The effect of hydrophobicity upon the direct mutagenicity of *N*-acyloxy-*N*-alkoxyamides — bilinear dependence upon Log*P*.

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Abstract

N-Acyloxy-*N*-alkoxyamides **1** are direct-acting mutagens for which a bilinear QSAR has been established, which predicts with accuracy their activity in the Ames reverse mutation assay in *Salmonella typhimurium* TA100, based upon their hydrophobicity (Log*P*), reactivity (pK_A of the carboxylic acid of the *N*-carboxyl group) and TAFT steric parameters. From activity data for 55 congeners and incorporating five mutagens bearing long-chain hydrocarbons on the alkoxyl and acyloxyl groups, designed for this study, a maximal LogP_o, is found to be Log*P* = 6.4. Mutagens with Log*P* < than this value show a linear hydrophobic dependence (h = 0.24) relating to their binding to bacterial DNA and those with Log*P* > LogP_o undergo lipid entrapment which masks the DNA binding effect. The QSAR has been used to differentiate between lipophilicity and steric inhibition to groove binding in a series of outliers bearing large *tert*-butyl groups as well as to confirm the enhancement to DNA binding of the naphthalene moiety, which is shown to be equivalent to about 3.5 Log*P* units.

Keywords: Mutagenicity; Direct-acting electrophilic amides; Ames Test; Hydrophobic effects; Bilinear QSAR; DNA binding.

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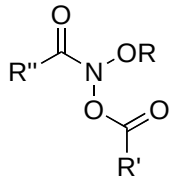
1. Introduction

N-Acyloxy-*N*-alkoxyamides (NAA) **1** are a class of direct-acting mutagens, which revert *Salmonella typhimurium* TA100 and TA98 without the need for microsomal (S9) activation.[1-11] They give excellent, linear dose response data and since their discovery in our laboratories in the early 1990's, we have studied the activity of a wide variety of congeners in TA100. As *N*-alkoxy analogues of one class of carcinogenic metabolites of aromatic amines (*N*-acetoxy-*N*-arylacetamides),[12,13] we initially set out to establish whether they too would be DNA damaging agents, which proved to be the case. However, while they are mutagenic, several members of the class have demonstrable anticancer activity in the NCI 60 antitumour screen.[10,14] Their ongoing evaluation as direct–acting mutagens has in part been to optimise their activity through classical structure-activity studies. However, they are proving to be ideal probes for investigating the influence of structure upon binding of small molecules to DNA, a prerequisite for their mutagenic activity, and about which little is known.[6,8-10]



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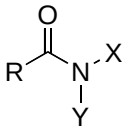

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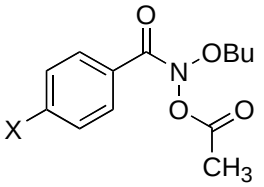
a) X=Y=OCH_3,R=CH_3
b) X=Y=CH_3,R=CH_3



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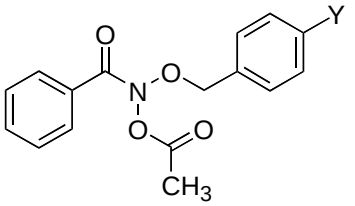

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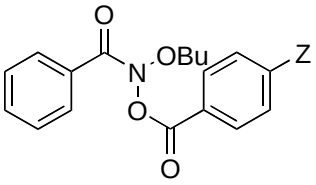

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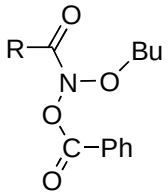
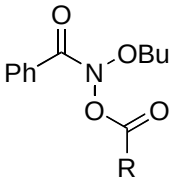


6

R=Me, Et, Pr^i, Bu^t, CH_2Bu^t, 1-adamantyl



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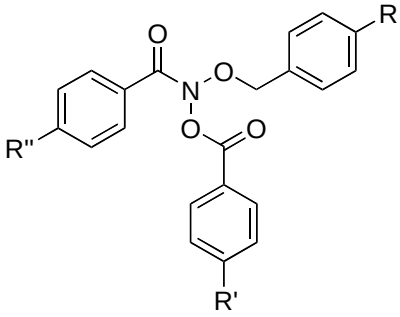
R=Me, Pr, Pr^i, (S)-2-Bu, CH_2Bu^t, Bu^t

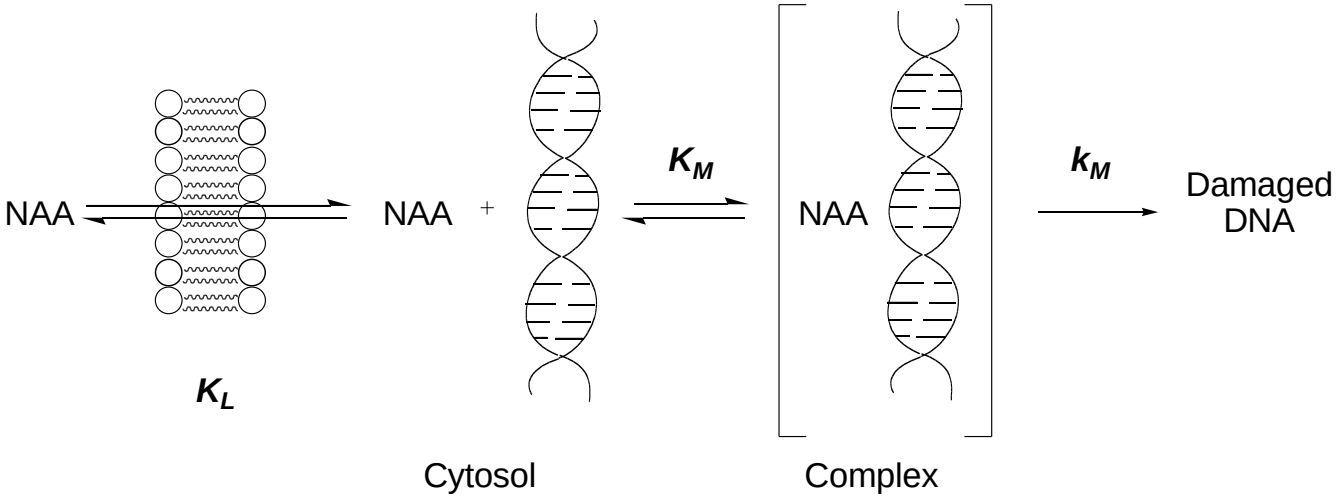


8

a) R= R''=Bu^t, R'=H
b) R= R'=Bu^t, R''=H
c) R'= R''=Bu^t, R=H
d) R=R'= R''=Bu^t

The class of anomeric amides **2**, of which *N*-acyloxy-*N*-alkoxyamides are a member, are amides that are substituted at nitrogen with two electronegative heteroatoms, X and Y, and the combined electron demand of the nitrogen substituents results in a radical alteration in the amide

properties.[10,11,15-18] The extent of amide resonance, so-called amidicity,[19-22] can be greatly reduced, depending upon the nature of the heteroatoms. The nitrogen atoms assume a pyramidal, rather than a planar geometry and the lone pair resides in an sp^3 hybrid orbital on nitrogen resulting in reduced delocalisation and consequently long *N—C(O)* bonds.[3,11,17,18,20] We have recently computed the amidicity of *N,N*-dimethoxyacetamide **2a** to be as low as 50% that *N,N*-dimethylacetamide **2b**.[20,23]



Scheme 1

N-Acyloxy-*N*-alkoxyamides are typical anomeric amides, having strongly pyramidal nitrogens, long *N—C* bonds and spectroscopic properties commensurate with this reduced resonance.[10,18] However, unlike *N,N*-dialkoxyamides, they bear an acyloxyl leaving group and while they are relatively stable under anhydrous conditions at room temperature, they have been shown to undergo both S_N1 and S_N2 reactions at the amide nitrogen with a range of nucleophiles.[3,5,8,10,11,15,24-30] Importantly, DNA itself acts as a nucleophile towards *N*-acyloxy-*N*-alkoxyamides resulting in their mutagenic behaviour. They have been shown by DNA damage studies to react with DNA at guanine *N*-7 and adenine *N*-3 and they must therefore have to enter the major and minor grooves of DNA. Furthermore, they do so intact since the nature of the acyloxyl leaving group, like the amide and alkoxyl side chains, has an impact upon their mutagenic behaviour.[7,31] Clearly there are a number of factors involved in control and limitation of mutagenic activity. Scheme 1 shows that after passing through the cell membrane (controlled by diffusion constant K_L, Scheme 1), they must generate an intimate NAA/DNA complex (controlled by equilibrium constant K_M, Scheme 1). Reaction with DNA and formation of the damaged DNA adduct (controlled by rate constant k_M, Scheme 1) must ensue leading to a mutation event.

