

Crystal structure of 3-amino-5-methyl-1,2,4-benzotriazine 1-oxide: Evidence for formation of a covalent attachment between a carbon-centered radical and the antitumor agent tirapazamine

Tarra Fuchs,⁽¹⁾ Charles L. Barnes,⁽¹⁾ and Kent S. Gates^{(1,2)*}

Received January 28, 2001

The compound 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine, **1**) is a potent antitumor agent. We isolated 3-amino-5-methyl-1,2,4-benzotriazine 1-oxide (**5**) from the reaction tirapazamine with methyl radical and characterized this compound by X-ray crystallography. The crystal structure of **5** provides clear evidence that this carbon-centered radical forms a covalent attachment with the drug tirapazamine. Given that tirapazamine generates carbon-centered DNA radicals as part of its mechanism of action, this finding may provide a chemical basis for understanding the small but significant levels of drug that become covalently associated with DNA during the damage process. The title compound $C_8H_8N_4O$ crystallized in the monoclinic space group $P2_1/c$ with unit cell parameters: $a = 15.670(8)$, $b = 7.381(4)$, $c = 13.491(7)$ Å, $\alpha = 90(1)$, $\beta = 95.623(9)$, $\gamma = 90(1)^\circ$, and $Z = 8$.

KEY WORDS: Crystal structure; tirapazamine; covalent adduct.

Introduction

The compound 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine, **1**) is currently undergoing clinical trials as a potential antitumor agent.¹ Tirapazamine derives its medicinal activity by selectively damaging DNA in oxygen-deficient tumor cells, but the chemical mechanisms of DNA damage by this drug are, as yet, only partially understood. It is clear

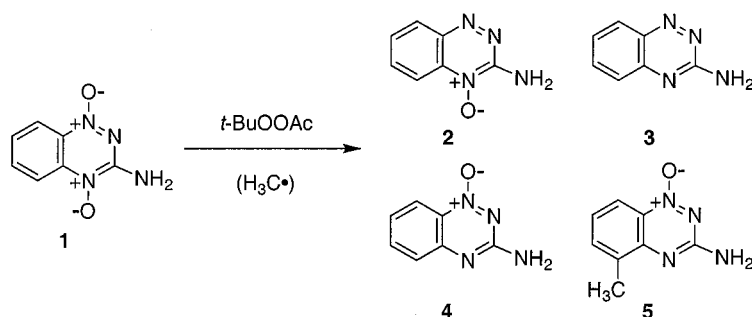
that tirapazamine undergoes enzymatic one-electron reduction in the cell to generate an oxygen-sensitive drug radical that ultimately leads to the abstraction of hydrogen atoms from the DNA backbone under low-oxygen conditions.^{2–4} In addition to initiating the formation of carbon-centered DNA radicals, tirapazamine and its metabolites can react further with these radicals, directly transferring an oxygen atom from an *N*-oxide functional group to the radical site.^{5–7} This reaction affords the net conversion of DNA radical lesions into toxic DNA strand breaks.^{5–7}

During the course of recent investigations into the interactions of carbon-centered radicals with the antitumor agent tirapazamine, we

⁽¹⁾ Department of Chemistry, University of Missouri – Columbia, Columbia, Missouri 65211.

⁽²⁾ Department of Biochemistry, University of Missouri – Columbia, Columbia, Missouri 65211.

* To whom correspondence should be addressed. E-mail: gatesk@missouri.edu.



Scheme 1. Reaction of tirapazamine (1) with methyl radical.

