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The role of steric effects in the direct mutagenicity of *N*-acyloxy-*N*-alkoxyamides

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Abstract

Electrophilic *N*-acyloxy-*N*-alkoxyamides are mutagenic in *Salmonella typhimurium* TA100 without the need for S9 metabolic activation and they react with DNA at guanine—N7 at physiological pH. Since these are direct-acting mutagens, structural factors influence binding and reactivity with DNA. Mutagenicity in TA100 can be predicted by a QSAR incorporating hydrophobicity (log *P*), stability to substitution reactions at nitrogen (pK_a of the leaving acid) and steric effects of *para*-aryl substituents (E_s). A number of mutagens exhibit activities that deviate markedly from the predicted values and they fall into two classes: di-*tert*-butylated *N*-benzoyloxy-*N*-benzyloxybenzamides, which – because of their size – are most probably excluded from the major groove or are unable to achieve a transition state for reaction with DNA, and *N*-benzoyloxy-*N*-butoxyalkylamides with branching α-to the amide carbonyl, which are resistant to S_N2 reactions at the amide nitrogen.
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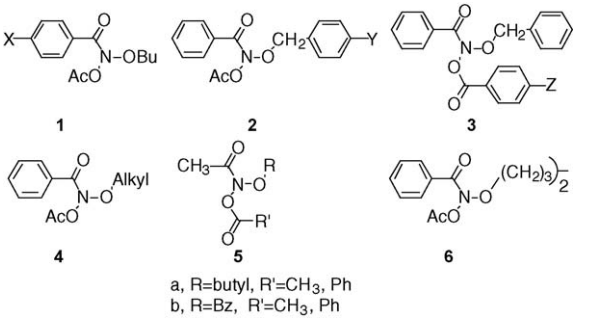
Keywords: Mutagenicity; Direct-acting; Ames Test; Steric effects; QSAR; Electrophiles; Amides

1. Introduction

N-Acyloxy-*N*-alkoxyamides are a class of direct-acting mutagens that we have shown to react with DNA in the major groove at guanine—N7 (G—N7) at physiological pH [1–7]. Results have pointed to an S_N2 reaction with DNA, which involves displacement of the *N*-acyloxy group by the N7 lone pair of guanine [7]. The mutagenicity of a wide range of congeners (**1–6**) has been measured in *Salmonella typhimurium* strain TA100 and mutagenic activity has been found to be dependent upon a number of variables.



Activity correlates with stability or pK_a of the carboxylic acid leaving group in that mutagens that are the most reactive towards S_N2 reactions are the least

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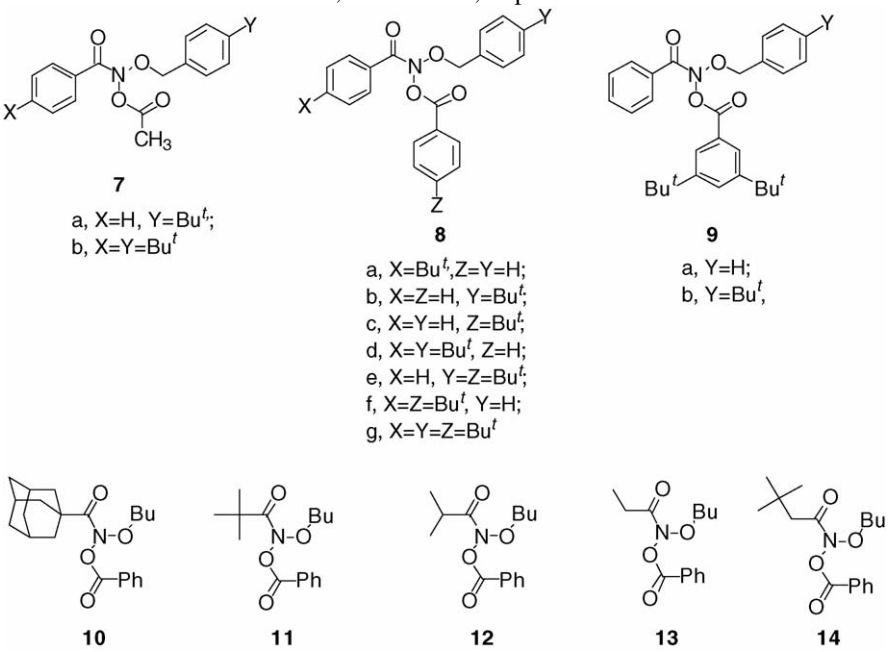
mutagenic [5,6]. Furthermore, there is an obvious dependence upon hydrophobicity of the intact mutagens (as estimated from log *P*, the log of the octanol/water partition coefficient). Since these mutagens react at G—N7 and require no metabolic activation, it is reasonable to propose that at physiological pH, the hydrophobic effect reflects binding of the molecules in the major groove of DNA. More recently, we have determined that steric effects also play a role and, using Taft steric parameters, E_s, for substituents attached to the *para* positions of benzene rings on each of the three side chains, the pK_a of the departing acid and log *P*, a quantitative structure–activity relationship has been derived, which predicts well the activity of a wide range of *N*-acyloxy-*N*-alkoxyamides (Eq. (1))[8].

Analysis of Eq. (1) indicates a strong dependence upon log *P*, significant correlations with pK_a and, where present, steric effects of groups at *para* positions on both the amide and benzyloxy side chains. However, statistically, steric effects due to substituents on the leaving group would appear to be less important. The dependence upon hydrophobicity contrasts with the hitherto prevailing view that a lack of correlation with log *P* is in fact a characteristic of direct-acting mutagens [9–11].

The relationship in Eq. (1) provides an excellent means of probing the effect of substructure upon mutagenic activity and therefore upon binding to DNA. In this paper, we describe two sets of mutagens for which the QSAR fails in that both are found to be significantly less mutagenic than predicted. The deviation from the predicted values can be ascribed on the one hand to distal and, on the other, to proximal steric effects.



$$\log \text{TA } 100^{1} = 0.29(\pm 0.03)\log P + 0.22(\pm 0.09)\text{p}K_a + 0.14(\pm 0.04)E_s^1 + 0.16(\pm 0.04)E_s^2 + 0.11(\pm 0.06)E_s^3 + 0.81(\pm 0.5)$$
$$n = 41, \quad \text{standard error} = 0.17, \quad r = 0.891, \quad F = 29.17 \qquad (1)$$

E_S^1, E_S^2 and E_S^3 represent steric effects on the benzamide, benzyloxy and benzoyloxy side chains, respectively.

[1] Log of mutagenic activity in *Salmonella typhimurium* TA100 at 1 μmol/plate taken from the gradients of revertant colonies versus dose in the linear range of dose response curves.

2. Materials and methods

2.1. Instrumentation

Melting points were determined on a Reichert Microscopic Hot-Stage and are uncorrected. Infrared spectra were recorded on a Perkin Elmer 1725× FT spectrophotometer. Nuclear Magnetic Resonance spectra (300 MHz) were recorded in $CDCl_3$ on a Bruker AC 300P FT NMR spectrometer. ESI mass spectra were obtained on a Varian 1200L Mass Spectrometer. Micro-analytical data were obtained from the Research School of Chemistry, Australian National University, Canberra.

