Antitumor Imidazotetrazines. 20.[1] Preparation of the 8-Acid Derivative of Mitozolomide and Its Utility in the Preparation of Active Antitumor Agents

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The preparation of 3-(2-chloroethyl)-4-oxo-3*H*-imidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxylic acid, a key derivative of mitozolomide in our exploration of the structure–activity relationships of this class of antitumor agents, is described. The facile conversion to the 8-carbonyl chloride gave a derivative that reacted preferentially with nucleophiles at the 8-position rather than at the reactive 4-oxo group, allowing the preparation of a wide range of ester, thioester, amide (including an amide derived from an amino acid), hydroxamic acid, hydrazide and sulfoximide, azide and diazoacetyl derivatives. The in vivo activity is presented of a range of these compounds against TLX5 lymphoma and L1210 leukemia cell lines.

Mitozolomide (1) has been shown to be an agent with clinical potential as a treatment for malignant melanoma,[2] but against this disease and other tumor types against which the compound has been examined in clinical trial, the therapeutic index of the drug has been insufficient to give tumor remission without causing serious concomitant side effects, particularly those of bone marrow depression.[3] During our search for congeners of mitozolomide that might show an improved therapeutic ratio, we have described the preparation and structure–activity profile of several series of compounds.[4–6] These earlier studies have been reviewed.[7]

With regard to 8-substitution, 8-carbamoyl, 8-sulfamoyl, and 8-sulfonyl derivatives were extremely potent compounds, and there was seen to be a change in potency across the range of test systems, with activity against solid tumors being retained, and an increase in activity observed against the leukemia L1210 test system relative to mitozolomide.[4] We were hence encouraged to explore further derivatives at this position of the imidazotetrazin-4-one skeleton. This paper describes compounds prepared in the series bearing an 8-carbonyl group, which became available to us following successful preparation of the 8-acid derivative of mitozolomide.

Chemistry

Our previously described syntheses of mitozolomide and its derivatives[4–6] have nearly always commenced with a preformed 4-aminoimidazole (e.g. 4), bearing in the 5-position a preformed group destined to become located at position 8 of the imidazotetrazine ring. Exceptions to this only occurred where such a 4-amino-5-substituted- or 4-diazo-5-substituted-imidazole was too unstable to participate in this scheme, notably in the cases of monoaryl- and monoalkyl-substituted carboxamides and sulfonamides. For example, the diazotization of 4-amino-*N*-phenyl-imidazole-5-carboxamide led to cyclization to an imidazo[4,5-*d*]-1,2,3-triazine. To overcome these side reactions, resort was made to protection of the amide groups which, although ultimately successful, resulted in long, tedious syntheses. It soon became apparent that a better approach to such 8-substituted carbamoyl derivatives and other interesting products would be via reaction of an activated

derivative of the 8-acid with nucleophiles. A potential problem with this approach would be that we had already established that the 4-carbonyl groups of our imidazotetrazinones were highly susceptible to attack by nucleophiles.[6,8] The question was: could we select a group at the 8-position which would preferentially react with nucleophiles?

The known instability[9] of 4-aminoimidazole-5-carboxylic acid precluded a direct synthesis of the 8-acid derivative of mitozolomide by our established route. The first attempt to make this derivative commenced, as usual, with the dimeric dinitroimidazopyrazinedione (2), which was transformed into the benzyl 4-nitroimidazole-5-carboxylate (3) by reaction with benzyl alcohol. Reduction of the nitro group gave the amino ester (4). Subsequent diazotization and cycloaddition of the resulting diazo ester (5) with 2-chloroethyl isocyanate gave the imidazotetrazine ester 6, which on hydrogenation gave the acid 7 in a rather impure form. This synthesis was not considered suitable for scale-up.

During attempts to validate decontamination procedures for apparatus and equipment containing mitozolomide (1), a degradative experiment (on preparative scale) employing a mildly alkaline bleach as destructive agent was performed. Following removal of undissolved, unreacted mitozolomide from the bleach solution, acidification of the

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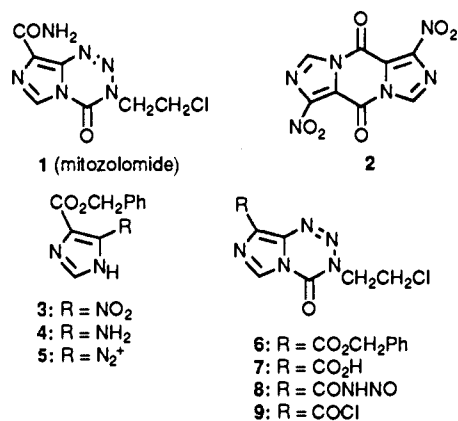(9) Rabinowitz, J. C. *J. Biol. Chem.* 1956, *218*, 175.

