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ARTICLE *in* JOURNAL OF MEDICINAL CHEMISTRY · JANUARY 2003

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Structure−Activity Relationships of 1,2,4-Benzotriazine 1,4-Dioxides as Hypoxia-Selective Analogues of Tirapazamine

Michael P. Hay,*,† Swarna A. Gamage,† Mary S. Kovacs,‡ Frederik B. Pruijn,† Robert F. Anderson,† Adam V. Patterson,† William R. Wilson,† J. Martin Brown,‡ and William A. Denny†

Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand, and Department of Radiation Oncology, Stanford University School of Medicine, Stanford, California

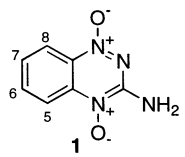
Received August 26, 2002

Tirapazamine (TPZ, 1,2,4-benzotriazin-3-amine 1,4-dioxide) is a bioreductive hypoxic cytotoxin currently in Phase II/III clinical trials in combination with radiotherapy and with cisplatin-based chemotherapy. As part of a program to develop TPZ analogues with improved solubility/potency and therapeutic indices, we synthesized 34 1,2,4-benzotriazin-3-amine 1,4-dioxides (BTO) to examine structure−activity relationships (SAR) for ring substitution. The electronic, hydrophobic, and steric parameters of substituents at the 5-, 6-, 7-, and 8-positions were systematically varied, and the aqueous solubility and one-electron reduction potentials [$E(1)$] of the analogues were determined. For each compound, we determined cell killing of mouse SCCVII tumor cells in vitro under aerobic and hypoxic conditions by clonogenic survival and determined their relative hypoxic toxicity (RHT; relative to TPZ) and hypoxic cytotoxicity ratio (HCR). A subset of compounds was independently evaluated using a 96-well SRB proliferation assay, the data from which correlated well with that derived by the clonogenic endpoint. Most substituents, except 5- and 8-dimethylamino and 8-diethylamino, gave analogues less soluble than TPZ. $E(1)$ values ranged from −240 mV through −670 mV (with TPZ having a value of −456 mV) and correlated well with the electronic parameter σ for substituents at the 5-, 6-, 7-, and 8-positions. Aerobic cytotoxic potency showed a strong positive correlation with $E(1)$ (i.e., electron-withdrawing substituents increased aerobic toxicity). Hypoxic cytotoxicity also generally increased with increasing $E(1)$, with a maximum (RHT up to 3.9-fold) seen in halo- and trifluoromethyl-substituted BTO derivatives having $E(1)$ between ca. −370 to −400 mV. Analogues with high HCRs (>50) all had $E(1)$s in the range −450 to −510 mV (weakly electron-donating substituents) with the exception of the 8-CF_3 analogue, which had an HCR of 112 against SCCVII despite a high $E(1)$ of −372 mV). The results suggest that ring-A substituents in BTO analogues can be used to predictably vary one-electron reduction potentials and also provide a much better definition than previously of the optimum range of these reduction potentials for a desirable biological activity profile (high HCR, RHT, and solubility).

Introduction

It is now established that many human tumors contain a significant fraction of hypoxic cells,[1,2] due to chaotic growth and an inefficient microvasculature system within the tumor, leading to large intercapillary distances and variable blood flow. Reduction in oxygen tension within tumors leads to radioresistance, with up to a 3-fold increase in radiation dose required to kill severely hypoxic tumor cells. The presence of tumor hypoxia and failure of local control by radiation therapy have been linked.[3] The phenomenon of tumor hypoxia has been exploited in the development of a class of anticancer agents termed 'bioreductive drugs',[4−6] which are selectively active against hypoxic cells in tumors.

Tirapazamine (TPZ, **1**) is the leading bioreductive agent,[7−9] currently undergoing advanced clinical trials for the potentiation of radiotherapy and cisplatin chemotherapy.[10] TPZ is reduced by one-electron reductases[11−15] to form a radical anion, and this oxygen-reversible process is the basis for its selective toxicity to hypoxic cells.[16] The radical anion is back-oxidized by molecular oxygen under aerobic conditions, generating superoxide radicals that mediate aerobic toxicity. It has been proposed that under hypoxic conditions the protonated radical anion,[17] or nitroxide[11] undergoes fragmentation to produce the hydroxyl radical.[18] A strong oxidizing species must be formed as TPZ causes DNA double-strand breaks under anoxic conditions, and these correlate with cytotoxicity.[19]



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Although a Phase III trial has shown that TPZ in combination with cisplatin significantly improves response rate and median survival in non-small-cell lung cancer,[20] mixed results have been seen in earlier-stage

* To whom correspondence should be addressed. E-mail: m.hay@auckland.ac.nz.
† The University of Auckland.
‡ Stanford University School of Medicine.

10.1021/jm020367+ CCC: $25.00
Published on Web 12/02/2002

trials of TPZ in combination with fractionated radiation against head and neck carcinomas and glioblastomas.[21,22] Drug toxicity prevented TPZ being administered with each fraction of radiation, which has been shown theoretically to be the optimum administration protocol.[23]

Previous structure−activity relationship (SAR) studies of A-ring-substituted 3-amino-1,2,4-benzotriazine 1,4-dioxides (BTO) have focused on a small set of 6- or 7-substituents [e.g., 6,7-dimethyl, 6-Cl, 7-Me, 7-alkoxy, and mixtures of 6(7)-OMe and 6(7)-Cl].[24] In this study, hypoxic toxicity was shown to increase with increasing $E_{1/2}$ (the half wave potential of two-electron reduction, measured polarographically). A more complex relationship was observed between hypoxic cell selectivity (HCR) and $E_{1/2}$, with TPZ having the maximum HCR. A subsequent paper examined the effect of a small set of electron-withdrawing substituents (F, Cl, CF_3, NO_2, Ph) in combination with a variety of 3-substituents.[8]

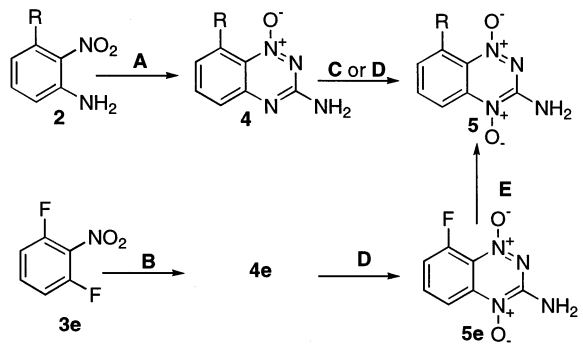
As part of a program to develop TPZ analogues with improved solubility/potency and therapeutic indices, we prepared a more diverse set of A-ring substituted BTOs to explore the SAR for A-ring substitution more thoroughly, using substituents at all four ring positions and without the confounding influence of substitution at the 3-position. We also measured one-electron reduction potentials [$E(1)$] by pulse radiolysis rather than $E_{1/2}$ values, as a more accurate determination of the one electron reduction potential. We synthesized 34 3-amino-BTOs, substituted at the 5-, 6-, 7-, and 8-positions with groups spanning a wide range of electronic, hydrophobic, and steric properties. The aqueous solubility, $E(1)$ values, and effect on the clonogenic survival of SCCVII cells under aerobic and hypoxic conditions of the compounds were determined, and hypoxic toxicity relative to TPZ (RHT), and the hypoxic cytotoxicity ratio (HCR), defined as the ratio of doses under aerobic and hypoxic conditions producing equal toxicity, were calculated. For a limited set of 12 compounds, including TPZ, a proliferation assay (SRB) was used to determine IC_{50} values, under oxic and anoxic conditions, against two cells lines (murine SCCVII and human adenocarcinoma HT-29), allowing a comparison between clonogenic survival (C_{10}) and proliferation (IC_{50}) based assays.

Chemistry

For the substituted 3-amino-BTOs, 11 substituents with widely different electronic, lipophilic, and steric substituent parameters were chosen (NEt_2, NMe_2, OMe, Me, F, Cl, CF_3, $MeSO_2$, *n*-$BuSO_2$, NO_2, and OH). The majority of the compounds were prepared in a two-step process; condensation−cyclization to form the 1-oxides, followed by oxidation of these to give the 1,4-dioxides. Two main condensation−cyclization reactions were used. One (Method A: Schemes 1−4)[25,26] employed 2-nitroanilines with cyanamide, followed by cyclization of the intermediate guanidine with NaOH. Generally, lower yields were noted for the more lipophilic compounds, reflecting poor solubility in the reaction medium. In several instances (7-CF_3, 7-NO_2, 5-Cl), it was necessary to use an alternative method (Method B: Schemes 2 and 4),[27] involving reaction of 2-chloronitrobenzenes (**7g**, **7j**, and **14e**, respectively) with guanidine, followed by cyclization under basic conditions. This gave 1-oxides in modest yield (15−45%). Similarly, reaction of 2,6-difluoronitrobenzene **3e** with guanidine followed by cyclization under basic conditions gave 1-oxide **4e** in moderate yield (Scheme 1).

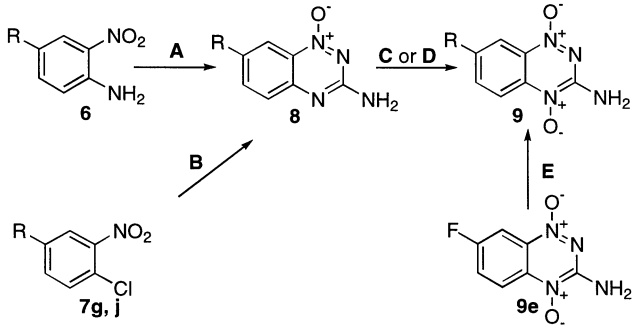
Sulfides **2h** and **2i** were prepared by nucleophilic displacement of 3-chloro-2-nitroaniline (**2f**) with the appropriate alkylthiolate salt (Scheme 5). Similarly, displacement of 5-chloro-2-nitroaniline (**10f**) with BuSLi gave **10i**. Subsequent studies showed that displacements of 8-chloro- and 6-chloro-1-oxides **4f** and **11f** with *n*BuSLi gave the corresponding sulfides **4i** and **11i**, whereas reaction of the 7-chloro-1-oxide **8f** with ^{n}BuSLi gave mixtures of starting material, product, and the corresponding nor-*N*-oxides from which pure **8h** could not be obtained. Reaction of the corresponding 7-bro-

Scheme 1[a]



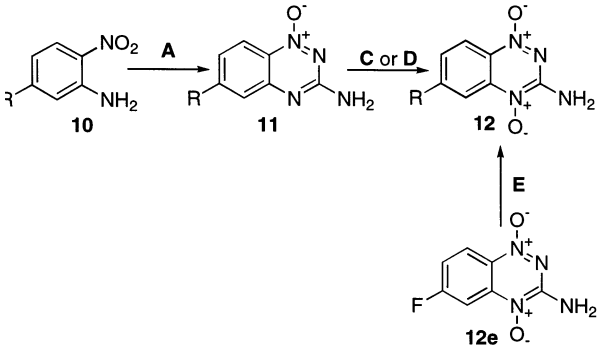
[a] Reagents: Method A, cyanamide, HCl, then NaOH; Method B, guanidine·HCl, KO*t*Bu, then NaOH; Method C, 30% H_2O_2, HOAc; Method D, 70% H_2O_2, CF_3CO_2H; Method E, R_2NH, MeCN.

Scheme 2[a]



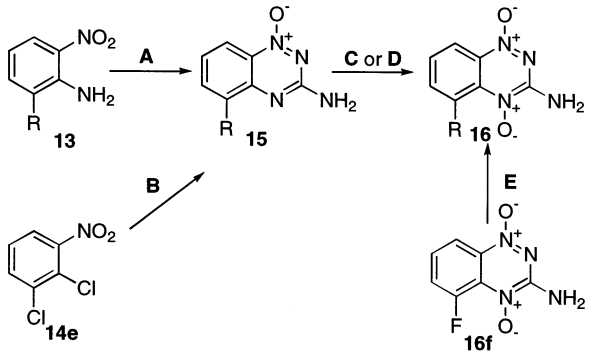
[a] Reagents: Method A, cyanamide, HCl, then NaOH; Method B, guanidine·HCl, KO*t*Bu, then NaOH; Method C, 30% H_2O_2, HOAc; Method D, 70% H_2O_2, CF_3CO_2H; Method E, R_2NH, MeCN.

Scheme 3[a]



[a] Reagents: Method A, cyanamide, HCl, then NaOH; Method B, guanidine·HCl, KO*t*Bu, then NaOH; Method C, 30% H_2O_2, HOAc; Method D, 70% H_2O_2, CF_3CO_2H; Method E, R_2NH, MeCN.

Scheme 4[a]



[a] Reagents: Method A, cyanamide, HCl, then NaOH; Method B, guanidine·HCl, KO*t*Bu, then NaOH; Method C, 30% H_2O_2, HOAc; Method D, 70% H_2O_2, CF_3CO_2H; Method E, R_2NH, MeCN.

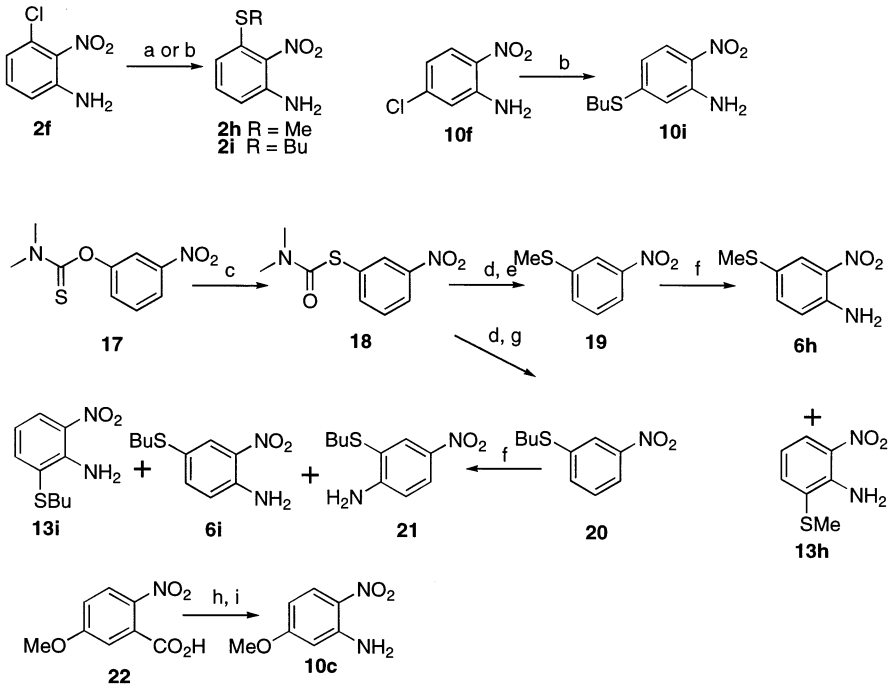
mide gave complete reaction, but was accompanied by significant amounts of 1-oxide reduction. Attempts to prepare 4-(methylsulfanyl)-2-nitroaniline (**6h**) by nucleophilic displacement of the 4-chloride **6f** were not successful. Reaction of 5-chloro-1-oxide **15e** with ^{n}BuSLi gave only starting material. A circuitous approach utilizing the Newman−Kwart rearrangement[28] and vicarious nucleophilic substitution (V_NS)[29,30] gave two of the required methylsulfanyl isomers **6h** and **13h** in sufficient purity (Scheme 5). Thus, isomerization of *O*-thiocarbamate **17** gave S-thiocarbamate **18**, which was hydrolyzed, and the intermediate thiol was alkylated with MeI to give sulfide **19**. V_NS Reaction of **19** with NH_2OMe·HCl gave nitroanilines **6h** and **13h**. A similar sequence from **18** gave butylsufanylnitroanilines **6i** and **13i** as well as the isomeric **21**. Curtius rearrangement of 5-methoxy-2-nitrobenzoic acid (**22**) gave nitroaniline **10c**.

Oxidation of the substituted 1-oxides with peracetic acid (Method C: Schemes 1−4) or trifluoroperacetic acid (Method D; Schemes 1−4) gave the corresponding 1,4-dioxides. The oxidations provided moderate yields of 6-, 7-, and 8-substituted 1,4-dioxides, with significant losses in the workup and purification of the products from reaction mixtures containing large amounts of peroxides. Treatment of the reaction mix with strong base led to decomposition of the product 1,4-dioxides. Sulfide groups were oxidized to sulfone groups simultaneously with N-oxidation. Dimethylamino- and diethylamino-BTO derivatives were prepared by displacement of the corresponding fluoro-1,4-dioxides with the appropriate amine (Method E: Schemes 1−4).

