Antitumour Imidazotetrazines. Part 31.[1] The Synthesis of Isotopically Labelled Temozolomide and a Multinuclear (^{1}H, ^{13}C, ^{15}N) Magnetic Resonance Investigation of Temozolomide and Mitozolomide

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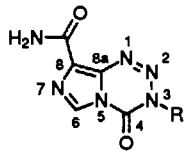
The antitumour drug temozolomide has been synthesized isotopically labelled with NMR active nuclei at a variety of sites and all its ^{13}C and ^{15}N NMR spectral resonances have been assigned. At low pH a site of protonation has been identified which accounts for the acid stability of the drug.

The antitumour imidazotetrazines temozolomide **1**[2] and mitozolomide **2**, are of considerable current interest and **1** is now in phase II clinical trials.[3] Previously, ^{11}C-methyl-**1** and ^{14}C(6)-**2** have been prepared;[4,5] clinical and biochemical investigations



R = CH_3 Temozolomide **1**
R = CH_2CH_2Cl Mitozolomide **2**

have now made necessary the preparation of specifically labelled ^{15}N and ^{13}C samples of the drug **1**. These have exhibited a variety of interesting properties in their NMR spectra, not least among them the observation and identification of a site of protonation at low pH.

Results and Discussion

Synthesis of Labelled Compounds.—Economical synthesis of specifically ^{13}C or ^{15}N labelled **1** has been accomplished by variations on the standard synthesis[6] (see Scheme 1). Labelling of N(2) of temozolomide **1**, Scheme (A), was achieved by diazotization using $Na^{15}NO_2$ in a procedure adjusted so that the nitrite was not required in excess. Methyl isocyanate labelled with [^{15}N]-**7a**, and [^{13}C]-**7b** was prepared by the pyrolysis of 3-methyl-1,1-diphenylurea **6**, easily obtained from the reaction of methylamine hydrochloride (available with ^{15}N or ^{13}C label) with the chlorocarbamate **5**.[7]

^{13}C *Assignments for* **1** *and* **2**.—A combination of CPD, DEPT135, SIMPLE[8] and COLOC[9] experiments allowed assignment of all the drug ^{13}C resonances (see Table 1). When the ^{13}C NMR spectra of **1** and **2** were acquired using CF_3CO_2D as solvent some perturbation of the resonances was observed: whilst in most cases these shifts were small enough to be accounted for by the change of solvent, in the imidazole ring larger shifts were observed particularly for C(8) which were strongly suggestive of protonation.

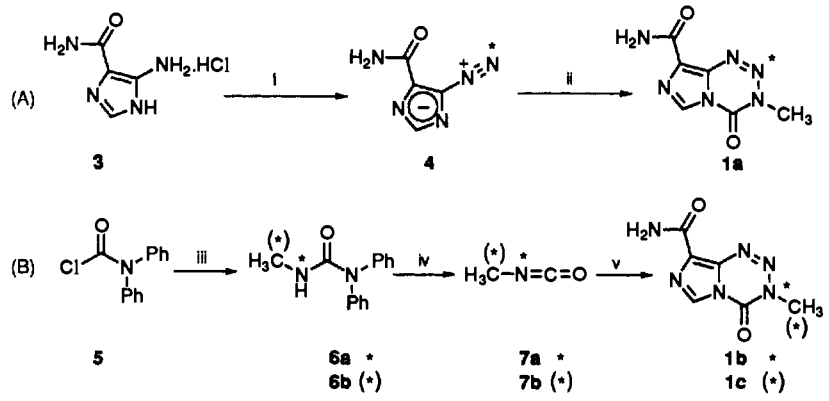
^{15}N *NMR Interpretation.*—Selected ^{15}N NMR data for compounds **1**, **1a**, **1b**, **2** and **8** are collated in Table 2: the resonances assigned N-(1), -(2), -(3) have chemical shifts very similar to related 1-aryl-3,3-dialkyltriazenes;[10] those assigned N(5) and N(7) are typical of imidazoles.[11]

