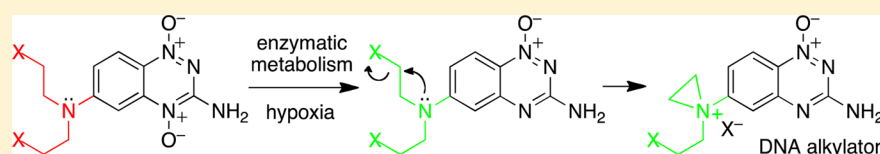


Toward Hypoxia-Selective DNA-Alkylating Agents Built by Grafting Nitrogen Mustards onto the Bioreductively Activated, Hypoxia-Selective DNA-Oxidizing Agent 3-Amino-1,2,4-benzotriazine 1,4-Dioxide (Tirapazamine)

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Supporting Information



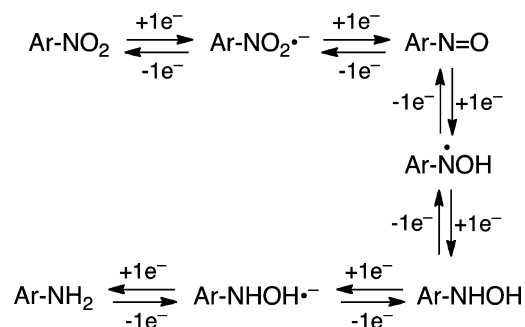
ABSTRACT: Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide) is a heterocyclic di-*N*-oxide that undergoes enzymatic deoxygenation selectively in the oxygen-poor (hypoxic) cells found in solid tumors to generate a mono-*N*-oxide metabolite. This work explored the idea that the electronic changes resulting from the metabolic deoxygenation of tirapazamine analogues might be exploited to activate a DNA-alkylating species selectively in hypoxic tissue. Toward this end, tirapazamine analogues bearing nitrogen mustard units were prepared. In the case of the tirapazamine analogue **18a** bearing a nitrogen mustard unit at the 6-position, it was found that removal of the 4-oxide from the parent di-*N*-oxide to generate the mono-*N*-oxide analogue **17a** did indeed cause a substantial increase in reactivity of the mustard unit, as measured by hydrolysis rates and DNA-alkylation yields. Hammett sigma values were measured to quantitatively assess the magnitude of the electronic changes induced by metabolic deoxygenation of the 3-amino-1,2,4-benzotriazine 1,4-dioxide heterocycle. The results provide evidence that the 1,2,4-benzotriazine 1,4-dioxide unit can serve as an oxygen-sensing prodrug platform for the selective unmasking of bioactive agents in hypoxic cells.

INTRODUCTION

The nitrogen mustard mechlorethamine (**1**), developed in the 1940s, was the first cancer chemotherapeutic agent.^{1,2} Analogues such as chlorambucil (**2**), melphalan (**3**), bendamustine (**4**), estramustine (**5**), uramustine (**6**), cyclophosphamide (**7**), and ifosfamide (**8**) see widespread clinical use today.^{3,4} Nitrogen mustards generate aziridinium ions that alkylate DNA at a variety of positions including N7-guanosine, N3-adenosine, N1-adenosine, and N3-cytosine.^{3,5–12} The predominant site of DNA alkylation by nitrogen mustards is the N7-atom of guanine residues.^{6,8,9}

Nitrogen mustards cause serious side effects that arise from the alkylation of DNA and other biomolecules in nonmalignant tissue.⁴ Consequently, there have been many efforts to develop “targeted” nitrogen mustards with improved potency and selectivity against cancer cells.^{13–21} One promising approach for the design of cancer-cell-selective mustards exploits selective enzymatic reduction of nitroaryl compounds in the oxygen-starved (hypoxic) cells found in solid tumors.^{22–32} One-electron reductases such as NADPH:cytochrome P450 reductase, cytochrome *b*₅ reductase, xanthine oxidase, and aldehyde oxidase can convert nitroaryl compounds to nitroso, hydroxylamino, and amino metabolites selectively under hypoxic conditions (Scheme 1).^{22–32} In normal tissue, O₂

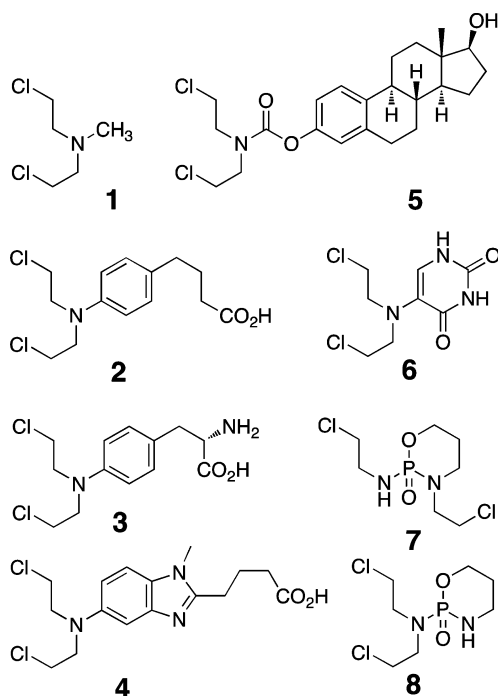
Scheme 1



blocks production of reduced metabolites via oxidation of the radical anion intermediates involved in this process (reverse reactions, Scheme 1).^{28,33,34} The initial nitro-to-nitroso conversion typically is a key oxygen-sensitive step in the bioreduction of nitroaryl compounds,^{26–28} but there is also evidence that the hydroxylamino-to-aniline step can be inhibited by O₂.²²

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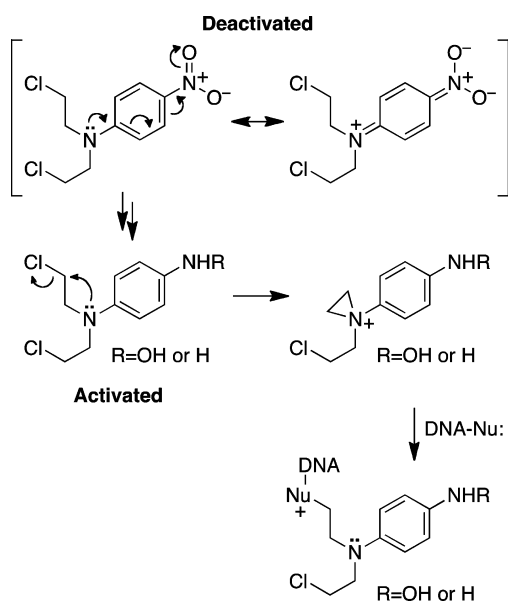
Published: July 16, 2014



It is well established that aziridinium ion formation by *N*-aryl nitrogen mustards is suppressed by electron-withdrawing substituents and favored by electron-releasing substituents on the aromatic ring.^{35,36} Thus, hypoxia-selective conversion of the electron-withdrawing nitro substituent (Hammett $\sigma = 0.78$) to the electron-donating hydroxylamino ($\sigma = -0.34$) or amino substituents ($\sigma = -0.66$) constitutes an “electronic switch” that can transform a deactivated *N*-aryl nitrogen mustard into an activated nitrogen mustard selectively in tumor tissue (Scheme 2).^{28,37} Two anticancer drug candidates, TH-302 and PR-104, that employ this design principle are currently undergoing phase I and II clinical trials.^{38,39}

In the pursuit of clinically useful hypoxia-selective DNA cross-linking agents, it may be important to explore the utility of oxygen-sensing units other than the nitroaryl motif. In this

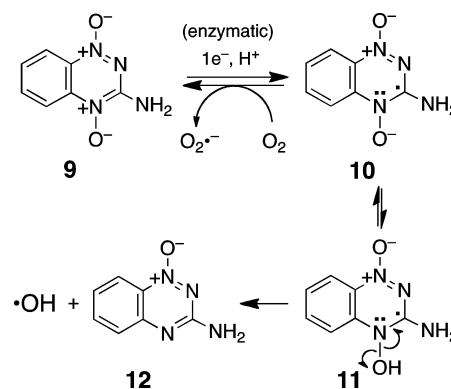
Scheme 2



regard, the 1,2,4-benzotriazine 1,4-dioxide scaffold deserves consideration. In terms of both basic and clinical research, the compound 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine, **9**) may be the best-characterized hypoxia-selective antitumor agent.^{40–43} On the basis of its potent hypoxia-selective cytotoxicity in preclinical testing,^{44–46} this compound was examined in a wide variety of phase I, II, and III clinical trials.^{40–43} In early clinical trials, the drug showed promise^{47,48} that was not realized in subsequent studies.⁴³ The disappointing clinical performance of **9** may stem, in part, from failure to stratify patients on the basis of the hypoxic character of their tumors.⁴⁹ In addition, pharmacokinetic models suggested that **9** may be metabolized in a small zone of hypoxic tumor cells, leaving a significant fraction of neighboring cancer cells untouched by the drug.⁴⁹ Second-generation analogues of **9** are in development.⁴⁹

Rational use of the 1,2,4-benzotriazine 1,4-dioxides as components in the design of hypoxia-selective DNA-alkylating agents rests upon an existing understanding of the mechanisms by which these agents selectively kill hypoxic cells. Intracellular one-electron reductases convert **9** to an oxygen-sensitive radical intermediate **10** (Scheme 3).^{50,51} We have presented evidence

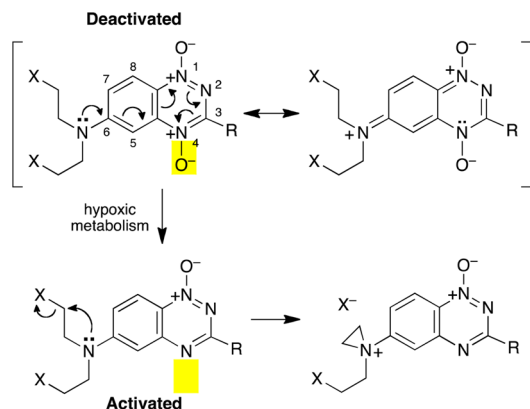
Scheme 3



that, under hypoxic conditions, the protonated drug radical **11** fragments to release the DNA-damaging agent, hydroxyl radical,^{52–57} though other mechanisms also have been considered.^{58–62} The deoxygenation product, 1,2,4-benzotriazine 1-oxide **12**, is the major metabolite generated by hypoxic metabolism of **9**,^{63–65} and other 1,2,4-benzotriazine 1,4-dioxides similarly are converted to the 1-oxide products.^{53,66} Compound **9** is not extensively metabolized to the mono-*N*-oxide **12** by oxygen-insensitive pathways involving two-electron reductases such as DT-diaphorase.^{67,68} The mono-*N*-oxide metabolite of tirapazamine is not cytotoxic on its own,^{45,69} although it does display useful oxygen mimetic (radiosensitizing) properties that potentiate the DNA strand-cleaving properties of hydroxyl radical.^{70–73}

We envisioned that electronic changes resulting from the deoxygenation of tirapazamine analogues might be exploited to activate DNA-alkylating functional groups selectively in hypoxic tissue. Indeed, the charge distribution in the mono-*N*-oxide metabolite **12** is quite different than that of the parent di-*N*-oxide **9**.⁵⁷ We describe the design, synthesis, and characterization of hypoxia-selective DNA-alkylating agents constructed by grafting nitrogen mustard units onto the 1,2,4-benzotriazine 1,4-dioxide scaffold (Scheme 4).