Table I. Preparative and Physical Data of Imidazotetrazinones

no.	R	general exptl method[a]	purification method[b]	yield, %	mp, °C	formula	anal.[c]	ν(C=O), cm⁻¹	NMR chem shifts, (Me₂SO), δ
7		A	see exptl	54	145	$C_7H_6ClN_5O_3$	na[d]	1755, 1710	4.00 (2 H, t, J = 6 Hz, CH₂N), 4.60
		B	see exptl	37	162	$C_7H_6ClN_5O_3\cdot H_2O$	C,H,Cl,N,H₂O[l]		(2 H, t, J = 6 Hz, CH₂Cl), and 8.70
		C	see exptl	86	166	$C_7H_6ClN_5O_3\cdot H_2O$	C,H,Cl,N,H₂O[l]	1760, 1710	(1 H, s, C6-H)
9		D	see exptl	100	69	$C_7H_5Cl_2N_5O_3$	na[d]	1730	
10a	OMe	E	r (aq acet)	68	113	$C_8H_8ClN_5O_3$	C,H,N	1780, 1750	4.00 (3 H, s, CH₃), 4.05 (2 H, t, J = 6 Hz, CH₂N), 4.65 (2 H, t, J = 6 Hz, CH₂Cl), and 8.95 (1 H, s, C6-H)
10b	OEt	E	r (aq acet)	70	96	$C_9H_{10}ClN_5O_3$	C,H,N	1780, 1740	1.35 (3 H, t, J = 7 Hz, CH₃), 4.00 (2 H, t, J = 6 Hz, CH₂N), 4.35 (2 H, q, J = 7 Hz, OCH₂), 4.65 (2 H, t, J = 6 Hz, CH₂Cl), and 8.9 (1 H, s, C6-H)
10c	OPr	E	r (aq acet)	63	92	$C_{10}H_{12}ClN_5O_3$	C,H,N	1760, 1730	1.00 (3 H, t, J = 6.5 Hz, CH₃), 1.75 (2 H, hex, J = 6.5 Hz, CH₂CH₃), 4.0 (2 H, t, J = 6 Hz, CH₂N), 4.3 (2 H, t, J = 6.5 Hz, CH₂O), 4.65 (2 H, t, J = 6 Hz, CH₂Cl), and 8.9 (1 H, s, C6-H)
10d	O^{i}Pr	E	r (aq acet)	58	113	$C_{10}H_{12}ClN_5O_3$	C,H,Cl,N	1760, 1720	1.35 (6 H, d, J = 5 Hz, 2 CH₃), 4.00 (2 H, t, J = 6 Hz, CH₂N), 4.65 (2 H, t, J = 6 Hz, CH₂Cl), 5.20 (1 H, sept, J = 5 Hz, CH(CH₃)₂), and 8.85 (1 H, s, C6-H)
10e	SEt	E	r (aq acet)	49	111–112	$C_9H_{10}ClN_5O_2S$	C,H,N	1760, 1660	1.3 (3 H, t, J = 5 Hz, CH₃), 3.05 (2 H, q, J = 5 Hz, SCH₂), 4.05 (2 H, t, J = 6 Hz, CH₂N), 4.65 (2 H, t, J = 6 Hz, CH₂Cl), and 8.9 (1 H, s, C6-H)
10f	NHMe	F	r (aq acet)	57	121	$C_8H_9ClN_6O_2\cdot H_2O$	C,H,N[l]	1750, 1640	2.80 (3 H, d, J = 5 Hz, CH₃), 4.00 (2 H, t, J = 6 Hz, CH₂N), 4.60 (2 H, t, J = 6 Hz, CH₂Cl), 8.35 (1 H, br q, J = 5 Hz, NH), and 8.9 (1 H, s, C6-H)
10g	NMe₂	F	r (aq acet)	42	116–117	$C_9H_{11}ClN_6O_2$	C,H,N	1720, 1610	3.05 (6 H, s, 2 CH₃, 4.05 (2 H, t, J = 6 Hz, CH₂N), 4.60 (2 H, t, J = 6 Hz, CH₂Cl), and 8.85 (1 H, s, C6-H)
10h	NHPr	F	r (aq acet)	64	111	$C_{10}H_{13}ClN_6O_2$	C,H,N	1760, 1650	0.9 (3 H, t, J = 7 Hz, CH₃), 1.5 (2 H, hex, J = 7 Hz, CH₂CH₃), 3.25 (2 H, q, J = 7 Hz, NHCH₂CH₂), 4.0 (2 H, t, J = 6 Hz, CH₂N), 4.6 (2 H, t, J = 6 Hz, CH₂Cl), 8.45 (1 H, t, J = 7 Hz, NH), and 8.9 (1 H, s, C6-H)