A number of the planned BTOs could not be prepared. The condensation reactions (Method A or B) failed to give 6-nitro- and 8-nitro-1-oxides. Oxidation of 5-substituted 1-oxides **15c**−**f** proved extremely difficult, with extensive decomposition in the reaction leading to low yields, and 5-CF_3, 5-SO_2Me, 5-SO_2Bu, and 5-NO_2 BTOs could not be prepared at all. Nucleophilic displacement of the 5-Cl-BTO **16e** with LiSMe gave only starting material. Attempts to prepare the proposed hydroxy-BTOs were not also successful. Demethylation of the 7- or 8-OMe-BTO analogues (**9c** and **5c**, respectively) with BBr_3 gave only complex mixtures, while reaction with LiSEt gave mixtures with reduction of both *N*-oxides. Reaction of the 7-OMe 1-oxide **8c** with pyridine hydrochloride gave intractable mixtures. While it was possible to prepare 7-hydroxy-1,2,4-benzotriazine 1-oxide, we were unable to find a protecting group (apart from OMe) capable of surviving the oxidation conditions required for 1,4-dioxide formation.

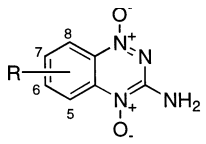
Physicochemical Measurements. The solubilities of the BTOs in α-MEM with 5% added fetal calf serum was determined by HPLC (Table 1). None of the analogues proved more soluble than TPZ itself (although increasing solubility was not a major goal of the current

Scheme 5[a]



[a] Reagents: (a) MeSLi, DMF; (b) nBuSLi, DMF; (c) 220 °C; (d) KOH, MeOH; (e) Me_2SO_4, KOH, MeOH; (f) NH_2OMe·HCl, KOtBu, CuCl, DMF; (g) nBuBr, K_2CO_3, DMF; (h) DPPA, Et_3N, tBuOH; (i) HCl, MeOH.

Table 1. Physicochemical Properties of Ring-A-Substituted BTO Analogues



no.	R	synth method	soln[a] (mM)	$E(1)$[b] (mV)	ref[c]	$\log P_{meas}$[d]	$\log P_{train}$[e]
1	H		8.9	−456 ± 8	MV	−0.34 ± 0.02	−0.33
5a	8-NEt_2	E	14.1	−554 ± 6	TeQ	0.82 ± 0.01	0.82
5b	8-NMe_2	E	9.0	−545 ± 10	TQ		−0.24
5c	8-OMe	A,C	6.4	−503 ± 8	MV		−0.42
5d	8-Me	A,C	1.3	−510 ± 7	MV	0.24 ± 0.03	0.24
5e	8-F	B,D	4.9	−400 ± 7	MV		−0.28
5f	8-Cl	A,C	2.2	−388 ± 8	MV	0.17 ± 0.005	0.17
5g	8-CF_3	A,C	0.5	−372 ± 12	MV	0.53 ± 0.03	0.48
5h	8-SO_2Me	A,D	0.9	−309 ± 12	BV	−1.38 ± 0.02	−1.38
5i	8-SO_2Bu	A,D	1.7	−314 ± 10	DQ		0.21
9a	7NEt_2	E	0.4	–	–		0.84
9b	7-NMe_2	E	1.2	−525 ± 8	TQ		−0.23
9c	7-OMe	A,C	2.0	−494 ± 8	TQ		−0.05
9d	7-Me	A,C	1.9	−474 ± 11	MV	0.13 ± 0.003	0.14
9e	7-F	B,C	0.9	−400 ± 8	MV		−0.28
9f	7-Cl	A,C	0.1	−397 ± 7	MV	0.46 ± 0.04	0.44
9g	7-CF_3	B,C	0.7	−345 ± 10	DQ	0.81 ± 0.05	0.81
9h	7-SO_2Me	A,D	0.5	−297 ± 10	DQ	−1.15 ± 0.05	−1.15
9i	7-SO_2Bu	A,D	0.3	−296 ± 10	DQ		0.44
9j	7-NO_2	B,C	0.3	−260 ± 10	DQ		−0.60
12a	6-NEt_2	E	1.5	−671 ± 6	TeQ		0.84
12b	6-NMe_2	E	0.4	−668 ± 6	TeQ		−0.23
12c	6-OMe	A,D	0.6	−558 ± 8	TQ		−0.05
12d	6-Me	A,C	3.0	−493 ± 8	MV	0.21 ± 0.03	0.08
12e	6-F	A,C	1.4	−443 ± 7	MV		−0.28
12f	6-Cl	A,C	0.4	−391 ± 8	MV		0.41
12g	6-CF_3	A,C	0.2	−335 ± 10	MV		0.24
12h	6-SO_2Me	A,D	0.2	−258 ± 11	DQ		−1.27
12i	6-SO_2Bu	A,D	0.1	−240 ± 16	DQ		0.32
16a	5-NEt_2	E	3.2	−489 ± 8	MV		0.84
16b	5-NMe_2	E	10.0	−481 ± 10	BV		−0.23
16c	5-OMe	A,C	3.0	−427 ± 10	BV		−0.42
16d	5-Me	A,C	2.4	–	–		0.13
16e	5-Cl	B,D	1.0	−401 ± 8	MV	0.07 ± 0.01	0.10
16f	5-F	A,D	7.5	−394 ± 7	MV		−0.28

[a] Determined in FCS + 5% α-medium. [b] One-electron potential in mV; determined by pulse radiolysis. [c] DQ; duroquinone (2,3,5,6-tetramethylbenzoquinone) = −260 ± 10 mV:[44] BV; benzyl viologen (1,1′-dibenzyl-4,4′-bipyridinium) = −375 ± 10 mV:[45] MV; methyl viologen (1,1′-dimethyl-4,4′-bipyridinium) = −447 ± 7 mV:[46] TQ; triquat (7,8-dihydrodipyrido-[1,2-*a*:2′,1′-*c*][1,4]diazepinediium) = −548 ± 7 mV:[46] TeQ, tetraquat (6,7,8,9-tetrahydrodipyrido[1,2-*a*:2′,1′-*c*][1,4]diazocinediium) = −635 ± 5 mV.[47] [d] In *n*-octanol/water. [e] Using the program ACDLogP v. 4.5.

study). Octanol/water partition coefficients at pH 7.4 were determined by a low volume shake flask method, with drug concentrations in both the octanol and buffer phases analyzed by HPLC as previously described.[31] These values were used to "train" ACD LogP prediction software (v. 4.5, Advanced Chemistry Development Inc., Toronto, Canada) and measured and predicted values are reported (Table 1).

Pulse radiolysis experiments were performed on The University of Auckland Dynaray 4 (4 MeV) linear accelerator (200 ns pulse length with a custom-built optical radical detection system). $E(1)$ values for the compounds were determined in anaerobic aqueous solutions containing 2-propanol (0.1 M) buffered at pH 7.0 (10 mM phosphate) by measuring the equilibrium constant[32] for the electron transfer between the radical anions of the compounds and the appropriate viologen or quinone reference standard. Data were obtained at three concentration ratios at room temperature (22 ± 2 °C) and used to calculate the ΔE between the compounds and references, allowing for ionic strength effects on the equilibria.

Biological Assays

The efficacy of the compounds in killing aerobic and hypoxic mouse SCCVII tumor cells in vitro was determined by clonogenic survival. Cytotoxicity was measured as the concentration required to reduce plating efficiency to 10% of controls (C_{10}), and the RHT was determined as the intraexperimental ratio of hypoxic TPZ C_{10} to hypoxic BTO C_{10} [RHT = C_{10}(TPZ)/C_{10}(BTO)]. The intraexperimental differential between the hypoxic and aerobic cytotoxicity for each compound was calculated as the HCR [= C_{10}(aerobic)/C_{10}(hypoxic)] (Table 2).

Twelve compounds (Table 3) were evaluated using a proliferation assay (Sulforhodamine B; SRB) to determine IC_{50} values under oxic and anoxic conditions. For each experiment, all 12 compounds were simultaneously tested under both conditions against both SCCVII and HT-29 cell lines. Thus each set of IC_{50} determinations is an intraexperiment comparison. Plates were stained as described previously[33] and IC_{50} values determined. Results were averaged for three independent experiments.

Table 2. Clonogenic Assay Data for BTO Analogues in SCCVII Cells

no.	R	$C_{10(hyp)}$[a] (μM)	$C10_{(aer)}$[b] (μM)	RHT[c]	HCR[d]
1	H	10.2 ± 0.5 (34)[e]	2644 ± 266 (9)[e]	1.0	258 ± 21 (9)[e]
5a	8-NEt_2	114	1904	0.089	16.7
5b	8-NMe_2	157	2769	0.065	17.6
5c	8-OMe	25.4	>135	0.40	>5.3
5d	8-Me	24.7	2538	0.41	102.9
5e	8-F	10.2	132	1.0	13.0
5f	8-Cl	2.58	102	3.9	39.4
5g	8-CF_3	5.56	626	1.8	112.5
5h	8-SO_2Me	50.8	338	0.20	6.7
5i	8-SO_2Bu	8.31	175	1.2	21.1
9a	7NEt_2	>72.5	>725	<0.14	
9b	7-NMe_2	237	3159	0.043	13.3
9c	7-OMe	19.3	1934	0.53	100.0
9d	7-Me	16.2	2031	0.63	125.0
9e	7-F	5.41	381	1.9	70.3
9f	7-Cl	9.48	271	1.1	28.6
9g	7-CF_3	9.14	305	1.1	33.3
9h	7-SO_2Me	31.7	251	0.32	7.9
9i	7-SO_2Bu	7.76	>89.6	1.3	>11.5
9j	7-NO_2	5.37	36.3	1.9	6.8
12a	6-NEt_2	>50.8	>1269	<0.20	
12b	6-NMe_2	>440	440	<0.023	<1.0
12c	6-OMe	127	nd[f]	0.080	
12d	6-Me	17.4	2901	0.58	166.7
12e	6-F	10.2	114	1.0	11.2
12f	6-Cl	6.47	140	1.6	21.6
12g	6-CF_3	8.23	110	1.2	13.3
12h	6-SO_2Me	26.1	<274	0.39	<10.5
12i	6-SO_2Bu	16.2	<102	0.63	<6.3
16a	5-NEt_2	67.7	58.7	0.15	0.87
16b	5-NMe_2	25.8	30.5	0.39	1.2
16c	5-OMe	58.0	47.9	0.18	0.83
16d	5-Me	38.1	<381	0.27	<10.0
16e	5-Cl	7.46	10.2	1.4	1.4
16f	5-F	10.2	<76.1	1.0	<7.5

[a] Concentration of drug for a one-log cell kill under hypoxia normalized against TPZ (**1**) in the same experiment. [b] Concentration of drug for a one-log cell kill under aerobic conditions normalized against TPZ (**1**) in the same experiment. [c] Intraexperimental Relative Hypoxic Toxicity; C_{10}(TPZ)/C_{10}(BTO). [d] Intraexperimental Hypoxic Cytotoxicity Ratio; C_{10}(AER)/C_{10}(HYPOX). [e] Mean ± SEM, with number of measurements in brackets. [f] Not determined due to poor solubility.

Results and Discussion

Physicochemical Measurements. The solubility of TPZ in medium containing 5% fetal calf serum was determined as 8.9 mM. This is in reasonable agreement with the solubility (13.5 mM) previously determined in 0.9% NaCl solution.[24] Three compounds (8-NEt_2-BTO **5a**, 8-NMe_2-BTO **5b**, and 5-NMe_2-BTO **16b**) showed increased solubility compared to TPZ, while the 7-Cl **9f** was ca. 100-fold less soluble. Generally, the 5- and 8-substituted BTOs were more soluble than the corresponding 6- and 7-substituted analogues, suggesting that a *peri* interaction with the *N*-oxides may reduce crystallinity via distortion of intermolecular hydrogen bonding. Generally, simple substituents on ring-A lowered solubility, emphasizing the need for extended substituents containing isolated polar groups to increase aqueous solubility.

The hydrophobicity of TPZ and eleven other BTOs were determined from HPLC analysis of drug distribution between octanol and aqueous buffer at pH 7.4 (Table 1). The measured value for TPZ (−0.34 ± 0.02) was in good agreement with the literature value (−0.32)[8] as was the value for the 7-Me-BTO **9d** (measured 0.13 ± 0.003; lit.[24] 0.2). The measured data, including literature values, were used to train ACDLogP software, which was then used to predict the values for the whole set of BTOs. There was a strong correlation between the measured ($logP_{meas}$) and calculated logP ($logP_{calc}$) without training (eq 1a) but this improved after training (eq 1b):

$$\log P_{meas} = 0.72 \log P_{calc} + 0.15$$

$$n = 12,\ r^2 = 0.903,\ F = 93 \qquad (1a)$$

$$\log P_{meas} = 1.01 \log P_{calc} + 0.012$$

$$n = 12,\ r^2 = 0.997,\ F = 2809 \qquad (1b)$$

The main improvements came from including **5h**, **9g**, and **9h** in the training set. For the whole set of BTOs, the trained logP was strongly correlated with the substituent hydrophobicity parameter π (eq 2):

$$\log P_{train} = 0.82\pi - 0.17$$

$$n = 3,\ 5r^2 = 0.847,\ F = 183 \qquad (2)$$

E(1) values were determined by pulse radiolysis for TPZ and all but two of the series (Table 1). The measured value for TPZ (−456 ± 8 mV) was in close agreement with the value of −0.45 ± 0.01 V previously reported.[17] The values range widely, from −240 mV for the 6-$BuSO_2$-BTO (**12i**) to −671 mV for the 6-NEt_2-BTO (**12a**). Regression analysis of substituents for each of the four positions (including TPZ; **1** in each case) gave

Table 3. IC_{50} Assay Data for BTO Compounds in SCCVII and HT-29 Cells

		IC_{50}[a] SCCVII				IC_{50}[a] HT-29			
no.	name	hypoxic	oxic	RHT[b]	HCR[c]	hypoxic	oxic	RHT[b]	HCR[c]
1	TPZ	2.4 ± 0.3	71.7 ± 8.7	1.0	32.9 ± 7.2	5.3 ± 0.2	196 ± 43	1.0	37.0 ± 7.5
5c	8-OMe-BTO	13.5 ± 2.9	288 ± 14	0.2	25.7 ± 7.0	28.8 ± 4.4	624 ± 151	0.2	21.9 ± 4.8
5e	8-F-BTO	1.2 ± 0.3	23.7 ± 4.6	1.9	13.9 ± 3.6	2.1 ± 0.2	8.7 ± 1.5	2.5	4.1 ± 0.4
5f	8-Cl-BTO	0.45 ± 0.14	8.2 ± 0.7	5.2	15.9 ± 5.8	0.55 ± 0.25	7.7 ± 4.0	9.6	13.6 ± 1.3
5h	8-$MeSO_2$-BTO	9.7 ± 0.5	15.5 ± 2.1	0.2	1.6 ± 0.3	15.1 ± 0.8	69.8 ± 14.9	0.4	4.5 ± 0.8
9e	7-F-BTO	1.1 ± 0.3	17.6 ± 1.4	2.2	19.3 ± 3.9	1.87 ± 0.25	47.8 ± 9.1	2.8	21.7 ± 2.8
9f	7-Cl-BTO	0.61 ± 0.07	12.2 ± 1.2	3.9	20.4 ± 2.7	0.73 ± 0.12	19.8 ± 4.4	7.2	28.5 ± 8.4
9g	7-CF_3-BTO	0.92 ± 0.15	23.9 ± 3.5	2.6	27.7 ± 5.0	2.1 ± 0.9	79.1 ± 15.2	2.5	35.7 ± 6.1
9j	7-NO_2-BTO	0.70 ± 0.09	2.8 ± 0.2	3.4	4.1 ± 0.3	0.79 ± 0.38	14.1 ± 4.0	6.7	14.8 ± 0.1
12d	6-Me-BTO	5.6 ± 0.7	217 ± 31	0.4	42.6 ± 10.4	12.9 ± 0.8	469 ± 84	0.4	35.8 ± 4.5
12f	6-Cl-BTO	0.98 ± 0.17	21.1 ± 3.2	2.4	22.5 ± 4.0	1.3 ± 0.07	41.2 ± 8.6	4.1	33.0 ± 7.7
16b	5-NMe_2-BTO	12.2 ± 1.5	2.1 ± 0.3	0.2	0.18 ± 0.03	23.3 ± 0.7	4.2 ± 1.2	0.2	0.18 ± 0.05

[a] IC_{50} values in μM (mean ± SEM, $n \geq 3$). [b] Interexperimental relative hypoxic toxicity; C_{10}(TPZ)/C_{10}(BTO). [c] Interexperimental hypoxic cytotoxicity ratio; C_{10}(AER)/C_{10}(HYPOX).

