# Antitumour imidazotetrazines. Part 39.[1] Synthesis of bis(imidazotetrazine)s with saturated spacer groups

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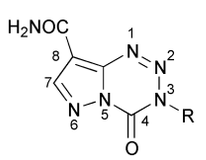
Bis(imidazotetrazine)s (**16**), related in structure to the antitumour agents mitozolomide (**1a**) and temozolomide (**1b**), but linked through the N(3)–N(3′) atoms of the imidazo[5,1-*d*][1,2,3,5]tetrazine ring-systems, are prepared by interaction of 5-diazoimidazole-4-carboxamide (**8**) and diisocyanates (**15**). The presence of the polymethylene linker with/without sulfur and oxygen heteroatoms does not substantially affect the acid stability, base-catalysed decomposition, antitumour activity or DNA base alkylation preference characteristic of the unlinked imidazotetrazines mitozolomide and temozolomide.

## Introduction

Of the N(3) substituted family of 8-carbamoylimidazo[5,1-*d*]-[1,2,3,5]tetrazin-4(3*H*)-ones only mitozolomide (**1a**) and temozolomide (**1b**) have pronounced antitumour activities *in vivo*.[2,3] Mitozolomide achieved chemotherapeutic notoriety in the 1980s. Although in a single dose it could elicit cures in most established mouse tumours,[2] sadly, in human trials it produced profound, life-threatening, bone marrow suppression (thrombocytopenia).[4] In contrast, the second-generation agent temozolomide, although less active in mouse tumours, can be used safely on an out-patients basis to treat human malignant brain tumours[5] for which use it is now approved and marketed throughout the world. The 3-ethyl analogue (**1c**) is inactive in all mouse tumour models.[3]




**1a:** R = $(CH_2)_2Cl$ mitozolomide
**b:** R = Me temozolomide
**c:** R = Et

The orally active temozolomide delivers a methylating reagent, methanediazonium ion, to nucleophilic sites on DNA. The cytotoxicity of temozolomide is principally associated with methylation at the O(6) position of guanine residues in the major groove of DNA[6,7] and is dependent on the mismatch repair pathway (MMR). This lesion is subjected to repair by $O^6$-methylguanine-DNA alkyltransferase (ATase) and, therefore, the susceptibility of a tumour to temozolomide depends on the relative expression of MMR and ATase in a cell.[8] Furthermore, other products of promiscuous DNA methylation, which give rise to strandbreaks, are identified and tagged for repair by poly(ADP-ribose)polymerase (PARP).[9] Modulation of these clinically compromising repair functions by specific inhibitors is being studied in an effort to extend the spectrum of susceptible tumour types.

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In recent papers we have reported on the outcome of efforts to target the imidazotetrazine moiety to specific DNA sequences. Thus, mitozolomide and temozolomide have been conjugated through the 8-carboxamide group to H-bonding motifs and spermidine[7] and peptidic DNA minor and major groove-recognition moieties.[10] Sadly, these initiatives have not led to any significant biological breakthroughs. For example, although conjugation of mitozolomide to the A–T selective DNA minor groove-binding octapeptide SPKKSPKK did enhance the potency of DNA interaction compared with that of the unconjugated drug, covalent alkylation still occurred in the *major* groove.[10] We have been able to rationalise these setbacks from our understanding of the molecular mechanism of action of temozolomide.[6] By analogy, the chemistry of breakdown of the temozolomide conjugates **2** probably involves nucleophilic attack by water at C(4) of the bicycle with ring-opening and loss of a molecule of carbon dioxide. The acyclic methyltriazene **3** thus formed then undergoes proteolytic fragmentation to afford the (conjugated) 5-aminoimidazole-4-carboxamide **4** with detachment of the reactive methanediazonium species **5** which methylates guanine residues of DNA **6** to generate the cytotoxic $O^6$-methylguanine lesion **7** (Scheme 1). Irrespective of the binding preference imposed by the targeting moiety, the short-lived methanediazonium species **5**[11] (pseudo-first order rate constant for hydrolysis = 1.8 $s^{-1}$ in unbuffered aqueous THF)[12] has time to diffuse to, and methylate, preferred nucleophilic sites within runs of guanine bases in the *major* groove. This process thwarts efforts to utilise the carboxamide moiety to target the imidazotetrazine methylating component to specific gene sequences.

In an alternative strategy to broaden the clinical utility of imidazotetrazines we have now synthesised bis(imidazotetrazine)s linked by saturated spacer groups. We anticipated that these new agents might forge DNA crosslinks which might not be reversed by known DNA repair mechanisms.

## Results and discussion

The most practicable route to 3-substituted imidazotetrazines is from the interaction of 5-diazoimidazole-4-carboxamide (**8**) and isocyanates (**9**) (Scheme 2).[2] In the present work we have prepared some previously unreported N(3)-substituted

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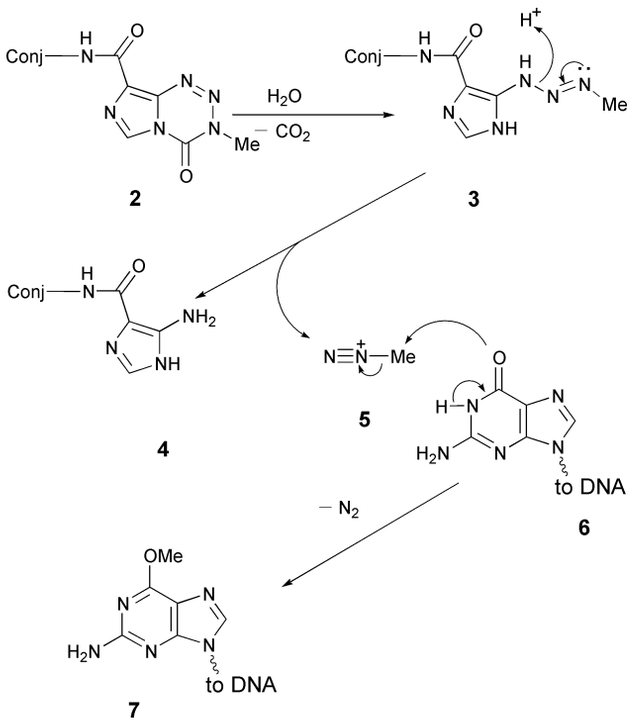
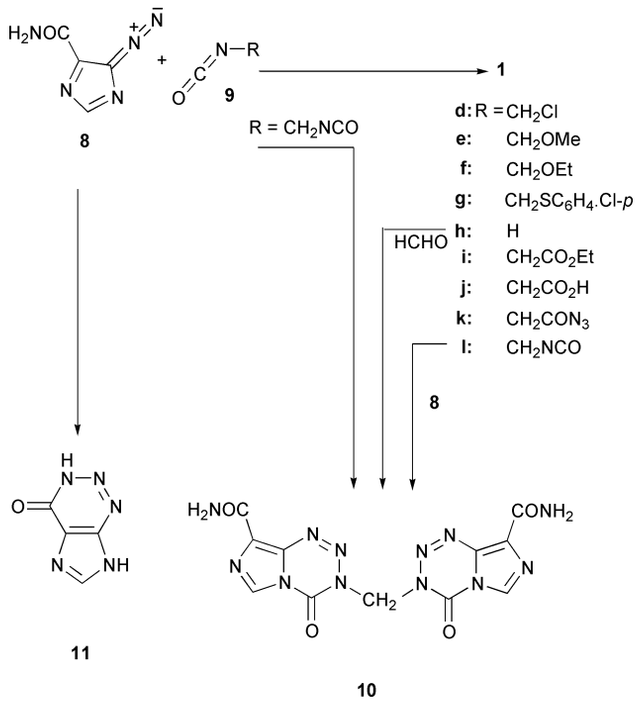



