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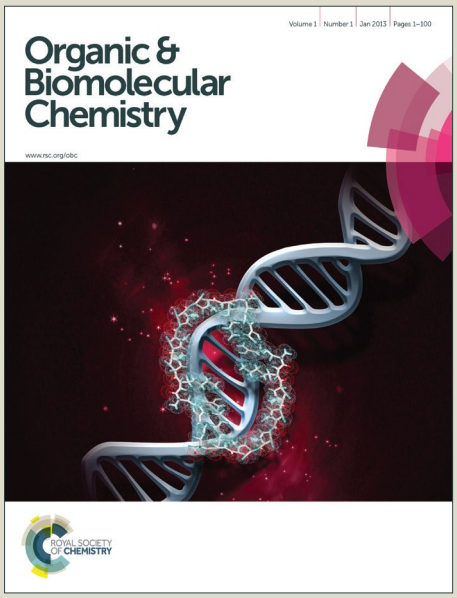

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Mutagenicity of *N*-Acyloxy-*N*-alkoxyamides as an indicator of DNA intercalation

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Part 1: Evidence for naphthalene as a DNA intercalator[†]

Tony. M. Banks, Samuel F. Clay, Stephen A. Glover[**] and Rhiannon S. Schumacher.

Chemistry Department, School of Science and Technology, University of New England, Armidale New South Wales 2351, Australia.

Abstract

N-Acyloxy-*N*-alkoxyamides are direct-acting mutagens in *S. typhimurium* TA100 with a linear dependence upon Log*P* that maximises at LogP_o = 6.4. Eight *N*-acyloxy-*N*-alkoxyamides (**2**-**9**) bearing a naphthalene group on any of the three side-chains and with LogP_o < 6.4 have been demonstrated to be significantly and uniformly more mutagenic towards *S. typhimurium* TA100 than 50 mutagens without naphthalene. The activity enhancement of **2**-**9** is likely due to intercalative binding of naphthalene to bacterial DNA as a number are also active in TA98, a frame-shift strain of *S. typhimurium*, which is modified by intercalators. DNA damage profiles for naphthalene-bearing mutagens confirm enhanced reactivity with DNA when naphthalene is incorporated and a different binding mode when compared to mutagens without naphthalene. The effect is independent of whether the naphthalene is attached to an electron-donating alkyl or electron-withdrawing acyl group, alkyl tether length or, in the case of **6** and **7**, the point of attachment to naphthalene. A new quantitative structure activity relationship has been constructed for all 58 congeners incorporating Log*P* and an indicator variable, *I*, for the presence (*I*=1) or absence (*I*=0) of naphthalene and from which the activity enhancing effect of a naphthalene has been quantified at between three and four Log*P* units. Contrary to conventional views, simple naphthalene groups could target molecules to DNA through intercalation.

[†] Electronic supplementary information (ESI) is available.

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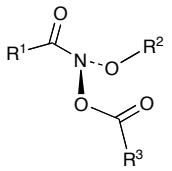
Introduction

N-Acyloxy-*N*-alkoxyamides **1** are members of the class of anomeric amides, amides substituted at nitrogen with two heteroatoms.[1, 2] These amides are unusual in that the nitrogen is strongly pyramidal and this, together with the inductive effect of the two heteroatoms, results in a much weaker resonance between the nitrogen lone pair and the carbonyl.[1-5] In this case, the amides are electrophilic agents, reacting with nucleophiles at the nitrogen with displacement of the acyloxyl group.[4, 6-12]



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Since they were first synthesised, they have been shown to be direct-acting mutagens and a wide number that have been tested in the Ames reverse mutation assay are mutagenic towards *Salmonella typhimurium* TA100 without the requirement for metabolic activation.[4, 7, 13-22] Well over a hundred congeners have now been synthesised and tested and several quantitative structure activity relationships (QSAR's) have evolved that can explain, with reasonable accuracy, their mutagenic activity in TA100.[4, 18-22] For congeners with Log$P$ < 6, a QSAR based on the activity of fifty congeners, equation (1), relates the activity to hydrophobicity (Log$P$) of the intact mutagen, the stability to extraneous S_N2 reactions through pK_A of the acyloxyl group and, in pertinent cases, through Taft steric parameters to the size of *para* substituents on benzyloxy (E_s^1), benzoyloxy (E_s^2) and benzamide (E_s^3) side chains.[21] The leave-one-out cross validation coefficient, Q^2 of 0.7 indicates good predictive ability.

$$\text{Log TA100} = 0.28\ (\pm0.03)\ \text{Log}P + 0.18\ (\pm0.08)\ \text{p}K_A + 0.13\ (\pm0.03)\ E_S^1 + 0.15\ (\pm0.04)\ E_s^2 + 0.11\ (\pm0.05)\ E_S^3 + 1.02\ (\pm0.4)$$

$$n = 50,\ r^2 = 0.7968,\ \text{adj. } r^2 = 0.7736,\ s = 0.16,\ F = 34.5;\ \text{LOOCV } Q^2 = 0.6987 \qquad (1)$$

The mutagenic activity of **1** undoubtedly derives from their electrophilic behaviour towards DNA, as our DNA damage studies have shown that they damage DNA at guanosine-N7 (N7-G) and adenosine-N3 (N3-A), the most nucleophilic centres in duplex DNA.[18, 23-27] Such damage renders DNA susceptible to cleavage reactions from which the reactive bases can be deduced.[18]

The QSAR in equation (1) permits identification of other structural factors that impact upon mutagenicity. For instance, branching in R^1 on the carbon α to the amide carbonyl in **1** results in loss of mutagenic activity, which is accounted for by a corresponding resistance to S_N2 reactivity at nitrogen. In those substrates, the limiting factor in Fig. 1 is a low rate of reactivity with DNA bases, ie a low k_M.[4, 20] In other cases, large mutagens with dimensions similar to those of the major groove of DNA show radically reduced activity when compared to the predictions based on equation (1). Such mutagens are the triaryl *N*-benzoyloxy-*N*-benzyloxybenzamides (**1**, R^1=R^3=Ph, R^2=Bn) bearing at least two *para tert*-butyl groups. In these cases, complexation with DNA is limiting due to a low K_M,[4, 20] although their higher hydrophobicity and a reduced K_L also plays a role.[22]

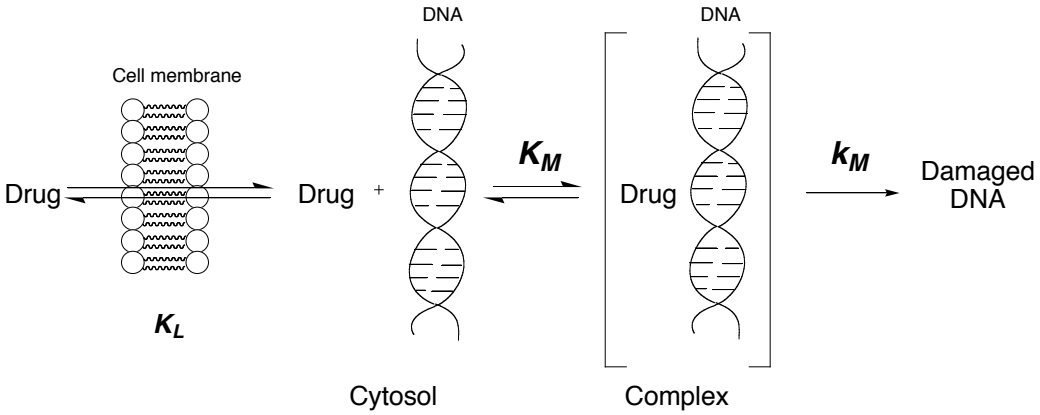

Fig. 1 Elemental processes in reaction of *N*-acyloxy-*N*-alkoxyamides with DNA.

Branching at R^3 on the α-carbon on the acyloxyl side chain also results in radically reduced activity but these mutagens readily undergo S_N2 reactions at nitrogen, a process unimpeded by steric bulk on the leaving group. Mutagens in this class are relatively small so K_m cannot be limiting. Here we have concluded that steric bulk prevents binding in the DNA grooves with an orientation that permits S_N2 reaction at the amide nitrogen.[4, 21] Finally, a series of *N*-acyloxy-*N*-alkoxyamides with branching in R^2 close to the alkoxyl group oxygen are slow to undergo S_N2 reaction at nitrogen,[11] but are nonetheless as mutagenic as predicted by equation (1). In this case, we have suggested that S_N2 reactivity is easier in the hydrophobic environment of the major groove where the disadvantage of desolvation (a powerful rate limiting factor in solution reactions) is not operative.[4, 21].

We have recently established that where *N*-acyloxy-*N*-alkoxyamides are much more hydrophobic (Log$P$ > 6), a bilinear relationship holds (equation (2)) with maximal Log$P$, LogP_0 of 6.4.[22]

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$$\text{LogTA100} = 0.243\ (\pm 0.02)\ \text{Log}P - 0.667\ (\pm 0.12)\ \text{Log}(\beta P + 1) + 0.106\ (\pm 0.08)\ pK_A + 0.088\ (\pm 0.03)\ E_S^1 + 0.092\ (\pm 0.04)\ E_S^2 + 0.022\ (\pm 0.05)\ E_S^3 + 1.466\ (\pm 0.41)$$

$$\text{Log}\beta = -6.639,\ n = 55,\ r^2 = 0.7458,\ \text{adj. } r^2 = 0.714,\ s = 0.18,\ F = 19.7017;\ \text{LOOCV } Q^2 = 0.5991, \quad (2)$$

The positive Log*P* dependency in this QSAR relates to hydrophobic binding to cytosolic DNA affecting K_M in Fig. 1, while above LogP_0 the negative dependence most probably relates to normal lipid entrapment, in this case in the bacterial membrane, thereby diminishing K_L in Fig. 1.

In earlier studies, we found that *N*-acetoxy-*N*-butoxy-2-naphthamide **2** exhibited mutagenicity, which is about an order of magnitude higher than is predicted by equation (1) and equation (2), an observation supported by DNA damage studies.[4, 17-19, 22] Relative to several *N*-acyloxy-*N*-alkoxybenzamides, *N*-acetoxy-*N*-butoxy-2-naphthamide, with a significantly lower Log*P*, clearly induced more damage to plasmid DNA at N7-G.[18] In this paper we demonstrate that incorporation of naphthyl group on any of the three side chains of **1** results in similarly enhanced levels of mutagenicity and DNA damage, which we demonstrate is most probably owing to intercalation of naphthyl at specific sites of DNA.

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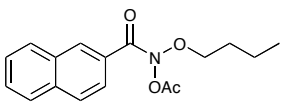
Results and Discussion

The enhanced DNA damage observed with **2**, as well as its increased mutagenic activity was attributed to the potential for the naphthyl moiety to intercalate between the bases of DNA, resulting in stronger drug-DNA interactions and increased K_M in Fig. 1. Through intercalative binding, an *N*-acyloxy-*N*-alkoxyamide would have a longer residence time on DNA than if DNA binding was simply under classical diffusion and hydrophobic control. It was postulated that this extended residence time could increase the time dependant probability for reaction with the bases of DNA.[28] To establish whether this tenet holds for other naphthalene-substituted mutagens, a series of congeners, **3**-**9**, each bearing a single naphthalene unit on one of the three side chains of **1** were synthesised and subjected to our standard mutagenicity assay using point mutation strain *S. typhimurium* TA100.



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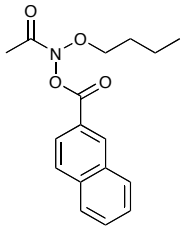

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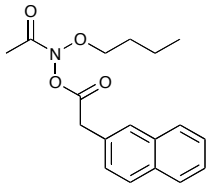

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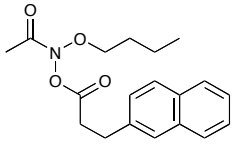

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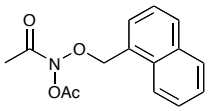

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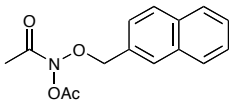

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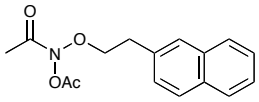

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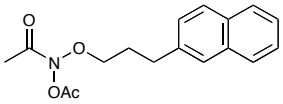

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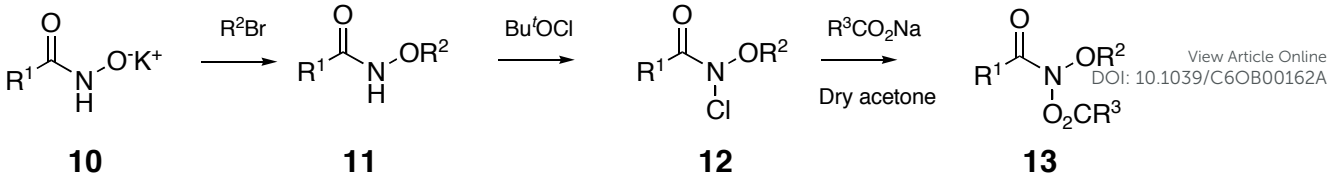
Synthetic procedures

The *N*-acyloxyl derivatives **13** of the appropriate hydroxamic ester **11** were obtained according to our established protocol (Scheme 1) whereby the *N*-chloro derivative **12** was treated with the required sodium carboxylate in anhydrous acetone.[7, 11, 15-18, 21, 22] Hydroxamic esters **11** were synthesised by reaction of the potassium salt of the hydroxamic acid **10** with the appropriate alkyl bromide,[29] or for **2**, by reaction of *n*-butoxyamine with 2-naphthoyl chloride. The synthesis of **8** and **9** required 2-(2'-naphthyl)ethyl and 3-(2'-naphthyl)propyl bromides, which were obtained by side chain elongation of 2-naphthylmethyl bromide (Scheme 2) and 2-naphthaldehyde respectively (Scheme 3).