10i	NH^{i}Pr	F	r (aq acet)	69	119–120	$C_{10}H_{13}ClN_6O_2$	C,H,N	1760, 1690	1.2 (6 H, d, J = 6 Hz, 2 CH₃), 4.05 (2 H, t, J = 6 Hz, CH₂N), 4.1 (1 H, m, CH), 4.65 (2 H, t, J = 6 Hz, CH₂Cl), 8.15 (1 H, br d, NHCH), and 8.9 (1 H s, C6-H)
10j	NH^{c}Pr	F	r (aq acet)	62	132	$C_{10}H_{11}ClN_6O_2\cdot H_2O$	C,H,N	1760, 1630	0.6–0.8 (4 H, m, 2 CH₂), 2.7–3.0 (1 H, m, CH), 4.0 (2 H, t, J = 6 Hz, CH₂N), 4.65 (2 H, t, J = 6 Hz, CH₂Cl), 8.5 (1 H, br d, NH), and 8.85 (1 H, s, C6-H)
10k	NH^{t}Bu	F	f (EtOAc)	77	126–128	$C_{11}H_{15}ClN_6O_2$	C,H,Cl,N	1750, 1675	1.45 (9 H, s, 3 CH₃), 4.00 (2 H, t, J = 6 Hz, CH₂N), 4.60 (2 H, t, J = 6 Hz, CH₂Cl), 7.60 (1 H, s, NH), and 8.85 (1 H, s, C6-H)
10l	NH^{c}Hex	F	r (aq acet)	66	130	$C_{13}H_{17}ClN_6O_2$	C,H,N	1750, 1660	1.0–2.0 (10 H, m, cyclohexyl 5 CH₂), 3.65–3.9 (1 H, m, CH), 4.0 (2 H, t, J = 6 Hz, CH₂N), 4.65 (2 H, t, J = 6 Hz, CH₂Cl), 8.15 (1 H, br d, J = 7 Hz, NH), and 8.9 (1 H, s, C6-H)
10m	NHCH₂^{t}BU	F	r (aq acet)	52	148	$C_{12}H_{17}ClN_6O_2$	C,H,N	1730, 1660	0.9 (9 H, s, 3 CH₃), 3.1 (2 H, d, J = 6 Hz, CH₂), 4.0 (2 H, t, J = 6 Hz, CH₂N), 4.6 (2 H, t, J = 6 Hz, CH₂Cl), 8.15 (1 H, br t, J = 6 Hz, NH), and 8.8 (1 H, s, C6-H)
10n	NHCH₂-CH=CH₂	F	r (aq acet)	50	121	$C_{10}H_{11}ClN_6O_2$	C,H,N	1780, 1660	3.8–4.2 (4 H, m, CH₂N and CH₂C=), 4.7 (2 H, t, J = 6 Hz, CH₂Cl), 5.0–5.3 (2 H, m, =CH₂), 5.7–5.9 (1 H, m, CH=, 8.6 (1 H, br t, NH), and 8.8 (1 H, s, C6-H)
10o	NHCH₂-CH₂Cl	F	f (pet./EtOAc) + r (EtOAc)	18	130–132	$C_9H_{10}Cl_2N_6O_2$	C,H,Cl,N	1730, 1660	3.60–3.80 (4 H, m, NHCH₂CH₂Cl), 4.00 (2 H, t, J = 6 Hz, CH₂N), 4.75 (2 H, t, J = 6 Hz, CH₂Cl), 8.20 (1 H, br s, NH), and 8.50 (1 H, s, C6-H)
10p	NHPh	F	r (aq acet)	66	166–167	$C_{13}H_{11}ClN_6O_2$	H,N;C[e]	1750, 1700	4.05 (2 H, t, J = 6 Hz, CH₂N), 4.65 (2 H, t, J = 6 Hz, CH₂Cl), 7.0–7.5 (3 H, m, Ar-H), 7.75–7.95 (2 H, m, Ar-H), 9.0 (1 H, s, C6-H), and 10.35 (1 H, s, NH)
10q	NHCH₂-CO₂Et	F	r (EtOAc)	43	114	$C_{11}H_{13}ClN_6O_4$	C,H;N[f]	1750, 1670	1.2 (3 H, t, J = 7 Hz, CH₃), 3.9–4.2 (6 H, m, CH₂N, CH₂O, and CH₂-C=O), 4.65 (2 H, t, CH₂Cl, J = 6 Hz, CH₂Cl), 8.80 (1 H, br t, J = 7 Hz, NH), and 8.85 (1 H, s, C6-H)
10r	NHOMe	F	f (EtOAc)	85	124–126	$C_8H_9ClN_6O_3$	H,N;C[g]	1750, 1690	3.80 (3 H, s, CH₃), 4.00 (2 H, t, J = 6 Hz, CH₂N), 4.60 (2 H, t, J = 6 Hz, CH₂Cl), 8.70 (1 H, s, C6-H), and 11.80 (1 H, s, NH)

