The oxidative dealkylation of tertiary amides: mechanistic aspects

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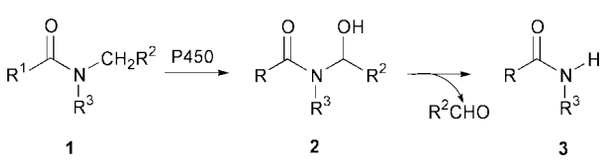
N-(But-3-enyl)-*N*-methylbenzamide **14a** undergoes microsomal oxidation by rat liver microsomes to yield both *N*-methyl- and *N*-(but-3-enyl)benzamides **18a** and **19**, the products of *N*-dealkylation. Cyclic products, that could be derived from a carbon-centred radical formed by hydrogen atom abstraction from the *N*-methyl group, were not observed. When generated independently, this carbon-centred radical underwent cyclisation, the 5-*exo-trig* mode being preferred to 6-*endo-trig* by a factor of 5. In contrast, *N*-(but-3-ynyl)-*N*-methylbenzamide **15** undergoes microsomal oxidation to yield the products of dealkylation **18a** and **23** and also *N*-benzoylpiperidone **24**. Dealkylation is preferred by factor of 3 and the piperidone accounts for *ca.* 45% of the reaction at the *N*-methyl group. Piperidone formation is consistent with the generation of a carbon-centred radical α- to the amide nitrogen atom during dealkylation and implies that cyclisation proceeds preferentially *via* the 6-*endo-dig* mode. Generated independently the radical undergoes cyclisation by both 5-*exo-dig* and 6-*endo-dig* modes, the former being favoured by a factor of 10. Similarly, *N*,*N*-dimethylacrylamide **26** and *N*-methyl-*N*-(3-pyridyl)acrylamide **27** undergo microsomal oxidation to form, *via* the 5-*endo-trig* cyclisation mode, 3-hydroxy-*N*-methyl-2-pyrrolidone **33** and 3-hydroxycotinine † **34**, respectively, confirming the intermediacy of a carbon-centred radical in the dealkylation process.

Attempts to trap *N*-acyliminium ions during microsomal dealkylation failed. Thus, although *N*,*N*-dimethylaniline **35** reacts in the presence of NaCN to form *N*-cyanomethyl-*N*-methylaniline **37** (Nu=CN), *N*,*N*-dimethylbenzamide undergoes dealkylation without forming *N*-cyanomethyl-*N*-methylbenzamide. Similarly, microsomal reaction of *N*,*N*-dimethylaniline in the presence of $NaBD_4$ gives rise to multiple incorporation of deuterium atoms into the methyl groups of the starting material, whereas *N*,*N*-dimethylbenzamide undergoes dealkylation but with no such deuterium incorporation into the starting material. Further, microsomal oxidation of *N*,*N*-dimethylsalicylamide **38** yields *N*-methylsalicylamide **40** with no evidence for the formation of *N*-methyl-2,3-dihydro-4*H*-1,3-benzoxazin-4-one **39**, the potential product of intramolecular cyclisation of the phenolic oxygen atom onto the putative *N*-aroylmethylene iminium ion.

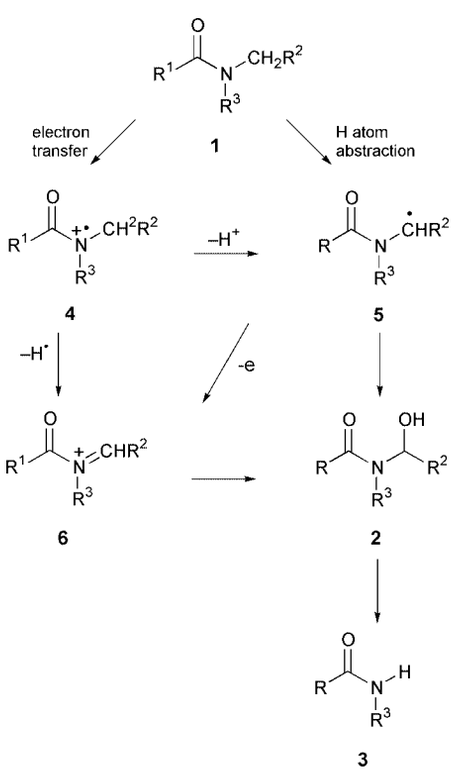
The oxidative dealkylation of tertiary amides **1** (Scheme 1), *e.g.*



1 **2** **3**

Scheme 1 The oxidative dealkylation of amides.

N,*N*-diethyl-3-toluamide (DET),[1] *N*,*N*-dimethylformamide (DMF),[2] *N*-methylpyrrolidone (NMP)[3] and cotinine,[4] by the cytochrome P450 enzyme system is of toxicological and mechanistic interest. *N*-Dealkylation to the corresponding secondary amide **3** is known to involve the formation of a *N*-hydroxymethyl amide **2** (Scheme 1)[3,5,6] but the mechanism by which this is formed from the parent tertiary amide remains uncertain. By analogy with tertiary amines,[7] *N*-hydroxymethyl amide formation could involve formation of the cation radical, **4**, the carbon-centred radical, **5**, or the iminium ion, **6** (Scheme 2). For tertiary amines, the mechanism has been generally considered to involve electron abstraction and formation of the cation radical **4**.[7–11] However, on the basis of kinetic deuterium isotope effect profiles over a number of compounds, the alternative pathway involving hydrogen atom abstraction to form **5** directly has been advanced.[12] Amides are less electron rich, and a mech-



1 **4** **5** **6** **2** **3**

Scheme 2

† The IUPAC name for 3-hydroxycotinine is 3-hydroxy-1-methyl-5-(3-pyridyl)-2-pyrrolidone.

