CHAPTER FOUR

4. Mutagenicity of alkyl *N*-acetoxybenzohydroxamates

4.1 The Ames test and factors controlling mutagenicity

The Ames test is a *salmonella*-mammalian liver assay developed by Dr. Bruce Ames.[240-243] The tester organism is the colon bacteria *salmonella typhimurium*, bearing a mutation that renders it unable to synthesise one of the enzymes responsible for the production of the amino acid histidine, which is a necessary component of proteins. As a result, the bacterium is unable to grow in a nutrient medium without an external supply of histidine. Spontaneous reversions occurring naturally restore the ability of the bacterium to produce the enzyme and allow the bacterium to form a colony. Chemicals that induce mutations increase the rate of change from the auxotrophic to the prototrophic form and this is reflected in an increase in the number of colonies that grow in the medium. Many chemicals are active mutagens only after metabolism by enzymatic processes in the body. To mimic the natural processes that occur in the liver, Ames added enzymes *via* an extract of rat-liver (S9) to the tester bacteria to convert the chemicals to their mutagenic metabolites. The enzymes are mainly cytochrome P-450 oxidases induced by deliberate administration of known carcinogens, such as Arochlor.

The rat liver extract, S9, is mixed with the tester bacteria and evenly spread on the agar medium. A DMSO solution of the suspected mutagen is added to the disk which is incubated for 2-3 days after which most of the *histidine negative* bacteria have died. Any DNA damage that results in histidine production caused by the chemical is detected as visible colonies. The mutagenic potential of the compound is proportional to the number of colonies produced. Direct acting mutagens yield positive test result without the addition of S9. Usually a linear dose-response is observed although at higher dose, toxicity may result in cell death.

The simplicity, sensitivity and accuracy[244] of the Ames test for screening large numbers of potential environmental mutagens and carcinogens has resulted in its rapid acceptance as a standard procedure in many governmental, industrial and academic laboratories throughout the world.

4.1.1 Transportation across the cell

Significant factors in the reactivity of alkylating agents is the accessibility to the nucleophilic sites on the DNA and the lypophilicity/hydrophobicity of the substrate.

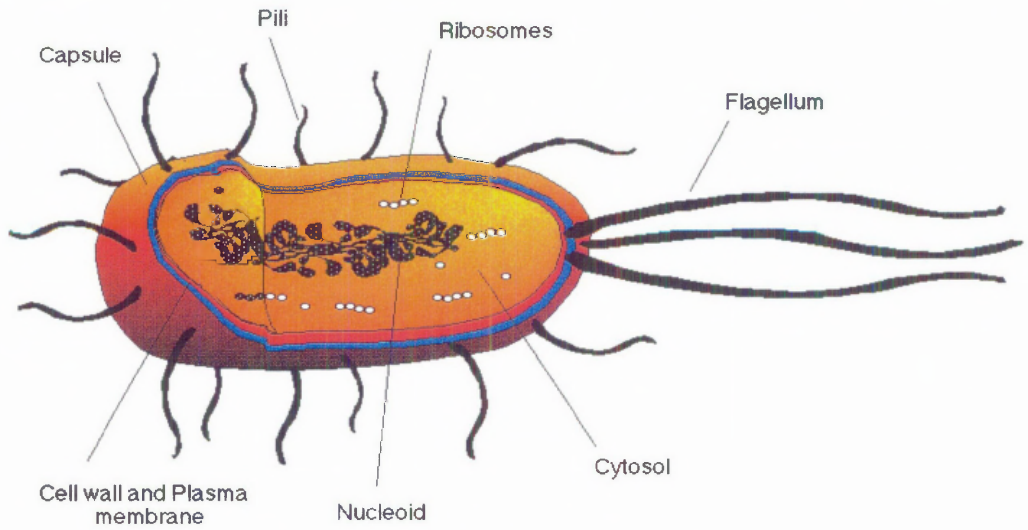


Figure 4-1 Prokaryotic cell[o]

The general cell structure for *salmonella typhimurium* is shown in Figure 4-1. As with all prokaryotic cells, the contents are protected by a relatively rigid cell wall. Within this wall is the cell membrane which is a very thin, highly flexible layer that is structurally very weak and it is through these layers that the food, waste and mutagenic compounds must pass. Most biological membranes contain phospholipids; compounds that contain highly hydrophobic and hydrophilic moieties which force the molecules to aggregate in a large micelle shell, called a phospholipid bi-layer. This thin, but highly specific bi-layer, is selectively permeable to small molecules such as alcohols, water, fatty acids and benzene which dissolve in the lipid phase of the matrix. Large polar molecules, such as sugars, amino acids and ions pass through the membrane through the action of membrane transport proteins. The phospholipid layer for the Gram-negative salmonella bacteria is relatively porous and consists of a complex polysaccharide called peptidoglycan.

Alkyl *N*-acyloxybenzohydroxamates **100**, **151** and **172** are relatively small, non charged and non-hydroxylic molecules that should pass through the cell wall and membrane

[o] Colours for illustration only.

layers, however hydrolysis and/or phosphorylation by the phosphotransferase transport system could occur in the lipid matrix rendering the substrate inactive.

Within the cell and bounded by the cell membrane is the cytoplasm, a complicated mixture of substances bathed in water, in which the functions of the cell are carried out. The major components of the cytoplasm are macromolecules (proteins, nucleic acids, polysaccharides, lipids), ribosomes, small organic molecules (mainly precursors of macromolecules) and various inorganic ions. Prokaryotic DNA is not enclosed in a nuclear membrane but is found as a highly aggregated DNA molecule called the nucleoid which is extensively folded and twisted to fit into the bacterial cell.

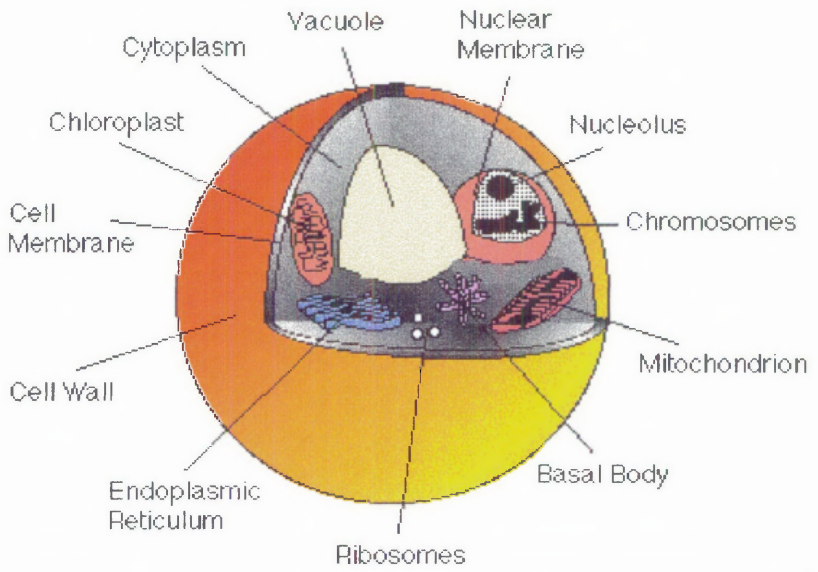


Figure 4-2 Eukaryotic Cell[P]

Eukaryotic cells are more complex inside the cell membrane, the genetic material being organised in chromosomes contained in the nucleus, a membrane-enclosed structure that functions as both a store house and factory for genetic information. The transport mechanisms across this lipid bi-layer are not as well understood, but are probably similar to the outer cell membrane.