Table I (Continued)

no.	R	general exptl method[a]	purification method[b]	yield, %	mp, °C	formula	anal.[c]	ν(C=O), cm^{-1}	NMR chem shifts, (Me$_2$SO), δ
10s	NHOCH$_2$Ph	F	f (tol/EtOAc) + r (pet./EtOAc)	20	139–141	C$_{14}$H$_{13}$ClN$_6$O$_3$	H,N;C[h]	1750, 1690	4.00 (2 H, t, J = 6 Hz, CH$_2$N), 4.60 (2 H, t, J = 6 Hz, CH$_2$Cl), 4.90 (2 H, s, OCH$_2$), 7.2–7.4 (5 H, m, Ar-H), 8.75 (1 H, s, C6-H), and 11.75 (1 H, br s, NH)
10t	NHOH	G	t (EtOAc/pet.)	69	139–141	C$_7$H$_7$ClN$_6$O$_3$	C,H,N	1750, 1660	4.05 (2 H, t, J = 6 Hz, CH$_2$N), 4.60 (2 H, t, J = 6 Hz, CH$_2$Cl), 8.90 (1 H, s, C6-H), 9.25 (1 H, br s, NH or OH), and 11.20 (1 H, br s, OH or NH)
10u	NHNHPh	F	r (aq acet)	71	156	C$_{13}$H$_{12}$ClN$_7$O$_2$	C,H,N	1750, 1680	4.0 (2 H, t, J = 6 Hz, CH$_2$N), 4.63 (2 H, t, J = 6 Hz, CH$_2$Cl), 6.65–6.85 (3 H, m, Ar-H), 7.16 (2 H, t, J = 8 Hz, Ar-H), 8.05 (1 H, br d, J = 2 Hz, NH), 8.9 (1 H, s, C6-H), and 10.4 (1 H, br d, J = 2 Hz, NH)
10v	NHNHC$_6$H$_3$-2,4-F$_2$	F	r (aq acet)	39	168	C$_{13}$H$_{10}$ClF$_2$N$_7$O$_2$	H,N;C[i]	1740, 1650	4.15 (2 H, t, J = 6 Hz, CH$_2$N), 4.75 (2 H, t, J = 6 Hz, CH$_2$Cl, 6.8–7.0 (2 H, m, Ar-H), 7.15 (1 H, t, J = 8 Hz, Ar-H), 7.8 (1 H, br s, NH), 8.9 (1 H, s, C6-H), and 10.4 (1 H, s, NH)
10w	NHNHC(=O)NHPh	F	r (aq acet)	71	169	C$_{14}$H$_{13}$ClN$_8$O$_3$·H$_2$O	C,H,N[l]	1745, 1665	4.0 (2 H, t, J = 6 Hz, CH$_2$N), 4.6 (2 H, t, J = 6 Hz, CH$_2$Cl), 7.0–7.5 (5 H, m, Ar-H), 8.26 (1 H, s, NH), 8.84 (1 H, s, NH), 8.96 (1 H, s, CH), and 10.24 (1 H, s, NH)
10x	NHNHC(=O)OMe	F	r (aq acet)	52	188	C$_9$H$_{10}$ClN$_7$O$_4$	C,H,N	1730, 1680	3.6 (3 H, s, NH*CH$_2$CH$_2$*Cl), 4.0 (2 H, t, J = 6 Hz, CH$_2$N), 4.6 (2 H, t, J = 6 Hz, CH$_2$Cl), 8.85 (1 H, s, C6-H), 9.2 (1 H, br s, NH), and 10.25 (1 H, br s, NH)
10y	N=S(=O)Me$_2$	F	r (aq acet)	78	165	C$_9$H$_{11}$ClN$_6$O$_3$S	C,H,N	1730, 1660	3.5 (6 H, s, 2 CH$_3$), 4.0 (2 H, t, J = 6 Hz, CH$_2$N), 4.6 (2 H, t, J = 6 Hz, CH$_2$Cl), and 8.7 (1 H, s, C6-H)
10z	N$_3$	H	r (aq acet)	81	115	C$_7$H$_5$ClN$_8$O$_2$	C,H,N	1760, 1720	4.05 (2 H, t, J = 6 Hz, CH$_2$N), 4.65 (2 H, t, J = 6 Hz, CH$_2$Cl), and 8.95 (1 H, s, C6-H)
13	CHN$_2$	I	r (aq acet)	65	69	C$_8$H$_6$ClN$_7$O$_2$·H$_2$O	H;C,N[j,l]	1750, 1730	4.05 (2 H, t, J = 6 Hz, CH$_2$N), 4.65 (2 H, t, J = 6 Hz, CH$_2$Cl), 6.85 (1 H, s, CH), and 8.9 (1 H, s, C6-H)
15	NHNO$_2$	J	r (aq acet)	95	160–161	C$_7$H$_6$ClN$_7$O$_4$·$\frac{1}{4}$H$_2$O	C,H,Cl,N[k,m]	1750, 1720	4.05 (2 H, t, J = 6 Hz, CH$_2$N), 4.70 (2 H, t, J = 6 Hz, CH$_2$Cl), 8.25 (1 H, br s, NH), and 9.05 (1 H, s, C6-H)

[a] General experimental methods are described by representative examples in the Experiment Section. [b] r = recrystallization, f = flash chromatography, t = trituration; solvents indicated in parentheses. [c] Analyses are within ±0.4% unless otherwise indicated. [d] Not analyzed. [e] Calcd: C, 48.9. Found: 48.4. This material was identical with that produced by an alternative procedure described in ref 4. [f] Calcd: N, 25.5. Found: 24.3. [g] Calcd: 35.2. Found: 34.5. [h] Calcd: 48.2. Found: 49.4. [i] Calcd: 42.2. Found: 41.6. [j] Calcd: 33.6; N, 34.3. Found: 34.1; N, 34.8. [k] for detailed data, see reference 12. [l] Monohydrate. [m] 0.25H$_2$O.

CONH$_2$ ... (tetrazine structure)

1 (mitozolomide)

(bis-nitroimidazotetrazine structure, NO$_2$ / NO$_2$)

2

CO$_2$CH$_2$Ph ... (imidazole structure)

3: R = NO$_2$
4: R = NH$_2$
5: R = N$_2^+$

(imidazotetrazine structure with CH$_2$CH$_2$Cl)

6: R = CO$_2$CH$_2$Ph
7: R = CO$_2$H
8: R = CONHNO
9: R = COCl

liquors gave the 8-acid **7** in pure form but moderate yield.

A better synthesis of the 8-acid **7** again commenced from mitozolomide itself, and used the nitrosylsulfuric acid or nitrosyl trifluoroacetic acid procedure for the hydrolysis of carbamoyl groups.[10] These processes involve the intermediate formation of a nitrosoamide (**8**) and are critically dependent on the degree of solvation of the acid.

(10) Wade, L. G., Jr.; Silvey, W. B. *Org. Synth. Prep. Int.* **1982**, 357.

With use of nitrosylsulfuric acid, the process could be scaled up to the 100 g batch scale.

The preparation of an activated derivative of the 8-acid for preferential reaction with nucleophiles centered on the 8-acid chloride **9**, which was readily formed from the 8-acid **7** by boiling with thionyl chloride, preferentially in the presence of a catalytic amount of DMF. To our satisfaction, this acid chloride (**9**) reacted preferentially with nucleophiles at the exocyclic carbonyl group and we were, therefore, able to prepare a large number of ester (**10a–d**), thioester (**10e**), amide (**10f–q**), hydroxamic acid (**10r–t**), and hydrazide (**10u–x**) derivatives under mild conditions without destruction of the rest of the molecule (Table I). Reaction of the acid chloride **9** with hydrazine itself did lead to degradation of the imidazotetrazine ring. We have shown previously that mitozolomide suffers attack by hydrazine at the carbonyl group of the tetrazinone ring to form (eventually) 4-azidoimidazole-5-carboxamide.[8] For the preparation of the hydroxamic acid **10t** it was necessary to use a protected nucleophile. In this case, O-benzylhydroxylamine served as a source of protected hydroxylamine, which gave the benzyl hydroxamic acid **10s** on reaction with **9**. This product (**10s**) was deprotected to give the 8-hydroxamic acid **10t** on catalytic hydrogenation. Of particular note was the preparation of the N-substituted

