ARTICLE

OBC
www.rsc.org/obc

Mutagenicity and DNA damage studies of *N*-acyloxy-*N*-alkoxyamides — the role of electrophilic nitrogen †

Tony M. Banks,[a] Antonio M. Bonin,[b] Stephen A. Glover[a] and Arungundrum S. Prakash

[a] *Division of Chemistry, School of Biological, Biomedical and Molecular Sciences, University of New England, Armidale 2351, N.S.W., Australia*

[b] *School of Chemistry, University of Sydney, NSW 2006, Australia*

[c] *National Research Centre for Environmental Toxicology, University of Queensland, 39 Kessels Rd., Coopers Plains, Qld 4108, Australia*

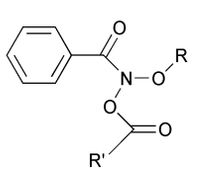
Received 10th February 2003, Accepted 16th May 2003
First published as an Advance Article on the web 2nd June 2003

N-Acyloxy-*N*-alkoxyamides are anomeric amides that are direct-acting mutagens. They have been shown to damage DNA in the major and the minor grooves in a pH and sequence-selective manner. In acidic media, they damage adenines at N3 in the minor groove but above neutral pH, only guanine is damaged at N7 in the major groove. Both the acyloxy leaving group and the alkoxy group at the amide nitrogen are responsible for their electrophilicity and *Salmonella* mutagenicities in TA100 and DNA damage data confirm that the mutagens react with DNA in an intact form, rather than by solvolysis to electrophilic nitrenium ions in the cytosol, or *in vitro*, prior to reacting with DNA. Hydrophobicity plays a role in both mutagenicity and DNA damage.

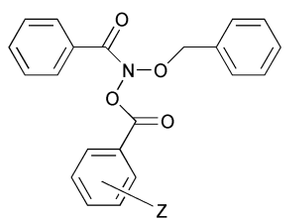
Introduction

N-Acyloxy-*N*-alkoxybenzamides **1** constitute a general class of electrophilic amides first synthesised by our group in 1989 and which we have shown to be direct-acting mutagens.[1–5] We have synthesised a wide range of these substances with structural variation on all three side chains[3,4,6] and, to date, almost all that we have tested have exhibited linear dose-response relationships in the TA100 strain of *Salmonella typhimurium* in the absence of metabolic activation. In a recent study, mutagenic activity of a diverse range of these compounds has been shown to correlate linearly with hydrophobicity but with a modest dependence (log TA100 = 0.23 log P +1.97) which we have interpreted to reflect the ease of non-covalent binding to bacterial DNA;[7] indirect mutagens show a much larger log P dependence of between 0.65 and 1.15 and both Debnath *et al.*[8] and more recently Tuppurainen[9] have suggested that the absence of a hydrophobic dependence may even be characteristic of direct-acting mutagens. Our results indicate that a weaker hydrophobic dependence is operative. In addition, for at least one series that have similar log P values, **2**, mutagenic activity is inversely correlated with chemical reactivity.[7] Compounds of type **1** and **2** undergo both S_N1 and S_N2 reactions at the amide nitrogens.[3,4,6] Acid-catalysed solvolysis leads to alkoxynitrenium ion formation and such reactions are facilitated by electron-withdrawing groups on the benzoate leaving group of **2** leading to a Hammett σ correlation with $\rho = +0.32$.[6] The amides are also susceptible to direct S_N2 displacement of carboxylate by a variety of nucleophiles including inorganic hydroxide[6] and azide,[10,11] organic amines,[12,13] glutathione and thiols.[14] With hydroxide[6] and *N*-methylaniline,[13] **2** afforded Hammett σ-correlations with reaction constants ρ = 1.69 and 0.55, respectively. Mutagenicity of Series **2**, on the other hand, correlated with Hammett σ-substituent constants with a negative slope (LogTA100 = -0.53σ).[7]

There are several notable exceptions to these activity trends. The presence of sterically demanding substituents such as *p-tert*-butyl groups reduce mutagenicity[15] while mutagens with a naphthyl substituent present on the side chains exhibit enhanced mutagenic activity in TA100 largely independent of other factors.[16] Both situations reflect different binding characteristics; bulky mutagens would be less capable of close association with DNA while naphthyl groups could intercalate between base pairs, thereby increasing residence times on DNA. As such, the mutagenicities of *N*-acyloxy-*N*-alkoxyamides are proving to be useful as probes of the influence of non-reactive organic substituents upon DNA binding.



1
R = alkyl, benzyl
R'=methyl, aryl



2
Z=*p*-Me, *p*-Bu^t, H, *p*-Cl, *p*-CHO, *p*-CN, *m*-and *p*-NO_2, *m*-MeO

N-Acyloxy-*N*-alkoxybenzamides can be expected to behave as molecular electrophiles towards DNA or generate electrophilic *N*-acyl-*N*-alkoxynitrenium ions which would be expected to attack DNA or other biological substrates. Hard and soft Lewis acids are known to damage DNA through covalent binding to nucleophilic centres on DNA, the most reactive of which is widely acknowledged as N7 on guanine residues (N7-G). Pullman and Pullman illustrated that the electrostatic potential in the region of N7-G is not only the most negative, but that the magnitude of the negative potential increases significantly in single-stranded and duplex DNA.[17] It is this site in the major groove that is most often modified by alkylating agents such as mustards, alkyl sulfonates or dialkyl sulfates.[18,19] These form primary alkyl cations, or, as in the case of aziridinium ions from nitrogen mustards, masked alkyl cations which are hard Lewis acids and would be expected to react at N7-G according to Pearson's hard-soft acid/base theory. Arylnitrenium ions, the ultimate carcinogenic metabolites formed from aromatic amines, are also nitrogen-centred electrophiles but these are highly delocalised[20] and one of their principal points of attachment to DNA is C8 on guanine (C8-G).[21,22] This is in accord with recent findings by McClelland which illustrated

† Electronic supplementary information (ESI) available: a partial sequence of the pBR322 DNA, solvolysis rate constants and primary bimolecular rate constants. See http://www.rsc.org/suppdata/ob/b3/b301618h/

DOI: 10.1039/b301618h

Table 1 Rate constants, log *P* values, Ames mutagenicities and predicted mutagenicities for *N*-benzoyloxy-*N*-benzyloxybenzamide **3**, *N*-benzyloxy-*N*-(4-methylbenzoyloxy)benzamide **4**, *N*-acetoxy-*N*-(4-phenylbenzyloxy)benzamide **5** and *N*-acetoxy-*N*-butoxy-2-naphthamide **6**

	3	**4**	**5**	**6**
$10^2K_H^{308}$/dm^3 mol^{-1} s^{-1} [a]	0.37	0.41	0.876	4.69[f]
$10^4K_2^{308}$/dm^3 mol^{-1} s^{-1} [b]	261	110	n.d.[g]	654[f]
Log *P* [c]	5.03	5.49	5.04	4.33
Log TA100/1 μmol plate^{-1} [d]	3.22	3.29	3.09	3.60
Calculated LogTA100/1 μmol plate^{-1} [e]	3.02	3.13	3.02	2.96

[a] Rate of $A_{Al}1$ acid-catalysed solvolysis in acetonitrile–water at 308 K.[3,4,6] [b] Rate of S_N2 reaction with *N*-methylaniline in methanol at 308 K.[13] [c] Computed value using ACD Laboratories software.[36] [d] Scaled relative to **9** (see experimental section); data from previous studies.[7] [e] Computed relative to **9** from the relationship in equation (1). [f] This study. [g] Not determined.

that the intermediate formed from direct attack of various arylnitrenium ions on C8-G is the initial species observed by laser flash photolysis experiments.[20,23,24] Alkoxyacylnitrenium ions are strongly delocalised onto electronegative oxygen and, as such, must be regarded as being harder than arylnitrenium ions.[25] If they are intermediates in the genotoxicity of *N*-acyloxy-*N*-alkoxyamides, they would be expected to attack DNA at N7-G. On the other hand, nucleophilic sites on the DNA bases could also react bimolecularly with **1** in an S_N2 reaction.

