iminium 13 did kill rapidly, but only at this much higher concentration. Therefore, it is not merely the iminium, nor merely the electrophilic sulfur, that is required to effect strong and rapid apoptosis.

Rather, this data taken along with observations by Wu et al. that both enantiomers of 1–4 are all actively apoptotic, make it likely that the observed high potency of dihydroxy-dimers derives from retrodimerization to electrophilic monomers (27).⁸ However, this correlation is imperfect. Recent work from Wu demonstrates cytotoxicity and rapid apoptosis in monomeric α -thioether quinolizidine iminium ions.⁵ And to our surprise, the proposed warhead 26 demonstrated low potency after 48 h incubation (Figure 7). Given its reactivity and polarity, we suspected this low activity of 26 might be due

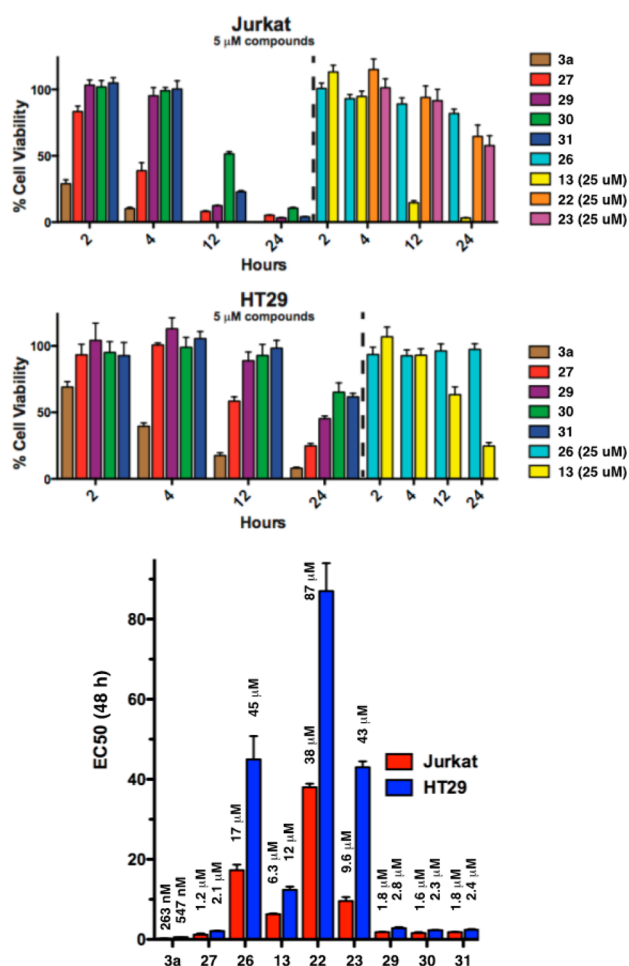


Figure 7. Dimers promote rapid apoptosis and kill at low concentrations, whereas monomeric iminiums do not.