**Table II**. In Vivo Antitumor Activity of Imidazotetrazinones

no.	tumor	mouse species	inoculum cells	inoculum route	drug route	day of drug dosing	drug[a] formu-lation	duration of experiment, days	drug dose, mg/kg	survivors to duration	T/C, %[b]
1 (mitozolomide)[c]	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	40	4/5	>458
									20	0/5	167
									10	0/5	163
									5	0/5	113
	L1210	B6D2F1	10^5	iv	po	3	B	38	40	1/7	>330
									20	0/7	217
									10	0/7	178
									5	0/7	133
7	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	320	0/5	223
									160	0/5	108
									80	0/5	106
									40	0/5	103
									20	0/5	102
	L1210	B6D2F1	10^5	ip	po	1	B	39	320	0/7	106
									160	0/8	107
									80	0/8	101
									40	0/8	102
10a	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	320	0/5	140
									160	0/5	123
									80	0/5	110
									40	0/5	110
									20	0/5	106
10b	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	160	0/5	113
									80	0/5	91
									40	0/5	104
									20	0/5	98
									10	0/5	89
	L1210	B6D2F1	10^5	ip	ip	1	B	22	200	0/7	137
									100	0/7	120
									50	0/7	113
10e	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	320	0/4	125
									160	0/5	119
									80	0/5	108
									40	0/5	104
									20	0/5	106
10f[c]	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	80	0/5	toxic[d]
									40	5/5	>555
									20	1/5	>154
									10	0/5	133
									5	0/5	104
	L1210	B6D2F1	10^5	ip	po	1	B	39	80	0/10	toxic
									40	2/10	>215
									20	10/10	>453
									10	2/10	>192
10g[c]	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	160	0/5	toxic
									80	3/5	>358
									40	5/5	>508
									20	0/5	161
	L1210	B6D2F1	10^6	ip	po	1	B	39	80	0/10	toxic
									40	4/10	>235
									20	8/10	>290
									10	0/10	197
10k	L1210	B6D2F1	10^5	iv	po	3	B	25	200	0/7	307
									100	0/7	149
									50	0/7	132
10l	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	320	0/5	toxic
									160	0/5	toxic
									80	1/5	>277
									40	0/5	125
									20	0/5	109
10o	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	80	0/5	toxic
									40	3/5	>361
									20	0/5	142
									10	0/5	103
	L1210	B6D2F1	10^5	iv	po	3	B	37	40	0/7	227
									20	0/7	183
									10	0/7	121
10p[c]	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	320	0/5	98
									160	0/5	98
									80	0/5	100
									40	0/5	103
									20	0/5	98
10q	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	640	0/5	139
									320	0/5	114
									160	0/5	105
									80	0/5	96[b]

Table II (Continued)

| no.	tumor	mouse species	inoculum cells	inoculum route	drug route	day of drug dosing	drug[a] formulation	duration of experiment, days	drug dose, mg/kg	survivors to duration	T/C, %[b]
10r	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	320	0/5	toxic
									160	3/5	>325
									80	0/5	185
									40	0/5	116
									20	0/5	103
	L1210	B6D2F1	10^5	iv	po	1	B	28	200	0/7	205
									100	0/7	179
									50	0/7	117
10s	L1210	B6D2F1	10^5	iv	po	1	B	28	200	0/7	98
									100	0/7	98
									50	0/7	98
10t	L1210	B6D2F1	10^5	iv	po	1	B	20	200	0/7	218
									100	0/7	187
10u	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	320	0/5	toxic
									160	0/5	169
									80	0/5	107
10z	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	160	0/5	toxic
									80	0/5	106
									40	0/5	96
									20	0/5	97
									10	0/5	101
15	TLX5	CBA/CA	2×10^5	sc	ip	3	A	60	320	0/5	toxic
									160	2/5	>354
									80	0/5	118
									40	0/5	101
									20	0/5	109

[a] A, 10% Me_2SO in arachis oil; B, suspension in solution of 1% (carboxymethyl)cellulose in water. [b] Mean death day treated animals/ mean death day control animals. [c] Data from ref 4. [d] Deaths predate those of controls.

amino acid derivative 10q, which may serve as an entry into peptide-bound mitozolomide derivatives and conceivably to monoclonal antibody-linked cytotoxic agents. The acid chloride 9 could also be used to react with other more unusual nucleophiles. Thus, reaction with dimethylsulfoximine gave the dimethylsulfoximide (10y), while reaction with azide anion gave the 8-azidocarbonyl derivative 10z.
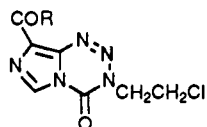

10 (for details of R (a – z), see Table I)

Attempts to effect a Curtius arrangement of the acid azide 10z in hot acetone, chloroform, benzene, or toluene failed to yield the expected 8-isocyanate (11) although evidence for the existence of an isocyanate was forthcoming in the mass spectrum of the acid azide, which showed the expected ion at m/z 240. However, the azide, when heated