Other relatively nucleophilic centres on DNA include N3 on guanine (N3-G), the exocyclic 2-amino group on guanine, (N2-G)[26–29] and N3 on adenine (N3-A) and reactivity at all these centres is dependent upon base sequence and accessibility;[29] purine N3 in the minor groove is regarded as less accessible to electrophiles than N7 of purines. It is well known that N7-G at the 5′-end of poly-G sequences is the most nucleophilic centre in DNA and will react more readily with electrophiles.[30–32] Base stacking of this kind is also responsible for preferential oxidative damage at 5′-guanines in poly-G sequences.[33–35]

To date, we have assumed that mutation of *Salmonella typhimurium* must involve modification of prokaryotic DNA in the cytosol by amides of type **1**. In this paper we describe DNA damage studies that point to site and groove preferences in the chemical modification by **1**. In addition, we provide unequivocal evidence for the requirement for activation of the nitrogen by both an alkoxy and an acyloxy functionality and evidence that alkoxynitrenium ions are unlikely to be formed *in vitro* or in the cytosol prior to reaction with DNA.

Results

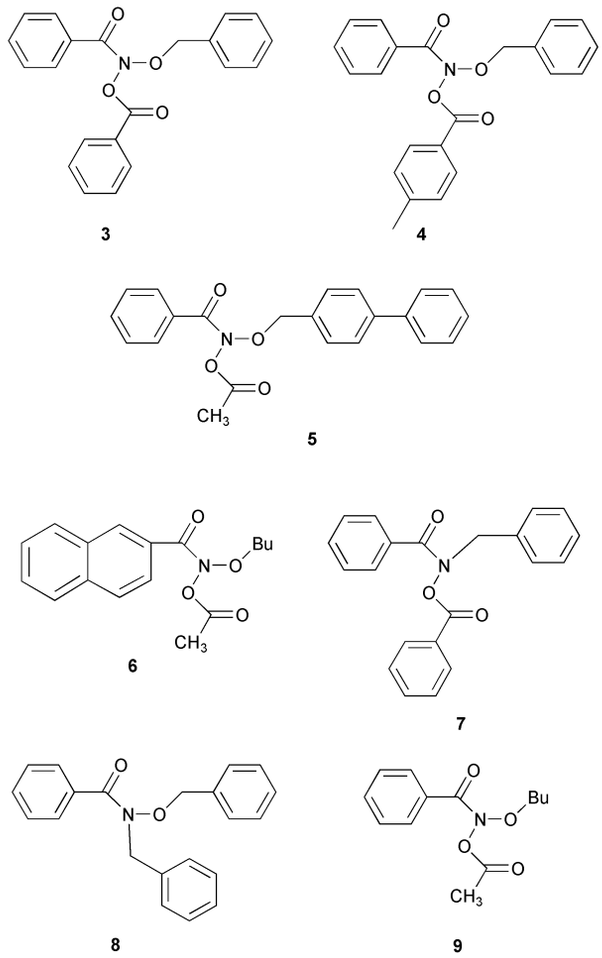
The mutagenicity of amides **1**–**6** has been described previously. Relative activities of **3**–**5** are adequately described by their hydrophobicity. The most recent QSAR for all mutagens of this class is given in equation (1)[7] and predicted, together with

$$\log \text{TA100} = 1.78 + 0.25 \log P. \quad (r = 0.889,\ s = 0.161,\ n = 35)\ddagger \qquad (1)$$

experimental values for all three are presented in Table 1. Table 1 also gives rate constants for both $A_{Al}1$ acid-catalysed ester solvolysis and S_N2 reactivity with *N*-methylaniline.

Based upon its log *P* value, the naphthamide **6** is predicted to be much less mutagenic than is observed. All four substrates reacted by both $A_{Al}1$ acid-catalysed solvolysis (S_N1 at nitrogen) and S_N2 reactions with *N*-methylaniline, and the naphthamide **6** is, if anything, predicted to be more reactive than the other three substrates. Since we have shown in the case of **2** that

‡ *r* = correlation coefficient; *s* = standard error; *n* = number of observations.



mutagenic activity is inversely correlated with reactivity, the mutagenicity of **6** would be expected to be lower than predicted and its enhanced activity cannot be ascribed to its greater survival under the assay conditions. We have interpreted this to indicate that the naphthyl group promotes a stronger association with DNA leading to a greater than expected mutagenicity.[7]

Fig. 1 illustrates the strand cleavage profile at pH 5.8 and 7.8 for the three polyaromatic mutagens **3**–**5** determined with untreated DNA at both pH's as reference. All three showed significant cleavage due to reaction at N7-G in the major groove. Comparison of the pH 5.8 and 7.8 lanes for each substance indicated that guanine damage appeared to be pH dependent with greater reaction at basic rather than acidic pH. The low pH lane for the naphthalene-containing substrate **6** did not load on this gel. However, the high pH reaction was similar to that of **3**–**5**. In addition, while all guanines were reactive, a sequence dependence was evident in a greater preference for the 5′-end of GG (G80-81) and GGG (G129-131) sequences. Cleavage at adenines was also evident but only in the low pH lanes (A56, 57, 61, 62, 65, 73, 83, 85, 87, 92, 94, 100, 132, 136 and 139). According to Pullman and Pullman,[17] the recognised order of reactivity of nucleophilic centres on DNA purines with electrophilic species is N7-G >> N3-G~N3-A > O6-G > N7-A and presumably adenine damage arises out of reaction at N3 in the minor groove. Adenine damage also appears to be substrate dependent. Relative to **3**, the biphenyl-containing substrate **5** is less reactive at most adenines but appears to react significantly at A83. The naphthamide **6** damages some adenines (*e.g.* A61, A62 and A92) even at basic pH and, relative to the corresponding lanes of benzamides **3**–**5**, guanine damage would appear to be more extensive.

The influence of pH upon base selectivity is illustrated for mutagen **3** in Fig. 2. All reactions were incubated for 16 h

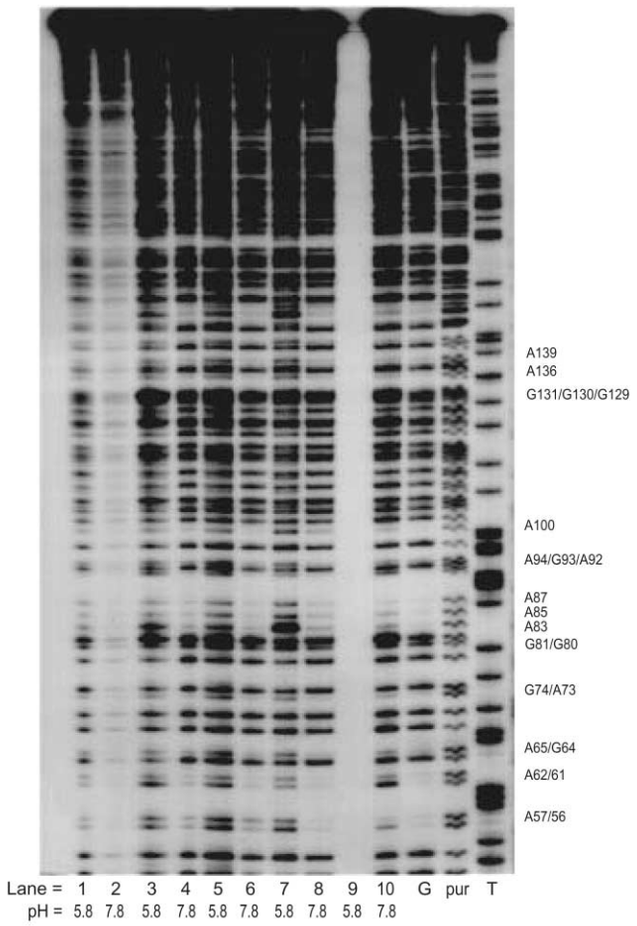


Fig. 1 Strand cleavage patterns obtained by the chemical treatment of the 3′-end-labelled EcoR1 to BamH1 fragment of pBR322 DNA with **4** (lanes 3 and 4), **3** (lanes 5 and 6), **5** (lanes 7 and 8) and **6** (lanes 9 and 10). All lanes incubated for 16 hours followed by heating in neutral buffer then hot piperidine. Lane 1 and 2 are the DMSO control lanes. Lanes 1, 3, 5, 7, 9 = pH 5.8. Lanes 2, 4, 6, 8, 10 = pH 7.8. Lanes 11, 12 and 13 are guanine, purines and thymine, respectively.