**Scheme 1**




**Scheme 2**

imidazotetrazines (**1d**–**g**) bearing a one-carbon fragment at N(3), which are potential reagents for coupling to an unsubstituted imidazotetrazinone (**1h**) to form the prototypic bis(imidazotetrazine) **10**. The chloromethyl isocyanate (**9**; R = $CH_2Cl$), cyclised with **8** in DMSO or HMPA to give the imidazotetrazine **1d**. Similarly, **8** and methoxymethyl isocyanate (**9**; R = $CH_2OMe$), ethoxymethyl isocyanate (**9**; R = $CH_2OEt$) and isocyanatomethyl 4-chlorophenyl sulfide (**9**; R = $CH_2SC_6H_4Cl$-*p*) afforded the imidazotetrazines (**1e**–**g**), respectively. In contrast, the sterically hindered *tert*-butyl and trichloromethyl isocyanates failed to produce the respective imidazotetrazines.

An obvious route to the bis(imidazotetrazine) **10** would involve interaction of the unsubstituted imidazotetrazin-4(3*H*)-one (**1h**) with formaldehyde (Scheme 2). The reported[13] synthesis of **1h** from diazoimidazolecarboxamide **8** and the sterically challenged trimethylsilyl isocyanate (**9**; R = TMS) has proven to be impossible to reproduce on a preparative scale and under a range of conditions only recovered starting materials or azahypoxanthine (**11**), the product of slow intramolecular cyclisation of **8**, were isolated. Other independent synthetic strategies to obtain the elusive imidazotetrazinone **1h** have not been successful.[14]

Ege *et al.*[15] have shown that N(3)-linked bis(pyrazolotetrazine)s can be prepared from diisocyanates and diazopyrazoles and to adapt this method to synthesise the bis(imidazotetrazine) **10** would require diisocyanatomethane (**9**; R = $CH_2NCO$) available from the potentially hazardous rearrangement of malonyl diazide. We did not consider it prudent to attempt this reaction even though it was apparently performed safely by the legendary Curtius himself.[16] In an alternative approach, the known ester **1i**, prepared from **8** and ethyl isocyanatoacetate (**9**; R = $CH_2CO_2Et$), was hydrolysed to the acetic acid derivative **1j**.[17] A logical sequence from **1j** to the isocyanatomethylimidazotetrazine **1l** would be the following: conversion of **1j** to the acid azide **1k**; Curtius rearrangement of the azide to **1l**; and finally, construction of the second imidazotetrazine ring by interaction of **1l** with a second tranche of diazoimidazolecarboxamide **8**. However this strategy foundered on our inability to secure a reliable and safe synthesis of the acid azide **1k** using a range of conditions. We therefore abandoned our efforts to synthesise the methylene-linked bis(imidazotetrazine) **10**.

Difunctional acids or esters **12**, depending on availability, were used as starting materials for the synthesis of a range of novel bis(imidazotetrazine)s. Esters were converted to acid hydrazides **13** and thence, by nitrosation, to acid azides **14**. The Curtius rearrangement of succinyl azide (**14a**)[18] in a mixture of refluxing chloroform–benzene was used safely to furnish the diisocyanate **15a**. The propensity of the diisocyanate to undergo polymerisation dictated that crude material obtained from the evaporated mixture was reacted without further purification; reaction with diazoimidazolecarboxamide **8** in DMSO at 25 °C afforded the ethylene-linked bis(imidazotetrazine) **16a** in poor yield (25%). Similarly prepared, from commercially available diisocyanates (**15b**–**e**), were the polymethylene-linked bis(imidazotetrazine)s (**16b**–**e**) in 60–97% yields (Scheme 3). The contrast in yields between **16a** and **16b**–**e** probably reflects the higher purity of the commercially available diisocyanates (**15b**–**e**), their ease of reactivity with **8** or the facility with which the products precipitated from the reaction mixture. For example, formation of **16a** took 28 days to reach completion, whereas synthesis of **16b**–**e** was complete in 12 hours. It is our experience that long reaction times inevitably lead to by-product formation [*e.g.* cyclisation of **8** to azahypoxanthine (**11**)].[19]

The diisocyanates (**15f**–**l**) bearing additional hetero atoms in the linker fragment were obtained as oils in yield of 50–77% by Curtius rearrangements of the precursor acid azides (**14f**–**l**) and were reacted directly with **8** in DMSO to furnish the bis(imidazotetrazine)s (**16f**–**l**) in moderate to good yields (Table 1).