In studies to date, we have deduced that mutagenic activity is influenced by the following:

1. overall hydrophobicity of the intact mutagen, which affects groove binding and K_M. Small molecules studied to date can permeate lipid membranes;[4,6,9]
2. reactivity due to the nature of the acyloxyl leaving group in that mutagenic activity is moderately dependent upon the pK_A of the departing carboxylic acid. While this may impact upon the ease of adduct formation (k_M), adventitius side-reactivity reduces the concentration of active mutagen;[4,6]
3. steric bulk at *para* positions of phenyl substituents on the benzamide, benzyloxyl and benzoyloxyl side chains in **3**, **4** and **5** lowers activity, presumably by impeding binding to DNA and K_M;[6]
4. steric bulk and branching close the reactive amide nitrogen on both the amide **6** and the acyloxyl **7** side chains,[8,9] which in both cases reduce activity, in the case of **6** due to steric impediment to S_N2 reactions at nitrogen,[8] reducing k_M and, in the case of **7**, due to apparent hindrance to groove binding reducing K_M;[9]
5. modest branching or bulkiness on the alkoxyl side chain would not appear to be an important determinant of activity;[9]
6. the presence of 4-*tert*-butylphenyl substituents on two or more side chains in triarylated mutagens **8a-d**, which reduce activity, either through excessive hydrophobicity or by impeding access to the major groove and thereby reducing K_M;[8]
7. naphthalene-bearing mutagens have enhanced activity, perhaps through weak intercalative binding to DNA (increasing K_m).[4,6,10]

In previous work we developed a quantitative structure-activity relationship (Equation 1), which models well the activity of some fifty mutagens of disparate structures and incorporates these effects through Log*P* (hydrophobicity), pK_A (reactivity) and E_S^1, E_s^2 and E_S^3 (distal steric effects of 4-phenyl substituents).[9,32]

Log TA100 = 0.28 (±0.03) Log*P* + 0.18 (±0.08) pK_A + 0.13 (±0.03) E_S^1 + 0.15 (±0.04) E_s^2 + 0.11 (±0.05) E_S^3 + 1.02 (±0.4)

n = 50, r^2= 0.80, adj. r^2 = 0.77, s = 0.16, F = 34.5; LOOCV Q^2=0.70

Equation 1

LogTA100 = 0.189 (±0.02) Log*P* + 2.08 (±0.08)

n = 50, r^2 = 0.67, adj. r^2 = 0.67, s = 0.19, F = 98.5; LOOCV Q^2 = 0.64

Equation 2

We have employed this training set to identify and understand structural characteristics that result in abnormally decreased mutagenic activity (such as described in points 4 and 6 above) or increased activity (such as described in point 7); we will shortly report in full that PAH-containing mutagens, including, unexpectedly, mononaphthalene-substituted derivatives strongly enhance the mutagenic activity of **1** through intercalative association with DNA.[6,10]

Log*P* dependence, though with a modest coefficient, shows a high degree of significance ($P = 2\text{x}10^{-13}$) , as does dependence upon E_S^1 and E_s^2 (P values of $1.6\text{x}10^{-4}$ and $2.2\text{x}10^{-4}$ respectively).[9] Dependence upon pK_A and E_S^3 are less statistically significant (0.03 and 0.04 respectively) but incorporation of the additional descriptors leads to improvement in the correlation and increased adj. r^2 relative to the correlation with Log*P* alone (Equation 2). Furthermore the similar leave-one-out cross validation Q^2 indicates the QSAR's good predictive capability.[33]

The dependence of mutagenic activity upon Log*P* by direct-acting *N*-acyloxy-*N*-alkoxyamides is unusual. Direct-acting mutagens are generally known to have mutagenic activity that is independent of the overall hydrophobicity of the molecule and Log*P* dependence is usually deemed to be a determinant of indirect-acting mutagenicity [34-37] where hydrophobicity is strongly involved in the metabolic activation of mutagens. QSAR for the interactions of organic compounds with DNA are often lacking positive hydrophobic terms.[38] Coupled with the fact that *N*-acyloxy-*N*-alkoxyamides react directly with DNA *in vitro*, [7,31] the measured mutagenic activity in TA100, points to the fact that the bacterial cellular target of *N*-acyloxy-*N*-alkoxyamides is DNA. Since enzymatic activation is not a factor, we have ascribed this Log*P* dependence to the importance of hydrophobic binding in the grooves of DNA. While the Log*P* dependence of drugs can relate to transport across lipid membranes, all the molecules upon which the QSAR (Equation 1) is based have modest Log*P* (90% with Log*P* < 6.0, median Log*P* of 3.8) and penetration of the cell is not regarded as a major factor with small molecules, particularly on account of the *S. typhimurium rfa* mutation intrinsic to the Ames reverse mutation assay[39-42].

In this paper we explore the impact of extending the range of Log*P* values over which mutagenicity of *N*-acyloxy-*N*-alkoxyamides had previously been obtained, to determine whether a maximal Log*P,* $\text{Log}P_o$, exists in the Ames assay in TA100 and what this may be. In addition, we have previously found that bulky mutagens such as **8** have radically reduced activity, which we attributed to steric inhibition to groove binding.[8] However, **8** also have Log*P* values > 8 and results from

this study clarify the role of hydrophobicity, as opposed to steric effects in their reduced activity. We also demonstrate how naphthalene incorporation can generate mutagenic activity equivalent to an *N*-acyloxy-*N*-alkoxyamide with a virtual Log*P* well above LogP_0.

2. Materials and Methods

2.1 Instrumentation

Infrared spectra were recorded using solution cells on a Perkin Elmer 1600 series FT IR spectrophotometer or on a Varian 660-IR FT IR spectrophotometer fitted with a Pike MIRacle attenuated total reflection (ATR) cell, neat or as chloroform solutions. Mass spectra were recorded on a Varian 1200L GC-LC-MS. ESI-MS analyses were carried out at a 30 V capillary voltage and 350° C/ 20 psi drying gas temperature/pressure, in HiPerSolv, 'Far UV' grade (BDH) methanol. EI-MS were carried out at 70 V capillary voltage using the same machine configured with a direct insertion probe (Varian, controlled by a Scientific Instrument Services Inc. direct exposure probe controller model PC-3). TOF ESI accurate mass determinations were obtained from the Mass Spectral Unit of the Australian National University. Nuclear magnetic resonance spectra were recorded in $CDCl_3$ on a Bruker Avance 300P FT NMR spectrometer with a 5-mm 1H inverse/BroadBand probe with a z-gradient, operating at 300.13 MHz (^{1}H), 75.46 MHz (^{13}C). Chemical shifts are reported in ppm downfield from TMS. Abbreviations used to indicate spectral multiplicities are:

s, (singlet), d, (doublet), t, (triplet), q, (quartet), qu, (quintet), sx, (sextet), m, (multiplet), br, (broad). Centrifugal chromatographic separations were performed on a 7294T model Harrison Research chromatatron with plates coated with 2.0 mm of silica gel 60 F_{254} (Merck).

2.2 Chemicals

Synthesis of *N*-acetoxy-*N*-butoxybenzamide **9f** has been described previously as has synthesis of the potassium salt of benzohydroxamic acid,[1,2] and the octyloxybenzamide precursor to **9c**.[4]

2.2.1. Preparation of *alkyl bromides.*

Alkyl bromides were prepared from the appropriate alcohols by stirring at room temperature in HBr/H_2SO_4. The mixture was extracted with chloroform and washed with conc. HCl, H_2O, 10% aqueous sodium carbonate, and H_2O. Concentration under reduced pressure yielded the alkyl bromides in good yield and high purity which were used without further purification.

1-Bromoheptane. 1-Heptanol (10.04g, 86.40 mmol) was refluxed in HBr (13.97 g, 174.63 mmol) for 150 h. The reaction was cooled then extracted with dichloromethane (3 x 20 mL). The dichloromethane layer was washed with conc. HCl, H_2O, 10% Na_2CO_3, H_2O, dried over Na_2SO_4 and concentrated under reduced pressure to yield 1-bromoheptane as a straw coloured oil (10.63 g, 59.02 mmol, 69%) with good purity. ν_{max}(neat)cm^{-1} 646.2(C-Br). δ_H 0.87(3H, t), 1.28(6H, br s),

1.41(2H, qu), 1.84(2H, qu), 3.39(2H, t) δ_C 14.00, 22.55, 28.14, 28.43, 31.63, 32.86, 33.92. m/z (EI): 177.9/179.1(M^+), 134.9/136.1(M-C_3H_7).

1-Bromooctane. 1-Octanol (6.16 g, 47. 25 mmol) was refluxed in HBr (12.41 g, 153.12 mmol) for 150 h. The reaction was cooled then extracted with dichloromethane (3 x 20 mL). The dichloromethane layer was washed with conc. HCl, H_2O, 10% Na_2CO_3, H_2O, dried over Na_2SO_4 and concentrated under reduced pressure to yield 1-bromooctane as a straw coloured oil (7.73 g, 40.25 mmol, 85%) with good purity. ν_{max}(neat)cm^{-1} 646.3(C-Br). δ_H 0.87(3H, t), 1.27(8H, br s), 1.37-1.43(2H, m), 1.84(2H, qu), 3.39(2H, t). δ_C 14.04, 22.61, 28.19, 28.73, 29.10, 31.75, 32.86, 33.93.