Steric hindrance drastically limits the access of exogenous electrophiles to nucleophilic sites. While the phosphodiester groups and sugar hydroxyls are exposed to electrophilic agents, the double helix of DNA ensures that purine and pyrimidine bases are partially or

---

[P] Colours for illustration only.

totally hindered from attack. Generally, only nucleophilic centres situated on the major or minor grooves or in the walls of the double helix remain accessible to electrophilic attack. Recent advances in computational algorithms and processor power have allowed the reactivity of the DNA macromolecule to be examined by exploration of the *electrostatic molecular potential* in which high nucleophilicity is associated with the most prominent potential minima.[245,246]

The binding of chemical carcinogens to proteins has been the cause of much speculation and experimentation[4] after Miller and Miller reported that carcinogenic compounds formed stable, covalently linked complexes with nucleic acids *in vivo*,[65] however this binding alone is not sufficient to account for the chemical induction of cancer.

Intercalation of substrates with DNA is a fundamental process in which planar or aromatic functional groups of a substrate associate directly with DNA by fitting into the minor or major grooves of the helix. The driving force for the intercalation of aromatic substrates occurs through the lowering of the energy of the system with π-π stacking and is believed to be an important consideration in the process of mutagenesis for aromatic amines. The degree of intercalation is also intertwined with the lypophilicity of the substrate which determines the ease of transport through the cell to the DNA.

Alkyl *N*-acyloxybenzohydroxamates must pass through much cellular material to interact with DNA. Once through the cell membrane, the driving force for binding with hydrophobic DNA would probably be the hydrophobic nature of the mutagen and possibly π-π driven intercalation. The later process is best if aromatics are electron deficient since this facilitates electrostatic attraction between electron rich (negatively charged) centres on the nucleotides and the electron deficient centres on the guest molecule. Thus acridines are outstanding intercalators although it is known that simple polycyclic aromatics may also intercalate effectively and the optimum size would appear to be three to four fused aromatics. Throughout the course of these studies, biological activity of substrates have been assayed by the Ames methodology. In this chapter the results of such assays are evaluated and point to some factors that may control the level of biological activity.

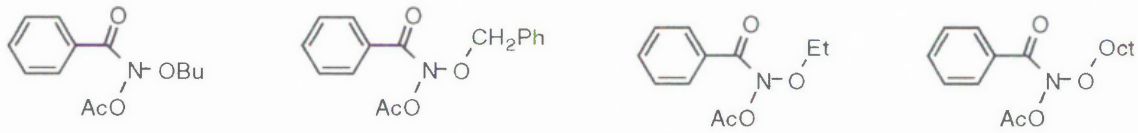
4.1.2 Assay procedure

Mutagenicity testing was carried out at the Australian Commonwealth "Toxicology Unit of Worksafe Australia" in Sydney by Dr Antonio M. Bonin. Pure samples of the appropriate alkyl *N*-acyloxybenzohydroxamate were placed in sealed glass vials under an

atmosphere of nitrogen gas. The samples were packed with dry ice and transported to the laboratories overnight and stored at -80 °C until the day of testing, when fresh DMSO solutions were prepared. The compounds were then subjected to the standard Ames test using TA100 *salmonella typhimurium* as the lawn culture. While TA98 and TA100 were used in preliminary tests, the latter was used as the reference strain to reduce costs, as both strains exhibited mutagenic responses when treated with DMSO solution of the mutagen. Metabolic activation was provided by a standard 10% S9 mix of Arochlor 1254-induced male (200-250 g) Sprague-Dawley rat livers.

4.2 Mutagenicity of butyl *N*-acetoxybenzohydroxamates

4.2.1 Butyl *N*-acetoxybenzohydroxamate



100a **151a** **187** **188**

Figure 4-3

Butyl *N*-acetoxybenzohydroxamate **100a** was one of the first alkyl *N*-acetoxybenzohydroxamates synthesised and subjected to the Ames test. Early "in house" mutagenicity testing with simple alkyl *N*-acetoxybenzohydroxamates (**100a**, **151a**, **187** and **188**) revealed that there was a positive induction of reversions and a linear dose-response relationship was evident in certain cases when moderate levels of mutagen (less than 200 µg/plate) were tested (Figure 4-4).[167] The tests indicated that the mutagens were active in TA98 (susceptible to frame-shift mutations) and TA100 (susceptible to point mutations).[247] In addition activity was evident with and without the administration of S9.[167]

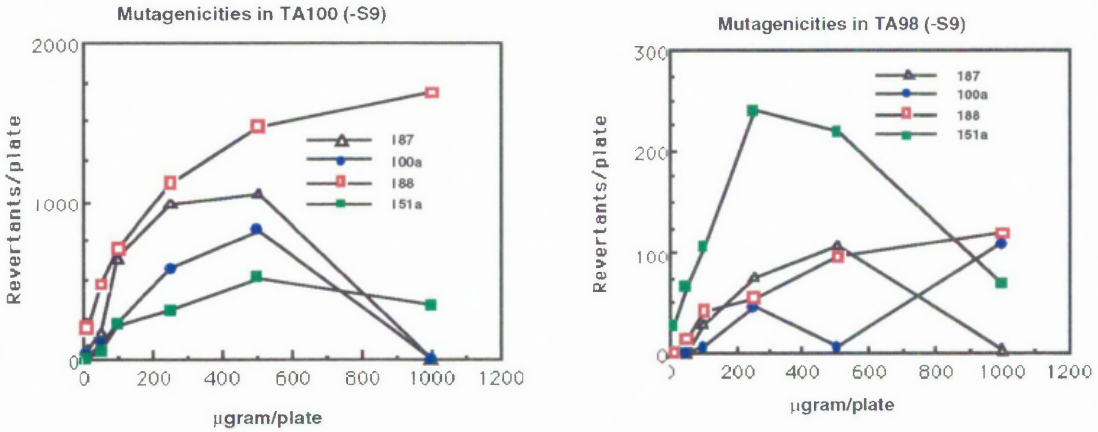


Figure 4-4[q]

Subsequently, data was obtained from the standardised tests performed by Dr Bonin who confirmed these trends in activity for butyl *N*-acetoxybenzohydroxamate **100a** (Figure 4-5); at low doses a linear dose-response was evident which was similar in both the presence and absence of S9. The dose-response relationship became non-linear at higher dosages due to toxicity and resulted in a flattening of the response curve. As such, the dose-response relationship was calculated from the linear section of each curve. Since TA100 salmonella was found to give an acceptable dose-response curve and was more sensitive it was used for all further mutagenicity testing.

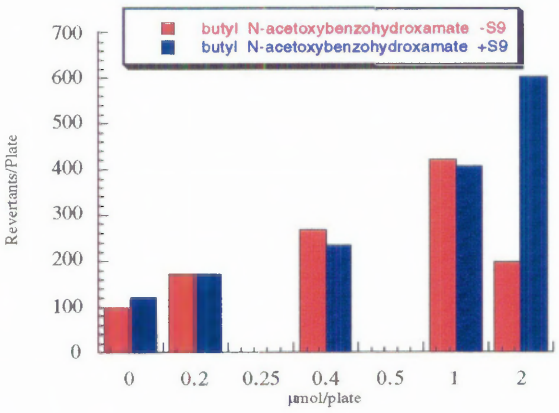


Figure 4-5 Mutagenicity of butyl *N*-acetoxybenzohydroxamate **100a** in TA100

---

[q] A spontaneous revertant rate of *ca.*160 and 35 for TA100 and TA98 respectively have been subtracted from the respective counts. Compounds are considered mutagenic with an induced revertant count of twice these levels at 500 µg/plate.