Scheme 4

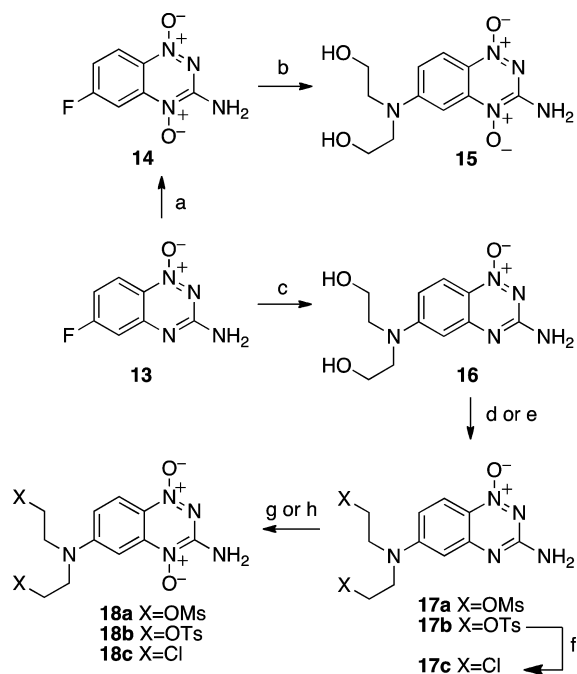


RESULTS AND DISCUSSION

Synthesis of 1,2,4-Benzotriazine 1,4-Dioxide Mustards. Our synthetic approaches to the desired tirapazamine-mustard derivatives were informed by previous reports describing the synthesis of tirapazamine and its analogues.^{74,75}

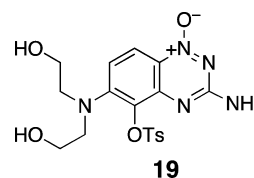
The compound 6-fluoro-1,2,4-benzotriazine 1-oxide **13** was prepared via condensation of 5-fluoro-2-nitroaniline with cyanamide.^{74,75} Oxidation of **13** with trifluoroacetic acid/80% H₂O₂ gave the di-*N*-oxide **14** in 43% yield (Scheme 5). Treatment with diethanolamine in acetonitrile afforded a 73% yield of **15**. Attempts to convert **15** to the tosyl mustard **17b** via treatment with tosyl chloride gave a complex mixture of products, from which we isolated **19** as a major component.

Scheme 5. Synthesis of 3-Amino-1,2,4-benzotriazine Mustards^a



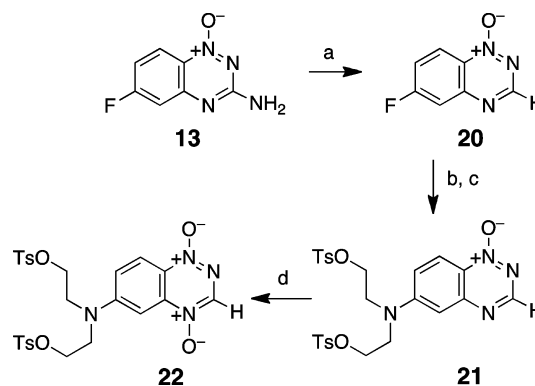
^aReagents and conditions: (a) CF₃COOH, 70% H₂O₂, 50 °C, 43%; (b) HN(CH₂CH₂OH)₂, CH₃CN, 73%; (c) HN(CH₂CH₂OH)₂, NMP, 100 °C, 94%; (d) mesyl chloride, TEA, DMF, 0 °C, 72%; (e) tosyl chloride, NaOH, THF/H₂O, 0 °C, 74%; (f) NaCl, DMF, 110 °C, 94%; (g) oxone, NaHCO₃, MeOH/H₂O, 50 °C, 15–30%; (h) *m*-CPBA, THF, 37%.

The structure of **19** was confirmed by single-crystal X-ray crystallographic analysis (Supplementary Figure S1). This product may arise via the initial attack of the 4-oxide unit of **15** on tosyl chloride, followed by a nucleophilic deoxygenative rearrangement.^{76,77} As an alternate route to the target mustards, **13** was treated with diethanolamine in *N*-methylpyrrolidone to give the mono-*N*-oxide **16** (94%). This product was converted in good yields to the ditosylate **17b** by treatment with tosyl chloride or to the dimesylate **17a** by treatment with mesyl chloride. Oxidation to the di-*N*-oxides **18** was effected by oxone or *m*-CPBA in modest yields (15–37%). The chlorinated mustard **17c** was synthesized in 94% yield via treatment of **17b** with lithium chloride in DMF, and **18c** was prepared in 30% yield by oxidation with oxone.



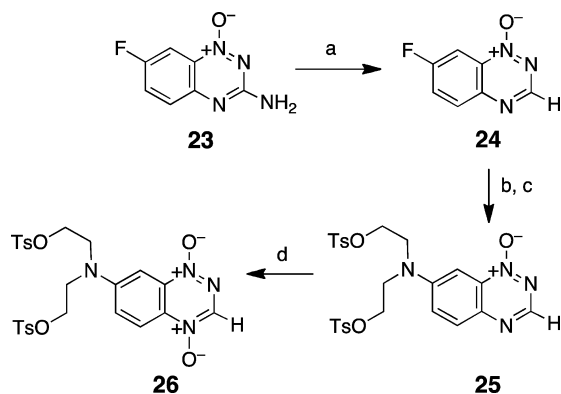
To investigate how the 3-amino substituent and the location of the mustard unit on the benzotriazine ring system affect reactivity of an appended nitrogen mustard unit, we sought to prepare the 3-desamino tirapazamine analogues **21**, **22**, **25**, and **26**. Toward this end, **13** was deaminated by treatment with *tert*-butylnitrite in dimethylformamide to give **20** (50% yield, Scheme 6).^{66,78} Nucleophilic aromatic substitution with

Scheme 6. Synthesis of 1,2,4-Benzotriazine 6-Mustards^a



^aReagents and conditions: (a) *t*-BuNO₂, DMF, 60 °C, 50%; (b) HN(CH₂CH₂OH)₂, CH₃CN; (c) TsCl, NaOH, THF 0 °C, 61% from 2 steps; (d) Oxone, NaHCO₃, MeOH, H₂O, 60 °C, 10%.

diethanolamine, followed by treatment with tosyl chloride, gave the mono-*N*-oxide mustard **21** in 67% yield for the two steps. Oxidation of **21** with oxone gave the di-*N*-oxide mustard **22** (10%). Synthesis of compounds with the mustard located on the 7-position of the benzotriazine ring system started with preparation of 7-fluoro-1,2,4-benzotriazine 1-oxide **23** via condensation of 6-fluoro-2-nitroaniline with cyanamide (Scheme 7).^{74,75} Treatment with *tert*-butylnitrite in dimethylformamide gave the deaminated analogue **24** in 50% yield. Subsequent reaction of this compound with diethanolamine, followed by tosyl chloride, gave a 57% yield of **25**. Finally, oxidation with either trifluoroacetic anhydride/70% H₂O₂ in CH₂Cl₂ or oxone gave the target di-*N*-oxide **26** in low (1%) yield. Formation of the desired compound **26**, in this case, was

Scheme 7. Synthesis of 1,2,4-Benzotriazine 7-Mustards^a

^aReagents and conditions: (a) *t*-BuNO₂, DMF, 60 °C, 50%; (b) HN(CH₂CH₂OH)₂, CH₃CN; (c) TsCl, NaOH, THF 0 °C, 57% from 2 steps; (d) TFA, 70% H₂O₂, CH₂Cl₂, 0 °C, 1%.

accompanied by extensive degradation during the reaction and workup.

Reactivity of the 1,2,4-Benzotriazine Oxide Mustards.

This work explores the idea that mustard units in the parent di-*N*-oxides will be relatively unreactive, while the mustard units in the mono-*N*-oxide deoxygenation products will be active alkylating agents. To learn whether the mustard units in the mono-*N*-oxides 17, 21, and 25 are in fact more reactive than those in the corresponding di-*N*-oxides (18, 22, 26) as illustrated in Scheme 4, we first examined the hydrolytic stability of these agents in an organic/aqueous solvent mixture. Hydrolysis of aromatic nitrogen mustards typically proceeds via a rate-determining formation of the aziridinium ion intermediate.^{5,36} We used ¹H NMR to monitor the hydrolysis of these mustard derivatives (~1 mM) in 50:50 CD₃CN/D₂O at 50 °C. This mixed organic/aqueous solvent system ensured solubility of the compounds at the concentrations required for the NMR experiments and also served to slow aziridinium ion formation and hydrolysis to easily measurable rates.⁷⁹ The disappearance of the compounds followed first-order kinetics (Figure 1). The mesylate mustard analogue of tirapazamine 18a disappeared with a half-life of 15.4 ± 0.6 d (Table 1), while the corresponding mono-*N*-oxide mustard 17a disappeared approximately 5-fold faster, with a half-life of 2.7 ± 0.4 d. The tosylate mustards displayed a similar trend, in which the di-*N*-oxide 18b was more stable (*t*_{1/2} = 15.4 ± 0.6 d) than the mono-*N*-oxide 17b (*t*_{1/2} = 5.5 ± 0.6 d). The similar reactivities of the