1-Bromodecane. 1-Decanol (10.14 g, 64.06 mmol) was refluxed in HBr (10.37 g, 128.42 mol) for 150 h. The reaction was cooled then extracted with dichloromethane (3 x 20 mL). The dichloromethane layer was washed with conc. HCl, H_2O, 10% Na_2CO_3, H_2O, dried over Na_2SO_4 and concentrated under reduced pressure to yield 1-bromodecane as a brown oil (10.91 g, 49.33 mmol, 77%) with good purity. ν_{max}(neat)cm^{-1} 647.0(C-Br). δ_H 0.87(3H, t), 1.26(12H, br s), 1.37-1.51(2H, br m), 1.85(2H, qu), 3.39(2H,t). δ_C 14.07, 22.65, 28.19, 28.77, 29.27, 29.44, 29.49, 31.87, 32.86, 33.95. *m/z* (EI): 220.2/222.1(M^+).

2.2.2 Preparation of potassium hydroxamates[43,44]

Potassium naphtho-2-hydroxamate. Potassium hydroxide (4.39 g, 78.3 mmol) in boiling methanol (20 mL) was added carefully to hydroxylamine hydrochloride (3.63 g, 52.2 mmol) in boiling methanol (25 mL) and the mixture cooled in an ice bath for 5 min. Ethyl-2-naphthoate (5.23 g, 26.1 mmol) in 10 mL methanol was added with shaking. The mixture was filtered immediately and washed with methanol. Crystallisation, upon refrigeration was complete within 48 h. Filtration, washing with methanol and drying under reduced pressure afforded potassium naphtho-2-hydroxamate (3.53 g, 15.6 mmol, 60%) as a yellow solid, which was used without further characterisation or purification.

2.2.3 Preparation of N-alkoxyamides[45]

Condensation of the potassium hydroxamic acid salt with the appropriate alkyl bromide and a 10% excess of sodium carbonate in 50% aqueous methanol gave the target *N*-alkoxyamide in good yield.

N-Hexyloxybenzamide. 1-Bromohexane (4.74 g, 28.72 mmol), potassium benzohydroxamate (5.03 g, 28.72 mmol) and sodium carbonate (3.35 g, 31.61 mmol) were stirred overnight in 50% aq. methanol (100 mL) and refluxed for 3 h. Excess methanol was removed under reduced pressure and the mixture extracted with dichloromethane, which was dried over anhydrous sodium sulfate,

filtered and concentrated under reduced pressure. Purification by flash chromatography using 25% ethyl acetate/hexane afforded pure *N*-hexyloxybenzamide as light brown oil. $\nu_{max}(CHCl_3)cm^{-1}$ 1680.6(C=O). δ_H 0.85(3H, t), 1.25(6H, br s), 1.64(2H, qu), 3.95(2H, t), 7.36(2H, t), 7.46(1h, t), 7.71(2H, d), 9.51(1H, br s). δ_C 14.17, 22.55, 25.48, 28.01, 31.61, 76.95, 127.18, 128.56, 131.87, 132.05, 166.43. *m/z* (ESI): 222.1490($M+H^1$); $C_{13}H_{20}NO_2$ requires 222.1494, 244.1313($M+Na^+$); $C_{13}H_{19}NO_2Na$ requires 244.1313.

N-Heptyloxybenzamide. Pure *N*-heptyloxybenzamide was obtained as a light brown oil. $\nu_{max}(CHCl_3)cm^{-1}$ 1681.2(C=O). δ_H 0.87(3H, t), 1.27(8H, br s), 1.62-1.79(2H, m), 4.01(2H, t), 7.41(2H, t), 7.48(1H, t), 7.71(2H, d), 8.76(1H, br s). δ_C 14.03, 22.56, 25.78, 28.06, 29.06, 31.71, 77.15, 127.07, 128.67, 131.97, 132.14, 166.3. m/z (ESI): 236.1651($M+H^+$); $C_{14}H_{22}NO_2$ requires 236.1651, 258.1473($M+Na^+$); $C_{14}H_{21}NO_2Na$ requires 258.1470.

N-Octyloxybenzamide.[4] Pure *N*-octyloxybenzamide as light brown oil. $\nu_{max}(CHCl_3)cm^{-1}$ 1682.2(C=O). δ_H 0.86(3H, t), 1.24(10H, br s), 1.58-1.76(2H, br m), 3.96(2H,t), 7.38(2H, t), 7.44(1H, t), 7.72(2H, d), 9.41(1H, br s) δ_C14.05, 22.61, 25.81, 28.06, 29.17, 29.37, 31.77, 76.98, 127.17, 128.55, 131.84, 132.13, 166.50. m/z (ESI): 250.1807($M+H^+$); $C_{15}H_{24}NO_2$ requires 250.1807, 272.1628($M+Na^+$); $C_{15}H_{23}NO_2Na$ requires 272.1626.

N-Nonyloxybenzamide. Pure *N*-nonyloxybenzamide was obtained as a brown low-melting solid. $\nu_{max}(CHCl_3)cm^{-1}$ 1684.2(C=O). δ_H 0.86(3H, t), 1.24(12H, br s), 1.60-1.74(2H, br m), 3.99(2H, t), 7.42(2H, t), 7.49(1H, t), 7.71(2H, d), 9.01(1H, br s). δ_C 14.14, 22.68, 25.82, 27.39, 28.05, 29.45, 29.51, 31.88, 77.10, 127.11, 127.79, 128.66, 131.98, 166.53. *m/z* (ESI): 264.1964($M+H^+$); $C_{16}H_{26}NO_2$ requires 264.1964, 286.1784($M+Na^+$); $C_{16}H_{25}NO_2Na$ requires 286.1783.

N-Decyloxybenzamide. Pure *N*-decyloxybenzamide was a light brown low melting solid. m.p. 44.5-45° C; $\nu_{max}(CHCl_3)cm^{-1}$ 1684.0(C=O). δ_H 0.87(3H, t), 1.25(14H, br s), 1.61-1.74(2H, br m), 4.00(2H, t), 7.40(2H, t), 7.50(1H, t), 7.71(2H, d), 8.81(1H, br s). δ_C 14.08, 22.66, 25.83, 28.07, 29.30, 29.42, 29.53, 31.88, 77.15, 127.08, 128.66, 131.96, 132.15, 166.61. *m/z* (ESI): 278.2120($M+H^+$); $C_{17}H_{28}NO_2$ requires 278.2120, 300.1933($M+Na^+$); $C_{17}H_{27}NO_2Na$ requires 300.1939.

N-Octyloxy-2-naphthamide. 1-Bromooctane (0.49 g, 2.54 mmol), potassium naphtho-2-hydroxamate (0.57 g, 2.54 mmol) and sodium carbonate (0.30 g, 2.80 mmol) were stirred for 2 h in 50% aq. methanol (100 mL) and refluxed for 5 h. the usual workup afforded pure *N*-octyloxy-2-naphthamide as light brown crystalline solid. m.p. 111-112° C; $\nu_{max}(CHCl_3)cm^{-1}$ 1684.1(C=O). δ_H 0.86(3H, t), 1.27(10H, br s), 1.69-1.76(2H, br m), 4.07(2H,t), 7.50-7.68(2H, m), 7.76(1H, d), 7.84-7.91(3H, m), 8.25(1H, s), 8.73(1H, br s). δ_C 14.07, 22.63, 25.86, 28.11, 29.19, 29.38, 31.78, 76.59,

123.45, 126.86, 127.73, 127.78, 127.87, 128.61, 128.90, 129.31, 132.55, 134.95, 166.76. *m/z* (ESI): 300.1965($M+H^+$); $C_{19}H_{26}NO_2$ requires 300.1964, 322.1783($M+Na^+$); $C_{19}H_{25}NO_2Na$ requires 322.1783.

2.2.4 Preparation of N-alkoxy-N-chloroamides

N-Alkoxyamides are quantitatively converted to *N*-chloro-*N*-alkoxyamides by reaction in the dark with a 5-10 fold excess of neat *tert*-butyl hypochlorite. *N*-Chloro-*N*-alkoxyamides generally presented as clean yellow oils, which required no further purification and were converted directly to *N*-acyloxy-*N*-alkoxyamides. Labile *N*-chloroamides were all characterised spectroscopically (IR, ^{1}H NMR, ^{13}C NMR).

N-Chloro-N-hexyloxybenzamide. $\nu_{max}(CHCl_3)cm^{-1}$ 1718.1(C=O). δ_H 0.84(3H, t), 1.22(6H, br s), 1.48-1.61(2H, br m), 4.10(2H, t), 7.43(2H, t), 7.55(1H, t), 7.75(2H, d). δ_C 13.99, 22.47, 25.53, 27.39, 31.39, 74.80, 128.34, 129.40, 131.22, 132.85, 174.30.