2.2. Chemicals

Syntheses of *N*-acetoxy-*N*-butoxybenzamide (**1**, X = H [124617-84-1]), *N*-acetoxy-*N*-(4-*tert*-butylbenzyloxy)benzamide(**2**, Y = Bu^t [139259-93-1]) [4], *N*-benzoyloxy-*N*-benzyloxybenzamide (**3**, Z = H [220168-39-8]) [5], *N*-benzoyloxy-*N*-(4-*tert*-butylbenzyloxy) benzamide (**8b** [37757-31-4]) [12] and *N*-benzyloxy-*N*-(4-*tert*-butylbenzoyloxy) benzamide (**8c** [220168-56-9]) [5] have been described previously as have those of the precursors to **7b**, **8d** and **8g**, *N*-(4-*tert*-butylbenzyloxy)-4-*tert*-butylbenzamide [637757-29-0] and *N*-(4-*tert*-butylbenzyloxy)-*N*-chloro-4-*tert*-butylbenzamide [637757-30-3] [12], **9a**, *N*-benzyloxybenzamide [3532-25-0] and *N*-benzyloxy-*N*-chlorobenzamide [3532-25-0] [4] and **9b**, *N*-(4-*tert*-butylbenzyloxy)benzamide [93949-93-0] and *N*-(4-*tert*-butylbenzyloxy)-*N*-chlorobenzamide [112403-68-6] [4].

2.2.1. General syntheses of N-alkoxybenzamides

2.2.1.1. Method 1 [13]. The appropriate *N*-alkoxybenzamide was synthesised in good yield by the reaction at room temperature (r.t.), overnight, of 1 equivalent of the potassium salt of an appropriate hydroxamic acid [14] with 1.5 equivalents of the relevant alkyl bromide and a 10% excess of sodium carbonate in a 50% aqueous solution of methanol. After refluxing for 3 h, excess methanol was removed in vacuo and the mixture extracted with dichloromethane (DCM), which was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. Crude mixtures were purified by column or centrifugal chromatography.

N-Benzyloxy-4-tert-butylbenzamide. Potassium 4-*tert*-butylbenzohydroxamate (6.11 g, 26.4 mmol), benzyl bromide (6.77 g, 39.6 mmol) and sodium carbonate (3.08 g, 29.0 mmol) were stirred overnight in 50% aqueous methanol (150 ml) and refluxed for 24 h. Excess methanol was removed in vacuo and the mixture extracted with DCM, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. Purification using centrifugal chromatography (15% EtOAc:85% petroleum spirit), produced *N*-benzyloxy-4-*tert*-butylbenzamide in 45.6% yield. m.p. 84–86 °C; ν_{max} ($CHCl_3$)/cm^{-1} 3300 (w) and 1680 (s). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.35 (9H, s, C(C$\underline{H}_3$)$_3$), 5.07 (2H, s, O$\underline{CH}_2$Ar), 7.42 (5H, m, Ar$\underline{H}$), 7.46 (2H, t, *o*'-Ar$\underline{H}$), and 7.63 (2H, d, *o*-ArH). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 31.5 (q, Ar-C(C$\underline{H}_3$)$_3$), 34.8 (s, Ar-$\underline{C}$(CH$_3$)$_3$), 77.1 (t, O$\underline{CH}_2$Ar), 125.3 (d, *m*-Ar$\underline{C}$-*t*Bu), 126.9 (d, *o*-Ar$\underline{C}$-*t*Bu), 127.3 (d, *o*-Ar$\underline{C}$-CO), 127.4 (s, *p*-Ar$\underline{C}$-CO), 128.7 (d, *m*-Ar$\underline{C}$-CO), 130.4 (s, Ar$\underline{C}$-CO), 140.9 (s, OCH$_2$-Ar$\underline{C}$), 150.5 (s, Ar$\underline{C}$-C(CH$_3$)$_3$), and 163.0 (s, Ar-$\underline{C}$O-NH). Calc. C 76.30, H 7.47, N 4.94%; found: C 76.30, H 7.20, N 4.78%.

N-Butoxypropanamide. Potassium propanohydroxamate (5.33 g, 41.9 mmol), butyl bromide (5.74 g, 41.9 mmol) and sodium carbonate (4.89 g, 46.1 mmol) were stirred overnight in 50% aqueous methanol (150 ml) and refluxed for 24 h. Excess methanol was removed in vacuo and the mixture extracted with DCM, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. Purification by centrifugal chromatography using 10% ethyl acetate/petroleum spirit, afforded pure *N*-butoxypropanamide (0.92 g, 6.34 mmol, 15%) as a pale yellow oil; ν_{max} ($CHCl_3$)/cm^{-1} 3188 (w, br), 1664 (s). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 0.76 (3H, t, CH_3), 0.99 (3H, t, COCH$_2$C$\underline{H}_3$), 1.22 (2H, sx, C$\underline{H}_2$CH$_3$), 1.47 (2H, quintet, OCH$_2$C$\underline{H}_2$), 2.01 (2H, q, CO$\underline{CH}_2$CH$_3$), 3.72 (2H, t, O$\underline{CH}_2$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 9.5 (q), 13.6 (q), 18.8 (t), 26.1 (t), 29.9 (t), 75.8 (t), 171.8 (s). MS(ESI): *m*/*z* = 168 (M + 23).

N-Butoxyisobutyramide. Potassium isobutyrohydroxamate (2.01 g, 14.2 mmol), butyl bromide (1.95 g, 14.2 mmol) and sodium carbonate (1.66 g, 15.7 mmol) were stirred overnight in 50% aqueous methanol (150 ml) and refluxed for 24 h. Excess methanol was removed in vacuo and the mixture extracted with DCM, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. Purification by centrifugal chromatography using 10% ethyl acetate/petroleum spirit, afforded pure *N*-butoxyisobutyramide (0.53 g, 3.33 mmol, 24%) as a pale yellow oil; ν_{max} ($CHCl_3$)/cm^{-1} 3188br (w), 1657 (s). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 0.80 (3H, t, CH_3), 1.02 (6H, d, CH(C$\underline{H}_3$)$_2$), 1.27 (2H, sx, C$\underline{H}_2$CH$_3$), 1.51 (2H, quintet, OCH$_2$C$\underline{H}_2$), 2.34 (1H, m, C$\underline{H}$(CH$_3$)$_2$), 3.77 (2H, t, O$\underline{CH}_2$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 13.8 (q), 19.0 (q), 30.0 (t), 32.2 (t), 76.6 (t), 174.9 (s). MS(ESI): *m*/*z* = 182 (M + 23).

2.2.1.2. Method 2. *N*-Butoxybenzamide (8.30 g, 46.3 mmol) in ethanol (45 ml) and conc. HCl (15 ml), was refluxed for 45 min. Excess ethanol was removed under reduced pressure, water added and the mixture extracted with ether. Concentration of the aqueous layer to dryness under reduced pressure afforded butoxyamine hydrochloride (3.99 g, 31.8 mmol, 69%) as a white solid, which was used without further purification.

An acid chloride (neat or in an ethereal solution) was added dropwise to an equimolar quantity of *N*-butoxyamine (hydrochloride) in dry ether containing a two molar excess of triethylamine such that the temperature remained below 5 °C. After stirring for 2 h, the ether layer was washed with water, 10% $NaHCO_3$, dried and concentrated to afford the *N*-alkoxyamide in good yield. Crude mixtures were purified by column or centrifugal chromatography.

N-Butoxyadamantane-1-carboxamide. Purification by centrifugal chromatography using 10% ethyl acetate/petroleum spirit, afforded pure *N*-butoxyadamantane-1-carboxamide (0.08 g, 0.32 mmol, 25%) as a pale yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1681 (s). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 0.75 (3H, t, CH_3), 1.22 (2H, sx, C$\underline{H}_2$CH$_3$), 1.45 (2H, quintet, OCH$_2$C$\underline{H}_2$), 1.55 (6H, t, CHC$\underline{H}_2$CH), 1.75 (6H, d, CHC$\underline{H}_2$C), 1.84 (3H, m, C$\underline{H}$), 3.72 (2H, t, O$\underline{CH}_2$), 9.45 (1H, s, N$\underline{H}$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 13.7 (q), 19.0 (t), 27.9 (t), 29.7 (t), 36.4 (t), 38.9 (d), 39.8 (s), 75.3 (t), 175.4 (s). MS(ESI): *m*/*z* = 274 (M + 23).