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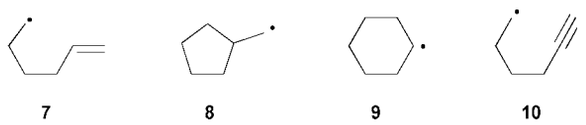
anism involving hydrogen atom abstraction to form the carbon-centred radical **5** has been implicated by substituent electronic effects and large intramolecular kinetic deuterium isotope effects (KDIEs).[6,13,14] However, the use of large KDIEs as evidence for such a pathway is questionable given the observation of values as large as 22 for *proton* loss from an aminium cation radical similar to **4**.[15,16] Moreover, our previous attempts to intercept a carbon-centred radical intermediate by the use of *N*-cyclopropylmethylamide substrates were unsuccessful.[17] Consequently, we have sought alternative evidence for the intermediacy of radical structures such as **5** and herein report the results of investigations involving substrates that have the potential to intercept such species by functioning as intramolecular traps. We also report our attempts to intercept the iminium ion intermediate **6**. Throughout our study we have used phenobarbital-induced rat liver microsomes. Phenobarbital induces isozymes (primarily P450 2B1) that catalyse amide dealkylation, and, for KDIEs at least, phenobarbital-induced rat liver microsomes give essentially the same results as purified P450 2B1.[13]

Results and discussion

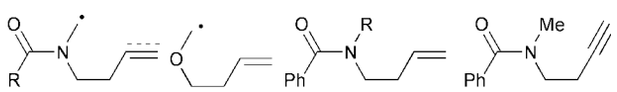
Trapping of carbon-centred radicals

The intramolecular interception of carbon-centred radicals by alkene and alkyne unsaturated systems is well-known and is commonly used to assemble cyclic molecules.[18] In this context, probably the most extensively studied system is the hex-5-enyl radical **7** which undergoes a 5-*exo*-*trig* rearrangement to the cyclopentylmethyl radical **8** with a rate constant of 2.3×10^5 s^{-1} at 25 °C.[19] The 5-*exo* cyclisation mode is preferred to the 6-*endo* alternative, which gives the cyclohexyl radical **9**, by a factor of *ca.* 50.[20] Similarly, the hex-5-ynyl radical **10** undergoes an analogous ring closure selectively *via* the 5-*exo* mode, though at a rate *ca*. 10 times slower than **7**.[21] By appropriate positioning



7 **8** **9** **10**

of the nitrogen atom, these cyclisations can be adapted to accommodate the putative radical, *i.e.* **5**, involved in amide dealkylation; this analysis leads to radicals **11** and **12**. Certainly,



11 alkene
12 alkyne

13

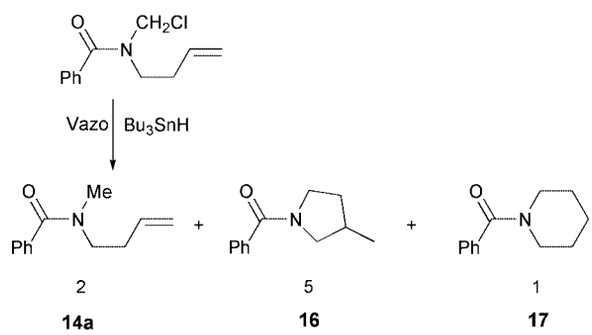
14 a R = Me
b R = CH_2Ph

15

radicals of general structure **11/12** have found use in synthesis, especially of fused pyrrolidine and piperidine systems.[22] In contrast to the hex-5-enyl system, the α-acylamino radical **11** cyclises by both 5-*exo* and 6-*endo* modes in a ratio of 2:1 and the corresponding radical **12** cyclises exclusively by the 6-*endo* mode. This change in selectivity has been attributed to a bond angle widening brought about by the incorporation of an sp^2 hybridised nitrogen atom into the chain containing the radical centre and the unsaturated system.[23] We are unaware of any previous measurements of the rate constants for cyclisation of radicals such as **11** or **12**. However, the related radical **13** derived from an ether cyclises about 5 times more slowly than **7**.[24] Although **13** is stabilised by *ca*. 20 kJ mol^{-1} with respect to a primary alkyl radical, and amines are stabilised by a further 15 kJ mol^{-1},[25] radical reactivity is affected more by stereoelectronic effects in the transition state than by stability.[26] Thus, the α-acylamino radical **11** might be expected to cyclise at a similar rate to **13**. Consequently, we employed *N*-(but-3-enyl)-*N*-methylbenzamide **14a**, *N*-(but-3-enyl)-*N*-benzylbenzamide **14b** and *N*-(but-3-ynyl)-*N*-methylbenzamide **15** as substrates to probe for oxidative dealkylation.

***N*-(But-3-enyl)-*N*-methylbenzamide 14a and *N*-benzyl-*N*-(but-3-enyl)benzamide 14b.** The AM1 SCF MO calculated heats of formation, ΔH_f, of **14a** and the potential α-acylamino radical intermediates derived from it, are contained in Table 1. The differences in ΔH_f between the parent amide and the methyl derived radical (103 kJ mol^{-1}) and the alkyl derived radical (100 kJ mol^{-1}) are very similar to those calculated previously for other *N*,*N*-dimethyl- (98–103 kJ mol^{-1}) and *N*-alkyl-*N*-methylbenzamides (81–92 kJ mol^{-1}).[17] The data reveal that, on thermodynamic grounds, hydrogen-atom abstraction from the *N*-alkyl group is slightly favoured over hydrogen-atom abstraction from the *N*-methyl group. Moreover, again on thermodynamic grounds, formation of cyclic products from the methyl-derived radical is significantly favoured with, as expected, the piperidin-4-yl radical being more stable than its pyrrolidin-3-ylmethyl counterpart.