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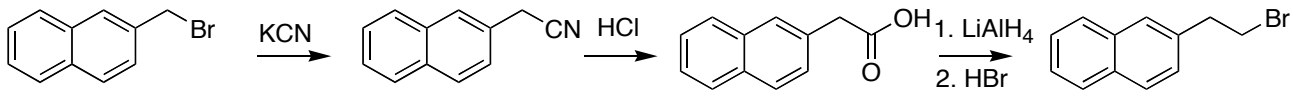
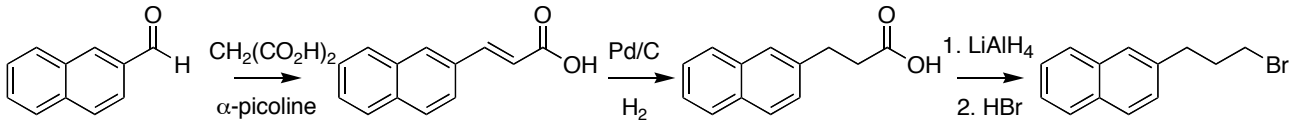
10 **11** **12** **13**

Scheme 1



Scheme 2



Scheme 3

Table 1 IR carbonyl absorption frequencies ($CHCl_3$) and ^{13}C chemical shifts ($CDCl_3$) for **2** and **9**

Compound	Amide C=O		Ester C=O	
	IR (cm^{-1})	^{13}C ($CDCl_3$)	IR (cm^{-1})	^{13}C ($CDCl_3$)
2	1724	174.0	1792	168.0[17]
3	1737	176.5	1757	164.3
4	1740	176.3	1789	168.3
5	1739	176.2	1798	164.6
6	1739	176.4	1793	167.9
7	1740	176.3	1793	168.0
8	1735	176.3	1793	168.0
9	1740	176.2	1794	167.9

Table 1 summarises the main infrared carbonyl stretch frequencies and carbonyl ^{13}C NMR shifts for mutagens **3**-**9** together with corresponding data for **2**. Their amide carbonyl stretch frequencies are typical of all other acetamide analogues, which are found between 1735 -1745 cm^{-1} and somewhat higher than aromatic amides such as **2**.[4] The data are characteristic of *N*-acyloxy-*N*-alkoxyamides where the very high amide carbonyl stretch frequency is due to the much reduced resonance owing to the pyramidal nature of the amide nitrogen and to destabilisation of the charge-separated, single bond form of the carbonyl on account of the gross electronegativity of the amide nitrogen group. The alkanoyloxyl carbonyls are uniformly high at around 1790 cm^{-1} and, typically, aryl conjugation

in **3** results in a lower frequency. The amide and ester carbonyl carbon ^{13}C NMR shifts are also characteristic. In this class of amides, most acetamide carbonyls resonate around δ176, while ester carbonyls generally resonate in the region of δ160-170.[4]

Mutation studies

Activity towards S. typhimurium TA100

Mutagens **2** – **9** were subjected to the Ames assay in *S. typhimurium* TA100 without microsomal activator S9, since all *N*-acyloxy-*N*-alkoxyamides are direct-acting. The standard, *N*-acetoxy-*N*-butoxybenzamide **14**, which we have consistently used to scale different test batches and which has provided a means of normalising activity of all mutagens in all our studies on *N*-acyloxy-*N*-alkoxyamides to date, was tested in conjunction with each batch of mutagens. The dose-response data for naphthalene bearing mutagens **2** to **9** together with that for **14** is presented in Tables 2-4.

Table 2 Dose response data for **2**, **3**, **6, 7** and **14** in *S. typhimurium* TA100 without S9.[a]

μmol/plate	**14**	μmol/plate	**2**	**3**	**6**	μmol/plate	**7**
0.000	211.3 (12)	0.000	209.7 (12)	209.7 (12)	209.7 (12)	0.000	211.3 (12)
0.250	441.0 (6)	0.015	224.7 (12)	238.3 (15)	241.7 (15)	0.060	727.7 (57)
0.500	634.3 (30)	0.030	418.0 (34)	517.0 (24)	420.3 (32)	0.125	1157.3 (59)
1.000	938.7 (69)	0.060	744.7 (57)	759.3 (21)	680.0 (23)	0.250	1442.0 (69)
1.500	[T]					0.500	1962.7 (40)
Gradient	717.71		9482.5	9844.4	8281.3		7554.7

[a] Counts displayed are average of three plates per dose with standard deviation in parentheses;T indicates toxicity at this dose

Table 3 Dose response data for **8**, **9** and **14** in *S. typhimurium* TA100 without S9.[a]

μmol/plate	**14**	μmol/plate	**8**	**9**
0.000	165 (24)	0.000	165 (24)	165 (24)
0.120	315 (28)	0.060	794 (13)	987 (37)
0.250	379 (21)	0.120	1276 (37)	1453 (61)
0.500	678 (43)	0.250	2044 (59)	2024 (69)
1.000	1311 (62)	0.500	2768 (129)	1773 (102)
2.000	1081 (90)	1.000	1651 (101)	[T]
Gradient	1143.1		9252.8	10731.0

[a] Counts displayed are average of three plates per dose with standard deviation in parentheses;T indicates toxicity at this dose

Table 4 Dose response data for **4**, **5** and **14** in *S. typhimurium* TA100 without S9.[a]

μmol/plate	**14**	μmol/plate	**4**	**5**
0.000	91 (9)	0.000	91 (9)	91 (9)
0.125	127 (16)	0.015	110 (14)	148 (16)
0.250	146 (28)	0.030	143 (10)	166 (20)
0.500	200 (21)	0.060	190 (21)	217 (11)
1.000	312 (7)	0.125	219 (14)[T]	271 (27)[T]
Gradient	217.0		1694.7	1980.4

[a] Counts displayed are average of three plates per dose with standard deviation in parentheses;T indicates toxicity at this dose

All compounds showed a good dose response with some deviation from linearity at concentration above 0.5 μmol/plate owing to toxicity (see ESI). The gradients were obtained from the linear region of the dose-response plots and scaled as usual to *N*-acetoxy-*N*-butoxybenzamide **14** (319 revertants/plate at 1 μmole). The scaled activity of *N*-acetoxy-*N*-butoxy-2-naphthamide in this study (LogTA100 = 3.62) was almost identical to that previously determined (LogTA100 = 3.56)[17]

All members of this naphthyl series are significantly more mutagenic than non-naphthyl substituted benzamides and acetamides upon which the linear QSAR in equation (1) is based. Computed Log*P* values, pK_A's (of the carboxylic acid corresponding to the leaving group) required for application of equation (1) or equation (2), gradients and scaled gradients (activities at 1 μmole/plate) are presented in Table 5.‡ All Log*P* values for **2**-**9** are well below LogP_O (LogP_O = 6.4),[22] therefore it is possible to apply the linear QSAR (equation (1)) to give the predicted mutagenicities, which are also presented in Table 5 together with the differences between theoretical and experimental activities.

‡ Taft steric parameters E_S^1,E_s^2 and E_S^3 are not applicable to **2**-**9** and are given a value of zero.

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Table 5 Experimental and predicted mutagenicity for **2**-**9** in *S. typhimurium* TA100 when LogTA100 is calculated using linear QSAR in equation (1).

Compound	Log*P*	pK_A	Gradient[a]	R^2	Scaled Gradient	Experimental LogTA100[b]	Predicted LogTA100	Diff.[c]
2	3.44	4.76	9483	0.95	4214	3.62	2.86	-0.76
3	3.44	4.16	9844	0.95	4375	3.64	2.75	-0.89
4	3.38	4.24	1695	0.99	2491	3.40	2.75	-0.65
5	3.80	4.57	1980	0.95	2911	3.46	2.93	-0.53
6	2.03	4.76	8281	0.97	3680	3.53	2.47	-1.06
7	2.03	4.76	7555	0.90	3358	3.57	2.47	-1.10
8	2.31	4.76	9253	0.98	2583	3.41	2.54	-0.87
9	2.73	4.76	10733	0.99	2995	3.48	2.66	-0.82

[a] Induced revertants at 1μmol/plate

[b] Log of scaled gradient

[c] Predicted LogTA100 – Experimental LogTA100

The experimental and predicted activities for the naphthalene series, together with the training set of 50 compounds without naphthalene as a substituent,[21, 22] are illustrated in Fig. 2 and show that the mutagenicities of all naphthalene-containing compounds are significantly under-predicted by equation (1). From differences in Table 5, the average discrepancy between experimental and predicted activities at 1μmol/plate is in the order of 1 LogTA100 unit. There is clearly an additional factor enhancing the mutagenic activity of *N*-acyloxy-*N*-alkoxyamides bearing naphthalene groups, which within this series, is operative irrespective of which side chain is functionalised, length of tethering groups and electronic effects; naphthalene is substituted by both +*I* alkyl groups (**4**-**9**) as well as –*M* carbonyl groups (**2** and **3**). 1- and 2-naphthylmethoxy substituents in **6** and **7** also produce similar enhancement.

The positive hydrophobic correlation with mutagenicity for the compounds previously tested (grey squares in Fig. 2) and embodied in the modest Log*P* term in equation (1), has been ascribed to a hydrophobic interaction between DNA and the groove-bound mutagens. However, in the case of the naphthalene series it is likely that the increased binding due to the naphthyl rings is due to intercalation in the DNA matrix. This type of binding involves π-stacking and charge transfer, both of which result in much stronger drug-DNA interactions and longer residence time on DNA. With substrates **2-9**, K_M (Fig. 1) would be larger leading to a higher probability of reaction with DNA.

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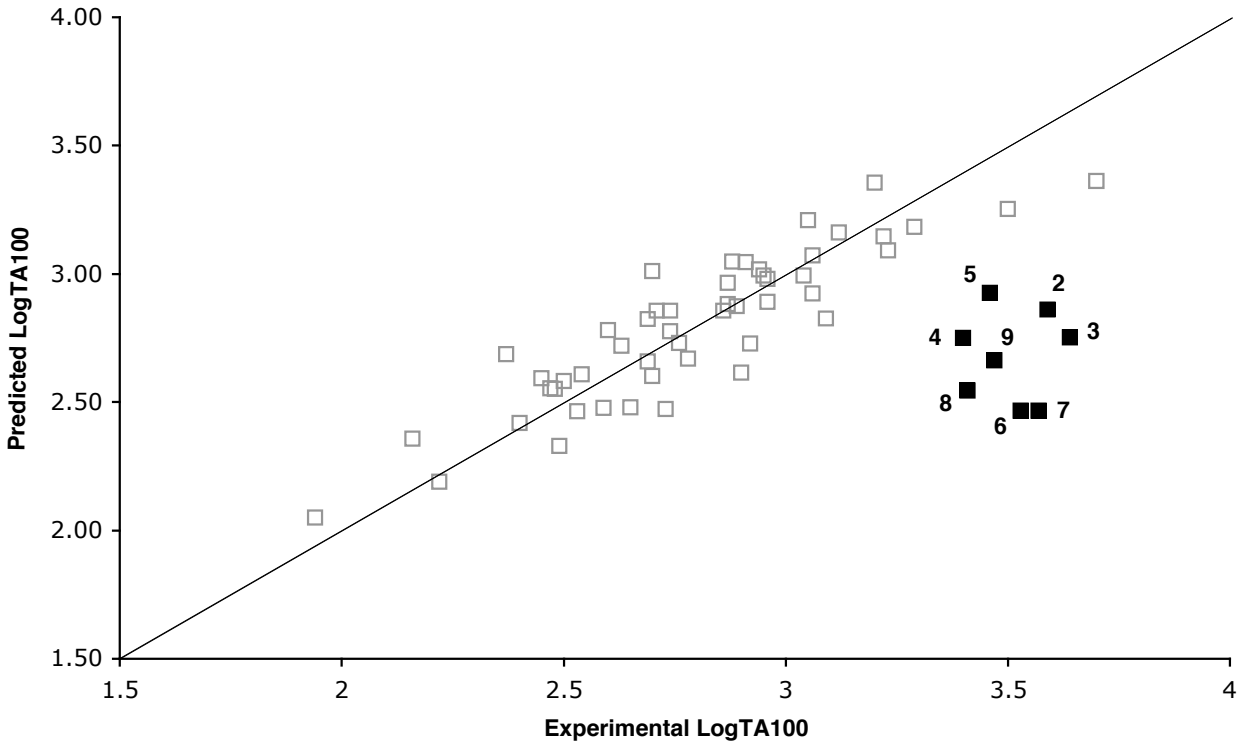


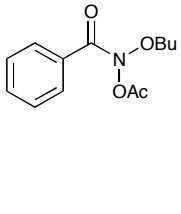
Fig. 2 Predicted vs experimental mutagenicities for *N*-acyloxy-*N*-alkoxyamides **2**-**9** (solid squares) and training set (open squares) in *S. typhimurium* TA100 when predicted by the linear QSAR in equation (1)

Activity towards S. typhimurium TA98

To support the TA100 results, four of the naphthalene series (**2**, **3**, **6** and **7**) with mono-substitituted naphthalene on the three possible side chains were also tested in *S. typhimurium* TA98, a frameshift mutation detection strain. Since the space requirement for an intercalated PAH (3.4 Å) is similar to that required by a single base pair,[30-36] intercalation of a PAH between the bases of DNA[37] reinstates the wild type reading frame in *S. typhimurium* through a +1 frame shift.[36, 38] Furthermore, to see significant reversion of the TA98 strain, a mutagen must be bifunctional.[39] Mutagenic potency of an intercalator can be increased 10 to 100-fold through its attachment to a side-chain capable of a covalent reaction with DNA, resulting in localisation of the intercalator.[36, 38-40] Accordingly, *N*-acyloxy-*N*-alkoxyamides bearing an intercalating PAH would be well set up to produce a positive response with the TA98 strain.