N-Butoxy-2,2-dimethylpropanamide [64214-68-2]. Purification by centrifugal chromatography using 10% ethyl acetate/petroleum spirit, afforded pure *N*-butoxy-2,2-dimethylpropanamide (0.11 g, 0.63 mmol, 42%) as a pale yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1683 (s). ^{1}H NMR ($CDCl_3$,

300 MHz) δ = 0.95 (3H, t, CH_3), 1.22 (9H, s, C($\underline{CH_3}$)$_3$), 1.42 (2H, sx, $\underline{CH_2}CH_3$), 1.63 (2H, quintet, $OCH_2\underline{CH_2}$), 3.91 (2H, t, $O\underline{CH_2}$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 13.8 (q), 19.1 (t), 27.2 (q), 30.1 (t), 38.0 (s), 76.4 (t), 176.2 (s). MS(ESI): *m*/*z* = 196 (M + 23).

N-Butoxy-3,3-dimethylbutyramide. Purification by centrifugal chromatography using 10% ethyl acetate/petroleum spirit, afforded pure *N*-butoxy-3,3-dimethylbutyramide (0.83 g, 4.43 mmol, 70%) as a pale yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1653 (s). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 0.77 (3H, t, CH_3), 0.94 (9H, s, C($\underline{CH_3}$)$_3$), 1.26 (2H, sx, $\underline{CH_2}CH_3$), 1.47 (2H, quintet, $OCH_2\underline{CH_2}$), 1.92 (2H, s, $\underline{CH_2}$C(=O)N), 3.76 (2H, t, $O\underline{CH_2}$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 13.7 (q), 18.9 (t), 29.7 (m), 30.0 (s), 30.7 (t), 46.1 (t), 75.9 (t), 169.5 (s). MS(ESI): *m*/*z* = 210 (M + 23).

2.2.2. Synthesis of N-alkoxy-N-chloroamides

A solution of the appropriate *N*-alkoxyamide and a 2–3 molar excess of *tert*-butyl hypochlorite [15] in CH_2Cl_2 was stirred in the dark under anhydrous conditions for 3–6 h. The progress of the reaction was monitored by thin-layer chromatography until complete, at which time the solvent and excess *tert*-butyl hypochlorite were removed in the dark under reduced pressure at 30 °C to afford the *N*-alkoxy-*N*-chloroamides in excellent yields (90–100%). The *N*-alkoxy-*N*-chloroamides were used without further purification.

2.2.2.1. N-Benzyloxy-N-chloro-4-tert-butylbenzamide. N-Benzyloxy-*N*-chloro-(4-*tert*-butyl)benzamide was produced as a yellow oil, in 90.2% yield. ν_{max} ($CHCl_3$)/cm^{-1} 1710 (C=O). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.36 (9H, s, C($\underline{CH_3}$)$_3$), 5.11 (2H, s, $O\underline{CH_2}$Ar), 7.32 (5H, m, Ar$\underline{H}$), 7.44 (2H, d, Ar$\underline{H}$), and 7.68 (2H, d, *o*-ArH).

2.2.2.2. N-Butoxy-N-chloropropanamide. N-Butoxy-*N*-chloropropanamide was produced as a yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1742br (s). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 0.97 (3H, t, CH_3), 1.19 (3H, t, $COCH_2\underline{CH_3}$), 1.43 (2H, sx, $\underline{CH_2}CH_3$), 1.67 (2H, quintet, $OCH_2\underline{CH_2}$), 2.59 (2H, q, $CO\underline{CH_2}CH_3$), 4.05 (2H, t, $O\underline{CH_2}$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 8.7 (q), 13.7 (q), 19.1 (t), 27.6 (t), 29.5 (t), 75.3 (t), 179.2 (s).

2.2.2.3. N-Butoxy-N-chloroisobutyramide. N-Butoxy-*N*-chloroisobutyramide was produced as a yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1735 (s). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 0.97 (3H, t, CH_3), 1.20 (6H, d, $CH(\underline{CH_3})_2$), 1.44 (2H, sx, $\underline{CH_2}CH_3$), 1.68 (2H, quintet, $OCH_2\underline{CH_2}$), 3.04 (1H, m, $\underline{CH}(CH_3)_2$), 4.08 (2H, t, $O\underline{CH_2}$).

2.2.2.4. N-Butoxy-N-chloroadamantane-1-carboxamide. *N*-Butoxy-*N*-chloroadamantane-1-carboxamide was produced as a yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1721 (s). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.01 (3H, t, CH_3), 1.48 (2H, sx, $\underline{CH_2}CH_3$), 1.70 (2H, quintet, $OCH_2\underline{CH_2}$), 1.73 (6H, t, $CH\underline{CH_2}CH$), 2.03 (9H, m, $CH\underline{CH_2}$C and $\underline{CH}$), 4.11 (2H, t, $O\underline{CH_2}$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 13.8 (q), 19.3 (t), 28.1 (t), 29.6 (t), 31.2 (t), 36.4 (d), 38.2 (s), 44.4 (s), 74.4 (t), 181.5 (s).

2.2.2.5. N-Butoxy-N-chloro-2,2-dimethylpropanamide. N-Butoxy-*N*-chloro-2,2-dimethylpropanamide was produced as a yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1727 (s). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 0.95 (3H, t, CH_3), 1.28 (9H, s, C($\underline{CH_3}$)$_3$), 1.43 (2H, sx, $\underline{CH_2}CH_3$), 1.66 (2H, quintet, $OCH_2\underline{CH_2}$), 4.08 (2H, t, $O\underline{CH_2}$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 13.6 (q), 19.2 (t), 27.1 (q), 29.4 (t), 41.0 (s), 774.5 (t), 182.1 (s).

2.2.2.6. N-Butoxy-N-chloro-3,3-dimethylbutyramide. N-Butoxy-*N*-chloro-3,3-dimethylbutyramide was produced as a yellow oil, which was used without further purification. ν_{max} ($CHCl_3$)/cm^{-1} 1742 (s). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 0.98 (3H, t, CH_3), 1.07 (9H, s, C($\underline{CH_3}$)$_3$), 1.46 (2H, sx, $\underline{CH_2}CH_3$), 1.68 (2H, quintet, $OCH_2\underline{CH_2}$), 2.67 (2H, s, $\underline{CH_2}$C(=O)N), 4.05 (2H, t, $O\underline{CH_2}$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 13.8 (q), 19.2 (t), 29.6 (m), 30.0 (s), 31.4 (t), 48.0 (t), 75.1 (t), 171.8 (s).

2.2.3. General synthesis of N-acyloxy-N-alkoxyamides

N-Alkoxy-*N*-chloroamides were acetoxylated or benzoyloxylated by treatment with 1.4 molar equivalents of anhydrous sodium acetate or the appropriate sodium benzoate in dry acetone, at room temperature, for 12–72 h in the dark. The reaction was monitored by thin-layer chromatography. Filtration and concentration in vacuo provided the *N*-acetoxy or *N*-benzoyloxy derivatives, frequently with quantitative conversion. Products were obtained in a pure state or were further purified by centrifugal chromatography (15% EtOAc:85% petroleum spirit or 10% EtOAc:90% petroleum spirit). In all cases, mutagens were characterised spectroscopically. For all *N*-benzoyloxy-*N*-benzyloxy benzamides, the ^{1}H and ^{13}C NMR chemical shift assignment for the benzoyloxy leaving group are indicated by the symbol (*).