followed by a heat and piperidine treatment. At pH 6.6 and above, only guanine is damaged. Analysis of densitometry profiles for lanes 4 to 9 (Fig. 3) indicates that all guanines are damaged and that damage increases with pH. However, at pH 5.8 (lane 2), the DNA is severely damaged as evidenced by the lack of DNA at the origin and increasing intensity towards the lower end of the gel. Densitometry on lane 2 clearly shows excessive damage at adenines 33–35, 43, 57, 65, 94, 117 and 132 and, in at least three clear cut cases, the adenine is 5′- to a guanine (G32, A33–35; G64, A65 and G93, A94). A far higher degree of minor groove damage at pH 5.8 was observed in this experiment when compared to the reaction of **3** at pH 5.8 in the previous study. Significant variability of the degree of minor groove damage was noted in this and in subsequent *in vitro* DNA damage studies using these mutagens and this will be the subject of a future publication.[37]

The requirement for biological activity of both alkoxy as well as acyloxy functionality at the amide nitrogen is evident from a comparison of the mutagenicities of **3** and the partially functionalised analogues **7** and **8**. **7** lacks an activating *N*-alkoxy group while in **8**, the *N*-acyloxy group is replaced by benzyl. The mutagenicities in TA100 for **3**, **7** and **8** are given in Table 2.

In line with previous studies on this class of compounds, *N*-benzoyloxy-*N*-benzyloxybenzamide **3** afforded an excellent dose response over the dose range 0–0.5 μmol plate^{-1} but both **7** and **8** were inactive over this range (Fig. 4). While this would be expected for **8** which lacks a suitable leaving group at nitrogen, the lack of activity for **7** must be attributed to the absence of an

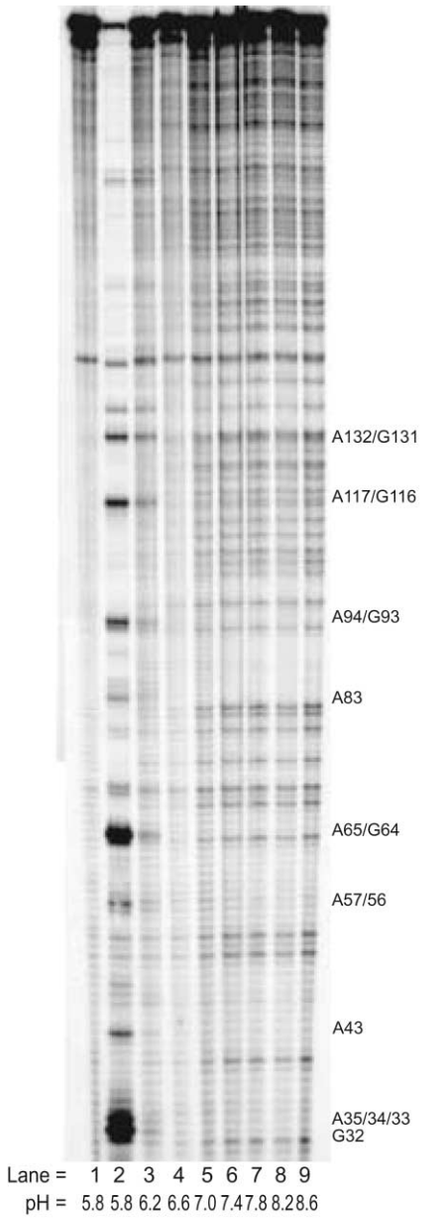


Fig. 2 Strand cleavage patterns obtained by the chemical treatment of the 3′-end-labelled EcoR1 to BamH1 fragment of pBR322 DNA with **3**. All lanes incubated for 16 hours followed by heating in neutral buffer then hot piperidine. Lane 1 is the DMSO control. Lanes 2–9 are pH 5.8, 6.2, 6.6, 7.0, 7.4, 7.8, 8.2 and 8.6, respectively.

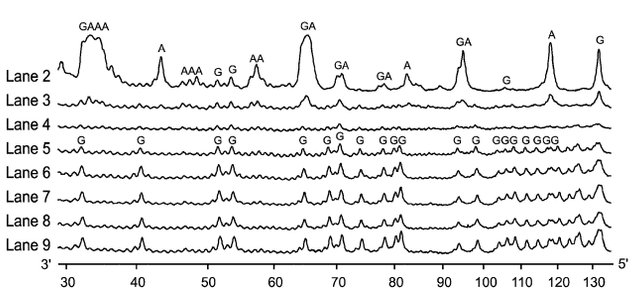


Fig. 3 Combined densitometry for lanes 2–9 (pH 5.8–8.6) from Fig. 2.

alkoxy group. Clearly, both a donor *N*-alkoxy group and an *N*-acyloxy leaving group are required for mutagenic activity.

DNA damage studies mirror these results. Fig. 5 illustrates the DNA cleavage patterns for untreated DNA, and DNA treated with **3**, **7** and **8** at pH 6.6, 7.4 and 8.6. DNA treated with **7** or **8** shows only faint damage which is little more than background. Lanes 2, 6 and 10, corresponding to DNA treated with mutagen **3**, show increasing damage with pH once again

Table 2 *Salmonella* mutagenicities of *N*-benzoyloxy-*N*-benzyloxybenzamide **3**, *N*-benzoyloxy-*N*-benzylbenzamide **7**, *N*-benzyl-*N*-benzyloxybenzamide **8** and *N*-benzoyloxy-*N*-butoxybenzamide **11** in TA100 [a,b]

μmol plate^{-1}	**9** [d,e,f]	μmol plate^{-1}	**3** [d]	**7** [d]	**8** [d]	μmol plate^{-1}	**11** [d,f]
0.000	211(12) [165(20)]	0.000	211(12)	211.3(12)	211(12)	0.000	211(12) [165(24)]
0.250 [0.120]	441(6) [312(19)]	0.060	471(27)	208.3(5)	214(8)	0.120	250(17) [512(13)]
0.500 [0.250]	634(30) [395(21)]	0.130	645(68)	213.3(17)	213(3)	0.250	550(60) [662(11)]
1.000 [0.500]	939(69) [712(46)]	0.250	947(31)	221.7(26)	199(4)	0.500	730(37) [1073(46)]
1.500 [1.000]	— [1269(92)]	0.500	1544(49)	219.0(14)	202(6)	1.000	1062(63) [1382(103)]
Dose response [c]	717.71 [1106.1]		2572.3	—	—	**Dose response** [c]	1117.5 [1733.4]

[a] Without S9 homogenate; mutagens in this class are direct-acting and do not require metabolic activation. [b] Solvent DMSO. [c] Revertants at 1 μmol plate^{-1} calculated from gradient of revertants plate^{-1} *versus* dosage. [d] 1 Standard deviation in parentheses. [e] Reference mutagen. [f] Mutagenicity of **11** was repeated; duplicate values for reference mutagen **9** and **11** in square brackets.

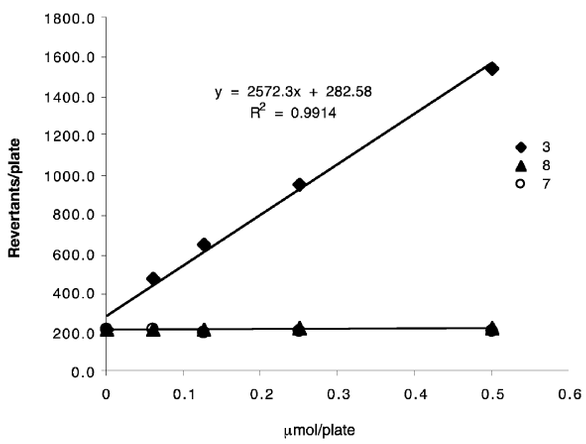


Fig. 4 Comparison of dose-response for **3**,**7** and **8** in TA100 without metabolic activation.

accentuated at the 5′-G of stacked GG sequences (Fig. 6). In addition, there is evidence for weak adenine damage.