Initial studies with TA98 were carried out with *N*-acetoxy-*N*-butoxy-2-naphthamide, **2**, together with four other substrates for comparison. **14** and **15** are *N*-acyloxy-*N*-alkoxyamides that are mutagenic in TA100,[17-22] but are lacking a naphthalene group, while **16** and **17** contain naphthalene

but lack a reactive nitrogen. Both of these lack a leaving group at nitrogen though *N,N*-dialkoxyamide **16** has a similar, pyramidal nitrogen and reduced resonance to that in **2**.[5, 41, 42] Hydroxamic ester **17** has a largely planar, strongly conjugated nitrogen. In earlier studies we have shown that an *N,N*-dialkoxybenzamide[13] and an *N*-alkylhydroxamic ester are non-mutagenic in TA100.[18] Dose-response data for TA98 studies are presented in Table 6.



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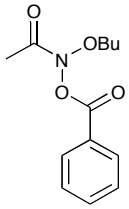

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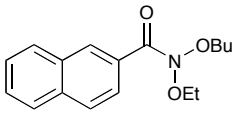

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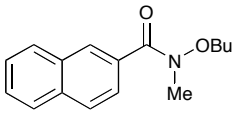

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Table 6 Dose-response data[a] for mutagenicity of **2**, **3**, **6** and **7** and **14**-**17** in *S. typhimurium* TA98

Test	Set[b]	μmol/plate					Gradient[c]	R^2
		0.00	0.03	0.06	0.125	0.25		
2	A	47 (7)	59 (6)	79 (10)	112 (5)	160 (17)	459	0.992
2	B	31 (1)	59 (8)	71 (14)	93 (19)	166 (16)	511	0.986
2	C	40 (7)	59 (8)	66 (4)	98 (16)	148 (11)	424	0.996
2	D	33 (4)	46 (8)	65 (3)	95 (7)	138 (11)	420	0.988
2	E	26 (3)	36 (3)	51 (5)	67 (14)	122 (11)	762	0.996
2	F	24 (2)	32 (2)	38 (1)	45 (1)	72 (4)	371	0.991
14	D	33 (4)			40 (8)	38 (2)	18 (20)	0.43
15	D	33 (4)			34 (7)	29 (5)	-16 (-17)	0.57
3	E	26 (3)	28 (3)	27 (2)	29 (5)	29 (5)	25 (15)	0.566
16	E	26 (3)	24 (1)	27 (1)	24 (4)	25 (2)	-3 (-2)	0.018
6	F	24 (2)	29 (1)	31 (4)	35 (7)	40 (3)	114 (141)	0.924
7	F	24 (2)	29 (5)	30 (6)	34 (1)	61 (6)	287 (355)	0.942
17	F	24 (2)	25 (2)	29 (2)	22 (3)	23 (4)	-15 (-18)	0.370

[a] Counts displayed are average of three plates per dose with standard deviation in parentheses

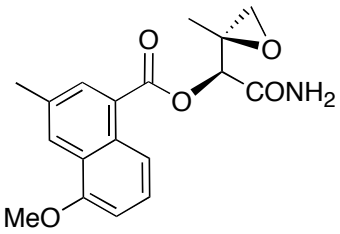
[b] Measurements for **2** were carried out independently in Sets A-C and in conjunction with **14** and **15** in Set D, **3** and **16** in Set E and **6, 7** and **17** in Set F

[c] Activities at 1 μmol/plate scaled to the activity of **2** = 459 in Set A in parentheses.

N-Acetoxy-*N*-butoxy-2-naphthamide **2**, bearing both an electrophilic nitrogen as well as intercalating naphthalene, was strongly and reproducibly active in TA98 over the dose range with

LogTA98 in excess of 2 in each case. Like **2**, naphthalene-bearing mutagens **6** and **7** were also active (LogTA98 > 2). All three possess a localising electrophilic centre and induce +1 frameshift mutations in the TA98 strain. However, **3** with the naphthalene on the leaving group is completely inactive in TA98, which we ascribe to the fact that the leaving group bearing the naphthalene is displaced by electrophilic attack on DNA resulting in loss of localisation and the duality of the mutagen-DNA interaction that enhances the TA98 response. Both naphthalene derivatives **16** and **17** were completely inactive reflecting their lack of an electrophilic nitrogen. The mutagens **14** and **15**, lacking a naphthyl group, while active in TA100, were as expected inactive in TA98. Comparison of the activity of **2** with inactivity of **14** indicates that the naphthyl, as opposed to the phenyl aromatic substituent, interacts with DNA in a different manner.

Based on the significantly enhanced activity of all the naphthalene-bearing mutagens in *S. typhimurium* TA100 and the activity of those tested in *S. typhimurium* TA98, we conclude that naphthyl groups most probably intercalate with DNA bases, thereby increasing the binding equilibrium constant K_M in Fig. 1 and residence time on DNA, with attendant increase in probability of electrophilic reaction with a proximal DNA base.



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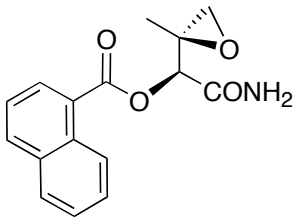

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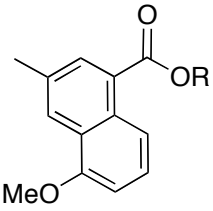

20a R=H
20b R=Me

There is significant debate in the literature as to the intercalative ability of naphthalenes. Many studies exploit the intercalation of naphthalene mono- and di- imides, well-known and established threading intercalators.[43-45] The naphthalene unit is broadly discussed as only acting as an intercalator when facilitated by the presence of positively charged side-chains,[46] or acyl substitution.[46, 47] There is recent evidence that derivatives of the "left half" of Azinomycin B modified to remove aziridine alkylating activity **18** and which have an acylated side-chain naphthalene motif similar to that of the *N*-acyloxy-*N*-alkoxyamides **2** and **3** bind intercalatively to DNA.[48] However the unsubstituted naphthalene system in the same structure **19** was shown to be ineffective in DNA binding.[48] The crucial role played by substituents in the intercalation of the naphthalene moiety in their study suggested that π-electron density distribution is likely to make a significant contribution to the intercalative potential of the naphthalene group. Computational

studies by Alcaro *et al*. have been unable to clearly define the role of the naphthalene moiety in Azinomycin, [49, 50] but Coleman *et al*.[51, 52] suggests that there is no intercalation of the naphthalene moieties in **18**, **20a** and **20b**. Casely-Hayford is equivocal on the intercalative binding ability of naphthalene component of azinomycins.[53] The results reported here appear to be the first to show an activating effect of a simple naphthalene moiety over and above straightforward hydrophobic groove-binding. While attractive electrostatic interactions are important with polarised intercalators such as polycyclic heteroaromatics or neutral polycyclics bearing a positively charged side chains,[35] these studies clearly show that a neutral, at best weakly polarised naphthyl group can intercalate to an extent that influences interaction and thereby reactivity with DNA bases. Notably, naphthyl groups with both electron-withdrawing acyl substituents (in **2** and **3**) and electron-donating alkyl substituents (in **4**-**9**) behave similarly in this study. The finding also represents a departure from the accepted view that PAH's with at least three fused rings provide an optimum surface area of 28Å^2 for intercalation.[30, 54-56] Secondary interactions such as hydrogen bonding can also promote intercalation of substructure and such bonding to the nitrogens of *N*-acyloxy-*N*-alkoxyamides could occur as they are probably more basic relative to normal amides on account of their amine-like character. However, the properties of the nitrogen in **16**, which was inactive in TA98 studies, are similar to those of **2**. Intercalation of the naphthalene groups of *N*-acyloxy-*N*-alkoxyamides **2**-**9** would possibly be driven by hydrophobicity and π-π-stacking resulting in van der Waals dispersion and weak electrostatic effects as well as HOMO-LUMO interactions.

Quantitative structure-activity relationships

QSARs in equation (1) and equation (2) can be modified to incorporate an indicator variable *I* which has the value *I*=1 for **2**-**9**, where naphthalene is present and *I*=0 for all other mutagens in our training set. Such an indicator variable has been used in QSAR as a descriptor to define the difference between congeners in a series of compounds with PAH of three or more fused rings (*I* = 1), and congeners with two or less rings (*I* = 0).[57, 58]

The revised linear and bilinear QSARs are presented in equation (3) and equation (4).

$$\text{Log TA100} = 0.26\ (\pm0.03)\ \text{Log}P + 0.17\ (\pm0.08)\ \text{p}K_A + 0.12\ (\pm0.03)\ E_S^1 + 0.14\ (\pm0.04)\ E_s^2 + 0.08\ (\pm0.05)\ E_S^3 + 0.83\ (\pm0.06)\ I + 1.12\ (\pm0.41)$$
$$n = 58,\ R^2 = 0.85,\ \text{adj. } R^2 = 0.83,\ s = 0.16,\ F = 48.2;\ \text{LOO CV } Q^2 = 0.85 \quad (3)$$

$$\text{LogTA100} = 0.23\ (\pm0.02)\ \text{Log}P - 0.65\ (\pm0.12)\ \text{Log}(\beta P + 1) + 0.11\ (\pm0.08)\ \text{pK}_A + 0.09\ (\pm0.03)\ E_S^1 + 0.09\ (\pm0.04)\ E_S^2 + 0.01\ (\pm0.05)\ E_S^3 + 0.85\ (\pm0.07)\ I + 1.48\ (\pm0.40)$$
$$\text{Log}\beta = -6.705,\ n = 63,\ R^2 = 0.81,\ \text{adj. } R^2 = 0.79,\ s = 0.18,\ F = 29.6;\ \text{LOO CV } Q^2 = 0.76 \quad (4)$$

From the coefficients in equation (4) a revised LogP_O value of 6.44 can be calculated, slightly higher than 6.37 calculated from Equation 2

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Calculated and predicted LogTA100 from equation (3) and equation (4) using $I = 1$ are given in Table 7. The differences between experimental and predicted mutagenicities are significantly diminished when compared to activities from equation (1) (Table 5).

Table 7 Experimental and predicted mutagenicities for **2**-**9** in *S. typhimurium* TA100 when LogTA100 is calculated using linear QSAR in equation (3) and bilinear QSAR in equation (4)

Mutagen		Linear QSAR		Bilinear QSAR	
	Experimental	Predicted	Difference[b]	Predicted	Difference[b]
2	3.59[a]	3.68	0.09	3.65	0.06
3	3.64	3.58	-0.06	3.59	-0.05
4	3.4	3.57	0.17	3.58	0.18
5	3.46	3.74	0.28	3.72	0.24
6	3.53	3.31	-0.22	3.33	-0.20
7	3.57	3.31	-0.26	3.33	-0.24
8	3.41	3.39	-0.02	3.39	-0.02
9	3.48	3.50	0.02	3.49	0.01

[a] An averaged value for **2** is used in derivation of equation (3) and equation (4)

[b] Predicted LogTA100 – Experimental LogTA100

Fig. 3 illustrates the fit of the predicted data for naphthalene-containing mutagens based on the linear QSAR in equation (3). Data produced from the bilinear QSAR in equation (4) yields a similar plot since the Log(βP+1) terms are negligible when Log$P$ < LogP_o.