2.2.3.1. N-Acetoxy-N-(p-tert-butylbenzyloxy)-p-tert-butylbenzamide **(7b** *[693809-12-0]).* Purification by centrifugal chromatography (15% EtOAc:85% petroleum spirit) provided the title compound as a yellow oil in 84% yield. ν_{max} ($CHCl_3$)/cm^{-1} 1783 (s, ester CO) and 1725 (s, amide CO). ^{1}H NMR ($CDCl_3$, 300 MHz) δ = 1.34 (9H, s, $\underline{Bu^t}$–Ar), 1.35 (9H, s, $\underline{Bu^t}$–Ar), 2.09 (3H, s, $COC\underline{H_3}$) 5.19 (2H, s, $OC\underline{H_2}$Ar), 7.37 (2H, d, Ar$\underline{H}$), 7.42 (2H, d, Ar$\underline{H}$), 7.45 (2H, d, Ar$\underline{H}$) 7.74 (2H, d, Ar$\underline{H}$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 18.8 ($CO\underline{C}H_3$), 31.1 ($C(\underline{C}'H_3)_3$), 31.3 ($C(\underline{CH_3})_3$), 34.6 ($\underline{C}'(CH_3)_3$) 35.1 ($\underline{C}(CH_3)_3$), 77.3 ($O\underline{C}H_2$Ar), 125.3 (*o*′-Ar$\underline{C}$H), 125.4 (*o*-Ar$\underline{C}$H), 128.7 (i-Ar$\underline{C}$), 129.2 (*m*- and *m*′-Ar$\underline{C}$H), 131.8 (i′-Ar$\underline{C}$), 151.8 (p′-Ar$\underline{C}$), 156.6 (*p*-Ar$\underline{C}$), 168.1 ($\underline{C}OCH_3$), 174.0 ($\underline{C}$ON).1.33 (9H, s, C[$\underline{CH_3}$]$_3$), 1.35 (9H, s, C[$\underline{CH_3}$]$_3$), 2.08 (3H, OC(O)$\underline{CH_3}$, s), 5.17 (2H, s, $O\underline{CH_2}$Ar), 7.32-7.45 (6H, m, Ar$\underline{H}$), and 7.71 (2H, d, *o*-Ar$\underline{H}$).

2.2.3.2. N-Benzoyloxy-N-benzyloxy-p-tert-butylbenzamide **(8a***[693809-10-8]).* Purification by centrifugal chromatography (15% EtOAc:85% petroleum spirit) provided the title

compound as a yellow oil in 42% yield. ν_{max} ($CHCl_3$)/cm^{-1} 1757 (s, ester CO) and 1724 (s, amide CO). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 1.33$ (9H, s, C[CH_3]$_3$), 5.30 (2H, s, OCH_2Ar), 7.30–7.52 (9H, m, ArH), 7.61 (1H, t, *p**-ArH), 7.98 (2H, d, *o*-ArH), and 8.10 (2H, d, *o**-ArH). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 31.3$ (q, Ar-C[CH_3]$_3$) 34.8 (s, Ar-C[CH_3]$_3$), 77.4 (t, OCH_2Ar), 125.4 (d, *m*-ArC-*t*Bu), 126.9 (d, *o*-ArC-*t*Bu), 127.4 (d, ArC), 128.3 (d, *m**-ArC), 128.6 (d, *m*-ArC), 130.1 (d, *o**-ArC), 130.4 (s, ArC-C(O)N), 130.7 (s, C*-C(O)ON), 133.4 (d, *p**-ArC), 140.6 (s, OCH_2-ArC), 150.8 (s, ArC-C[CH3]3), 157.3 (s, Ar-CO-NH), and 173.4 (s, OC*OAr).

*2.2.3.3. N-Benzoyloxy-N-(p-tert-butylbenzyloxy)-p-tert-butylbenzamide (***8d** *[693809-14-2]).* Purification by centrifugal chromatography (15% EtOAc:85% petroleum spirit) provided the title compound as a yellow oil in 74% yield. ν_{max} ($CHCl_3$)/cm^{-1} 1756 (s, ester CO) and 1730 (s, amide CO). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 1.29$ (9H, s, C[CH_3]$_3$), 1.34 (9H, s, C[CH_3]$_3$), 5.26 (2H, s, OCH_2Ar), 7.34 (4H, s, *o′,m′*-ArH-*t*Bu), 7.38-7.47 (4H, m, ArH), 7.61 (1H, t, *p**-ArH), 7.74 (2H, d, *o*-ArH), and 7.98 (2H, d, *o**-ArH). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 31.1$ (q, Ar-C[CH_3]$_3$), 31.3 (q, ArC[CH_3]$_3$), 34.6 (s, Ar-C[CH_3]$_3$), 35.1 (s, Ar-C[CH_3]$_3$), 77.5 (t, OCH_2Ar), 125.3 (d, *m*-ArC), 125.4 (d, *m*-ArC), 125.6 (d, *o*-ArC), 127.6 (d, *o*-ArC-C(O)N), 128.5 (d, *m**-ArC), 129.1 (d, *o**-ArC), 129.3 (s, ArC-C(O)N), 130.0 (s, ArC-C(O)ON), 131.9 (d, *p**-ArC), 133.8 (s, ArC-CH_2ON), 151.7 (s, ArC-*t*Bu), 156.6 (s, ArC-*t*Bu), 164.3 (s, Ar-C(O)N), and 174.2 (s, OC*(O)Ar).

*2.2.3.4. N-(p-Tert-butylbenzoyloxy)-N-(p-tert-butylbenzyloxy)benzamide (***8e** *[693809-16-4]).* Purification by centrifugal chromatography (15% EtOAc:85% petroleum spirit) provided the title compound as a yellow solid in 63% yield. ν_{max} ($CHCl_3$)/cm^{-1} 1754 (s, ester CO) and 1726 (s, amide CO). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 1.29$ (9H, s, C[CH_3]$_3$), 1.35 (9H, s, C*[CH_3]$_3$), 5.25 (2H, s, OCH_2Ar), 7.35 (4H, s, *o′,m′*-ArH-*t*Bu), 7.38-7.47 (4H, m, ArH), 7.53 (1H, t, *p*-ArH), 7.83 (2H, d, *o*-ArH), and 7.90 (2H, d, *o**-ArH). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 31.0$ (q, Ar-C[CH_3]$_3$), 31.3 (q, ArC*[CH_3]$_3$), 34.6 (s, Ar-C[CH_3]$_3$), 35.2 (s, Ar-C*[CH_3]$_3$), 77.4 (t, OCH_2Ar), 124.5 (d, *m**-ArC), 125.4 (d, *m*-ArC-*t*Bu), 125.6 (d, *o*-ArC), 127.0 (s, ArC*-C(O)ON), 128.2 (d, *o*-ArC), 129.1 (d, *m*-ArC), 129.9 (d, *o**-ArC), 131.9 (s, ArC-C(O)N), 132.6 (s, OCH_2-ArC), 151.7 (s, ArC-C[CH_3]$_3$), 157.8 (s, *p**-ArC), 164.2 (s, Ar-C(O)N), and 174.4 (s, OC*(O)Ar).