N-Chloro-N-heptyloxybenzamide. $\nu_{max}(CHCl_3)cm^{-1}$ 1716.9(C=O). δ_H 0.86(3H, t), 1.23(8H, br s), 1.48-1.67(2H, br m), 4.10(2H, t), 7.43(2H, t), 7.55(1H, t), 7.75(2H, d). δ_C 14.00, 22.49, 25.78, 27.42, 28.84, 31.59, 74.81, 128.30, 129.37, 131.28, 132.76, 174.21.

N-Chloro-N-octyloxybenzamide. $\nu_{max}(CHCl_3)cm^{-1}$ 1717.9(C=O). δ_H 0.86(3H, t), 1.22(10H, br s), 1.49-1.68(2H, br m), 4.10(2H, t), 7.43(2H, t), 7.55(1H, t), 7.75(2H, d). δ_C 14.04, 22.59, 25.82, 27.42, 29.05, 29.14, 31.70, 74.81, 128.29, 129.37, 131.28, 132.76, 174.21.

N-Chloro-N-nonyloxybenzamide. $\nu_{max}(CHCl_3)cm^{-1}$ 1719.8(C=O). δ_H 0.86(3H, t), 1.21(12H, br s), 1.52-1.68(2H, br m), 4.10(2H, t), 7.43(2H, t), 7.55(1H, t), 7.74(2H, d). δ_C 14.13, 22.67, 25.83, 26.82, 27.41, 29.20, 29.38, 31.84, 74.81, 128.34, 129.40, 131.23, 132.84, 174.30.

N-Chloro-N-decyloxybenzamide. $\nu_{max}(CHCl_3)cm^{-1}$ 1718.6(C=O). δ_H 0.87(3H, t), 1.22(14H, br s), 1.51-1.68(2H, br m), 4.10(2H, t), 7.43(2H, t), 7.54(1H, t), 7.75(2H, d). δ_C 14.07, 22.65, 25.82, 27.42, 29.18, 29.25, 29.40, 29.44, 31.86, 74.81, 128.30, 129.38, 131.29, 132.77, 174.22.

N-Chloro-N-octyloxy-2-naphthamide. $\nu_{max}(CHCl_3)cm^{-1}$ 1718.9(C=O). δ_H 0.84(3H, t), 1.16(10H, br m), 1.58(2H, br p), 4.16(2H, t), 7.55-7.62(2H, m), 7.77(1H, d), 7.86-7.94(3H, m), 8.35(1H, s). δ_C 14.01, 22.57, 25.88, 26.81, 27.47, 29.02, 31.65, 74.83, 125.12, 126.91, 127.80, 128.06, 128.36, 128.49, 129.23, 130.95, 132.30, 135.32, 174.33.

2.2.5 Preparation of sodium carboxylate salts

Method 1. The carboxylic acid was dissolved/suspended in a small volume of methanol. An equimolar volume of 10 M sodium hydroxide solution was added with stirring. If the salt failed to

precipitate upon standing or refrigeration, the methanol and water were removed under reduced pressure to yield the appropriate sodium salt, which was used without further purification.

Method 2 The carboxylic acid was stirred in dry diethyl ether to which a molar equivalent of sodium hydride was slowly added. After the addition the flask was sealed, flushed with nitrogen and allowed to stir until evolution of hydrogen gas ceased. Filtration of the precipitate afforded the appropriate sodium salt, which was used without further purification.

Sodium hexanoate. Hexanoic acid (5.16 g, 44.4 mmol) was dissolved in methanol (10 mL). 10 M Sodium hydroxide (4.44 mL, 44.4 mol) was added while stirring. After 30 min solvent was removed under reduced pressure to afford sodium hexanoate as a white solid (4.89 g, 35.4 mmol, 80%).

Sodium heptanoate. Heptanoic acid (5.04 g, 38.7 mmol) was dissolved in methanol (15 mL). 10 M Sodium hydroxide (3.87 mL, 38.7 mmol) was added while stirring. After 30 min the solid that formed was filtered and washed with two portions (10 mL) of methanol then dried under reduced pressure to afford sodium heptanoate as a white solid (0.93 g, 6.12 mmol, 16%).

Sodium octanoate. Octanoic acid (5.05 g, 34.9 mmol) was dissolved in dry ether (40 mL). NaH (0.80 g, 33.2 mmol) was added carefully with stirring and the flask purged with N_2. Stirring at room temperature continued until evolution of $H_{2(g)}$ ceased. Sodium octanoate was filtered, washed with dry diethyl ether and dried under reduced pressure to yield a white solid (4.20 g, 25.3 mmol, 76%).

Sodium nonanoate. Nonanoic acid (5.01 g, 31.6 mmol) was dissolved in methanol (15 mL). 10 M Sodium hydroxide (3.16 mL, 31.6 mmol) was added while stirring. After 30 min the solid that was formed was filtered and washed with two portions (10 mL) of methanol then dried under reduced pressure to afford sodium nonanoate as a white solid (2.07 g, 11.5 mmol, 36%).

Sodium decanoate. Decanoic acid (6.18 g, 35.9 mmol) was dissolved in methanol (15 mL). 10 M Sodium hydroxide (3.59 mL, 35.9 mmol) was added while stirring. After 30 min the solid that was formed was filtered and washed with two portions (10 mL) of methanol then dried under reduced pressure to afford sodium decanoate as a white solid (3.59 g, 18.5 mmol, 51%).

2.2.6. Preparation of N-acyloxy-N-alkoxyamides

General Procedure. Reaction of sodium carboxylates with *N*-chloro-*N*-alkoxyamides in anhydrous acetone gave the desired mutagen in variable yields of between 10-75%. Purification was by centrifugal chromatography, and the labile mutagens were all characterised spectroscopically by IR, ^{1}H NMR and ^{13}C NMR and by ESI-MS.

*N-Hexanoyloxy-N-hexyloxybenzamide (**9a**)*. *N*-Chloro-*N*-hexyloxybenzamide (0.61 g, 2.40 mmol) was stirred with sodium hexanoate (0.46 g, 3.36 mmol) in dry acetone (10 mL) in the dark and followed by TLC until completion (approx. 27 h). The reaction mixture was filtered to remove NaCl, and the filtrate concentrated under reduced pressure to give the crude product. Centrifugal chromatography with 10% ethyl acetate/hexane afforded pure *N*-hexanoyloxy-*N*-hexyloxybenzamide as a yellow oil (0.10 g, 0.31 mmol, 13%). $\nu_{max}(CHCl_3)cm^{-1}$ 1778.8(C=O), 1723.5(C=O). δ_H 0.85(6H, t), 1.25(10H, br m), 1.60(4H, m), 2.32(2H, t), 4.15(2H, t), 7.40(2H, t), 7.52(1H, t), 7.74(2H, d). δ_C 13.77, 13.94, 22.15, 22.48, 24.30, 25.44, 28.02, 30.98, 31.46, 32.08, 75.71, 128.17, 129.02, 132.00, 132.54, 171.10, 174.41. *m/z* (ESI): 335.4(M^+), 336.4($M+H^+$), 358.3($M+Na^+$).

*N-Heptanoyloxy-N-heptyloxybenzamide (**9b**)*. *N*-Chloro-*N*-heptyloxybenzamide (0.54 g, 1.99 mmol) was stirred with sodium heptanoate (0.42 g, 2.78 mmol) in dry acetone (10 mL) in the dark and followed by TLC until completion (approx. 18 h). The reaction mixture was filtered to remove NaCl, and the filtrate concentrated under reduced pressure to give the crude product. Centrifugal chromatography with 10% ethyl acetate/hexane afforded pure *N*-heptanoyloxy-*N*-heptyloxybenzamide as a yellow oil (0.22 g, 0.60 mmol, 30%). $\nu_{max}(CHCl_3)cm^{-1}$ 1779.1(C=O), 1722.7(C=O). δ_H 0.86(6H, t), 1.23(16H, br m), 1.60(2H, m), 2.32(2H, t), 4.15(2H, t), 7.40(2H, t), 7.52(1H, t), 7.74(2H, d). δ_C 13.95, 14.01, 22.36, 22.54, 24.58, 25.73, 28.06, 28.52, 28.94, 31.28, 31.67, 32.12, 75.70, 128.17, 129.01, 131.99, 132.53, 171.09, 174.39. *m/z* (ESI): 363.5(M^+), 364.5($M+H^+$).

*N-Octanoyloxy-N-octyloxybenzamide (**9c**)*. *N*-Chloro-*N*-octyloxybenzamide (0.48 g, 1.69 mmol) was stirred with sodium octanoate (0.39 g, 2.37 mmol) in dry acetone (10 mL) in the dark and progress followed by TLC until completion (approx. 24 h). The reaction mixture was filtered to remove NaCl, and the filtrate concentrated under reduced pressure to give the crude product. Centrifugal chromatography with 10% ethyl acetate/hexane afforded pure *N*-octanoyloxy-*N*-octyloxybenzamide as a yellow oil (0.17 g, 0.43 mmol, 25%).$\nu_{max}(CHCl_3)cm^{-1}$ 1778.8(C=O), 1725.9(C=O). δ_H 0.87(6H, t), 1.23(18H, br m), 1.55-1.64(4H, m), 2.32(2H, t), 4.15(2H, t), 7.40(2H, t), 7.52(1H, t), 7.74(2H, d). δ_C 14.00, 14.02, 22.53, 22.60, 24.62, 25.77, 28.06, 28.77, 28.80, 29.12,

29.24, 31.54, 31.74, 32.12, 75.70, 128.17, 129.02, 131.99, 132.53, 171.09, 174.40. *m/z* (ESI): 391.5(M^+), 414.5($M+Na^+$).