In dose-response plots such as these in Figure 4-5, the background reversion rate is available from the intercept and may be subtracted for the purposes of comparative studies. Interestingly, a linear dose-response relationship was observed when the concentration of applied mutagen without S9 activation, was less than 1.0 μmol per plate but above these doses the toxicity was evident with a diminution of revertants. The addition of the S9 agent however reduced the toxicity of the mutagen such that the linear dose-response was evident over the entire dosage tested. Neglecting the points after 1 μmol/plate for the mutagen without S9 activation allowed the dose-response at 1 μmol/plate to be calculated as the slope, which in this case was 319±30 (r = 0.9911).

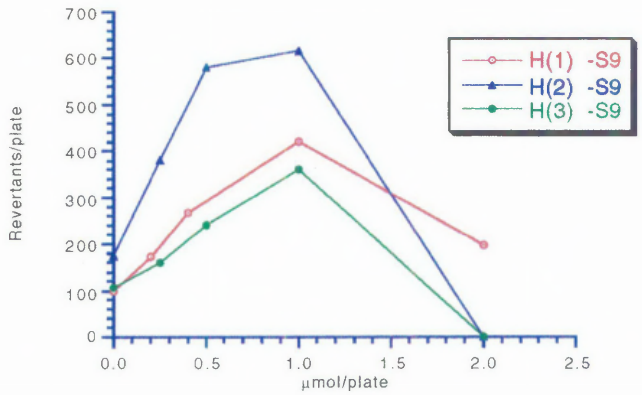


Figure 4-6 Mutagenicity of butyl *N*-acetoxybenzohydroxamate **100a** in three successive studies

During the course of testing the butyl *N*-acetoxy (*para*-substituted)benzohydroxamate series, butyl *N*-acetoxybenzohydroxamate was tested independently three times with TA100 and the results are shown in Figure 4-6. The discrepancies are due to uncontrollable differences in the batches of TA100 bacteria which were cultured immediately prior to treatment with mutagen. Ideally a series of compounds should be tested from the same batch of salmonella for an accurate comparison but this was impractical in this study. Generally, samples from each series were synthesised, purified and dispatched in batches, several days or even several weeks apart. Therefore to offset the differences in responses between batches butyl *N*-acetoxybenzohydroxamate was used as the standard; responses for the standard enabled modification of data for new substrates by a factor based on the ratio of the activities of the standard at 1 μmol/plate in different batches.

Table 4-1 Dose-response data for **100a**, **100b**, and **100d**.(Set 1)

µmol/plate	Stand[a] -S9	Stand[a] +S9	MeO -S9	MeO +S9	Me -S9	Me +S9
0.00	99	121	99	121	99	121
0.20	173	173	277	230	157	173
0.40	267	234	409	326	252	193
1.00	421	407	730	661	420	321
2.00	198	602	0	1000	454	435

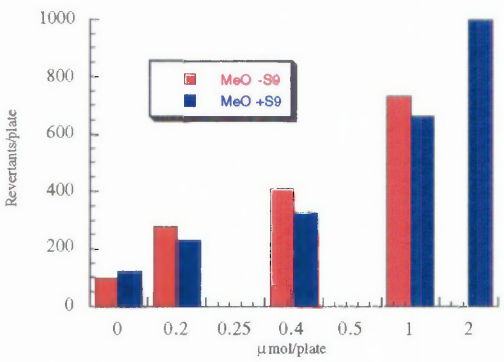
Table 4-2 Dose-response data for **100a**, **100e**, and **100c**.(Set 2)

µmol/plate	Stand[a] -S9	Stand[a] +S9	Bu^t -S9	Bu^t +S9	Ph -S9	Ph +S9
0.00	175	182	175	182	175	182
0.25	381	252	403	306	707	290
0.50	581	360	461	323	0	363
1.00	616	493	0	440	0	415
2.00	0	761	0	0	0	553

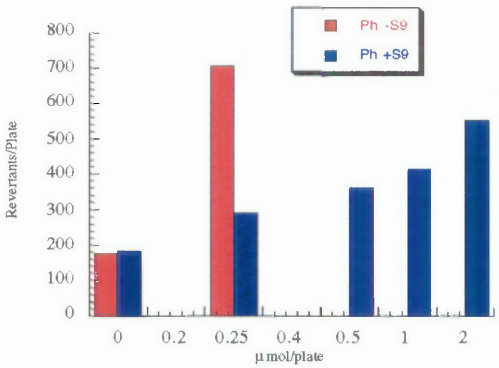
Table 4-3 Dose-response data for **100a**, **100g**, **100f**, and **100h**.(Set 3)

µmol/plate	Stand[a] -S9	Br -S9	Br +S9	Cl -S9	Cl +S9	NO_2 -S9	NO_2 +S9
0.00	122	108	122	107	122	107	122
0.25	164	226	171	172	171	146	166
0.50	237	307	242	330	242	167	236
1.00	373	362	346	376	346	256	325
2.00	565	0	483	0	483	349	424

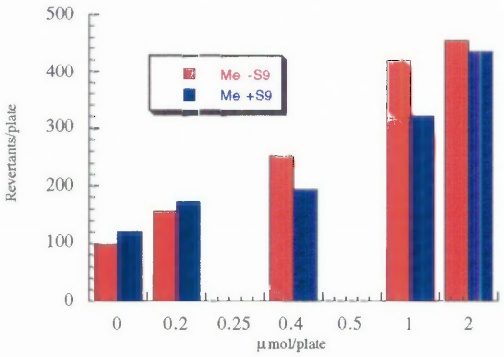
[a] Standard was butyl *N*-acetoxy benzohydroxamate **100a**



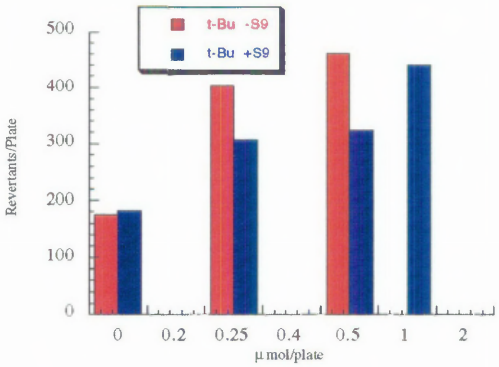
(i)



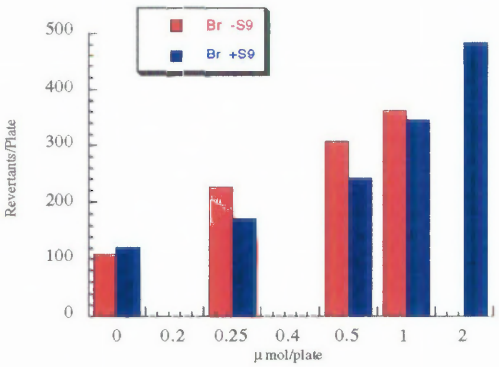
(ii)



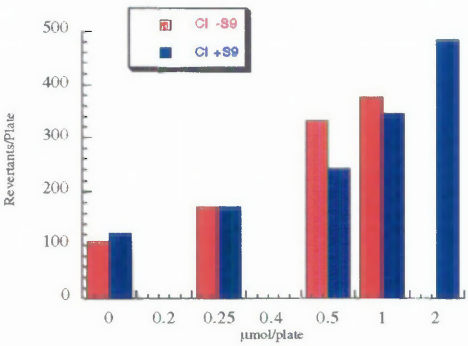
(iii)



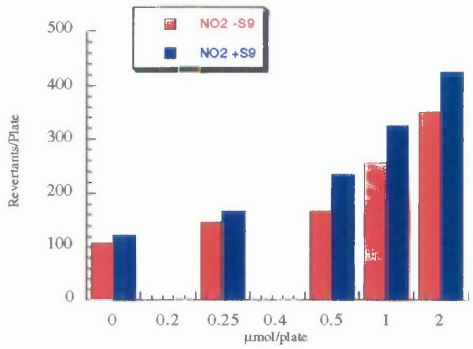
(iv)



(v)



(vi)



(vii)

Figure 4-7 Dose-response for **100b-h**.