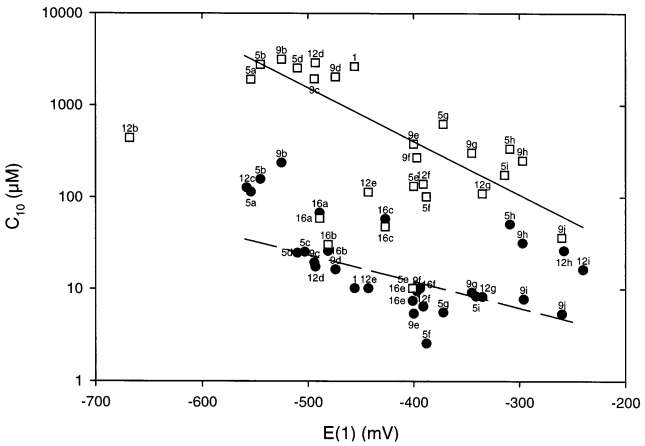


Figure 1. Cytotoxicity of BTO analogues against SCCVII cells (clonogenic assay) versus one electron reduction potential: aerobic, □; hypoxic, ●; solid line eq 4a, dashed line eq 5a.

the following equations:

$$\text{5-sub: } E(1)/\text{mV} = -453 + 161\sigma_m$$

$$n = 7,\ r^2 = 0.976,\ F = 160 \quad (3a)$$

$$\text{6-sub: } E(1)/\text{mV} = -454 + 282\sigma_p$$

$$n = 10,\ r^2 = 0.987,\ F = 596 \quad (3b)$$

$$\text{7-sub: } E(1)/\text{mV} = -424 + 171\sigma_p$$

$$n = 10,\ r^2 = 0.933,\ F = 111 \quad (3c)$$

$$\text{8-sub: } E(1)/\text{mV} = -492 + 287\sigma_m$$

$$n = 10,\ r^2 = 0.946,\ F = 106 \quad (3d)$$

In all cases, the $E(1)$ values were predicted very well by the σ values of the substituents, with no significant steric effects, even for 5- and 8-substituents. In both the 5- and 8-substituted series, the correlation fitted best to σ_m, with the 8-substituents showing the stronger dependence. In the 6- and 7-substituted series, the data were fitted better by the σ_p parameter, with a stronger dependence for the 6-substituents, which also spanned the largest $E(1)$ range of more than 400 mV. These well-defined relationships make it possible to adjust $E(1)$ quite precisely by appropriate substitution of the 3-amino-BTO nucleus.

Clonogenic Assay. Twenty-five of the thirty-four BTO analogues were sufficiently soluble to obtain aerobic cytotoxicity data in SCCVII cells, using a clonogenic assay (concentration of drug for a one-log cell kill; C_{10} in μM) (Figure 1). The aerobic $\log C_{10}$ values showed a linear dependence on $E(1)$, as shown by eq 4a:

$$\log(\text{aerobic}C_{10}) = -0.14 - 5.90(\pm 0.90)E(1) \times 10^{-3} - 1.41(\pm 0.19)D \quad (4a)$$

$$n = 25,\ r^2 = 0.79,\ F = 42$$

This analysis includes a dummy variable (D), to account for the fact that the four sterically strained 5-substituted analogues (**16a**−**c**, **16e)** are much (about 25-fold) more cytotoxic than predicted by $E(1)$ alone, possibly due to accelerated metabolic activation. It excludes the 6-NMe_2-BTO (**12b**), which was measured at its solubility limit and had a very low $E(1)$ value. Addition of various hydrophobicity parameters (π for the substituents, or logP values) to eq 4a gave small but significant improvements (with π being best, eq 4b), showing that, for this set of analogues, aerobic activity was not very dependent on hydrophobicity.

$$\log(\text{aerobic}C_{10}) = -0.12 - 6.81(\pm 0.90)E(1) \times 10^{-3} - 1.36(\pm 0.18)D - 0.23(\pm 0.11)\pi \quad (4b)$$

$$n = 25,\ r^2 = 0.83,\ F = 33$$

Equations 4a and 4b show that (aerobic) potency in the clonogenic assay is favored by higher reduction potential. This is reasonable, since higher $E(1)$s will lead to more rapid (aerobic) metabolism. Previous work[34] demonstrated a linear correlation (slope 10.4 V^{-1}) between the (logarithm of) oxygen consumption rate in CHO cells and the $E_{1/2}$ for sixteen BTOs, based on other data.[24] The regression coefficient in eq 4 (5.9 V^{-1}) is in reasonable agreement with that, suggesting that under aerobic conditions reactive oxygen species, formed by redox-cycling of the BTO radical anion with oxygen, are mainly responsible for toxicity.

Hypoxic C_{10} values could be determined for all but three of the BTO analogues studied, and $E(1)$ values were measured for all but one of these (the 5-Me-BTO **16d**, which could be prepared only in very low yield). The hypoxic C_{10} values of the remaining thirty-one compounds in SCCVII cells were less well predicted by a linear relationship with $E(1)$ (equation 5a and Figure 1):

$$\log(\text{hypoxic}C_{10}) = 0.07 - 2.88(\pm 0.08)E(1) \times 10^{-3} \quad (5a)$$

$$n = 31,\ r^2 = 0.30,\ F = 12$$

The significance of the QSAR was slightly improved by the addition of hydrophobicity parameters (logP, π, MR), with the best being π:

$$\log(\text{hypoxic}C_{10}) = -0.47 - 4.32(\pm 0.05)E(1) \times 10^{-3} - 0.37(\pm 0.11)\pi \quad (5b)$$

$$n = 31,\ r^2 = 0.51,\ F = 15$$

Unlike the aerobic toxicity, there appeared to be a loss of hypoxic potency at both low (< ca. −500 mV) and high (>−370 mV) ends of the $E(1)$ range (Figure 1). This is reflected by the less strong linear correlation between $E(1)$ and hypoxic C_{10}. The most potent compounds were halo-substituted analogues; 8-Cl-BTO (**5f**), 7-F-BTO (**9e**), 6-Cl-BTO (**12f**), 5-Cl-BTO (**16e**), and 8-CF_3-BTO (**5g**). BTO analogues with a dimethylamino or diethylamino substituent at any position were considerably less potent than TPZ under hypoxia, also resulting in very low RHT values. With the exception of 5-Cl-BTO (**16e**), analogues with a 5-substituent were less potent than TPZ under hypoxia, and all 5-substituted BTO analogues were considerably more potent than TPZ under aerobic conditions. This resulted in low RHT

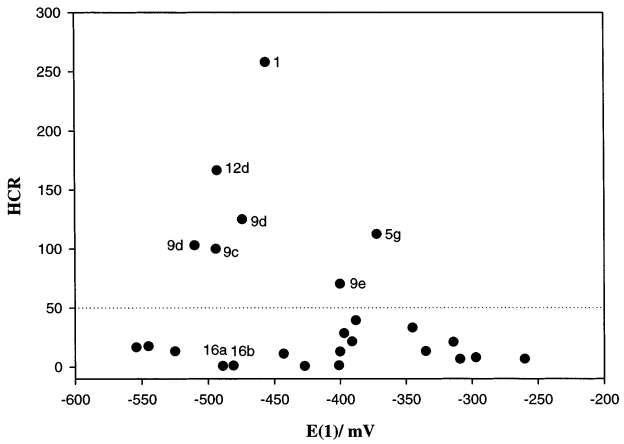


Figure 2. Hypoxic selectivity of BTO analogues against SCCVII cells (clonogenic assay) versus one electron reduction potential.

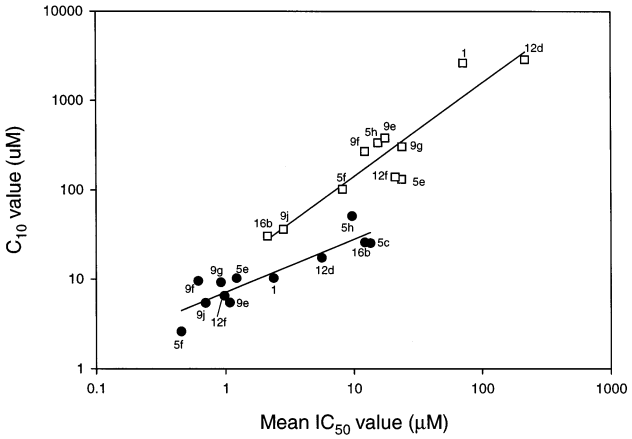


Figure 3. Comparison of clonogenic assay (C_{10} values) with proliferation assay (IC_{50} values, SRB) for SCCVII cells.

values and no hypoxic selectivity (Figures 1 and Table 2). Analogues contributing to the falloff in potency at high reduction potential include methyl sulfones **5h**, **9h**, **12h**, and butyl sulfones **9i** and **12i**. The low logP values for the methyl sulfones (−1.38, −1.15, and −1.27, respectively) may contribute to poor uptake by cells resulting in reduced potency. Another potential factor contributing to reduced hypoxic potency for analogues with high reduction potentials may be an increased rate of metabolic depletion. It is notable that none of the BTO analogues gave an overall increase in hypoxic potency × solubility compared to TPZ (**1**).

There was no clear relationship between HCR and *E*(1) in the clonogenic assay, (Figure 2). None of the BTO analogues displayed hypoxic selectivity greater than TPZ. However, a cluster of compounds with good hypoxic selectivity (HCR ≥ 50) [8-Me-BTO (**5d**), 7-OMe-BTO (**9c**), 7-Me-BTO (**9d**) and 6-Me-BTO (**12d**)] had weakly electron-donating substituents, giving *E*(1) values in the range of −510 to −450 mV. Other compounds with *E*(1) values within this range that did not show good selectivity were 5-NEt_2-BTO (**16a**) and 5-NMe_2-BTO (**16b**), further emphasizing that 5-substituted BTO analogues are poor hypoxia-selective cytotoxins. Two other analogues 8-CF_3-BTO (**5g**) and 7-F-BTO (**9e**) displayed good selectivity suggesting hypoxic selectivity is influenced by a variety of factors as well as *E*(1).

The most common side effects of TPZ observed in clinical trial[21,22,35] were neutropenia, fatigue, muscle cramps, and nausea and vomiting. The mechanisms of these toxicities are poorly understood, especially whether they arise from metabolism to the TPZ radical in normal tissues under physiological hypoxia, aerobic redox cycling leading to reactive oxygen species, or mitochondrial TPZ metabolism. One possible strategy to reduce normal tissue toxicity is to increase the hypoxic selectivity of BTO analogues. The clonogenic assay data identifies a range of *E*(1) values (−450 to −510 mV) where maximal HCR was observed. It is expected that compounds with lower *E*(1) values will have decreased rates of metabolism under aerobic conditions, and the higher selectivity may translate into improved therapeutic indices. Much of the metabolism of TPZ occurs outside the nucleus[36] and does not result in DNA double strand breaks, and mitochondrial toxicity induced by TPZ[37] is probably involved in the pathogenesis of muscle cramps. Thus another strategy to improve therapeutic indices is to target the BTO analogue to the nucleus via a DNA binding moiety. Indeed, preliminary studies show that DNA-targeting does increase the potency of BTO analogues.[38]

Comparison of C_{10} and IC_{50} Values in SCCVII Cells. Clonogenic survival (C_{10}) assays have the advantage of being a direct measure of cell sterilization, but are time-consuming. Proliferation assays, such as the SRB assay, enable higher throughput screening but are potentially subject to artifacts related to different rates of cell death or cell cycle arrest for different treatments. To assess the suitability of rapid IC_{50} assays for further development of TPZ analogues, we utilized a subset of BTO compounds with a range of potencies and differential toxicities, as determined by clonogenic survival, and compared IC_{50} values using the SRB assay (Table 3). Despite the differences in exposure time (1 h vs 4 h), endpoint (C_{10} vs IC_{50}) and method for achieving hypoxia, the SAR remained consistent, with the data for all three parameters derived from both assays showing good agreement (oxic cytotoxic potency, hypoxic cytotoxic potency and HCR; $R = 0.93$, 0.90, and 0.80, respectively; $P < 0.002$). log−log regression analysis of the data suggests the SRB assay is an adequate surrogate for clonogenic survival, at least in the SCCVII cell line (Figure 3). TPZ is more toxic under aerobic conditions in the IC_{50} assay than would be predicted by the C_{10} assay. This anomaly is not observed under hypoxic conditions resulting in a higher hypoxic selectivity being displayed by TPZ in the clonogenic assay (258-fold; Table 2) than in the IC_{50} assay (33-fold; Table 3). However, for the other compounds in the study HCR values for the two assays were in closer agreement (Figure 3).

Comparison of SCCVII and HT-29 Cell Line Screens. To evaluate potential intercell line variations in BTO analogue SAR, the SRB assay was also used to compare the murine cell line SCCVII with the human adenocarcinoma cell line HT-29. Highly significant log−log correlations between the two cell lines were seen for all measured parameters (oxic cytotoxic potency, hypoxic cytotoxic potency, and HCR; $R = 0.90$, 0.99, and 0.92, respectively; $P < 0.001$). This is illustrated for the relationship between hypoxic IC_{50} in the two lines in Figure 4. Thus the SAR for both the aerobic and anoxic

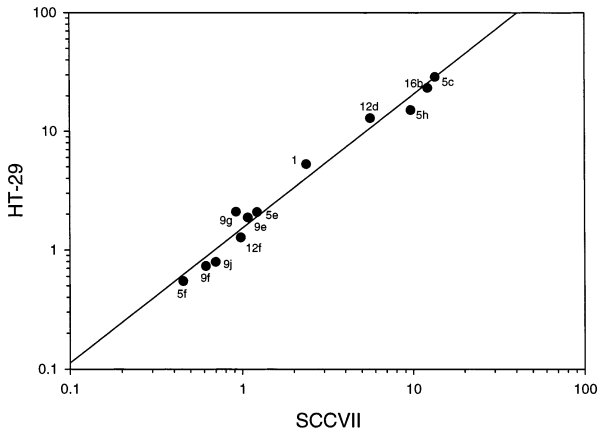


Figure 4. Comparison of Hypoxic IC_{50} values: HT-29 cells versus SCCVII cells.

toxicity of BTO analogues is very similar in both cell lines evaluated.

Conclusions

The use of substituted BTO analogues with a wide range of electronic and lipophilic properties allowed a better definition than previously of the influences of these properties on cytotoxicity. One-electron reduction potentials were highly dependent on substituent electronic properties, and aerobic C_{10}s in the clonogenic assay were highly dependent on $E(1)$. However, all 5-substituted analogues were exceptionally toxic under aerobic conditions, which lowered their HCR ratios. Hypoxic C_{10}s were less well predicted, but again reduction potential was the most important parameter. In each case, potency was slightly increased with increasing lipophilicity.

Overall, the results suggest that ring-A substituents in BTO analogues can be used to predictably vary one-electron reduction potentials, and that high hypoxic cytotoxicity ratios (HCRs > 50) are best achieved by weakly electron-donating substituents that result in $E(1)$ values between −450 to −510 mV. This defines a narrow range of substituent electronic properties required to optimize the 1,2,4-benzotriazine-3-amine 1,4-dioxide nucleus as a hypoxic cell cytotoxin and allows the rational design of analogues that have improved pharmacological properties (e.g., solubility and distribution) while retaining high HCR and RHT values. Our efforts to improve the therapeutic indices of BTO analogues by targeting compounds to the nucleus and reducing the amount of unproductive metabolism will be the subject of pending publication.

In addition, the excellent agreement between clonogenic and proliferation assays for both hypoxic and aerobic cytotoxic potency demonstrate the utility of the IC_{50} assays for the higher throughput screening of hypoxic cytotoxins, especially for SAR comparisons within related series of analogues, where the influences of other mechanisms of action are likely to be similar.