*2.2.3.5. N-(p-Tert-butylbenzoyloxy)-N-benzyloxy-p-tert-butylbenzamide (***8f** *[693809-16-4]).* Purification by centrifugal chromatography (15% EtOAc:85% petroleum spirit) provided the title compound as a yellow oil in 47% yield. ν_{max} ($CHCl_3$)/cm^{-1} 1757 (s, ester CO) and 1726 (s, amide CO). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 1.32$ (9H, s, C[CH_3]$_3$), 1.35 (9H, s, C*[CH_3]$_3$), 5.29 (2H, s, OCH_2Ar), 7.31-7.48 (9H, m, ArH), 7.77 (2H, d, *o*-ArH), and 7.93 (2H, d, *o**-ArH). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 31.1$ (q, Ar-C[CH_3]$_3$), 31.5 (q, ArC*[CH_3]$_3$), 34.4 (s, Ar-C[CH_3]$_3$), 35.0 (s, Ar-C*[CH_3]$_3$), 77.4 (t, OCH_2Ar), 125.2 (d, *m**-ArC), 125.4 (d, *m*-ArC), 126.8 (d, *o*-ArC-C(O)N), 127.1 (d, ArC), 127.6 (s, ArC-C(O)ON), 128.5 (d, *m*-ArC), 129.9 (d, *o**-ArC), 130.1 (s, ArC-C(O)N), 139.8 (s, ArC-CH_2ON), 150.7 (s, *p*-ArC), 154.6 (s, *p**-ArC), 160.3 (s, Ar-C(O)N), and 174.2 (s, OC*(O)Ar).

*2.2.3.6. N-(3,5-Di-tert-butylbenzoyloxy)-N-benzyloxybenzamide (***9a** *[693809-20-0]).* Purification by centrifugal chromatography (15% EtOAc:85% petroleum spirit) provided the title compound as a yellow oil in 51% yield. ν_{max} ($CHCl_3$)/cm^{-1} 1759 (s, ester CO) and 1729 (s, amide CO). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 1.33$ (18H, s, C[CH_3]$_3$), 5.31 (2H, s, OCH_2Ar), 7.32–7.45 (7H, m, ArH), 7.52 (1H, t, *p*-ArH), 7.70 (1H, fine triplet, *p**-ArH), 7.78 (2H, d, *o*-ArH), and 7.82 (2H, d, *o**-ArH). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 31.4$ (2q, Ar-C[CH_3]$_3$), 35.2 (s, Ar-C[CH_3]$_3$), 76.9 (t, OCH_2Ar), 124.2 (d, *o**-ArC), 127.1 (d, *p**-ArC), 127.3 (d, *o'*-ArC), 127.4 (d, *p-ArC),* (128.6 (d, *m*-ArC), 129.8 (s, ArC*-C(O)ON), 131.3 (d, *p*-ArC), 132.9 (s, ArC-C(O)N), 139.8 (s, ArC-CH_2ON), 149.7 (s, ArC-*t*Bu), 164.3 (s, Ar-C(O)N), and 174.2 (s, OC*(O)Ar).

*2.2.3.7. N-(3,5-Di-tert-butylbenzoyloxy)-N-(p-tert-butylbenzyloxy)benzamide (***9b***).* Purification by centrifugal chromatography (15% EtOAc:85% petroleum spirit) provided the title compound as a yellow oil in 58% yield. ν_{max} ($CHCl_3$)/cm^{-1} 1760 (s, ester CO) and 1728 (s, amide CO). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 1.29$ (9H, s, C[CH_3]$_3$), 1.34 (18H, s, C[CH_3]$_3$), 5.26 (2H, s, OCH_2Ar), 7.29 (4H, s, o′, m′-ArH-tBu), 7.38-7.44 (2H, m, ArH), 7.51 (1H, t, *p*-ArH), 7.66 (1H, fine triplet, p*-ArH), 7.81 (2H, d, *o*-ArH), and 7.82 (2H, d, *o**-ArH). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 31.3$ (C(C′H_3)$_3$ and C(C*H_3)$_3$), 34.6 (C′ (CH_3)$_3$), 34.9 (C*(CH_3)$_3$), 77.4 (OCH_2Ar), 124.3 (m'-ArCH), 125.4, 126.8, 128.3, 129.2 and 132.6 (ArCH) 131.8 and 132.1 (I-ArC or i′-ArC), 151.0 and 151.7 (i*-ArC and p′-ArC), 151.4 (C*(CH_3)$_3$), 164.9 (CON), 174.5 (OCOAr).

*2.2.3.8. N-Benzoyloxy-N-butoxypropanamide (***13***).* Purification by centrifugal chromatography using 10% ethyl acetate/petroleum spirit afforded pure *N*-benzoyloxy-*N*-butoxypropanamide (0.41 g, 1.55 mmol, 47%) as a pale yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1745 s (s, amide C=O), 1766 (s, ester C=O). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 0.90$ (3H, t, CH_3), 1.18 (3H, t, $COCH_2CH_3$), 1.36 (2H, sx, CH_2CH_3), 1.67 (2H, quintet, OCH_2CH_2), 2.61 (2H, q, $COCH_2CH_3$), 4.14 (2H, t, OCH_2), 7.45 (2H, t, *m*-Ar-H), 7.59 (1H, t, *p*-Ar-H), 8.05 (2H, d, *o*-Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 8.1$ (q), 13.7 (q), 19.0 (t), 27.1 (t), 30.1 (t), 75.9 (t), 127.4 (s), 128.6 (d), 130.0 (d), 134.0 (d), 164.1 (s), 180.1 (s). MS(ESI): $m/z = 288$ (M + 23).

*2.2.3.9. N-Benzoyloxy-N-butoxyisobutyramide (***12***).* Purification by centrifugal chromatography using 10% ethyl acetate/petroleum spirit afforded pure *N*-benzoyloxy-*N*-

butoxyisobutyramide (0.19 g, 0.68 mmol, 25%) as a pale yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1736 (s, amide C=O), 1767 (s, ester C=O). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 0.93$ (3H, t, CH_3), 1.24 (6H, d, $CH(\underline{CH_3})_2$), 1.41 (2H, sx, $\underline{CH_2}CH_3$), 1.69 (2H, quintet, $OCH_2\underline{CH_2}$), 3.01 (1H, m, $\underline{CH}(CH_3)_2$), 4.19 (2H, t, $O\underline{CH_2}$), 7.47 (2H, t, *m*-Ar-H), 7.61 (1H, t, *p*-Ar-H), 8.08 (2H, d, *o*-Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 13.7$ (q), 18.9 (q), 30.1 (t), 32.6 (t), 76.0 (t), 127.4 (s), 128.6 (d), 130.0 (d), 134.0 (d), 164.0 (s), 182.6 (s). MS(ESI): $m/z = 302$ (M + 23).

*2.2.3.10. N-Benzoyloxy-N-butoxyadamantane-1-carboxamide (**10**).* Purification by centrifugal chromatography using 10% ethyl acetate/petroleum spirit afforded pure *N*-benzoyloxy-*N*-butoxyadamantane-1-carboxamide (0.16g, 0.43 mmol, 22%) as a pale yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1721 (s, amide C=O), 1755 (s, ester C=O). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 0.95$ (3H, t, CH_3), 1.45 (2H, sx, $\underline{CH_2}CH_3$), 1.71 (2H, quintet, $OCH_2\underline{CH_2}$), 1.76 (6H, t, $CH\underline{CH_2}CH$), 2.06 (3H, m, $\underline{CH}$), 2.10 (6H, d, $CH\underline{CH_2}C$), 4.20 (2H, t, $O\underline{CH_2}$). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 13.8$ (q), 19.2 (t), 28.0 (t), 30.1 (t), 36.5 (d), 38.0 (s), 43.2 (s), 75.0 (t), 127.9 (s), 128.6 (d), 130.0 (d), 133.7 (d), 164.1 (s), 182.2 (s). MS(ESI): $m/z = 394$ (M + 23).