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

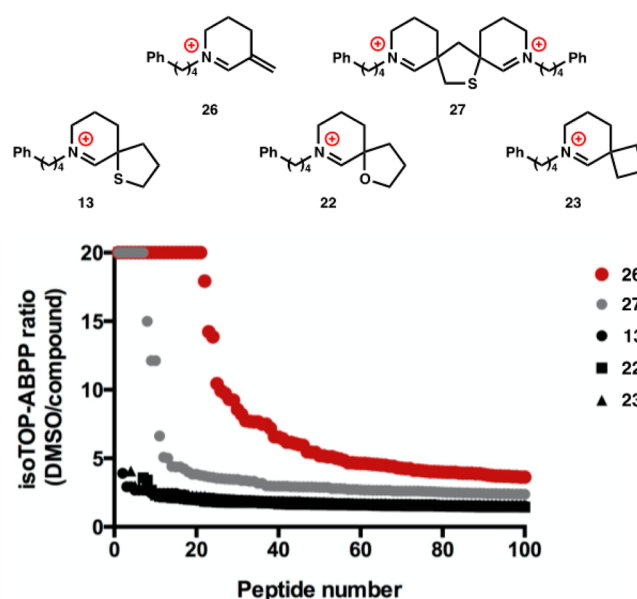


Figure 8. isoTOP-ABPP of Jurkat cell lysate treated with dimer, warhead, or unsaturated monomers. Jurkat cell lysate was treated with dimer (25 μM), warhead (50 μM), or unsaturated monomers (50 μM) for 1 h at ambient temperature. isoTOP-ABPP was performed as previously described.^{41,43} Shown on *x*-axis are top-100 changing Cys-peptides for each tested compound, where change is defined as a high isoTOP-ABPP ratio (DMSO/compound).

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 pK_a 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.

We anticipate that these observations of sulfur electrophilicity in the iminium thiaspirane 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.⁶⁷

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.6b00113.

Detailed experimental procedures, spectral data, chromatograms and X-ray crystallography (PDF)

Complete isoTOP-ABPP proteomics data (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: rshenvi@scripps.edu.

Present Address

†National Center for Advancing Translational Sciences, National Institutes of Health, 9800 Medical Center Drive, Rockville, Maryland 20850, USA.

Author Contributions

‡N.T. and D.J.J. contributed equally.

Funding

Financial support for this work was provided by the NIH (GM105766 and CA132630). Additional support was provided by Eli Lilly, Novartis, Bristol-Myers Squibb, Amgen, Boehringer-Ingelheim, the Sloan Foundation, and the Baxter Foundation.

Notes

The authors declare the following competing financial interest(s): A provisional patent was filed in collaboration with Ben Gurion University (J. Gopas) on the use of the compounds in this manuscript for treatment of inflammation.

■ ACKNOWLEDGMENTS

We thank Dr. Milan Gembicky, Dr. Curtis Moore, and Professor Arnold L. Rheingold for X-ray crystallographic analysis, and Professors Jacob Gopas and Avi Golan for helpful discussions.

■ REFERENCES

- (1) For reviews, see: LaLonde, R. T.; Wong, C. Properties of sulfur containing Nuphar alkaloids. *Pure Appl. Chem.* **1977**, *49*, 169–181.
- (2) LaLonde, R. T. Intramolecular iminium ion-sulfide charge-transfer association: a recurring theme in the study of thiaspirane alkaloids. *Acc. Chem. Res.* **1980**, *13*, 39–44.
- (3) Vichkanova, S. A.; Adgina, V. V.; Fedorchenko, T. S.; Rubinchik, M. A. Study of antibacterial and antifungal activity of lutenuirine in experiments in vitro. *Farmakol. Toksikol.* **1969**, *32*, 620–623.
- (4) Okamura, S.; Nishiyama, E.; Yamazaki, T.; Otsuka, N.; Taniguchi, S.; Ogawa, W.; Hatano, T.; Tsuchiya, T.; Kuroda, T. Action mechanism of 6, 6'-dihydroxythiobinupharidine from *Nuphar japonicum*, which showed anti-MRSA and anti-VRE activities. *Biochim. Biophys. Acta, Gen. Subj.* **2015**, *1850*, 1245–1252.
- (5) Cullen, W. P.; LaLonde, R. T.; Wang, C. J.; Wong, C. F. Isolation and in-vitro antifungal activity of 6,6'-dihydroxythiobinupharidine. *J. Pharm. Sci.* **1973**, *62*, 826–827.

- (6) Matsuda, H.; Shimoda, H.; Yoshikawa, M. Dimeric sesquiterpene thioalkaloids with potent immunosuppressive activity from the rhizome of *Nuphar pumilum*: structural requirements of Nuphar alkaloids for immunosuppressive activity. *Bioorg. Med. Chem.* **2001**, *9*, 1031–1035.