While mutagenicity and DNA damage studies confirm the requirement for an amide nitrogen activated towards displacement of carboxylate through the geminal alkoxy group, from these studies no distinction can be drawn between S_N1 or S_N2 displacement reactions with DNA. The former process would invoke the intermediacy of highly electrophilic *N*-acyl-*N*-alkoxynitrenium ions that, like one ultimate carcinogenic form of arylamine metabolites, *N*-acetyl-*N*-arylnitrenium ions, would be expected to react with DNA. Arylnitrenium ions react at near diffusion rates with purine nucleosides yielding mostly the C8 adduct and these adducts have been formed from *in vitro* and *in vivo* studies with DNA. However, this depends upon their lifetimes in water. Most arylnitrenium ions react rapidly with water ($k_w = 1 \times 10^5$–1×10^7) even though they are highly delocalised.[20,38–40] Precursors such as 2-aminofluorene or 4-aminobiphenyl, which form nitrenium ions that are configurationally more resistant to attack by water, react more selectively with bionucleophiles and are more mutagenic.[38,41–43] In contrast, *N*-acyl-*N*-alkoxynitrenium ions are far less delocalised and are likely to be very reactive with water. This has been illustrated in the analysis of the products from the acid-catalysed solvolysis of *N*-acyloxy-*N*-alkoxyamides where *N*-acyl-*N*-alkoxynitrenium ions, formed by $A_{Al}1$ solvolysis, are rapidly quenched by water.[3,4,6] They are unlikely to survive long enough to reach target DNA during *in vitro* DNA studies or in the Ames mutagenicity assay.

A comparative study of the reactivities, mutagenicities and DNA damage profiles of four mutagens **3**, **9**–**11** confirms that *N*-acyloxy-*N*-alkoxyamides bind intact with DNA.

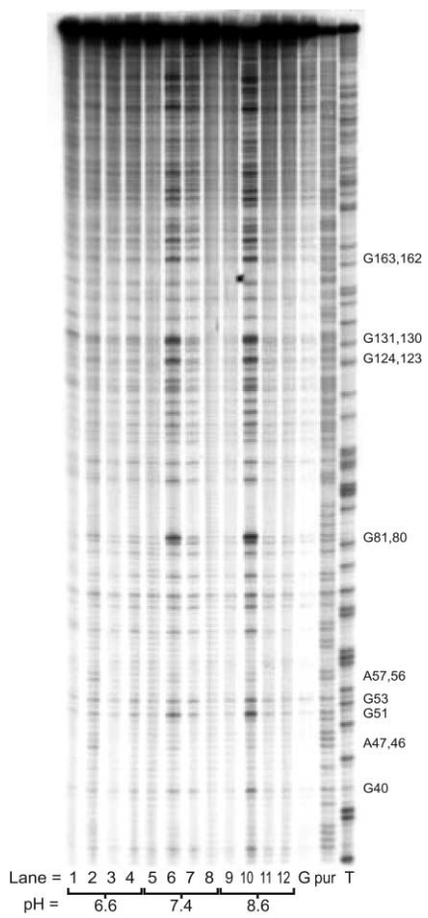
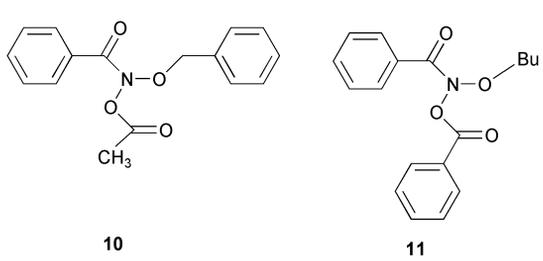


Fig. 5 Strand cleavage patterns obtained by the chemical treatment of the 3′-end-labelled EcoR1 to BamH1 fragment of pBR322 DNA. Lanes 1–4 = pH 6.6, 5–8 = pH 7.4 and 9–12 = pH 8.6. Lanes 1, 5 and 9 are DMSO controls. Lanes 2, 6 and 10 were incubated with **3**. Lanes 3, 7 and 11 were incubated with **7**. Lanes 4, 8 and 12 were incubated with **8**. All lanes incubated for 16 hours followed by heating in neutral buffer then hot piperidine. Lanes 13, 14 and 15 are guanine (faint), purines and thymine, respectively.



10 **11**

Table 3 Rate constants, log *P* values, Ames mutagenicities and predicted mutagenicities for *N*- benzoyloxy-*N*- benzyloxybenzamide **3**, *N*-acetoxy-*N*-butoxybenzamide **9**, *N*-acetoxy-*N*-benzyloxybenzamide **10** and *N*-benzoyloxy-*N*-butoxybenzamide **11**

	9	**10**	**11**	**3**
$10^2 K_H^{308}$/l mol^{-1} s^{-1} [a]	2.6	0.68	1.57[f]	0.41
$10^4 K_2^{308}$/l mol^{-1} s^{-1} [b]	581	174[f]	2553[f]	261
Log *P* [c]	3.1	3.28	4.85	5.03
Log TA100/1 μmol plate^{-1} [d]	2.50	2.63	2.70[f]	3.05[f]
Calculated Log TA100/1 μmol plate^{-1} [e]	2.54	2.59	2.98	3.02

[a] Rate of $A_{Al}1$ acid-catalysed solvolysis in acetonitrile–water at 308 K.[3,4,6] [b] Rate of S_N2 reaction with *N*-methylaniline in methanol at 308 K.[13] [c] Computed value using ACD Laboratories software.[36] [d] Scaled relative to **9** (see Experimental section); data for **10** and **11** from previous studies.[7] [e] Computed relative to **9** from the relationship in equation (1). [f] This study.

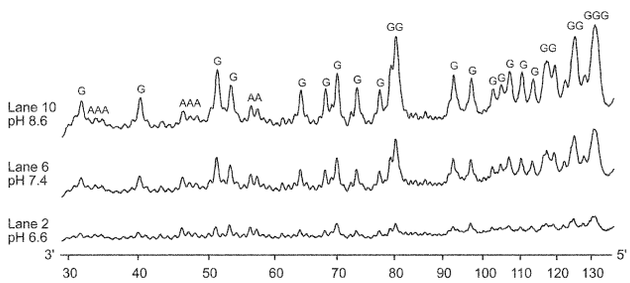


Fig. 6 Densitometry of lanes 2, 6 and 10 (for **3**) from Fig. 5.

Table 3 illustrates comparative $A_{Al}1$ (S_N1) and S_N2 reaction rate constants at 308 K for mutagens **3**, **9**–**11**, together with their mutagenicities at 1 μmol plate^{-1}, computed log *P* values and predicted mutagenicities. Replicate Ames mutagenicity data for the new mutagen **11** was highly reproducible (after scaling to **9**) and is presented together with that for **3**, **7** and **8** in Table 2. While relative reactivities are similar for all four substances with the exception of **11**, which undergoes S_N2 reactions with *N*-methylaniline much more rapidly than **3**, **9** or **10**, mutagenicities vary significantly and correlate with their hydrophobicity in line with our previously reported dependence. Clearly, overall mutagenicity levels are dependent upon the hydrophobicity of the full structure. If nitrenium ion intermediates were to be formed prior to association with DNA, **9** and **11** would form the same nitrenium ion, as would **3** and **10** and mutagenicities would have been expected to be comparable for each pair of substrates.

In vitro DNA damage studies support this assertion. Fig. 7 illustrates damage for **3**, **9**–**11** and untreated DNA at pH 6.6, 7.4 and 8.6. **3** and **10** showed normal guanine (all guanines) and adenine damage (*e.g.* A56, A57, A61, A62, A65, A83, A85 and A87), but guanine damage in the case of **3** strongly increases with pH whereas the corresponding damage with **10** does not. This is best illustrated in densitometry on the well-resolved lower regions of the gel (Fig. 8 (b) and (d)). Similarly, **9** and **11** both damage guanines without discernable adenine damage but their guanine damage profiles are different. Guanine damage increases from low to high pH for **11** but not significantly for **9** (Fig. 8 (a) and (c)). Solvolysis of *N*-benzoyloxy-*N*-benzyloxybenzamide **3** and *N*-acetoxy-*N*-benzyloxybenzamide **10** would yield the same *N*-benzoyl-*N*-benzyloxynitrenium ion which would have led to identical pH and selectivity profiles from both reactions. Similarly, *N*-acetoxy-*N*-butoxybenzamide **9** and *N*-benzoyloxy-*N*-butoxybenzamide **11** would have formed a common *N*-benzoyl-*N*-butoxynitrenium ion. Thus, it is apparent that reactivity profiles are determined in each case by the intact mutagen leading to four distinct DNA damage patterns.