*2.2.3.11. N-Benzoyloxy-N-butoxy-2,2-dimethylpropanamide (**11**[693809-37-9]).* Purification by centrifugal chromatography using 10% ethyl acetate/petroleum spirit afforded pure *N*-benzoyloxy-*N*-2,2-dimethylpropanamide (0.17 g, 0.58 mmol, 60%) as a pale yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1728 (s, amide C=O), 1763 (s, ester C=O). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 0.94$ (3H, t, CH_3), 1.37 (9H, s, $C(\underline{CH_3})_3$), 1.39 (2H, sx, $\underline{CH_2}CH_3$), 1.69 (2H, quintet, $OCH_2\underline{CH_2}$), 4.19 (2H, t, $O\underline{CH_2}$), 7.49 (2H, t, *m*-Ar-H), 7.63 (1H, t, *p*-Ar-H), 8.10 (2H, d, *o*-Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 13.8$ (q), 19.2 (t), 27.1 (q), 30.0 (t), 40.6 (s), 75.1 (t), 127.9 (s), 128.6 (d), 129.9 (d), 133.8 (d), 164.0 (s), 182.8 (s). MS(ESI): $m/z = 316$ (M + 23).

*2.2.3.12. N-Benzoyloxy-N-butoxy-3,3-dimethylbutyramide (**14**).* Purification by centrifugal chromatography using 10% ethyl acetate/petroleum spirit afforded pure *N*-benzoyloxy-*N*-butoxy-3,3-dimethylbutyramide (0.29 g, 0.94 mmol, 52%) as a pale yellow oil. ν_{max} ($CHCl_3$)/cm^{-1} 1735 (s, amide C=O), 1763 (s, ester C=O). ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 0.83$ (3H, t, CH_3), 1.03 (9H, s, $C(\underline{CH_3})_3$), 1.34 (2H, sx, $\underline{CH_2}CH_3$), 1.60 (2H, quintet, $OCH_2\underline{CH_2}$), 2.44 (2H, s, $\underline{CH_2}C(=O)N$), 4.07 (2H, t, $O\underline{CH_2}$) 7.36 (2H, t, *m*-Ar-H), 7.50 (1H, t, *p*-Ar-H), 7.99 (2H, d, *o*-Ar-H). ^{13}C NMR ($CDCl_3$, 75 MHz) $\delta = 13.6$ (q), 19.0 (t), 29.5 (m), 30.0 (s), 30.9 (t), 45.5 (t), 75.6 (t), 127.4 (s), 128.6 (d), 129.5 (d), 133.9 (d), 163.8 (s), 177.2 (s). MS(ESI): $m/z = 330$ (M + 23).

2.3. Mutagenicity assays

S. typhimurium strain TA100 was obtained from Professor B.N. Ames, University of California, Berkeley, U.S.A. and cultured as described [16] 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 and 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. The efficacy of the strain in each set of experiments was verified using 10 μg of 4-nitroquinoline-*N*-oxide in 10 μl DMSO, which gave a 4+ reaction in a spot test [16].

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 (**1**, X = H) (log TA100 = 2.5) [6], which was always analysed in parallel with new mutagens.

2.4. Bimolecular rate studies

Rate constants for the bimolecular reaction between *N*-methylaniline and various mutagens were determined using ^1H NMR spectroscopy. Mutagen (2–10 mg) in d_4-methanol (400 μl) in an ultra-high precision NMR tube, was equilibrated at the required temperature in the probe of an NMR spectrometer. The sample was shimmed, removed from the probe and a micropipette was used to add a minimum of twice the molar equivalent of *N*-methyl aniline (2–20 μl). The exact time of mixing and the initial concentrations of both compounds were noted. After brief shimming, a series of acquisitions were accumulated at a preset time interval, and the extent of reaction was monitored by analysing the disappearance of both starting materials according to peak areas/heights of characteristic signals in the mutagen and the *N*-methyl resonance of the *N*-methylaniline. Initial substrate concentrations were obtained by back-extrapolation of concentration plots for both reagents to the initial time of mixing, t_o.

2.5. Calculations

Log *P* (log [octanol/water partition coefficient]) values were calculated by Ghose–Crippen methods as part of computation of optimised geometries at the AM1 semi-empirical level using SPARTAN [17].

3. Results

Mutagenicities of two series of mutagens are provided in Table 1 together with relevant data for related species from earlier studies. The first series comprises congeners

Table 1
Mutagenicities of the *N*-acyloxy-*N*-alkoxyamides in TA100 using **1**(X = H) as the normalisation standard

Set 1	μmol/plate	0.00	0.03	0.06	0.12	0.25	0.50	1.00	Slope[d]	Normalised slope[e]	Log TA100[f]	Diff.[g]
1 X = H	(Set 1)[a]	186.7 (15)			241.3(12)	353.7(8)		807.3(160	626	319	2.5	0.1
	(Set 2)[b]	211.7(13)	190.7(18)	240.3(9)	299.0(6)	426.3(13)	795.3(31)		940	319		
	(Set 3)[c]	193.7(13)			235.3(10)	347.7(37)	554.0(38)	845.3(23)	676	319		
7b	(Set 2)	211.7(13)	327.3(57)	488.3(28)	516.0(22)	516.0(22)	620.3(23)		2306	783	2.89	−0.04
8a	(Set 2)	211.7(13)	1217.7(50)	1338.7(45)	1364.3(40)	1500.3(65)	1621.0(39)		9391	3186	3.50	−0.24
8b	(Set 1)	186.7(15)	215.7(19)	323.3(10)	438.3(10)	472.7(23)	521.7(26)		2204	1123	3.05	0.16
8c	(Set 1)	186.7(15)	251.014)	443.014)	525.7(22)	608.7(41)	708(70.5)		2827	1441	3.20	0.19
8d	(Set 2)	211.7(13)	201.3(6)	230.0(5)	233.0(10)	274.0(19)	335.7(22)		130	44	1.64	1.65
8e	(Set 1)	186.7(15)	189.0(13)	189.3(12)	230.3(13)	237.7(10)	254.3(9)		142	72	1.86	1.56
8f	(Set 1)	186.7(15)	201.7(10)	220.7(6)	284.0(18)	381.3(48)	495.7(8)		805	410	2.51	0.97
8g	(Set 2)	211.7(13)	204.3(9)	185.7(10)	182.3(10)	187.0(5)	199.3(6)		n.d.r.[h]	–	–	–
9a	(Set 1)	186.7(15)	192.3(9)	195.0(7)	206.7(6)	233.3(19)	300.3(10)		227	116	2.07	2.15
9b	(Set 2)	211.7(13)	193.3(13)	184.3(10)	193.0(12)	188.3(12)	208.0(10)		n.d.r.[h]	–	–	–
10 ($\log P = 5.12$; $pK_A = 4.2$)	(Set 3)	194 (13)			254 (11)[i]	395 (12)	687 (12)	882 (10)	693	327	2.51	0.74
11 ($\log P = 4.37$; $pK_A = 4.2$)	(Set 3)	194 (13)			203 (5)[i]	195 (8)	198 (17)	202 (14)	n.d.r.[h]	–	–	–
12 ($\log P = 3.66$; $pK_a = 4.2$)	(Set 3)	194 (13)			261 (66)[i]	356 (8)	461 (11)	616 (20)	541	255	2.41	0.42
13 ($\log P = 3.1$;$pK_a = 4.2$)	(Set 3)	194 (13)			253 (13)[i]	358 (33)	478 (8)	716 (29)	522	246	2.39	0.28
14 ($\log P = 4.31$; $pK_a = 4.2$)	(Set 3)	194 (13)			250 (3)[i]	299 (10)	449 (11)	494 (9)	510	241	2.38	0.64
5a (R′ = Ph)[j]										447	2.65	−0.17

[a] **1**, X = H tested as reference with mutagens of Set 1.
[b] **1**, X = H tested as reference with mutagens of Set 2.
[c] **1**, X = H tested as reference with mutagens of Set 3.
[d] Slope calculated from the gradient of the induced revertants per plate vs. the dosage in the linear range.
[e] Members of each set scaled through its respective reference to 319 revertants per plate for **1**, X = H for comparison with previous studies.
[f] Log of the normalised slope which represents relative activity at 1 μmol/plate.
[g] Computed activity from (1) – measured activity.
[h] No dose response; non-mutagenic.
[i] Revertants at 0.125 μmol/plate in Set 3.
[j] Data from Refs. [8] and [19].