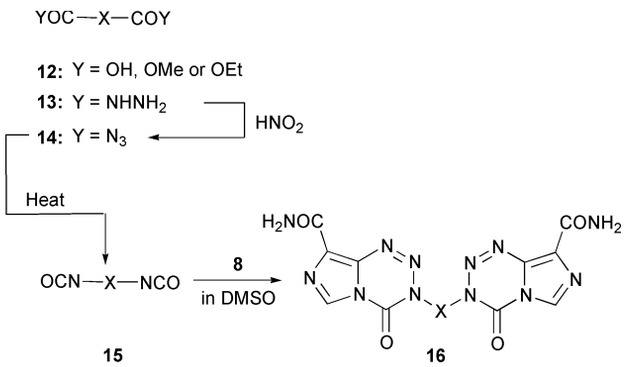
The crude bis(imidazotetrazine)s (**16a**–**l**) always contained contaminants absorbing in the 1600–1700 $cm^{-1}$ region of the IR spectrum—possibly triazine-1,3,5-triones formed by trimerisation of the isocyanates. No suitable crystallisation solvent was found to remove these contaminants but a brief washing of the bis(imidazotetrazine) with petroleum–ether followed by formic acid proved to be an effective process to furnish pure compounds; these were characterised by FAB MS, $^1H$ and $^{13}C$ NMR, and in some cases C, H, N microanalyses. The $^1H$ NMR spectrum of the hexamethylene-linked imidazotetrazine **16c** clearly shows that the structure is symmetrical about the 3,4-hexamethylene bond.

The availability of the sulfide-linked imidazotetrazine **16f**

**Table 1** Yields and physical characteristics of bis(imidazotetrazine)s **16**

| Compound | Yield (%) | Mp/°C [a] | Formula | MW [b] | Microanalysis found (required) | | | $^1$H NMR [c] | $^{13}$C NMR [d] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | C | H | N | | |
| **16a** | *e* | 165–167 | $C_{12}H_{10}N_{12}O_4$ | 386 | 37.31 (37.30) | 2.61 (2.59) | 43.49 (43.52) | 8.86 (2 H, s, 6-H, 6′-H), 7.88 (2 H, s, 2 × NH), 7.72 (2 H, s, 2 × NH), 4.78 (4 H, s, 2 × $CH_2$) | — |
| **b** | 82 | 155–158 | $C_{14}H_{14}N_{12}O_4$ | 414 | 40.57 (40.58) | 3.41 (3.80) | 40.57 (40.58) | 8.81 (2 H, s, 6-H, 6′-H), 7.86 (2 H, s, 2 × NH), 7.70 (2 H, s, 2 × NH), 4.34 (4 H, br, 2 × $CH_2$), 1.94 (4 H, br s, 2 × $CH_2$) | 162.4, 139.9, 135.3, 131.4, 129.5, 49.2, 26.0 |
| **c** | 42 | 167–169 | $C_{16}H_{18}N_{12}O_4$ | 442 | 43.54 (43.44) | 4.10 (4.07) | 37.96 (38.00) | 8.81 (2 H, s, 6-H, 6′-H), 7.86 (2 H, s, 2 × NH), 7.70 (2 H, s, 2 × NH), 4.28 (4 H, t, *J* 7.0, 2 × $CH_2$), 1.18 (4 H, br, 2 × $CH_2$), 1.43 (4 H, br, 2 × $CH_2$) | 161.7, 139.2, 134.7, 130.7, 128.8, 48.9, 28.2, 25.6 |
| **d** | 44 | 160 | $C_{18}H_{22}N_{12}O_4$ | 470 | 45.55 (45.96) | 4.71 (4.68) | 35.70 (35.74) | 8.80 (2 H, s, 6-H, 6′-H), 7.85 (2 H, s, 2 × NH), 7.68 (2 H, s, 2 × NH), 4.30 (4 H, t, *J* 7.5, 2 × $CH_2$), 1.83 (4 H, br, 2 × $CH_2$), 1.27 (8 H, br, 4 × $CH_2$) | — |
| **e** | 60 | 165 | $C_{22}H_{30}N_{12}O_4$ | 526 | 49.85 (50.19) | 5.74 (5.70) | 31.72 (31.94) | 8.67 (2 H, s, 6-H, 6′-H), 7.85 (2 H, s, 2 × NH), 7.65 (2 H, s, 2 × NH), 4.15 (4 H, br, 2 × $CH_2$), 1.62 (4 H, br, 2 × $CH_2$), 1.17 (16 H, br, 8 × $CH_2$) | — |
| **f** | 79 | 130–132 | $C_{12}H_{10}N_{12}O_4S$ | 418 | 34.05 (34.45) | 2.64 (2.41) | — | 8.89 (2 H, s, 6-H, 6′-H), 7.84 (2 H, br, 2 × NH), 7.27 (2 H, br, 2 × NH), 5.65 (4 H, s, 2 × $CH_2$) | 161.3, 138.7, 134.2, 131.2, 129.2, 50.0 |
| **g** | 33 | 168–170 | $C_{12}H_{10}N_{12}O_5$ | 402 | 35.66 (35.82) | 2.21 (2.49) | — | 8.94 (2 H, s, 6-H, 6′-H), 7.89 (2 H, br, 2 × NH), 7.75 (2 H, s, 2 × NH), 5.88 (4 H, s, 2 × $CH_2$) | 161.7, 139.7, 134.4, 131.9, 130.0, 76.8 |
| **h** | 63 | 160–162 | $C_{14}H_{14}N_{12}O_6$ | 446 | 37.57 (37.67) | 3.19 (3.16) | — | 8.87 (2 H, s, 6-H, 6′-H), 7.86 (2 H, br, 2 × NH), 7.73 (2 H, br, 2 × NH), 5.65 (4 H, s, 2 × $CH_2$), 3.79 (4 H, s, 2 × $CH_2$) | 161.9, 139.9, 134.7, 131.9, 130.0, 78.5, 68.9 |
| **i** | 24 | 144 | $C_{14}H_{14}N_{12}O_4S_2$ | 478 [f] | — | — | — | 8.87 (2 H, s, 6-H, 6′-H), 7.88 (2 H, br, 2 × NH), 7.72 (2 H, br, 2 × NH), 5.51 (4 H, s, 2 × $CH_2$), 3.03 (4 H, s, 2 × $CH_2$) | 162.3, 139.5, 135.1, 130.1, 50.8, 31.6 |
| **j** | 50 | 164–166 | $C_{14}H_{14}N_{12}O_4S$ | 446 | 37.53 (37.67) | 3.05 (3.16) | — | 8.89 (2 H, s, 6-H, 6′-H), 7.89 (2 H, br, 2 × NH), 7.76 (2 H, br, 2 × NH), 4.57 (4 H, t, *J* 6.8, 2 × $CH_2$), 3.12 (4 H, t, *J* 6.8, 2 × $CH_2$) | 162.0, 139.5, 134.8, 131.4, 129.4, 48.4, 29.6 |
| **k** | 47 | 141 | $C_{15}H_{16}N_{12}O_4S_2$ | 492 [g] | — | — | — | 8.85 (2 H, s, 6-H, 6′-H), 7.83 (2 H, br, 2 × NH), 7.70 (2 H, br, 2 × NH), 4.53 (4 H, t, *J* 6.0, 2 × $CH_2$), 4.00 (2 H, s, $CH_2$), 3.07 (4 H, t, *J* 6.0, 2 × $CH_2$) | — |
| **l** | 86 | 146–148 | $C_{14}H_{14}N_2O_4S_2$ | 478 | 34.90 (35.15) | 2.67 (2.93) | — | 8.86 (2 H, s, 6-H, 6′-H), 7.86 (2 H, br, 2 × NH), 7.73 (2 H, br, 2 × NH), 4.62 (4 H, t, *J* 6.4, 2 × $CH_2$), 3.24 (4 H, t, *J* 6.4, 2 × $CH_2$) | 161.9, 139.6, 134.7, 131.4, 129.4, 48.2, 35.7 |