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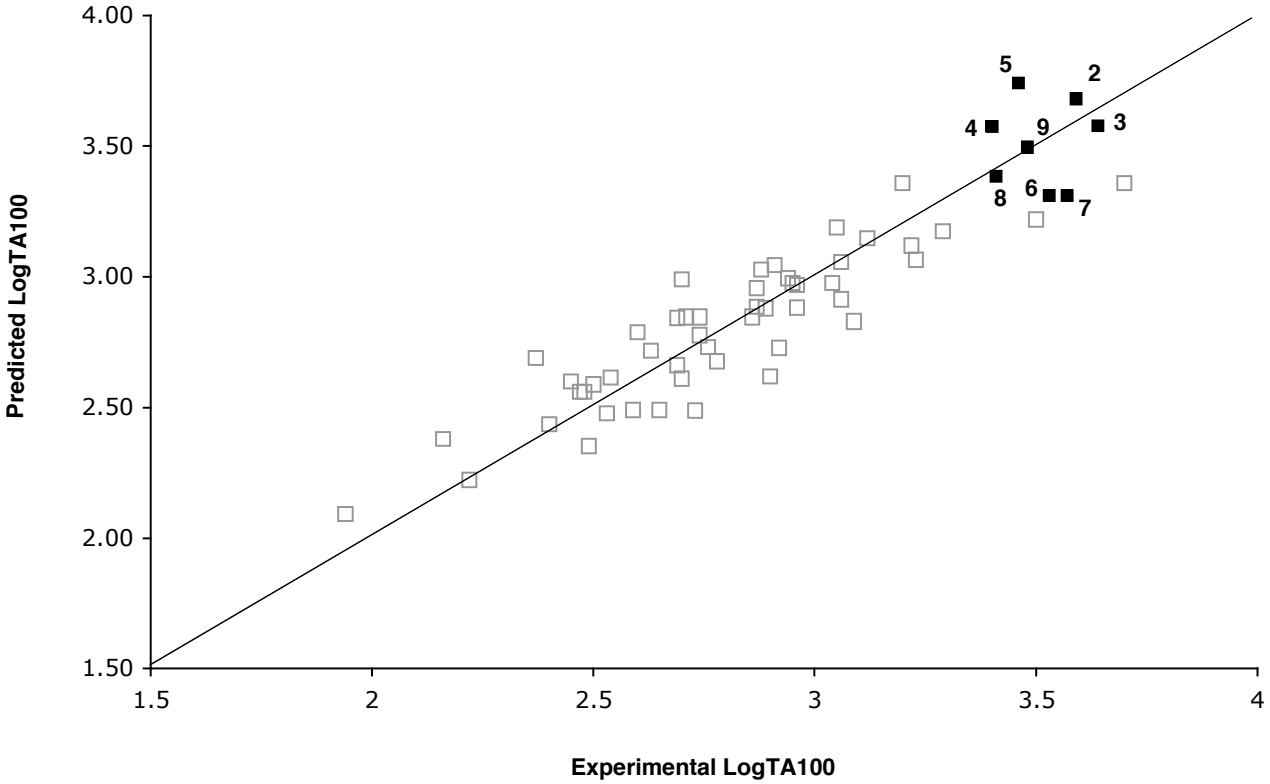

Fig. 3 Predicted vs experimental mutagenicities for *N*-acyloxy-*N*-alkoxyamides **2**-**9** (solid squares) and training set (open squares) in *S. typhimurium* TA100 when predicted by the linear QSAR in equation (3).

The correlations with Log*P* and *I* in equation (3) and equation (4) are highly significant (*P*-values of 10^{-13} and 10^{-17} respectively; see ESI), which makes it possible to quantify the changes in activity due to intercalation by naphthalene. From a comparison of the respective coefficients of dependence upon Log*P* and *I* in equation (3) and equation (4), the presence of one naphthalene group generates increased activity equivalent to between 3 and 4 Log*P*. Though Log*P* of **2**-**9** fall well below the $\text{Log}P_{\text{O}}$ for *N*-acyloxy-*N*-alkoxyamides they exhibit mutagenic activity equivalent to mutagens with an 'effective' Log*P* somewhat above $\text{Log}P_{\text{O}}$.

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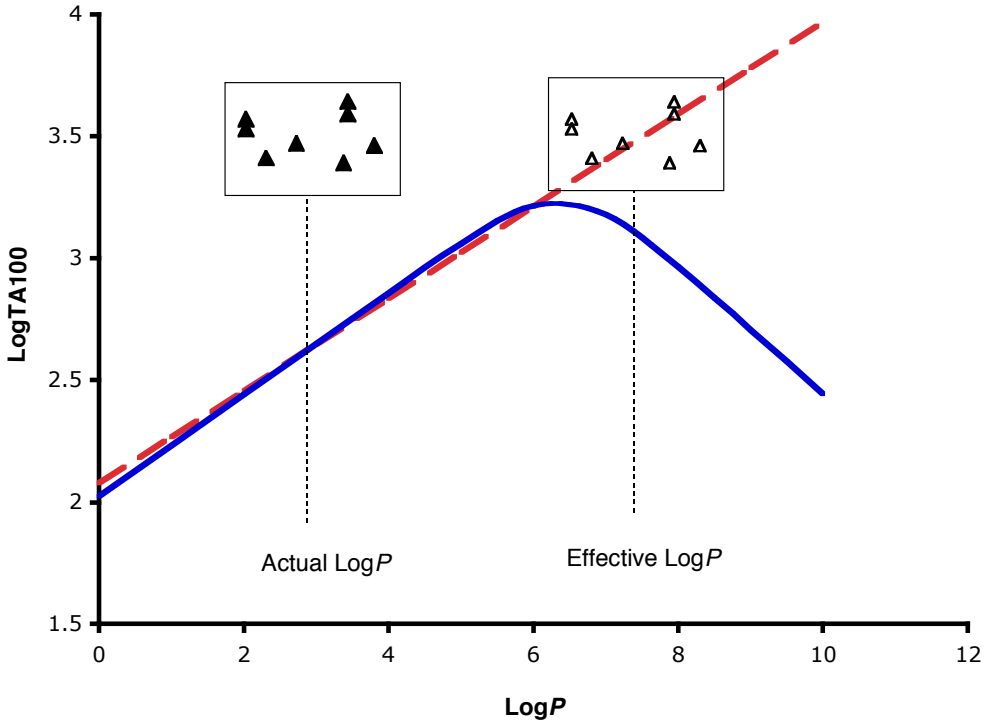

Fig. 4 Plot of the linear (equation (5), broken line) and bilinear (equation (6), solid line) dependence of LogTA100 on Log*P* and the experimental activities of naphthalene-containing compounds **2**-**9** at calculated (black triangles) and effective Log*P* range (open triangles).

This is depicted in Fig. 4. Log*P* is the most important element of the linear and bilinear QSARs in equation (1) and equation (2) and satisfactory linear and bilinear dependencies upon Log*P* alone have previously been derived for the *N*-acyloxy-*N*-alkoxyamides in our training set (equation (5) and equation (6) respectively).[22] From equation (6), a $\text{Log}P_0$ of 6.38 has been found. Fig. 4 shows the relationship between these two functions of Log*P* over the range Log*P* = 1-10, together with the experimental activities for naphthalene-bearing mutagens **2**-**9** (solid triangles).

$$\text{LogTA100} = 0.19\ (\pm 0.02)\ \text{Log}P + 2.08\ (\pm 0.08)$$
$$n = 50,\ r^2 = 0.67,\ \text{adj. } r^2 = 0.67,\ s = 0.19,\ F = 98.5;\ \text{LOOCV } Q^2 = 0.6381 \qquad (5)$$

$$\text{LogTA100} = 0.21\ (\pm 0.02)\ \text{Log}P - 0.47\ (\pm 0.10)\ \text{Log}(\beta P + 1) + 2.02\ (\pm 0.08)$$
$$\text{Log}\beta = -6.466,\ n = 55,\ r^2 = 0.68,\ \text{adj. } r^2 = 0.66,\ s = 0.19,\ F = 36.0;\ \text{LOOCV } Q^2 = 0.64 \qquad (6)$$

It is clear from Fig. 4 that the mutagenic activities of *N*-acyloxy-*N*-alkoxyamides-containing naphthalene groups are significantly higher than predicted by the bilinear function of Log*P*. When

mapped onto the extrapolated linear relationship, the naphthalene substrates have mutagenicities that would be generated if Log*P* were in the range of 6.5-8.5, an increase on their calculated hydrophobicity of about 4 Log*P* units. At these Log*P*, activities would normally be sharply reduced due to lipid entrapment.[22] However, in the case of **2**-**9**, incorporation of naphthalene into the structure of *N*-acyloxy-*N*-alkoxyamides results in Log*P* values which are low enough to avoid the negative effects associated with high Log*P*, such as membrane localisation and reduced active concentrations, while generating activity that would be associated with much greater Log*P* values. Importantly, in this system, a mutagen bearing a naphthalene with a maximal Log*P* ≈ LogP_O (6.4) could display activity equivalent to a molecule with a virtual Log*P* of around 9-10. In drug terms, activity well above the Lipinski limit for *N*-acyloxy-*N*-alkoxyamides becomes possible.[59] When actual Log*P* > LogP_0, lipid entrapment would be expected to negate the activity enhancing effect of naphthalene substitution.[22] In support of this, we recently showed that the mutagenic activity of a highly hydrophobic analogue of naphthamide **2**, bearing *N*-octyloxyl and *N*-octanoyloxyl groups and with a computed Log*P* = 7.9 is well predicted by bilinear QSAR in Equation 2 (experimental and predicted LogTA100 were 3.0 and 3.1, respectively).

DNA damage studies

The cellular target of *N*-acyloxy-*N*-alkoxyamides is undoubtedly DNA to which they bind and with which they ultimately react electrophilically. In DNA damage studies on plasmid DNA at pH 7.8, naphthamide **2** has already been shown to react more prolifically than several benzamides with higher hydrophobicity, reacting at N7-G in the major groove and N3-A in the minor groove.[18] In these studies a 375 base pair fragment of plasmid pBR322 DNA, which is 3' end–labelled with ^{32}P, is treated with mutagens. The DNA is susceptible to cleavage by hot piperidine treatment at damaged bases and labelled fragments are detectable along with sequencing lanes in autoradiograms following electrophoresis. The damage is compared with reference profiles for unreacted DNA. Using the same DNA damage studies, a direct comparison between **2** and **14** has been made, which demonstrates their very different damage profiles. In addition, using a range of naphthalene-bearing mutagens **3**, **6**-**7**, these studies confirm reactivity that is comparable to, or higher than that of **2**.

The DNA damage profiles for naphthamide **2** and its benzamide analogue, **14** at three pH's are compared in Fig. 5. While the purine damage for benzamide **14** and naphthamide **2** was mainly guanine damage at neutral and basic pH and typical of other groove-binding *N*-acyloxy-*N*-alkoxyamides,[18] benzamide **14** exhibited additional, more intense adenine damage at low pH, noticeably at A33, A46, A47, A56, A61, A92 and A105 (Fig. 5, lane 7). However, the DNA damage profile for the naphthamide **2** at low pH (Fig. 5, lane 4) differs very significantly from that of **14**. Damage at purines immediately preceded by a 3'- cytosine and in one case, a 3'-thymine, are strongly damaged. This is evident at C39/G40, C42/A43, T52/G53, C69/G70, C77/G78, C82/A83, C101/G102, C144/G145, C150/G151 and C161/G162 (Fig. 5, red labels). The corresponding bases were at most weakly affected by **14** at this pH. The preponderance of $^{3'}$C-$^{5'}$G sequence selectivity would suggest a preference for naphthalene intercalation between $^{3'}$CG||$^{5'}$GC base pairs leading to reaction at the N7-$^{5'}$G in the major groove. The strong A43 and A83 bands would indicate a second preference for intercalation between $^{3'}$CG||$^{5'}$AT leading to N3-$^{5'}$A damage in the minor groove.