Discussion

Mutagens **3**, **4** and **5** show similar reactivity towards DNA in line with their mutagenicities that correlate linearly with their hydrophobicities. This is demonstrated by the similarities in their calculated log TA100 values based upon octanol–water

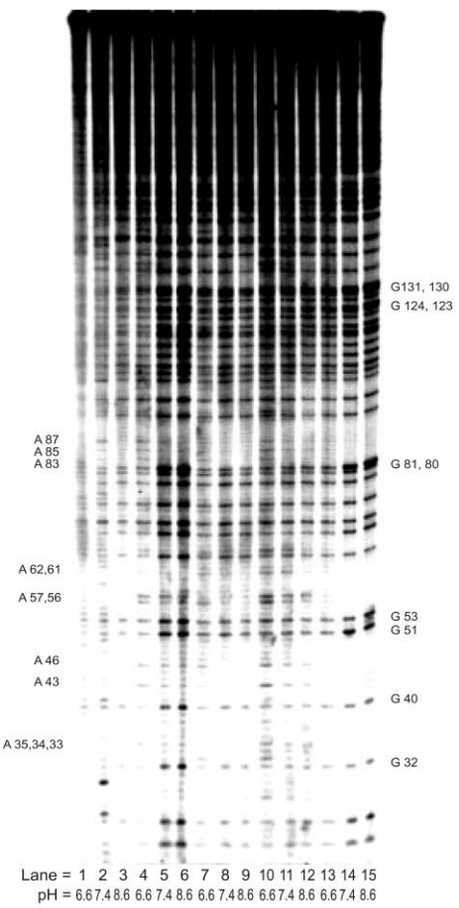


Fig. 7 Strand cleavage patterns obtained by the chemical treatment of the 3′-end-labelled EcoR1 to BamH1 fragment of pBR322 DNA with **3** (lanes 4–6), **9** (lanes 7–9), **10** (lanes 10–12) and **11** (lanes 13–15). All lanes incubated for 16 hours followed by heating in neutral buffer then hot piperidine. Lane 1–3 are the DMSO control. Lanes 1, 4, 7, 10, 13 = pH 6.6. Lanes 2, 5, 8, 11, 14 = pH 7.4. Lanes 3, 6, 9, 12, 15 = pH 8.6

partition coefficients, log *P* (Table 1).[7] The naphthamide analogue **6** has a lower calculated log *P* (4.38) but its significant DNA reactivity is in line with the mutagenicity of this substrate, which is nearly double that of **3** and has been attributed to an intercalative association with DNA. In a future paper, we will show that a series of naphthalene-containing substrates show similarly increased reactivities relative to other members of this class of mutagens, in support of an alternative intercalative binding mode with DNA.[37]

The mutagenic behaviour of **3**–**6**, as well as that of other mutagens,[7] while clearly dependent upon hydrophobicity or possibly intercalation in the case of **6**, has, to date, not been unequivocally ascribed to reactivity at nitrogen or, more specifically, to either an S_N1 or an S_N2 process. These results point to the fact that the process whereby these mutagens damage DNA is clearly electrophilic and requires the presence of, not only a leaving group, but an activating alkoxy group at the amide

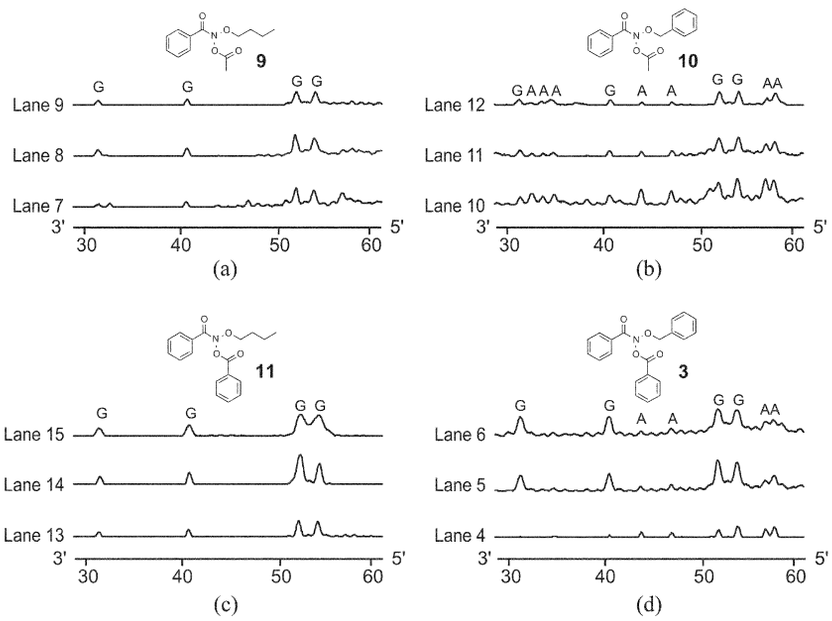


Fig. 8 Densitometry of (a) lanes 7, 8 and 9 (for **9**), (b) lanes 10, 11 and 12 (for **10**), (c) 13, 14 and 15 (for **11**), and (d) 4, 5 and 6 (for **3**) from Fig. 7.

nitrogen. The limiting reagents, *N*-benzoyloxy-*N*-benzylbenzamide **7** (log *P* = 4.58) and *N*-benzyl-*N*-benzyloxybenzamide **8** (log *P* = 5.02), which are computed to have similar hydrophobicities to **3**, are inactive towards DNA in both the DNA damage studies as well as in *Salmonella* mutagenicity tests.

The role of an alkoxy or an amino group in anomeric weakening of an *N*—*O* acyl, *N*—*O* alkyl or *N*—halogen bond has been described previously.[5,10,44–48] In these anomeric amides, the nitrogen is highly pyramidalised, a consequence of the combined electron demand of the two electronegative oxygen atoms which is better met with longer sp^3 hybrid orbitals at nitrogen (Fig. 9(a)). The lone pair is consequently also sp^3 and hence most, if not all, resonance delocalisation onto the carbonyl is lost. Infrared carbonyl stretch frequencies of all these mutagens reflect this; they absorb in the region of 1720–1740 cm^{-1} which is completely atypical of simple amides.[5] In addition, we have evidence from theoretical studies,[5,44,47] and more recently from X-ray analysis,[49] that the conformation about the *N*—*O* alkyl bond facilitates an anomeric interaction between the p-type lone pair on oxygen and the *N*—*O* acyl σ* orbital in a classical anomeric interaction (Fig. 9(b)). This destabilisation of the *N*—*O* acyl bond, together with the sp^3 hybridisation of nitrogen, facilitates "carbon-type" substitution reactions at the amide nitrogen. These mutagens can be expected to react with nucleophilic centres on DNA by either an S_N1 or an S_N2 process.

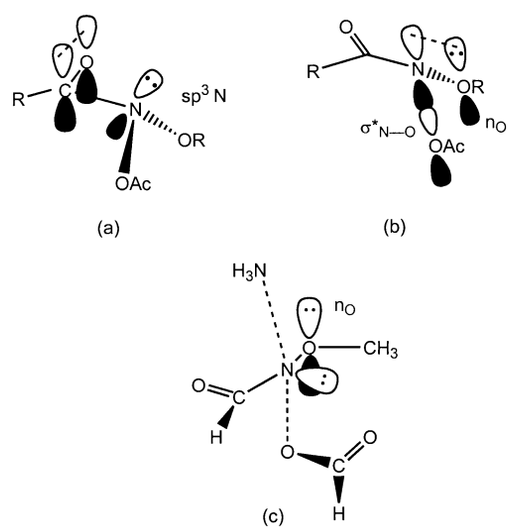
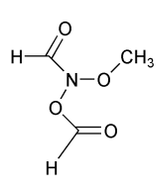
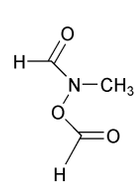


Fig. 9 (a) Pyramidal nitrogen and (b) anomeric interaction in *N*-acyloxy-*N*-alkoxyamides; (c) HF/6-31G* transition state for reaction of ammonia with *N*-formyloxy-*N*-methoxyformamide.