Experimental Section

Analyses were carried out in the Microchemical Laboratory, University of Otago, Dunedin, NZ. Melting points were determined on an Electrothermal 2300 Melting Point Apparatus. NMR spectra were obtained on a Bruker AM-400 spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C spectra. Spectra were obtained in $CDCl_3$ unless otherwise specified, and are referenced to Me_4Si. Chemical shifts and coupling constants were recorded in units of ppm and hertz, respectively. Assignments were determined using COSY, HSQC, and HMBC two-dimensional experiments. Mass spectra were determined on a VG-70SE mass spectrometer using an ionizing potential of 70 eV at a nominal resolution of 1000. High-resolution spectra were obtained at nominal resolutions of 3000, 5000, or 10000 as appropriate. All spectra were obtained as electron impact (EI) using PFK as the reference unless otherwise stated. Solutions in organic solvents were dried with anhydrous Na_2SO_4. Solvents were evaporated under reduced pressure on a rotary evaporator. Thin-layer chromatography was carried out on aluminum-backed silica gel plates (Merck 60 F_{254}) with visualization of components by UV light (254 nm) or exposure to I_2. Column chromatography was carried out on silica gel, (Merck 230−400 mesh). All compounds designated for biological testing were analyzed at >99% purity by reverse phase HPLC using a Philips PU4100 liquid chromatograph, a Phenomenex BondClone 10-C18 stainless steel column (300 mm × 3.9 mm i.d.) and a Philips PU4120 diode array detector. Chromatograms were run using various gradients of aqueous (1 M NaH_2PO_4, 0.75 M heptanesulfonic acid, 0.5 M dibutylammonium phosphate, and MilliQ water in a 1:1:1:97 ratio) and organic (80% MeOH/MilliQ water) phases. DCM refers to dichloromethane, DME refers to dimethoxyethane, DMF refers to dry dimethylformamide, ether refers to diethyl ether, EtOAc refers to ethyl acetate, EtOH refers to ethanol, MeOH refers to methanol, and pet. ether refers to petroleum ether, boiling range 40−60 °C; THF refers to tetrahydrofuran dried over sodium benzophenone ketyl. All solvents were freshly distilled. Nitroanilines **2d**, **2f**, **2g**, **6c**, **6d**, **6e**, **6f**, **10d**, **10e**, **10f**, **10g**, **13d**, **13f**, chloronitrobenzenes **7g**, **7j**, **13e**, and 2,6-difluoronitrobenzene **3e**, were commercially available. Nitroanilines **2c**,[39] **10h**,[29] and **13c**[29] were prepared as previously described. The synthesis of nitroanilines **2h**, **2i**, **6h**, **6i**, **10c**, **10i**, **13g**, and **13h** is described herein.

3-(Methylsulfanyl)-2-nitroaniline (2h). A solution of LiSMe (1.19 g, 22.0 mmol) in DMF (20 mL) was added dropwise to a stirred solution of 3-chloro-2-nitroaniline (**2f**) (3.17 g, 18.4 mmol) in DMF (80 mL) at 20 °C and the mixture stirred for 2 h. The mixture was poured into water (300 mL) and extracted with EtOAc (2 × 150 mL). The combined organic fraction was washed with water (2 × 100 mL) and brine (50 mL) and dried and the solvent evaporated. The residue was chromatographed, eluting with 20% EtOAc/pet. ether, to give (i) starting material (0.51 g, 16%) and (ii) sulfide (**2h**) (2.36 g, 70%) as red crystals, mp (EtOAc/pet. ether) 70−72 °C; ^{1}H NMR δ 7.21 (t, J = 8.2 Hz, 1 H, H-5), 6.55 (d, J = 8.2 Hz, 2 H, H-4, H-6), 5.92 (br s, 2 H, NH_2), 2.42 (s, 3 H, SCH_3); ^{13}C NMR δ 146.0, 141.6, 133.3, 131.0, 113.9 (2), 17.0; Anal. ($C_7H_8N_2O_2S$) C, H, N.

3-(Butylsulfanyl)-2-nitroaniline (2i). A solution of LiSBu (1.39 g, 14.5 mmol) in DMF (10 mL) was added dropwise to a stirred solution of 3-chloro-2-nitroaniline (**2f**) (2.08 g, 12.05 mmol) in DMF (50 mL) at 20 °C and the mixture stirred for 2 h. The mixture was poured into water (300 mL) and extracted with EtOAc (2 × 150 mL). The combined organic fraction was washed with water (2 × 100 mL) and brine (50 mL) and dried and the solvent evaporated. The residue was chromatographed, eluting with 20% EtOAc/pet. ether, to give sulfide **2i** (2.47 g, 91%) as a red oil, ^{1}H NMR δ 7.17 (dd, J = 8.2, 8.0 Hz, 1 H, H-5), 6.62 (d, J = 8.0 Hz, 1 H, H-4), 6.54 (dd, J = 8.2, 1.0 Hz, 1 H, H-6), 5.74 (br s, 2 H, NH_2), 2.87 (t, J = 7.4 Hz, 2 H, CH_2S), 1.64−1.72 (m, 2 H, CH_2), 1.45−1.53 (m, 2 H, CH_2), 0.95 (s, 3 H, CH_3); ^{13}C NMR δ 145.3, 140.1, 133.0, 132.4, 115.0, 113.9, 33.0, 29.8, 22.2, 13.6; MS (EI) m/z 226 (M^+, 35%), 106 (100); HRMS (EI) calcd for $C_{10}H_{14}N_2O_2S$ (M^+) m/z 226.0776, found 226.0773.

4-(Methylsulfanyl)-2-nitroaniline (6h) and 6-(Methylsulfanyl)-2-nitroaniline (13h). *O*-(3-Nitrophenyl) dimethylthiocarbamate[28] (**17**) (14.05 g, 62.1 mmol) was heated at

235−240 °C for 3 h under N_2, cooled to 20 °C, to give crude *S*-(3-nitrophenyl) dimethylthiocarbamate (**18**) which was heated at reflux temperature with KOH solution (7.5 M, 410 mL, 3.1 mol) and MeOH (200 mL) for 2 h. The mixture was cooled to 20 °C, Me_2SO_4 (59 mL, 0.62 mol) added dropwise, and the mixture stirred at 20 °C for 16 h. The mixture was partitioned between EtOAc (300 mL) and water (300 mL), the organic fraction washed with water (3 × 100 mL) and brine (100 mL) and dried, and the solvent evaporated. The residue was chromatographed, eluting with 10% EtOAc/pet. ether, to give 1-(methylsulfanyl)-3-nitrobenzene (**19**) (9.6 g, 91%) as a soft solid, ^{1}H NMR δ 8.05 (dd, J = 2.0, 1.9 Hz, 1 H, H-2), 7.96 (dd, J = 8.2, 2.0, 0.9 Hz, 1 H, H-4), 7.53 (ddd, J = 7.9, 1.9, 1.0 Hz, 1 H, H-6), 7.43 (dd, J = 8.2, 7.9 Hz, 1 H, H-5), 2.56 (s, 3 H, SCH_3); ^{13}C NMR δ 148.6, 141.6, 131.9, 129.4, 120.3, 119.6, 15.4.

A solution of NH_2OMe.HCl (2.83 g, 34.0 mmol) and nitrobenzene **19** (4.79 g, 28.3 mmol) in DMF (100 mL) was added dropwise to a stirred mixture of KOtBu (13.0 g, 116.6 mmol) and CuCl (0.28 g, 2.83 mmol) in DMF (50 mL) at 5 °C. The mixture was stirred at 20 °C for 3 h and quenched with saturated aqueous NH_4Cl solution (100 mL). The mixture was extracted with EtOAc (3 × 100 mL), the combined organic fraction dried, and the solvent evaporated. The residue was chromatographed, eluting with 10% EtOAc/pet. ether, to give (i) 6-(methylsulfanyl)-2-nitroaniline (**13h**) (2.11 g, 40%) as a red oil, ^{1}H NMR δ 8.11 (dd, J = 8.7, 1.5 Hz, 1 H, H-3), 7.66 (dd, J = 7.4, 1.5 Hz, 1 H, H-5), 6.92 (br s, 2 H, NH_2), 6.66 (dd, J = 8.7, 7.4 Hz, 1 H, H-4), 2.38 (s, 3 H, SCH_3); ^{13}C NMR δ 145.0, 140.7, 132.5, 126.6, 124.5, 115.9, 18.2; MS (EI) *m*/*z* 184 (100, M^+), 169 (10), 150 (30); HRMS calcd for $C_7H_8N_2O_2S$ (M^+) *m*/*z* 184.0307, found 184.0304; (ii) 4-(methylsulfanyl)-2-nitroaniline (**6h**) (0.8 g, 15%) as a red oil, ^{1}H NMR δ 8.05 (d, J = 2.3 Hz, 1 H, H-3), 7.34 (dd, J = 8.7, 2.3 Hz, 1 H, H-5), 6.76 (d, J = 8.7 Hz, 1 H, H-6), 6.05 (br s, 2 H, NH_2), 2.46 (s, 3 H, SCH_3); ^{13}C NMR δ 143.1, 136.8, 132.2, 125.8, 125.1, 119.5, 18.0.

4-(Butylsulfanyl)-2-nitroaniline (6i) and 6-(Butylsulfanyl)-2-nitroaniline (13i). A mixture of crude **18** (7.0 g, 31 mmol) and KOH (7.5 M, 41 mL, 310 mmol) in MeOH (200 mL) was heated a reflux temperature for 2 h. The mixture was cooled to 5 °C and the pH adjusted to 2 with concd HCl. The precipitate was collected, washed with water (20 mL), dissolved in EtOAc (200 mL), and dried, and the solvent was evaporated. The residue was dissolved in DMF (100 mL), K_2CO_3 (5.15 g, 37.3 mmol) added, and the mixture stirred at 20 °C for 30 min. *n*-Butyl bromide (4.0 mL, 37.3 mmol) was added and the mixture stirred at 80 °C for16 h. The mixture was cooled and the solvent evaporated. The residue was partitioned between EtOAc (300 mL) and water (300 mL), the organic fraction washed with water (3 × 100 mL) and brine (100 mL) and dried, and the solvent evaporated. The residue was chromatographed, eluting with 10% EtOAc/pet. ether, to give 4-(butylsulfanyl)-2-nitrobenzene (**20**) (5.53 g, 84%) as a yellow oil, ^{1}H NMR δ 8.10 (dd, J = 2.0, 2.0 Hz, 1 H, H-2), 7.97 (ddd, J = 8.1, 2.0, 0.9 Hz, 1 H, H-4), 7.57 (ddd, J = 8.1, 2.0, 0.9 Hz, 1 H, H-6), 7.42 (dd, J = 8.1, 8.1 Hz, 1 H, H-5), 3.01 (dd, J = 7.4, 7.3 Hz, 2 H, CH_2S), 1.64−1.71 (m, 2 H, CH_2), 1.43−1.53 (m, 2 H, CH_2), 0.95 (t, J = 7.3 Hz, 3 H, CH_3); ^{13}C NMR δ 148.6, 140.5, 133.5, 129.4, 121.9, 120.1, 32.6, 30.7, 21.9, 13.6; MS (EI) *m*/*z* 211 (M^+, 60%), 155 (100); HRMS (EI) calcd for $C_{10}H_{13}NO_2S$ (M^+) *m*/*z* 211.0667, found 211.0661.

A solution of NH_2OMe·HCl (2.61 g, 31.2 mmol) and **20** (5.5 g, 26.0 mmol) in DMF (40 mL) was added dropwise to a stirred mixture of KOtBu (12.0 g, 106.7 mmol) and CuCl (0.26 g, 2.6 mmol) in DMF (50 mL) at 5 °C. The mixture was stirred at 20 °C for 6 h and quenched with saturated aqueous NH_4Cl solution (300 mL). The mixture was extracted with EtOAc (3 × 100 mL), the combined organic fraction dried, and the solvent evaporated. The residue was chromatographed, eluting with 5% EtOAc/pet. ether, to give (i) 6-(butylsulfanyl)-2-nitroaniline (**13i**) (2.34 g, 40%) as a red oil, ^{1}H NMR δ 8.12 (dd, J = 8.7, 1.5 Hz, 1 H, H-3), 7.66 (dd, J = 7.3, 1.5 Hz, 1 H, H-5), 7.00 (br s, 2 H, NH_2), 6.64 (dd, J = 8.7, 7.3 Hz, 1 H, H-4), 2.75 (dd, J = 7.5, 7.2 Hz, 2 H, CH_2S), 1.51−1.58 (m, 2 H, CH_2), 1.37−1.46 (m, 2 H, CH_2), 0.90 (dd, 7.4,7.2 Hz, 3 H, CH_3); ^{13}C NMR δ 145.8, 142.5, 132.5, 127.0, 122.7, 115.6, 35.0, 31.6, 21.7, 13.6; MS (EI) *m*/*z* 226 (M^+,100%), 209 (15), 192 (25); HRMS (EI) calcd for $C_{10}H_{14}N_2O_2S$ (M^+) *m*/*z* 226.0776, found 226.0770; (ii) 4-(butylsulfanyl)-2-nitroaniline (**6i**) (1.12 g, 19%) as a red oil, ^{1}H NMR δ 8.16 (d, J = 2.2 Hz, 1 H, H-3), 7.39 (dd, J = 8.7, 2.2 Hz, 1 H, H-5), 6.76 (d, J = 8.7 Hz, 1 H, H-6), 6.06 (br s, 2 H, NH_2), 2.83 (dd, J = 7.3, 7.2 Hz, 2 H, CH_3S), 1.56−1.62 (m, 2 H, CH_2), 1.38−1.48 (m, 2 H, CH_2), 0.91 (dd, J = 7.4, 7.3 Hz, 3 H, CH_3); ^{13}C NMR δ 143.5, 139.1, 132.2, 128.3, 123.9, 119.3, 35.3, 31.2, 21.8, 13.6; MS (EI) *m*/*z* 226 (M^+,100%), 170 (80); HRMS (EI) calcd for $C_{10}H_{14}N_2O_2S$ (M^+) *m*/*z* 226.0776, found 226.0772; and (iii) 2-(butylsulfanyl)-4-nitroaniline (**21**) (0.76 g, 13%) as a red oil, ^{1}H NMR δ 8.16 (d, J = 2.6 Hz, 1 H, H-3), 8.00 (dd, J = 9.1, 2.6 Hz, 1 H, H-5), 6.68 (d, J = 9.1 Hz, 1 H, H-6), 5.07 (br s, 2 H, NH_2), 2.79 (dd, J = 7.2, 6.6 Hz, 2 H, CH_2S), 1.52−1.60 (m, 2 H, CH_2), 1.38−1.47 (m, 2 H CH_2), 0.91 (t, J = 7.3 Hz, 3 H, CH_3); ^{13}C NMR δ 153.5, 138.7, 131.4, 125.7, 118.1, 113.0, 34.7, 31.5, 21.7, 13.6.

5-Methoxy-2-nitroaniline (10c). A mixture of 5-methoxy-2-nitrobenzoic acid (**22**) (10 g, 50.7 mmol), diphenylphosphoryl azide (DPPA) (11.5 mL, 53.3 mmol), and Et_3N (7.4 mL, 53.3 mmol) in t-BuOH (200 mL) was heated at reflux temperature for 16 h. The solution was cooled to 20 °C and the solvent evaporated. The residue was dissolved in DCM (300 mL) and washed with water (2 × 100 mL), saturated aqueous $KHCO_3$ (100 mL), and brine (50 mL), dried and the solvent evaporated. The residue was suspended in MeOH (250 mL), concd HCl (50 mL) added, and the mixture stirred at 20 °C for 96 h. The solvent was evaporated and the residue suspended in saturated aqueous $KHCO_3$ (400 mL) and stirred for 30 min. The suspension was filtered and the solid washed with water (20 mL) and dried at 80 °C under reduced pressure. The solid was chromatographed, eluting with a gradient (20−30%) of EtOAc/pet. ether, to give **10c** (8.26 g, 98%), as a yellow solid, mp 128−130 °C (lit.[29] mp 130−132 °C); ^{1}H NMR δ 8.07 (d, J = 9.5 Hz, 1 H, H-3), 6.28 (dd, J = 9.5, 2.6 Hz, 1 H, H-4), 6.21 (br s, 2 H, NH_2), 6.15 (d, J = 2.6 Hz, 1 H, H-6), 3.83 (s, 3 H, OCH_3); ^{13}C NMR δ 165.4, 147.1, 128.5, 126.9, 106.7, 99.4, 55.7.

5-(Butylsulfanyl)-2-nitroaniline (10i). A solution of LiS-nBu (3.34 g, 34.8 mmol) in DMF (30 mL) was added dropwise to a stirred solution of 5-chloro-2-nitroaniline (**10f**) (5.0 g, 30.0 mmol) in DMF (50 mL) at 20 °C and the mixture stirred for 2 h. The mixture was poured into water (300 mL) and extracted with EtOAc (2 × 150 mL). The combined organic fraction was washed with water (2 × 100 mL) and brine (50 mL) and dried and the solvent evaporated. The residue was chromatographed, eluting with 20% EtOAc/pet. ether, to give **10i** (6.12 g, 90%) as a red solid, mp (EtOAc/pet. ether) 91−93 °C; ^{1}H NMR δ 8.00 (d, J = 7.5 Hz, 1 H, H-3), 6.52−6.56 (m, 2 H, H-4, H-6), 6.11 (br s, 2 H, NH_2), 2.96 (dd, J = 7.4, 7.3 Hz, 2 H, CH_2S), 1.66−1.73 (m, 2 H, CH_2), 1.44−1.53 (m, 2 H, CH_2), 0.96 (s, 3 H, CH_3); ^{13}C NMR δ 149.0, 144.8, 129.6, 126.4, 115.2, 113.4, 31.3, 30.6, 22.0, 13.6; Anal. ($C_{10}H_{14}N_2O_2S$) C, H, N.