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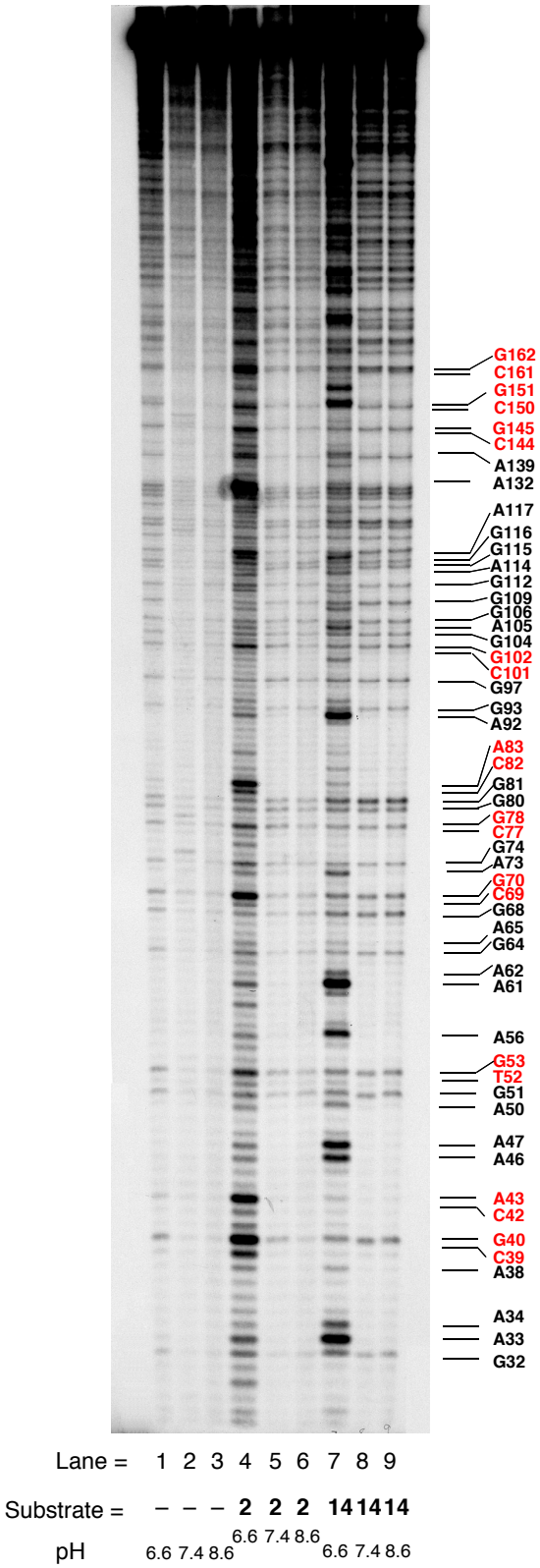

Fig. 5 Strand cleavage patterns obtained by the chemical treatment of the 3´-end-labelled EcoR1 to BamH1 fragment of pBR322 DNA with **2** (lanes 4-6) and **14** (lanes 7-9). All lanes incubated for 16 hours. Lane 1-3 are the DMSO control. Lanes 1, 4, 7= pH 6.6, lanes 2, 5, 8 = pH 7.4 and lanes 3, 6, 9= pH 8.6. Base labels from previous sequencing of the DNA fragment.[18]

Fig. 6 shows the strand cleavage pattern for **2**, **3** and **7** with naphthalene on different side chains and at three different pH's. Damage was more significant at pH 6.6 in each case. While at pH 7.4 and 8.6, strand cleavage occurs due to damage at N7-G, at the lower pH, characteristic damage at both N7-G and N3-A is evident (G40, A43, G53, G70, G78, A83 and G102). From lanes 4, 7 and 10 it is clear that cleavage patterns for all three are similar and that **3** and **7** are more reactive than **2**. From depletion of the uncut fragment and larger fragments at the top of lane 7 in the autoradiogram, as well as the high intensity of smaller fragments towards the bottom of this lane, the degree of damage is noticeably greater for **3** where multiple hits are evident. In turn, damage detected for **7** is more intense than that for **2**.

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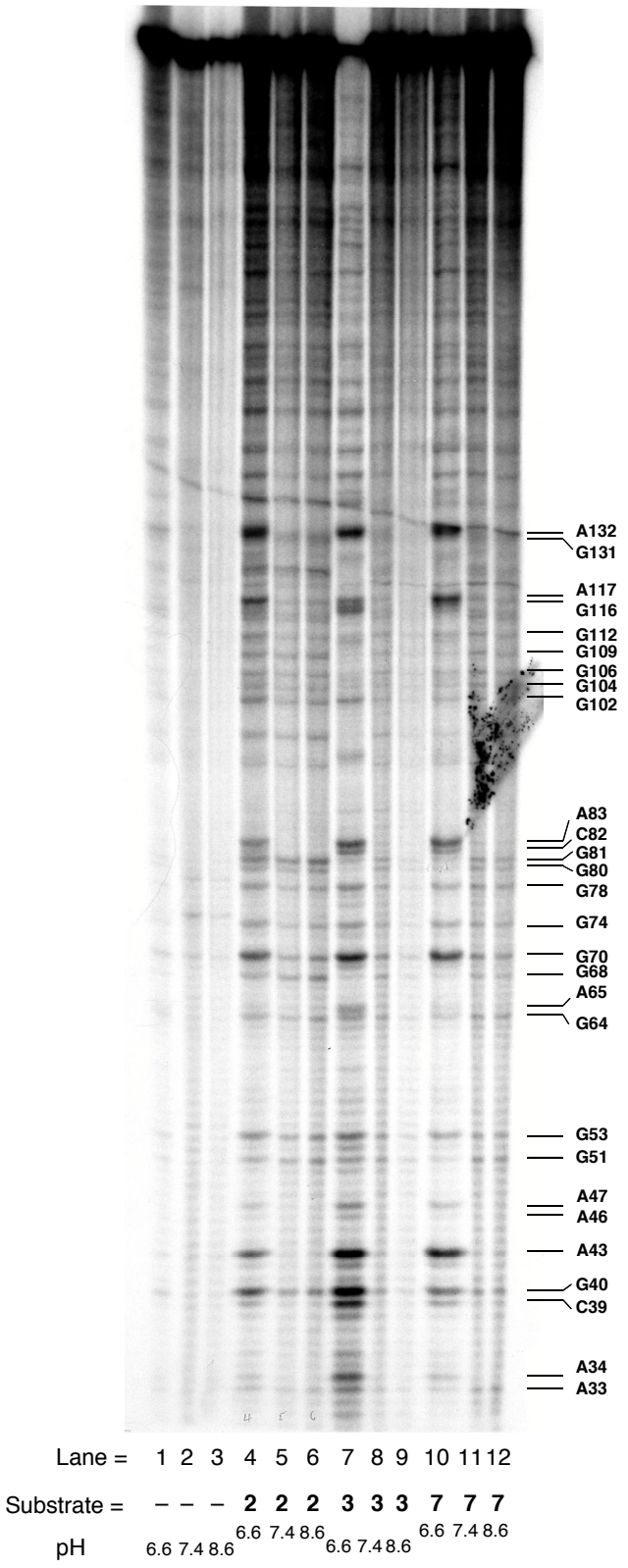

Fig. 6 Strand cleavage patterns obtained by the chemical treatment of the 3´-end-labelled EcoR1 to BamH1 fragment of pBR322 DNA with **2** (lanes 4-6), **3** (lanes 7-9) and **7** (lanes 10-12). All lanes incubated for 16 hours. Lane 1-3 are the DMSO control. Lanes 1, 4, 7, 10 = pH 6.6, lanes 2, 5, 8, 11 = pH 7.4 and lanes 3, 6, 9, 12 = pH 8.6. Base labels from previous sequencing of the DNA fragment.[18]

Fig. 7 shows a comparison of the DNA damage of mutagens **6**, **8** and **9** with that of **7**. Like **7**, all three are more reactive at lower pH. At higher pH, damage is again at N7-G and from comparison of lanes 9 and 10 with lanes 7 and 8 at pH 7.4 and lanes 14 and 15 with lanes 12 and 13 at pH 8.6 the more hydrophobic mutagens **8** and **9** bearing tethered naphthalene appear to be more reactive. At lower pH, **7** would appear to be more reactive than **6**, **8** and **9**. The damage pattern is by and large independent of position of substitution at naphthalene or of the nature of the tether. In this experiment, in addition to the weaker but characteristic damage at low pH, all four exhibit much strong fragmentation at A34/33, A65, and A94, which may be the result of multiple hits as evidenced by the depletion of DNA at the origin and preponderance of smaller fragments. This consequential damage has been noted in a previous study of *N*-benzoyloxy-*N*-benzyloxybenzamide (**1**, R^1=R^3=Ph, R^2=Bn).[18] In addition to this, the nature and variability of DNA damage in these studies can be attributed to revertible N3-A damage, which is time and pH dependent,[60] so direct comparison of band intensities in autoradiograms from different studies is unwise. However, while Fig. 6 and Fig. 7 are not directly comparable, consideration of the comparisons in each study indicates that the most reactive mutagen at low pH is **3** followed by **7**, and both are more reactive than **2**, **6**, **8** and **9**. From experimental data in Table 5 this ordering broadly correlates with their mutagenicities in point mutation studies with *S. typhimurium* TA100 where mutagenic activities of **3** and **7** are marginally greater than that of **6**, **8** and **9**.

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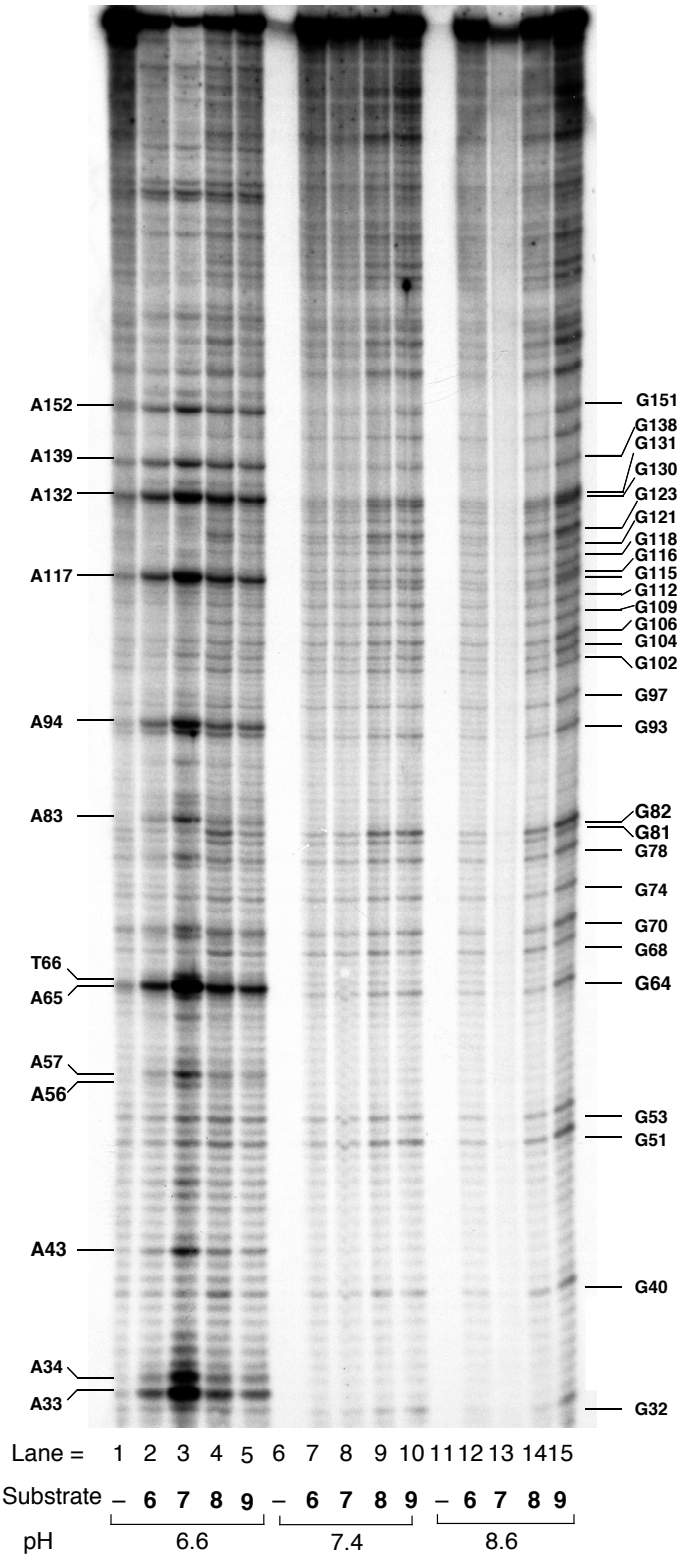

Fig. 7 Strand cleavage patterns obtained by the chemical treatment of the 3´-end-labelled EcoR1 to BamH1 fragment of pBR322 DNA with **6** (lanes 2, 7, 11), **7** (lanes 3, 8, 13), **8** (lanes 4, 9, 14) and **9** (lanes 5, 10, 15). All lanes incubated for 16 hours. Lane 1, 6, 11 are the DMSO control. Lanes 1-5 = pH 6.6, lanes 2-10 = pH 7.4 and lanes 11-15 = pH 8.6. Base labels from previous sequencing of the DNA fragment.[18]

Conclusion

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Compelling evidence has been presented showing that mutagenic activity of *N*-acyloxy-*N*-alkoxyamides in point mutation strain *S. typhimurium* TA100 is strongly and uniformly enhanced when a naphthalene group is present on any of the three side chains. We attribute this to a hitherto unrecognised ability of naphthalene to increase residence time in the major and minor grooves of DNA through intercalation. This is supported by activity in the frame-shift strain *S. typhimurium* TA98. DNA damage studies on a range of structures bearing naphthalene demonstrate comparable enhanced, sequence specific damage at guanine and adenine upon naphthyl substitution. Comparison of damage profiles of *N*-acetoxy-*N*-butoxy-2-naphthamide and its benzamide analogue confirms a different DNA binding mode, which is indicative of preferred intercalation between $^{3'}$CG—G$^{5'}$C or $^{3'}$CG—A$^{5'}$T base pairs.