Mutagenicities in TA100 (Table 3) and the DNA damage profiles of **3**, **9**–**11** (Fig. 7) suggest that nitrenium ions are not the reactive species that interact with DNA as the mutagens clearly associate with DNA intact. These results indicate that the reaction with N7-G and N3-A is most probably best described as an S_N2, rather than an S_N1 process. The difference between the mutagenicity and DNA damage reactivity of **3** and **7**, where the anomerically destabilising oxygen atom is absent, can be accounted for on the basis of either S_N1 or S_N2 reactivity. However, recent *ab initio* calculations on model S_N2 reactions of ammonia with *N*-formyloxy-*N*-methoxyformamide **12** and *N*-formyloxy-*N*-methylformamide **13** predict an activation barrier twice as large where *N*-methyl (E_A = 140 kJ mol^{-1}) as opposed to *N*-methoxy (E_A = 72 kJ mol^{-1}) is present.[47] A similar outcome was computed for the S_N2 reaction with azide.[10] Furthermore, the computed transition state geometry for the reaction of ammonia with *N*-formyloxy-*N*-methoxyformamide (Fig. 9(c)), allows maximal overlap between the p-type lone pair on the methoxy oxygen and the departing *N*—*OCHO* bond pointing to the importance of this interaction in promoting such reactions by nucleophiles at the amide nitrogen.

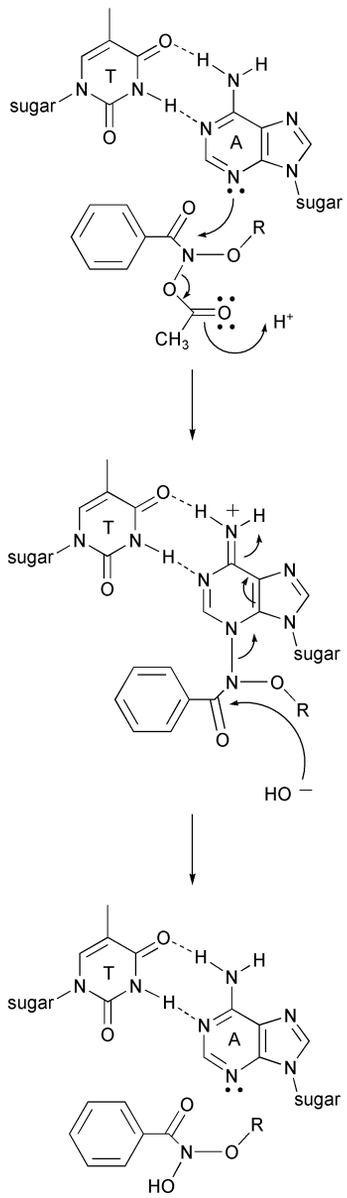


12



13

Analysis of reactivity patterns of **3** at low pH (Figs. 1–3) indicates that the mutagen damages DNA in pH-dependent fashion. Minor groove damage at N3-A, prevalent from reactions at acidic pH (Figs. 1–3), is disfavoured in neutral to basic buffers. By analogy with alkylation reactions, the ammonium ion adduct from N3-A attack, leads to direct fragmentation of DNA upon application of heat (90 °C).[50,51] At 37 °C and under basic reaction conditions, this adduct might be unstable undergoing hydrolysis to hydroxamic acid resulting in regeneration of

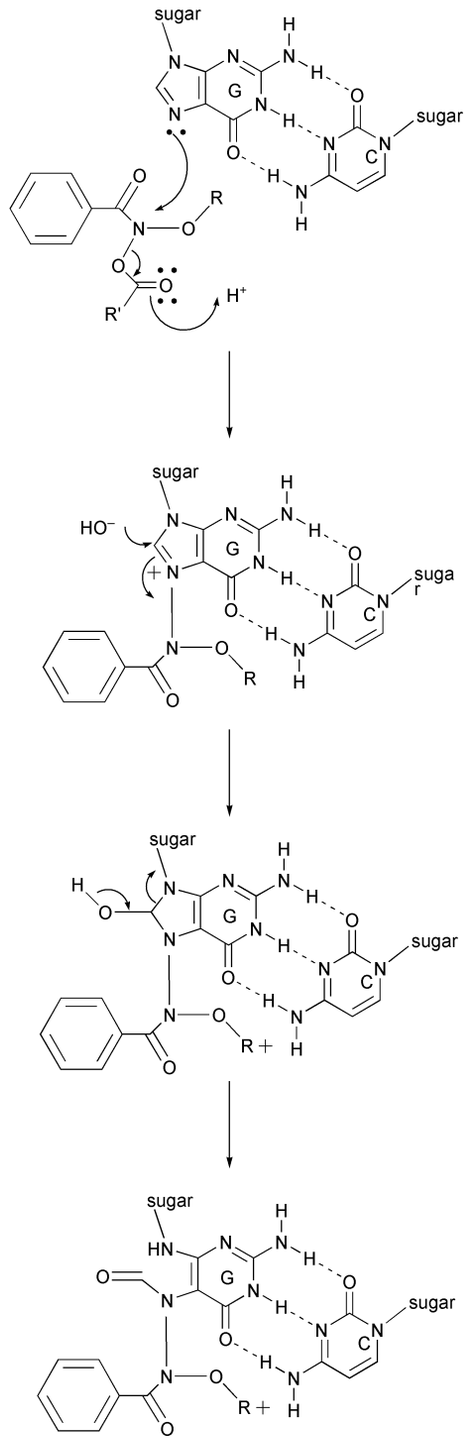


Scheme 1

adenine (Scheme 1). Evidence will be presented elsewhere which supports this proposal.[37]

Guanine damage is ascribed to the reaction of mutagens at N7-G in the major groove which leads to lability of the purine–deoxyribose bond resulting in DNA cleavage upon treatment with hot piperidine.[52] While the trend is not as evident with all mutagens, the pH dependence of guanine damage, particularly evident with mutagens **3**–**5** and **11** is interesting. For instance, with *N*-benzoyloxy-*N*-benzyloxybenzamide **3**, a marked increase in damage is evident with increasing pH in Figs. 1, 2, 5 and 7. While the N7-G adduct, like the N3-A adduct in the minor groove, may be labile to base, hydrolysis is more likely to involve attack on C8 of the purine which becomes highly susceptible to nucleophilic attack upon functionalisation of N7 (Scheme 2). Thus, in neutral to basic buffer, hydrolysis and ring-opening of the imidazole ring, which corresponds to the first step in the DNA cleavage sequence, would occur.

The difference between the *N*-benzoyloxy and *N*-acetoxy mutagens is not clear although the pH dependence may well be more evident with more strongly hydrophobic mutagens. While, in Figs. 7 and 8, **9** and **10** show relatively similar damage across the pH profile, **5**, which also bears an *N*-acetoxy leaving group, but which has a greater overall hydrophobicity (log *P* =5.04 as opposed to 3.1 and 3.28), also exhibits greater guanine damage at higher pH (Fig. 1).



Scheme 2

Experimental

Chemicals

General syntheses of mutagens **3**–**6**, **9** and **10** have been described elsewhere[3,4,6,7] as has the synthesis of *N*-benzoyloxy-*N*-benzylbenzamide **7**.[10] *N*-Benzoyloxy-*N*-butoxybenzamide **11** and **8** were synthesised for the first time in this study. Pet. spirit was the fraction boiling between 60 and 80 °C. DCM is dichloromethane. 40% (19 : 1) acrylamide–bisacrylamide was purchased from Bio-Rad Laboratories Pty Ltd. pBr322, Klenow fragment, *Bam*H1, *Eco*R1, BSA (bovine serum albumin) and NE buffer 2, were purchased from (New England Biolabs) Genesearch Pty Ltd. α-dATP([α-^{32}P]-deoxyadenosine-5′-triphosphate (10 μCi μl^{-1}), isoblue stabilized) was purchased from ICN Biomedicals Inc.