Prior to investigating the oxidative dealkylation of **14a**, we studied the efficiency of the *N*-(but-3-enyl) group as a trap for the *N*-methylene carbon-centred radical **11** (R=Ph). This radical was generated from the reaction of *N*-(but-3-enyl)-*N*-chloromethylbenzamide with Bu_3SnH in the presence of 1,1′-azobis(cyclohexanecarbonitrile) (Vazo). GCMS analysis of this reaction identified three products; *N*-benzoyl-3-methylpyrrolidine **16** (*via* 5-*exo* cyclisation), *N*-benzoylpiperidine **17** (*via* 6-*endo* cyclisation) and *N*-(but-3-enyl)-*N*-methylbenzamide **14a** (trapping of the methylene radical by Bu_3SnH) (Scheme 3).



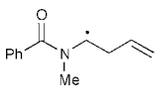
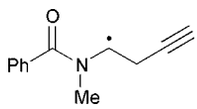
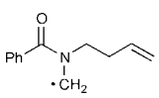
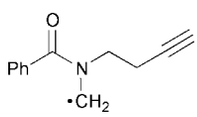
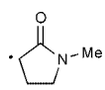
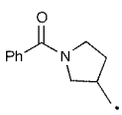
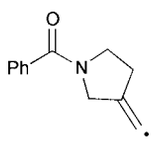
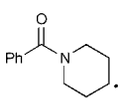
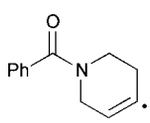
2 **5** **1**
14a **16** **17**

Scheme 3

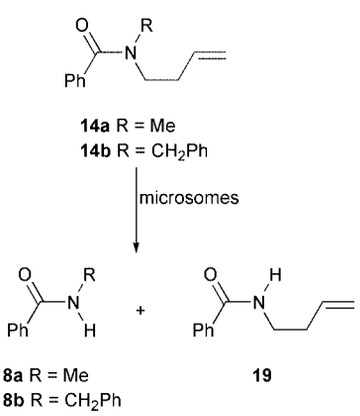
These products are formed in the ratio 5:1:2; thus, formation of cyclic products is favoured by 3:1 and 5-membered ring formation is favoured over 6-membered ring formation by 5:1. Interestingly, the ratio of *exo* to *endo* closure observed here for the formation of a monocyclic system appears to be larger than those obtained for formation of fused ring systems.[22] Presumably for these fused systems the development of ring strain as the transition state forms contributes to a greater proportion of *endo* cyclisation. If we assume that the rate constant for the reaction of the radical **11** (R=Ph) with Bu_3SnH is approximately that for a primary alkyl radical, *i.e.* 6.6×10^6 s^{-1} at 80 °C,[19] then the rate constants for the 5-*exo* and 6-*endo* modes of cyclisation for **11** (R=Ph) are *ca.* 10^7 and 2×10^6 s^{-1}, respectively. This would imply that, at 80 °C, **11** (R=Ph) cyclises *via* the 5-*exo* mode *ca.* 6 times faster than the hex-5-enyl radical. However, given that the isostructural radical **13** cyclises 5 times more slowly than the hex-5-enyl radical, it is clear that the assumption that **11** behaves like a primary alkyl radical in its reaction with Bu_3SnH is probably not correct, especially as the stabilised benzyl radical reacts about 50 times more slowly.[19] Clearly there is a need for an accurate determination of the rate constants for the reaction of α-aminoacyl radicals **11** with Bu_3SnH.

Microsomal incubation of *N*-(but-3-enyl)-*N*-methylbenzamide **14a** afforded *N*-methylbenzamide **18a** (from dealkyl-

Table 1 Heats of formation, ΔH_f, calculated by the AM1 method for the amides **14**, **15**, **26** and their derived radicals

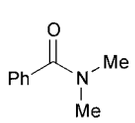
Compound or radical	ΔH_f/ kJ mol^{-1}	Compound or radical	ΔH_f/ kJ mol^{-1}	Compound or radical	ΔH_f/ kJ mol^{-1}
14a	30	**15**	187	**26**	−58.6
	130		276		39.5
	133		291		30.3
	85		250		
	75		243		

ation) and *N*-(but-3-enyl)benzamide **19** (from demethylation) in a ratio of 2:1 (total metabolism 15%) (Scheme 4). No evidence



Scheme 4

for any cyclised product was found. An initial rate study of the demethylation and dealkylation reactions gave rise to the kinetic parameters in Table 2. Comparison of the V_{max} values‡ *per* metabolisable hydrogen atom with those for *N*,*N*-dimethylbenzamide **20** demonstrates that oxidative demethylation of



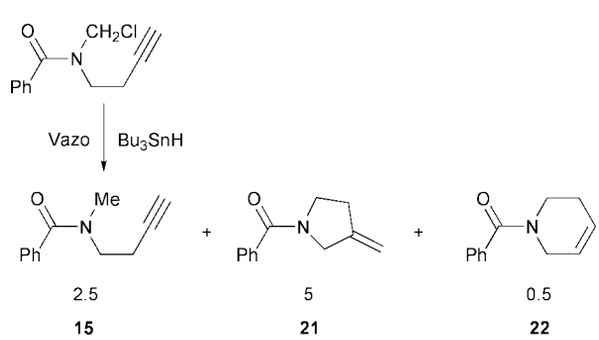
20

14a proceeds normally. Moreover, the V_{max} value *per* metabolisable hydrogen atom for dealkylation is that expected for a simple alkyl group.[17] Thus, the absence of intramolecular trapping of radical **11** (R=Ph) is not due to poor metabolism of the substrate.