11

12
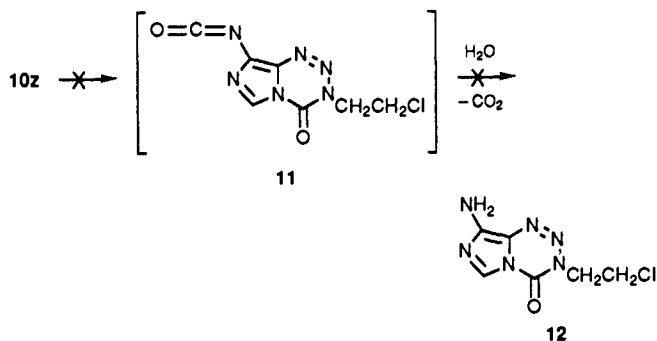
without solvent, exploded at 115 °C. Alternative efforts to synthesize the 8-amine 12 from mitozolomide under Hofmann degradation conditions were thwarted by the lability of imidazotetrazinones at pH values >8.[6,8] The reagent phenyl iodosyl bis(trifluoroacetate), which can effect "Hofmann reactions" under acidic conditions,[11] failed

to convert mitozolomide into the required amine 12. Reaction of the acid chloride 9 with ethereal diazomethane afforded the diazoacetyl derivative 13. Unfortunately, the potentially interesting series of imidazotetrazines (e.g. 14), where the carbonyl substituent is separated from the imidazole ring by a methylene group, could not be prepared from 13 by Wolff degradation without degradation of the bicyclic ring structure. The unexpected stability of the

13   **14**
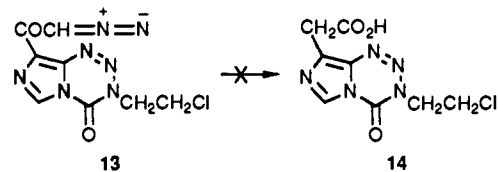
imidazo[5,1-d]-1,2,3,5-tetrazine ring structure toward strong acids, previously commented upon,[6,8] was further highlighted in the present work. Attempts to nitrate mitozolomide (1) in a concentrated nitric acid–sulfuric acid mixture led not to the expected 6-nitro derivative but the 8-(N-nitrocarbamoyl)imidazotetrazine 15. The presence of a characteristic singlet at δ 9.05 for the imidazole H-6 proton in the product confirmed the nitroamide structure.

15
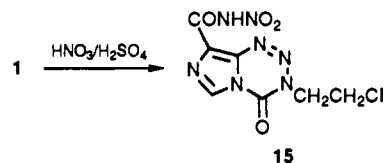
Results and Discussion

Most of the compounds prepared in this work were first evaluated against the mouse TLX5 lymphoma cell line in vitro. The activities expressed as an IC_{50} dose varied only over a 20-fold range (0.75–15.8 μM), the most active compound being the diazoacetyl derivative 13 and the least

(11) Fuller, W. D.; Goodman, M.; Verlander, M. S. *J. Am. Chem. Soc.* 1985, *107*, 5821.

active the carboxylic acid **7**. No clear structure–activity correlation emerged from this study. Mitozolomide (**1**) (IC_{50} = 2.30 ± 0.3 μM) was equi-cytotoxic with the monomethylcarboxamide analogue **10f** (IC_{50} = 3.0 ± 0.4 μM) whereas the dimethylcarboxamide **10g** was significantly less active (IC_{50} = 14.6 ± 1.1 μM). We have examined this difference in detail and have shown that the cytotoxicity of dimethylcarboxamide **10g** can be increased 5-fold by preincubation with murine hepatic microsomes, during which process a demethylation occurs to generate significant amounts of the monomethylcarboxamide.[1]

In in vivo tests (Table II) against the murine TLX5 lymphoma and L1210 leukemia, mitozolomide (**1**) and its monomethylcarboxamide (**10f**) and dimethylcarboxamide (**10g**) analogues were approximately equi-active, thus supporting the proposal that the dimethylcarboxamide (**10g**) can function as a prodrug.[1] Where sufficient material was available for in vivo tests the cyclohexylamide **10l**, the 2-chloroethylamide **10o**, the methyl hydroxamate **10r**, and the *N*-nitroamide **15** all showed high activity, with cures being elicited against the TLX5 lymphoma test system. The *tert*-butylamide **10k** and hydroxamic acid **10t** both showed good activity against the L1210 leukemia but the *O*-benzyl hydroxamate **10s** was inactive. The methyl ester **10a**, ethyl ester **10b**, ethyl thioester **10e**, *N*-phenylamide **10p**, and the acid azide **10z** were all inactive against the TLX5 lymphoma. The free acid **7** and the *N*-phenyl-hydrazide **10u** showed some activity against the TLX5 lymphoma but only at toxic doses leading to significant weight loss in the treated mice. From this and previously published work,[4,6] we are able to conclude that in antitumor imidazotetrazinones the substituent of choice at N-3 is 2-chloroethyl, at C-6 a hydrogen atom or small alkyl group is desirable, and at C-8 high in vivo activity extends through a range of unsubstituted and substituted carboxamides, sulfonamides, and sulfones.

It has been shown recently[13] that even a small bicyclic heterocycle like mitozolomide achieves some sequence specificity in alkylating DNA, preferring to chloroethylate the N-7 positions of the inner guanines in a run of four contiguous guanines. Guanine N-7 and O-6 residues, which are also vulnerable to attack by alkylating imidazotetrazinones,[14] occupy positions in the major groove of DNA. We propose to adapt the chemistry described in this paper to prepare imidazotetrazinones linked to peptide and protein moieties. Although the prototype derivative, the glycinate ester **10q**, proved to be inactive against the TLX5 lymphoma in vivo, we will persevere in efforts to exploit the major groove sequence recognition potential of this class of compound.