Based upon our extensive data for activity in TA100, we have established a working QSAR that incorporates an indicator variable, *I,* for the presence (*I*=1) or absence (*I*=0) of naphthalene intercalator. We will presently demonstrate that the fitting of mutagenic activity of *N*-acyloxy-*N*-alkoxyamides towards TA100 to this new QSAR is a reasonably accurate test of whether other, substituted naphthyl substituents facilitate intercalation or whether other PAH substructures can intercalate. For instance, several mutagens with a biphenyl substituent, and the related fluorenyl as a substituent, fit our QSAR with *I*=0 from which we conclude that both biphenyl and fluorenyl do not intercalate. On the other hand, mutagens with fluorenone and pyrene fit with *I*=1 indicating that these substructures, like naphthalene do intercalate. Since we have always evaluated the activity of new mutagens in TA100 in conjunction with standard **14**, with appropriate synthesis of substituted *N*-acyloxy-*N*-alkoxyamides and **14**, the methods described above could be executed in other laboratories to discriminate between intercalating and non-intercalating substructures.

The results presented herein point very strongly to the enhanced association of molecules to prokaryotic DNA when a naphthalene unit is incorporated as a substituent. In these studies, the value to binding with DNA is worth between 3-4 Log*P* units such that a mutagen bearing naphthalene with Log*P* close to optimum LogP_0 can have DNA binding that would be generated by Log*P* well in excess of LogP_0 without lipid entrapment. We suggest that the enhanced binding to DNA due to naphthalene intercalation may be put to similar advantage in the design of drugs, which like *N*-acyloxy-*N*-alkoxyamides, target DNA and may as a consequence result in activity beyond their limiting LogP_0 or Lipinski limit.

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Experimental

Chemicals

Infrared spectra were recorded on a Perkin–Elmer1600 series Fourier-transform (FT)-IR spectrophotometer solutions in chloroform. Mass spectra were recorded on a Varian 1200L liquid chromatograph-mass spectrometer at a 30-V capillary voltage and 350 °C, 138 kPa drying gas temperature and pressure, in HiPerSolv acetonitrile/water. 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). Syntheses of mutagens **2** and **14** have been described elsewhere[15, 17] Mutagens **3**-**9** and **15** were all synthesised as part of this study. For DNA damage studies 40% (19:1) Acrylamide/bisacrylamide was purchased from Bio-Rad Laboratories Pty Ltd. pBr322, Klenow fragment, *Bam*H1, *Eco*R1, BSA (bovine serum albumin) and NE buffer 2, were purchased from (New England Biolabs) Genesearch Pty Ltd. α-dATP([α-^{32}P]-deoxyadenosine-5'-triphosphate (10μCi/μl), isoblue stabilized) was purchased from ICN Biomedicals Inc.

General Synthesis of *N*-alkoxyamides

Condensation of the potassium salt of acetohydroxamic acid with an appropriate alkyl bromide, prepared from the parent alcohol (see ESI), and a 10% excess of sodium carbonate in 50% aqueous methanol gave the appropriate *N*-alkoxyamide in good yield.[29]

Potassium acetohydroxamate.[61] Potassium hydroxide (56.10 g, 1.00 mol) in boiling methanol (140 ml) was added to hydroxylamine hydrochloride (46.70 g, 0.67 mol) in boiling methanol (240 ml) and the mixture cooled in an ice bath for 5 minutes. Ethyl acetate (29.08g, 0.33 mol) was added with shaking the mixture filtered immediately and reduced to dryness *in vacuo* to give potassium acetohydroxamate as a straw-coloured viscous oil (31.00 g, 83%) which was used without further purification.

***N*-Butoxyacetamide.** Potassium acetohydroxamate (10.00 g, 0.0884 mol), butyl bromide (12.11 g, 0.0884 mol) and sodium carbonate (10.3 g, 0.01 mol) were stirred overnight in 50% aq. MeOH (160 ml) and refluxed for 3 hours. Removal of MeOH *in vacuo* followed by extraction with chloroform provided the crude hydroxamate. *N*-Butoxyacetamide was obtained as a straw coloured oil after purification by repeated fractional distillations under reduced pressure (4.9 g, 42%). b.p. 70°C @

0.2mm Hg; $\nu_{max.}$(neat)/cm^{-1} 3190br (NH), 1678s (C=O), 1370s, 1086s; δ_H(300 MHz; $CDCl_3$) 1.05 (3H, t, CH_3), 1.50 (2H, m, $\underline{CH_2}CH_3$), 1.72 (2H, m, $OCH_2\underline{CH_2}$), 2.11 (3H, br d, $COCH_3$), 4.01 (2H, br s, OCH_2), 8.40 (1H, br s, NH); δ_C(75 MHz; $CDCl_3$) 13.7 (q), 18.9 (t), 19.7 (br), 29.9 (t), 76.3 (t), 167.9 (s); *m/z* (ESI) 170 ([M+K$^+$], 50%), 154 ([M+Na$^+$], 100%); *m/z* (EI) 131.094093 ([M$^+$], $C_6H_{13}NO_2$ requires 131.094629).

***N*-(2-Naphthylmethoxy)acetamide.** 2-Bromomethylnaphthalene (10.00 g, 45.2 mmol), potassium acetohydroxamate (7.68 g, 67.8 mmol) and sodium carbonate (7.2 g, 68 mmol) were stirred overnight in 50% aq. MeOH (160 ml) and refluxed for 4 hours. Removal of MeOH *in vacuo* followed by extraction with DCM provided impure *N*-(2-naphthylmethoxy)acetamide. Purification was achieved by centrifugal chromatography with ethyl acetate/pet spirit. *N*-(2-Naphthylmethoxy)acetamide was obtained as a white solid (3.7 g, 38%), mp 113-115°C (from benzene); (Found: C, 72.01%; H, 6.17%; N, 6.34%. $C_{13}H_{13}NO_2$ requires C, 72.54%; H, 6.09%; N, 6.51%); ν_{max}($CHCl_3$)/cm^{-1} 3401w (NH), 3235br (NH), 1691s (C=O); δ_H(300 MHz; $CDCl_3$, 50°C) 1.92 3H, br, CH_3), 5.03 (2H, br, CH_2), 7.50-7.53 (3H, m, Ar-H), 7.83-7.88 (4H, m, Ar-H), 8.32 (1H, br, NH); δ_C(75 MHz; $CDCl_3$, 50°C) 19.6 (q br), 78.7 (br), 126.4 (d br), 125.5 (d), 127.7 (d), 128.0 (d), 128.5 (br), 132.6 (s very br), 133.2 (s), 133.5 (s), 168.2 (very br). ^{13}C incomplete due to extreme line broadening even at 50°C; *m/z* (ESI) 238 ([M+Na$^+$], 100%), 141 (15%).

***N*-(1-Naphthylmethoxy)acetamide.** 1-Bromomethylnaphthalene (10.00 g, 45.2 mmol), potassium acetohydroxamate (7.68 g, 67.8 mmol) and sodium carbonate (7.2 g, 68 mmol) were stirred overnight in 50% aq. MeOH (160 ml) and refluxed for 4 hours. Removal of MeOH *in vacuo* followed by extraction with DCM provided impure *N*-(1-naphthylmethoxy)acetamide. Purification was achieved by centrifugal chromatography with ethyl acetate/pet spirit. *N*-(1-Naphthylmethoxy)acetamide was obtained as a white solid (2.8 g, 29%), mp 119-121°C (from benzene); ν_{max}($CHCl_3$)/cm^{-1} 3401w (NH), 1695s (C=O); δ_H(300 MHz; $CDCl_3$) 1.88 (3H, br s, $\underline{CH_3}$), 5.39 (2H, s, $\underline{CH_2}$), 7.38-7.54 (4H, m, Ar-H), 7.92 (2H, m, Ar-H), 8.31 (1H, br s, Ar-H); δ_C(75 MHz; $CDCl_3$, 50°C) 19.7 (v br), 77.2 (v br), 124.2 (v br), 125.1 (d), 126.2 (d), 126.8 (d), 128.6 (br), 128.8 (br), 130.0 (br s), 133.8 (s), 167.6 (v br). ^{13}C incomplete due to extreme line broadening even at 50°C; *m/z* FAB 216 [M+H$^+$], 238 ([M+Na$^+$],100%), 141 (13%), (Found: 215.09435 ([M$^+$], $C_{13}H_{13}NO_2$ requires 215.09462).

***N*-(2-(2'-Naphthyl)ethoxy)acetamide.** 2-(2'-Naphthyl)ethyl bromide (2.20 g, 9.3 mmol), potassium acetohydroxamate (1.06 g, 9.4 mmol) and sodium carbonate (1.2 g, 11 mmol) were stirred overnight in 50% aq. MeOH (160 ml) and refluxed for 4 hours. Removal of MeOH *in vacuo*

followed by extraction with DCM provided impure *N*-(2-(2'-naphthyl)ethoxy)acetamide. Purification was achieved by centrifugal chromatography with ethyl acetate/pet spirit. *N*-(2-(2-Naphthyl)ethoxy)acetamide was obtained as a white solid. (1.09 g, 51%), mp 146-149°C (from benzene); $\nu_{max}(CHCl_3)/cm^{-1}$ 3401 (NH), 1695s (C=O), 1368s; δ_H(300 MHz; $CDCl_3$) 1.90 (3H, br s, CH_3), 3.14 (2H br t, $ArCH_2$), 4.21 (2H br s, OCH_2), 7.36 (1H, d), 7.47 (2H, m), 7.69 (1H, s), 7.80 (3H, m), 8.34 (1H, br s, NH); δ_C(75 MHz; $CDCl_3$) 19.6 (br q), 34.7 (t), 76.8 (br t), 125.5 (d), 126.1 (d), 127.3 (d), 127.3 (d), 127.5 (d), 127.6 (d), 128.2 (d), 132.3 (s), 133.6 (s), 135.4 (br s), 168.1 (br s); *m/z* (ESI) 268 ([M+K^+], 30%), 252 ([M+Na^+], 100%), 155 (10%), *m/z* (EI) 229.110193 ([M^+], $C_{14}H_{15}NO_2$ requires 229.110279).

***N*-(3-(2'-Naphthyl)-1-propoxy)acetamide.** A solution of 3-(2'-naphthyl)-1-propyl bromide (1.7 g, 6.7 mmol) potassium acetohydroxamate (0.7 g, 6.7 mmol) and sodium carbonate (0.85 g, 8 mmol) were stirred overnight in 50% aq. MeOH (40 ml) and refluxed for 4 hours. Removal of MeOH *in vacuo* followed by extraction with DCM provided impure *N*-(3-(2'-naphthyl)-1-propyloxy)acetamide. Purification was achieved by centrifugal chromatography with ethyl acetate/pet spirit. *N*-(3-(2'-Naphthyl)-1-propyloxy)acetamide as a light brown solid (1.06 g, 68 %), mp 90-91°C (from benzene); (Found: C, 73.63%; H, 7.01%; N, 5.61%. $C_{20}H_{24}N_2O_4$ requires C, 74.05%; H, 7.04%; N, 5.76%); $\nu_{max}(CHCl_3)/cm^{-1}$ 3401 (NH), 1694s (C=O), 1369; δ_H(300 MHz, $CDCl_3$) 1.95 (3H, br d, CH_3), 2.08 (2H, qu, $CH_2\underline{CH_2}CH_2$) 2.90 (2H br t, $ArCH_2$), 3.95 (2H br s, OCH_2), 7.34 (1H, d), 7.46 (2H, m), 7.64 (1H, s), 7.80 (3H, m), 8.39 (1H, br s, NH); δ_C(75 MHz $CDCl_3$) 19.6 (br q), 29.4 (t), 32.1 (t), 75.8 (br t), 125.3 (d), 126.0 (d), 126.5 (d), 127.2 (d), 127.4 (d), 127.6 (d), 128.1 (d), 132.1 (s), 133.6 (s), 138.8 (br s), 168.0 (br s); *m/z* (ESI) 266 ([M+Na^+], 100%), 244 ([M+H^+], 10).