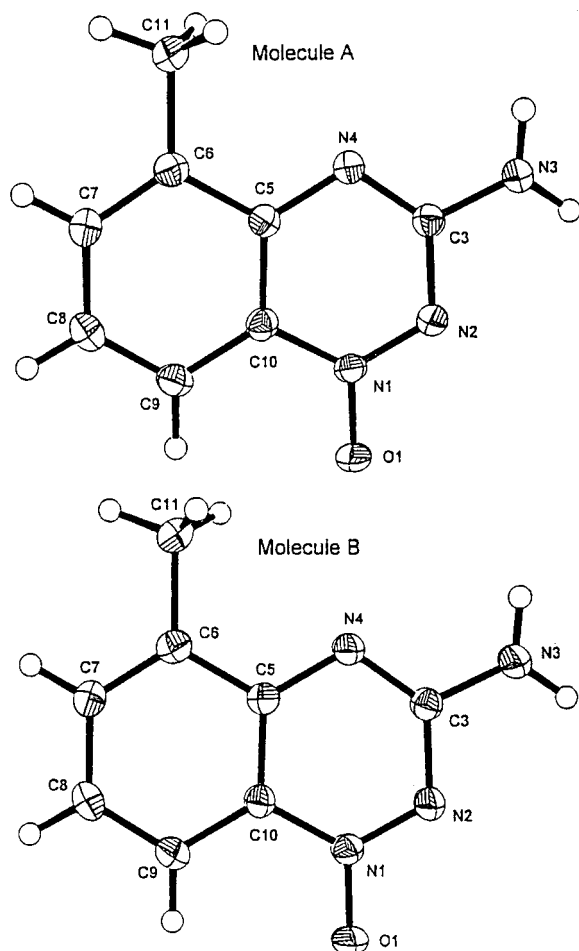


Fig. 1. ORTEP diagram, with atom labeling, of the two independent molecules of the asymmetric unit cell.

examined the reaction of this drug with methyl radical ($\text{H}_3\text{C}\cdot$) generated by thermolysis of *t*-butyl peracetate.⁸ This reaction affords a mixture of the deoxygenated products **2**, **3**, and **4** (Scheme 1).⁸ In addition, we isolated from this reaction a 15% yield of a mono-*N*-oxide product in which

Table 1. Crystal Data and Structure Refinement

Compound	$\text{C}_8\text{H}_8\text{N}_4\text{O}$
CCDC no.	CCDC-1003/6066
Color/shape	Colorless/prism
Formula weight	176.18
Temperature, K	173(2)
Crystal system	Monoclinic
Space group	$P2_1/c$
Unit cell dimensions	$a = 15.670$ (8) Å $b = 7.381$ (4) Å $c = 13.491$ (7) Å $\beta = 95.623$ (9)°
Volume, Å ³	1553.0(4)
<i>Z</i>	8
D_{calc} , g/cm ³	1.507
Absorption coefficient, mm ⁻¹	0.107
Diffractometer/scan	Bruker SMART/CCD area detector
Radiation/wavelength, Å	0.71073
θ range for data, deg	2.6–27.1
Reflections measured	9174
Independent/observed reflections	3394 ($R_{\text{int}} = 0.018$)/2678 [$I > 2\sigma(I)$]
Absorption correction ¹⁰	Semiempirical
T_{min} , T_{max}	0.84, 1.00
Data/restraints/parameters	3394/0/237
Goodness of fit on F^2	1.049
Final <i>R</i> indices [$I > 2\sigma(I)$]	$R1 = 0.0444$, $wR2 = 0.1246$
<i>R</i> indices (all data)	$R1 = 0.0575$, $wR2 = 0.1327$

Table 2. Atomic Coordinates (10^4) and Equivalent Isotropic Displacement Parameters (10^3Å^2)

	<i>x</i>	<i>y</i>	<i>z</i>	U_{eq}^a
O (1A)	3973 (1)	1832 (1)	6767 (1)	31 (1)
N (1A)	3629 (1)	3385 (2)	6667 (1)	22 (1)
C (10A)	2753 (1)	3547 (2)	6371 (1)	21 (1)
N (4A)	2937 (1)	6815 (2)	6440 (1)	23 (1)
C (5A)	2433 (1)	5334 (2)	6253 (1)	21 (1)
C (7A)	1040 (1)	4042 (2)	5814 (1)	26 (1)
C (9A)	2232 (1)	2003 (2)	6206 (1)	25 (1)
N (2A)	4133 (1)	4788 (2)	6854 (1)	25 (1)
N (3A)	4294 (1)	7840 (2)	6977 (1)	29 (1)
C (6A)	1539 (1)	5565 (2)	5947 (1)	23 (1)
C (8A)	1375 (1)	2273 (2)	5934 (1)	27 (1)
C (11A)	1192 (1)	7455 (2)	5805 (1)	30 (1)
C (3A)	3750 (1)	6471 (2)	6746 (1)	22 (1)
O (1B)	1043 (1)	5463 (1)	3172 (1)	32 (1)
N (2B)	849 (1)	2520 (2)	3211 (1)	24 (1)
N (1B)	1365 (1)	3908 (2)	3353 (1)	23 (1)
C (5B)	2541 (1)	1943 (2)	3848 (1)	21 (1)
N (4B)	2020 (1)	473 (2)	3695 (1)	23 (1)
C (10B)	2236 (1)	3730 (2)	3675 (1)	22 (1)
C (7B)	3936 (1)	3215 (2)	4305 (1)	27 (1)
C (3B)	1210 (1)	836 (2)	3384 (1)	22 (1)
C (6B)	3425 (1)	1701 (2)	4179 (1)	24 (1)
N (3B)	644 (1)	-528 (2)	3219 (1)	29 (1)
C (8B)	3620 (1)	4982 (2)	4120 (1)	28 (1)
C (11B)	3763 (1)	-180 (2)	4377 (1)	32 (1)
C (9B)	2769 (1)	5263 (2)	3807 (1)	25 (1)