Synthesis of *N*-benzoyloxy-*N*-butoxybenzamide 11

***N*-Butoxy-*N*-chlorobenzamide.** *N*-Butoxybenzamide[3] (1.5 g, 7.76 mmol) and neat *tert*-butyl hypochlorite (2.53 g, 23.3 mmol) was stirred for 10 minutes in the dark. Removal of excess hypochlorite *in vacuo* provided the title compound which was used immediately without further purification. $\nu_{max}(CHCl_3)cm^{-1}$ 1718s (C=O), 1239m; δ_H(300 MHz, $CDCl_3$) 0.90 (3H, t, CH_3), 1.32 (2H, m, CH_2CH_3), 1.58 (2H, m, OCH_2CH_2), 4.14 (2H, t, OCH_2), 7.46 (2H, t, *m*-Ar–H), 7.57 (1H, t, *p*-Ar–H), 7.79 (2H, d, *o*-Ar–H); δ_C(75 MHz $CDCl_3$) 13.6 (q), 19.0 (t), 29.4 (t), 74.5 (t), 128.3 (d), 129.3 (d), 131.2 (s), 132.8 (d), 174.2 (s).

***N*-Benzoyloxy-*N*-butoxybenzamide.** Sodium benzoate (0.89 g, 6.15 mmol) was stirred at room temperature with *N*-butoxy-*N*-chlorobenzamide (1.0 g, 4.4 mmol) in dry acetone for 24 hours.[6] The solvent was removed *in vacuo* and purification was achieved by centrifugal chromatography with ethyl acetate–pet spirit (0.91 g, 66%). The oil was characterised spectroscopically. $\nu_{max}(CHCl_3)cm^{-1}$ 1758s (C=O), 1728s (C=O), 1238s; δ_H(300 MHz, $CDCl_3$) 0.91 (3H, t, CH_3), 1.39 (2H, sextet, CH_2CH_3), 1.69 (2H, quintet, OCH_2CH_2), 4.31 (2H, t, OCH_2), 7.43 (2H, t, *m*-Ar–H), 7.46 (2H, t, *m*′-Ar–H), 7.54 (lH, t, *p*-Ar–H), 7.62 (1H, t, *p*′-Ar–H), 7.87 (2H, d, *o*-Ar–H), 8.03 (2H, d, *o*′-Ar–H); δ_C(75 MHz, $CDCl_3$) 13.7 (q), 19.0 (t), 30.1 (t), 75.6 (t), 127.4 (d), 128.3 (d), 128.6 (d), 129.1 (d), 130.0 (d), 131.9 (s), 132.7 (d), 134.0 (s), 164.3 (s), 174.5 (s).

Compound **8** was synthesised by benzylation of *N*-benzyloxybenzamide the synthesis of which was described previously.[3]

Synthesis of *N*-benzyl-*N*-benzyloxybenzamide 8. *N*-Benzyloxybenzamide (1.00 g, 0.0044 mol) and benzyl bromide (0.75 g, 0.0044 mol) were dissolved in 10% aqueous methanol (20 ml). Potassium hydroxide (0.35 g, 0.0062 mol) was added and the solution was stirred for 24 h. The methanol was removed *in vacuo* and water was added (25 ml). The solution was extracted with DCM (3 × 20 ml) which was washed with dilute HCl (20 ml), H_2O (20 ml), 10% Na_2CO_3 (20 ml) and dried over $MgSO_4$. The pure product was obtained by centrifugal chromatography using ethyl acetate–pet spirit (0.47 g, 34%) as eluant (Found: C, 79.88; H, 6.15; N, 4.29%. $C_{21}H_{19}NO_2$ requires C, 79.47; H, 6.03; N, 4.41%); $\nu_{max}(CHCl_3)cm^{-1}$ 1636s (C=O); δ_H(300 MHz, $CDCl_3$) 4.56 (2H, s, NCH_2), 4.94 (2H, s, OCH_2), 6.97 (2H, d, Ar–H), 7.28 (3H, m, Ar–H), 7.36–7.46 (8H, m, Ar–H), 7.70 (2H, d, Ar–H); δ_C(75 MHz, $CDCl_3$) 51.5 (t), 77.1 (t), 127.8 (d), 128.0 (d), 128.3 (d), 128.4 (d), 128.6 (d), 128.7 (d), 128.8 (d), 129.5 (d), 130.6 (d), 134.1 (s), 134.5 (s), 136.4 (s). Carbonyl carbon absent.

Labelling and isolation of a plasmid DNA fragment

A 375 base pair EcoRI to BamHI fragment of plasmid pBR322 DNA was 3′ end labelled at the EcoRI site using Klenow fragment and [α-32P]dATP(3000 Ci $mmol^{-1}$) according to a published procedure.[51] The fragment was isolated on a 4% non-denaturing polyacrylamide gel. A partial sequence of the pBR322 DNA used in this work is presented as supplementary material to this paper.

DNA reaction studies[32]

The compound to be tested was prepared as a 50 mmol stock solution in dry DMSO. The labelled DNA was prepared in a solution of TE buffer such that the radiation level was 15000 cpm μl^{-1}. A typical alkylation reaction mixture consisted of 1 µl of labelled DNA in 97 µl of 10 mM phosphate buffer and 2 µl of compound stock solution (final volume 100 µl). Samples were incubated for 16 h at 37 °C whereupon they were immediately precipitated with ethanol and lyophilised.

Depurination and subsequent strand breaks at all purines modified at N7-G, N3-A and N7-A[50,52,53] were effected by heating at 90° (15 minutes) in water and then at 90° in 1.1 M piperidine.

Where appropriate, sequencing lanes were produced by the Maxam–Gilbert sequencing reactions.[50,51] Samples were denatured at 95 °C and loaded onto a 0.4 mm 6% denaturing acrylamide–bisacrylamide (19 : 1) polyacrylamide gel which was run at a constant 75 W until the xylene cyanol had migrated 26 cm from the bottom of the loading wells (*ca.* 2 h). At the completion of the run, an autoradiogram was generated with Kodak XAR-5 film (−80 °C for 15–20 hours) using a Kodak Biomax MS intensifying screen.

Mutagenicity assays

Salmonella typhimurium strain TA100 was obtained from Professor B. N. Ames, University of California, Berkeley, U.S.A. and cultured as described[54] with the exception that fresh broth cultures were incubated at 37 °C in a shaking water bath for 10 h prior to use in each assay. Top agar, supplemented with a trace of histidine and biotin, was dispensed in 2 ml volumes into 5 ml plastic vials and maintained at 45 °C in a water bath. Before pouring on the surface of minimal agar plates, 0.1 ml of the broth culture, 0.1 ml of the test chemical dissolved in dry, analytically pure DMSO were added to each vial. Triplicate plates at each dose level were incubated at 37 °C for 72 h before counting revertant colonies with an Artek model 880 counter.

Assays at different dose levels were carried out in triplicate together with negative controls. Responses for each compound at 1 µmol $plate^{-1}$ were obtained from least squares analysis of linear regions of the plots of mean revertants/plate *versus* dose. Comparative data were derived by scaling mutagenicities at 1 µmol $plate^{-1}$ to that of a single standard, *N*-acetoxy-*N*-butoxybenzamide **9** (Log TA100 = 2.5),[7] which was always analysed in parallel with new mutagens.