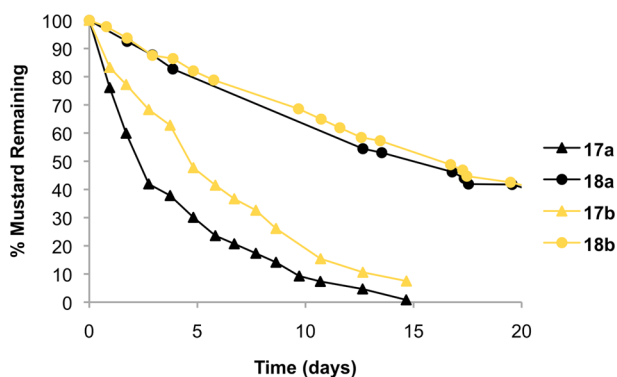
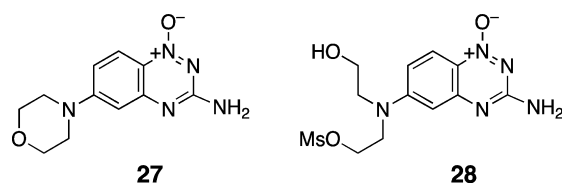


Figure 1. Rate of decay for compounds 17a, 17b, 18a, and 18b in 1:1 acetonitrile-*d*₆/D₂O measured by ¹H NMR at 50 °C.

tosyl (17b/18b) and mesyl (17a/18a) systems is consistent with previous analyses showing that tosylate and mesylate are comparable leaving groups in aqueous/organic solvent mixtures.⁸⁰ The chlorinated mustard analogues 17c and 18c were quite stable, with 17c displaying an estimated half-life of more than 51 d under the conditions of our NMR experiment (85% of the starting material remained after 10 d). Compound 18c showed no detectable reaction over the course of 10 d. Due to the sluggishness of these reactions we did not examine the hydrolysis of 17c and 18c further.

The products resulting from decomposition of the nitrogen mustards in these NMR experiments were identified as the expected diol (16) and the morpholino compound (27), presumably derived from intramolecular attack of the hydroxyethyl arm of the half-mustard intermediates (28) on the adjacent mustard group. Analogous metabolites have been observed for the clinically used mustard 2.⁸¹



The desamino analogues 21 and 22, bearing a tosyl mustard at the 6-position, disappeared with half-lives that were the same within experimental error (Table 1). The half-lives of these compounds (*t*_{1/2} ≈ 13 d) were very similar to that of the deactivated dioxide tosyl mustard 18b in the 3-amino series. The desamino analogues 25 and 26, bearing the tosyl mustard unit on the 7-position, also disappeared at the same rate within experimental error (Table 1). The decomposition rates of the 7-mustards 25 and 26 (*t*_{1/2} ≈ 6.5 d) was approximately two times faster than the decomposition of the desamino 6-mustards 21 and 22 (*t*_{1/2} ≈ 13 d). In the Conclusion, we consider possible reasons why there is no significant difference between the reactivities of the desamino mono-*N*-oxide and di-*N*-oxide mustards.

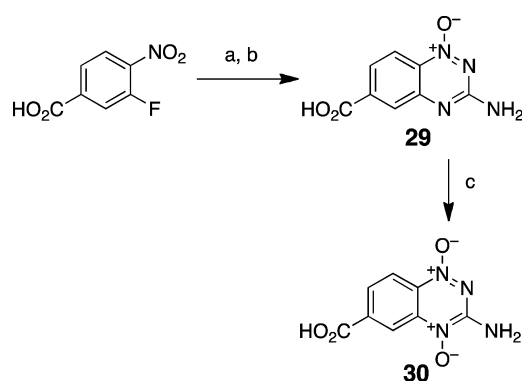
We also measured decomposition of the mesyl mustards 17a and 18a (250 μM) at 50 °C in a predominantly aqueous solvent mixture composed of sodium phosphate buffer (25 mM, pH 7) containing DMF (2.5% v/v). We used HPLC analysis to monitor the disappearance of the starting mustards (17a and 18a). As expected, the hydrolysis rates of 17a and 18a were substantially faster in this solvent as compared to the 50:50 CD₃CN/D₂O mixture employed in the NMR experiments.⁷⁹ Compounds 17a and 18a disappeared with half-lives of 12 ± 0.5 h and 96 ± 21 h, respectively, in phosphate-buffered water. Under these conditions, the “activated” mono-*N*-oxide metabolite 17a reacted approximately seven times faster than the parent dioxide 18a. Comparison with authentic synthetic standards revealed that the major products generated in the decomposition of 17a were the diol 16 and the morpholino 27, both presumably formed via the half-mustard 28 (Supplementary Figure S2).

Measurement of the Electron-Withdrawing Power of the Triazine Ring Systems in 17 and 18. We felt it would be useful to quantitatively measure the electron-withdrawing power of the triazine ring systems in 17 and 18. Electronic effects exerted by various substituents on an aromatic ring typically are assessed using Hammett sigma (σ) constants.⁸² Hammett σ values are determined by measuring the affect that a substituent exerts on the acidity of benzoic acid, where

Table 1. Decay Rates of 1,2,4-Benzotriazine Mustards in Acetonitrile- d_6 /D $_2$ O (1:1) at 50 °C Measured by ^1H NMR

compd	R ₁	R ₂	R ₃	oxidation	k (d ⁻¹)
17a	H ₂	N(CH ₂ CH ₂ OMs) ₂	H	1-oxide	0.259 ± 0.060
18a	NH ₂	N(CH ₂ CH ₂ OMs) ₂	H	1,4-dioxide	0.045 ± 0.002
17b	NH ₂	N(CH ₂ CH ₂ OTs) ₂	H	1-oxide	0.127 ± 0.014
18b	NH ₂	N(CH ₂ CH ₂ OTs) ₂	H	1,4-dioxide	0.045 ± 0.002
21	H	N(CH ₂ CH ₂ OTs) ₂	H	1-oxide	0.050 ± 0.004
22	H	N(CH ₂ CH ₂ OTs) ₂	H	1,4-dioxide	0.055 ± 0.006
25	H	H	N(CH ₂ CH ₂ OTs) ₂	1-oxide	0.107 ± 0.013
26	H	H	N(CH ₂ CH ₂ OTs) ₂	1,4-dioxide	0.106 ± 0.001

$\sigma_{\text{substituent}} = \log(K_{\text{a}}(\text{substituted benzoic acid})/K_{\text{a}}(\text{benzoic acid}))$. Therefore, we prepared the carboxylic acid derivatives **29** and **30** via reaction of guanidine with 3-fluoro-4-nitrobenzoic acid under basic conditions, as shown in Scheme 8.⁸³

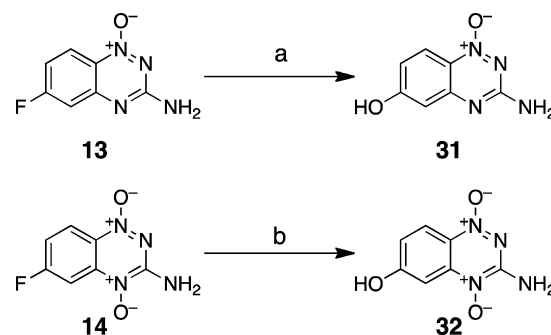
Scheme 8. Synthesis of 6-Carboxy-1,2,4-benzotriazines^a

^aReagents and conditions: (a) guanidine, THF, EtOH 90 °C; (b) KOtBu, THF, 90 °C, 95% after 2 steps; (c) TFA, 70% H₂O, 50 °C, 36%.

We measured the acidity of **29** by monitoring the changes in its UV-vis spectrum as a function of pH (Supplementary Figure S3). From the measured pK_a of 2.9, we were able to calculate a σ value of 1.3 for the mono-*N*-oxide **29**. This establishes the 1,2,4-triazine-1-oxide ring as a strongly electron-withdrawing substituent comparable to a *p*-sulfonyl cyanide group (-SO₂CN).⁸² Unfortunately, we were not able to measure the pK_a for the di-*N*-oxide **30** because the UV-vis spectral changes associated with protonation of the carboxylic acid group were obscured by another process, perhaps involving protonation of the oxygen in the 4-oxide group.

Electronic properties of substituents can also be measured using a σ^- parameter that is obtained by determining the effect of substituents on the acidity of phenol.^{82,84} While the σ^- parameter is probably less relevant than σ to the aziridinium ion-forming reactions that are the subject of this work,³⁶ we felt this measurement would nonetheless provide a useful evaluation of the electronic properties of the triazine mono-oxide and dioxide ring systems. Therefore, we synthesized the phenol derivatives **31** and **32** by reaction of the corresponding fluoro compounds with basic hydrogen peroxide, followed by

workup with sodium thiosulfate (Scheme 9).⁸⁵ We then measured the acidity of these compounds by monitoring the

Scheme 9. Synthesis of 6-Hydroxy-1,2,4-benzotriazines^a

^aReagents and conditions: (a) NaOH, 30% H₂O₂, H₂O/NMP 60 °C, 50%; (b) NaOH, 30% H₂O₂, H₂O/NMP 50 °C, 51%.