Method A: Condensation of 2-Nitroanilines with Cyanamide. 2-Nitroaniline (4.3 mmol) and cyanamide (22 mmol) were melted together at 100 °C cooled to ca. 50 °C and concd HCl (5 mL) added carefully. The mixture was stirred until the exotherm subsided then stirred at 100 °C for 2 h. If necessary, more cyanamide (22 mmol) was added and the mixture stirred at 100 °C for 4 h. The mixture was cooled to 20 °C and made strongly basic with 7.5 M NaOH solution (ca. 50 mL) and the mixture heated at 100 °C for 1 h then cooled to 20 °C and diluted with water (100 mL). The precipitate was filtered, washed with water (2 × 10 mL) and ether (2 × 10 mL) and dried. If necessary, the solid was chromatographed, eluting with a gradient (2−5%) of MeOH/$CHCl_3$, to give the corresponding 1,2,4-benzotriazin-3-amine 1-oxide.

Method B: Condensation of 2-Nitrohalobenzenes with Guanidine. Guanidine hydrochloride (104 mmol) was added to a stirred solution of KOtBu (104 mmol) in absol EtOH (80 mL) and the mixture stirred at 20 °C for 1 h. The mixture was filtered and the filtrate added slowly to a stirred solution of 2-nitrohalobenzene (26 mmol) in absol EtOH (50 mL). The mixture was heated at reflux temperature for 72 h then cooled,

acidified with concd HCl and the solvent evaporated. The residue was suspended in 0.5 M HCl and the precipitate was filtered. The aqueous fraction was washed with $CHCl_3$, basified with aq NH_3, and extracted into EtOAc. The organic fraction was dried and the solvent evaporated. The residue was suspended in 10% aq NaOH and heated at 100 °C for 2 h. The precipitate was filtered, washed with water (2 × 10 mL), ether (2 × 10 mL) and dried. If necessary, the solid was chromatographed, eluting with a gradient (2−5%) of MeOH/$CHCl_3$, to give the corresponding 1,2,4-benzotriazin-3-amine 1-oxide.

8-Methoxy-1,2,4-benzotriazin-3-amine 1-Oxide (4c). Method A using **2c** gave **4c** (51%) as a yellow powder, mp (H_2O) 235−239 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.62 (dd, J = 8.3, 8.0 Hz, 1 H, H-6), 7.15 (br s, 2 H, NH_2), 7.02 (d, J = 8.3 Hz, 1 H, H-7), 6.80 (d, J = 8.0 Hz, 1 H, H-5), 3.83 (s, 3 H, OCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 160.0, 153.3, 151.4, 135.4, 122.6, 117.0, 105.1, 56.4; Anal. ($C_8H_8N_4O_2$) C, H, N.

8-Methyl-1,2,4-benzotriazin-3-amine 1-Oxide (4d). Method A using **2d** gave **4d** (100%) as a yellow powder, mp (DMF) 265 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 7.59 (dd, J = 8.3, 7.3 Hz, 1 H, H-6), 7.35 (d, J = 8.0 Hz, 1 H, H-5), 7.18 (s, 2 H, NH_2), 7.10 (dd, J = 7.2, 0.8 Hz, 1 H, H-7), 2.79 (s, 3 H, CH_3); Anal. ($C_8H_8N_4O$) C, H, N.

8-Fluoro-1,2,4-benzotriazin-3-amine 1-Oxide (4e). Method B using **3e** gave **4e** (49%) as a yellow powder, mp DCM/pet. ether) 270−278 °C (dec) (lit.[40] mp 271 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 7.69 (ddd, J = 10.9, 8.3, 5.2 Hz, 1 H, H-6), 7.45 (br s, 2 H, NH_2), 7.31 (dd, J = 9.6, 1.0 Hz, 1 H, H-5), 7.09 (ddd, J = 12.0, 8.0, 1.0 Hz, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 160.2 (d, J = 5.3 Hz), 153.5 (d, J = 264.3 Hz), 151.1 (d, J = 3.2 Hz), 135.2 (d, J = 4.4 Hz), 121.7 (d, J = 4.5 Hz), 121.1, 110.0 (d, J = 20.8 Hz); HRMS (EI$^+$) calcd for $C_7H_5FN_4O$ (M^+) m/z 180.0360, found 180.0441.

8-Chloro-1,2,4-benzotriazin-3-amine 1-oxide (4f). Method A using **2f** gave **4f** (30%) as a yellow powder, mp (DMF) 280−290 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 7.63 (dd, J = 8.4, 7.8 Hz, 1 H, H-6), 7.45 (dd, J = 8.6, 1.0 Hz, 1 H, H-7), 7.42 (br s, 2 H, NH_2), 7.36 (dd, J = 7.6, 1.1 Hz, 1 H, H-5); HRMS (EI) calcd for $C_7H_5N_4O^{35}Cl$ (M^+) m/z 196.0152, found 196.0152; calc for $C7H_5N_4O^{37}Cl$ (M^+) m/z 198.0122, found 198.0124.

8-Trifluoromethyl-1,2,4-benzotriazin-3-amine 1-Oxide (4 g). Method A using **2g** gave **4g** (75%) as a yellow powder, mp (DCM/pet. ether) 280−286 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 7.78−7.87 (m, 3 H, H-5, H-6, H-7), 7.55 (br s, 2 H, NH_2); Anal. ($C_8H_5F_3N_4O$) C, H, N, F.

8-(Methylsulfanyl)-1,2,4-benzotriazin-3-amine 1-Oxide (4h). Method A using **2h** gave **4h** (68%) as a yellow powder, mp (H_2O) 271−275 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.63 (dd, J = 8.3, 8.0 Hz, 1 H, H-6), 7.28 (s, 2 H, NH_2), 7.17 (d, J = 8.3 Hz, 1 H, H-5), 6.98 (d, J = 8.0 Hz, 1 H, H-7), 2.39 (s, 3 H, SCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 159.9, 151.1, 137.1, 134.7, 127.9, 120.1, 118.7, 15.7; Anal. ($C_8H_8N_4OS$) C, H, N.

8-(Butylsulfanyl)-1,2,4-benzotriazin-3-amine 1-Oxide (4i). Method A using **2i** gave **4i** (26%) as a red/brown solid, mp (MeOH/$CHCl_3$) 233−236 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.61 (dd, J = 8.2, 7.4 Hz, 1 H, H-6), 7.27 (s, 2 H, NH_2), 7.16 (dd, J = 8.2, 0.8 Hz, 1 H, H-5), 7.04 (dd, J = 7.4, 0.8 Hz, 1 H, H-7), 2.86 (t, J = 7.3 Hz, 2 H, CH_2S), 1.62−1.70 (m, 2 H, CH_2), 1.44−1.52 (m, 2 H, CH_2), 0.93 (s, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 159.8, 151.1, 136.4, 134.7, 128.0, 120.1, 119.1, 31.0, 28.8, 21.7, 13.5; Anal. ($C_{11}H_{14}N_4OS$) C, H, N.

7-Methoxy-1,2,4-benzotriazin-3-amine 1-Oxide (8c). Method A using **6c** gave **8c** (93%) as a yellow powder, mp (HOAc) 269−271 °C [lit.[26] mp (HOAc) 271 °C]; ^{1}H NMR [$(CD_3)_2SO$] δ 7.48−7.53 (m, 3 H, H-5, H-6, H-8), 7.10 (br s, 2 H, NH_2), 3.88 (s, 3 H, OCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 159.3, 156.3, 144.9, 129.7, 128.3, 127.3, 97.9, 55.8.

7-Methyl-1,2,4-benzotriazin-3-amine 1-Oxide (8d). Method A using **6d** gave **8d** (74%) as a yellow powder, mp (DMF) 270 °C (dec) [lit.[27] mp (Methylcellosolve) 282 °C]; ^{1}H NMR [$(CD_3)_2SO$] δ 7.94 (s, 1 H, H-8); 7.64 (dd, J = 8.7, 1.9 Hz, 1 H, H-6), 7.46 (d, J = 8.6 Hz, 1 H, H-5), 7.21 (s, 2 H, NH_2), 2.42 (s, 3 H, CH_3); Anal. ($C_8H_8N_4O$) C, H, N.

7-Fluoro-1,2,4-benzotriazin-3-amine 1-Oxide (8e). Method A using **6e** gave **8e** (78%) as a yellow powder, mp (DMF) 280−290 °C (dec) [lit.[40] mp 290 °C (dec)]; ^{1}H NMR [$(CD_3)_2SO$] δ 7.89 (dd, J = 8.6, 2.9 Hz, 1 H, H-8), 7.76 (ddd, J = 9.3, 8.8, 2.9 Hz, 1 H, H-6), 7.62 (dd, J = 9.3, 5.2 Hz, 1 H, H-5), 7.35 (br s, 2 H, NH_2); Anal. ($C_7H_5FN_4O$) C, H, N, F.

7-Chloro-1,2,4-benzotrazin-3-amine 1-Oxide (8f). Method A using **6f** gave **8f** (39%) as a yellow powder, mp (DCM/pet. ether) 309 °C (dec) [lit.[27] (HOAc) 306−308 °C]; ^{1}H NMR [$(CD_3)_2SO$] δ 8.14 (d, J = 1.7 Hz, 1 H, H-8), 7.80 (dd, J = 8.8, 1.9 Hz, 1 H, H-6), 7.56 (d, J = 9.0 Hz, 1 H, H-5), 7.48 (br s, 2 H, NH_2).

7-Trifluoromethyl-1,2,4-benzotriazin-3-amine 1-Oxide (8 g). Method B using **7g** gave **8g** (30%) as a yellow powder, mp (DCM/pet. ether) 290 °C (dec) [lit.[40] (acetone/toluene) 301−302 °C]; ^{1}H NMR [$(CD_3)_2SO$] δ 8.38 (br s, 1 H, H-8), 8.01 (dd, J = 8.9, 2.0 Hz, 1 H, H-6), 7.72 (br s, 2 H, NH_2), 7.68 (d, J = 8.9 Hz, 1 H, H-5); ^{13}C NMR [$(CD_3)_2SO$] δ 161.1, 150.6, 130.7 (J = 2.9 Hz), 129.3, 127.5, 123.4 (q, J = 272.0 Hz), 123.6 (q, J = 32.1 Hz), 118.0 (q, J = 10.9 Hz).

7-(Methylsulfanyl)-1,2,4-benzotriazin-3-amine 1-Oxide (8h). Method A using **6h** gave **8h** (56%) as a red solid, mp (MeOH/$CHCl_3$) 245−247 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.79 (d, J = 2.1 Hz, 1 H, H-8), 7.67 (dd, J = 8.9, 2.1 Hz, 1 H, H-6), 7.47 (d, J = 8.9 Hz, 1 H, H-5), 7.28 (s, 2 H, NH_2), 2.58 (s, 3 H, SCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 159.9, 147.0, 135.7, 134.6, 130.1, 126.5, 113.4, 14.6; Anal. ($C_8H_8N_4OS$) C, H, N.

7-(Butylsulfonyl)-1,2,4-benzotriazin-3-amine 1-Oxide (8i). Method A using **6i** gave **8i** (68%) as a red solid, mp (MeOH/$CHCl_3$) 215−217 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.87 (d, J = 2.1 Hz, 1 H, H-8), 7.68 (dd, J = 8.9, 2.1 Hz, 1 H, H-6), 7.46 (d, J = 8.9 Hz, 1 H, H-5), 7.07 (br s, 2 H, NH_2), 3.04 (dd, J = 7.3, 7.2 Hz, 2 H, CH_2S), 1.55−1.63 (m, 2 H, CH_2), 1.37−1.45 (m, 2 H, CH_2), 0.88 (t, J = 7.3 Hz, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 160.0, 147.3, 135.9, 133.9, 130.0, 126.4, 115.8, 31.6, 30.1, 21.2, 13.4; Anal. ($C_{11}H_{14}N_4OS$) C, H, N.

7-Nitro-1,2,4-benzotriazin-3-amine 1-Oxide (8j). Method B using **7j** gave **8j** (15%) as a yellow powder, mp (DMF) 269−272 °C [lit.[27] mp (pyridine/EtOH) 290 °C]; ^{1}H NMR [$(CD_3)_2SO$] δ 8.82 (d, J = 2.6 Hz, 1 H, H-8), 8.44 (dd, J = 9.4, 2.6 Hz, 1 H, H-6), 8.03 (br s, 2 H, NH_2), 7.64(d, J = 9.3 Hz, 1 H, H-5).

6-Methoxy-1,2,4-benzotriazin-3-amine 1-Oxide (11c). Method A using **10c** gave **11c** (63%) as a yellow powder, mp ($CHCl_3$) 265−270 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.04 (d, J = 9.5 Hz, 1 H, H-8), 7.24 (br s, 2 H, NH_2), 6.95 (dd, J = 9.5, 2.6 Hz, 1 H, H-7), 6.86 (d, J = 2.6 Hz, 1 H, H-5), 3.91 (s, 3 H, OCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 164.7, 160.7, 151.3, 125.0, 121.5, 117.0, 103.8, 56.0; MS (EI$^+$) m/z 192 (M^+, 100%), 176 (5); HRMS calcd for $C_8H_8N_4O_2$ (M^+) m/z 192.0647, found 192.0653; Anal. ($C_8H_8N_4O_2$) C, H, N.

6-Methyl-1,2,4-benzotriazin-3-amine 1-Oxide (11d). Method A using **10d** gave **11d** (87%) as a yellow powder, mp (DMF) 263 °C (dec) [lit.[41] mp (HOAc) 284−286 °C]; ^{1}H NMR [$(CD_3)_2SO$] δ 8.02 (d, J = 8.8 Hz, 1 H, H-8), 7.33 (s, 1 H, H-5), 7.27(br s, 2 H, NH_2), 7.18 (dd, J = 8.8, 1.7 Hz, 1 H, H-7), 2.42 (s, 3 H, CH_3); Anal. ($C_8H_8N_4O$) C, H, N.

6-Fluoro-1,2,4-benzotriazin-3-amine 1-Oxide (11e). Method A using **10e** gave **11e** (61%) as a yellow powder, mp (DCM/pet. ether) 276−280 °C (lit.[40] mp 268 °C); ^{1}H NMR [$(CD_3)_2SO$] δ 8.21 (dd, J = 9.5, 5.9 Hz, 1 H, H-8), 7.50 (br s, 2 H, NH_2), 7.30 (dd, J = 10.0, 2.6 Hz, 1 H, H-5), 7.21 (ddd, J = 8.8, 7.5, 2.6 Hz, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 164.5 (d, J = 254.6 Hz), 160.8, 150.6 (d, J = 16.1 Hz), 127.4, 123.4 (d, J = 11.1 Hz), 114.1 (d, J = 26.2 Hz), 109.5 (d, J = 23.1 Hz); Anal. ($C_7H_5FN_4O$) C, H, N, F.

6-Chloro-1,2,4-benzotrazin-3-amine 1-Oxide (11f). Method A using **10f** gave **11f** (53%) as a yellow solid, mp (DCM/pet. ether) >320 °C [lit.[41] mp (HOAc) >300 °C]; ^{1}H NMR [$(CD_3)_2SO$] δ 8.13 (d, J = 9.2 Hz, 1 H, H-8), 7.60 (d, J = 2.11 Hz, 1 H, H-5), 7.53 (br s, 2 H, NH_2), 7.33 (dd, J = 9.2, 2.1 Hz, 1 H, H-7).

6-Trifluoromethyl-1,2,4-benzotriazin-3-amine 1-Oxide (11 g). Method A using **10g** gave **11g** (29%) as a yellow solid, mp (DCM/pet. ether) 280−284 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 8.31 (d, J = 8.9 Hz, 1 H, H-8), 7.85 (br s, 1 H, H-5), 7.65 (br s,

2 H, NH_2), 7.56 (dd, J = 8.9, 1.6 Hz, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 160.8, 148.4, 134.8 (q, J = 32.7 Hz), 131.5, 123.5 (q, J = 4.0 Hz), 123.1 (q, J = 273.1 Hz), 122.0, 119.3; Anal. ($C_8H_5F_3N_4O$) C, H, N, F.