N-Nonanoyloxy-N-nonyloxybenzamide ***(9d)***. *N*-Chloro-*N*-nonyloxybenzamide (0.64 g, 2.16 mmol) was stirred with sodium nonanoate (0.55 g, 3.03 mmol) in dry acetone (10 mL) in the dark and progress was monitored by TLC until completion (approx. 6 h). The reaction mixture was filtered to remove NaCl, and the filtrate concentrated under reduced pressure to give the crude product. Centrifugal chromatography with 10% ethyl acetate/hexane afforded pure *N*-nonanoyloxy-*N*-nonyloxybenzamide as a yellow oil (0.13 g, 0.30 mmol, 14%). $\nu_{max}(CHCl_3)cm^{-1}$ 1779.2(C=O), 1724.4(C=O). δ_H 0.86(6H, t), 1.23(22H, br m), 1.57-1.66(4H, m), 2.31(2H, t), 4.15(2H, t), 7.40(2H, t), 7.51(1H, t), 7.74(2H, d). δ_C 14.05, 14.08, 22.63, 24.63, 25.78, 28.07, 28.87, 29.01, 29.08, 29.15, 29.21, 29.30, 29.43, 31.75, 31.84, 32.13, 75.70, 128.17, 129.02, 132.00, 132.54, 171.10, 174.40. *m/z* (ESI): 419.3(M^+), 420.3($M+H^+$), 442.3($M+Na^+$).

N-Decanoyloxy-N-decyloxybenzamide ***(9e)***. *N*-Chloro-*N*-decyloxybenzamide (0.56 g, 1.80 mmol) was stirred with sodium decanoate (0.49 g, 2.52 mmol) in dry acetone in the dark and the reaction was followed by TLC until completion (approx. 27 h). The reaction mixture was filtered to remove NaCl, and the filtrate concentrated under reduced pressure to give the crude product. Purification by preparative TLC with 10% ethyl acetate/hexane afforded pure *N*-decanoyloxy-*N*-decyloxybenzamide as a yellow oil. $\nu_{max}(CHCl_3)cm^{-1}$ 1778.9(C=O), 1725.6(C=O). δ_H 0.87(6H, t), 1.23(26H, br m), 1.57-1.64(4H, m), 2.32(2H, t), 4.15(2H, t), 7.40(2H, t), 7.52(1H, t), 7.74(2H, d). δ_C 14.06, 14.09, 22.64, 24.63, 25.78, 26.03, 28.06, 28.86, 29.13, 29.28, 29.48, 31.84, 32.13, 75.71, 128.17, 129.02, 132.00, 132.53, 171.10, 174.40. *m/z* (ESI): 447.3(M^+), 448.4($M+H^+$), 470.3($M+Na^+$).

N-Octanoyloxy-N-octyloxy-2-naphthamide ***(10b)***. *N*-Chloro-*N*-octyloxy-2-naphthamide (0.31 g, 0.94 mmol) was stirred with sodium acetate (0.22 g, 1.31 mmol) in dry acetone (10 mL) in the dark and followed by TLC until completion (approx. 22 h). The reaction mixture was filtered to remove NaCl, and the filtrate concentrated under reduced pressure to give the crude product. Centrifugal chromatography with 10% ethyl acetate/hexane afforded pure *N*-octanoyloxy-*N*-octyloxy-2-naphthamide as a yellow oil. $\nu_{max}(CHCl_3)cm^{-1}$ 1723.8(C=O), 1778.7(C=O). δ_H 0.83(6H, t), 1.21(18H, br m), 1.55-1.69(4H, m), 2.31(2H, t), 4.18(2H, t), 7.51-7.60(2H, m), 7.77-7.92(4H, m), 8.33(1H, d). δ_C 14.03, 22.52, 22.60, 24.67, 25.83, 27.48, 28.12, 28.79, 29.03, 29.13, 29.26, 31.47, 31.73, 32.17, 75.75, 124.89, 126.83, 127.78, 127.99, 128.34, 129.23, 130.43, 130.95, 132.25, 135.24, 171.23, 174.53. *m/z* (ESI): 441.3(M^+), 442.3($M+H^+$), 464.2($M+Na^+$).

2.3 Mutagenicity assays.

N-Acyloxy-*N*-alkoxyamides, freshly isolated as a single band from centrifugal chromatography were stored in a freezer and were analysed for purity by proton NMR prior to dissolution in dry, analytically pure DMSO. All Ames tests were carried out in *S. typhimurium* TA100 without metabolic activation according to standard protocols.[40,46] For scaling and direct comparison of new data with that previously obtained for all *N*-acyloxy-*N*-alkoxyamides from which QSAR (Equation 1) was derived, a standard, *N*-acetoxy-*N*-butoxybenzamide (**9f**) was tested in conjunction with new mutagens. Its activity at 1μmol/plate was used to scale the new data to that of previous data from these laboratories using the original activity for **9f** of 319 revertants at 1 μmol/plate.[4,6-9] For each new substrate the gradient over the linear dose response range, which is equal to the mutagenicity at 1μmol per plate, was scaled and reported as LogTA100.[‡] All compounds showed a good dose-response over the ranges reported, with toxicity appearing only at higher doses. At doses above those reported in Table 2, there was obvious precipitation of the more hydrophobic drugs when added to the top agar in the plate incorporation assay. Though all test compounds were soluble in DMSO, in which they were administered, the quantity of DMSO in top agar (constant at 100μL in 2mL according to protocol) was insufficient to maintain complete dissolution at higher doses.

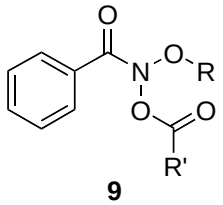
2.4 Quantitative structure-activity relationships

For predicted activities, Log *P* values were calculated using the Ghose-Crippen algorithm within MacSpartan'08.[47,48] These, together with all QSAR parameters used to establish Equations 1, 2 and 6 and 8 are presented as supplementary material.

[‡] Log_{10} of the activity in TA100 at 1μmol/plate

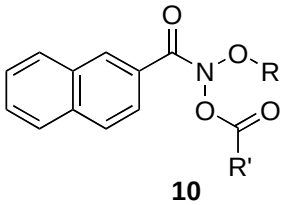
3. Results and discussion

Initially Log*P* calculations were employed to find structures of *N*-acyloxy-*N*-alkoxyamides with a common motif, which could support sequential, small structural changes to generate a series of compounds with a range of Log*P* values extending from within and beyond those previously employed (Log*P*<6) and which could be readily synthesised using our standard methodologies. These criteria were satisfied by a series of *N*-acyloxy-*N*-alkoxybenzamides bearing long chain hydrocarbons, of varying lengths, on the alkoxyl and acyloxyl side-chains (**9a-e**).



9

a) R=$(CH_2)_5CH_3$, R'=$(CH_2)_4CH_3$
b) R=$(CH_2)_6CH_3$, R'=$(CH_2)_5CH_3$
c) R=$(CH_2)_7CH_3$, R'=$(CH_2)_6CH_3$
d) R=$(CH_2)_8CH_3$, R'=$(CH_2)_7CH_3$
e) R=$(CH_2)_9CH_3$, R'=$(CH_2)_8CH_3$
f) R=Bu, R'=CH_3



10

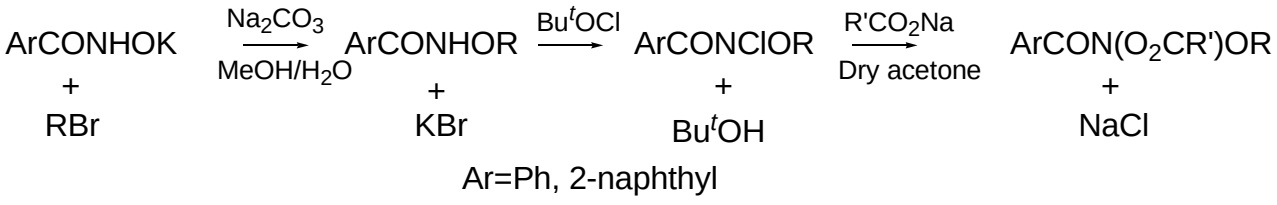
a) R=$(CH_2)_3CH_3$, R'=CH_3
b) R=$(CH_2)_7CH_3$, R'=$(CH_2)_6CH_3$

Table 1 gives the Log*P* values calculated for **9a-e** and a 2-naphthamide analogue of **9c**, **10b**.