[a] All compounds melted with vigorous decomposition. [b] Determined by FAB MS. [c] Spectra recorded at 250 MHz in $(CD_3)_2SO$. [d] Spectra recorded at 62.9 MHz in $(CD_3)_2SO$. [e] Purity of starting isocyanate not known. [f] FAB MS *m/z* 479.0781. $C_{14}H_{14}N_{12}O_4S$ requires *m/z* 479.0777 ($M + H^+$). [g] FAB MS *m/z* 493.0937. $C_{15}H_{16}N_{12}O_4S_2$ requires *m/z* 493.0929 ($M + H^+$).




**a:** X = $(CH_2)_2$
**b:** $(CH_2)_4$
**c:** $(CH_2)_6$
**d:** $(CH_2)_8$
**e:** $(CH_2)_{12}$
**f:** $CH_2SCH_2$
**g:** $CH_2OCH_2$
**h:** $CH_2O(CH_2)_2OCH_2$
**i:** $CH_2S(CH_2)_2SCH_2$
**j:** $(CH_2)_2S(CH_2)_2$
**k:** $(CH_2)_2SCH_2S(CH_2)_2$
**l:** $(CH_2)_2SS(CH_2)_2$

**Scheme 3**

offered the prospect of developing a new reductive desulfurisation route to temozolomide (**1b**). Firstly, the sulfide was heated with ethanol-washed Raney nickel in refluxing trifluoroethanol, but no temozolomide was detected, despite the fact that the target molecule is stable under the reaction conditions. Back and Yang[20] have used nickel boride, prepared by the reduction of $Ni^{2+}$ salts with sodium borohydride, to effect efficient desulfurisation of sulfide substrates. Again, no temozolomide was formed using this reagent and only tetrazinone ring-opening was observed.

The acid stability of the imidazotetrazine nucleus of **1a–c** is a characteristic property of this class of agent.[2,6] The stabilities of representative bis(imidazotetrazine)s in phosphate buffer were compared by a UV spectrophotometric method.[6] At pH 5.0 there was no measurable decomposition of any compound (data not shown). The $t_{\frac{1}{2}}$ values for mitozolomide (**1a**) and temozolomide (**1b**) at pH 7.4 and 37 °C were 1.03 and 1.30 h, respectively (Table 2). Lengthening the linker from a hexa- (**16c**) to an octa-methylene unit (**16d**) had little effect on $t_{\frac{1}{2}}$ (2.77 and 3.27 h, respectively); compound **16l** with a disulfide residue attached to the ethylene group at N(3) was more unstable with a $t_{\frac{1}{2}}$ (1.28 h) comparable to that of mitozolomide.

Scrutiny of the UV curves reveals underlying differences in the decomposition mechanisms depending on the nature of the N(3)-attached group. The overall UV curves for temozolomide (**1b**) and the hexamethylene-linked bis(imidazotetrazine) (**16c**) (Fig. 1A and B) are nearly identical, with the decay of the bands at 328 and 331 nm, respectively, and the emergence of a peak at 270 nm indicating that decomposition probably follows the accepted pathway[6]—*via* the alkyltriazenes **17** or **18**, leading to the formation of 5-aminoimidazole-4-carboxamide (AIC) **19** and, the generation of the rapidly solvolysed methanediazonium **5** or a hexane-1,6-bis(diazonium) reactive species **20**, respectively (Scheme 4). This proposal is corroborated by the presence of a sharp isosbestic point at 295 nm in both cases indicating that the only UV-absorbing species in the decomposition mixtures from **1b** and **16c** are the starting materials and

**Table 2** Decomposition of imidazotetrazines and bis(imidazotetrazine)s at pH 7.4 in phosphate buffer

| Compound | $t_{\frac{1}{2}}$/h 25 °C | $t_{\frac{1}{2}}$/h 37 °C | Analytical wavelength/nm |
|---|---|---|---|
| **1a** | 4.41 | 1.03 | 328 |
| **1b** | — | 1.30 | 328 |
| **16c** | — | 2.77 | 331 |
| **16d** | — | 3.27 | 331 |
| **16l** | 5.07 | 1.28 | 331 |

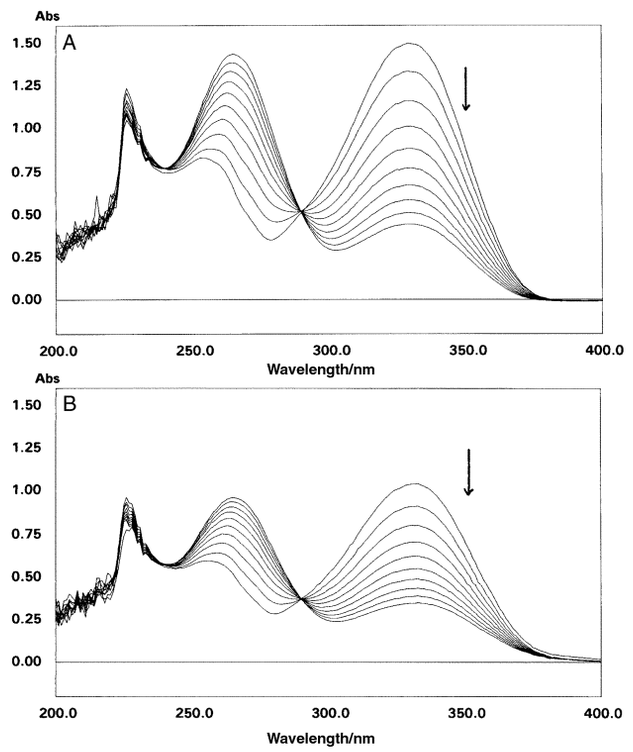
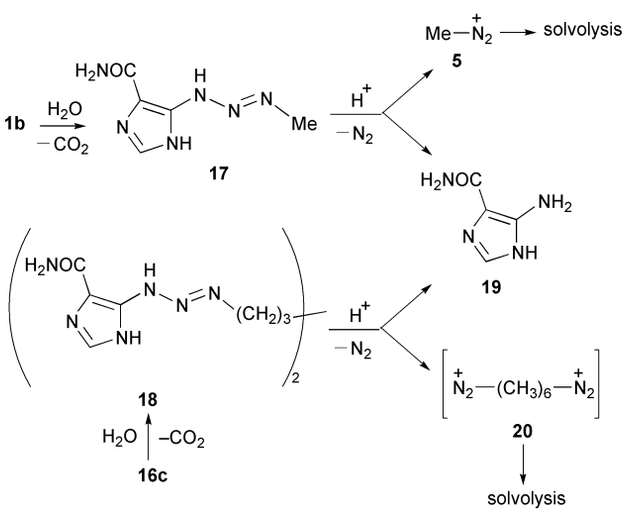