Figure 4-7(i-vii) show the dose-response graphs[r] for the series of butyl *N*-acetoxybenzohydroxamates **100b-h** over the range 0-2.0 µmol/plate with and without S9. All displayed a linear dose-response region when the concentration of applied mutagen was less than 1.0 µmol/plate but in some cases the number of revertants reduced above this dosage. For comparative purposes, S9 deficient test results were used. From the linear regions the slope for each compound was obtained and divided by the slope obtained for butyl *N*-acetoxybenzohydroxamate tested along with that particular set. The normalised slopes (with respect to butyl *N*-acetoxybenzohydroxamate **100a**, from a linear dose region) are recorded in Table 4-4 and are presented in column graph format in Figure 4-8.

[r] Raw data with background reversion rate included. In column graphs, dose axis is non-linear.

Table 4-4 Normalised mutagenicity

Subst.	Set	Corrected Slope¶	Normalised Slope	r
100a H	1	319(30)	1	0.9981
100d Me	1	323(22)	1.0 (0.1)	0.9953
100b MeO	1	612(51)	1.9 (0.2)	0.9932
100a H	2	812(7)	1	0.9999
100c Ph	2	2126(1)	2.6(0.0)	1.0000
100e Bu^t	2	572(196)	0.7(0.2)	0.9459
100a H	3	258(11)	1	0.9927
100f Cl	3	281(74)	1.1(0.3)	0.9372
100g Br	3	397 (44)	1.5(0.2)	0.9940
100h NO_2	3	122(9)	0.5(0.1)	0.9922

¶ Corrected slope was calculated from the slope of the revertants per plate versus dosage over the linear range (generally 0 - 1.0 μmol/plate).

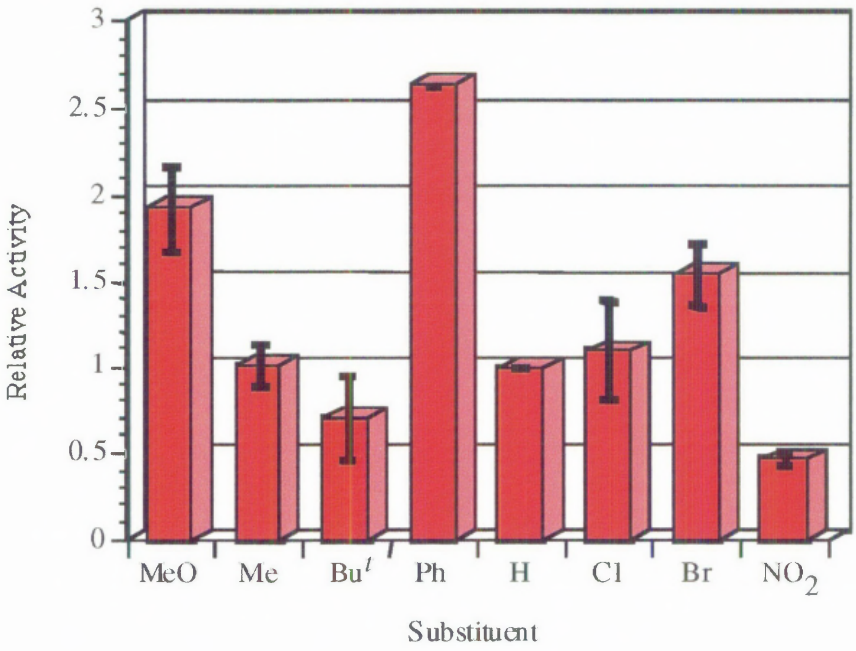


Figure 4-8 Relative mutagenicities for **100a-h** at 1μmol/plate (100a=1)

Mutagenicities across the series show no obvious trend. The ordering in Figure 4-8 corresponds to Hammett σ substituent constants and reflects the electronic effects of *para* substituents. While the *p*-methoxylated mutagen **100b** is more active and the *p*-nitro **100h** is less active than parent **100a**, the *p*-chloro **100f** and *p*-bromo **100g** compounds are clearly against the trend, whereas *p*-phenyl **100c** is significantly more mutagenic than **100a**. This cannot be due to electronic effects.

The ability of the substrate to mutate DNA is dependent upon several features; transport through the cell wall, electronic effects, hydrolysis, intercalation with DNA, specialised local pocket features, enzymatic destruction processes, etc. The biphenyl functional group could however increase activity through lipophilicity or hydrophobicity thus enabling more facile passage through the cell wall or increasing the binding with DNA (*vide infra*).

4.3 Mutagenicity of benzyl *N*-acetoxybenzohydroxamates 151

The mutagenicity levels for **151a-i** were ascertained in TA100 *salmonella typhimurium* and are given in Table 4-5 to Table 4-7 and illustrated in Figure 4-9(i-ix).

Table 4-5 Dose-response data for **151b**, **151d**, **151i**.

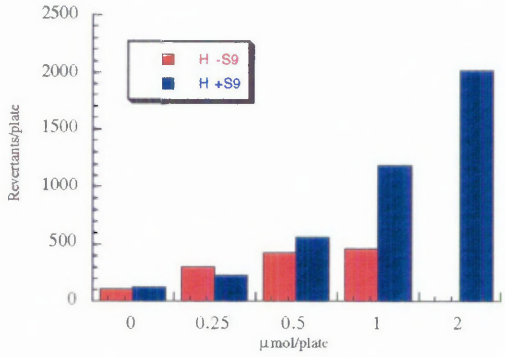
µmol/plate	PhO -S9	PhO +S9	Ph -S9	Ph +S9	NO_2 -S9	NO_2 +S9
0.000	175	182	175	182	175	182
0.250	805	474	1268	904	2090	444
0.500	0	1170	1751	1365	2153	788
1.000	0	676	2328	0	0	1105
2.000	0	0	2590	0	0	0

Table 4-6 Dose-response data for **151f**, **151a**, **151g**.

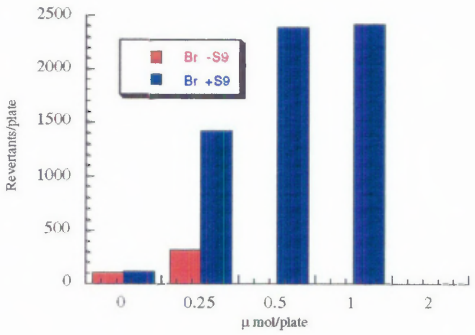
µmol/plate	Bu^t -S9	Bu^t +S9	H -S9	H +S9	Br -S9	Br +S9
0.000	175	182	108	117	108	117
0.250	539	0	296	231	314	1419
0.500	626	0	427	557	0	2388
1.000	715	0	458	1187	0	2418
2.000	629	0	0	2015	0	0

Table 4-7 Dose-response data for **151h**, **151c**, **151e**.