As *N*-benzyl-*N*-methylbenzamide is known to undergo metabolism of the *N*-benzyl group approximately 5 times more readily than the methyl group[27] we decided to examine the dealkylation of *N*-benzyl-*N*-(but-3-enyl)benzamide **14b**. In this case, prior chemical cyclisation using the Bu_3SnH method was not possible because we were unable to synthesise the appropriate *N*-chloroalkyl substrate. Microsomal dealkylation of this substrate (total metabolism 35%) gave rise to *N*-benzylbenzamide **18b** and *N*-(but-3-enyl)benzamide **19**, the products of *N*-debenzylation and *N*-dealkylation respectively, in a relative ratio of 3:1 (Scheme 4). Again, cyclic products were not observed. The kinetic parameters for the two reactions are contained in Table 2 and these show the compound to be behaving normally. Thus, if the carbon-centred radical intermediate exists, we conclude that the *N*-alkenyl system is an unsuitable trap for it.

***N*-(But-3-ynyl)-*N*-methylbenzamide 15.** The heats of formation, ΔH_f, calculated by the AM1 method for **15** and its derived α-acylamino radicals are given in Table 1. As for **14a** these indicate that the thermodynamically favoured radical is that derived from the alkyl rather than the methyl group. However, cyclisation of the methyl derived radical **12** is highly favoured, the 1,2,5,6-tetrahydropyridin-4-yl radical being the more stable.

The radical **12** was generated from *N*-(but-3-ynyl)-*N*-chloromethylbenzamide and Bu_3SnH. GCMS analysis identified three products; *N*-benzoyl-3-methylenepyrrolidine **21** (5-*exo* cyclisation), *N*-benzoyl-1,2,5,6-tetrahydropyridine **22** (6-*endo* cyclisation) and *N*-(but-3-ynyl)-*N*-methylbenzamide **15** (trapping of the methylene radical by Bu_3SnH) (Scheme 5). These products



Scheme 5

were formed in the ratio 5.0:0.5:2.5; thus, formation of cyclic products is favoured by 2:1, and 5-*exo* cyclisation is preferred to 6-*endo* cyclisation by 10:1. We can use these ratios, together

‡ V_{max} is derived from the Michaelis–Menten equation $v_i = V_{max}[S]/(K_M + [S])$, where v_i is the initial rate of reaction and [S] the initial substrate concentration.

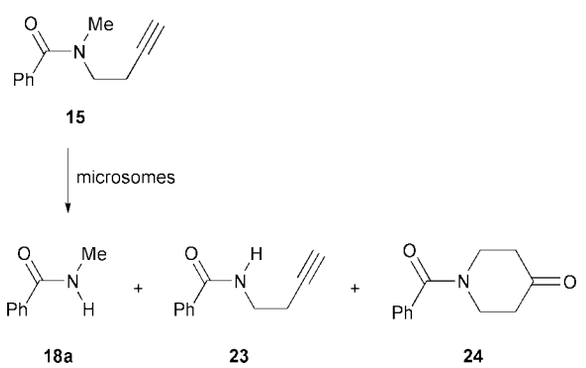
Table 2 Kinetic constants for the microsomal dealkylation of some tertiary amides[a]

Compound	Demethylation		Dealkylation	
	V_{max}/mM h^{-1} (nmol P450)$^{-1}$	(V_{max}/K_M)/h^{-1} (nmol P450)$^{-1}$	V_{max}/mM h^{-1} (nmol P450)$^{-1}$	(V_{max}/K_M)/h^{-1} (nmol P450)$^{-1}$
20	3.10 ± 0.12 (0.52)	1.50 ± 0.17		
	3.07 ± 0.11 (0.51)[b]	1.50 ± 0.20[b]		
	3.08 ± 0.13 (0.51)[c]	1.48 ± 0.13[c]		
14a	1.73 ± 0.09 (0.58)	3.81 ± 0.44	1.16 ± 0.06 (0.58)	1.45 ± 0.15
14b			2.50 ± 0.11 (1.25)[d]	3.10 ± 0.15[d]
			1.00 ± 0.05 (0.50)[e]	1.11 ± 0.10[e]
15	1.20 ± 0.08[f]	0.70 ± 0.08[f]	1.10 ± 0.06 (0.55)	1.22 ± 0.12
	0.75 ± 0.03[g] (0.65)[h]	0.60 ± 0.05[g]		
26	1.07 ± 0.08 (0.18)	0.58 ± 0.08		
27			2.05 ± 0.12 (1.03)	1.51 ± 0.15
38	2.57 ± 0.09 (0.43)	0.63 ± 0.07		

[a] Values in parentheses are *per* H atom. [b] In the presence of 1 mM NaCN. [c] In the presence of 5 mM $NaBD_4$. [d] Loss of benzyl group. [e] Loss of but-3-enyl group. [f] Loss of methyl group. [g] Cyclisation to **24**. [h] Combined value for demethylation and cyclisation.