- (7) Matsuda, H.; Yoshida, K.; Miyagawa, K.; Nemoto, Y.; Asao, Y.; Yoshikawa, M. Nuphar alkaloids with immediately apoptosis-inducing activity from *Nuphar pumilum* and their structural requirements for the activity. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1567–1573.

- (8) Korotkov, A.; Li, H.; Chapman, C. W.; Xue, H.; MacMillan, J. B.; Eastman, A.; Wu, J. Total syntheses and biological evaluation of both enantiomers of several hydroxylated dimeric Nuphar alkaloids. *Angew. Chem., Int. Ed.* **2015**, *54*, 10604–10607.

- (9) Li, H.; Korotkov, A.; Chapman, C. W.; Eastman, A.; Wu, J. Enantioselective Formal Syntheses of 11 Nuphar Alkaloids and Discovery of Potent Apoptotic Monomeric Analogues. *Angew. Chem., Int. Ed.* **2016**, *55*, 3509–3513.

- (10) Dul'tsina, S. M.; Zhizhina, G. P.; Kruglyak, S. A.; Vermel, E. M.; Emanuel, N. M. Kinetics of the destruction of tumor cells under the influence of chemotherapeutic preparations in vitro. *Vopr. Onkol.* **1966**, *12*, 60–66.

- (11) Matsuda, H.; Morikawa, T.; Oda, M.; Asao, Y.; Yoshikawa, M. Potent anti-metastatic activity of dimeric sesquiterpene thioalkaloids from the rhizome of *Nuphar pumilum*. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4445–4449.

- (12) Kawahara, Y.; Shimoda, H.; Yoshikawa, M. (Morishita Jintan Co. Ltd. Japan) Hair growth stimulants containing sesquiterpene dimer thioalkaloids. JP 2000–233085, August 1, 2000.

- (13) Ozer, J.; Eisner, N.; Ostrozhenkova, E.; Bacher, A.; Eisenreich, W.; Benharroch, D.; Golan-Goldhirsh, A.; Gopas, J. *Nuphar lutea* thioalkaloids inhibit the nuclear factor κ B pathway, potentiate apoptosis and are synergistic with cisplatin and etoposide. *Cancer Biol. Ther.* **2009**, *8*, 1860–1868.

- (14) LaLonde, R. T.; Tsai, A. I.-M.; Wang, C. J.; Wong, C.; Lee, G. Preparation and in vitro antifungal activities of some quinolizidine derived hemiaminals and β -tert-amino sulfides. *J. Med. Chem.* **1976**, *19*, 214–219.

- (15) LaLonde, R. T.; Wong, C. F.; Das, K. C. Thiospiran, C₃₀, *Nuphar* alkaloids. Structure and evidence for intramolecular sulfur-immunium ion interactions. *J. Am. Chem. Soc.* **1973**, *95*, 6342–6349.

- (16) LaLonde, R. T.; Wong, C. F.; Das, K. C. The stereospecificity of α -thiohemiaminal reduction. A 300 MHz proton magnetic resonance investigation of deuteride reduction products from 6,6'-dihydroxythiobinupharidine and 6-hydroxythiobinupharidine. *Can. J. Chem.* **1974**, *52*, 2714–2716.

- (17) LaLonde, R. T.; Wong, C. F. Hemiaminal derivatives of neothiobinupharidine. *J. Org. Chem.* **1976**, *41*, 291–294.

- (18) Cf. the neocarzinostatin chromophore: Myers, A. G. Proposed structure of the neocarzinostatin chromophore-methyl thioglycolate adduct; a mechanism for the nucleophilic activation of neocarzinostatin. *Tetrahedron Lett.* **1987**, *28*, 4493–4496.

- (19) Jansen, D. J.; Shenvi, R. A. Synthesis of (–)-neothiobinupharidine. *J. Am. Chem. Soc.* **2013**, *135*, 1209–1212.