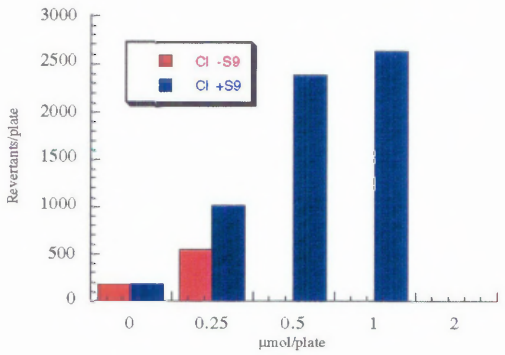
µmol/plate	Cl -S9	Cl +S9	MeO -S9	MeO +S9	Me -S9	Me -S9
0.000	175	182	175	182	175	182
0.250	543	1010	461	586	555	644
0.500	0	2383	821	923	682	1263
1.000	0	2626	0	1118	0	1230
2.000	0	0	0	1271	0	1334



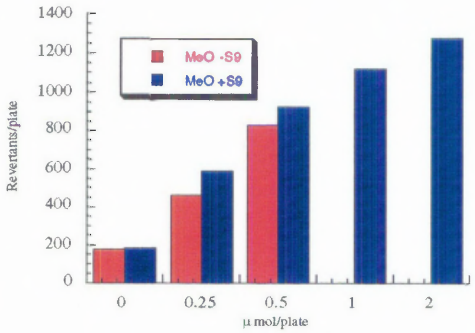
(i)



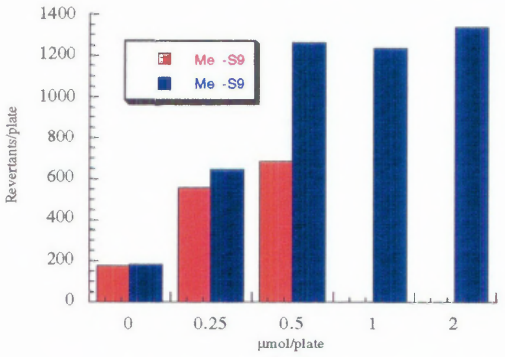
(ii)



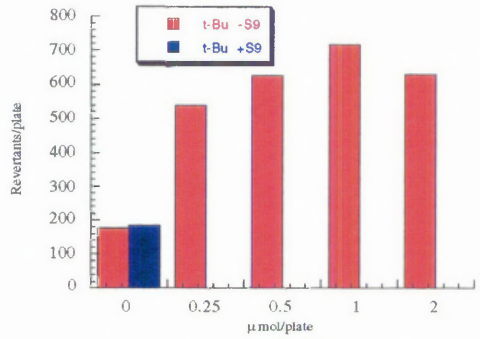
(iii)



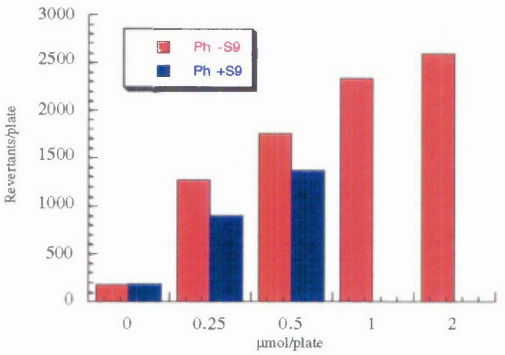
(iv)



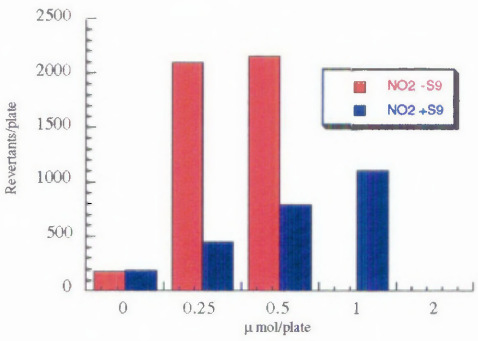
(v)



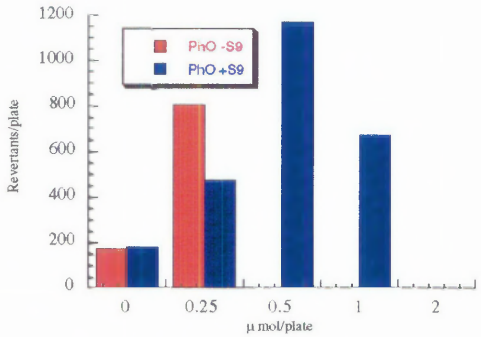
(vi)



(vii)



(viii)



(ix)

Figure 4-9 Dose-response for **151a-i.**

The raw mutagenicity levels revealed that apart from benzyl *N*-acetoxybenzohydroxamate, these compounds were significantly more mutagenic than the previous series but displayed higher toxicity towards the salmonella bacteria above 0.5 µmol/plate. Furthermore, while linearity could be detected at low dose, no clear trend in activity was evident. The dose mutagenicity data for benzyl *N*-acetoxybenzohydroxamate **151a** displayed an excellent linear relationship when S9 enzymes were present (r^2=0.995) over the range 0-2 µmol/plate whereas in the absence of applied enzymes the response at higher doses was modified by toxicity (Figure 4-9i). At 2.0 µmol/plate the compound displayed no net mutagenicity (Figure 4-10).

Similar results were found for other compounds (Figure 4-9(ii-ix)).

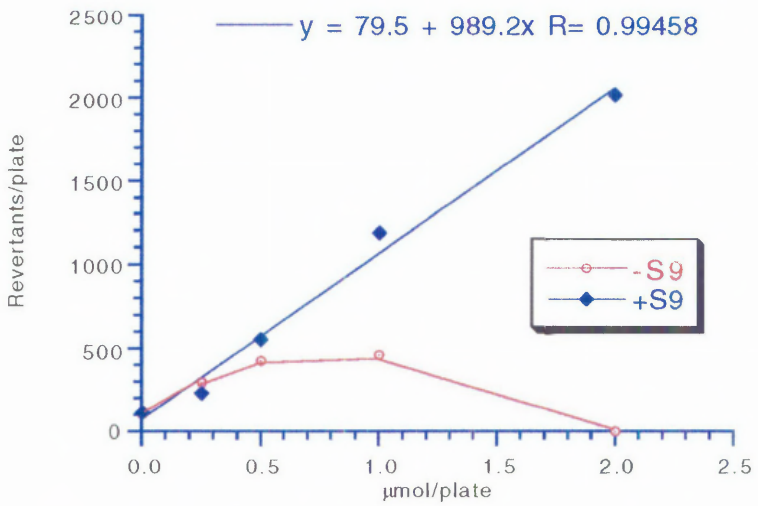


Figure 4-10 Mutagenicity of benzyl *N*-acetoxybenzohydroxamate **151a**.

To enable some comparison to be made, the data for all compounds was extrapolated back to the 0.25 μmol/plate level where reasonable linear-dose relationships held. Furthermore to allow comparison with the results obtained for the previous alkyl *N*-acetoxybenzohydroxamate series **100** the measured mutagenicity levels are normalised with respect to the standard compound, butyl *N*-acetoxybenzohydroxamate **100a**, which was tested in parallel with these batches. A comparison of the normalised mutagenicity levels for this series without S9, is shown below (Figure 4-11).