6-(Methylsulfonyl)-1,2,4-benzotriazin-3-amine 1-Oxide (11h). Method A using **10h** gave **11h** (55%) as a yellow powder, mp (MeOH/$CHCl_3$) 248−250 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.99 (d, J = 8.3 Hz, 1 H, H-8), 7.30 (br s, 2 H, NH_2), 7.16−7.19 (m, 2 H, H-5, H-7), 2.59 (s, 3 H, SCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 160.7, 149.2, 149.1, 127.3, 122.9, 119.8, 118.0, 14.0; Anal. ($C_8H_8N_4OS$) C, H, N.

6-(Butylsulfanyl)-1,2,4-benzotriazin-3-amine 1-Oxide (11i). Method A using **10i** gave (i) starting material (**10i**) (60%) and (ii) **11i** (30%) as a red solid, mp (MeOH/$CHCl_3$) 180−182 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.99 (d, J = 9.1 Hz, 1 H, H-8), 7.31 (br s, 2 H, NH_2), 7.21 (d, J = 2.0 Hz, 1 H, H-5), 7.17 (dd, J = 9.1, 2.0 Hz, 1 H, H-7), 3.12 (dd, J = 7.3, 7.2 Hz, 2 H, CH_2S), 1.61−1.68 (m, 2 H, CH_2), 1.41−1.49 (m, 2 H, CH_2), 0.92 (s, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 160.6, 149.1, 147.9, 127.3, 123.4, 119.9, 118.8, 30.1, 29.9, 21.3, 13.4; Anal. ($C_{11}H_{14}N_4OS$) C, H, N.

5-Methoxy-1,2,4-benzotriazin-3-amine 1-Oxide (15c). Method A using **13c** gave **15c** (66%) as a yellow powder, mp (HOAc) 267 °C (dec) [lit.[41] mp (HOAc) >270 °C]; ^{1}H NMR [$(CD_3)_2SO$] δ 7.65−7.69 (m, 1 H, H-7), 7.39 (br s, 2 H, NH_2), 7.23−7.27 (m, 2 H, H-6, H-8), 3.92 (s, 3 H, OCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 159.7, 153.3, 141.5, 130.0, 123.9, 113.4, 110.7, 55.9; Anal. ($C_8H_8N_4O_2$) C, H, N.

5-Methyl-1,2,4-benzotriazin-3-amine 1-Oxide (15d). Method A using gave **13d** gave **15d** (89%) as a yellow solid, mp (DCM/pet. ether) 253−255 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.98 (d, J = 8.6, Hz, 1 H, H-8), 7.64 (d, J = 7.1 Hz, 1 H, H-6), 7.33 (br s, 2 H, NH_2), 7.23 (dd, J = 8.6, 7.1 Hz, 1 H, H-7), 2.49 (s, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 156.7, 148.0, 135.1, 134.3, 129.8, 124.0, 117.4, 16.8; Anal. ($C_8H_8N_4O$) C, H, N.

5-Chloro-1,2,4-benzotriazin-3-amine 1-Oxide (15e). Method B using **14e** gave **15e** (45%) as a yellow solid, mp (HOAc) 251−254 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.11 (dd, J = 8.7, 1.0 Hz, 1 H, H-8), 7.95 (dd, J = 7.6, 1.0 Hz, 1 H, H-6), 7.67 (br s, 2 H, NH_2), 7.29 (dd, J = 8.7, 7.6 Hz, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 160.1, 145.7, 135.1, 131.1, 128.6, 123.6, 119.1; Anal. ($C_7H_5ClN_4O$) C, H, N.

5-Fluoro-1,2,4-benzotriazin-3-amine 1-Oxide (15f). Method A using **13f** gave **15f** (43%) as a yellow powder, mp (DMF) 252−256 °C (lit.[40] mp 278 °C) ^{1}H NMR [$(CD_3)_2SO$] δ 7.96 (dd, J = 8.7, 1.0 Hz, 1 H, H-8), 7.66 (ddd, J = 10.3, 7.9, 1.2 Hz, 1 H, H-6), 7.61 (br s, 2 H, NH_2), 7.28 (ddd, J = 8.6, 8.0, 5.1 Hz, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 160.1 (d, J = 5.6 Hz), 154.9 (d, J = 253.1 Hz), 139.7 (d, J = 16.1 Hz), 131.1 (d, J = 4.2 Hz), 122.8 (d, J = 7.6 Hz), 119.4 (d, J = 17.7 Hz), 115.8 (d, J = 4.4 Hz); Anal. ($C_7H_5FN_4O$) C, H, N, F.

Method C: Oxidation of 1-Oxides with 30% H_2O_2/HOAc. Hydrogen peroxide (30%, 12 mL) was added dropwise to a suspension of 1-oxide (2.60 mmol) in HOAc (25 mL) and the suspension stirred at 50 °C for 4 h. The solution was diluted with water (100 mL) and carefully neutralized with solid $NaHCO_3$. The solution was extracted with $CHCl_3$ (5 × 50 mL), the combined organic extracts were dried, and the solvent was evaporated. The residue was chromatographed, eluting with a gradient (0−5%) of MeOH/$CHCl_3$, to give the corresponding 1,2,4-benzotriazin-3-amine 1,4-dioxide.

Method D: Oxidation of 1-Oxides with 70% H_2O_2/CF_3CO_2H. Hydrogen peroxide (70%, 4 mL) (CAUTION) was added dropwise to a stirred suspension of 1-oxide (1.65 mmol) in trifluoroacetic acid (5 mL) and the mixture stirred at 50 °C for 7 days. The mixture was diluted with water (50 mL) and carefully neutralized with solid $NaHCO_3$. The precipitate was filtered, washed with water (2 × 5 mL), and dried to give the corresponding 1,2,4-benzotriazin-3-amine 1,4-dioxide.

Method E: Amination of Fluoro-1,2,4-benzotriazin-3-amine 1,4-Oxides. Fluoro-1,2,4-benzotriazin-3-amine 1,4-dioxide (0.4 mmol) was dissolved in acetonitrile (8 mL), and a solution of 40% aq dimethylamine (3 mL) or diethylamine (3 mL) was added and stirred at 20 °C for 24 h. The solvent was evaporated and the residue chromatographed, eluting with a gradient (0−3%) of MeOH/$CHCl_3$ to give dialkylamino-1,2,4-benzotriazin-3-amine 1,4-dioxide.

8-Diethylamino-1,2,4-benzotriazin-3-amine 1,4-Dioxide (5a). Method E using **5e** gave **5a** (87%) as a purple powder, mp (DCM/pet. ether) 154−156 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.77 (br s, 2 H, NH_2), 7.66 (t, J = 8.2 Hz, 1 H, H-6), 7.56 (d, J = 8.0 Hz, 1 H, H-5), 6.93 (d, J = 7.5 Hz, 1 H, H-7), 3.24 (q, J = 6.9 Hz, 4 H, 2 × CH_2), 1.05 (t, J = 6.9 Hz, 6 H, 2 × CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 150.9, 145.9, 141.7, 135.2, 126.3, 115.6, 107.0, 46.6 (2), 12.3(2); Anal. ($C_{11}H_{15}N_5O_2$) C, H, N.

8-Dimethylamino-1,2,4-benzotriazin-3-amine 1,4-Dioxide (5b). Method E using **5e** gave **5b** (80%) as a purple powder, mp (DCM/pet. ether) 170−172 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.77 (br s, 2 H, NH_2), 7.65 (t, J = 8.3 Hz, 1 H, H-6), 7.51 (dd, J = 7.8, 0.8 Hz, 1 H, H-5), 6.87 (dd, J = 8.1, 0.8 Hz, 1 H, H-7), 2.87 [s, 6 H, $N(CH_3)_2$]; ^{13}C NMR [$(CD_3)_2SO$] δ 150.4, 147.4, 140.9, 135.0, 124.4, 111.7, 105.3, 43.7 (2); Anal. ($C_9H_{11}N_5O_2$) C, H; N: calc 31.7, found 31.0%.

8-Methoxy-1,2,4-benzotriazin-3-amine 1,4-Dioxide (5c). Method C using **4c** gave **5c** (54%) as an orange powder, mp (H_2O) 208−213 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 7.90 (br s, 2 H, NH_2), 7.80 (dd, J = 8.5, 8.0 Hz, 1 H, H-6), 7.68 (d, J = 8.5 Hz, 1 H, H-5), 7.03 (dd, J = 8.0 Hz, 1 H, H-7), 3.94 (s, 3 H, OCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 153.8, 150.7, 140.4, 135.7, 123.3, 107.9, 107.4, 56.8; Anal. ($C_8H_8N_4O_3$·1/2H_2O) C, H; N: calcd 25.8, found 25.3%.

8-Methyl-1,2,4-benzotriazin-3-amine 1,4-Dioxide (5d). Method C using **4d** gave **5d** (34%) as an orange powder, mp (DCM/pet. ether) 225 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.04 (d, J = 8.7 Hz, 1 H, H-5), 7.93 (br s, 2 H, NH_2), 7.77 (dd, J = 8.6, 7.3 Hz, 1 H, H-6), 7.35 (d, J = 7.2 Hz, 1 H, H-7), 2.85 (s, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 150.5, 139.8, 134.5, 134.3, 130.6, 129.1, 115.2, 23.1; Anal. ($C_8H_8N_4O_2$) C, H; N: calcd 29.15, found 28.4.

8-Fluoro-1,2,4-benzotriazin-3-amine-1,4-Dioxide (5e). Method D using **4e** gave **5e** (67%) as a red powder, mp (DCM/pet. ether) 210−211 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.12 (br s, 2 H, NH_2), 7.94 (d, J = 8.8 Hz, 1 H, H-5), 7.85 (ddd, J = 9.0, 8.4, 4.6 Hz, 1 H, H-6), 7.35 (dd, J = 12.2, 7.5 Hz, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 153.6 (d, J = 264.1 Hz) 151.3, 140.1, 135.3 (d, J = 9.7 Hz), 122.4 (d, J = 7.6 Hz), 113.1 (d, J = 5.4 Hz), 112.4 (d, J = 21.5 Hz); Anal. ($C_7H_5FN_4O_2$) C, H, F; N: calcd 28.6, found 28.1%.

8-Chloro-1,2,4-benzotriazin-3-amine 1,4-Dioxide (5f). Method C using **4f** gave **5f** (16%) as a red powder, mp (DMF) 231 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.13 (d, J = 8.7 Hz, 1 H, H-5), 8.12 (br s, 2 H, NH_2), 7.80 (t, J = 8.2 Hz, 1 H, H-6), 7.62 (d, J = 7.6 Hz, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 151.1, 140. 9, 134.4, 129.3, 128.1, 126.4, 116.7; Anal. ($C_7H_5ClN_4O_2$) C, H, N, Cl.

8-Trifluoromethyl-1,2,4-benzotriazin-3-amine 1,4-Dioxide (5g). Method C using **4g** gave **5g** (27%) as a red powder, mp (DCM/pet. ether) 265−275 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 8.49 (d, J = 8.1 Hz, 1 H, H-7), 8.20 (br s, 2 H, NH_2), 7.97−8.04 (m, 2 H, H-5, H-6); ^{13}C NMR [$(CD_3)_2SO$] δ 151.4, 140.4 (q, J = 9.0 Hz), 133.8, 127.6, 127.2 (q, J = 7.9 Hz), 122.8, 122.5 (q, J = 271.5 Hz), 121.7 (q, J = 32.9 Hz); Anal. ($C_8H_5F_3N_4O_2$) C, H, N, F.

8-(Methylsulfonyl)-1,2,4-benzotriazin-3-amine 1,4-Dioxide (5h). Method D using **4h** gave **5h** (38%) as a red solid, mp (H_2O) 290−300 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.48 (d, J = 8.6 Hz, 1 H, H-5), 8.32 (d, J = 7.0 Hz, 1 H, H-7), 8.27 (br s, 2 H, NH_2), 8.03 (dd, J = 8.6, 7.0 Hz, 1 H, H-6), 3.61 (s, 3 H, SO_2CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 151.6, 140.3, 135.4, 133.9, 130.1, 127.5, 123.5, 45.1; MS (FAB$^+$) m/z 257 (MH^+, 5%), 242 (1); HRMS (FAB$^+$) calcd for $C_8H_9N_4O_4S$ (MH^+) m/z 257.0345, found 257.0345.

8-(Butylsulfonyl)-1,2,4-benzotriazin-3-amine 1,4-Dioxide (5i). Method D using **4i** gave **5i** (21%) as a red solid, mp (water) 220−230 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.49 (dd, J = 8.6, 0.7 Hz, 1 H, H-5), 8.30 (dd, J = 7.5, 0.6 Hz, 1 H, H-7), 8.26 (br s, 2 H, NH_2), 8.04 (dd, J = 8.6, 7.5 Hz, 1 H, H-6), 3.83 (s, 2 H, SO_2CH_2), 1.58−1.64 (m, 2 H, CH_2), 1.32−1.41 (m, 2 H, CH_2), 0.85 (t, J = 7.3 Hz, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 151.6,

140.4, 133.8, 133.7, 130.0, 127.6, 123.6, 55.6, 24.6, 20.8, 13.3; MS (EI^+) *m/z* 298 (M^+, 2%), 282 (12), 253 (10), 201 (30), 162 (100); HRMS (EI^+) calcd for $C_{11}H_{14}N_4O_4S$ (M^+) *m/z* 298.0736, found 298.0746.

7-Diethylamino-1,2,4-benzotriazin-3-amine 1,4-Dioxide (9a). Method E using **9e** gave **9a** (5%) as a purple powder, mp (DCM/pet. ether) 323 °C (dec); ^1H NMR [$(CD_3)_2SO$] δ 7.98 (d, J = 9.7 Hz, 1 H, H-5), 7.60 (dd, J = 9.7, 2.9 Hz, 1 H, H-6), 7.50 (br s, 2 H, NH_2), 6.97 (d, J = 2.7 Hz, 1 H, H-8), 3.47 (q, J = 7.0 Hz, 4 H, 2 × CH_2), 1.15 (t, J = 7.0 Hz, 6 H, 2 × CH_3), ^{13}C NMR [$(CD_3)_2SO$] δ 149.0, 148.9, 131.8, 131.2, 124.2, 118.1, 94.6, 44.1 (2), 12.0 (2); HRMS (EI) calcd for $C_{11}H_{15}N_5O_2$ (M^+) *m/z* 249.1226, found 249.1231.

7-Dimethylamino-1,2,4-benzotriazin-3-amine 1,4-Dioxide (9b). Method E using **9e** gave **9b** (76%) as a purple powder, mp (DCM/pet. ether) 205−215 °C (dec); ^1H NMR [$(CD_3)_2SO$] δ 7.99 (d, J = 9.6 Hz, 1 H, H-5), 7.63 (dd, J = 9.6, 2.7 Hz, 1 H, H-6), 7.55 (br s, 2 H, NH_2), 6.96 (d, J = 2.7 Hz, 1 H, H-8), 3.06 [s, 6 H, $N(CH_3)_2$], ^{13}C NMR [$(CD_3)_2SO$] δ 149.2; 148.5, 131.5, 131.5, 124.4, 117.7, 95.6, 39.9 (2); Anal. ($C_9H_{11}N_5O_2$·1/2H_2O) C, H, N.

7-Methoxy-1,2,4-benzotriazin-3-amine 1,4-Dioxide (9c). Method C using **8c** gave **9c** (30%) as a red powder, mp (HOAc) 210−214 °C [lit.[42] mp (H_2O) 213-214 °C]; ^1H NMR [$(CD_3)_2SO$] δ 8.07 (d, J = 9.4 Hz, 1 H, H-5), 7.87 (br s, 2 H, NH_2), 7.59 (dd, J = 9.4, 2.6 Hz, 1 H, H-6), 7.47 (d, J = 2.6 Hz, 1 H, H-8), 3.92 (s, 3 H, OCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 157.9, 150.5, 134.3, 131.0, 127.7, 118.7, 99.1, 56.1; Anal. ($C_8H_8N_4O_3$) C, H, N.

7-Methyl-1,2,4-benzotriazin-3-amine 1,4-Dioxide (9d). Method C using **8d** gave **9d** (25%) as a red powder, mp (DMF) 215 °C [lit.[26] mp (H_2O) 220 °C]; ^1H NMR [$(CD_3)_2SO$] δ 8.05 (d, J = 8.8 Hz, 1 H, H-5), 8.01 (s, 1 H, H-8), 7.93 (br s, 2 H, NH_2), 7.79 (dd, J = 8.8, 1.7 Hz, 1 H, H-6), 2.48 (s, 3 H, CH_3); Anal. ($C_8H_8N_4O_2$) C, H; N: calcd 29.2, found 28.4%

7-Fluoro-1,2,4-benzotriazin-3-amine 1,4-Dioxide (9e). Method C using **8e** gave **9e** (53%) as a red powder, mp (DCM/pet. ether) 240−250 °C (dec) [lit.[40] mp >300 °C]; ^1H NMR [$(CD_3)_2SO$] δ 8.20 (dd, J = 9.5, 5.1 Hz, 1 H, H-8), 8.03 (br s, 2 H, NH_2), 7.98 (dd, J = 8.7, 2.7 Hz, 1 H, H-5), 7.88 (ddd, J = 10.2, 7.4, 2.0 Hz, 1 H, H-6); ^{13}C NMR [$(CD_3)_2SO$] δ 105.9 (d, J = 27.4), 120.2 (d, J = 9.1 Hz), 125.1 (d, J = 26.7 Hz), 130.7 (J = 10.1 Hz), 136.0, 151.2 (d, J = 5.0 Hz), 159.5 (d, J = 249.2 Hz); Anal. ($C_7H_5FN_4O_2$) C, H, N, F.