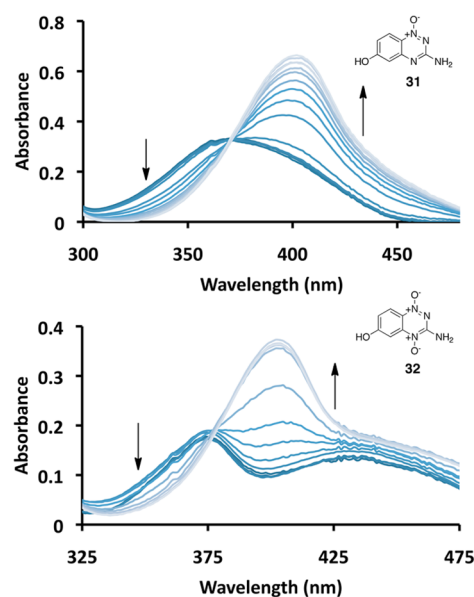


Figure 2. UV-vis spectra of compounds **31** (top) and **32** (bottom) from pH 3.4 to 9.0 (**31**) and pH 2.4 to 9.0 (**32**).

changes in their UV–vis spectra as a function of pH (Figure 2). The pK_a of **31** was found to be 6.3, and that of **32** was 5.3 (Supplementary Figure S4). The pK_a measurements allowed us to calculate σ^- values of 1.81 for the 1,2,4-triazine 1-oxide “substituent” in **31** and 2.31 for the 1,2,4-triazine 1,4-dioxide “substituent” in **32**. These values indicate that both the triazine mono-*N*-oxide and the di-*N*-oxide rings are strongly electron-withdrawing. By way of comparison, the sulfonyl perfluoropropane group ($-\text{SO}_2(\text{CF}_2)_2\text{CF}_3$) has a σ^- value of 1.75, and the diazonium group ($-\text{N}_2^+$) has a σ^- value of 3.43.⁸² Importantly, there is a substantial difference (0.5) between the σ^- values of the 1,2,4-triazine 1-oxide and 1,2,4-triazine 1,4-dioxide substituents in **31** and **32**. This is similar to the difference between σ^- values of the nitro group (1.27) and the acetyl group (0.84).⁸² Overall, the results validate the idea that transformation of the 1,4-di-*N*-oxide (**9**) to the 1-*N*-oxide (**12**) is accompanied by a substantial change in electron density at the 6-position of the benzo ring.⁵⁷

DNA Alkylation by 17a. We next examined the abilities of **17a** and **18a** to alkylate DNA. Nitrogen mustards alkylate DNA primarily at the N7-position of guanine residues, with smaller amounts of reaction also occurring at the N3-position of adenine residues.^{6,8,9,86} The resulting lesions can be converted to strand breaks by treatment of the DNA with warm piperidine (Maxam–Gilbert workup).⁸⁷ We incubated mustards **17a** and **18a** with the 5'-³²P-labeled DNA duplex **33** in HEPES buffer (50 mM, pH 7) containing NaCl (100 mM) and DMF (10% v/v), followed by piperidine workup (Figure 3). The resulting labeled DNA fragments were resolved on a 20% denaturing polyacrylamide gel and visualized by phosphorimager analysis. We found that treatment of DNA with the dioxide **18a**, followed by piperidine workup, generated little strand cleavage at the guanine residues in the labeled strand of duplex **33**. The mono-*N*-oxide **17a** generated significantly higher yields of alkali-labile lesions at both guanine residues in duplex **33**. Specifically, compound **17a** generated a 30-fold higher yield of alkylation at G2 than **18a** (lanes 3 and 4, Figure 3B). The control diol **16** did not induce strand cleavage that was significantly above background (lane 2, Figure 3B). We compared DNA alkylation by **17a** to that by the clinically used nitrogen mustard chlorambucil (**2**, lanes 4 and 5, Figure 3B). Chlorambucil generated piperidine-labile lesions predominantly at the guanine residues in duplex **31**, alongside weaker cleavage that may arise from alkylation at adenine residues in the DNA.⁸⁸

Compound **17a** showed a marked selectivity for alkylation at G2 of duplex **33** that is distinct from the sequence specificity of **2** (Figure 3B). This observation inspired us to examine the alkylation of duplex **34** containing a larger number of guanine residues embedded in different sequence contexts. Again, compound **17a** showed a strong sequence preference for alkylation of a guanine residue residing in a 5'-GT sequence (G1 in duplex **34**, Figure 3C). Mechlorethamine (**1**) displayed relatively low sequence specificity compared to **17a**, with 5'-GGG as a favored sequence and 5'-GC as a disfavored sequence, consistent with literature reports (lane 5, Figure 3C).⁸⁶ Possible origins of the sequence specificity displayed by **17a** are discussed in the Conclusions below. These experiments provided evidence that the anticipated hypoxic metabolite **17a** is a significantly better DNA-alkylating agent than the parent di-*N*-oxide **18a**.

Hypoxia-Selective, In Vitro Metabolic Conversion of 15 to 16. Successful deployment of the activated mustard **17a**

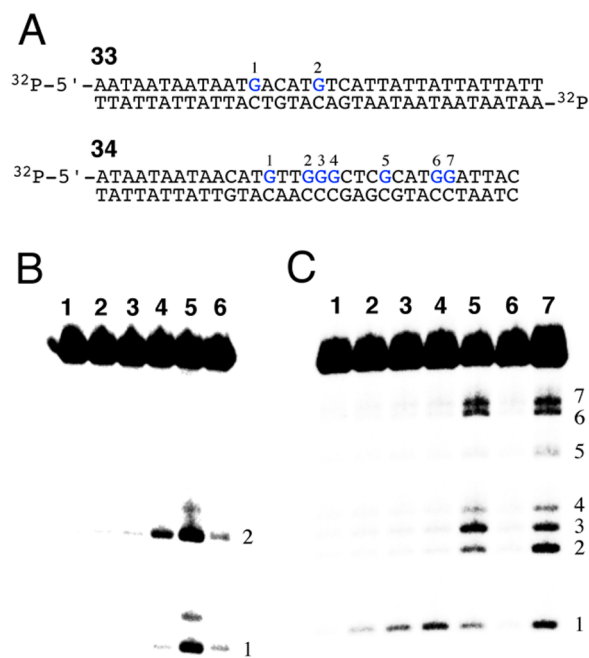


Figure 3. DNA Alkylation by **17a**. Conditions: HEPES buffer (50 mM, pH 7), NaCl (100 mM), mustard (1 mM unless otherwise specified), 10% DMF, 37 °C for 3 d followed by piperidine workup (1 M, 95 °C 30 min). Following incubation labeled DNA fragments were resolved on a 20% denaturing polyacrylamide gel. Labeled DNA was visualized by phosphorimager analysis. (A) ³²P-labeled duplexes **33** and **34** used to examine DNA-alkylating properties of mustards. (B) Lane 1: duplex **33** (no mustard), Lane 2: duplex **33** with **16**, Lane 3: duplex **33** with **18a**, Lane 4: duplex **33** with **17a**, Lane 5: duplex **33** with **2**, Lane 6: G-sequencing of duplex **33**. (C) Lane 1: duplex **34** (no mustard), Lanes 2–4: duplex **34** with **17a** at 0.25, 0.5, and 1 mM concentrations, Lane 5: duplex **34** with **1** (0.25 mM), Lane 6: duplex **34** with **2** (0.25 mM), and Lane 7: G-sequencing of duplex **34**.

requires hypoxia-selective enzymatic reduction of the dioxide **18a**. Thus, it was important to examine whether the dialkylamine substituent at the 6-position of the tirapazamine analogue perturbs bioreductive, deoxygenative metabolism of the 1,2,4-benzotriazine 1,4-dioxide “core”. To simplify product analysis in these experiments, we examined the in vitro hypoxic metabolism of an analogue, **15**, bearing the chemically stable bis(2-hydroxyethyl)amino substituent. We employed recombinant human NADPH:cytochrome P450 reductase as the bioreductive enzyme because this, or a closely related enzyme, is thought to be responsible for bioactivation of **9** and related compounds in mammalian cells.^{68,89–91}

Compound **15** was incubated with NADPH:cytochrome P450 reductase (1 U/mL) and NADPH (500 μM) in sodium phosphate buffer (25 mM, pH 7) under hypoxic conditions. HPLC analysis of the resulting metabolites revealed conversion of the di-*N*-oxide **15** to the corresponding mono-*N*-oxide **16** in approximately 16% yield (Figure 4). A parallel experiment carried out under aerobic conditions produced no detectable yields of **16** (Supplementary Figure S6). A separate control experiment showed that tirapazamine is a better substrate for NADPH:cytochrome P450 reductase than is **15** under our reaction conditions (47% conversion of **9** to the mono-*N*-oxide versus 16% for **15**). Likely this is because the electron-donating bis(2-hydroxyethyl)amino group in **15** decreases the electron-affinity of the 1,2,4-benzotriazine 1,4-dioxide core. Nonetheless,

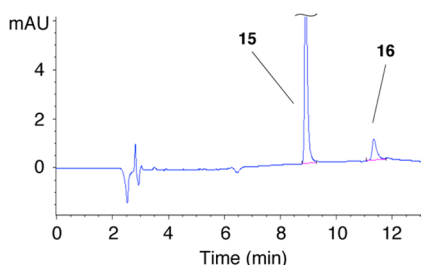


Figure 4. HPLC chromatogram showing the *in vitro* metabolic conversion of **15** to **16** by NADPH:cytochrome P450 reductase under anaerobic conditions.

the results provide evidence that **15** can undergo hypoxia-selective metabolism in a manner similar to that of the parent compound tirapazamine (Figure S7).

CONCLUSIONS

In the work described here, we explored the idea that the hypoxia-selective enzymatic reduction of 1,2,4-benzotriazine 1,4-dioxides to the corresponding 1,2,4-benzotriazine 1-oxide metabolites can be exploited for the selective generation of DNA-alkylating species in the oxygen-poor cells found in tumor tissue. In the case of the tirapazamine analogue **18a** bearing a nitrogen mustard unit at the 6-position, it was found that removal of the 4-oxide from the parent di-*N*-oxide to generate the mono-*N*-oxide analogue **17a** does indeed cause a substantial increase in reactivity of the mustard unit, as measured by the rates of mustard hydrolysis. Hammett σ and σ^- values measured for the 3-amino-1,2,4-triazine 1-oxide and 3-amino-1,2,4-triazine 1,4-dioxide “substituents” in **17a** and **18a**, respectively, confirmed that removal of the 4-oxide causes a significant decrease in the electron-withdrawing effects exerted by the triazine ring system on substituents at the 6-position of the benzo ring.

In contrast to the tirapazamine analogues **17** and **18**, the reactivities of the mustard units in the desamino series **21**, **22**, **25**, and **26** were unaffected by the presence (or absence) of the 4-oxide group. It is interesting to consider why the reactivities of the mustard groups in the 3-desaminotirapazamine analogues (**21**, **22**, **25**, and **26**) are *not* “switched” by deoxygenation. First, it may be noteworthy that the N=N(O) group in **17** and **18** is cross-conjugated with the mustard nitrogen at the 6-position and the 3-amino group. Thus, in these tirapazamine analogues, the 3-amino group may serve to mitigate the electron-withdrawing properties of the N=N(O) group. On the other hand, in analogues **21** and **22** lacking the 3-amino group, the N=N(O) group may exert a strong electron-withdrawing (deactivating) effect on the mustard unit in the 6-position that altogether prevents participation of the nitrogen lone pair in aziridinium ion formation. The hydrolysis rates of **25** and **26** are higher than those of **21** and **22** but still are unaffected by the presence or absence of the 4-oxide unit. Evidently, the N=N(O) unit in the meta position relative to the mustard unit is less electron-withdrawing than when it resides in the para position. In this regard, the N=N(O) group behaves similarly to the nitro group. The observation that the reactivity of the mustard unit in the 7-position is not affected by the presence or absence of the 4-oxide unit may be rationalized by the fact that the nitrogen lone pair of the mustard substituent in this location is not “through conjugated” with the 4-oxide unit.