Table 1. Calculated Ghose-Crippen Log*P* values,[47] and pK_A of R'CO_2H[49] for **9a-e** and **10b**

Compound	Substituents (R, R´)	Log*P*	pK_A
9a	Hexyl, Pentyl	5.18	4.86
9b	Heptyl, Hexyl	6.02	4.78
9c	Octyl, Heptyl	6.85	4.78
9d	Nonyl, Octyl	7.69	4.78
9e	Decyl, Nonyl	8.52	4.79
10b	Octyl, Heptyl	7.85	4.78

9a-e and **10b** were synthesised according to Scheme 2 from *N*-chlorohydroxamic esters and the sodium salts of the appropriate carboxylic acids according to previously published procedures.[2,4,7-9,24,29] Hydroxamic esters bearing the required alkoxyl groups were derived from the corresponding alkyl bromide and potassium benzo- and 2-naphthohydroxamates.[45,50]



Scheme 2

The mutagens were subjected to mutagenicity testing and Table 2 provides the revertant counts for compounds **9a-e** and **10b** in *S. typhimurium* TA100, along with the response for the *N*-acetoxy-*N*-butoxybenzamide standard **9f**, which was tested in conjunction with each batch of compounds.

Table 2. Dose-response data[a] for mutagenicity of **9f, 9a-e** and **10b** in *S. typhimurium* TA100

$R^1ON(OCOR^2)COR^3$ (R^1, R^2, R^3)	Set	Dose[b]	0.00	0.125	0.25	0.50	1.00
9f (Bu, Me, Ph)	A		97 (16)	114 (8)	158 (19)	249 (37)	359 (19) [T]
	B		138 (9)	181 (16)	244 (15)	302 (21)	199 (13) [T]
	C		109 (21)	111 (6)	113 (5)	164 (15)	246 (20)
	D		104(5)	111(6)	120(3)	164(15)	246(20)
9a (Hex, Pent, Ph)	A		97 (16)	441 (24)	660 (73)	885 (64) [T]	928 (54) [T]
		Dose[b]	0.00	0.03	0.06	0.125	0.25
9b (Hep, Hex, Ph)	B		138 (9)	195 (8)	280 (7)	369 (29)	470 (8) [T]
9c (Oct, Hep, Ph)	C		109 (21)	132 (2)	151 (3)	171 (17)	178 (8) [T]
9d (Non, Oct, Ph)	B		138 (9)	149 (7)	195 (18)	180 (10) [T]	174 (5) [T]
9e (Dec, Non, Ph)	C		109 (21)	112 (2)	123 (17)	151 (14)	149 (10) [T]
10b(Oct,Hep, 2-Np)	D		104(5)	121(2)	132(3)	142(4) [T]	164 (3) [T]

[a] Counts displayed are average of three plates per dose with standard deviation in parentheses
[b] All doses expressed as μmol/plate; [T] Denotes toxicity at this dose

Though toxic at higher doses, lower doses afforded linear dose-responses. All test batches (Set A-D) were scaled using the corresponding activity of *N*-acetoxy-*N*-butoxybenzamide (**9f**) for comparison to data in the training set,[¶] and experimental and scaled activities for for **9a-e** and **10b**, together with experimental and calculated LogTA100 data from the linear QSAR in Equation 1 are presented in Table 3.

[¶] Activities were standardised using the activity of *N*-acetoxy-*N*-butoxybenzamide **9f (**319 revertants/plate at 1μmol/plate).

Table 3. Experimental mutagenicity data for **9f** and **9a-e** and **10b** in *S. typhimurium* TA100 and their predicted LogTA100 calculated using Equation 1

Compound	Set	Gradient[a]	R^2	Scaled Gradient[b]	Experimental LogTA100	Calculated LogTA100	Diff.[c]
9f	A	273	0.99	319	2.50	2.58	0.08
	B	333	0.97	319	2.50	2.58	0.08
	C	147	0.96	319	2.50	2.58	0.08
	D	149	0.98	319	2.50	2.58	0.08
9a	A	2255	0.98	2635	3.42	3.37	-0.05
9b	B	1866	0.98	1788	3.25	3.59	0.34
9c	C	486	0.95	1055	3.02	3.82	0.80
9d	B	964	0.89	923	2.95	4.05	1.10
9e	C	351	0.96	762	2.88	4.29	1.41
10b	D	471	0.98	1011	3.00	4.10	1.10

[a] Induced Revertants at 1μmol/plate
[b] 319 x (gradient test compound/gradient $\mathbf{9f}_{A\text{-}D}$)
[c] Predicted LogTA100 – Experimental LogTA100

While the activity of the least hydrophobic mutagen **9a** (Log*P* = 5.18) is well predicted, there is a significant and incremental difference between the predicted and experimental mutagenicities of **9b-e** as the value of Log*P* rises above Log*P* = 5. Figure 1 illustrates the observed and predicted LogTA100 for the training set and **9a-e** and demonstrates the extent to which these LogTA100 values become radically over-predicted by the linear QSAR (Equation 1). It is likely that this is caused by membrane localisation of the strongly hydrophobic mutagens**.** Lipid entrapment results in a lower concentration of drugs penetrating cell membranes, thus reducing the effective drug concentration available to interact with the target, which in the case of mutagens *N*-acyloxy-*N*-alkoxyamides is cytosolic DNA. Above Log*P* ~ 6 there is a decreased concentration of the compound reaching the DNA of *S. typhimurium* TA100.[36] Drug-like compounds are more than likely to have a Log*P* < 5 and, similarly, direct-acting mutagens acting on *S. typhimurium* would be expected to conform to this rule.[51] With respect to the mutagenicity of *N*-acyloxy-*N*-alkoxyamides, there is clearly an optimum Log*P* value in the range of Log*P*'s covered by mutagens **9a-e**.

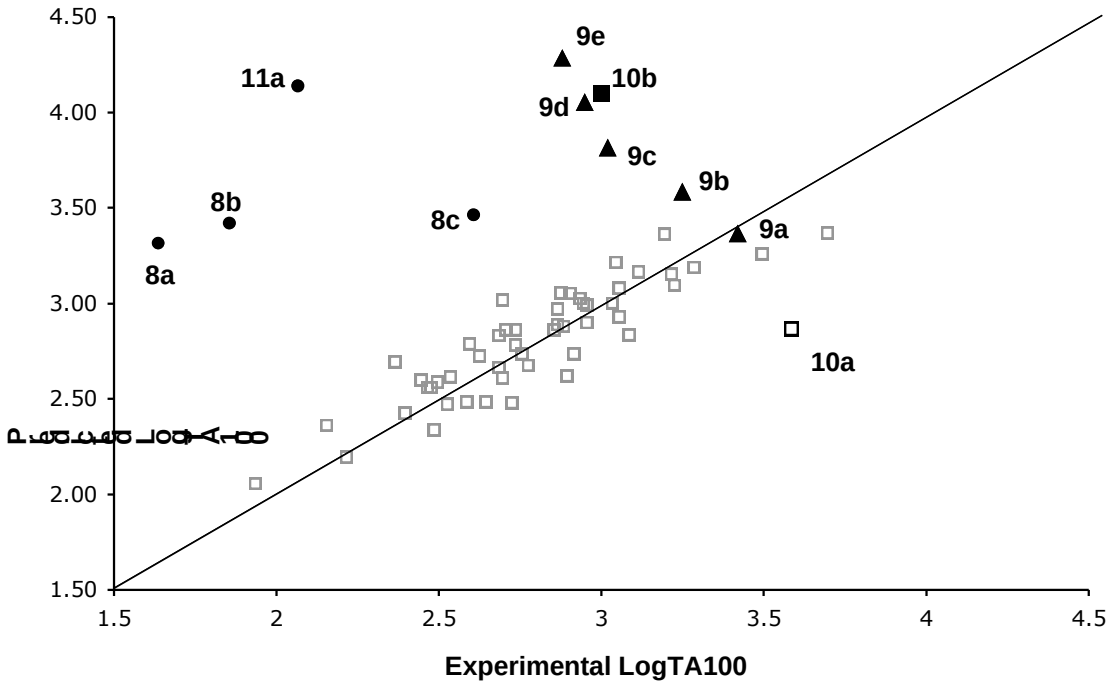


Figure 1. Predicted *vs* experimental mutagenicities for **9a-e** (triangles), **10a** (open square) and **10b** (filled square), **8a-c**, **11a** (closed circles) and training set (grey squares) in *S. typhimurium* TA100 using the linear QSAR in Equation 1

To establish a QSAR that encompasses the activities of increasingly hydrophobic mutagens **9b-e**, we chose Kubinyi's bilinear model (Equation 3)[52-54] for a number of reasons. While this approach and the traditional parabolic treatment (Equation 4) account for lipid entrapment,[36,54] and both methods generate an estimate of optimum LogP_0 where the gradient is zero, the bilinear method is a theoretically derived model,[53] and the positive, initial slope can be compared with the slope of linear QSAR.[55] In Equation 3, a, b and c are linear terms calculated through linear multiple regression, and β is a no*n*-linear term calculated by an iterative procedure. In cases where Log*P* is sufficiently small, β*P* becomes negligible; thus the Log (β*P* + 1) term reduces to zero, converting the model to a linear form (Equation 5).

$$\text{Log TA100} = a\,\text{Log}P + b\,\text{Log}(\beta P + 1) + c$$

Equation 3

$$\text{Log TA100} = a\,(\text{Log}P)^2 + b\,\text{Log}P + c$$

Equation 4

LogTA100 = aLog*P* + c

Equation 5

Equation 1 shows that while there are additional factors contributing to the mutagenic activity of *N*-acyloxy-*N*-alkoxyamides, the most significant contribution is that of Log*P*. As such, initially we formulated the bilinear model in Equation 6 for mutagenic activity of *N*-acyloxy-*N*-alkoxyamides using Log*P* as the sole descriptor and compared its predictive ability to that of Equation 2, which gives the linear dependence of Log TA100 upon Log*P* for our training set.