General procedure for chlorination of *N*-alkoxyamides

N-Chlorination of *N*-alkoxyamides was achieved in quantitative yields by stirring with a 3-fold excess of neat *tert*-butyl hypochlorite at room temperature or 0^0. Rarely did the reaction require longer than 10-15 minutes for completion. Removal of excess *tert*-butylhypochlorite and *tert*-butanol under reduced pressure afforded *N*-alkoxy-*N*-chloroamides generally as clean yellow oils, which required no further purification and were characterised spectroscopically. *N*-Chlorination was evident from characteristically high carbonyl stretch frequencies in the range 1720-1745 cm^{-1}, resonance of the methylenoxyl protons slightly downfield of the corresponding protons in the parent *N*-alkoxyamide and carbonyl resonances in the region of 175 ppm, 7 ppm downfield of the parent

hydroxamic ester.[1, 2] On account of their thermal and photochemical lability they were converted directly to their *N*-acyloxy derivatives.

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***N*-Butoxy-*N*-chloroacetamide.** *N*-Butoxyacetamide (0.40 g, 3.1 mmol) and neat *tert*-butyl hypochlorite (0.99 g, 9.2 mmol) was stirred for 10 minutes in the dark. Removal of excess hypochlorite *in vacuo* provided the title compound which was used immediately without further purification. $\nu_{max.}$(neat)/cm^{-1} 1740s (C=O), 1215s; δ_H(300 MHz; $CDCl_3$) 0.92 (3H, t,$CH_2\underline{CH_3}$), 1.39 (2H, m, $CH_3\underline{CH_2}$), 1.64 (2H, m, $OCH_2\underline{CH_2}$), 2.24 (3H, s, $COCH_3$), 4.01 (2H, t, OCH_2); δ_C(75 MHz; $CDCl_3$) 13.6 (q), 19.1 (t), 21.4 (q), 29.4 (t), 75.3 (t), 175.3 (s).

***N*-Chloro-*N*-(2-naphthylmethoxy)acetamide.** *N*-(2-Naphthylmethoxy)acetamide (0.50 g, 2.3 mmol) and neat *tert*-butyl hypochlorite (0.76 g, 7.0 mmol) was stirred for 10 minutes in the dark. Removal of excess hypochlorite *in vacuo* provided the title compound which was used immediately without further purification. ν_{max}($CHCl_3$)/cm^{-1} 1733s (C=O); δ_H(300 MHz; $CDCl_3$) 2.13 (3H, s, CH_3), 5.19 (2H, s, CH_2), 7.55 (3H, m, Ar-H), 7.89 (4H, m, Ar-H); δ_C(75 MHz; $CDCl_3$) 21.7 (q), 77.4 (t), 126.6 (d), 126.9 (d), 126.9 (d), 127.8 (d), 128.2 (d), 128.6 (d), 129.6 (d), 130.9 (s), 133.1 (s), 133.5 (s), 175.5 (s).

***N*-Chloro-*N*-(1-naphthylmethoxy)acetamide.** *N*-(1-Naphthylmethoxy)acetamide (0.50 g, 2.3 mmol) and neat *tert*-butyl hypochlorite (0.76 g, 7.0 mmol) was stirred for 10 minutes in the dark. Removal of excess hypochlorite *in vacuo* provided the title compound which was used immediately without further purification. ν_{max}($CHCl_3$)/cm^{-1} 1733s (C=O), 1362m, 1220br; δ_H(300 MHz; $CDCl_3$) 2.01 (3H, s, CH_3), 5.49 (2H, s, CH_2), 7.49 1H, t, Ar-H), 7.54-7.65 (3H, m, Ar-H), 7.92 (1H, d, Ar-H), 7.94 (1H, d, Ar-H), 8.17 (1H, d, Ar-H); δ_C(75 MHz; $CDCl_3$) 21.5 (q), 75.1 (t), 123.5 (d), 125.2 (d), 126.2 (d), 127.0 (d), 128.9 (d), 129.3 (d), 129.7 (d), 130.5 (d), 132.0 (s), 133.8 (s), 175.6 (s).

***N*-Chloro-*N*-(2-(2'-naphthyl)ethoxy)acetamide.** *N*-(2-(2'-Naphthyl)ethoxy)acetamide (0.65 g, 2.8 mmol) and neat *tert*-butyl hypochlorite (0.92 g, 8.5 mmol) was stirred for 10 minutes in the dark. Removal of excess hypochlorite *in vacuo* provided the title compound which was used immediately without further purification. ν_{max}($CHCl_3$)/cm^{-1} 1733s (C=O), 1362s, 1217s; δ_H(300 MHz; $CDCl_3$) 2.11 (3H, s, CH_3), 3.20 (2H, t, $ArCH_2$), 4.39 (2H, br s, OCH_2), 7.38 (1H, d, Ar-H), 7.50 (2H, m, Ar-H), 7.71 (1H, s, Ar-H), 7.83 (3H, m, Ar-H); δ_C(75 MHz; $CDCl_3$) 21.4 (q), 34.1 (t), 75.7 (t), 125.7 (d), 126.3 (d), 127.0 (d), 127.4 (d), 127.5 (d), 127.7 (d), 128.3 (d), 132.4 (s), 133.6 (s), 134.6 (s), 175.6 (s).

_N_-Chloro-_N_-(3-(2'-naphthyl)propoxy)acetamide. _N_-(3-(2'-Naphthyl)-1-propyloxy)acetamide (0.20 g, 0.8 mmol) and neat *tert*-butyl hypochlorite (0.3 g, 2.4 mmol) was stirred for 10 minutes in the dark. Removal of excess hypochlorite *in vacuo* provided the title compound which was used immediately without further purification. $\nu_{max}(CHCl_3)/cm^{-1}$ 1732s (C=O), 1362s, 1221s; δ_H(300 MHz; $CDCl_3$) 2.14 (2H, qu, $CH_2\underline{CH_2}CH_2$), 2.33 (3H, s, CH_3), 2.93 (2H, t, $ArCH_2$), 4.12 (2H, s, OCH_2), 7.37 (1H, d), 7.50 (2H, m, Ar-H), 7.68 (1H, s, Ar-H), 7.85 (3H, m, Ar-H); δ_C(75 MHz; $CDCl_3$) 21.6 (q), 29.0 (t), 32.2 (t), 74.7 (t), 125.5 (d), 126.2 (d), 126.6 (d), 127.1 (d), 127.7 (d), 127.9 (d), 128.2 (d), 132.2 (s), 133.7 (s), 138.3 (s), 175.5 (s).

General procedures for synthesis of sodium salts of carboxylic acids

Method 1. The appropriate carboxylic acid was dissolved/suspended in a small volume of methanol. An equimolar volume of 10 M sodium hydroxide solution was added with stirring. The salts were isolated by precipitation upon standing or refrigeration or, in cases where this was slow, the methanol and water were removed under reduced pressure to yield the appropriate sodium salt in near quantitative yields.

Method 2. The appropriate 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.

Sodium 1-naphthoate. 1-Naphthoic acid (1.45 g, 8.45 mmol) was dissolved in methanol (15 mL) and 10 M sodium hydroxide (845.0 μL, 8.45 mmol) was added while stirring. After 30 min the solvent was removed under reduced pressure to yield sodium 1-naphthoate as a brown solid (1.54 g, 7.93 mmol, 94%), which was used without further characterisation or purification.

Sodium 2-naphthoate. 2-Naphthoic acid (1.02 g, 5.91 mmol) was dissolved in methanol (15 mL) and 10 M sodium hydroxide (591.18 μL, 5.91 mmol) was added while stirring. After stirring overnight the solvent was removed under reduced pressure to yield sodium 2-naphthoate as a light brown solid (1.12 g, 5.72 mmol, 97%), which was used without further characterisation or purification.

Sodium 2-naphthylacetate. 2-Naphthylacetic acid (0.26 g, 1.40 mmol) was dissolved in diethyl ether (10 mL). NaH (0.03 g, 1.40 mmol) was added carefully with stirring and the flask purged with

N_2. Stirring at room temperature continued until evolution of hydrogen gas ceased. Sodium 2-naphthylacetate was filtered, washed with dry diethyl ether and dried under reduced pressure to yield a tan solid (0.21 g, 1.01 mmol, 72%), which was used without further characterisation or purification.

Sodium 3-(2'-naphthyl)propanoate. 3-(2-Naphthyl)propanoic acid (0.27 g, 1.35 mmol) was dissolved in diethyl ether (10 mL). NaH (0.03 g, 1.35 mmol) was added carefully with stirring and the flask purged with N_2. Stirring at room temperature continued until evolution of hydrogen gas ceased. Sodium 3-(2-naphthyl)propanoate was filtered, washed with dry diethyl ether and dried under reduced pressure to yield a tan solid (0.19 g, 0.86 mmol, 63%), which was used without further characterisation or purification.

General procedure for acyloxylation of hydroxamic esters [11, 17, 20]

N-Acyloxy-*N*-alkoxyamides were all synthesised by Finkelstein chemistry. 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. Ester carbonyls fall typically in the range of 1767-1800 cm^{-1} (alkanoyloxyls) or 1750-1767 cm^{-1} for aroyloxyls). Acetamide carbonyls frequencies fall within the range of 1736-1746 cm^{-1} while their ^{13}C NMR resonances are uniformly around 176-177 ppm.[1, 2]

***N*-Butoxy-*N*-(2-naphthoyloxy)acetamide 3.** *N*-Butoxy-*N*-chloroacetamide (0.40 g, 2.44 mmol) was stirred with sodium 2-naphthoate (0.66 g, 3.42 mmol) in dry acetone (10 mL) in the dark and the progress of the reaction followed by TLC until completion (approx. 16 h). The 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*-butoxy-*N*-(2-naphthoyloxy)acetamide as a yellow oil. $\nu_{max}(CHCl_3)$/cm^{-1} 1757s (OC=O), 1737s (NC=O); δ_H(300 MHz; $CDCl_3$) 0.92(3H, t, $CH_2\underline{CH_3}$), 1.43(2H, sx, $\underline{CH_2}CH_3$), 1.72(2H, p, $OCH_2\underline{CH_2}$), 2.34(3H, s, $COCH_3$), 4.19(2H, t, OCH_2), 7.63(2H, m), 7.89-8.01(3H, m), 8.09(1H, d), 8.67(1H, s); δ_C (75 MHz; $CDCl_3$) 13.7(q), 19.1(t), 21.4 (q), 30.1 (t), 76.0 (t), 124.4 (s), 125.1 (d), 127.0 (d), 127.9 (d), 128.6 (d), 128.9 (d), 129.5 (d), 132.1 (d), 132.4 (s), 136.0 (s), 164.3 (s), 176.5 (s); *m/z* (ESI) 301.3 [M^+], 302.4 [$M+H^+$].

***N*-Butoxy-*N*-(2-naphthylacetoxy)acetamide 4.** *N*-Butoxy-*N*-chloroacetamide (0.08 g, 0.51 mmol) was reacted with sodium 2-naphthylacetate (0.15 g, 0.72 mmol) in dry acetone (10 mL) in the dark

and monitored by TLC until completion (approx. 16 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*-butoxy-*N*-(2-naphthylacetoxyoxy)acetamide as a yellow oil. $\nu_{max}(CHCl_3)/cm^{-1}$ 1789s (OC=O), 1740s (NC=O); δ_H(300 MHz; $CDCl_3$) 0.86(3H, t, $CH_2\underline{CH_3}$), 1.29(2H, sx, $CH_2\underline{CH_3}$), 1.56(2H, p, $OCH_2\underline{CH_2}$), 2.23(3H, s, $COCH_3$), 3.94 (2H, s, $ArCH_2$), 4.04(2H, t, OCH_2), 7.62(2H, m), 7.89-8.04(3H, m), 8.07(1H, d), 8.66(1H, s); δ_C (75 MHz; $CDCl_3$) 13.6 (q), 19.0 (t), 21.2 (q), 30.0 (t), 39.2 (t), 75.9 (t), 126.1 (d), 126.3 (d), 127.1 (d), 127.7 (d), 127.8 (d), 128.4 (d), 128.7 (d), 129.8 (s), 132.7 (s), 133.5 (s), 168.3 (s), 176.3 (s); *m/z* (ESI) 316.3 [$M+H^+$], 338.2 [$M+Na^+$].

***N*-Butoxy-*N*-(3-(2'-naphthyl)propanoyloxy)acetamide 5.** *N*-Butoxy-*N*-chloroacetamide (0.05 g, 0.32 mmol) was stirred with sodium 3-(2-naphthyl)propanoate (0.10 g, 0.45 mmol) in dry acetone (10 mL) in the dark and followed by TLC until completion (approx. 16 h). The mixture was filtered to remove NaCl, and the filtrate concentrated under reduced pressure to give the crude product. Centrifugal chromatography with 15% ethyl acetate/hexane afforded pure *N*-butoxy-*N*-(3-(2-naphthyl)propanoyloxy)acetamide as a yellow oil. $\nu_{max}(CHCl_3)/cm^{-1}$ 1798s (OC=O), 1739s (NC=O); δ_H(300 MHz; $CDCl_3$) 0.89(3H, t, $CH_2\underline{CH_3}$), 1.31(2H, sx, $CH_2\underline{CH_3}$), 1.56(2H, p, $OCH_2\underline{CH_2}$), 2.25(3H, s, $COCH_3$), 3.22(2H, t, $COCH_2$), 3.89(2H, t, CH_2Ar), 3.94(2H, t, OCH_2), 7.38(1H, d), 7.43-7.51(2H, m), 7.69(1H, s), 7.79-7.82(3H, m); δ_C (75 MHz; $CDCl_3$) 13.7 (q), 18.9 (t), 21.2 (q), 29.9 (t), 30.8 (t), 33.5 (t), 75.7 (t), 125.6 (d), 126.1 (d), 126.6 (d), 126.8 (d), 127.1 (d), 127.6 (d), 127.9 (d), 132.3 (s), 133.6 (s), 135.2 (s), 164.6 (s), 176.2 (s).