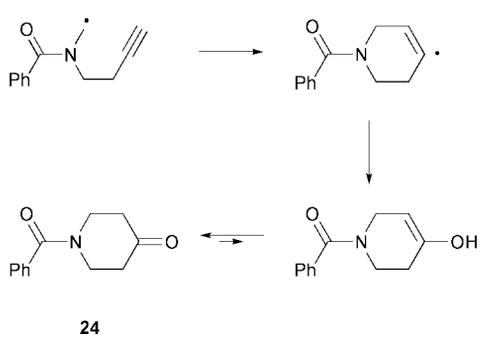
with the rate constant for reaction of a primary alkyl radical with Bu_3SnH (noting the caveat above) and the concentration of Bu_3SnH (1.67 M) to estimate the rates of the 5-*exo* and 6-*endo* cyclisations as 8×10^6 and 8×10^5 s^{-1}, respectively.

Microsomal dealkylation of *N*-(but-3-ynyl)-*N*-methylbenzamide **15** gave rise to three products (18%) in a ratio of 2 : 1 : 1, identified by comparison with synthetic standards as *N*-methylbenzamide **18a**, *N*-(but-3-ynyl)benzamide **23** and *N*-benzoylpiperidin-4-one **24**, respectively (Scheme 6). The formation of



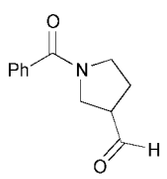
Scheme 6

24 provides the first clear evidence for the formation of α-aminoacyl radicals **5** in the dealkylation of amides by cytochrome P450 (Scheme 7). Even so, the formation of the latter

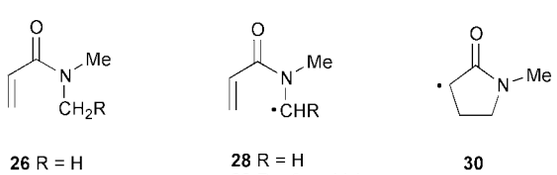


Scheme 7

compound is interesting on two counts. First, no evidence for the formation of the product of the kinetically preferred 5-*exo* mode of cyclisation, *N*-benzoyl-3-formylpyrrolidine **25**, was obtained. Second, the estimated rate of "oxygen rebound", that is, the rate at which the haem-hydroxy unit transfers the hydroxy group to a carbon-centred radical, is *ca.* 10^{10}–10^{11} s^{-1}.[28,29] This is far in excess of our crude estimates of the radical cyclisation rates. To accommodate both observations we conclude that the enzyme must exert a constraining influence on the radical in the active site. Such an effect has been reported previously for the 2,2,4,4-tetramethylcyclopropylmethyl radical which is known to undergo ring opening with a rate constant of 5×10^9 s^{-1}, yet the parent hydrocarbon underwent P450 oxidation without rearrangement.[29] An additional factor could be that a radical such as **12** is significantly stabilised by the adjacent nitrogen atom such that it behaves like a secondary alkyl radical. Secondary alkyl radicals are generally less reactive than their primary counterparts,[19] and the stabilisation afforded by the adjacent nitrogen atom is much greater than that afforded by a corresponding alkyl group.[25] An initial rate study of the demethylation, dealkylation and cyclisation reactions gave the kinetic parameters in Table 2. These reveal that hydrogen atom abstraction from the substrate partitions normally between the demethylation and dealkylation channels, and that cyclisation of **12** (R=Ph) accounts for *ca.* 40–45% of reaction at the methyl group.



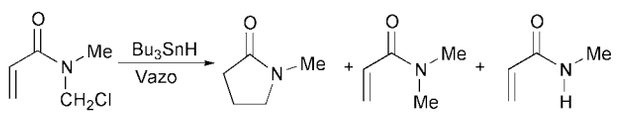
***N*,*N*-Dimethylacrylamide 26 and *N*-methyl-*N*-(3-pyridylmethyl)acrylamide 27.** An alternative approach for trapping intramolecularly a carbon-centred radical such as **5** is to position the trapping group within the acyl rather than the *N*-alkyl fragment. This led us to examine the acrylamides **26** and **27**. As



well as following the normal mode of dealkylation, the corresponding radicals **28** and **29** could well cyclise in a 5-*endo-trig* mode to form pyrrolidin-2-ones. Although 5-*endo-trig* cyclisation is uncommon, it has been used for the assembly of pyrrolidin-2-ones by linking an electrophilic α-acyl radical to an electron-rich *N*-vinylamide.[30] In such cases, 5-*endo* cyclisation leads to the thermodynamic product, the 4-*exo* mode being kinetically favoured but reversible.[31] Cyclisation of **28** and **29** can be thought of as involving an electron-rich radical with an electron-deficient alkene. The heats of formation, ΔH_f, for

the radical **28**, its cyclic counterpart **30** and for *N*,*N*-dimethylacrylamide **26** calculated by the AM1 method are contained in Table 1. The difference, 98.1 kJ mol^{-1}, between the ΔH_f values for **26** and the radical **28** is of similar magnitude to those for other *N*-methylamides[6,17] and to those for the other amides in Table 1. Significantly, the cyclic radical **30** is 9.2 kJ mol^{-1} thermodynamically more stable over its acyclic counterpart.

Prior to investigating the microsomal metabolism of **26**, we studied the ability of the acryloyl system for trapping the carbon-centred radical **28**. This radical was generated from *N*-chloromethyl-*N*-methylacrylamide by reaction with Bu_3SnH in the presence of Vazo. GCMS analysis of the product mixture revealed three products (Scheme 8); *N*,*N*-dimethylacrylamide

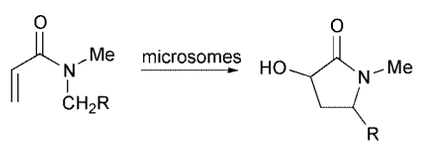


31 **26** **32**

Scheme 8

26, *N*-methyl-2-pyrrolidone **31**, and *N*-methylacrylamide **32**. We are uncertain of the origin of the latter compound but it may well have been carried through from the synthesis of the *N*-chloromethylamide. Quantification of **26** and **31** indicated that the pyrrolidone product is favoured by 4 to 1.