^a U_{eq} is defined as one third of the trace of the orthogonalized U_{ij} tensor.

a methyl group was incorporated into the aromatic carbon ring of the drug. Here we report X-ray crystallographic studies that conclusively establish the structure of this product as 3-amino-5-methyl-1,2,4-benzotriazine 1-oxide (**5**) (Scheme 1, Fig. 1).

The crystal structure of **5** reported here provides clear evidence that a carbon-centered radical can react with tirapazamine to form a stable covalent attachment. Tirapazamine generates carbon-centered DNA radicals as part of its mechanism of action²⁻⁴ and our findings suggest that reaction of the drug with these radical centers could lead to covalent drug-DNA adducts. Thus, our results may provide a chemical basis for understanding the small but significant levels of drug that become irreversibly (covalently) associated with DNA during tirapazamine-mediated DNA damage.⁴

Table 3. Selected Bond Lengths (Å) and Angles (deg) for Molecules A and B

	A	B
Bond length		
O(1)–N(1)	1.268 (2)	1.268 (2)
N(1)–N(2)	1.312 (2)	1.307 (2)
N(1)–C(10)	1.397 (2)	1.398 (2)
C(10)–C(5)	1.415 (2)	1.414 (2)
N(4)–C(3)	1.325 (2)	1.325 (2)
N(4)–C(5)	1.358 (2)	1.362 (2)
N(2)–C(3)	1.381 (2)	1.376 (2)
N(3)–C(3)	1.339 (2)	1.346 (2)
Bond angle		
O(1)–N(1)–N(2)	116.89 (12)	116.92 (12)
O(1)–N(1)–C(10)	120.24 (12)	120.18 (12)
N(2)–N(1)–C(10)	122.87 (12)	122.89 (12)
N(1)–C(10)–C(9)	120.94 (13)	120.86 (13)
N(1)–C(10)–C(5)	116.12 (12)	116.27 (13)
C(3)–N(4)–C(5)	115.35 (13)	115.39 (13)
N(4)–C(5)–C(10)	122.43 (13)	122.04 (13)
N(1)–N(2)–C(3)	116.34 (12)	116.46 (12)
N(4)–C(3)–N(3)	119.94 (13)	119.80 (14)
N(4)–C(3)–N(2)	126.83 (13)	126.93 (13)
N(3)–C(3)–N(2)	113.22 (13)	113.26 (13)

Experimental

The methylated mono-*N*-oxide derivative **5** was initially isolated from the reaction of tirapazamine with methyl radical.⁸ Although it was not fully characterized previously, NMR and mass spectroscopic data suggested that the compound might be 3-amino-5-methyl-1,2,4-benzotriazine 1-oxide (**5**). Thus, we sought an independent synthesis of this molecule by the general method of Mason and Tennant.⁹ Accordingly, the reaction of 2-methyl-6-nitroaniline with cyanamide gave a dark yellow solid that was purified by column chromatography on silica gel (eluted with 1:1 ethyl acetate–hexane) to yield compound **5** as a yellow powder: TLC $R_f = 0.6$ (silica gel eluted with 1:1 ethyl acetate–hexane); ¹H NMR (250 MHz, DMSO-*d*₆): δ 7.97 (d, 8.5 Hz, 1H), 7.63 (d, 7.0 Hz, 1H), 7.31–7.21 (m, 3H, overlap of NH₂ protons and aromatic C–H proton), 2.47 (s, 3H); ¹³C NMR (63 MHz, DMSO-*d*₆): δ 159.6, 148.0, 135.2, 134.4, 129.9, 124.1, 117.5, 16.8. The properties of the material obtained from the independent synthesis were identical in all regards to those