***N*-Acetoxy-*N*-(1-naphthylmethoxy)acetamide 6.** Sodium acetate (0.23 g, 2.8 mmol) was stirred at room temperature with *N*-chloro-*N*-(1-naphthylmethoxy)acetamide (0.5 g, 2.0 mmol) in dry acetone for 24 hours. The solvent was removed *in vacuo* and purification was achieved by centrifugal chromatography with ethyl acetate/pet spirit (0.26 g, 48%). $\nu_{max}(CHCl_3)/cm^{-1}$ 1793s (OC=O), 1739s (NC=O); δ_H(300 MHz; $CDCl_3$) 2.02 (3H, s, CH_3), 2.06 (3H, s, CH_3), 5.54 (2H, s, CH_2), 7.46-7.63 (4H, m, Ar-H), 7.91 (2H, d, Ar-H), 8.14 (1H, d, Ar-H); δ_C(75 MHz; $CDCl_3$) 18.4 (q), 21.2 (q), 76.1 (t), 123.8 (d), 125.3 (d), 126.1 (d), 126.7 (d), 128.7 (d), 129.3 (d), 130.0 (d), 130.4 (s), 131.9 (s), 133.7 (s), 167.9 (s), 176.4 (s); *m*/z (ESI) 273.3 [M^+], 274.4 [$M+H^+$].

***N*-Acetoxy-*N*-(2-naphthylmethoxy)acetamide 7.** Sodium acetate (0.23 g, 2.8 mmol) was stirred at room temperature with *N*-chloro-*N*-(2-naphthylmethoxy)acetamide (0.5 g, 2.0 mmol) in dry acetone for 16 hours. The solvent was removed *in vacuo* and purification was achieved by centrifugal chromatography with ethyl acetate/pet spirit (0.36 g, 66%). $\nu_{max}(CHCl_3)/cm^{-1}$ 1793s (OC=O), 1740s

(NC=O); δ_H(300 MHz; $CDCl_3$) 2.12 (3H, s, CH_3), 2.15 (3H, s, CH_3), 5.23 (2H, s, CH_2), 7.50-7.56 (3H, m, Ar-H), 7.84-7.90 (4H, m, Ar-H); δ_C(75 MHz; $CDCl_3$) 18.5 (q), 21.3 (q), 78.1 (t), 126.4 (d), 126.6 (d), 126.8 (d), 127.7 (d), 128.2 (d), 128.4 (d), 129.1 (d), 132 1 (s), 133.1 (s), 133.4 (s), 168.0 (s), 176.3 (s); *m*/z (ESI) 273.3 [M^+], 274.3[$M+H^+$], 296.3[$M+Na^+$].

***N*-Acetoxy-*N*-(2-(2'-naphthyl)ethoxy)acetamide 8.** Sodium acetate (0.22 g, 2.7 mmol) was stirred at room temperature with *N*-chloro-*N*-(2-(2'-naphthyl)ethoxy)acetamide (0.5 g, 1.9 mmol) in dry acetone for 18 hours. The solvent was removed *in vacuo* and purification was achieved by centrifugal chromatography with ethyl acetate/pet spirit (0.29 g, 53%). ν_{max}($CHCl_3$)/cm^{-1} 1793s (OC=O), 1735s (NC=O), 1366s, 1184s; δ_H(300 MHz; $CDCl_3$) 2.10 (3H, s, CH_3CON), 2.14 (3H, s, CH_3COO), 3.20 (2H, t, $OCH_2\underline{CH_2}$), 4.43 (2H, t, OCH_2), 7.38 (1H, d, Ar-H), 7.49 (2H, m, Ar-H), 7.71 (1H, s, Ar-H), 7.82 (3H, m, Ar-H); δ_C(75 MHz; $CDCl_3$) 18.5 (q), 21.1 (q), 34.6 (t), 76.3 (t), 125.6 (d), 126.2 (d), 127.2 (d), 127.4 (d), 127.5 (d), 127.7 (d), 128.2 (d), 132.3 (s), 133.6 (s), 134.9 (s), 168.0 (s), 176.3 (s).

***N*-Acetoxy-*N*-(3-(2'-naphthyl)-1-propoxy)acetamide 9.** Sodium acetate (0.21 g, 2.5 mmol) was stirred at room temperature with *N*-chloro-*N*-(3-(2'-naphthyl)-1-propyloxy)acetamide (0.5 g, 1.8 mmol) in dry acetone for 24 hours. The solvent was removed *in vacuo* and purification was achieved by centrifugal chromatography with ethyl acetate/pet spirit (0.34 g, 63%). ν_{max}($CHCl_3$)/cm^{-1} 1794s (OC=O), 1736s (NC=O), 1184s; δ_H(300 MHz; $CDCl_3$) 2.14 (2H, qui, $CH_2\underline{CH_2}CH_2$), 2.20 (3H, s, CH_3CON), 2.29 (3H, s, CH_3COO), 2.91 (2H, t, $ArCH_2$), 4.15 (2H, t, OCH_2), 7.36 (1H, d, Ar-H), 7.47 (2H, m, Ar-H), 7.65 (1H, s, Ar-H), 7.82 (3H, m, Ar-H); δ_C(75 MHz; $CDCl_3$) 18.6 (q), 21.2 (q), 29.5 (t), 32.1 (t), 75.1 (t), 125.4 (d), 126.1 (d), 126.6 (d), 127.1 (d), 127.4 (d), 127.6 (d), 128.1 (d), 132.1 (s), 133.6 (s), 138.4 (s), 167.9 (s), 176.2 (s).

***N*-Benzoyloxy-*N*-butoxyacetamide 15.** Sodium benzoate (0.61 g, 4.2 mmol) was stirred at room temperature with *N*-butoxy-*N*-chloroacetamide (0.5 g, 3.0 mmol) in dry acetone for 40 hours. The solvent was removed *in vacuo* and purification was achieved by centrifugal chromatography with ethyl acetate/pet spirit (0.54 g, 72%). $\nu_{max.}$(neat)/cm^{-1} 1765s (OC=O), 1738s (NC=O), 1363m, 1241s; δ_H(300 MHz; $CDCl_3$) 0.94 (3H, t, CH_3), 1.42 (2H, m, $\underline{CH_2}CH_3$), 1.71 (2H, m, $OCH_2\underline{CH_2}$), 2.33 (3H, s, $COCH_3$), 4.17 (2H, t, OCH_2), 7.49 (2H, t, Ar-H), 7.64 (1H, t, Ar-H), 8.10 (2H, d, Ar-H); δ_C(75 MHz; $CDCl_3$) 13.7 (q), 19.0 (t), 21.3 (q), 30.1 (t), 76.0 (t)), 127.3 (s), 128.7 (d), 130.1 (d), 134.1 (dH) 164.1 (s), 176.5 (s).

***N*-Butoxy-*N*-ethoxy-2-naphthamide 16.**[41] *N*-Butoxy-2-naphthamide (0.12 g, 0.48 mmol) was dissolved in ethanol (10 mL). PIFA ([Bis(trifluoroacetoxy)iodo]benzene) (0.31 g, 0.73 mmol) dissolved in ethanol (10 mL) was added and the reaction was stirred in the dark for 5 min. Whereupon 10% aq. $NaHCO_3$ (40 mL) was added to quench the reaction. Ethanol was removed under reduced pressure and product extracted from the base solution with dichloromethane, which was washed with brine and dried over Na_2SO_4. Concentration under reduced pressure yielded the crude product. Centrifugal chromatography with 10% ethyl acetate/hexane afforded the pure *N*-butoxy-*N*-ethoxy-2-naphthamide as a brown oil. $\nu_{max}(CHCl_3)/cm^{-1}$ 1700s (C=O); δ_H(300 MHz; $CDCl_3$) 0.85(3H, t, $(CH_2)_2\underline{CH_3}$), 1.25(3H, t, $OCH_2\underline{CH_3}$), 1.32(2H, sx, $CH_2\underline{CH_2}CH_3$), 1.59(2H, p, $\underline{CH_2}CH_2CH_3$), 4.05(2H, q, $O\underline{CH_2}CH_3$), 4.14(2H, t, $O\underline{CH_2}CH_2$), 7.55(2H, m), 7.84-7.93(4H, m), 8.38(1H, s); δ_C (75 MHz; $CDCl_3$) 13.7 (q), 13.7 (q), 19.1 (t), 30.2 (t), 69.1 (t), 73.0 (t), 125.3 (d), 126.6 (d), 127.6 (d), 127.7 (d), 128.0 (d), 129.2 (d), 129.9 (s), 130.5 (d), 132.3 (s), 135.1 (s), 174.2 (s); *m/z* (ESI): 287.2(M^+) (Found: 310.1425 [$M+Na^+$], $C_{17}H_{21}NO_3Na$ requires 310.1419).

***N*-Butoxy-*N*-methyl-2-naphthamide 17.** *N*-Butoxy-2-naphthamide (0.41 g, 1.67 mmol) and iodomethane (0.24 g, 1.67 mmol) were dissolved in 10% aq. methanol (20 mL) and cooled in an ice bath. Potassium hydroxide (0.09 g, 1.67 mmol) was added to the solution and the mixture stirred for 24 h. Methanol was removed under reduced pressure followed by addition of distilled water (25 mL). The solution was extracted with dichloromethane, which was washed with dil. HCL, H_2O, 10% Na_2CO_3 and dried over $MgSO_4$ before concentration under reduced pressure. Centrifugal chromatography with 15% ethyl acetate/hexane afforded the pure *N*-butoxy-*N*-methyl-2-naphthamide as a brown oil. $\nu_{max}(CHCl_3)/cm^{-1}$ 1654 (C=O); δ_H(300 MHz; $CDCl_3$) 0.72(3H, t, $CH_2\underline{CH_3}$), 1.15(2H, sx, $\underline{CH_2}CH_3$), 1.39(2H, p, $OCH_2\underline{CH_2}$), 3.42(3H, s, NCH_3), 3.71(2H, t, OCH_2), 7.52(2H, m), 7.74(1H, d), 7.86(3H, m), 8.23(1H, s); δ_C (75 MHz; $CDCl_3$) 14.0 (q), 18.9 (t), 29.9 (t), 34.6 (q), 73.7 (t), 124.8 (d), 125.7 (d), 126.8 (d), 127.7 (d), 127.9 (d), 128.3 (d), 129.2 (d), 131.6 (s), 132.5 (s), 134.2 (s), 169.8 (s); *m/z* (ESI): 258($M+H^+$) (Found: 258.1495, $C_{16}H_{20}NO_2$ requires 258.1494).

Mutagenicity assays.

All Ames tests were carried out in *S. typhimurium* TA100 and TA98 without metabolic activation according to standard protocols.[17, 21, 22, 37, 62] For scaling and direct comparison of new data for **2**-**9** with that previously obtained in TA100 for all *N*-acyloxy-*N*-alkoxyamides from which the QSAR in equation (1) was derived, standard *N*-acetoxy-*N*-butoxybenzamide **14** was tested in conjunction with new mutagens. For each new substrate the gradient over the linear dose response range, which

is equal to the mutagenicity at 1μmol/plate, was scaled to the activity of **14** (gradient 319 revertants at 1 μmol/plate) and reported as LogTA100.‡ Similarly, in TA98 responses of all test compounds at 1 μmol/plate were normalised with respect to *N*-acetoxy-*N*-butoxy-2-naphthamide **2** (gradient= 459 at 1 μmol/plate), which was tested concurrently.

For prediction of activities of **2**-**9** in TA100 from linear and bilinear equations 1-6, Log *P* values were calculated using the Ghose-Crippen algorithm within MacSpartan'08.[63] Data for linear QSAR in equation (1) and bilinear QSAR in Equation 2 were reported previously.[22]

DNA damage studies

A 375 base pair EcoRI to BamHI fragment of plasmid pBR322 DNA was 3' end labelled at the EcoRI site using Klenow fragment and [α-32P]dATP(3000 Ci/mmol) according to a published procedure.[64] The fragment was isolated on a 4% non-denaturing polyacrylamide gel. A partial sequence of the pBR322 DNA used in this work, as determined in an earlier publication,[18] is reproduced as supplementary information. DNA damage studies on mutagens **2**, **3**, **6**-**9** were carried out using previously published protocols[18, 65]

‡ Log_{10} of the induced revertants in TA100 at 1μmol/plate

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