Microsomal incubation of *N*,*N*-dimethylacrylamide **26** gave rise to a single new product (20% conversion) that was identified by comparison with an authentic standard as *N*-methyl-3-hydroxy-2-pyrrolidone **33** (Scheme 9). Significantly,



26 R = H
27 R = 3-pyridyl

33 R = H
34 R = 3-pyridyl

Scheme 9

no *N*-methylacrylamide, the expected product of *N*-demethylation, was found. The initial rates for the formation of **33** from **26** followed typical Michaelis–Menten kinetics, from which the V_{max} and V_{max}/K_M values in Table 2 were obtained. These are unremarkable, but reveal a rate *ca*. one-third of that for *N*,*N*-dimethylbenzamide, which probably reflects the greater hydrophilicity of the acrylamide.[17] Microsomal incubation of the corresponding *N*-methyl-*N*-pyridylmethylacrylamide **27** also gave rise to only one product (30% conversion), identified by comparison with a synthetic standard as the well-known nicotine metabolite *trans*-3-hydroxycotinine **34**[32] (Scheme 9). The formation of the *trans* diastereomer may imply stereocontrol of product formation by the enzyme, but we did not examine this issue further. Though hydrogen atom loss could occur from either α-carbon atom, abstraction from the NCH_2Ar system is clearly preferred. Michaelis–Menten treatment of the initial rates for this transformation yields the V_{max} and V_{max}/K_M values given in Table 2. Comparison of these values with those for **26** indicate that **27** is metabolised 2–3 times faster, as would be expected for a substrate that is more lipophilic and which also contains a benzylic system.

Attempts to trap an iminium ion

Next we examined the possible intermediacy of the iminium ion **6**. Our first approach was to attempt intermolecular trapping using either NaCN or $NaBD_4$, our second involved intramolecular trapping using a phenol/phenoxide ion.

Attempted trapping by NaCN. Cyanide ion is known to trap iminium ions generated from tertiary amines.[33–35] Prior to

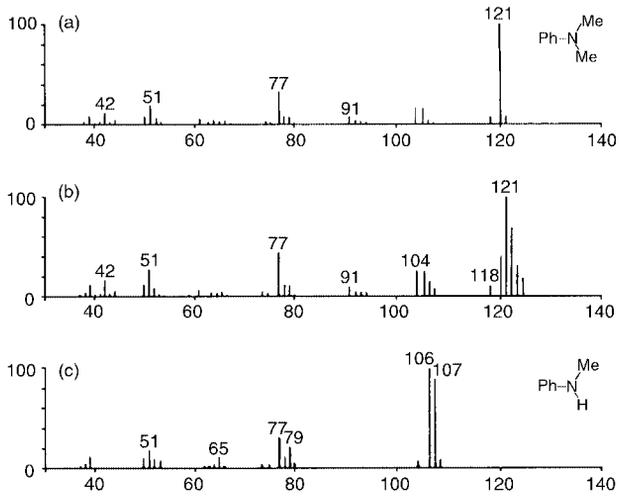
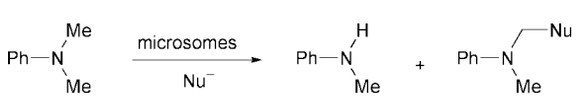


Fig. 1 GCMS of an extract from the microsomal uncubation of 10 mM *N*,*N*-dimethylaniline in the presence of 5 mM $NaBD_4$: (a) *N*,*N*-dimethylaniline standard, (b) *N*,*N*-dimethylaniline recovered from the incubation, (c) *N*-methylaniline recovered from the incubation.

examining *N*,*N*-dimethylbenzamide, oxidation of *N*,*N*-dimethylaniline **35**, a substrate reported to be metabolised *via* an iminium ion,[36] was performed in the presence of NaCN. As CN^- is a well-known P450 inhibitor, incubations were optimised using *N*,*N*-dimethylaniline and NaCN concentrations of 10 and 1 mM, respectively, at which concentration CN^- does not appreciably inhibit the P450-dependent metabolism. GCMS analysis revealed *N*-methylaniline **36** and *N*-cyanomethyl-*N*-methylaniline **37** (Scheme 10) as the products in a



35 **36** **37** Nu = CN or D

Scheme 10

relative ratio of 1 : 1 (40% conversion). Clearly sodium cyanide is an efficient trap for the iminium ion species. Moreover, in the presence of CN^- the values of V_{max} and V_{max}/K_M, (2.68 ± 0.05) mM h^{-1} (nmol P450)$^{-1}$ and (2.51 ± 0.13) h^{-1} (nmol P450)$^{-1}$, respectively, are almost identical to those, (2.70 ± 0.06) mM h^{-1} (nmol P450)$^{-1}$ and (2.81 ± 0.17) h^{-1} (nmol P450)$^{-1}$, respectively, obtained in its absence.