7-Chloro-1,2,4-benzotriazin-3-amine 1,4-Dioxide (9f). Method C using **8f** gave **9f** (50%) as a red powder, mp (DMF) 248−250 °C (dec) [lit.[26] (HOAc) 269 °C]; ^1H NMR [$(CD_3)_2SO$] δ 8.21 (d, J = 2.1 Hz, 1 H, H-8), 8.14 (m, 3 H, H-5, NH_2), 7.94 (dd, J = 9.2, 2.2 Hz, 1 H, H-6); ^{13}C NMR [$(CD_3)_2SO$] δ 151.5, 137.4, 135.5, 131.1, 130.8, 119.3, 119.4; Anal. ($C_7H_5ClN_4O_2$) C, H, N, Cl.

7-Trifluoromethyl-1,2,4-benzotriazin-3-amine 1,4-Dioxide (9g). Method C using **8g** gave **9g** (76%) as red crystals, mp (DCM/pet. ether) 256−260 °C (lit.[40] >300 °C); ^1H NMR [$(CD_3)_2SO$] δ 8.45 (br s, 1 H, H-8). 8.40 (br, 2 H, NH_2), 8.30 (d, J = 8.9 Hz, 1 H, H-5), 8.18 (dd, J = 9.1, 1.8 Hz, 1 H, H-6); ^{13}C NMR [$(CD_3)_2SO$] δ 152.5, 139.8, 130.3 (q, J = 2.8 Hz), 130.0, 126.0 (q, J = 33.4 Hz), 123.1 (q, J = 272.0 Hz), 119.4 (q, J = 4.4 Hz), 119.2; Anal. ($C_8H_5F_3N_4O_2$) C, H, N, F.

7-(Methylsulfonyl)-1,2,4-benzotriazin-3-amine 1,4-Dioxide (9h). Method D using **8h** gave **9h** (55%) as a red solid, mp (water/EtOH) 306−310 °C; ^1H NMR [$(CD_3)_2SO$] δ 8.61 (br s, 1 H, H-8), 8.40−8.53 (m, 2 H, H-5, H-6), 8.31 (br s, 2 H, NH_2), 3.37 (s, 3 H, SO_2CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 152.7, 140.0, 137.8, 131.6, 129.9, 121.8, 119.8, 43.1; Anal. ($C_8H_8N_4O_4S$·1/2EtOH) C, N; H: calcd 4.0, found 3.3

7-(Butylsulfonyl)-1,2,4-benzotriazin-3-amine 1,4-Dioxide (9i). Method D using **8i** gave **9i** (22%) as a red solid, mp (water) 255−262 °C; ^1H NMR [$(CD_3)_2SO$] δ 8.55 (d, J = 1.5 Hz, 1 H, H-8), 8.50 (br s, 2 H, NH_2), 8.31 (d, J = 9.0 Hz, 1 H, H-5), 8.26 (dd, J = 9.0, 1.5 Hz, 1 H, H-6), 3.46 (dd, J = 7.9, 7.7 Hz, 2 H, SO_2CH_2), 1.49−1.57 (m, 2 H, CH_2), 1.30−1.38 (m, 2 H, CH_2), 0.83 (br s, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 152.7, 140.1, 136.1, 132.1, 130.1, 122.6, 119.1, 54.0, 24.1, 20.6, 13.4; Anal. ($C_{11}H_{14}N_4O_4S$) C, H, N.

7-Nitro-1,2,4-benzotriazin-3-amine 1,4-Dioxide (9j). Method C using **8j** gave **9j** (22%) as a red powder, mp (DMF) 242−243 °C (dec) [lit.[43] mp (DMF/acetone) 286−288 °C]; ^1H NMR [$(CD_3)_2SO$] δ 8.84 (d, J = 2.4 Hz, 1 H, H-8), 8.56 (m, 3 H, H-6, NH_2), 8.27 (d, J = 9.5 Hz, 1 H, H-5); ^{13}C NMR [$(CD_3)_2SO$] δ 153.1, 144.1, 140.5, 129.8, 128.3, 119.2, 118.1; Anal. ($C_7H_5N_5O_4$) C, H, N.

6-Diethylamino-1,2,4-benzotriazin-3-amine 1,4-Dioxide (12a). Method E using **12e** gave **12a** (69%) an orange powder, mp (DCM/pet. ether) 209 °C (dec); ^1H NMR [$(CD_3)_2SO$] δ 7.96 (d, J = 9.8 Hz, 1 H, H-8), 7.71 (br s, 2 H, NH_2), 7.13 (dd, J = 9.8, 2.0 Hz, 1 H, H-7), 6.87 (d, J = 1.8 Hz, 1 H, H-5), 3.55 (q, J = 6.8 Hz, 4 H, 2 × CH_2), 1.19 (t, J = 6.9 Hz, 6 H, 2 × CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 152.0, 151.3, 139.8, 122.8, 122.4, 115.5, 90.2, 44.5, 12.1; Anal. ($C_{11}H_{15}N_5O_2$) C, H, N.

6-Dimethylamino-1,2,4-benzotriazin-3-amine 1,4-Dioxide (12b). Method E using **12e** gave **12b** (83%) as a red powder, mp (DCM/pet. ether) 238 °C (dec); ^1H NMR [$(CD_3)_2SO$] δ 7.98 (d, J = 9.8 Hz, 1 H, H-8), 7.75 (br s, 2 H, NH_2), 7.17 (dd, J = 9.8, 2.7 Hz, 1 H, H-7), 6.85 (d, J = 2.7 Hz, 1 H, H-5), 3.16 [s, 6 H, $N(CH_3)_2$]; ^{13}C NMR [$(CD_3)_2SO$] δ 154.0, 151.4, 139.5, 122.6, 122.4, 115.7, 90.8, 39.9; Anal. ($C_9H_{11}N_5O_2$) C, H, N.

6-Methoxy-1,2,4-benzotriazin-3-amine 1,4-Dioxide (12c). Method D using **11c** gave **12c** (30%) as a red powder, mp (EtOAc/MeOH) 234−236 °C; ^1H NMR [$(CD_3)_2SO$] δ 8.11 (d, J = 9.6 Hz, 1 H, H-8), 8.02 (br s, 2 H, NH_2), 7.41 (d, J = 2.6 Hz, 1 H, H-5), 7.15 (dd, J = 9.6, 2.6 Hz, 1 H, H-7), 3.99 (s, 3 H, OCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 164.9, 151.8, 140.1, 125.7, 123.2, 119.1, 96.7, 56.6; Anal. ($C_8H_8N_4O_3$) C, H, N.

6-Methyl-1,2,4-benzotriazin-3-amine 1,4-Dioxide (12d). Method C using **11d** gave **12d** (57%) as a red powder, mp (DMF) 225−229 °C; ^1H NMR [$(CD_3)_2SO$] δ 8.09 (d, J = 8.9 Hz, 1 H, H-8), 8.00 (br s, 2 H, NH_2), 7.95 (s, 1 H, H-5), 7.40 (dd, J = 9.0, 3.1 Hz, 1 H, H-7), 2.51 (s, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 151.4, 146.9, 138.2, 128.9, 128.8, 120.9, 115.6, 21.6; Anal. ($C_8H_8N_4O_2$) C, H, N.

6-Fluoro-1,2,4-benzotriazin-3-amine 1,4-Dioxide (12e). Method C using **11e** gave **12e** (40%) as an orange powder, mp (DMF) 241−246 °C (dec) (lit.[40] mp >300 °C); ^1H NMR [$(CD_3)_2SO$] δ 8.30 (dd, J = 9.5, 5.2 Hz, 1 H, H-8), 8.20 (br s, 2 H, NH_2), 7.83 (dd, J = 9.2, 2.6 Hz, 1 H, H-5), 7.43−7.48 (m, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 164.8 (d, J = 256.3 Hz), 151.8, 139.8 (d, J = 12.1 Hz), 128.1, 125.2 (d, J = 11.1 Hz), 116.4 (d, J = 26.7 Hz), 101.9 (d, J = 28.5 Hz); Anal.($C_7H_5FN_4O_2$) C, H, N, F.

6-Chloro-1,2,4-benzotrazin-3-amine 1,4-Dioxide (12f). Method C using **11f** gave **12f** (20%) as a red powder, mp (DMF) 230−240 °C (dec); ^1H NMR [$(CD_3)_2SO$] δ 8.21 (m, 3 H, H-8, NH_2), 8.11 (d, J = 2.2 Hz, 1 H, H-5), 7.56 (dd, J = 9.2, 2.4 Hz, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 150.9, 140.4, 138.7, 129.4, 127.0, 123.5, 115.9; HRMS (EI^+) calcd for $C_7H_5{}^{35}ClN_4O_2$ (M^+) *m/z* 212.0101, found 212.0102; calcd for $C_7H_5{}^{37}ClN_4O_2$ (M^+) *m/z* 214.0072, found 214.0073.

6-Trifluoromethyl-1,2,4-benzotriazin-3-amine 1,4-Dioxide (12 g). Method C using **11g** gave **12g** (24%) as a red powder, mp (DCM/pet. ether) 238−242 °C; ^1H NMR [$(CD_3)_2SO$] δ 8.40 (d, J = 9.2 Hz, 1 H, H-8), 8.37 (br s, 1 H, H-5), 8.30 (br s, 2 H, NH_2), 7.81 (dd, J = 9.1, 1.8 Hz, 1 H, H-7); Anal. ($C_8H_5F_3N_4O_2$–1/2H_2O) C, H, N, F.

6-(Methylsulfonyl)-1,2,4-benzotriazin-3-amine 1,4-Dioxide (12h). Method D using **11h** gave **12h** (67%) as a red powder, mp (DMF/MeOH) 281 °C (dec); ^1H NMR [$(CD_3)_2SO$] δ 8.57 (d, J = 1.9 Hz, 1 H, H-5), 8.42 (d, J = 9.1 Hz, 1 H, H-8), 8.34 (br s, 2 H, NH_2), 7.96 (dd, J = 9.1, 1.9 Hz, 1 H, H-7), 3.41 (s, 3 H, SO_2CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 152.1, 146.0, 138.1, 132.2, 123.6, 123.0, 117.2, 42.8; Anal. ($C_8H_8N_4O_4S$) C, H; N, calcd 21.9; found 21.2%.

6-(Butylsulfonyl)-1,2,4-benzotriazin-3-amine 1,4-Dioxide (12i). Method D using **11i** gave **12i** (41%) as a red solid, mp (EtOH/H_2O) 203−206 °C; ^1H NMR [$(CD_3)_2SO$] δ 8.52 (s, 1 H, H-5), 8.42 (d, J = 9.0 Hz, 1 H, H-7), 8.34 (br s, 2 H, NH_2), 7.91 (d, J = 9.0 Hz, 1 H, H-8), 3.51 (s, 2 H, SO_2CH_2), 1.50−

1.57 (m, 2 H, CH_2), 1.31−1.40 (m, 2 H, CH_2), 0.83 (t, J = 7.3 Hz, 3 H, CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 152.1, 144.4, 138.2, 132.3, 123.6, 123.4, 118.0, 53.7, 24.1, 20.6, 13.3; Anal. ($C_{11}H_{14}N_4O_4S\cdot$ 1/2EtOH) C, H, N.

5-Diethylamino-1,2,4-benzotriazin-3-amine 1,4-Dioxide (16a). Method E using **16f** gave **16a** (96%) as a deep red gum, ^{1}H NMR [$(CD_3)_2SO$] δ 7.86 (br s, 2 H, NH_2), 7.76 (dd, J = 8.7, 1.1 Hz, 1 H, H-8), 7.36 (br dd, J = 8.7, 7.7 Hz, 1 H, H-7), 7.22 (dd, J = 7.7, 0.8 Hz, 1 H, H-6), 3.24 (q, J = 7.0 Hz, 4 H, 2 × CH_2), 1.00 (t, J = 7.0 Hz, 6 H, 2 × CH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 152.1, 141.8, 132.9, 132.8, 126.6, 124.3, 113.2, 46.6 (2), 11.4 (2); HRMS (EI$^+$) calcd for $C_{11}H_{15}N_5O_2$ (M^+) m/z 249.1226, found 249.1231.

5-Dimethylamino-1,2,4-benzotriazin-3-amine 1,4-Dioxide (16b). Method E using **16f** gave **16b** (58%) as a purple powder, mp (DCM/pet. ether) 143−146 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.87 (br s, 2 H, NH_2), 7.71 (d, J = 8.6 Hz, 1 H, H-8), 7.35 (t, J = 8.2 Hz, 1 H, H-7), 7.18 (d, J = 7.8 Hz, 1 H, H-6), 2.81 [s, 6 H, $N(CH_3)_2$]; ^{13}C NMR [$(CD_3)_2SO$] δ 152.1, 141.8, 132.7, 126.9, 119.5, 114.7, 112.0, 44.9 (2); Anal. ($C_9H_{11}N_5O_2$) C, H, N.

5-Methoxy-1,2,4-benzotriazin-3-amine 1,4-Dioxide (16c). Method C using **15c** gave **16c** (5%) as an orange powder, mp (H_2O) 195−200 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.88 (br s, 2 H, NH_2), 7.78 (dd, J = 8.7, 1.2 Hz, 1 H, H-8), 7.43 (dd, J = 8.7, 7.8 Hz, 1 H, H-7), 7.32 (d, J = 7.8 Hz, 1 H, H-6), 3.90 (s, 3 H, OCH_3); ^{13}C NMR [$(CD_3)_2SO$] δ 152.3, 150.9, 132.2, 131.4, 128.6, 115.2, 112.6, 57.3; MS (EI) m/z 208 (M^+, 5%), 192 (100); HRMS (EI) calcd for $C_8H_8N_4O_3$ (M^+) m/z 208.0596, found 208.0594.

5-Methyl-1,2,4-benzotriazin-3-amine 1,4-Dioxide (16d). Method C using **15d** gave **16d** (4%) as a red powder, mp (H_2O) 226−232 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.08 (d, J = 8.7 Hz, 1 H, H-8), 7.94 (br s, 2 H, NH_2), 7.61 (d, J = 7.2 Hz, 1 H, H-6), 7.41 (dd, J = 8.6, 7.3 Hz, 1 H, H-7), 3.00 (s, 3 H, CH_3); HRMS (EI) calcd for $C_8H_8N_4O_2$ (M^+) m/z 192.0647, found 192.0644.

5-Chloro-1,2,4-benzotriazin-3-amine 1,4-Dioxide (16e). Method D using **15e** gave **16e** (18%) as a red solid, mp (MeOH/$CHCl_3$) 219 °C (dec); ^{1}H NMR [$(CD_3)_2SO$] δ 8.19 (d, J = 8.8 Hz, 1 H, H-8), 8.14 (br s, 2 H, NH_2), 7.90 (d, J = 7.7 Hz, 1 H, H-6), 7.45 (dd, J = 8.8, 7.7 Hz, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 152.6, 137.2, 135.2, 132.6, 126.4, 122.4, 121.0; MS (EI$^+$) m/z 214 (M^+, 4%), 212 (M^+, 12), 198 (35), 196 (100); HRMS (EI$^+$) calcd for $C_7H_5{}^{35}ClN_4O_2$ (M^+) m/z 212.0101, found 212.0093, calcd for $C_7H_5{}^{37}ClN_4O_2$ (M^+) m/z 214.0072, found 214.0065; Anal. ($C_7H_5ClN_4O_2$) C, H, N.

5-Fluoro-1,2,4-benzotriazin-3-amine 1,4-Dioxide (16f). Method D using **15f** gave **16f** (50%)as a red powder, mp (DCM/pet. ether) 236−239 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.11 (br s, 2 H, NH_2), 8.03 (dd, J = 8.3, 1.2 Hz, 1 H, H-8), 7.69 (ddd, J = 12.7, 7.9, 1.1 Hz, 1 H, H-6), 7.48 (ddd, J = 8.7, 8.1, 4.3 Hz, 1 H, H-7); ^{13}C NMR [$(CD_3)_2SO$] δ 153.7 (d, J = 259.8 Hz), 152.4, 138.4, 129.9 (d, J = 7.7 Hz), 126.3 (d, J = 6.9 Hz), 120.1 (d, J = 19.8 Hz), 117.4 (d, J = 5.8 Hz); Anal. ($C_7H_5FN_4O_2$) C, H, N, F.