**Fig. 1** Decomposition (absorbance values *versus* wavelength in nm) of imidazotetrazines in phosphate buffer at pH 7.4: A, temozolomide (**1b**); B, the bis(imidazotetrazine) (**16c**). Spectra were recorded at intervals of 15 min for A; 30 min for B.




**Scheme 4**

**19**. Presumably, the electronic 'insulation' of the two imidazotetrazine rings by the hexamethylene linkage in **16c** ensures that each bicycle undergoes independent ring-opening to the unstable bis(triazene) **18** and thence further decomposition.

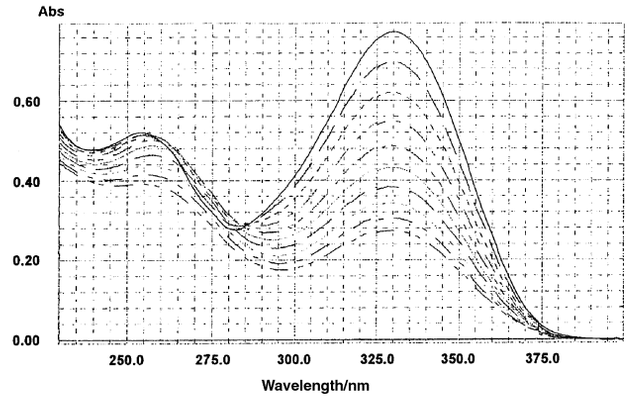
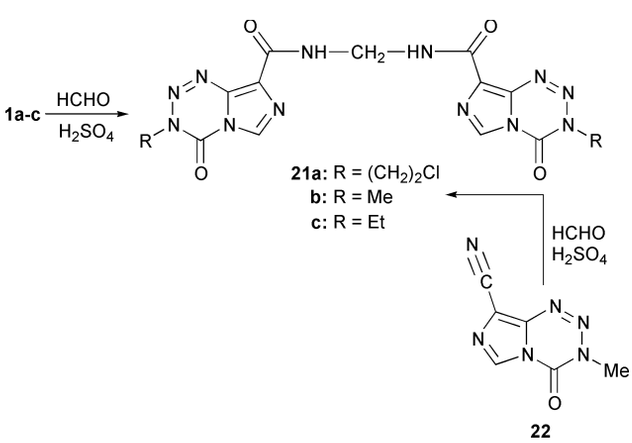



**Fig. 2** Decomposition (absorbance values *versus* wavelength in nm) of bis(imidazotetrazine) **16l** in phosphate buffer at pH 7.4. Spectra were recorded at intervals of 5 min.

The decomposition curves of **16l** are significantly different; no isosbestic point is observed and no peak develops at 270 nm (Fig. 2). Presumably, the electronic influence of the β-disulfide residue dictates that an alternative ring-opening mechanism may apply in this case.

Bis(imidazotetrazine)s of a different type (**21a**–**c**) were formed from the imidazotetrazines (**1a**–**c**) and formaldehyde in concentrated sulfuric acid at 30 °C (Scheme 5). These compounds were isolated as hydrates. The cyano analogue of temozolomide (**22**)[1] also formed the methylene-linked carboxamide **21b** when reacted similarly. Presumably, the cyano group undergoes hydrolysis in the reaction conditions prior to interaction with formaldehyde. Unfortunately, the scope of this reaction was limited: no reaction occurred between **1a** and **1b** with acetaldehyde, propionaldehyde, butyraldehyde, valeraldehyde, chloroacetaldehyde, tribromoacetaldehyde or phenylacetaldehyde.




**Scheme 5**

The bis(imidazotetrazine) **16l** was selected for further evaluation in a *Thermus aquaticus* (*Taq*) DNA polymerase assay[10,21] to determine the preferred base sequences within DNA modified by covalent alkylation. Sites of modification were determined following a 2 hour incubation of *Bam*HI linearised pBR322 DNA with the test agent. Following annealing of a 5′ end-labelled primer complementary to bases 621–640 of the *Bam*HI-*Sal*I fragment of pBR322 extension with the thermostable *Taq* DNA polymerase produced a full length fragment of 263 base pairs. Covalent modification of the DNA by drug serves to block the progress of the polymerase causing termination of chain elongation. The products of linear amplification were run on a sequencing gel to reveal sites of covalent modification. In the partial DNA sequence shown (Fig. 3), clearly guanine-rich sequences are the preferred sites of