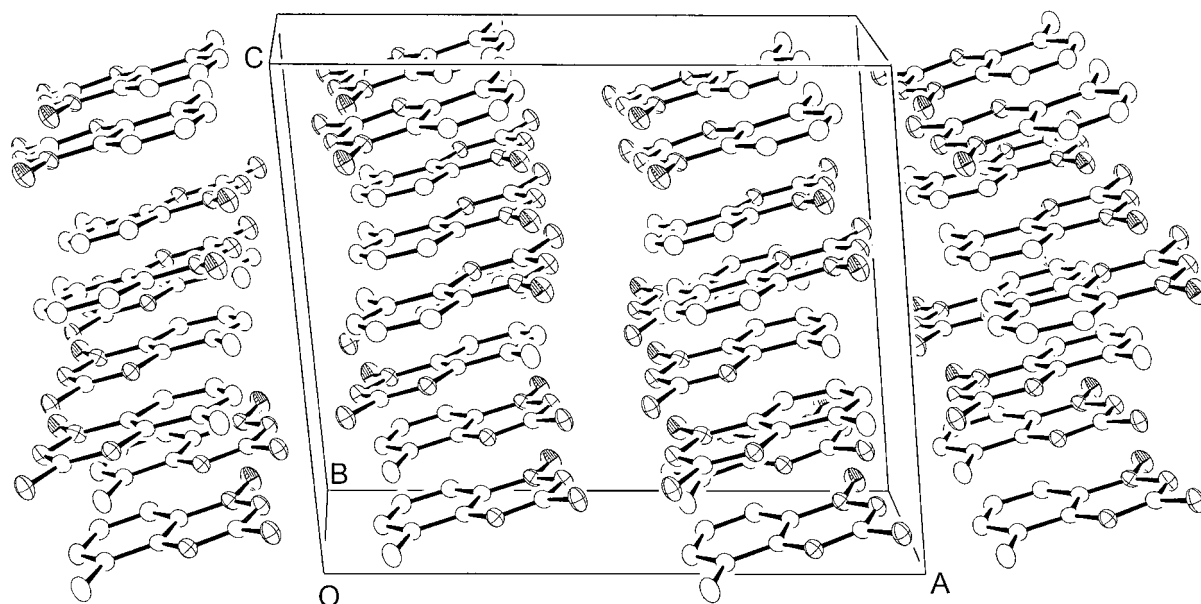


Fig. 2. Packing diagram. Carbon atoms drawn as plain ellipsoids, nitrogen atoms drawn with principle and boundary ellipsoids, oxygen atoms drawn with octant shading.

of the methylated mono-*N*-oxide isolated from the reaction of methyl radical with tirapazamine.

Crystallography

From the independent synthesis of **5**, crystals suitable for X-ray diffraction analysis were prepared by slow evaporation of a dilute solution of the compound in 1:1 ethyl acetate–hexane. Intensity data were collected on a Bruker SMART system at 173 K. The crystal structure and the details of data collection and structure refinement are given in Table 1. The structure was solved

by direct methods and refined using the SHELX suite of programs.^{11,12}

Results and discussion

There are two independent molecules in the asymmetric unit, related by an approximate inversion center at $x = 0.249$, $y = 0.364$, $z = 0.505$. That this inversion center is only approximate is confirmed by the following: (1) the mean planes of the two molecules make an angle of about 3.5° ; (2) in refinement, there are no correlations between parameters greater than 0.5; (3) the

Table 4. H-Bond Parameters

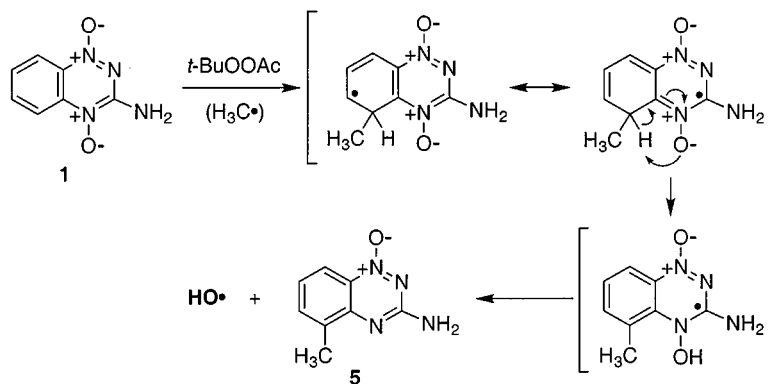
D–H	$d(\text{D–H})$ (Å)	$d(\text{H} \cdots \text{A})$ (Å)	$\angle\text{DHA}$ (deg)	$d(\text{D} \cdots \text{A})$ (Å)	A
N(3A)–H(3A1)	0.880	2.135	166.67	2.998(2)	O (1A) ^a
N(3B)–H(3B1)	0.880	2.169	164.69	3.027(2)	O (1B) ^b
N(3A)–H(3A2)	0.880	2.307	159.32	3.146(2)	O (1A) ^c
N(3A)–H(3A2)	0.880	2.546	125.68	3.142(2)	N (2A) ^c
N(3B)–H(3B2)	0.880	2.353	155.22	3.174(2)	O (1B) ^d
N(3B)–H(3B2)	0.880	2.662	122.60	3.223(2)	N (2B) ^d

^a Acceptor at x , $y + 1$, z .