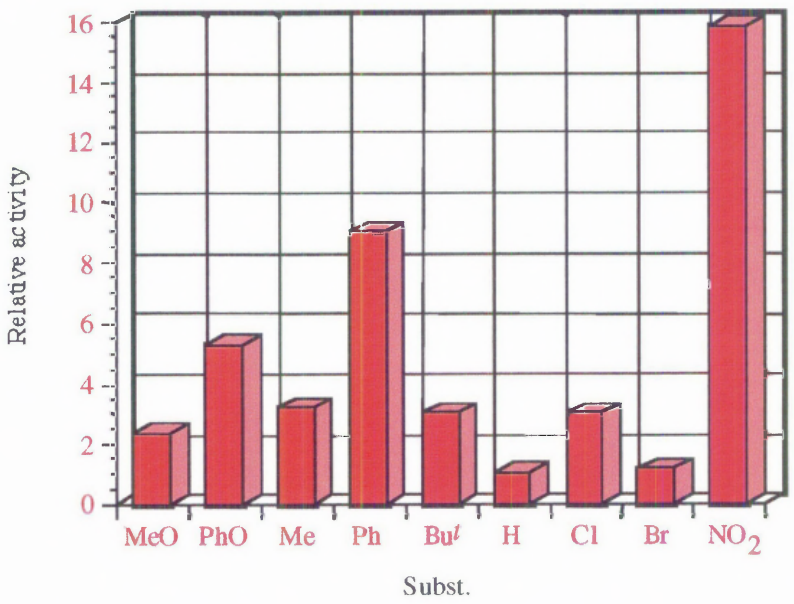


Figure 4-11 Relative mutagenicities for **151a-i** at 0.25μmol/plate (100a=1)

Across the electronic series it is evident that no clear trend is apparent. The data for *para*-phenoxy **151b** and *para*-phenyl **151d** substrates indicate greater activity relative to the unsubstituted compound. Once again, aromatic character would appear to enhance mutagenicity. It is significant that, while studies outlined elsewhere in this thesis indicate that these two substrates **151b** and **151d** together with *p*-methoxy mutagen **151c** do not form nitrenium ions even under acid-catalysis, but rather generate *para*-substituted benzyl cations, they are nonetheless significantly mutagenic. Either the actual chemical interaction with DNA leading to the mutagenic activity occurs by an alternative process or the substrates behave as alkylating agents through benzyl cation formation. The extreme mutagenicity of the *p*-nitro compound **151i** is interesting and will be addressed in Section 4.5.2. In this series, S9 appeared to enhance activity and particularly so in the case of the *p*-chloro **151h** and the *p*-bromo **151g** substrates. The role of S9 in this enhancement as well as in the general detoxification of substrates is beyond the scope of this study.

4.4 Mutagenicity of benzyl *N*-benzoyloxybenzohydroxamates

The mutagenicity of a series of benzyl *N*-benzoyloxybenzohydroxamates **172a-e**, **172g** and **172h**, was measured for dose levels of 0 - 0.25 µmol/plate in TA100, with and without enzymatic activation. The low levels of applied mutagen were employed to ensure adequate mutagenicity levels without toxicity to the bacterial strain.

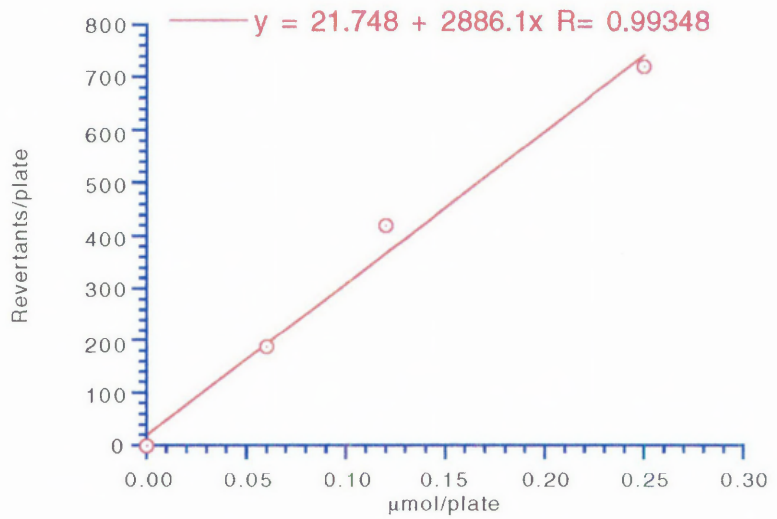


Figure 4-12 Dose-response relationship for **172a**

Figure 4-12 displays the dose-response relationship for **172a** and reflects the characteristically excellent results obtained for this series of compounds. Table 4-8 and Table 4-9 record the results for **172b-e**, **172g** and **172h**.

Table 4-8 Dose-response data for **100a**, **172b**, **172c**. (Set I)

µmol/plate	Stand -S9	H -S9	MeO -S9	MeO +S9	Me -S9	Me +S9
0.00	0(8)	0(8)	0(10)	0(7)	0(10)	0(8)
0.03			27(9)	100(12)	40(10)	86(5)
0.06		188(12)	51(11)	154(14)	127(6)	158(17)
0.12		420(24)	156(37)	296(7)	328(150)	371(10)
0.25	78(4)	720(78)	324(77)	696(59)	1286(38)	1008(67)
0.50	355(59)					
1.00	448(54)					

Table 4-9 Dose-response data for **172h**, **172g**, **172e**, **172d**. (Set II)

µmol/plate	NO_2 -S9	NO_2 +S9	CN -S9	CN +S9	CHO -S9	CHO +S9	Cl -S9	Cl +S9
0.00	0(10)	0(10)	0(10)	0(7)	0(10)	0(7)	0(10)	0(7)
0.03	10(8)	26(6)	29(7)	14(8)	31(6)	36(21)	93(18)	21(6)
0.06	51(12)	107(12)	119(8)	93(25)	132(17)	111(10)	138(14)	91(11)
0.12	80(35)	116(19)	179(13)	190(19)	180(15)	239(12)	307(7)	268(20)
0.25	174(24)	264(22)	332(40)	356(29)	274(28)	366(17)	480(18)	612(8)