Kinetic studies

$A_{Al}1$ acid-catalysed solvolysis. The procedure for determining rates of acid-catalysed solvolysis has been described previously[3,4,6] and involves dissolving a small quantity of the *N*-acyloxy-*N*-alkoxyamide (typically between 10–40 mg) in 26% (D_2O)–(CD_3CN) in an NMR tube. The mixture was preheated and shimmed in the probe of the NMR spectrometer before an appropriate volume of sulfuric acid–D_2O solution was injected to initiate solvolysis. ^1H NMR spectra were then acquired at intervals using an automated program. Rates were obtained by integration of appropriate resonances. Solvolysis rate constants were obtained at four different temperatures in the range of 293 K to 323 K with correlation coefficients close to unity and are presented in Table S4 of supplementary material to this paper. †

S_N2 reactions with *N*-methylaniline. Rate constants for the bimolecular reaction between *N*-acyloxy-*N*-alkoxybenzamides **6**, **10** and **11** and *N*-methylaniline were determined using ^1H NMR spectroscopy.[12,13] 5–20 mg of *N*-acyloxy-*N*-alkoxybenzamide in 400 µl of methanol-d_4 was equilibrated in a 5 mm NMR tube at the required temperature prior to addition of *N*-methylaniline (5–30 µl). The exact time of mixing was noted. ^1H NMR spectra were collected using an automated program after which progress of the reaction was monitored by integration of representative NMR resonances of the substrate and the *N*-methyl resonance of *N*-methylaniline. Initial substrate concentrations were obtained by back extrapolation of concentration plots for both reagents to the initial time of mixing, t_o. Primary bimolecular rate constants were obtained at four temperatures between 290 K and 325 K and are presented in Table S5 of supplementary material to this paper. †

Acknowledgements

The authors are grateful to the Australian Research Council for a Large Grant and to the University of New England for a Ph.D. scholarship to Tony Banks.

References

1 R. G. Gerdes, S. A. Glover, J. F. Ten Have and C. A. Rowbottom, *Tetrahedron Lett.*, 1989, **30**, 2649.
2 J. J. Campbell, S. A. Glover and C. A. Rowbottom, *Tetrahedron Lett.*, 1990, **31**, 5377.
3 J. J. Campbell, S. A. Glover, G. P. Hammond and C. A. Rowbottom, *J. Chem. Soc., Perkin Trans. 2*, 1991, 2067.
4 A. M. Bonin, S. A. Glover and G. P. Hammond, *J. Chem. Soc., Perkin Trans. 2*, 1994, 1173.
5 S. A. Glover, *Tetrahedron*, 1998, **54**, 7229.
6 A. M. Bonin, S. A. Glover and G. P. Hammond, *J. Org. Chem.*, 1998, **63**, 9684.
7 T. M. Banks, A. M. Bonin, J. J. Campbell, S. A. Glover, G. P. Hammond, A. S. Prakash and C. A. Rowbottom, *Mutat. Res.*, 2001, **494/1–2**, 115.
8 A. K. Debnath, A. J. Shusterman, R. L. Lopez de Compadre and C. Hansch, *Mutat. Res.*, 1994, **305**, 63.
9 K. Tuppurainen, *Chemosphere*, 1999, **38**, 3015.
10 S. A. Glover and G. Mo, *J. Chem. Soc., Perkin Trans. 2*, 2002, 1728.
11 S. A. Glover and A. Rauk, *J. Chem. Soc., Perkin Trans. 2*, 2002, 1740.
12 J. J. Campbell and S. A. Glover, *J. Chem. Soc., Perkin Trans. 2*, 1992, 1661.
13 J. J. Campbell and S. A. Glover, *J. Chem. Res. (S)*, 1999, **8**, 474; J. J. Campbell and S. A. Glover, *J. Chem. Res. (M)*, 2075.
14 M. Adams, S. A. Glover and D. J. Tucker, unpublished results.
15 A. M. Bonin, A.-M. E. Gillson and S. A. Glover, unpublished results.
16 T. M. Banks, A. Bonin and S. A. Glover, unpublished results.
17 A. Pullman and B. Pullman, *Int. J. Quantum Chem., Symp.*, 1980, **7**, 245.
18 M. Gniazdowski and C. Cera, *Chem. Rev.*, 1996, **96**, 619.
19 S. R. Rajski and R. M. Williams, *Chem. Rev.*, 1998, **98**, 2723.
20 R. A. McClelland, *Tetrahedron*, 1996, **52**, 6823.
21 F. A. Beland and F. F. Kadlubar, *Environ. Health Perspect.*, 1985, **62**, 19.
22 W. G. Humphreys, F. F. Kadlubar and F. P. Guengerich, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 8278.
23 R. A. McClelland and P. Sukhai, *J. Chem. Soc., Perkin Trans. 2*, 1996, 1529.
24 R. A. McClelland, A. Ahmad, A. P. Dicks and V. E. Licence, *J. Am. Chem. Soc.*, 1999, **121**, 3303.
25 S. A. Glover and A. P. Scott, *Tetrahedron*, 1989, **45**, 1763.
26 D. E. Thurston and D. S. Bose, *Chem. Rev.*, 1994, **94**, 433.
27 N. Cooper, D. R. Hagan, A. Tiberghien, T. Ademefun, C. S. Matthews, P. W. Howard and D. E. Thurston, *Chem. Commun.*, 2002, 1764.
28 A. Kamal, G. Ramesh, N. Laxman, P. Ramulu, O. Srinivas, K. Neelima, A. K. Kondapi, V. B. Sreenu and H. A. Nagarajaram, *J. Med. Chem.*, 2002, **45**, 4679.
29 W. A. Denny, *Expert Opin. Ther. Pat.*, 2000, **10**, 459.
30 K. W. Kohn, J. A. Hartley and W. B. Mattes, *Nucleic Acids Res.*, 1987, **15**, 10531.
31 M. A. Warpehoski and L. H. Hurley, *Chem. Res. Toxicol.*, 1988, **1**, 315.
32 A. S. Prakash, W. A. Denny, T. A. Gourdie, K. K. Valu, P. D. Woodgate and L. P. G. Wakelin, *Biochemistry*, 1990, **29**, 9799.
33 H. Sugiyama and I. Saito, *J. Am. Chem. Soc.*, 1996, **118**, 7063.
34 N. Nakamura and I. Saito, *Tetrahedron Lett.*, 2000, **41**, 7917.
35 F. Pratt, K. Houk and C. S. Foote, *J. Am. Chem. Soc.*, 1998, **120**, 845.
36 'ACD/Log *P* Web Service 5.0', Advanced Chemistry Development Inc.
37 T. M. Banks, S. A. Glover and A. S. Prakash, unpublished results.
38 R. A. Davidse, M. J. Kahley, R. A. McClelland and M. Novak, *J. Am. Chem. Soc.*, 1994, **116**, 4513.
39 R. A. McClelland, P. A. Davidse and G. Hadzialic, *J. Am. Chem. Soc.*, 1995, **117**, 4173.
40 P. Ramlall and R. A. McClelland, *J. Chem. Soc., Perkin Trans. 2*, 1999, 225.
41 M. Novak, M. J. Kahley, J. Lin, S. A. Kennedy and L. A. Swanegan, *J. Am. Chem. Soc.*, 1994, **116**, 11626.
42 M. Novak and S. A. Kennedy, *J. Am. Chem. Soc.*, 1995, **117**, 574.
43 M. Novak, A. J. VandeWater, A. J. Brown, S. A. Sanzenbacher, L. A. Hunt, B. A. Kolb and M. E. Brooks, *J. Org. Chem.*, 1999, **64**, 6023.
44 S. A. Glover and A. Rauk, *J. Org. Chem.*, 1996, **61**, 2337.
45 S. A. Glover, G. Mo and A. Rauk, *Tetrahedron*, 1999, **55**, 3413.
46 S. A. Glover and A. Rauk, *J. Org. Chem.*, 1999, **64**, 2340.
47 S. A. Glover, *Arkivok*, 2001 Issue in Honour of Prof. O. S. Tee, ms. OT-308C (http://www.arkat-usa.org/ark/journal/Volume2/Part3/Tee/OT-308C/OT-308.htm).
48 S. A. Glover, G. Mo, A. Rauk, D. Tucker and P. Turner, *J. Chem. Soc., Perkin Trans. 2*, 1999, 2053.
49 A.-M. E. Gillson, S. A. Glover and P. Turner, *Org. Biomol. Chem.*, submitted.
50 A. M. Maxam and W. Gilbert, *Proc. Natl. Acad. Sci.*, 1977, **74**, 560.
51 A. M. Maxam and W. Gilbert, *Methods Enzymol.*, 1980, **65**, 499.
52 W. B. Mattes, J. A. Hartley and K. W. Kohn, *Biochim. Biophys. Acta*, 1986, **868**, 71.
53 W. B. Mattes, J. A. Hartley and K. W. Kohn, *Nucleic Acids Res.*, 1986, **14**, 2971.
54 D. M. Maron and B. N. Ames, *Mutat. Res.*, 1983, **113**, 173.