Clonogenic Assays. Tumor cells were plated in glass Petri dishes at 2 × 10^5 to 1 × 10^6 cells per dish, 1−2 days prior to the experiment. TPZ and analogues were made up into solution immediately before the experiment and added to the cells in complete medium. Hypoxia (less than 200 ppm O_2) was achieved by exposing the glass dishes in prewarmed, airtight aluminum jigs to a series of five rapid evacuations and flushings with 95% nitrogen + 5% CO_2 in a 37 °C water bath on a shaking platform (controls were flushed with 95% air + 5% CO_2). After the fifth evacuation and flushing, the platform (with water bath and jigs) was shaken for 5 min, then one more evacuation and flushing was performed, and the jigs were transferred to a shaker in a 37 °C incubator for the remainder of the 1 h drug exposure. The oxygen concentrations in the medium and gas phases were checked using an oxygen electrode (Animas, Phoenixville, PA) in a specially modified aluminum jig that allowed monitoring of both gas and liquid phases. Following the 1 h exposure to TPZ or BTO analogue the aluminum vessels were opened, the cell monolayers washed twice in medium, trypsinized, and plated for clonogenic survival in plastic Petri dishes. Ten to fourteen days later the dishes were stained with crystal violet (0.25% in 95% ethanol), and colonies containing more than 50 cells were counted.

IC_{50} Assays. Drug exposures were performed in 96-well plates (Nunc) using either a 37 °C humidified incubator (20% O_2, 5% CO_2) or in the incubator compartment (37 °C) of an anaerobic chamber (Shell Lab) where palladium catalyst scrubbed gas (90% N_2, 5% H_2, 5% CO_2) ensures severe anoxia (<0.001% O_2). For each experiment, all 12 compounds were simultaneously tested under both conditions against both SCCVII and HT-29 cell lines. Thus each set of IC_{50} determinations is an intraexperiment comparison. Cell cultures were grown in αMEM (Gibco) containing 5% heat inactivated FCS and maintained in exponential growth phase. For each individual experiment an appropriate number of cells were seeded (SCCVII = 200; HT-29 = 1000) into wells in αMEM + 10% FCS + 10 mM added glucose + 100 μM 2′-deoxycytidine (2′dCyd), and allowed to attach for 3 h. High glucose (final concentration 17mM) and the presence of 2′-dCyd minimize hypoxia-induced cell cycle arrest. Replicates were then exposed to BTOs, using 2-fold serial dilutions in triplicate, for a further 4 h. Subsequently cells were washed free of compound using complete media (without glucose/2′-dCyd) and allowed to grow for 5 (oxic) or 6 (anoxic) days. Plates were stained as described previously[33] and IC_{50} values determined. Results were averaged for three independent experiments.

Acknowledgment. The authors thank Dr Maruta Boyd, Sarath Liyanage, Li Fong Leong, and Karin Tan for technical assistance. The authors thank Degussa Peroxide Ltd, Morrinsville, NZ for the generous gift of 70% hydrogen peroxide. This work was supported by the US National Cancer Institute under Program Project Grant 1PO1-CA82566-01A1 (M.P.H., S.A.G., M.S.K., F.B.P., K.O.H.), the Health Research Council of New Zealand (R.F.A., W.R.W., A.V.P.), the Auckland Division of the Cancer Society of New Zealand (W.A.D.), and the Australian Institute of Nuclear Sciences and Engineering (M.P.H.).

References

(1) Kennedy, A. S.; Raleigh, J. A.; Perez, G. M.; Calkins, D. P.; Thrall, D. E.; Novotny, D. B.; Varia, M. A. Proliferation and hypoxia in human squamous cell carcinoma of the cervix − first report of combined immunohistochemical assays. *Int. J. Radiat. Oncol. Biol. Phys.* **1997**, *37*, 897−905.

(2) Movsas, B.; Chapman, J. D.; Horwitz, E. M.; Pinover, W. H.; Greenberg, R. E.; Hanlon, A. L.; Iyer, R.; Hanks, G. E. Hypoxic regions exist in human prostate carcinoma. *Urology* **1999**, *53*, 11−18.

(3) Brizel, D. M.; Dodge, R. K.; Clough, R. W.; Dewhirst, M. W. Oxygenation of head and neck cancer: changes during radiotherapy and impact on treatment outcome. *Radiother., Oncol.* **1999**, *53*, 113−117.

(4) Brown, J. M.; Siim, B. G. Hypoxia-specific cytotoxins in cancer therapy, *Semin. Radiat. Oncol.* **1996**, *6*, 22−36.

(5) Denny, W. A.; Wilson, W. R.; Hay, M. P. Recent developments in the design of bioreductive drugs. *Br. J. Cancer* **1996**, *74* (Suppl. XXVII), 32−38.

(6) Stratford, I. J.; Workman, P. Bioreductive drugs into the next millennium. *Anti-Cancer Drug Des.* **1998**, *13*, 519−528.

(7) Brown, J. M. SR 4233 (tirapazamine): a new anticancer drug exploiting hypoxia in solid tumors. *Br. J. Cancer* **1993**, *67*, 1163−1170.

(8) Kelson, A. B.; McNamara, J. P.; Pandey, A.; Ryan, K. J.; Dorie, M. J.; McAfee, P. A.; Menke, D. R.; Brown, J. M.; Tracy, M. 1,2,4-Benzotriazine 1,4-dioxides. An important class of hypoxic cytotoxins with antitumor activity. *Anti-Cancer Drug Des.* **1998**, *13*, 575−592.

(9) Lee, W. W.; Brown, J. M.; Grange, E. W.; Martinez, A. P.; Tracy, M.; Pollart, D. J. 1,2,4-Benzotriazine oxides as radiosensitizers and selective cytotoxic agents. PCT Patent WO9104028, April 1991.

(10) Denny, W. A.; Wilson, W. R. Tirapazamine: a bioreductive anticancer drug that exploits tumour hypoxia. *Exp. Opin. Invest. Drugs* **2000**, *9*, 2889−2901.

(11) Lloyd, R. V.; Duling, D. R.; Rumyantskeva, G. V.; Mason, R. P.; Brisdon, P. K. Microsomal reduction of 3-amino-1,2,4-benzotriazine-1,4-dioxide to a free radical. *Mol. Pharmacol.* **1991**, *40*, 440−445.
(12) Walton, M. I.; Wolf, C. R.; Workman, P. The role of cytochrome P450 and cytochrome P450 reductase in the reductive bioactivation of the novel benzotriazine di-*N*-oxide hypoxic cytotoxin 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233, WIN 59075) by mouse liver. *Biochem. Pharmacol.* **1992**, *44*, 251−259.
(13) Patterson, A. V.; Barham, H. M.; Chinje, E. C.; Adams, G. E.; Harris, A. L.; Stratford, I. J. Importance of P450 reductase activity in determining sensitivity of breast tumour cells to the bioreductive drug, tirapazamine (SR 4233). *Br. J. Cancer* **1995**, *72*, 1144−1150.
(14) Patterson, A. V.; Saunders, M. P.; Chinje, E. C.; Patterson, L. H.; Stratford, I. J. Enzymology of tirapazamine metabolism: a review. *Anti-Cancer Drug Des.* **1998**, *13*, 541−573.
(15) Fitzsimmons, S. A.; Lewis, A. D.; Riley, R. J.; Workman, P. Reduction of 3-amino-1,2,4-benzotriazine-1,4-di-*N*-oxide (tirapazamine, WIN 59075, SR4233) to a DNA damaging species − a direct role for NADPH-cytochrome P450 oxidoreductases. *Carcinogenesis* **1994**, *15*, 1503−1510.
(16) Baker, M. A.; Zeman, E. M.; Hirst, V. K.; Brown, J. M. Metabolism of SR 4233 by Chinese hamster ovary cells: basis of selective hypoxic cytotoxicity. *Cancer Res.* **1988**, *48*, 5947−5952.
(17) Priyadarsini, K. I.; Tracy, M.; Wardman, P. The one electron reduction potential of 3-amino-1,2,4-benzotriazine 1,4-dioxide (Tirapazamine): a hypoxia-selective bioreductive drug. *Free Radical Res.* **1996**, *25*, 393−399.
(18) Daniels, J. S.; Gates, K. S. DNA cleavage by the antitumor agent 3-amino-1,2,4-benzotriazine 1,4-dioxide (SR4233). Evidence for involvement of hydroxyl radical. *J. Am. Chem. Soc.* **1996**, *118*, 3380−3385.
(19) Wang, J.; Biedermann, K. A.; Brown, J. M. Repair of DNA and chromosome breaks in cells exposed to SR 4233 under hypoxia or to ionizing radiation. *Cancer Res.* **1992**, *52*, 4473−4477.
(20) von Pawel, J.; von Roemeling, R.; Gatzemeier, U.; Boyer, M.; Elisson, L. O.; Clark, P.; Talbot, D.; Rey, A.; Butler, T. W.; Hirsh, V.; Olver, I.; Bergman, B.; Ayoub, J.; Richardson, G.; Dunlop, D.; Arcenas, A.; Vescio, R.; Viallet, J.; Treat, J. Tirapazamine plus cisplatin versus cisplatin in advanced non small-cell lung cancer: A report of the international CATAPULT I study group. Cisplatin and tirapazamine in subjects with advanced previously untreated non small-cell lung tumors. *J. Clin. Oncol.* **2000**, *18*, 1351−1359.
(21) Lee, D. J.; Trotti, A.; Spencer, S.; Rostock, R.; Fisher, C.; von Roemeling, R.; Harvey, E.; Groves, E. Concurrent tirapazamine and radiotherapy for advanced head and neck carcinomas: a Phase II study. *Int. J. Radiat. Oncol., Biol., Phys.* **1998**, *42*, 811−815.
(22) Del Rowe, J.; Scott, C.; Werner-Wasik, M.;. Bahary, J. P.; Curran, W. J.; Urtasun, R. C.; Fisher, B. Single-arm, open-label phase II study of intravenously administered tirapazamine and radiation therapy for glioblastoma multiforme. *J. Clin. Oncol.* **2000**, *18*, 1254−1259.
(23) Brown, J. M.; Koong, A. Therapeutic advantage of hypoxic cells in tumors: a theoretical study. *J. Natl Cancer Inst.* **1991**, *83*, 178−185.
(24) Zeman, E. M.; Baker, M. A.; Lemmon, M. J.; Pearson, C. I.; Adams, J. A.; Brown, J. M.; Lee, W. W.; Tracy, M. Structure−activity relationships for benzotriazine di-*N*-oxides. *Int. J. Radiat. Oncology Biol. Phys.* **1989**, *16*, 977−981.
(25) Arndt, F. Ring-closing between nitro and amino groups forming triazines. *Ber.* **1913**, *46*, 3522−3529.
(26) Mason, J. C.; Tennant, G. Heterocyclic *N*-oxides. Part VI. Synthesis and nuclear magnetic resonance spectra of 3-aminobenzo-1,2,4-triazines and their mono- and di-*N*-oxides. *J. Chem. Soc. (B)* **1970**, 911−916.
(27) Pazdera, P.; Potacek, M. 4-Substituted 2-nitrophenylguanidines I. Synthesis and cyclization of 4-substituted 2-nitrophenylguanidines. *Chem. Papers* **1988**, *42*, 527−537.
(28) Newman, M. S.; Karnes, H. A. The conversion of phenols to thiophenols via dialkylthiocarbamates. *J. Org. Chem.* **1966**, *31*, 3980−3984.
(29) Seko, S.; Miyake, K.; Kawamura, N. A convenient copper-catalyzed direct amination of nitroarenes with *O*-alkylhydroxylamines. *J. Chem. Soc., Perkin Trans. 1* **1999**, 1437−1444.
(30) Makosza, M.; Bialecki, M. Nitroarylamines via the vicarious nucleophilic substitution of hydrogen: amination, alkylamination, and arylamination of nitroarenes with sulfenamides. *J. Org. Chem.* **1998**, *63*, 4878−4888.
(31) Siim, B. G.; Hicks, K. O.; Pullen, S. M.; van Zijl, P. L.; Denny, W. A.; Wilson, W. R. Comparison of aromatic and tertiary amine *N*-oxides of acridine DNA intercalators as bioreductive drugs − Cytotoxicity, DNA binding, cellular uptake, and metabolism. *Biochem. Pharmacol.* **2000**, *60*, 969−978.
(32) Patel, K. B.; Willson, R. L. Semiquinone free radicals and oxygen. Pulse radiolysis study of one electron equilibria. *J. Chem. Soc., Faraday Trans.* **1973**, *69*, 814−819.
(33) Wilson, W. R.; Thompson, L. H.; Anderson, R. F., Denny, W. A. Hypoxia-selective antitumor agents. 2. Electronic effects of 4-substituents on the mechanisms of cytotoxicity and metabolic stability of nitracrine analogues. *J. Med. Chem.* **1989**, *32*, 31−38.
(34) Siim, B. G.; Wilson, W. R. Efficient redox cycling of nitroquinoline bioreductive drugs due to aerobic nitroreduction in chinese hamster cells. *Biochem. Pharmacol.* **1995**, *50*, 75−82.
(35) Rischin, D.; Peters, L.; Hicks, R.; Hughes, P.; Fisher, R.; Hart, R.; Sexton, M.; D′Costa, I.; von Roemeling, R. Phase I trial of concurrent tirapazamine, cisplatin, and radiotherapy in patients with advanced head and neck cancer. *J. Clin. Oncol.* **2001**, *19*, 535−542.
(36) Evans, J. W.; Yudoh, K.; Delahoussaye, Y. M.; Brown, J. M. Tirapazamine is metabolized to its DNA damaging radical by intranuclear enzymes. *Cancer Res.* **1998**, *58*, 2098−2101.
(37) Wouters, B. G.; Delahoussaye, Y. M.; Evans, J. W.; Birrel, G. W.; Dorie, M. J.; Wang, J.; MacDemed, D.; Chiu, R. K.; Brown, J. M. Mitochondrial dysfunction after aerobic exposure to the hypoxic cytotoxin tirapazamine. *Cancer Res.* **2001**, *61*, 145−152.
(38) Delahoussaye, Y. M.; Hay, M. P.; Pruijn, F. B.; Denny, W. A.; Brown, J. M. Unpublished results.
(39) Shigyo, H.; Sato, S.; Shibuya, K.; Takahashi, Y.; Yamaguchi, T.; Sonoki, H.; Ohta, T. Synthesis and antiarrhythmic activity of disubstituted phenylpyridine derivative. *Chem. Pharm. Bull.* **1993**, *41*, 1573−1582.
(40) Suzuki, H.; Kawakami, T. A convenient synthesis of 3-amino-1,2,4-benzotriazine 1,4-dioxide (SR 4233) and related compounds via nucleophilic aromatic substitution between nitroarenes and guanidine base. *Synthesis* **1977**, 855−857.
(41) Friebe, W. G.; Scheuer, W.; Tibes, U. Benzotriazines, pharmaceutical compositions, and methods of using same. US Patent 5856325, Jan 1999.
(42) Robbins, R. F.; Schofield, K. Polyazabicyclic compounds. Part II. Further derivatives of benzo-1,2,4-triazine. *J. Chem. Soc.* **1957**, 3186−3194.
(43) Lee, W. W.; Brown, J. M.; Grange, E. W.; Martinez, A. P.; Tracy, M.; Pollart, D. J. 1,2,4-Benzotriazine oxides as radiosensitizers and selective cytotoxic agents. US Patent 5624925, April 1997.
(44) Wardman, P. Reduction potentials of one-electron couples involving free radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **1989**, *18*, 1637−1755.
(45) Van Leeuwen, J. W.; Van Dijk, C.; Veeger, C. A pulse radiolysis study of the reduction of flavodoxin from *Megasphaera elsdenii* by viologen radicals. *Eur. J. Biochem.* **1983**, *135*, 601−607.
(46) Stekhan, E.; Kuwana, T. Spectroelectrochemical study of mediators. I. Bipyridylium salts and their electron-transfer rates to cytochrome C. *Ber. Bunsen-Ges. Physik Chem.* **1974**, *78*, 253−259.
(47) Anderson, R. F. Electron transfer and equilibria between pyridinyl radicals and FAD. *Ber. Bunsen-Ges. Physik Chem.* **1976**, *80*, 969−972.

JM020367+