Experimental Section

Chemistry. General experimental details have been given in earlier parts of this series.[4,6]

Method A. Preparation of 3-(2-Chloroethyl)-4-oxo-imidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxylic Acid (7). A stirred solution of triethylamine (9.1 mL, 113 mmol) in benzyl alcohol (91 mL) was treated portionwise with 1,6-dinitro-5*H*,10*H*-diimidazo[1,5-*a*:1',5'-*d*]pyrazine-5,10-dione[4] (**2**) (9.07 g, 33.3 mmol) over 5 min. The temperature rose to 50 °C and the mixture was held at 60 °C after the addition was complete (15 min). The solution was cooled, acidified with saturated ethereal

(12) Baig, G. U.; Stevens, M. F. G.; Lunt, E.; Newton, C. G.; Pedgrift, B. L.; Smith, C.; Straw, C. G.; Walsh, R. J. A.; Warren, P. J. *U.K. Patent* 2,125,402B, 1985.

(13) Hartley, J. A.; Mattes, W. B.; Vaughan, K.; Gibson, N. W. *Carcinogenesis* **1988**, *9*, 669.

(14) Gibson, N. W.; Hartley, J. A.; LaFrance, R. J.; Vaughan, K. *Carcinogenesis* **1986**, *7*, 259.

hydrogen chloride solution, and diluted with ether. The resulting precipitate was collected, washed with water, and recrystallized from ethanol, giving benzyl 5-nitroimidazole-4-carboxylate (**3**) (9.95 g, 56%), as pale yellow crystals, mp 205–207 °C dec. Anal. (C, H, N).

A mixture of **3** (1.0 g, 4.0 mmol) and platinum oxide (0.1 g) in methanol (18 mL) and *N*,*N*-dimethylformamide (12 mL) was shaken under a hydrogen atmosphere (20 min) when hydrogen uptake was complete. The mixture was filtered through Celite and evaporated, giving benzyl 5-aminoimidazole-4-carboxylate (**4**) (0.91 g, 100%) as a brown oil, used directly in the next stage: NMR (d_6-DMSO) δ 5.2 (2 H, s, CH$_2$), 7.2 (1 H, s, C2-H), and 7.3 (5 H, m, Ar-H).

A solution of **4** (4.89 g, 22.5 mmol) in dilute hydrochloric acid (1 M, 54 mL) was treated with charcoal and filtered. The yellow filtrate was added dropwise to a stirred solution of sodium nitrite (2.04 g, 29.6 mmol) in water (15 mL) over 5 min, while the temperature of the solution was kept at 5–10 °C. The resulting gummy suspension was extracted with ethyl acetate (3 × 125 mL). The combined organic extracts were dried (MgSO$_4$) and concentrated, giving benzyl 5-diazoimidazole-4-carboxylate (**5**) (4.73 g, 92%) as a yellow oil: IR (film) 2200 cm^{-1}.

A solution of crude **5** (0.88 g, 3.9 mmol) in ethyl acetate (23 mL) was treated with 2-chloroethyl isocyanate (3.68 g, 34.9 mmol) and allowed to stand at room temperature (18 h). The solution was evaporated to dryness below 35 °C (0.1 mm) and the resulting residue purified by flash chromatography (silica, petroleum ether/ethyl acetate 1:1) to give benzyl 3-(2-chloroethyl)-3*H*-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxylate (**6**) (0.43 g, 33.3%) as pale yellow crystals: mp 110–112 °C; IR (KBr) 1745 and 1725 cm^{-1}; NMR (d_6-DMSO) δ 4.0 (2 H, t, *J* = 6 Hz, CH$_2$N), 4.6 (2 H, t, *J* = 6 Hz, CH$_2$Cl), 5.4 (2 H, s, CH$_2$O), 7.3 (5 H, m, Ar-H), and 8.7 (1 H, s, Ar-H). Anal. (C, H, N).

A mixture of **6** (0.36 g, 1.1 mmol) and palladium on charcoal (10%, 0.036 g) was stirred under an atmosphere of hydrogen (90 min). The mixture was diluted with acetone (7 mL) and filtered, and the filtrate was concentrated. The residue was triturated with ethyl acetate to give 3-(2-chloroethyl)-3*H*-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxylic acid (**7**) (0.14 g, 54%) in the form of a colorless solid: mp 143 °C dec; IR (KBr) 1755, 1710 cm^{-1}.

Method B. Preparation of 3-(2-Chloroethyl)-4-oxo-imidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxylic Acid (7). An ice-cooled, stirred suspension of mitozolomide (2.0 g, 8.2 mmol) in water (100 mL) was slowly treated (10 min) with an aqueous solution of commercial bleach (10.5% available chlorine, 100 mL). After 30 min, the mixture was filtered from unreacted mitozolomide (0.29 g, 14.5%), the filtrate was acidified to pH 1 (concentrated hydrochloric acid), and the precipitated solid was collected, giving **7** monohydrate as a colorless solid (0.79 g, 37%): mp 162 °C; IR 1760, 1710 cm^{-1}. Anal. Found: C, 32.4; H, 2.97; Cl, 13.7; N, 26.8; H$_2$O, 6.2%. C$_7$H$_6$ClN$_5$O$_2$·H$_2$O requires: C, 32.1; H, 3.06; Cl, 13.6; N, 26.8; H$_2$O 6.9%.

Method C. Preparation of 3-(2-Chloroethyl)-4-oxo-imidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxylic Acid (7). A suspension of mitozolomide (10.0 g, 0.04 mol) in concentrated sulfuric acid (50 mL) was carefully treated with a solution of sodium nitrite (10 g, 0.145 mol) in water (25 mL), and the mixture was stored at 35 °C (2.5 h). The mixture was poured onto ice, and the precipitate was collected to give **7** monohydrate, as a buff-colored powder (8.34 g, 77%): mp 166 °C.