The DNA-alkylating properties of the nitrogen mustard unit in the mono-*N*-oxide **17a** are “switched on” compared to those of the parent di-*N*-oxide **18a**. Under our assay conditions, the activated analogue **17a** generates approximately 30-fold greater yields of guanine alkylation than does **18a**. The mono-*N*-oxide **17a** displays a striking and unexpected preference for reaction at guanine residues located in 5'-GT sequences. In contrast, typical mustards such as mechlorethamine (**1**) alkylate guanine residues with modest sequence selectivity (lane 5 of Figure 3C).⁸⁶ We speculate that the sequence specificity of **17a** arises via formation of Hoogsteen-type hydrogen bonds between the 3-amino-1,2,4-benzotriazine ring system and the adenine residue in the target 5'-GT/5'-AC sequence. Such an interaction could deliver the mustard unit of **17a** to the N7-atom of the guanine residue in the major groove of the duplex (Figure 5). A similar scenario has been proposed to explain the

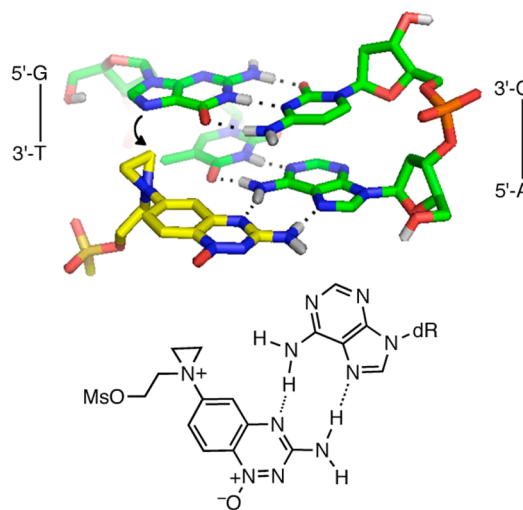


Figure 5. Molecular model depicting Hoogsteen base pairing by **17a** with the adenine residue in a 5'-GT/5'-AC sequence.

unusual 5'-GC sequence specificity for uramustine **6**.⁸⁶ Finally, we provided evidence that the dialkylamino substituent on the 6-position of the 3-amino-1,2,4-benzotriazine 1,4-dioxide ring in analogues **18** is compatible with the hypoxia-selective enzymatic deoxygenative metabolism required to deploy the activated mustards such as **17a**.

The compounds described here result from the combination of two well-studied anticancer drugs. The drug tirapazamine has oxygen sensing properties and generates oxidative DNA damage selectively under hypoxic conditions,^{44–46,52–57} while the nitrogen mustards are clinically used DNA-alkylating agents.³ Our work provides evidence that a judicious union of these two anticancer drug motifs can yield new agents with the potential to deliver both DNA-alkylating and DNA-oxidizing power selectively to oxygen-poor tumor tissue. A potential disadvantage of tirapazamine is that the agent kills only the small subset of tumor cells in which bioreductive metabolism occurs. Addition of a DNA-alkylating unit to the tirapazamine scaffold may yield agents with an ability to diffuse into—and kill—neighboring cells that exist under conditions of both more modest and more severe hypoxia. More generally, our results provide evidence that the 1,2,4-benzotriazine 1,4-dioxide unit can serve as an oxygen-sensing prodrug platform for the selective release or activation of various bioactive agents in hypoxic tissue.

EXPERIMENTAL SECTION

Materials and Methods. All chemicals were used as purchased. The compound 5-fluoro-2-nitroaniline was purchased from Ak Scientific. The compound 3-fluoro-4-nitrobenzoic acid was purchased from Oakwood Chemical. Human cytochrome P450 reductase was purchased from Sigma-Aldrich. For the kinetic measurements in the NMR, S500 select series NMR tubes from Norell were used. NMR spectra were recorded at 500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR. Oligonucleotides were obtained from Integrated DNA Technologies. T4 polynucleotide kinase was purchased from New England Biolabs. [γ - ^{32}P]-ATP (6000 Ci/mmol) was purchased from PerkinElmer. Compounds **9**, **12**, **13**, **14**, and **23** were prepared according to literature methods.^{65,74,75,78,92} Mass spectra were recorded using ESI-QTOF MS.

Synthesis of 3-Amino-6-(bis(2-hydroxyethyl)amino)-1,2,4-benzotriazine 1,4-Oxide (15). Compound **14** (240 mg, 1.3 mmol) was suspended in a mixture of acetonitrile (35 mL), water (1 mL), and diethanolamine (1.4 g, 10 equiv). The suspension was stirred at room temperature for 2 d protected from light. The solvent was removed by rotary evaporation, the orange residue resuspended in cold ethanol, and the solid collected by vacuum filtration. The solid was washed with cold ethanol followed by diethyl ether and dried in an oven overnight at 70 °C to give **15** as an orange powder (270 mg, 73%): mp 210–212 °C dec; ^1H NMR (500 MHz, DMSO- d_6) δ 7.94 (d, J = 9.8 Hz, 1H), 7.71 (bs, 2H), 7.20 (dd, J = 9.9, 2.7 Hz, 1H), 6.96 (d, J = 2.7, 1H), 4.93 (t, J = 5.1 Hz, 2H), 3.64 (bs, 8H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 153.7, 151.8, 140.1, 123.0, 122.8, 116.5, 91.2, 58.2, 53.7; HRMS (ESI) m/z calculated for $\text{C}_{11}\text{H}_{16}\text{N}_5\text{O}_4$ ($\text{M} + \text{H}^+$) 282.1197, found 282.1201.

Synthesis of 3-Amino-6-(bis(2-hydroxyethyl)amino)-1,2,4-benzotriazine 1-Oxide (16). Compound **13** (1.97 g, 11 mmol) and diethanolamine (5.75 g, 5 equiv) were suspended in 1-methyl-2-pyrrolidinone (18 mL) and heated at 100 °C for 18 h. The resulting orange suspension was cooled to room temperature, triturated with cold ethanol (40 mL), and collected by vacuum filtration. The solid was washed with dichloromethane and dried in an oven overnight at 70 °C to give **16** as an orange powder (2.73 g, 94%). (For further purification the solid was suspended in ethanol, briefly heated with stirring, cooled, and collected by vacuum filtration): mp 205–210 °C dec; ^1H NMR (500 MHz, DMSO- d_6) δ 7.88 (d, J = 9.7 Hz, 1H), 6.99 (dd, J = 9.7, 2.4 Hz, 2H), 6.83 (bs, 2H), 6.41 (d, J = 2.3, 1H), 4.85 (t, J = 5.2 Hz, 2H), 3.60–3.56 (m, 8H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 160.6, 153.3, 150.8, 122.0, 120.9, 114.4, 99.7, 58.0, 53.2; HRMS (ESI) m/z calculated for $\text{C}_{11}\text{H}_{16}\text{N}_5\text{O}_3$ ($\text{M} + \text{H}^+$) 266.1248, found 266.1252.

Preparation of 3-Amino-6-(bis(2-((methylsulfonyl)oxy)ethyl)amino)-1,2,4-benzotriazine 1-Oxide (17a). Compound **16** (500 mg, 1.9 mmol) was dissolved in dimethylformamide (4.5 mL), followed by the addition of triethylamine (800 μL , 3 equiv) and cooling in an ice bath. Methanesulfonyl chloride (370 μL , 2.5 equiv) was added dropwise over 15 min as an ice-cold solution in dimethylformamide (0.5 mL), and the resulting mixture was stirred at room temperature for 2.5 h. The solution was poured into 30 mL of cold water with stirring and refrigerated overnight at 4 °C. The resulting orange precipitate was collected by vacuum filtration. The solid was washed with water and diethyl ether and dried in a desiccator to give **17a** (570 mg, 72%): ^1H NMR (500 MHz, DMSO- d_6) δ 7.94 (d, J = 9.6 Hz, 1H), 7.06 (dd, J = 9.7, 1.8 Hz, 2H), 6.98 (bs, 2H), 6.56 (s, 1H), 4.38 (t, J = 5.3 Hz, 4H), 3.90 (t, J = 5.2 Hz, 4H), 3.17 (s, 6H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 161.0, 152.8, 151.1, 123.1, 121.6, 114.4, 101.6, 67.4, 49.7, 37.1; HRMS (ESI) m/z calculated for $\text{C}_{13}\text{H}_{20}\text{N}_5\text{O}_7\text{S}_2$ ($\text{M} + \text{H}^+$) 422.0799, found 422.0800.

Preparation of 3-Amino-6-(bis(2-(tosyloxy)ethyl)amino)-1,2,4-benzotriazine 1-Oxide (17b). Compound **16** (126 mg, 0.5 mmol) was suspended in a biphasic mixture of tetrahydrofuran (2 mL) and NaOH (1.5 mL of a 4 M solution in water) and cooled in an ice bath. To this orange biphasic mixture was added dropwise an ice-cold solution of *p*-toluenesulfonyl chloride (200 mg, 2.2 equiv) in tetrahydrofuran (3 mL). The resulting suspension was stirred vigorously with cooling in an ice bath for 5 h. During this time the

mixture turned yellow. The mixture was then poured into an ice-water slurry (10 mL) and stirred for 20 min to give a bright yellow powder. This solid was collected by vacuum filtration, washed with water and then diethyl ether, and then dried in a desiccator in to give **17b** (202 mg, 74%): ^1H NMR (500 MHz, DMSO- d_6) δ 7.73 (d, J = 9.7 Hz, 1H), 7.60 (d, J = 8.2 Hz, 4H), 7.24 (d, J = 8.1 Hz, 4H), 6.97 (bs, 2H), 6.63 (dd, J = 9.7, 2.5 Hz, 1H), 6.15 (d, J = 7.5 Hz, 1H), 4.13 (t, J = 5.1 Hz, 4H), 3.64 (t, J = 5.1 Hz, 4H), 2.25 (s, 6H); ^{13}C NMR (125 MHz, DMSO) δ 160.6, 151.4, 150.4, 144.9, 131.6, 129.9, 127.4, 122.5, 120.6, 113.7, 101.1, 67.29, 48.9, 20.9. HRMS (ESI) m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_5\text{O}_7\text{S}_2$ ($\text{M} + \text{H}^+$) 574.1425, found 574.1433.