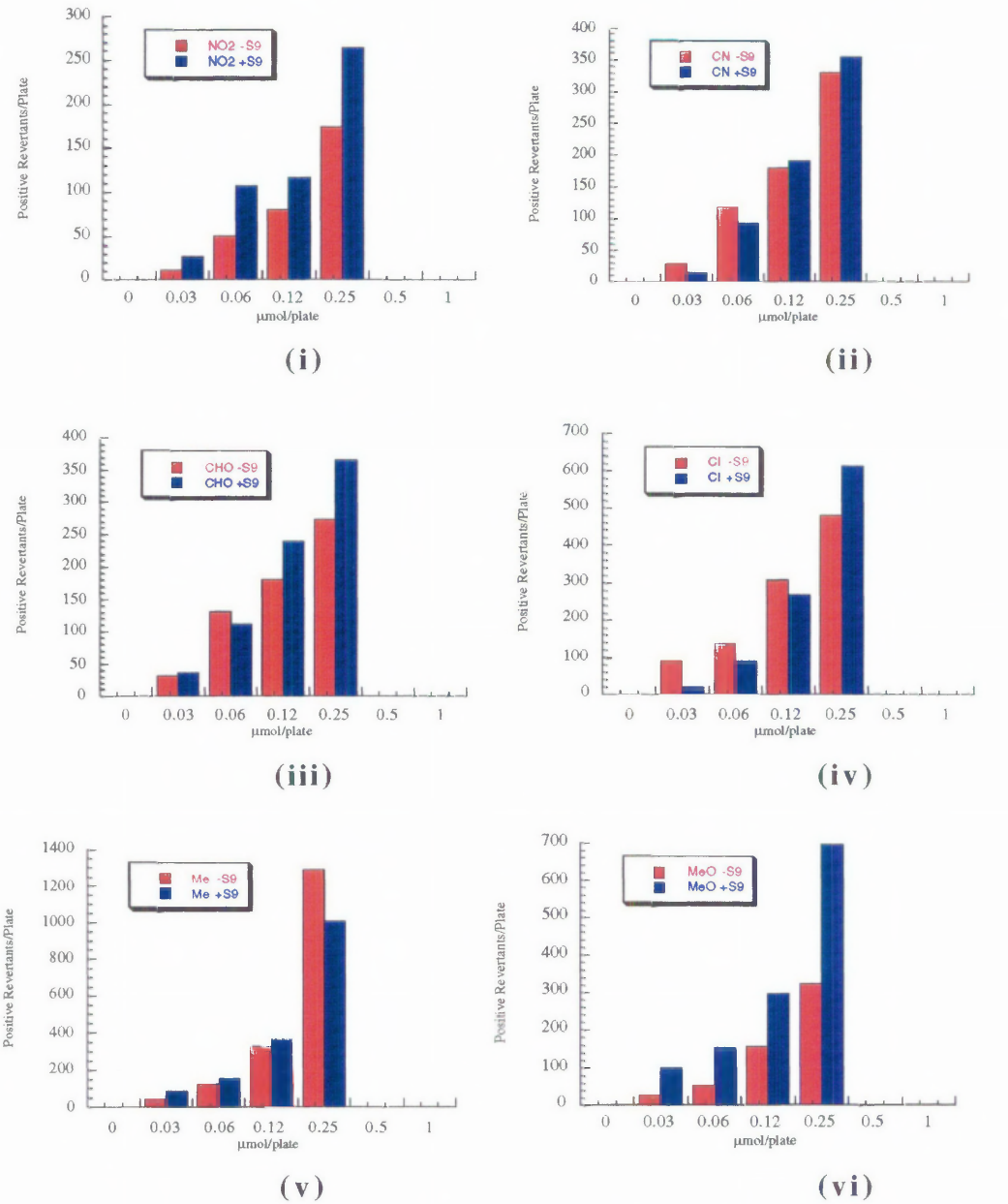


Figure 4-13 Positive dose-response data for **172b-e**, **172g** and **172h**

The data in Table 4-8 and Table 4-9 are the positive dose-responses for benzyl *N*-benzoyloxybenzohydroxamates **172a-e**, **172g** and **172h** in TA100 *salmonella typhimurium* after the background reversion rate has been subtracted. Each dose level was tested with three plates of bacteria and the average level and *population standard deviation errors* (σ_n) were calculated. Butyl *N*-acetoxybenzohydroxamate **100a** was tested together with the series and the linear dose-response region is described by: Slope = 474±119; Y Intercept = 13±68.412; and r = 0.9421. Dose-response plots for each substrate are given in Figure 4-13(i-vi).

The linear dose-response region for each compound (-S9) was calculated and normalised, with appropriate analysis of errors to give the normalised response relative to butyl *N*-acetoxybenzohydroxamate 100a (Table 4-10). The data is presented in column graph format in Figure 4-14.

Table 4-10 Normalised mutagenicities for **172a-e, 172g** and **172h**.

Substrate	Normalised Mutagenicity	Error
MeO	2.32	0.66
Me	7.03	1.90
H	6.84	1.77
Cl	4.51	1.27
CHO	2.76	0.85
CN	3.30	1.00
NO_2	1.52	0.42

† based on **100a** displaying 474 positive reversions/plate.

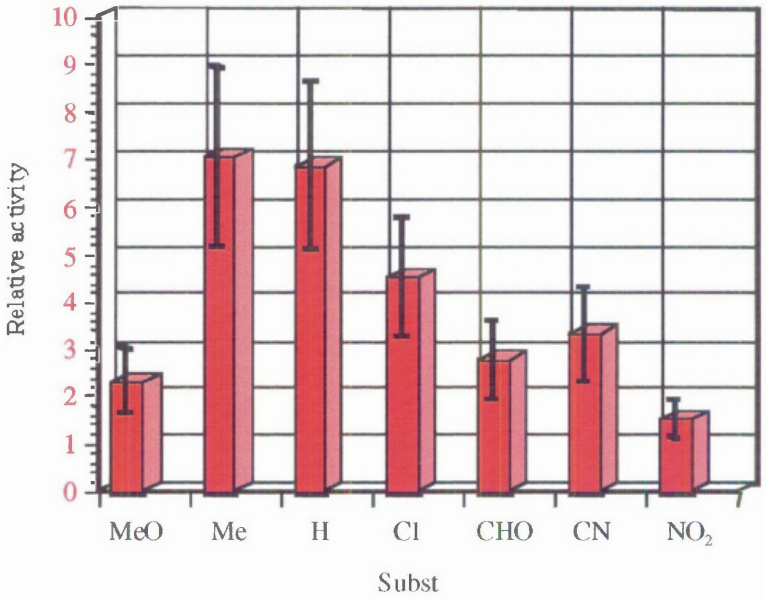


Figure 4-14 Relative mutagenicities of **172a-e, 172g** and **172h** at 1μmol/plate (100a=1)

Figure 4-14 illustrates that the most mutagenic compounds were the *para*-methyl **172c** and parent benzyl *N*-benzoyloxybenzohydroxamate **172a**, while the least mutagenic were the *p*-nitro **172h** and *p*-methoxy **172b** compounds respectively. Excluding the *p*-methoxy results, mutagenicity would appear to increase with electron-donor capacity of the *para* substituent.

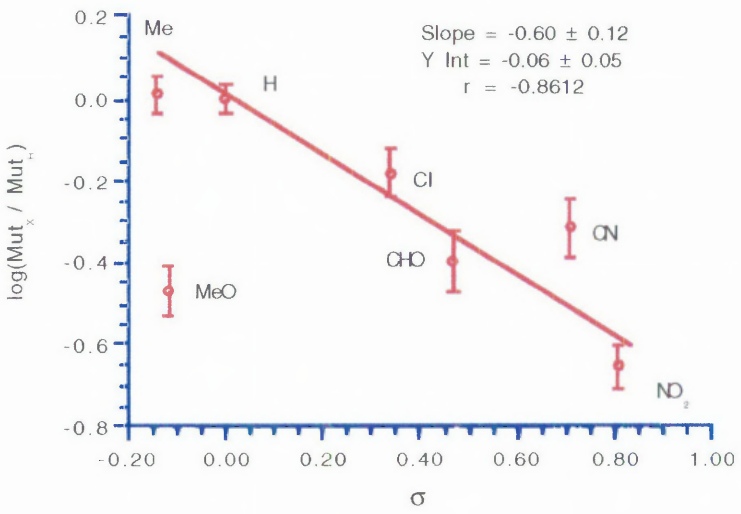


Figure 4-15 Hammett mutagenicity relationship

The quality of the data obtained for this series and the apparent trends in Figure 4-14 was encouraging and justified further qualitative analysis.

While this data is preliminary and should be treated with caution, without the methoxy substrate the relative mutagenicities appear to correlate with Hammett σ substituent constants (Figure 4-15) with a low but significant ρ value of -0.60±0.12 (r=0.86). It is noteworthy that both nitrenium ion formation in acid-catalysed solvolysis (ρ=+0.32), as well as S_N2 reactivity with base (ρ= +0.55) and aromatic amines[239] (ρ=+1.3) yield positive correlations in the same series of compounds. The activity therefore appears to be correlating with stability. An interpretation of the above might be that the lower the reactivity, the higher the probability that the mutagens survive to reach the target sites of DNA or other receptors.