Using an identical experimental protocol, microsomal incubation of *N*,*N*-dimethylbenzamide **20** afforded *N*-methylbenzamide **18a** as the only product; no evidence for the formation of *N*-cyanomethyl-*N*-methylbenzamide was obtained under any experimental conditions, even though this compound was found to be stable to the conditions of both the experiment and the analytical system. Confirmation that NaCN does not interfere with substrate metabolism, and therefore with possible iminium ion formation, was obtained from a kinetic investigation (Table 2) for which the V_{max} and V_{max}/K_M values were identical to those in the absence of NaCN.

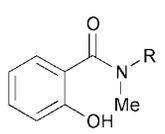
Attempted trapping by sodium borodeuteride. Using *N*,*N*-dimethylaniline **35** (0.5 and 10 mM) as substrate, microsomal incubation in the presence of $NaBD_4$ (1 to 10 mM) gave rise to GCMS traces like that in Fig. 1. These reveal the presence of the substrate and of *N*-methylaniline **36**, the product of *N*-demethylation. The major peak in the mass spectrum of *N*,*N*-dimethylaniline itself is the (M − 1) peak at *m*/*z* 120; therefore, the *m*/*z* 121 peak in the spectrum of *N*,*N*-dimethylaniline recovered from microsomal metabolism can be assigned to the incorporation of one D atom **37** (Nu = D) (Scheme 10). Moreover, the presence of peaks at *m*/*z* 122, 123 and 124 thus

signifies the incorporation of two, three and four deuterium atoms, respectively. This result is good evidence for the formation of an iminium ion intermediate that is subsequently reduced to deuterated starting material by $NaBD_4$. The presence of (M + 2) to (M + 4) species implies that the so-formed deuterated *N*,*N*-dimethylanilines undergo further P450 oxidation.

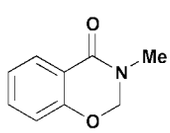
Microsomal incubation of *N*,*N*-dimethylbenzamide **20**, performed in the presence of $NaBD_4$ under identical conditions to those used for *N*,*N*-dimethylaniline, gave *N*-methylbenzamide **18a** and *N*,*N*-dimethylbenzamide. Comparison of the mass spectrum of the *N*,*N*-dimethylbenzamide recovered from the incubation with that of a synthetic standard revealed no deuterium incorporation. To eliminate the possibility that $NaBD_4$ might inhibit microsomal dealkylation of the amide substrate, we analysed the metabolism of both *N*,*N*-dimethylaniline and *N*,*N*-dimethylbenzamide quantitatively by the initial rate method in the presence of 5 mM $NaBD_4$. For *N*,*N*-dimethylaniline, Michaelis–Menten analysis of the data gave rise to V_{max} and V_{max}/K_M values of (2.70 ± 0.11) mM h^{-1} (nmol P450)$^{-1}$ and (2.81 ± 0.29) h^{-1} (nmol P450)$^{-1}$ in the absence of $NaBD_4$ and (2.20 ± 0.09) mM h^{-1} (nmol P450)$^{-1}$ and (1.91 ± 0.21) h^{-1} (nmol P450)$^{-1}$ in its presence. The data for *N*,*N*-dimethylbenzamide are contained in Table 2. These data indicate that metabolism of *N*,*N*-dimethylbenzamide is unaffected by the presence of $NaBD_4$, whereas the metabolism of *N*,*N*-dimethylaniline is somewhat inhibited by the presence of the BD_4^- ion.

These results from the attempts to trap an iminium ion with NaCN and $NaBD_4$ can be interpreted either as indicating that microsomal metabolism of tertiary amides does not occur *via* iminium ion formation or that intermolecular trapping of such an intermediate is not efficient. The latter seems unlikely, given the effective trapping of iminium ion intermediates during the metabolism of *N*,*N*-dimethylaniline. Nevertheless, we attempted to intercept the putative iminium ion using an intramolecular trap.

***N*,*N*-Dimethyl-2-hydroxybenzamide 38.** *N*,*N*-Dimethyl-2-hydroxybenzamide **38** is a substrate that has the potential to



38 R = Me
40 R = H



39

trap, intramolecularly, an iminium ion intermediate to form the cyclic product, 3-methyl-4-oxo-2,3-dihydro-4*H*-1,3-benzoxazine **39**. Indeed, at pH 7.4 it is possible to estimate that approximately 30% of the substrate exists as the phenoxide ion.[37] However, microsomal oxidation of **38** gives rise to the formation of one new product (14% conversion) that was identified by GCMS comparison with a synthetic standard as *N*-methyl-2-hydroxybenzamide **40**, the product of simple *N*-demethylation. The cyclic product **39** was not observed even though we verified independently that it was possible to isolate and identify a synthetic standard of **39** when subjected to the same experimental and analytical conditions. Indeed, we also employed HPLC analysis of the incubations of **38** and these confirmed that no cyclisation product was formed. Values of V_{max} and V_{max}/K_M for the *N*-demethylation of **38** are contained in Table 2. Comparison of these values with those for *N*,*N*-dimethylbenzamide confirms that increased substrate polarity results in a decreased value of V_{max}/K_M[17] almost entirely due to a two-fold increase in K_M.