Stepwise iteration yielded the bilinear function in Equation 6

LogTA100 = 0.208 (±0.02) Log*P* – 0.472 (±0.10) Log(β*P* + 1) + 2.02 (±0.08)

Logβ = -6.466, n = 55, r^2 = 0.68, adj. r^2 = 0.66, s = 0.19, F = 36.0; LOOCV Q^2 = 0.64

Equation 6

Table 4 shows that by using the bilinear model the activity of the more highly hydrophobic compounds **9c-e** are well predicted when compared to the linear model and the prediction for **9a** and **9b** are similar. A maximal LogP_0 of 6.36 can be deduced from the values of β and coefficients *a* and *b* from Equation 6 using Equation 7.[53]

$P_O = a / \beta(b - a)$ (only for b > a)

Equation 7

Table 4. Experimental and predicted mutagenicities for **9a-f** in *S. typhimurium* TA100 when LogTA100 is calculated using linear QSAR (Equation 2) and bilinear QSAR (Equation 6)

Compound	Experimental Log TA100	Calculated Log TA100 (Linear)[a]	Linear diff.[b]	Calculated Log TA100 (Bilinear)[c]	Bilinear diff.[b]
9f	2.50	2.54	0.04	2.53	0.03
9a	3.42	3.06	-0.36	3.09	-0.33
9b	3.25	3.22	-0.03	3.22	-0.03
9c	3.02	3.37	0.35	3.20	0.18
9d	2.95	3.53	0.58	3.04	0.09
9e	2.88	3.69	0.81	2.83	-0.05

[a] Calculated according to Equation 2
[b] Predicted LogTA100 – Experimental LogTA100
[c] Calculated according to Equation 6

The coefficient of the linear component of Equation 6 (0.207 ±0.02) compares very well with the Log*P* dependence of the activities of the training set in Equation 2 (0.189 ±0.02). The mean absolute errors for the training set using Equation 2 and Equation 6 are 0.144 and 0.142 respectively indicating that the bilinear model nicely accounts for the activities where Log*P* < LogP_0. The LOO cross validation coefficient of 0.64 indicates the good predictive ability of this QSAR.

A number of congeners bearing bulky *para* substituted phenyl systems in the training set were over-predicted by Equation 6 presumably due to the no*n*-incorporation of steric effects. Recalculation of the bilinear model and including appropriate steric parameters E_S^1, E_s^2, and E_S^3 and computed or experimental pK_A's for the carboxylic acid corresponding to the acyloxyl group gave, after iteration, the more comprehensive bilinear QSAR in Equation 8, which has a measurably improved adj. r^2 when compared to that of Equation 6.

LogTA100 = 0.243 (±0.02) Log*P* - 0.667 (±0.12) Log(β*P* + 1) + 0.106 (±0.08) pK_A + 0.088 (±0.03) E_S^1 + 0.092 (±0.04) E_S^2 + 0.022 (±0.05) E_S^3 + 1.466 (±0.41)

Logβ = -6.639, n = 55, r^2 = 0.75, adj. r^2 = 0.72, s = 0.18, F = 19.7; LOOCV Q^2 = 0.60,

Equation 8

From Equation 8 and Equation 7, the maximal LogP_0 is 6.4.

Using Equation 8 to calculate predicted mutagenicity gives the satisfactory distribution shown in Figure 2. Notably the highly hydrophobic mutagens **9c-e** are well predicted but for mutagens with

Log$P$ < LogP_0, and where the bilinear term is negligible, the coefficients are not unreasonable when compared to the linear correlation in Equation 1. The mean absolute errors for the training set from Equation 8 (0.127) is similar to that from Equation 1 (0.12). Statistically, the Log$P$, Log(βP + 1) and the E_s^1 and E_S^2 terms are again significant. The pK_A and E_S^3 terms are less important in Equation 8 than in Equation 1 although removal of both from the correlation results in little change in the standard error (Supplementary material). LOO cross validation indicates the good predictive ability of Equation 8.

Mutagens with Log$P$ > LogP_0 would be expected to penetrate lipid membranes to a much lower degree resulting in diminution of concentration at the DNA target (K_L in Scheme 1 is limiting) Structural factors influencing DNA binding (impacting on K_M and k_M in Scheme 1) would be less evident in this case. The observed activity of naphthalene-containing mutagens confirms this. Previously, we have shown that the presence of a naphthalene unit on any side chain of **1** results in a mutagenicity (LogTA100) about one order of magnitude greater than predicted by the linear QSAR (Equation 1), which we have attributed to the intercalative binding resulting in increased association with DNA (increased K_M Scheme 1).[4,6,7,10] By way of example, naphthamide mutagen **10a**, with a Log$P$ of 3.44 has an experimental activity (TA100 = 4214) 5.8 times greater than that predicted by linear QSAR in Equation 1 (TA100 = 724) (Table 5, Figure 1). Upon application of full bilinear QSAR (Equation 8) its activity is still under-predicted by LogTA100=0.78 (Table 5, Figure 2). Naphthamide mutagen **10b**, on the other hand, has a computed Log$P$ of 7.85, significantly higher than LogP_0. Like **9d**, which has a similar Log$P$, its activity is greatly reduced when compared to its predicted LogTA100 from linear QSAR in Equation 1 (Table 5, Figure 1). However, its activity is predicted well by the full bilinear QSAR in Equation 8 (Table 5, Figure 2). The activity enhancement due to the naphthamide substructure is, as predicted, negated owing to lipid entrapment and dominance of a limiting K_L in Scheme 1.

Comparing **10a** (Log$P$ = 3.44, LogTA100 = 3.59) and **9f** (Log$P$ = 2.44, LogTA100 = 2.5), based on Equation 8 the activity enhancement generated by the increased hydrophobicity (Log$P$ = 1), which is due to the additional aromatic ring, is LogTA100 = 0.24. The increase in experimental activity is much greater (LogTA100 = 1.09) and the component due to other factors is LogTA100 = 0.85, which equates with the impact of about an additional 3.5 Log$P$ units. We will demonstrate the generality of this finding for other naphthalene-substituted mutagens in a future publication. Importantly, a mutagen with Log$P$ = 5 but bearing a single naphthalene unit might not suffer lipid entrapment and would generate mutagenicity equivalent to a substrate with Log$P$ ~ 8.