4.5 Summary of mutagenicity effects

4.5.1 Hydrophobic effects

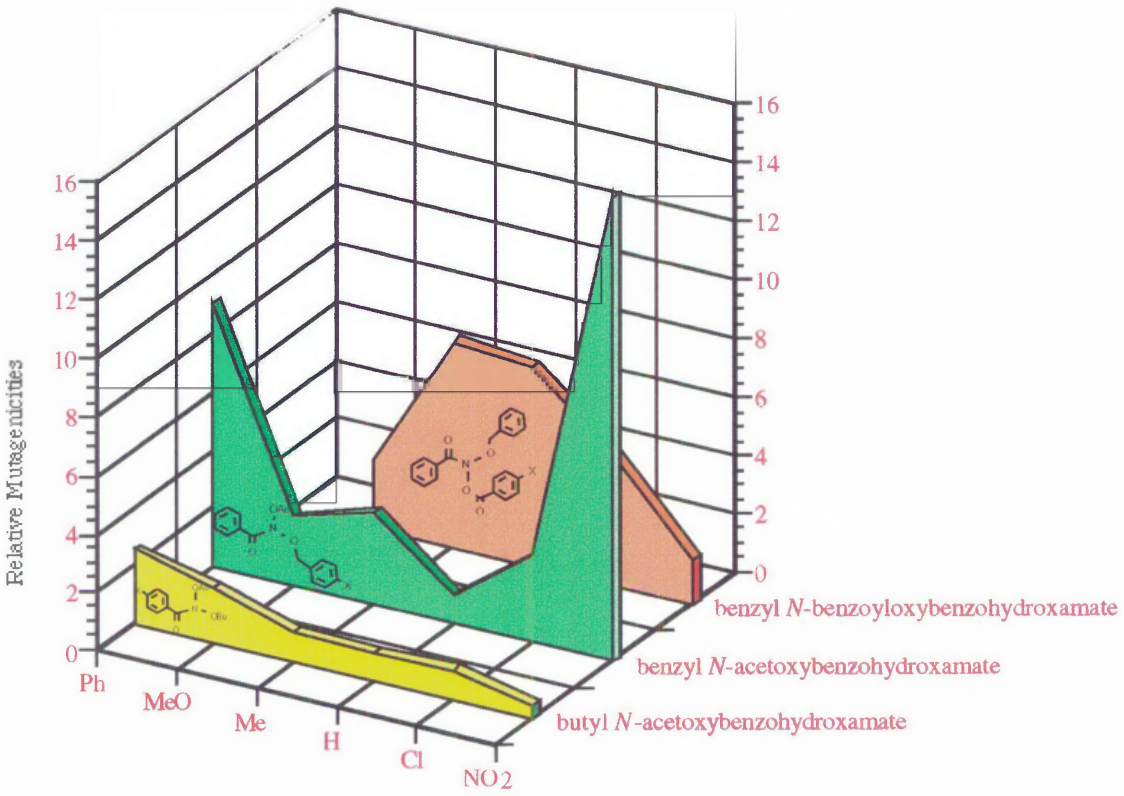


Figure 4-16 Normalised mutagenicity across the **100**, **151** and **172** benzohydroxamate series

While comparisons, when common *para* substituents are present, are difficult across the three benzohydroxamate series the average mutagenicity tended to increase from butyl *N*-acetoxybenzohydroxamates **100** through benzyl *N*-acetoxybenzohydroxamates **152** to benzyl *N*-benzoyloxybenzohydroxamates **172**. This can be seen in Figure 4-16 which compares the normalised mutagenicity (as area) with structure. Qualitatively, as the hydrophobicity increases with the number of phenyl rings, the area under the curve similarly increase.

In addition, in the two cases where *p*-phenyl substituents were tested, **100c** and **151d**, there is a significant increase in the mutagenicity when compared to the corresponding unsubstituted substrate, **100a** and **151a**, respectively. The biphenyl group would be expected to increase the hydrophobicity of the mutagen relative to phenyl.

4.5.2 Nitro-aromatics

The moderate mutagenicity of butyl *N*-acetoxy-*p*-nitrobenzohydroxamate **100h** is interesting in that there is a known alternative mechanism by which arylnitro groups can cause mutations.

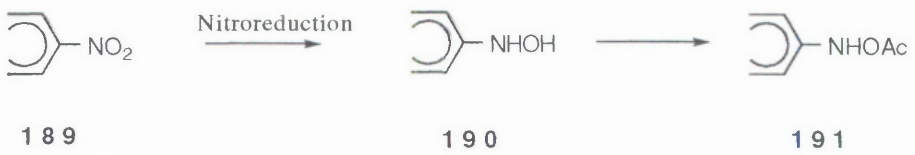


Figure 4-17 Metabolic activation of nitro-PAHs

Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) are among the most potent mutagens as determined by the Ames tests with *salmonella typhimurium*. The genotoxicity of nitro-PAHs has been extensively reported[248] and the accepted mode of metabolism involves reduction of the nitro group **189** to nitroso-, *N*-hydroxylamino-, and amino derivatives. Of these metabolites, the *N*-hydroxylamino intermediate **190** is believed to be responsible for the carcinogenicity and mutagenicity and can be further activated *via O*- **191** and *N*-acetylation (Figure 4-17). The active electrophilic metabolites from the reduction of nitro-PAHs are thus similar to those formed from the oxidation of aromatic amines.

The mutagenic activity of the nitro-PAH compounds is strongly influenced by several structural factors such as isomeric position of the nitro group, conformation of the nitro

group with respect to the plane of the aromatic ring, physical dimension of the aromatic rings and the ability to resonance stabilise the electrophilic metabolites.

The mutagenic activity of butyl *N*-acetoxy-*p*-nitrobenzohydroxamate **100h** was tested in TA100 *Salmonella typhimurium* which is a strain of bacteria that contains nitroreductase components that efficiently reduce nitro-PAHs.[249] Our results indicate that the nitro group is not reduced by the reductase in this strain as this compound displayed the least activity of the series. Nitroreductase activation would have greatly increased the mutagenic potency of this compound but this was not evident. The electron-withdrawing carbonyl moiety *para* to the nitro group may impede nitroreductase activity or, alternatively, reduce the electrophilicity of intermediates from metabolic modification.

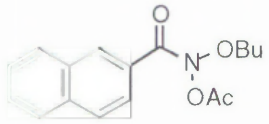
It is perhaps significant that the *p*-nitrobenzoyloxy substrate **172h** also exhibited modest mutagenicity relative to the rest of the series. Here too the nitro group is *para* to an electron deficient carbonyl. In the case of the benzyloxy series however, the *p*-nitrobenzyl *N*-acetoxybenzohydroxamate **151i** is extremely mutagenic when compared to the unsubstituted member of that series. Here, the *para*-nitro aromatic moiety is most probably far less deactivated by the oxymethylene *para* substituent. Further experiments with substrates bearing *meta*-nitro groups or nitro-reductase-free TA100 would assist in explaining these anomalous results.

4.5.3 Summary of mutagenicity data

Every member of this new class of alkyl *N*-acyloxybenzohydroxamates tested to date is mutagenic. There is no clear cut correlation with electronic effects of substituents when the benzoyl **100** and benzyloxy **151** series are considered.

In the case of the benzoyloxy series **172**, the mutagenicity can be regarded as correlating with the inverse of reactivity and hence correlating with stability. Recent results from these laboratories have consolidated this correlation.[250]

Several factors point to hydrophobicity as an activating factor. Mutagenicities generally increase across the series **100** through **151** to **172** (that is, with increasing aromatic content). Biphenyl substitution also markedly enhances activity relative to the corresponding phenylated substrate. Recent results also show that a butyl *N*-acetoxy-2-naphthohydroxamate **192** (5500 revertants at 1 μmol/plate), is more than ten times as active as **100a** (477 revertants at 1 μmol/plate).[204]



192

Figure 4-18

DNA damage studies[225] have recently indicated that alkyl *N*-acyloxybenzamides react at N-7 of guanine and, as such, must react in the major groove of DNA in which this, the most nucleophilic site, is exposed to electrophiles. Thus the observed increase in activity with hydrophobicity may be related to the increased capacity of these substrates to bind hydrophobically in the major groove of DNA.