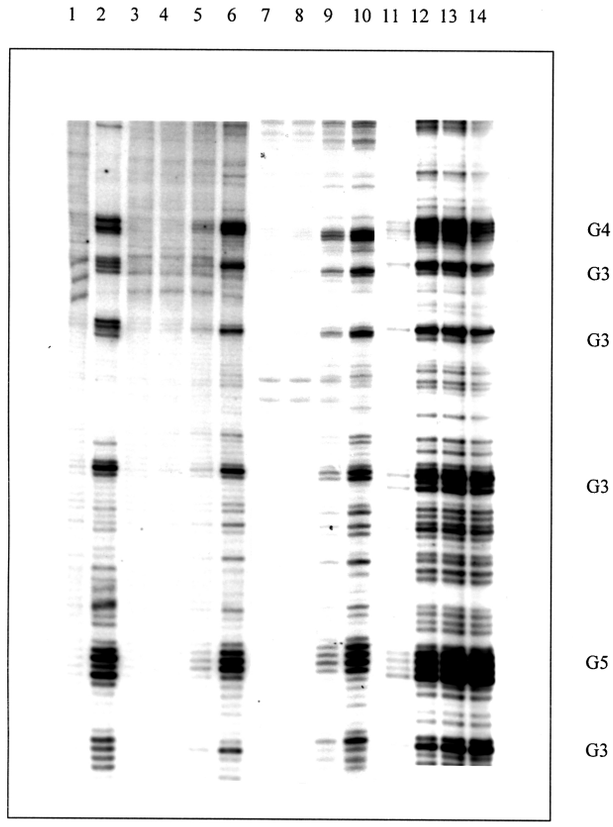



**Fig. 3** Partial autoradiogram of 6% denaturing sequencing gel showing the blocks to *Taq* DNA polymerase after treatment with agents: lane 1, control of unmodified *Bam*HI-*Sal*I fragment of pBR322 DNA; lane 2, cisplatin at 1 μM; lanes 3–6, mitozolomide (**1a**) at 1, 10, 100 μM and 1 mM, respectively; lanes 7–10, temozolomide (**1b**) at 1, 10, 100 μM and 1 mM, respectively; lanes 11–14, the bis(imidazotetrazine) **16l** at 30, 100, 300 μM and 1 mM, respectively. Bands marked G3–G5 refer to sequences of 3–5 consecutive guanine residues.

alkylation with the reference compounds cisplatin, mitozolomide (**1a**) and temozolomide (**1b**) (lanes 2–10).

The polymerase stop assay showed that the bis(imidazotetrazine) **16l** has broadly the same sequence selectivity as the chloroethylating and methylating agents **1a** and **1b**. This is unsurprising since the sites of reaction of DNA with small molecular weight electrophiles are principally determined by the nucleophilicity of microenvironments within DNA. However there was a significant increase in the efficiency of DNA modification by the bis(imidazotetrazine) **16l** (compare lanes 12 and 14 with lanes 9 and 10 in Fig. 3). This can be related to the stability of ultimate electrophilic species derived from intermediates in the different decomposition pathways. Thus the longer-lived electrophiles derived from the active drugs **1a** and **1b** can locate and react with DNA whereas the ethanediazonium ion generated from inactive **1c** can undergo a competitive elimination reaction with evolution of ethene.[22] The particularly effective alkylation by **16l** provides indirect evidence that the decomposition pathway in this case may lead to an episulphonium ion as the ultimate electrophile. Such ions are considerably more stable than electrophiles derived from the prodrugs **1a**–**c**[23] and presumably more effective DNA alkylating agents.

The new bis(imidazotetrazine)s also showed comparable *in vitro* activities in the National Cancer Institute (USA) 60 human tumour cell panel[24] with mitozolomide (**1a**). Mean $GI_{50}$ values (mean concentrations to inhibit cell growth of all cell lines in the panel by 50%) were in the 10–100 μM range with evidence of some selectivity against cells of leukaemic, CNS and melanoma origin ($GI_{50}$ values 1–5 μM), which is typical of the profile of this class of agent. However, no compelling case

can be made at the moment for considering these bifunctional alkylating analogues as alternatives to the clinically useful monofunctional temozolomide.

## Experimental

Melting points are uncorrected. $^1$H and $^{13}$C NMR spectra were acquired on a Bruker ARX 250 instrument observing $^1$H at 250.13 MHz and $^{13}$C at 62.9 MHz. $^1$H and $^{13}$C spectra were referenced to tetramethylsilane and coupling constants are recorded in Hz. Analytical TLC was performed using Merck Kieselgel 60 $F_{254}$ plates and flash chromatography was carried out using silica columns, Kieselgel 60 mesh grade 24; compounds were visualised with UV irradiation. IR spectra were recorded on a Mattson Instruments Galaxy Series FTIR 2020 instrument. Mass spectra were recorded on either a Bio-ion 20 plasma desorption instrument or a VG Autospec. Fast atom bombardment (FAB) mass spectra were recorded on a VG Autospec.

Chemicals, solvents and diisocyanates **15b–e** were purchased from Sigma-Aldrich Chemical Company Ltd. or Lancaster Synthesis Ltd. The materials for the linear *Taq* stop PCR were provided by Professor J. A. Hartley, UCL Middlesex Hospital. Mitozolomide and temozolomide were synthesised by the method of Stevens *et al.*[2] and had $^1$H, $^{13}$C NMR and IR spectra identical to authentic samples. Chloromethyl isocyanate was prepared from chloroacetyl chloride by the Curtius rearrangement of the corresponding acid azide.[25] Methoxymethyl and ethoxymethyl isocyanates were prepared from chloromethyl methyl ether or chloromethyl ethyl ether, respectively, and silver isocyanate, according to the method of Jones and Powers.[26]

### 8-Carbamoyl-3-(chloromethyl)imidazo[5,1-*d*][1,2,3,5]tetrazin-4(3*H*)-one 1d

Chloromethyl isocyanate (**9**; R = $CH_2Cl$) (0.5 g) in diethyl ether (5 mL) was stirred with 5-diazoimidazole-4-carboxamide (**8**; 0.20 g) in HMPA (1 mL) at 25 °C, in the dark, under nitrogen, for 12 h. The white product (0.2 g, 59%) which precipitated from the reaction mixture had mp 130 °C (vigorous decomp.); $\nu_{max}$ (KBr)/cm$^{-1}$ 3428, 3092, 1750 (C=O), 1674 (C=O), 1366, 1057, 743; $\delta_H$ (DMSO-$d_6$) 6.28 (2 H, s, $CH_2$), 7.77 (1 H, br s, NH), 7.91 (1 H, br s, NH), 8.95 (1 H, s, 6-H); $\delta_C$ (DMSO-$d_6$) 56.3 ($CH_2$), 130.3 (C-6), 132.2 (C-8), 134.6 (C-8a), 139.9 (C-4), 161.4 ($CONH_2$) (Found: C, 31.58; H, 2.27; N, 36.46. $C_6H_5ClN_6O_2$ requires: C, 31.52; H, 2.20; N, 36.76%).