Unambiguous assignment of the $CONH_2$ at −274.89 ppm was possible by acquiring with full ^{1}H NOE to show the signal as a negative triplet; N(2) and N(3) were uniquely identified by isotopic labelling. Distinguishing between the resonances of N(5) and N(7) was less straightforward: ^{1}H–^{15}N HMBC showed equivalent connections to H(6); also, the length of synthesis and the equivalence of four nitrogen atoms in the starting material[12] meant specific labelling of imidazole **3** was impractical. Carboxylic acid **8** showed negligible chemical-shift differences relative to **1** except for the signal at −101.43 ppm which shifted 4 ppm downfield which implies that this is N(7), closest to the structural alteration.

Of particular interest are the ^{15}N spectra of imidazotetrazines **1** and **2** acquired in CF_3CO_2H solution. For **1**, the signal at −105.64 ppm in Me_2SO was considerably shifted to −168.14. Furthermore, when the data were acquired with full ^{1}H NOE the signal became negative, showing a proton attached to that nitrogen atom. The cation thus formed must be **9** since protonation at N(5) would disrupt the aromaticity of both rings. This provides further evidence for the assignment of N(7) and N(5). Cation **9** also accounts for the resistance of imidazotetrazinones to acid hydrolysis.[1]

The ^{15}N nucleus is relatively insensitive to electronic effects away from its immediate environment[13,14] and attempts have been made to correlate ^{15}N chemical shifts with partial atomic charges, for a discussion see ref. 13. Fig. 1 shows that for **1** there is indeed quite a good correlation between chemical shift and partial atomic charge (theoretical Lowdin charges for the nitrogen atoms of **1** calculated[15] using the molecular mechanics package AMBER).

$^nJ_{NC}$ *Coupling Constants.*—The preparation of ^{15}N-labelled compounds affords the direct observation of N–X couplings in the spectra of the X-nuclei. Fig. 2 illustrates the newly measured ^{13}C–^{15}N couplings found in the current work. The 1-bond couplings are broadly illustrative of the dependence of the magnitude of the coupling constant on the hybridization of the atoms involved in the bond (Fermi contact mechanism) although effects due to resonance increase the coupling to adjacent carbonyl carbons.[11,13,14] This is most marked in methyl isocyanate **7a** which has $^1J_{CN}$ of 47.8 Hz, an anomalously large coupling only exceeded by 2,4,6-trimethylbenzonitrile oxide[16] with $^1J_{NC}$ of 77.5 Hz, which indicates significant triple-bond character in the C–N bond.



Scheme 1 *Reagents and conditions:* i, $Na^{15}NO_2$, HCl, 0–5 °C; ii, CH_3NCO, Me_2SO, room temp.; iii, (*)CH_3–*NH_2·HCl, NaOH, H_2O–EtOAc; iv, heat to 240 °C; v, [^{14}N]-4, Me_2SO

Table 1 ^{13}C Chemical-shift data (δ) for compounds **1** and **2**

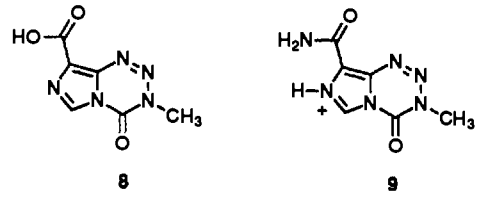
Compound	Solvent	C-4	C-6	C-8	C-8a	CH_3	CH_2N	CH_2Cl	$CONH_2$
1	A	140.0	129.2	131.4	135.4	36.9	—	—	162.3
1	B	138.5	130.9	125.3	136.1	38.5	—	—	162.3
2	A	139.3	129.3	131.7	134.2	—	50.3*	41.6†	161.5
2	B	139.4	131.9	127.7	136.3	—	53.3	41.6	163.6

Solvents: A, $(CD_3)_2SO$ at 310 K; B, CF_3CO_2D at 310 K. * δ_H 4.67; † δ_H 4.03.