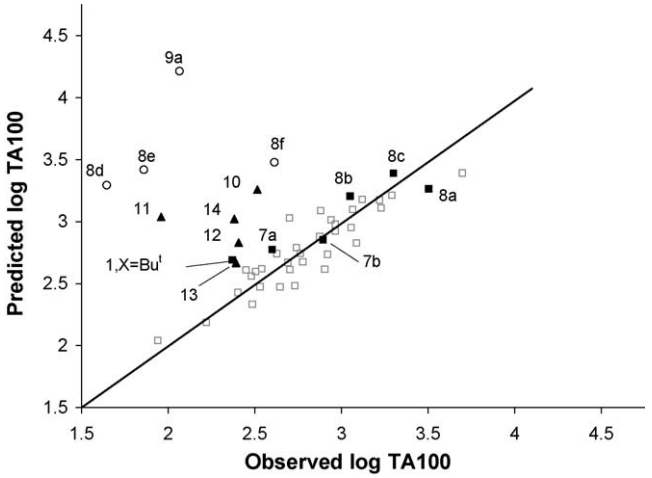


Fig. 1. Predicted (using Eq. (1)) vs. actual mutagenicities ($\log_{10}$ TA100) at 1 μmol/plate.

7–**9** in which at least one aryl ring is substituted in the *para* positions with a *tert*-butyl group.

In the second series of mutagens (**10**–**14**), the structures are characterised by their aliphatic amide side chains and by their varying degrees of branching adjacent to the amide carbonyl.

In all cases, the mutagens were tested without microsomal activation since they are direct-acting and the mutagens in this class that we have tested to date have yielded similar activities both with and without S9 [6]. All the mono-*tert*-butylated mutagens (**8a**–**c**) and the di-*tert*-butylated *N*-acetoxybenzyloxybenzamide (**7b**) were toxic above doses of 0.25 μmol/plate, but gave linear dose–response curves below this dose level. Tri-*tert*-butylated mutagens **8g** and **9b** were inactive at any dose and the di-*tert*-butylated mutagens **8d**–**f** and **9a** gave shallow but linear dose–response curves over the full dose range. Mutagen **8c** was tested again in this series and gave a normalised activity (log TA100 = 3.2) that was similar to that previously recorded (log TA100 = 3.4). For comparison with computed activity, an average value of log TA100 = 3.3 was used.

Mutagens **10**, **11** and **13** gave excellent dose–response curves, but **12** and **14** were toxic at higher doses (1 μmol/plate).

In all cases, new mutagens were tested in conjunction with *N*-acetoxy-*N*-butoxybenzamide (**1**, X = H) whose activity at 1 μmol/plate was used to scale activities relative to those from previous studies.[2]

4. Discussion

Fig. 1 depicts predicted versus observed mutagenic activities for the full set of mutagens from which the QSAR in Eq. (1) was derived (Fig. 1, all squares). The differences between computed and experimental activities for a range of *tert*-butylated mutagens are given in Table 1. The mutagenicities of all mono 4-*tert*-butylated mutagens from previous studies (**1**, X = Bu^t, **7a**, **8c**) and those in this study (**8a** and **8b**) are predicted well according to the QSAR in Eq. (1), as is that for *N*-acetoxy 4′-*tert*-butylbenzyloxy-4-*tert*-butylbenzamide (**7b**) (Fig. 1, closed squares). In particular, the activities of the triarylated mutagens **8a**–**c** bearing a single *tert*-butyl group are adequately predicted. Binding and reaction of these mutagens with DNA in the major groove would appear to be unimpeded by the presence, on one aryl ring, of the bulky *tert*-butyl group. The observed mutagenicities for the four di-*tert*-butylated *N*-benzoyloxy-*N*-benzyloxybenzamides (**8d**–**f** and **9a**) deviate significantly from the predicted values (Table 1, Fig. 1, open circles,) and only **8f** can be regarded as mutagenic, although its experimental activity is approximately an order of magnitude lower than expected. The triarylated mutagens **8g** and **9b** were inactive.[3]

Lack of reactivity towards S_N2 reactions at nitrogen would in itself account for low mutagenic activity.[4]

[2] At 1 μmol/plate **1** (X = H) was scaled to 319 induced revertants and the scaling factor was used to standardise mutagenicities in the same set to those from previous studies [6,8].

[3] Mutagen **8g** partially precipitated in the Ames assay however mutation rates were at background level.

[4] Hydroxamic esters lacking an acyloxy group at nitrogen are inactive. Steric inhibition to nucleophilic attack at nitrogen would similarly be expected to reduce reactivity.

Table 2
Bimolecular rate constants for the reaction of *N*-acyloxy-*N*-alkoxyamides with *N*-methylaniline in d_4-methanol

Substrate	$10^4 k^{303}$ ($l\,mol^{-1}\,s^{-1}$)
3, Z = H	926.2[a]
8a	798.5
8b	578.5
8c	517.1
8d	369.8
8e	353.0
8f	156.6
	$10^4 k^{313}$ ($l\,mol^{-1}\,s^{-1}$)
5a, R′ = Ph	97.2[b]
13	8.1
14	2.2
10	–[c]
11	–[c]
12	–[c]

[a] From Ref. [25].
[b] From Ref. [19].
[c] No reaction at this temperature.

However, the *tert*-butyl groups on **8d–g** and **9a,b** are well removed from the reactive nitrogen and, as well, S_N2 reactions of mutagens **8d–f** with *N*-methylaniline in methanol at 303 K occur with relatively similar rate constants to those of **8a–c** (Table 2).

The steric impediment to reaction with DNA, which is accounted for in the QSAR through Taft steric parameters E_S^1, E_S^2 and E_S^3, presupposes that the mutagen can enter the major groove, where bulkiness of substituents presumably impacts upon alignment of the mutagen for reaction with guanine. The radical decrease in activity of mutagens **8d–g** and **9a,b** suggests that, in these cases, the drugs cannot react with DNA since they are either too large to enter the major groove or, once in the groove, are unable to achieve the transition state for reaction with G—N7. A comparison of the activity of **7b** with that of **8d**, or the mono *tert*-butylated *N*-benzoyloxy-*N*-benzyloxybenzamides (**8a–c**) with the di-*tert*-butylated substrates (**8d–f**) suggests that there is a critical bulk beyond which, even with conformational flexibility, inclusion in the major groove or reaction of the nucleotide at the electrophilic amide nitrogen becomes difficult. The computed transition state for S_N2 reaction at nitrogen requires the "backside" attack of the nucleophile on the nitrogen and approximately on line with the bond to the acyloxy leaving group [18]. With such constraints the transition state may be reached with one *tert*-butyl group on one of the three benzene rings but may be unattainable with two such substituents. While all di-*tert*-butylated substrates exhibit no or low activity, **8f** is less affected. While this may be attributed to the particular configuration, it is also the least reactive towards S_N2 reactions (Table 2), which, on account of the inverse relationship between mutagenicity and reactivity, would generate higher activity.