### 8-Carbamoyl-3-(methoxymethyl)imidazo[5,1-*d*][1,2,3,5]tetrazin-4(3*H*)-one 1e

Methoxymethyl isocyanate (**9**; R = $CH_2OMe$) (0.5 g) in diethyl ether (5 mL), was cyclised with **8** (0.20 g) in DMSO (1 mL) under similar conditions (see above). The white methoxymethylimidazotetrazine (0.1 g, 31%) had mp 143–145 °C (vigorous decomp.); $\nu_{max}$ (KBr)/cm$^{-1}$ 3091, 1744 (C=O), 1688 (C=O), 1607, 1262, 910; $\delta_H$ (DMSO-$d_6$) 3.41 (3 H, s, $CH_3$) 5.61 (2 H, s, $CH_2$), 7.72 (1 H, br s, NH), 7.86 (1 H, br s, NH), 8.88 (1 H, s, 6-H); $\delta_C$ (DMSO-$d_6$) 57.0 ($CH_3$), 79.4 ($CH_2$), 129.6 (C-6), 131.4 (C-8), 134.4 (C-8a), 139.7 (C-4), 161.6 ($CONH_2$) (Found: C, 37.29; H, 3.64; N, 37.28. $C_7H_8N_6O_3$ requires: C, 37.50; H, 3.60; N, 37.49%).

### 8-Carbamoyl-3-(ethoxymethyl)imidazo[5,1-*d*][1,2,3,5]tetrazin-4(3*H*)-one 1f

Similarly prepared, from ethoxymethyl isocyanate (**9**; R = $CH_2OEt$) and **8** in DMSO, the cream ethoxymethylimidazotetrazine (36%) had mp 245–248 °C (vigorous decomp.); $\nu_{max}$ (KBr)/cm$^{-1}$ 3090, 1740 (C=O), 1680 (C=O), 1458, 1258, 1096; $\delta_H$ (DMSO-$d_6$) 1.13 (3 H, t, *J* 6.4, $CH_3$), 3.66 (2 H, q, *J* 6.4, $CH_2$) 5.64 (2 H, s, $NCH_2O$), 7.72 (1 H, br s, NH), 7.86 (1 H, br s, NH), 8.87 (1 H, s, 6-H); $\delta_C$ (DMSO-$d_6$) 15.1 ($CH_3$), 51.0 ($CH_2$), 62.8 ($CH_2$), 129.6 (C-6), 131.1 (C-8), 134.5 (C-8a), 140.3 (C-4), 162.9 ($CONH_2$) (Found: C, 40.19; H, 4.26; N, 35.15. $C_8H_{10}N_6O_3$ requires: C, 40.34; H, 4.23; N, 35.28%).

### 8-Carbamoyl-3-(4-chlorophenylthiomethyl)imidazo[5,1-*d*]-[1,2,3,5]tetrazin-4(3*H*)-one 1g

Isocyanatomethyl 4-chlorophenyl sulfide (**9**; R = $CH_2SC_6H_4Cl$-*p*) was obtained from chloromethyl 4-chlorophenyl sulfide and silver cyanate according to the method of Jones and Powers.[26] The isocyanate had $\nu_{max}$ (KBr)/cm$^{-1}$ 2255–2264 (N=C=O) and was used in an ethereal solution without purification and reacted with **8** in DMSO (see above). The cream thiomethylimidazotetrazine (87%) had mp 145–147 °C (decomp.); $\nu_{max}$ (KBr)/cm$^{-1}$ 3090, 1736 (C=O), 1680 (C=O), 1609, 1479, 1366, 1269, 1096, 1044; $\delta_H$ (DMSO-$d_6$) 5.80 (2 H, s, $CH_2$), 7.51 (4 H, dd, *J* 8.5, arom C–H), 7.71 (1 H, br s, NH), 7.85 (1 H, br s, NH), 8.88 (1 H, s, 6-H); $\delta_C$ (DMSO-$d_6$) 53.1 ($CH_2$), 129.3 (arom C–H), 129.5 (C-6), 131.5 (C-8), 131.8 (arom C), 133.1 (arom C), 134.0 (arom CH), 134.2 (C-8a), 138.6 (C-4), 161.5 ($CONH_2$) (Found: C, 42.64; H, 2.72; N, 24.86. $C_{12}H_9ClN_6O_2S$ requires: C, 42.80; H, 2.69; N, 24.96%).

### Synthesis of isocyanates 15a and 15f–l: general method

The acid hydrazides **13a** and **l3f–l** were prepared from the corresponding methyl or ethyl esters **12a**, **12f–l** in 95% ethanol containing an excess of hydrazine hydrate. The hydrazides (0.5 g) were introduced into a vigorously stirred emulsion of carbon tetrachloride (5 mL), water (5 mL) and 10 M hydrochloric acid (1.0 mL) at 0–5 °C. Sodium nitrite (2.2 mol equiv.) in water (5 mL) was added dropwise and the mixture was stirred at 0 °C for 1 h. The organic layer was separated and the aqueous layer was extracted with benzene (2 × 10 mL). The combined organic fractions containing the diazides were dried ($CaCl_2$) and heated under reflux (2–4 h) to complete conversion to the isocyanates. The solvent was then removed by vacuum evaporation to furnish the diisocyanates as oils which showed $\nu_{max}$ (film) at 2240–2260 (N=C=O) cm$^{-1}$. The diisocyanates were used without further purification.

### General method for the synthesis of bis(imidazotetrazine)s 16 linked through the N(3)–N(3′) positions

To the crude diisocyanate **15** (3.0 mmol) in DMSO (5 mL) was added 5-diazoimidazole-4-carboxamide **8** (6.0 mmol) and the mixture was stirred (28 d to prepare **16a**; 12 h to prepare **16b–l**) at 0 °C under a nitrogen atmosphere. The mixture was diluted with ice–water and the beige solid bis(imidazotetrazine), which precipitated from the reaction mixture, was collected and purified by sequential washing with petroleum–ether, formic acid and water. Yields and physical characteristics of compounds **16a–l** are recorded in Table 1.