Synthesis of 3-Amino-6-(bis(2-chloroethyl)amino)-1,2,4-benzotriazine 1-Oxide (17c). Compound **17b** (200 mg, 0.35 mmol) and LiCl (148 mg, 10 equiv) were dissolved in dimethylformamide (1.5 mL) and heated at 110 °C for 2 h under a N_2 atmosphere before being cooled on ice and mixed with ice-cold water (20 mL). The resulting yellow precipitate was collected by vacuum filtration, washed with water and diethyl ether, and then dried in a desiccator to give **17c** (104 mg, 94% yield): ^1H NMR (500 MHz, DMSO- d_6) δ 7.95 (d, J = 9.6 Hz, 1H), 7.04 (dd, 9.7, 2.6 Hz, 1H), 6.98 (bs, 2H), 6.48 (d, J = 2.5 Hz, 1H), 3.89 (t, J = 6.6 Hz, 4H), 3.80 (t, J = 6.6 Hz, 4H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 160.7 (d, J = 253.90 Hz), 152.1, 150.8, 122.8, 121.4, 113.9, 101.1, 51.8, 40.9; HRMS (ESI) m/z calculated for $\text{C}_{11}\text{H}_{14}\text{Cl}_2\text{N}_5\text{O}$ ($\text{M} + \text{H}^+$) 302.0570, found 302.0576.

Preparation of 3-Amino-6-(bis(2-((methylsulfonyl)oxy)ethyl)amino)-1,2,4-benzotriazine 1,4-Dioxide (18a). Compound **17a** (70 mg, 0.17 mmol) and NaHCO_3 (86 mg, 6 equiv) were suspended in methanol (25 mL). Oxone (245 mg, 1.2 equiv) was added, followed by water (10 mL). The mixture was stirred at 50 °C under a N_2 atmosphere for 24 h. Thin layer chromatographic analysis indicated that most of the reaction progress occurred within the first 5 h. The reaction was cooled to room temperature, and the white solid was removed by vacuum filtration and washed with CH_2Cl_2 (10 mL). The filtrate was extracted with CH_2Cl_2 (2 \times 125 mL), dried with anhydrous sodium sulfate, and evaporated under reduced pressure, and the resulting residue was subjected to column chromatography on silica gel eluted with a gradient of 1–5% methanol in CH_2Cl_2 to give **18a** as an orange solid (11 mg, 15%): ^1H NMR (500 MHz, DMSO- d_6) δ 8.01 (d, J = 9.8 Hz, 1H), 7.82 (bs, 2H), 7.28 (dd, J = 9.9, 2.7 Hz, 2H), 7.09 (d, J = 2.7, 1H), 4.43 (t, J = 5.3 Hz, 4H), 3.97 (t, J = 5.3 Hz, 4H), 3.19 (s, 6H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 153.0, 151.9, 140.1, 123.7, 123.2, 116.2, 92.8, 67.2, 49.7, 37.1; HRMS (ESI) m/z calculated for $\text{C}_{13}\text{H}_{20}\text{N}_5\text{O}_8\text{S}_2$ ($\text{M} + \text{H}^+$) 438.0748, found 438.0746.

Preparation of 3-Amino-6-(bis(2-(tosyloxy)ethyl)amino)-1,2,4-benzotriazine 1,4-Dioxide (18b). Compound **17b** (501 mg, 0.9 mmol) was dissolved in tetrahydrofuran (100 mL) and cooled with stirring in an ice bath. To this mixture was added *m*-CPBA (300 mg, \sim 1.5 equiv of a 77% maximum purity material), and the reaction was stirred overnight in an ice bath. An additional portion of *m*-CPBA was added, and the reaction was stirred at room temperature for an additional 3 d. The solution was concentrated under vacuum and poured into cold water, and the solid was collected by vacuum filtration. The material was purified by column chromatography on silica gel eluted with a gradient of 1–3% methanol in CH_2Cl_2 to give **18b** as a yellow solid (194 mg, 37%): ^1H NMR (500 MHz, DMSO- d_6) δ 7.84 (bs, 2H), 7.80 (d, J = 9.8 Hz, 1H), 7.60 (d, J = 8.2 Hz, 4H), 7.22 (d, J = 8.1 Hz, 4H), 6.86 (dd, J = 9.8, 2.7 Hz, 1H), 6.65 (d, J = 2.7 Hz, 1H), 4.19 (t, J = 5.0 Hz, 4H), 3.67 (t, J = 4.9 Hz, 4H), 2.24 (s, 6H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 151.6, 151.5, 144.9, 139.3, 131.6, 129.9, 127.4, 123.0, 122.1, 115.6, 92.2, 67.1, 48.9, 20.8. HRMS (ESI) m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_5\text{O}_8\text{S}_2$ ($\text{M} + \text{H}^+$) 590.1374, found 590.1371.

Preparation of 3-Amino-6-(bis(2-chloroethyl)amino)-1,2,4-benzotriazine 1,4-Dioxide 18c. Compound **17c** (50 mg, 0.17 mmol) and NaHCO_3 (42 mg, 3 equiv) were suspended in methanol (25 mL). Oxone (122 mg, 1.2 equiv) was added, followed by water (10 mL). The mixture was stirred at 50 °C under a N_2 atmosphere. The reaction mixture was filtered while still warm, and the solids were washed with methanol (5 mL). The filtrate was cooled to room

temperature, diluted with water (50 mL), and extracted with methylene chloride (3 × 100 mL). Column chromatography on silica gel eluted with a gradient of 2–6% methanol in CH₂Cl₂ gave **18c** as an orange solid (16 mg, 30%): ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.01 (d, *J* = 9.8, Hz, 1H), 7.81 (bs 2H), 7.27 (dd, *J* = 9.8, 2.7 Hz, 1H), 7.05 (d, *J* = 2.7 Hz, 1H), 3.96 (t, *J* = 6.7 Hz, 4H), 3.85 (t, *J* = 6.7 Hz, 4H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 152.3, 151.5, 139.7, 123.4, 123.0, 115.8, 92.2; HRMS (ESI) *m/z* calculated for C₁₁H₁₄Cl₂N₃O₂ (M + H⁺) 318.0519, found 318.0519.

Synthesis of 3-Amino-5-tosyl-6-(bis(2-hydroxyethyl)amino)-1,2,4-benzotriazine 1-Oxide (19). Compound **15** (52 mg, 0.18 mmol) was suspended in a stirred solution of pyridine in an ice bath, and tosyl chloride (172 mg, 5 equiv) was added. The mixture was stirred in an ice bath for 12 h, poured into ice-cold water (20 mL), and extracted with methylene chloride (5 × 25 mL). The combined organic fractions were washed with brine and dried over Na₂SO₄, and solvent was removed by rotary evaporation. Column chromatography on silica gel eluted with 1% methanol in methylene chloride **19** as an orange solid (6 mg, 10%): ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.85 (d, *J* = 9.8 Hz, 1H), 7.75 (d, *J* = 8.2 Hz, 2H), 7.739 (d, *J* = 8.2 Hz, 2H), 7.19 (d, *J* = 9.8 Hz, 1H), 6.94 (bs 2H), 4.65 (t, *J* = 5.1 Hz, 2H), 3.47–3.40 (m, 8H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 160.1, 148.2, 145.9, 145.5, 132.6, 129.5, 128.8, 126.6, 123.6, 118.8, 118.7, 58.4, 54.3, 21.3; HRMS (ESI) *m/z* calculated for C₁₈H₂₂N₃O₆S (M + H⁺) 436.1285, found 436.1293. Crystals suitable for X-ray crystallography were prepared by vapor diffusion with ethyl acetate and hexane. The crystal structure and crystallographic data for **19** are shown in Supplementary Figure S1 and Table S1, respectively.

Synthesis of Desamino-tirapazamine Derivatives 6-Fluoro-1,2,4-benzotriazine 1-Oxide (20) and 7-Fluoro-1,2,4-benzotriazine 1-Oxide (24). Following the general procedure of Boyd et al.⁹³ **23** (930 mg, 5 mmol) was dissolved in anhydrous dimethylformamide (50 mL), and the mixture was degassed by bubbling argon through the solution for 30 min. To this mixture was added *tert*-butyl nitrite (3.5 mL, 5 equiv, 90%) by syringe, and the mixture was heated in a 60 °C oil bath for 2 h under an atmosphere of argon gas. The reaction was cooled, and the solvent was removed under vacuum. The resulting dark residue was taken up in ethyl acetate (300 mL), mixed with brine (150 mL), and stirred vigorously. The phases were allowed to separate, the organic layer was washed with brine (2 × 150 mL) and dried over anhydrous sodium sulfate, and the solvent was removed by rotary evaporation. Column chromatography on silica gel eluted with a gradient of 0–10% ethyl acetate in CH₂Cl₂ gave **24** as a pale white solid (438 mg, 50%): mp 127–129 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.20 (s, 1H), 8.24 (dd, *J* = 9.3, 5.3 Hz, 1H), 8.20 (dd, *J* = 8.4, 2.8 Hz, 1H), 8.08 (m, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 162.6 (d, *J* = 253.9), 154.0 (d, *J* = 2.6 Hz), 144.9, 136.0, 132.5 (d, *J* = 9.4 Hz), 126.7 (d, *J* = 25.9 Hz), 105.2 (d, *J* = 29.3 Hz); HRMS (ESI) *m/z* calculated for C₇H₃FN₃O (M + H⁺) 166.0411, found 166.0419. Compound **20** was prepared from **13** (980 mg) by the same method (447 mg, 50%): mp 160–162 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.19 (s, 1H), 8.50 (dd, *J* = 9.6, 5.5 Hz, 1H), 7.99 (dd, *J* = 9.1, 2.7 Hz, 1H), 7.82 (m, 1H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 166.1 (d, *J* = 256.7), 155.5, 149.3 (d, *J* = 15.1 Hz), 133.1, 123.8 (d, *J* = 11.2 Hz), 121.8 (d, *J* = 26.6 Hz), 113.3 (d, *J* = 22.7 Hz); HRMS (ESI) *m/z* calculated for C₇H₃FN₃O (M + H⁺) 166.0411, found 166.0417.