The mutagens **8** and **9** and most of the mutagens we have studied to date are arylamide derivatives. Only a handful of alkylamides have been investigated and these were universally the acetamides (**5**) [8,19]. The activity of *N*-benzoyloxy-*N*-butoxyacetamide (**5a**, R′ = Ph) is included in Table 1 for comparison. Remarkably, the deviations from the predicted mutagenicities increase through the series $CH_3 < Et < Pr^i < Bu^t$, the latter being totally inactive. Clearly branching at the α-carbon is an important factor controlling activity. The bulky adamantamide derivative (**10**) shows weak activity that deviates substantially from the predicted activity. Comparison of the activity of the pivalamide (**11**) with that of the 3,3-dimethylbutyramide (**14**) also indicates a diminishing steric influence as the *tert*-butyl group is moved one methylene away from the carbonyl.

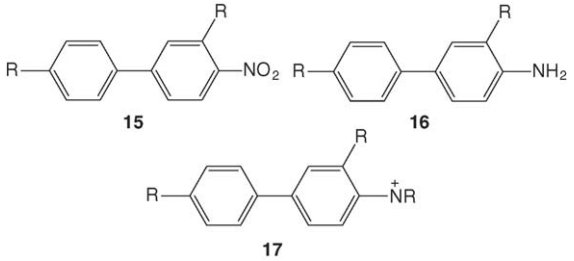
Unlike the mutagens **8d–g** and **9a,b** the overall dimensions of substrates **10–14** would not be expected to impede their access to the major groove. We attribute the systematic reduction in activities to an increase in the activation barrier for reaction at G—N7 as a consequence of branching at the α-carbon. The rate constants for S_N2 reaction of these substrates with *N*-methylaniline at 313 K (Table 2) broadly support this. The rate constant for reaction of propanamide **13** is an order of magnitude smaller than that of the acetamide (**5a**, R′ = Ph) [19] while **12** with the isopropyl side-chain and **11** with the *tert*-butyl side-chain are unreactive with *N*-methylaniline. In addition, while the pivalamide **11** is unreactive, the neohexamide **14**, in which the branching is one methylene removed from the reactive nitrogen is susceptible to S_N2 reaction with *N*-methylaniline, albeit with a smaller rate constant than **13**. The adamantane carboxamide mutagen (**11**) is also resistant to S_N2 reactions at nitrogen.

Clearly there are differences between reactivity and mutagenic activity, in that the isobutyramide substrate (**12**), which exhibits impaired mutagenicity, is unreactive towards S_N2 reactions with *N*-methylaniline and the adamantamide (**10**), though resistant to S_N2 reaction with *N*-methylaniline at matching temperatures, is still marginally mutagenic. However, such reactions are also dependent upon the nature of the nucleophile and the solvent environment. For instance, while the *tert*-butyl-containing amide side-chain in **11** completely inhibits attack by *N*-methylaniline at nitrogen, simi-

larly substituted *N*-chloroamides react readily with azide which is known to be a stronger nucleophile [20]. Differences between experimental S_N2 reaction rates and the mutagenicity data may also be related to the influence of the hydrophobic environment within the major groove; solvent organisation about a polar S_N2 transition state accounts for substantially negative entropies of activation in the reactions of these mutagens with *N*-methylaniline [7,18,21]. Such a contribution would presumably be different or absent in reactions at G—N7. Overall, though, relative mutagenicities in this series would appear to be controlled by steric inhibition of the substitution reaction at the amide nitrogen rather than by any steric inhibition to binding in the major groove.

These mutagens are pyramidal amides in that their nitrogens are completely sp^3 hybridised [12,22]. As a consequence, their S_N2 reactivity with neutral nucleophiles like *N*-methylaniline parallels that of α-haloketones with amines, which are also strongly affected by steric effects on the α′-carbon [23]. In general, the rates of S_N2 reactions are strongly adversely influenced by steric effects and branching β to the reactive centre and the same appears to be true for *N*-acyloxy-*N*-alkoxyamides **10**–**14**. Rates of reaction of α-haloketones are also radically different with ionic nucleophiles relative to neutral amino nucleophiles, which react much more slowly. This has been attributed to a tighter transition state with ionic nucleophiles like acetate and azide leading to a classical S_N2 transition in which there is additional stability through orbital overlap with the carbonyl carbon $2p_z$ orbital [23,24].

Steric inhibition of mutagenicity by remote [26,27] and proximal alkyl substituents [28,29] has recently been established by Boche and coworkers who demonstrated that the presence of increasingly bulky groups on the remote 4′ position or the 3 position of 4-nitro- (**15**) and 4-aminobiphenyls (**16**) caused significant or complete loss of activity in *Salmonella* strains TA100 and TA98. These effects are more difficult to interpret than ours since in both cases enzymatic activation is involved (nitroreductase in the case of **15** and cytochrome P-450 oxidases in the case of **16**) and in addition, intercalative binding of the ultimate carcinogenic nitrenium ions, **17**, formed from both precursors is also a possibility. In the case of the nitro compounds, the diminution in activity was ascribed to steric inhibition of intercalation into DNA rather than binding to nitroreductase, but steric inhibition to metabolic oxidation of the amino group of **16** was observed. Inhibition to reaction with G-C8 was also proposed to explain reduced mutagenicities. In our system, metabolic activation is not required. Furthermore, there is no suggestion that these simple mutagens intercalate with DNA [8].[5] It is clear from series **8** that bulky distal groups can have a significant influence upon the actual groove binding with bacterial DNA.



Boche showed that bulky groups adjacent to the nitro or amino group also have a marked effect upon mutagenic activity. In both cases this has been ascribed to steric inhibition of their respective metabolic activation steps. The respective activities of mutagens **10**–**14** indicate that steric effects at positions close to the reactive centre of mutagens have a marked influence upon actual reactivity with DNA.

5. Conclusions

N-Acyloxy-*N*-alkoxyamides, being direct-acting mutagens, can probe the impact of molecular structure on interactions with DNA. Through QSAR (1) it is possible to highlight important structural factors that both target small molecules to DNA, or impede binding or reaction with DNA components. In this study we illustrate that their mutagenicity can be modified by steric effects of two kinds. Where the steric bulk reaches a critical level (di-*tert*-butylated triaryl mutagens), regardless of their susceptibility to S_N2 reactions at nitrogen, mutagenicity is severely retarded owing to an inability to enter the major groove and achieve the requisite proximity to G—N7 for modification to take place. Presumably, in these cases achievement of a transition state for S_N2 displacement at the mutagen nitrogen would require energetically unfavourable conformational changes that are too severe.

Mutagenicity is also moderated by lack of reactivity of the electrophilic amides. In the case of (**10**–**14**), an increasing steric environment close to the amide

[5] In Ref. [8] we reported that naphthyl containing mutagens, and one bearing a pyrene group, produced mutagenicities that are one order of magnitude higher than predicted by QSAR (1), which is consistent with intercalative binding. Interestingly, biphenyl substituents appear to give rise to only a small increase in mutagenicity, which does not signify a strong intercalative binding to bacterial DNA in Salmonella typhimurium TA100.

nitrogen reduces mutagenic activity systematically and broadly in parallel to the loss of S_N2 reactivity with *N*-methylaniline. This remarkable correspondence further emphasises that the activities of these mutagens strongly reflect their chemical and physical interactions with DNA.

We envisage that the QSAR (1) may be used in a similar fashion to elaborate on the influence of other structural elements upon DNA binding. For instance, the intercalative ability of small polycyclic aromatics, incorporated into the *N*-acyloxy-*N*-alkoxyamide structure, has been found to enhance mutagenicity and we have been able to estimate that incorporation of a naphthalene or pyrene unit appears to be worth ca. 3log *P* units to DNA binding.

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