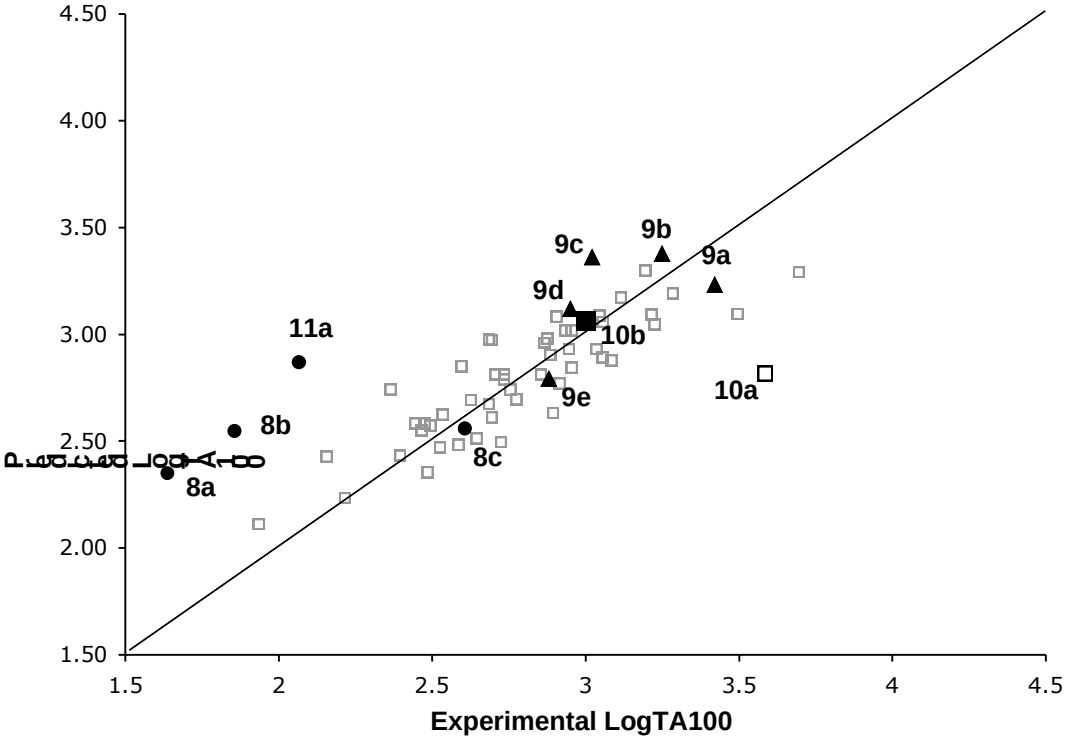
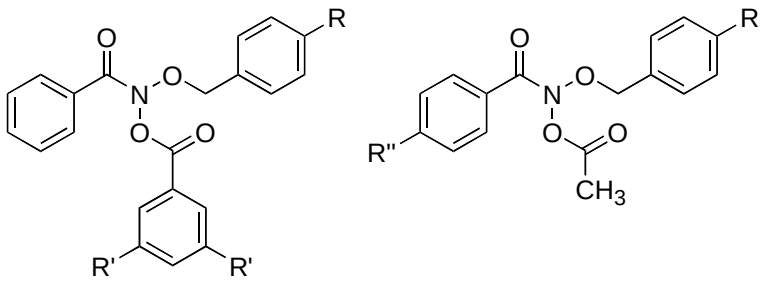


Figure 2. Predicted vs experimental mutagenicities for **9a-e** (triangles), **10a** (open square) and **10b** (filled square), **8a-c, 11a** (closed circles) and training set (grey squares) in *Salmonella* TA100 using the full bilinear QSAR in Equation 8**.**

Table 5. Experimental and predicted mutagenicities for standard **9f**, naphthamides **10a**, **10b** and di-*tert*-butylate substrates **8a-c**, **11a** and in *S. typhimurium* TA100 when LogTA100 is calculated using linear QSAR in Equation 1 and bilinear QSAR in Equation 8

Compound (Log*P*)	Experimental Log TA100	R^2	Predicted Log TA100 (Linear)[a]	Diff. (Linear)[b]	Predicted Log TA100 (Bilinear)[c]	Diff. (Bilinear)[a]
9f (2.44)	2.50	-	2.58	0.08	2.57	0.07
8a (8.24)	1.64	0.99	3.31	1.67	2.34	0.70
8b (8.24)	1.86	0.90	3.41	1.55	2.54	0.68
8c (8.24)	2.61	0.99	3.46	0.85	2.55	0.06
12 (6.3)	2.89	0.99	2.87	-0.02	2.90	0.01
11a (8.24)	2.07	0.99	4.13	2.06	2.86	0.79
10a (3.44)	3.59	0.95	2.86	-0.73	2.81	-0.78
10b (7.85)	3.00	0.91	4.10	1.10	3.06	0.06

[a] Calculated according to Equation 1
[b] Predicted LogTA100 – Experimental LogTA100
[c] Calculated according to Equation 8



11 **12**

a) R'=Bu^t, R=H
b) R= R'=Bu^t

We earlier reported the synthesis and testing of a series of di- (**8a-c**, **11a**) and tri- (**8d** and **11b**) *tert*-butylated *N*-benzoyloxy-*N*-benzyloxybenzamides along with di-*tert*-butylated *N*-acetoxy-*N*-benzyloxybenzamide **12**.[6,8] Apart from **12**, which is not triarylated and, with a Log*P* = 6.3 is well predicted by the linear and bilinear QSARs in Equation 1 and Equation 8, these mutagens exhibited exceptionally low activity. No dose-response could be obtained for the tri-*tert*-butylated congeners (**8d**, **11b**) due to precipitation and **8a-c**, and **11a** gave very low dose-responses rendering them essentially no*n*-mutagenic. We originally proposed that the di-*tert*-butyl or tri-*tert*-butyl tri-aryl systems were unable to react with DNA due to overall dimensions of these compounds, which could exclude them from the grooves of DNA. We previously reported AM1 calculations, which indicated that the minimum energy conformation of these di-*tert*-butylated *N*-benzoyloxy-*N*-benzyloxybenzamides are likely to have cross-sections that approach the width of the major groove (12 Å). Distances between *tert*-butyl groups in the crystal structure of **8d** are *ca* 17, 15 and 5Å.[18] Alternatively, the significant Log*P* values for these di-*tert*-butylated, triaryl compounds (Log*P* = 8.24) may also cause membrane localisation of these more lipophilic substrates.

With the development of the bilinear QSAR in Equation 8 it becomes possible to discriminate between these two possible causes for reduced mutagenicity. Using their Log*P* values and appropriate E_S^1, E_s^2 and E_S^3 values for *tert*-butyl (-2.78)[36,56], activities were predicted from linear QSAR Equation 1 and bilinear QSAR Equation 8 (Table 5). There is a significant deviation of all of these compounds from the unity line when the linear QSAR in Equation 1 is used as a predictive tool (Table 5, Figure 1). However, when their high hydrophobicity is taken into account, the activity of three mutagens (**8a**, **8b** and **11a**) still deviate negatively by close to an order of magnitude relative to the predicted activity (Table 5, Figure 2). **8c** becomes well predicted using the bilinear QSAR indicating that the deviation of this compound from the linear QSAR can largely be attributed to the impact of additional hydrophobicity. The over-prediction of the mutagenic activity of **8a**, **8b** and **11a** shows that while some of the reduction in their mutagenic activity can be

attributed to hydrophobic effects alone there is clearly an additional mechanism resulting in their impaired activity. Membrane localisation can be eliminated as the sole reason; it is only a contributor to the overall diminution in activity. Size exclusion from the major groove of DNA and/or inability to attain a suitable transition state for reaction with DNA bases will both radically reduce the mutagenic activity of *N*-acyloxy-*N*-alkoxyamides. From the difference between **8c** and the other di-*tert*-butyl congeners **8a** and **8b**, it is also clear that absence of a *t*-butyl group on the benzyloxyl side-chain, as in **8c**, is important in differentiating steric exclusion from the groove and hydrophobic inhibition of mutagen. Groove exclusion is potentially responsible for the additional reduction in mutagenic activity of **8a**, **8b**.

In the case of the di-*tert*-butyl congeners it is likely that for **8c** a smaller K_L (Scheme 1) is the determining factor, where its high hydrophobicity influences the rate of diffusion through the cell membrane. However, for **8a**, **8b** and **11a** both K_L and K_M are discriminating. Not only is diffusion influenced by increased hydrophobicity, but the formation of the NAA/DNA complex is impeded through size exclusion.

Thus, the bilinear QSAR for *N*-acyloxy-*N*-alkoxyamides can be used to further explore and understand the mechanisms underlying mutagenic activity of selected *N*-acyloxy-*N*-alkoxyamides, in which steric and hydrophobic effects may be important.

4. Conclusion.

The mutagenic response of *N*-acyloxy-*N*-alkoxyamides to changes in hydrophobicity is dual in nature. Where Log*P* is below LogP_0, which has been established to be Log*P*= 6.4, the positive, linear dependence relates to binding of the mutagens with the target, in this case cytosolic DNA. The activity of mutagens with Log*P* above LogP_0 is adversely affected by hydrophobicity and can more than likely be ascribed to well known lipid entrapment. In effect, this process diminishes mutagen concentration reaching the DNA thereby masking the smaller, positive influence of hydrophobicity on binding to DNA. The limiting Log*P* is in line with the Lipinski rules for drug behaviour although LogP_0 is a little higher for bacterial *S. typhimurium*.[51] A bilinear dependence upon hydrophobicity has been demonstrated of which the rising portion is similar to the linear QSAR for *N*-acyloxy-*N*-alkoxyamides with low Log*P* (<6). With this extended relationship we have been able to discriminate between influences of structure that relate to hydrophobicity and other factors. Where restrictions on DNA groove binding are significant, such as size exclusion, loss in activity can be partitioned between that imparted by lipid entrapment and that imposed by steric bulk. The positive impact of a single naphthalene group upon binding to DNA is shown to be equivalent to that of about an additional three Log*P* units.

The smaller, positive dependence upon hydrophobicity of DNA binding, as well as other dependencies on steric factors and the inverse dependence upon reactivity, are measurable with QSAR due to a coherent testing strategy throughout all mutagenicity studies on *N*-acyloxy-*N*-alkoxyamides; by co-testing all new congeners with a single reference standard and normalising new data, we have largely been able to compensate for variations in testing conditions. We strongly recommend this strategy where possible. As shown in this study, the confidence in data used to establish our training set enables deviations from the QSAR to be detected from which meaningful interpretations can be made.

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