Table 2 ^{15}N Chemical-shift data (δ) of imidazotetrazinones

Compound	Solvent	N-1	N-2	N-3	N-5	N-7	$CONH_2$
1	A	−33.99	20.02	−180.44	−199.82	−105.64	−274.89 (−ve t)
1a	A	−32.55 [trace]	20.12				
1b	A			−180.30			
1*	B	−45.81	40.62	−174.50	−199.52	−168.14 (−ve s)	−271.79 (−ve t)
2†	A	−31.88	18.19	−175.62	−200.38	−105.14	−274.22
2*	B	−42.65	36.49	−170.92	−200.09	−155.56	−271.90
8‡	A	−33.98	22.35	−197.10	−199.73	−101.43	

Solvents: A, Me_2SO at 305 K; B, CF_3CO_2H at 305 K. ( ) effect of acquiring with full ^{1}H NOE. * Assigned by analogy with **1** in Me_2SO, note the protonation of N-7. † Assigned by analogy with **1**. ‡ Assigned by analogy with **1**, note the shift of N-7 relative to **1** and absence of $CONH_2$.



8 **9**

The $^2J_{NC}$ and $^3J_{NC}$ values measured in **1a** and **1b** show a combination of effects: the presence of a lone pair of electrons on the ^{15}N atom increases the coupling to carbon atoms close in space to the lone pair [$^2J_{N(2)CH_3}$ 7.0 Hz, whereas $^2J_{N(2)C(4)}$ 2.3 Hz]; the presence of multiple, equivalent coupling pathways [$^3J_{N(3)C(8a)}$ 7.2 Hz, whereas $^3J_{N(3)C(6)}$ 1.1 Hz and $^3J_{N(2)C(8)}$ 2.7 Hz] and conformational effects [$^3J_{N(3)C(8a)}$ 7.2 Hz whereas $^3J_{N(2)C(8)}$ 2.7 Hz]. Unusually, a 4-bond coupling, $^4J_{N(2)C(6)}$ 0.9 Hz was also resolved.

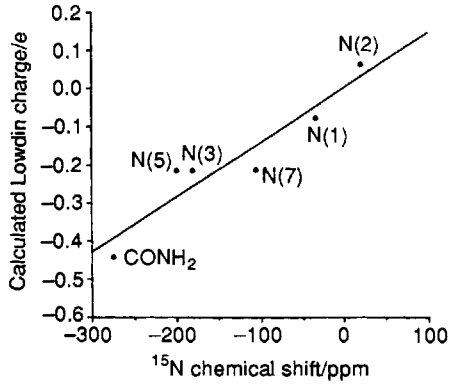


Fig. 1 Plot of ^{15}N chemical shift against the calculated Lowdin charge for the nitrogen atoms of **1**

Experimental

General.—Temozolomide **1**, mitozolomide **2** and 5-amino-imidazole-4-carboxamide hydrochloride **3** were obtained from Aston Molecules Ltd, Birmingham, UK, [^{15}N]-methylamine hydrochloride (95 atom%) was purchased from Euriso-top through Fluorochem Ltd, Glossop, Derbyshire, UK and other reagents from the Aldrich Chemical Company. The carboxylic acid **8** was prepared from **1** by the method of Horspool *et al.*[17]

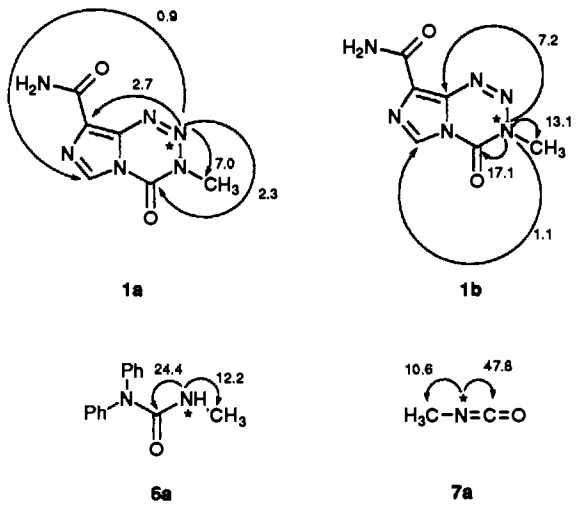


Fig. 2 ^{15}N–^{13}C coupling constants (absolute value in Hz) observed in ^{15}N-labelled compounds