### *N*,*N*′-Bis[3,4-dihydro-4-oxo-3-(2-chloroethyl)imidazo[5,1-*d*]-[1,2,3,5]tetrazin-8-ylcarbonyl]diaminomethane (21a)

Mitozolomide (**1a**; 1.0 g, 4.12 mmol) and 37% formaldehyde solution (0.18 mL, 2.47 mmol) were stirred in concentrated sulfuric acid (20 mL) for 5 h at 25 °C. The white diaminomethane (0.41 g, 40%), precipitated when the solution was poured into ice–water, had mp 108–112 °C; $\nu_{max}$ (KBr)/cm$^{-1}$ 3331, 3092, 1738 (C=O), 1661, 1254; $\delta_H$ (DMSO-$d_6$) 4.02 (4 H, t, *J* 6.0, 2 × $CH_2Cl$), 4.64 (4 H, t, *J* 6.0, 2 × $NCH_2$), 4.97 (2 H, t, *J* 5.6, $NCH_2N$), 8.92 (2 H, t, *J* 5.6, 2 × NH), 8.93 (2 H, s, 2 × 6-H); $\delta_C$ (DMSO-$d_6$) 41.7 ($CH_2Cl$), 44.3 ($NCH_2N$), 50.3 ($NCH_2$), 129.6 (C-6, C-6′), 130.3 (C-8, C-8′), 134.5 (C-8a, C-8a′), 139.2 (C-4, C-4′), 159.8 (C=O) (Found: C, 35.03; H, 3.03; N, 32.63. $C_{15}H_{14}Cl_2N_{12}O_4.H_2O$ requires C, 34.86; H, 3.13; N, 32.62%).

### *N*,*N*′-Bis(3,4-dihydro-4-oxo-3-methylimidazo[5,1-*d*][1,2,3,5]-tetrazin-8-ylcarbonyl)diaminomethane (21b)

Similarly prepared, from temozolomide (**1b**) and formaldehyde in sulfuric acid, this diaminomethane (64%) had mp 134–136 °C; $\nu_{max}$ (KBr)/cm$^{-1}$ 3387, 1761, 1736, 1655, 1578, 1458, 1252; $\delta_H$ (DMSO-$d_6$) 3.87 (6 H, s, 2 × $CH_3$), 4.96 (2 H, t, *J* 5.9, $CH_2$), 8.86 (2 H, s, 2 × 6-H), 8.87 (2 H, t, *J* 5.9, 2 × NH); $\delta_C$ (DMSO-$d_6$) 36.5 ($CH_3$), 44.2 ($CH_2$), 128.9 (C-6, C-6′), 129.7 (C-8, C-8′), 135.0 (C-8a, C-8a′), 139.3 (C-4, C-4′), 159.9 (C=O) (Found: C, 34.84; H, 3.52; N, 37.23. $C_{13}H_{12}N_{12}O_4$·2.5$H_2O$ requires C, 35.06; H, 3.85, N, 37.75%).

The same diaminomethane (62%) was obtained from 8-cyano-3-methylimidazo[5,1-*d*][1,2,3,5]tetrazin-4(3*H*)-one **22**[1] with formaldehyde in concentrated sulfuric acid.

### *N*,*N*′-Bis(3,4-dihydro-4-oxo-3-ethylimidazo[5,1-*d*][1,2,3,5]-tetrazin-8-ylcarbonyl)diaminomethane (21c)

Similarly prepared, from **1c** and formaldehyde in sulfuric acid, this diaminomethane (40%) had mp 177–179 °C; $\nu_{max}$ (KBr)/cm$^{-1}$ 3418, 4123, 1742, 1655, 1572, 1460, 1277, 1250; $\delta_H$ (DMSO-$d_6$) 1.39 (6 H, t, *J* 7.0, 2 × $CH_3$), 4.33 (4 H, q, *J* 7.0, 2 × C$H_2$CH$_3$), 4.96 (2 H, t, *J* 4.9, NC$H_2$N), 8.86 (2 H, s, 2 × 6-H), 8.87 (2 H, t, *J* 4.9, 2 × NH); $\delta_C$ (DMSO-$d_6$) 14.0 ($CH_3$), 44.2 ($CH_2$), 44.6 ($CH_2$), 129.1 (C-6, C-6′), 129.7 (C-8, C-8′), 135.0 (C-8a, C-8a′), 138.9 (C-4, C-4′), 159.98 (C=O) (Found: C, 38.47; H, 4.19; N, 35.88. $C_{15}H_{16}N_{12}O_4$·2.25$H_2O$ requires C, 38.42; H, 4.41; N, 35.84%).

### Linear *Taq* polymerase stop PCR assay

The sequence specificity of covalent DNA modification by mitozolomide (**1a**), temozolomide (**1b**) and the disulfide-linked bis(imidazotetrazine) **16l** was determined by a polymerase stop assay.[21]

pBR322 Plasmid DNA (125 μL, 160 μg mL$^{-1}$) was linearised with *Bam*HI restriction enzyme (3 μL), in 10 × Reaction 3 buffer (15 μL) and water (7 μL), and precipitated with NaOAc (3 M, 15 μL) and ethanol (95%, 495 μL). Linearised DNA (0.5 μg) was treated with drug at concentrations in the range 1–1000 μM as detailed in Fig. 3 for 2 hours at 37 °C (solutions were made up to a final volume of 50 μL with buffer: 25 mM tris(2-hydroxyethyl)amine, 1 mM EDTA, pH 7.2). The DNA was treated with NaOAc (3 M, 10 μL), water (10 μL) and precipitated with ethanol (95%, 300 μL), washed and lyophilised. The *Bam*HI-*Sal*I fragment was used as a template for extension of a 20-base oligonucleotide primer of sequence 5′-TATGCGACTCCTGCATTAGG-3′. The primer was 5′-end labelled with [γ-$^{32}$P] ATP using T4 polynucleotide kinase. The linear amplification of DNA was performed in a total volume of 100 μL containing 0.5 μg DNA, 10 μL 10 × buffer (670 mM Tris pH 8.4, 20 mM $MgCl_2$), 0.25 ng labelled primer, 250 μM dNTP mix and 1 U *Taq* polymerase. The samples were mixed, overlaid with 2 drops of mineral oil and then incubated in a thermal cycler. The amplification procedure was carried out for 30 cycles, each consisting of 1 min denaturation at 95 °C, 2 min annealing at 60 °C and 2 min chain elongation at 72 °C. Following amplification, the samples were precipitated with NaOAc (3 M, 1 μL) and ethanol (95%, 300 μL), washed and lyophilised. The samples were taken up in formamide dye (4 μL), denatured at 90 °C (2 min) and removed onto ice. The DNA fragments were separated on 0.4 mm, 6% polyacrylamide gels (80 mL sequagel 6, 20 mL sequagel complete) with a Tris–boric acid–EDTA buffer system at 55 °C, 3000 V for approximately 3 h. Gels were transferred to filter paper, dried and visualised by autoradiography.

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