- (20) Achmatowicz, O.; Bellen, Z. Alkaloids of *Nuphar luteum* (L) Sm. Isolation of alkaloids containing sulphur. *Tetrahedron Lett.* **1962**, *3*, 1121–1124.

- (21) Achmatowicz, O.; Bellen, Z. Alkaloids of *Nuphar luteum* (L) Sm. II. Isolation of alkaloids containing sulfur. *Roczniki Chim.* **1962**, *36*, 1815–1825.

- (22) Achmatowicz, O.; Wrobel, J. T. Alkaloids from *Nuphar luteum*. III. A new alkaloid, neothiobinupharidine. Spectroscopic studies on the structure of thiobinupharidine and neothiobinupharidine. *Tetrahedron Lett.* **1964**, *5*, 129–136.

- (23) Achmatowicz, O.; Banaszek, H.; Spittler, G.; Wrobel, J. T. Alkaloids from *Nuphar luteum*. IV. Mass spectroscopy of thiobinupharidine, neothiobinupharidine, and their desulfuration products. *Tetrahedron Lett.* **1964**, *5*, 927–934.

- (24) Birnbaum, G. I. Structure of neothiobinupharidine. *Tetrahedron Lett.* **1965**, *6*, 4149–4152.

- (25) Il'inskaya, T. N.; Kuzovkov, A. D.; Monakhova, T. G. Alkaloids of *Nuphar luteum*. *Khim. Priro. Soed.* **1967**, *3*, 178–182.
- (26) Birnbaum, G. Crystal and molecular structure of neothiobinupharidine dihydrobromide tetrahydrate. *Acta Crystallogr.* **1967**, *23*, 526–535.
- (27) Wrobel, J. T. Thionupharoline a new alkaloid from yellow water-lily. *Roczniki Chim.* **1970**, *44*, 457–458.
- (28) Wrobel, J. T.; Galuszko, K. Furan chemistry. IV. IR spectra of monosubstituted derivatives. *Roczniki Chim.* **1970**, *44*, 769–776.
- (29) LaLonde, R. T.; Wong, C. F.; Cullen, W. P. Two new *Nuphar* alkaloids, 6,6'-dihydroxythionupharidine-A and -B. *Tetrahedron Lett.* **1970**, *11*, 4477–4480.
- (30) Wrobel, J. T.; Iwanow, A.; Szychowski, J.; Poplawski, J.; Yu, C. K.; Martin, T. I.; MacLean, D. B. Neothiobinupharidine sulfoxide, a new alkaloid of *Nuphar luteum*. *Can. J. Chem.* **1972**, *50*, 1968–1971.
- (31) LaLonde, R. T.; Wong, C. F. Sulfur containing alkaloids from *Nuphar luteum*. *Phytochemistry* **1972**, *11*, 3305–3306.
- (32) Wong, C. F.; LaLonde, R. T. 6- and 6'-hydroxyneothiobinupharidine. Monohemiaminals from *Nuphar luteum* and their possible role in thiaspirane *Nuphar* alkaloid biogenesis. *Experientia* **1975**, *31*, 15–16.
- (33) LaLonde, R. T.; Wong, C. Thiobinupharidines epimeric at C-1 and C-1'. *Can. J. Chem.* **1975**, *53*, 3545–3550.
- (34) Wrobel, J. T.; Iwanow, A.; Wojtasiewicz, K. New hemiaminals from *Nuphar luteum*. 6,6'-Dihydroxyneothiobinupharidine and monohydroxyhemiaminals. *Bull. Acad. Polym. Sci.* **1975**, *23*, 735–737.
- (35) Wrobel, J.; Iwanow, A.; Wojtasiewicz, K. New sulfoxides from *Nuphar luteum*. Thiobinupharidine and thionupharidine sulfoxides. *Bull. Acad. Polym. Sci.* **1976**, *24*, 99–100.
- (36) Iwanow, A.; Wojtasiewicz, K.; Wrobel, J. T. Sulfoxides of thiobinupharidine thiohemiaminals from *Nuphar lutea*. *Phytochemistry* **1986**, *25*, 2227–2231.