Method D. Preparation of 3-(2-Chloroethyl)-4-oxo-imidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl Chloride (9). A mixture of **7** monohydrate (10 g, 0.04 mol), thionyl chloride (80 mL), and *N*,*N*-dimethylformamide (5 drops) was heated under reflux (2.5 h). The resulting solution was concentrated under reduced pressure. Toluene (50 mL) was added, and the residue was again evaporated to give **9** (9.9 g, 99%) as a light brown crystalline solid: mp 69 °C; MS *m*/*z* 261/263 (M$^{•+}$).

Method E. Preparation of Methyl 3-(2-Chloroethyl)-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxylate (10a). A solution of **9** (1 g, 3.8 mmol) and methanol (5 mL) was stirred at ambient temperature (2.5 h). The resulting precipitate was recrystallized from 90% aqueous acetone to give **10a** (0.68 g, 67%) as a colorless solid: mp 113 °C; IR 1780, 1750 cm^{-1}. Analysis (C, H, Cl, N).

Method F. Preparation of 3-(2-Chloroethyl)-*N*-methyl-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxamide (10f). A

solution of **9** (1.6 g, 6.1 mmol) in tetrahydrofuran (5 mL) was treated with a solution of aqueous methylamine (40% w/w, 0.95 g, 12.3 mmol) in tetrahydrofuran (5 mL). The mixture was stirred (30 min), and the precipitate was recrystallized from 90% aqueous acetone, giving **10f** monohydrate (0.96 g, 57%) as a colorless solid: mp 121 °C; IR 1750, 1640 cm^{-1}. Anal. (C, H, N).

Method G. Preparation of 3-(2-Chloroethyl)-4-oxo-imidazo[5,1-*d*]-1,2,3,5-tetrazine-8-hydroxamic Acid (10t). A mixture of **10s** (0.8 g, 2.3 mmol) and palladium on charcoal (10%, 0.1 g) in ethyl acetate (120 mL) and *N,N*-dimethylformamide (25 mL) was stirred at room temperature under an atmosphere of hydrogen until uptake ceased. The solution was filtered and concentrated and the residue triturated with ethyl acetate/petroleum ether, giving **10t** (0.4 g, 68%) as a colorless solid: mp 139–141 °C. Anal. (C, H, Cl, N).

Method H. Preparation of 3-(2-Chloroethyl)-4-oxo-imidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carbonyl Azide (10z). A solution of **9** (1 g, 3.8 mmol) in 90% aqueous acetone (10 mL) was treated with sodium azide (0.25 g, 3.8 mmol) at room temperature. The mixture was stirred at ambient temperature (3.5 h) and the resulting suspension was treated with petroleum ether (10 mL). The precipitate was collected and recrystallized from 90% aqueous acetone, giving **10z** (0.83 g, 81%) as a colorless solid: mp 115 °C; IR 2175, 1760, 1720 cm^{-1}. Anal. (C, H, N).

Method I. Preparation of 3-(2-Chloroethyl)-8-diazo-acetylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (13). A solution of diazomethane (2.1 g, 23.8 mmol) in ether (75 mL) was slowly added to **9** at 0 °C. The mixture was stirred at 0 °C (0.5 h) and the precipitate was collected and crystallized from 90% aqueous acetone, giving **13** (0.66 g, 65%) as a buff solid: mp 69 °C; IR 2100, 1750, 1720 cm^{-1}. Anal. Found: C, 34.1; H, 2.9; N, 34.8. $C_8H_8N_7O_2Cl \cdot H_2O$ requires: C, 33.6; H, 2.8; N, 34.3.

Method J. Preparation of 3-(2-Chloroethyl)-*N*-nitro-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazine-8-carboxamide (15). An ice-cold suspension of mitozolomide (0.24 g) in concentrated sulfuric acid (2.5 mL) was treated dropwise with concentrated nitric acid (d = 1.42; 1 mL). The mixture was maintained at 4 °C (1 h) and then poured on to ice. The precipitate was collected, washed with water, and recrystallized from 90% aqueous acetone, giving **15** (0.28 g, 95%) as a colorless solid: mp 160–161 °C. Anal. (C, H, Cl, N).

Biology. In vitro testing was conducted on cells obtained from the peritoneal cavity of CBA/CA mice bearing a routine passage of TLX5 lymphoma. Cells were mixed with sterile saline at 37 °C, sedimented on a Hereus 6000 Labofuge bench centrifuge (500G), and washed with erythrocyte lysis buffer to remove red cells debris. Tumor cells were centrifuged and resuspended in RPMI 1640 media containing 17% horse serum. TLX5 cells were maintained in exponential phase at a density of 2×10^4 cells/mL under an atmosphere of 10% CO_2 in air at 37 °C with RPMI 1640 media supplemented with 17% horse serum as the culture medium.

Aliquots (2 mL) of TLX5 cells were plated out at approximately 2×10^4/mL into multiwell dishes, and drug solutions (in DMSO) were added at a concentration of drug at 0.625–20 mg/L in amounts so that the final concentration of DMSO did not exceed 0.2%. After 72 h of incubation at 37 °C under an atmosphere of air/CO_2 (90:10), cells were counted with a Coulter Laboratories ZM or ZBI electronic coulter counter. Cytotoxicity was expressed as a concentration (IC$_{50}$) required to inhibit cell numbers by 50% relative to controls after 72 h of incubation.

In vivo testing was carried out on two mouse tumor types. The L1210 leukemia test was performed at Rhône-Poulenc, France, under the protocols described previously.[4] The test against the TLX5 lymphoma was carried out at Aston University under the previously described protocols.[4] We have already established that compounds in the imidazotetrazine series active on one tumor type were effective on the other.