Synthesis of 6-(Bis(2-(tosyloxy)ethyl)amino)-1,2,4-benzotriazine 1-Oxide (21) and 7-(Bis(2-(tosyloxy)ethyl)amino)benzo[e][1,2,4]triazine 1-Oxide (25). Compound **20** (361 mg, 2.2 mmol) and diethanolamine (500 mg, 2.5 equiv) were dissolved in acetonitrile (7 mL), and the mixture was heated in an 80 °C oil bath overnight. The reaction mixture was cooled, and solvent was removed under reduced pressure by rotary evaporation. The resulting solid was suspended in ethanol (10 mL), briefly heated to 80 °C with stirring, and then cooled to 0 °C, and the resulting precipitate was collected by vacuum filtration. The orange solid was washed with cold ethanol and diethyl ether and dried in an oven overnight at 70 °C. The resulting solid (200 mg) was dissolved in a biphasic mixture of tetrahydrofuran (9 mL) and NaOH (2.4 mL of a 4 M solution in water) and cooled in

an ice bath. To this orange biphasic mixture was added dropwise an ice-cold solution of *p*-toluenesulfonyl chloride (455 mg, 3 equiv) in tetrahydrofuran (1.5 mL). The resulting mixture was stirred vigorously in an ice bath for 1.5 h. During this time the mixture turned yellow. The mixture was then poured into an ice–water slurry (100 mL) and stirred for 30 min to give a yellow precipitate. The solid was collected by vacuum filtration, then washed with water and diethyl ether, and dried in a desiccator to give **21** (356 mg, 61% overall yield over two steps): ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.87 (s, 1H), 7.96 (d, *J* = 9.2 Hz, 1H), 7.57 (d, *J* = 8.3 Hz, 4H), 7.19 (d, *J* = 8.0, 4H), 7.14 (dd, *J* = 9.8, 2.6 Hz, 1H), 6.64 (d, *J* = 2.7 Hz, 1H), 4.18 (t, *J* = 5.1 Hz, 4H), 3.71 (t, *J* = 5.1 Hz, 4H), 2.20 (s, 6H); ¹³C NMR (500 MHz, DMSO-*d*₆) δ 154.8, 152.1, 149.2, 145.2, 131.9, 130.2, 127.8, 127.4, 120.7, 120.3, 103.7, 67.6, 49.3, 21.2. HRMS (ESI) *m/z* calculated for C₂₅H₂₇N₄O₇S₂ (M + H⁺) 559.1316, found 559.1320. Compound **25** was prepared in from **24** by heating for 4 days using the same conditions (390 mg, 57% overall yield over two steps): ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.89 (s, 1H), 7.73 (d, *J* = 9.4 Hz, 1H), 7.56 (d, *J* = 8.2 Hz, 4H), 7.44 (dd, *J* = 9.5, 2.8, 1H), 7.16 (d, *J* = 8.1 Hz, 4H), 6.86 (d, *J* = 2.8 Hz, 1H), 4.19 (t, *J* = 5.0 Hz, 4H), 3.69 (t, *J* = 5.0 Hz, 4H), 2.17 (s, 6H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 150.2, 148.6, 145.2, 140.7, 135.8, 132.0, 130.1, 129.5, 127.7, 125.0, 94.8, 67.5, 49.1, 21.2. HRMS (ESI) *m/z* calculated for C₂₅H₂₇N₄O₇S₂ (M + H⁺) 559.1316, found 559.1317.

Preparation of 6-(Bis(2-(tosyloxy)ethyl)amino)-1,2,4-benzotriazine 1,4-Dioxide (22). Compound **21** (20 mg, 0.04 mmol) and NaHCO₃ (30 mg, 10 equiv) were suspended in methanol (6.25 mL). Oxone (110 mg, 5 equiv) was added, followed by water (2.5 mL), and the mixture was stirred at 50 °C under an atmosphere of nitrogen gas for 16 h. The reaction was cooled to room temperature and extracted with methylene chloride (5 × 10 mL). The organic layers were combined, washed with brine, and dried over anhydrous sodium sulfate. Column chromatography on silica gel eluted with 1% MeOH in CH₂Cl₂ gave compound **22** as an red-orange solid (2 mg, 10%) with 50% recovery of starting materials: ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.15 (s, 1H), 7.96 (d, *J* = 9.8 Hz, 1H), 7.57 (d, *J* = 8.2 Hz, 4H), 7.25–7.18 (m, 5H), 6.84 (d, *J* = 2.8 Hz, 1H), 4.21 (t, *J* = 5.0 Hz, 4H), 3.70 (t, *J* = 4.9 Hz, 4H), 2.25 (s, 6H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 152.0, 145.3, 142.5, 140.7, 132.0, 130.2, 127.8, 127.3, 122.4, 120.9, 84.6, 67.4, 49.1, 21.2. HRMS (ESI) *m/z* calculated for C₂₅H₂₇N₄O₈S₂ (M + H⁺) 575.1265, found 575.1263.

Synthesis of 7-(Bis(2-(tosyloxy)ethyl)amino)-1,2,4-benzotriazine 1,4-Dioxide (26). Using a procedure adapted from Pchalek and Hay,⁹⁴ trifluoroacetic anhydride (300 μL) and methylene chloride (1.5 mL) were mixed with stirring in an ice bath, and 70% H₂O₂ (105 μL) was added dropwise. The mixture was stirred for 10 min and then allowed to warm to room temperature. This solution was cooled in an ice bath and slowly added to an ice-cold solution of **25** (110 mg, 0.2 mmol) in methylene chloride (10 mL). The reaction was stirred in an ice bath for 30 min before being diluted with methylene chloride (100 mL) and washed with cold water, cold NaHCO₃ (saturated), and then brine. The organic layer was dried over anhydrous sodium sulfate, and column chromatography on silica gel eluted with 0.5% MeOH in CH₂Cl₂ gave **26** as a red solid (1 mg, 1%): ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.06 (s, 1H), 7.96 (d, *J* = 9.7 Hz, 1H), 7.57 (d, *J* = 8.3 Hz, 4H), 7.41 (dd, *J* = 9.7, 2.7, 1H), 7.22 (d, *J* = 8.0 Hz, 4H), 6.85 (d, *J* = 2.6 Hz, 1H), 4.19 (t, *J* = 5.0 Hz, 4H), 3.67 (t, *J* = 4.9 Hz, 4H), 2.25 (s, 6H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 149.3, 144.9, 138.8, 135.6, 131.8, 131.7, 129.9, 127.4, 123.2, 119.5, 96.1, 67.1, 48.6, 20.9. HRMS (ESI) *m/z* calculated for C₂₅H₂₇N₄O₈S₂ (M + H⁺) 575.1265, found 575.1275.

Synthesis of 3-Amino-6-*N*-morpholino-1,2,4-benzotriazine 1-Oxide (27). Compound **13** (503 mg, 2.8 mmol) and morpholine (0.72 mL, 3 equiv) were suspended in 1-methyl-2-pyrrolidinone (4 mL) and heated to 100 °C overnight. The resulting orange suspension was cooled to room temperature, diluted with water (40 mL), and filtered. The precipitate was washed with water and diethyl ether and then dried in an oven overnight at 70 °C. The orange-yellow powder was collected by vacuum filtration to give **27** (650 mg, 94%): mp 245–250 °C dec; ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.94 (d, *J* = 9.6 Hz,

1H), 7.16 (dd, $J = 9.7, 2.5$ Hz, 2H), 7.00 (bs, 2H), 6.60 (d, $J = 2.5, 1$ Hz), 3.73 (t, $J = 4.7$ Hz, 4H), 3.38 (t, $J = 4.8$ Hz, 4H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 160.7, 155.3, 150.8, 123.3, 120.9, 115.2, 103.0, 65.8, 46.8; HRMS (ESI) m/z calculated for $\text{C}_{11}\text{H}_{14}\text{N}_5\text{O}_2$ ($M + \text{H}^+$) 248.1142, found 248.1142.

Synthesis of 3-Amino-6-carboxy-1,2,4-benzotriazine 1-Oxide (29). Using a procedure adapted from the method of Lighthart et al.,⁹⁵ NaOH (528 mg, 10 equiv) was dissolved in hot ethanol (30 mL), guanidine hydrochloride (1.2 g, 10 equiv) was added, and the resulting mixture was stirred for 20 min. The reaction was cooled to room temperature and filtered to remove the white precipitate. To the filtrate were added tetrahydrofuran (20 mL) and 3-fluoro-4-nitrobenzoic acid (230 mg, 1.2 mmol), and the resulting heterogeneous mixture was refluxed for 4 h. Potassium *tert*-butoxide (150 mg, 10 equiv) was added, and the mixture was stirred at reflux for an additional 2 h. After the reaction was complete, the tetrahydrofuran was decanted off and water (60 mL) was added with vigorous stirring. Acidification with HCl (1 M) resulted in precipitation of a yellow solid that was collected by vacuum filtration and washed with dilute HCl, followed by minimal amounts of water and diethyl ether. The solid was then dried in an oven overnight at 70 °C to give **29** (244 mg, 95% yield): mp >280 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 13.63 (bs, 1H), 8.20 (d, $J = 8.9$ Hz, 1H), 7.98 (d, $J = 1.5$ Hz, 1H), 7.74 (dd, $J = 2$ Hz, 1.6 Hz, 1H), and 7.52 (s, 2H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 166.1, 160.6, 148.6, 137.1, 131.7, 127.4, 123.6, 120.7; HRMS (ESI) m/z calculated for $\text{C}_8\text{H}_7\text{N}_4\text{O}_3$ ($M + \text{H}^+$) 207.0513, found 207.0519.