- (37) Yoshikawa, M.; Murakami, T.; Ishikado, A.; Wakao, S.; Murakami, N.; Yamahara, J.; Matsuda, H. Crude drugs from aquatic plants. VI. On the alkaloid constituents of Chinese *Nupharis Rhizoma*, the dried rhizoma of *Nuphar pumilum* (TIMM.) DC. (Nymphaeaceae): structures and rearrangement reaction of thiohemiaminal type *Nuphar* alkaloids. *Heterocycles* **1997**, *45*, 1815–1824.
- (38) Lalonde observed sulfonylation of an intermediate sulfenic acid formed by thermolysis of neothiobinupharidine sulfoxide: LaLonde, R. T.; Wong, C. F. *Nuphar* thiaspirane sulfoxides. ¹³C nuclear magnetic resonance determined configuration and utilization in the interconversion of thiaspirane stereochemical types. *Can. J. Chem.* **1978**, *56*, 56–61.
- (39) Singh, R.; Whitesides, G. M. In *Sulfur-Containing Functional Groups; Supplement S*; Patai, S., Ed.; J. Wiley and Sons, Ltd: New York, 1993.
- (40) As in the reduction of vinblastine analogs for example: Magnus, P.; Ladlow, M.; Elliott, J. Models for a Hypothetical Mechanism of Action of the Anticancer Agent Vinblastine. *J. Am. Chem. Soc.* **1987**, *109*, 7929.
- (41) For other strategies to synthesize the *nuphar* pharmacophore, see ref 41–43, ref 8, and ref 19. LaLonde, R. T.; Florence, R. A.; Horenstein, B. A.; Fritz, R. C.; Silveira, L.; Clardy, J.; Krishnan, B. S. Variable reaction pathways for the action of polysulfide on Michael acceptors. *J. Org. Chem.* **1985**, *50*, 85–91.
- (42) Del Mazza, D.; Reinecke, M. G. Reactions of α,β -unsaturated ketones with hydrogen sulfide. γ -Keto sulfides or tetrahydrothiopyranols? *J. Org. Chem.* **1981**, *46*, 128–134.
- (43) Lu, P.; Herrmann, A. T.; Zakarian, A. Toward the synthesis of *Nuphar* sesquiterpene thioalkaloids: stereodivergent rhodium-catalyzed synthesis of the thiolane subunit. *J. Org. Chem.* **2015**, *80*, 7581–7589.
- (44) Norman, M. H.; Heathcock, C. H. Improved synthesis of N-benzyl-5-ethyl-1,2,3,4-tetrahydropyridine. *J. Org. Chem.* **1988**, *53*, 3370–3371.
- (45) Kamber, B.; Rittel, W. A new, simple method for the synthesis of cysteine peptides. *Helv. Chim. Acta* **1968**, *51*, 2061–2064.
- (46) Moyer, M. P.; Feldman, P. L.; Rapoport, H. Intramolecular N-H, O-H and S-H insertion reactions. Synthesis of heterocycles from α -diazo β -keto esters. *J. Org. Chem.* **1985**, *50*, 5223.
- (47) Zuend, S. J.; Jacobsen, E. J. Mechanism of Amido-Thiourea Catalyzed Enantioselective Imine Hydrocyanation: Transition State Stabilization via Multiple Non-Covalent Interactions. *J. Am. Chem. Soc.* **2009**, *131*, 15358–15374.
- (48) Bunting, J. W.; Stefanidis, D. A systematic entropy relationship for the general-base catalysis of the deprotonation of a carbon acid. A quantitative probe of transition-state solvation. *J. Am. Chem. Soc.* **1990**, *112*, 779–786.
- (49) Hall, H. K., Jr. Correlation of the Base Strengths of Amines. *J. Am. Chem. Soc.* **1957**, *79*, 5441–5444.
- (50) Brown, H. C. et al. In *Determination of Organic Structures by Physical Methods*; Braude, E. A., Nachod, F. C., Ed.; Academic Press: New York, 1955.