1D ^{15}N NMR spectra were recorded on a Bruker AC250 spectrometer at 25.36 MHz as previously described;[10] 2D ^{1}H–^{15}N HMBC on a Bruker AMX500 at 499.88 MHz. All other NMR spectra were recorded on a Bruker ARX250 spectrometer observing ^{1}H at 250.13 MHz and ^{13}C at 62.9 MHz. ^{1}H and ^{13}C chemical shifts are quoted downfield from tetramethylsilane, ^{15}N chemical shifts relative to external nitromethane. All J values are in Hz.

$^{15}N(2)$-*Temozolomide* **1a**.—A solution of compound **3** (0.429 g, 2.64 mmol) in 1 mol dm^{-3} HCl (4 cm^3) was passed through a cotton wool filter and delivered below the surface of a stirred, ice-cold solution of $Na^{15}NO_2$ (97 atom%) (0.2 g, 2.86 mmol) in water (4 cm^3), over 50 min. A heavy yellow precipitate was formed and the addition was stopped as soon as a pale pink colour persisted.[6] The reaction mixture was stirred on ice for a further 5 min after which the solids were filtered off, washed with a small amount of cold water, ethanol and ether and then dried *in vacuo* to give **4** (0.27 g, 74%) [ν_{max}/cm^{-1} 2200s; $\delta_N(Me_2SO)$ −27.82], which was used without further purification.

Methyl isocyanate (83 mg, 1.46 mmol) was added to a slurry of compound **4** (100 mg, 0.73 mmol) in Me_2SO (1 cm^3). After being stirred in the dark at room temperature for 36 h the mixture was diluted with ethyl acetate (10 cm^3) and the solids were filtered off and washed with a small amount of cold ethyl acetate. Compound **1a** was obtained as a white solid (0.87 g, 61%), m.p. 204 °C (Found: M^+, 195.0531. $C_6H_6N_5{}^{15}NO_2$ requires M, 195.0522). The absence of $(M - 1)^+$ in the mass spectrum suggests close to 100% ^{15}N incorporation.

3-[^{13}C]-*Methyl*-1,1-*diphenylurea* **6b**.—Sodium hydroxide (5 g) was added over 30 min to a vigorously stirred mixture of [^{13}C]-methylamine hydrochloride (99 atom%; 4.0 g, 58.4 mmol), water (5 cm^3), diphenylcarbamoyl chloride (13.54 g, 58.4 mmol) and toluene (20 cm^3). The mixture was heated at 50 °C for 40 min and then cooled for 20 min, after which the precipitate was filtered off, washed with water (5 × 10 cm^3) and dried *in vacuo* overnight at 50 °C to yield **6b** (11.98 g, 89%) (Found: C, 74.3; H, 6.1; N, 12.3. $^{12}C_5{}^{13}CH_6N_6O_2$ requires C, 74.42; H, 6.21; N, 12.33%); $\delta_H(CDCl_3)$ 7.3 (10 H, m, 2 × Ph), 4.51 (1 H, br s, NH), 2.81 (1.5 H, d, $^{1}J_{CH}$ 138.1, CH_3) and 2.79 (1.5 H, d, $^{1}J_{CH}$ 138.1, CH_3); $\delta_C(CDCl_3)$ 156.7 (CO), 142.8 (quaternary aromatic C), 129.3 (CH), 127.3 (CH), 126.0 (CH) and 27.4 (s, very strong, CH_3); m/z 227 (M^+) and 169 (100) $(M\text{-}CH_3N^{13}CO)^+$.