Synthesis of 3-Amino-6-carboxy-1,2,4-benzotriazine 1,4-Dioxide (30). Compound **29** (40 mg, 0.2 mmol) was suspended in trifluoroacetic acid (1 mL) and H_2O_2 (70%, 0.8 mL), and the mixture was stirred at 50 °C for 48 h. After the reaction was complete (as judged by thin layer chromatography), the solvent was removed by rotary evaporation under reduced pressure, and the resulting residue was triturated with ethanol (5 mL). The resulting suspension was chilled, and the red precipitate was collected by vacuum filtration. The solid was washed with cold ethanol and dried under vacuum to provide compound **30** (16 mg, 36%): mp 260 °C dec; ^1H NMR (500 MHz, DMSO- d_6) δ 13.84 (bs, 1H), 8.61 (s, 1H), 8.28 (d, $J = 8.8$ Hz, 1H), 8.19 (bs, 2H), and 7.94 (d, $J = 8.7$ Hz, 1H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 165.6, 151.8, 138.3, 136.3, 132.1, 125.8, 122.2, 118.8; HRMS (ESI) m/z calculated for $\text{C}_8\text{H}_7\text{N}_4\text{O}_4$ ($M + \text{H}^+$) 223.0462, found 223.0467.

Synthesis of 3-Amino-6-hydroxy-1,2,4-benzotriazine 1-Oxide (31). Using a procedure adapted from Cantrell et al.⁹⁶ compound **13** (504 mg, 2.8 mmol) was suspended in 1-methyl-2-pyrrolidinone (4 mL), and an aqueous solution of NaOH (400 mg in 20 mL, 4 equiv) was added. To this mixture, a solution of H_2O_2 (30%, 480 μL , 2 equiv) was added, and the mixture was stirred for 4 h at 60 °C. The reaction was cooled to room temperature and filtered to remove undissolved starting material. $\text{Na}_2\text{S}_2\text{O}_3$ (700 mg) was added to decompose peroxides. The resulting solution was acidified with HCl (1 M), and the resulting orange-yellow precipitate was collected by vacuum filtration. The solid was then washed with water and diethyl ether and dried in an oven overnight at 70 °C to give **31** (360 mg, 50%): mp 210 °C dec; ^1H NMR (500 MHz, DMSO- d_6) δ 10.94 (bs, 1H), 8.00 (d, $J = 9.3$ Hz, 1H), 7.13 (bs, 2H), 6.84 (dd, $J = 9.3$ Hz, 1.9 Hz, 1H), and 6.68 (d, $J = 1.9$ Hz, 1H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 163.8, 160.6, 151.1, 124.4, 122.0, 117.2, 106.1; HRMS (ESI) m/z calculated for $\text{C}_7\text{H}_7\text{N}_4\text{O}_2$ ($M + \text{H}^+$) 179.0564, found 179.0566.

Synthesis of 3-Amino-6-hydroxy-1,2,4-benzotriazine 1,4-Dioxide (32). Compound **14** (160 mg, 0.83 mmol) was suspended in 1-methyl-2-pyrrolidinone (1.2 mL), and NaOH (120 mg, 4 equiv, in 6 mL of water) was added. To this mixture was added H_2O_2 (30%, 144 μL , 2 equiv), followed by stirring for 1.5 h at 50 °C. The reaction was cooled to room temperature and acidified with HCl (1 M). The resulting precipitate was collected by vacuum filtration and washed with water and diethyl ether. Drying under vacuum gave **32** as a dark red solid (81 mg, 51%): mp 202 °C dec; ^1H NMR (500 MHz, DMSO- d_6) δ 11.51 (bs, 1H), 8.08 (d, $J = 9.5$ Hz, 1H), 7.92 (bs, 2H), 7.31 (d, $J = 2.5$ Hz, 1H), and 7.04 (dd, $J = 9.5$ Hz, 2.5 Hz, 1H); ^{13}C NMR (125 MHz, DMSO- d_6) δ 164.1, 151.5, 140.1, 125.1, 123.7, 119.2, 98.0;

HRMS (ESI) m/z calculated for $\text{C}_7\text{H}_7\text{N}_4\text{O}_3$ ($M + \text{H}^+$) 195.0513, found 195.0516.

Measurement of Mustard Hydrolysis Rates Using ^1H NMR. Solutions containing the 1,2,4-benzotriazine nitrogen mustards (1 mM final concentration) were prepared in $\text{CD}_3\text{CN}/\text{D}_2\text{O}$ (1:1) containing disodium maleate (5 mM) as an internal standard. Solutions were placed in a capped S500 Norell NMR tube and warmed in a 50 °C water bath. At various time points the NMR tubes were removed from the heat bath and cooled in a room temperature water bath, and ^1H NMR spectra were obtained using a 500 MHz NMR equipped with a 5 mm HCN cryo-probe. The probe temperature was 298 K during the experiment. Total number of scans acquired was 32 with repetition delay of 4.1719923 s to ensure that the integration of resonances in various compounds in the mixture was quantitatively accurate. The amount of unreacted mustard at each time point was assessed by measuring the integration of the CH_2 resonances of the starting material in comparison to the CH_2 peak of the internal standard at each time point. The pseudo-first-order rates for the hydrolysis reactions were obtained by a least-squares fit to the equation $\ln A/A_0 = -kt$.

Measurement of Mustard Hydrolysis Rates and Decomposition Products Using HPLC. Solutions of the mono- or di-*N*-oxide mustards (250 μM) were prepared in sodium phosphate buffer (25 mM, pH 7) containing 2.5% DMF (v/v). The samples were incubated at 50 °C for 24 h. At various time-points aliquots were removed and frozen at -20 °C for later analysis. The samples were then analyzed by HPLC using a Varian Microsorb-MV C-18 column (100 Å sphere size, 5 μm pore size, 250 mm length, and 2.6 mm i.d. eluted with a gradient mobile phase composed of solvent A (0.5% AcOH in H_2O) and solvent B (methanol). For compound **17a** a gradient of 25–50% B over 5 min, followed by 50–100% B over 5 min, followed by 100% B for 5 min was used. The mobile phase was returned to 25% B over 5 min and held at 25% B for 5 min post-run. For compound **18a** the mobile phase was held at 25% B for 5 min following injection, before increasing to 50% B over 5 min and holding at 50% B for 4 min. The mobile phase was then returned to 25% B over 1 min and held at 25% B for 5 min post-run. The products were detected by monitoring absorbance at 280 nm. The identity of the major hydrolysis products was confirmed by comparison to authentic synthetic standards.

Determination of pK_a 's for **29, **31**, and **32**.** UV-vis spectra were taken for compounds **29**, **30**, **31**, and **32** (50 μM) in solutions with pH values from 0 to 9 using HCl solutions and universal buffers prepared as in Britton and Robinson except containing 0.5% DMF (v/v).⁹⁷ Buffer pH was determined with via pH meter. Absorbance values were measured at 256 and 282 nm for **29**, 260 and 270 nm for **30**, 345 and 400 nm for **31**, and 360 and 400 nm for compound **32**. Changes in absorbance were plotted against pH and the pK_a values were calculated by fitting the data to the equation: $pK_a = \text{pH} + \log(d_M - d)/(d - d_1)$, where d_M is the absorbance for the un-ionized species (starting absorbance), d_1 is the absorbance of the ionized species (final absorbance), and d is the absorbance at any point in the pH titration.⁹⁸ Taking the midpoint of the titration data gave a very similar result. To confirm that pK_a values measured reflected protonation of the 6-COOH or 6-OH substituents in compounds **29**, **31**, and **32**, pH-absorbance measurements were performed with **9** and **12** to show that these control compounds displayed no significant changes in absorbance in the pH regions of interest.

Examination of the DNA-Alkylating Properties of **17a and **18a**.** The 2'-deoxyoligonucleotides (**33** and **34**, Figure 3) were labeled on the 5'-end with ^{32}P and annealed to their complements using standard methods.⁹⁹ DNA duplexes were mixed with the indicated compounds (1 mM final concentrations) and incubated at 37 °C for 3 d in HEPES buffer (50 mM, pH 7) containing NaCl (100 mM) and DMF (10% v/v). The DNA was then ethanol precipitated, washed with 80% ethanol twice, and briefly dried in a SpeedVac concentrator (5 min at room temperature). The DNA was resuspended in piperidine (1 M in water) and heated to 95 °C for 30 min. The piperidine solution was removed under vacuum in a SpeedVac concentrator, and the resulting residue was resuspended in formamide

loading buffer,⁹⁹ warmed briefly, and loaded onto a 20% denaturing polyacrylamide gel. The gel was electrophoresed for 3 h at 1500 V to resolve the labeled DNA fragments. The labeled DNA fragments in the gel were visualized using phosphorimaging.

Hypoxia-Selective Enzymatic Reduction of *N*-Mustard Containing 1,2,4-Benzotriazine 1,4-Dioxides with Cytochrome P450.

For experiments involving in vitro hypoxic metabolism, sodium phosphate buffer and HPLC-grade water were degassed by bubbling argon through the solutions for at least 30 min inside a glovebag filled with argon gas. Stock solutions of compounds **15** or **9** in DMF were degassed by three cycles of freeze–pump–thaw inside pyrex tubes. The tubes were then torch-sealed, scored, and transferred to the argon-purged glovebag. The enzyme NADPH:cytochrome P450 reductase was used without degassing. The enzyme substrate NADPH was dissolved in degassed water inside the glovebag. In the glovebag, solutions containing sodium phosphate buffer (25 mM, pH 7), the di-*N*-oxide **15** (100 μ M), NADPH (500 μ M), and NADPH:cytochrome P450 reductase (1 unit/mL) and DMF (1% v/v) were incubated in a sealed microcentrifuge tube at room temperature protected from light for 24 h. A similar reaction was prepared using nondegassed solutions and incubated open to air as a aerobic control. After 24 h, the solutions were passed through Millipore (YM-3) centrifuge filters (30 min, 7500 \times g). The filtrate was analyzed by HPLC using a Varian Microsorb-MV C-18 column (100 Å sphere size, 5 μ m pore size, 250 mm length, and 2.6 mm i.d.). The column was eluted with a mobile phase composed of solvent A (0.5% AcOH in H₂O) and solvent B (methanol). The column was eluted at a flow rate of 0.9 mL/min for 2 min at 10% B, a gradient of 10–50% B over 8 min, 50% B for 5 min, then returned to 10% B over 5 min and held at 10% B for 5 min post-run. Compounds were detected by their absorbance at 420 nm, and the identity of products **15** and **16** was confirmed by comparison to authentic synthetic standards.

■ ASSOCIATED CONTENT

Supporting Information

Crystallographic information for compound **19**. HPLC analysis. UV–vis spectra associated with p*K*_a determinations. Spectroscopic data for all compounds. This materials is available free of charge via the Internet at <http://pubs.acs.org>

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Notes

The authors declare no competing financial interest.

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