- (51) Anslyn, E. V.; Dougherty, D. A. *Modern Physical Chemistry*; University Science Books: Sausalito, 2006; p 445.
- (52) Eldin, S.; Jencks, W. P. Lifetimes of Iminium Ions in Aqueous Solution. *J. Am. Chem. Soc.* **1995**, *117*, 4851–4857.
- (53) Kessar, S. V.; Singh, P.; Singh, K. N.; Singh, S. K. Facile α -deprotonation–electrophilic substitution of quinuclidine and DABCO. *Chem. Commun.* **1999**, 1927–1928.
- (54) Jarvis, C. L.; Richers, M. T.; Breugst, M.; Houk, K. N.; Seidel, D. Redox-Neutral α -Sulfonylation of Secondary Amines: Ring-Fused *N,S*-Acetals. *Org. Lett.* **2014**, *16*, 3556–3559.
- (55) See Jarboe, S. G.; Terrazas, M. S.; Beak, P. The Endocyclic Restriction Test: The Geometries of Nucleophilic Substitutions at Sulfur(VI) and Sulfur(II). *J. Org. Chem.* **2008**, *73*, 9627–9632 and references therein for considerations of geometry in sulfur(II) transfer.
- (56) Meister, A.; Anderson, M. E. Glutathione. *Annu. Rev. Biochem.* **1983**, *52*, 711–760.
- (57) Park, C.-M.; Weerasinghe, L.; Day, J. J.; Fukuto, J. M.; Xian, M. Persulfides: current knowledge and challenges in chemistry and chemical biology. *Mol. BioSyst.* **2015**, *11*, 1775–1785.
- (58) Benavides, G. A.; Squadrito, G. L.; Mills, R. W.; Patel, H. D.; Isbell, T. S.; Patel, R. P.; Darley-Usmar, V. M.; Doeller, J. E.; Kraus, D. W. Hydrogen sulfide mediates the vasoactivity of garlic. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 17977–17982.
- (59) Because of peak broadening and instability on the column, the rate of retrodimerization of 3a could not be reliably measured. It appeared comparable to 27.
- (60) On the basis of integration of LCMS peak areas calibrated to standardized references.
- (61) Crouch, S. P.; Kozlowski, R.; Slater, K. J.; Fletcher, J. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J. Immunol. Methods* **1993**, *160*, 81–88.
- (62) Bradbury, D. A.; Simmons, T. D.; Slater, K. J.; Crouch, S. P. Measurement of the ADP:ATP ratio in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. *J. Immunol. Methods* **2000**, *240*, 79–92.
- (63) Brown, S. B.; Bailey, K.; Savill, J. Actin is cleaved during constitutive apoptosis. *Biochem. J.* **1997**, *323*, 233–237.
- (64) Weerapana, E.; Speers, A. E.; Cravatt, B. F. Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)—a general method for mapping sites of probe modification in proteomes. *Nat. Protoc.* **2007**, *2*, 1414–1425.
- (65) Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B.; Cravatt, B. F.; Bachovchin, D. A.; Mowen, K.; Baker, D. Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* **2010**, *468*, 790–795.
- (66) Wang, C.; Weerapana, E.; Blewett, M. M.; Cravatt, B. F. A chemoproteomic platform to quantitatively map targets of lipid-derived electrophiles. *Nat. Methods* **2014**, *11*, 79–85.
- (67) Abo, M.; Weerapana, E. A Caged Electrophilic Probe for Global Analysis of Cysteine Reactivity in Living Cells. *J. Am. Chem. Soc.* **2015**, *137*, 7087–7090.