[^{13}C]-*Methyl Isocyanate* **7b**.—Urea **6b** (11.75 g, 51.7 mmol) was placed in an Aldrich short path-length distillation apparatus and the receiver tubes cooled in isopropyl alcohol–solid CO_2. The white solid was heated slowly to 250 °C using a silicone oil-bath and, over 3 h, a colourless liquid was collected (1.4 g, 69%); $\delta_H(CDCl_3)$ 3.02 (d, $^{1}J_{CH}$ 143.2, CH_3); $\delta_C(CDCl_3)$ 28.0 (s, CH_3) (C=O not observed).

3-[^{13}C]-*Methyltemozolomide* **1c**.—This compound was prepared as for **1a** from [^{14}N]-**4** (3.94 g, 28.7 mmol) and **7b** (2.5 g, 43.0 mmol) as a pale pink solid (5.23 g, 93%), m.p. 185 °C (decomp.); $\delta_H[(CD_3)_2SO]$ 8.83 (1 H, s, 6-H), 7.82 (1 H, br s, NH), 7.69 (1 H, br s, NH) and 3.86 (3 H, d, $^{1}J_{CH}$ 143.8, CH_3).

3-*Methyl*-1,1-*diphenyl*-[3-^{15}N]-*urea* **6a**.—This compound was prepared as for **6b** from [^{15}N]-methylamine (95 atom%; 0.5 g, 7.30 mmol) as a white solid (1.48 g, 89%), m.p. 150 °C; $\delta_H(CDCl_3)$ 7.3 (10 H, m, 2 × Ph), 4.48 (1 H, dq, $^{1}J_{NH}$ 90.0, $^{3}J_{HH}$ 4.7, NH, exchanged with D_2O) and 2.81 (3 H, dd, $^{2}J_{NH}$ 1.0, $^{3}J_{HH}$ 4.8, became d, J 1.0 on exchanging with D_2O, CH_3); $\delta_C(CDCl_3)$ 156.8 (d, $^{1}J_{CN}$ 24.4, CO), 142.9 (Ar-C), 129.3 (CH), 127.4 (CH), 126.1 (CH) and 27.4 (d, $^{1}J_{CN}$ 12.2, CH_3); m/z 227 (5) (M^+), 170 (12), 169 (100), 168 (40), 167 (23) and 58 (9) (Found: M^+, 227.1072. $C_{14}H_{14}N^{15}NO$ requires M, 227.1076).

Methyl [^{15}N]-*Isocyanate* **7a**.—The urea **6a** (1.40 g, 6.16 mmol) was thermolysed as in the preparation of compound **7b** to give **7a** as a colourless liquid (0.13 g, 36%); $\delta_H[(CD_3)_2SO]$ 3.06 (d, $^{2}J_{NH}$ 2.9); $\delta_C[(CD_3)_2SO]$ 121.5 (d, $^{1}J_{CN}$ 47.8, C=O) and 28.1 (d, $^{1}J_{CN}$ 10.6, CH_3).

[3-^{15}N]-*Temozolomide* **1b**.—This compound was prepared as for **1a** from [^{14}N]-**4** (142 mg, 1.1 mmol) and **7a** (60 mg, 1 mmol) but with addition of further unlabelled methyl isocyanate (2 mmol) at the end of the reaction time. The mixture was stored for a further 3 h to give **1b** as a pale pink solid (127 mg, 63%), m.p. > 190 °C (decomp.); $\delta_H[(CD_3)_2SO]$ 8.80 (1 H, s, 6-H), 7.77 (1 H, s, NH), 7.65 (1 H, s, NH) and 3.87 (3 H, d, $^{2}J_{NH}$ 1.6, CH_3) (Found: M^+, 195.0514. $C_6H_6N_5{}^{15}NO_2$ requires M, 195.0522). From the mass spectrum the isotopic incorporation was estimated as 86% $^{15}N(3)$.

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