^b Acceptor at x , $y - 1$, z .

^c Acceptor at $1 - x$, $(1/2) + y$, $(3/2) - z$.

^d Acceptor at $-x$, $(-1/2) + y$, $(1/2) - z$.



Scheme 2. Possible mechanism for the formation of 3-amino-5-methyl-1,2,4-benzotriazine 1-oxide (**5**) from the reaction of tirapazamine (**1**) with methyl radical.

program MISSYM¹³ reports no extra symmetry. Final atomic coordinates are given in Table 2, bond lengths and angles are given in Table 3, and Fig. 1 gives perspective views of the two molecules. A packing diagram is shown in Fig. 2 and H-bond parameters are given in Table 4. The essentially planar molecules, A and B, pack as infinite ribbons composed exclusively of A or B molecules linked via H-bonds from the amino groups to the oxygen atoms and propagated along the *b* direction via translation. Each ribbon is linked to a twofold screw related ribbon by weaker interactions, the double ribbons again composed exclusively of A or B molecules. These assemblies are then layered along *c* to complete the packing.

Unambiguous assignment of the sites of N-oxidation and benzo ring modification using NMR spectroscopy can be problematic for substituted 1,2,4-benzotriazine *N*-oxides.^{8,9} Thus, the X-ray crystal structure reported here provides important confirmation of the chemical structure of compound **5**. The bond angles and bond lengths found in **5** do not differ markedly from that of related benzotriazine *N*-oxides that have been characterized crystallographically.^{8,14} While several pathways for the formation of **5** from the reaction of *t*-butyl peracetate with tirapazamine can be envisioned; the mechanism shown in Scheme 2 provides a reasonable rationale for the observed regiochemistry of the methylation reaction.

Acknowledgments

We are grateful to the American Cancer Society (RPG-00-028-01 to KSG) for support of this work. The X-ray diffractometer used in this study was obtained with funding from National Science Foundation (CHE 9011804).

References

1. Brown, J.M. *Cancer Res.* **1999**, *59*, 5863–5870.
2. Daniels, J.S.; Gates, K.S. *J. Am. Chem. Soc.* **1996**, *118*, 3380–3385.
3. Fitzsimmons, S.A.; Lewis, A.D.; Riley, R.J.; Workman, P. *Carcinogenesis* **1994**, *15*, 1503–1510.
4. Laderoute, K.L.; Wardman, P.; Rauth, M. *Biochem. Pharmacol.* **1988**, *37*, 1487–1495.
5. Hwang, J.-T.; Greenberg, M.M.; Fuchs, T.; Gates, K.S. *Biochemistry* **1999**, *38*, 14248–14255.
6. Daniels, J.S.; Gates, K.S.; Tronche, C.; Greenberg, M.M. *Chem. Res. Toxicol.* **1998**, *11*, 1254–1257.
7. Jones, G.D.D.; Weinfeld, M. *Cancer Res.* **1996**, *56*, 1584–1590.
8. Fuchs, T.; Chowdhary, G.; Barnes, C.L.; Gates, K.S. *J. Org. Chem.* **2001**, *66*, 107–114.
9. Mason, J.C.; Tennant, G. *J. Chem. Soc. B* **1970**, 911–916.
10. Sheldrick, G.M., *Sadabs, Program for Empirical Absorption Correction of Area Detector Data*; University of Gottingen: Germany, 1996.
11. Sheldrick, G.M., *SHELXS-97, Program for the Solution of Crystal Structures*; University of Gottingen: Germany, 1997.
12. Sheldrick, G.M., *SHELXL-97, Program for the Refinement of Crystal Structures*; University of Gottingen: Germany, 1997.
13. Le Page, Y. *J. Appl. Cryst.* **1988**, *21*, 983–984.
14. Daniels, J.S.; Chatterji, T.; MacGillivray, L.R.; Gates, K.S. *J. Org. Chem.* **1998**, *63*, 10027–10030.