These observations appear to be consistent with the P450-catalysed dealkylation of tertiary amides proceeding *via* an intermediate such as **5** (Scheme 2) rather than the iminium ion **6** (though our data do not definitively rule out such an intermediate). Moreover, a comparison of the kinetic deuterium isotope effects between the P450-catalysed and electrochemical *N*-dealkylation of amides appears to rule out the cation radical **4** as an intermediate on the dealkylation pathway.[13,38] Thus, the most likely pathway for *N*-dealkylation of amides appears to be **1** ⟶ **5** ⟶ **2** ⟶ **3**. Until very recently, the general understanding of P450 catalysed oxidations of C–H bonds, of which amide dealkylation is an example, has been that the active oxidant is an electrophilic haem iron(V)–oxo species that can abstract either an electron or a hydrogen atom from the substrate.[39] If this is correct, then formation of the radical **5** could be ascribed to hydrogen atom abstraction from the amide substrates. However, the recent work of Newcomb *et al.*[40] on hydrocarbon hydroxylation implies that neither discrete radical nor carbocationic intermediates are formed in P450 oxidations. Rather, oxidation is brought about by both iron–oxo and iron–hydroperoxo species, both of which involve insertion reactions ("O" for the former, "OH^+" for the latter). If the latter mechanism is operative in the present study, then the formation of cyclic products from acyclic substrates would seem to require strained bicyclic bridged structures. Clearly there is a need to study amide dealkylation further using substrates that incorporate the hypersensitive radical probe structures designed by these workers.

Experimental

Instrumentation

NMR spectra were recorded using JEOL FX 90Q and JNM-EX400 FT spectrometers. Chemical shifts, δ, are given in ppm and coupling constants, *J*, in Hz. UV/vis and IR spectra were recorded using Kontron Uvikon 810P and Nicolet 205 FTIR spectrophotometers, respectively. HPLC was carried out using a Varian 5000 instrument equipped with a Jones chromatography 25 cm × 5 mm C18 analytical column, a Cecil 2112 variable wavelength detector and a Waters integrator. Diode array HPLC was performed using a Varian 9060 polychrom diode array detector. GCMS was performed using a Hewlett-Packard 5890 gas chromatograph equipped with an SGE 25Q C3 BP5 12 m × 0.22 mm id column connected to a VG MassLab 20–250 mass spectrometer. Melting points were recorded using a Pye model 290 melting point apparatus. Elemental analyses were obtained from Medac Ltd., Brunel Science Centre, Cooper's Hill Lane, Englefield Green, Egham, UK TW20 0JZ.

Substrates and reagents

The following compounds were purchased commercially: *N*-benzoylpiperidine **17**, *N*-methylbenzamide **18a**, *N*,*N*-dimethylbenzamide **20**, *N*-benzoylpiperidin-4-one **24**, *N*,*N*-dimethylacrylamide **26**, *N*-methyl-2-pyrrolidone **31**, *N*-benzylbenzamide, (*S*)-cotinine, *N*-benzyl-3-pyrrolidone, piperidin-4-one hydrochloride, *N*,*N*-dimethylaniline, *N*-methylaniline, 3-(aminomethyl)pyridine, lithium diisopropylamide, molybdenum trioxide, 25% (w/v) hydrogen peroxide, hexamethylphosphoric triamide, *n*-butyllithium, 4-bromobut-1-ene, benzyl bromide, methyltriphenylphosphonium iodide, methoxymethyltriphenylphosphonium bromide, tributyltin hydride, 1,1′-azobis(cyclohexanecarbonitrile), iodomethane, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, tetraphenylporphyrin iron(III) chloride, and *tert*-butylhydroperoxide.

The following known compounds were synthesised analytically and chromatographically pure: (±)-*N*-benzoyl-3-methylpyrrolidine **16**,[41] *N*-benzoyl-1,2,5,6-tetrahydropyridine **22**,[42] (5*S*)-3′-hydroxycotinine **34**,[32] *N*,*N*-dimethyl-2-hydroxybenzamide **38**,[43] 3-methyl-2,3-dihydro-4*H*-1,3-benzoxazin-4-one **39**,[44] *N*-methyl-2-hydroxybenzamide **40**,[45] *N*-(but-3-enyl)-benzamide **19**,[46] *N*-methylacrylamide **32**,[47] *N*-cyanomethyl-*N*-

methylaniline **37** (Nu = CN),[48] and *N*-cyanomethyl-*N*-methylbenzamide.[49]

N-Hydroxymethyl-[6] and *N*-chloromethyl-*N*-methylbenzamide[50] are unstable and were synthesised as required. Other *N*-chloromethylamides are also unstable and were synthesised as required by the literature method:[50] *N-(but-3-enyl)-N-chloromethylbenzamide*: ν_{max}/cm^{-1} 3010, 1650, 1510, 1500, 1480, 1380; δ_H 2.1 (2H, m, $CH_2C{=}C$), 3.3 (2H, m, NCH_2), 5.0 (2H, m, $H_2C{=}C$), 5.30 (2H, s, NCH_2Cl), 5.6 (1H, m, CH=C), 7.28 (5H, s, Ar); *m/z* (%) 224 (5), 222 (15) ($M^{+\bullet}$), 173 (40) ($M^{+\bullet} - CH_2Cl$), 105 (44), 77 (100); *N-(but-3-ynyl)-N-chloromethylbenzamide*: ν_{max}/cm^{-1} 3280, 3050, 2100, 1650, 1580, 1550, 1520, 1500, 1480, 1400, 1350, 1300, 1280; δ_H 2.0 (1H, t, $J = 3$, HC≡C), 2.5 (2H, dt, $J = 3$, 5, $CH_2C{\equiv}C$), 3.0 (2H, t, $J = 5$, NCH_2), 5.30 (2H, s, NCH_2Cl), 7.0–7.5 (5H, m, Ar); *m/z* (%): 222 (4), 220 (10) ($M^{+\bullet}$), 171 (55) ($M^{+\bullet} - CH_2Cl$), 105 (35), 77 (100); *N-chloromethyl-N-methylacrylamide*: ν_{max}/cm^{-1} 3060, 1670, 1590, 1410, 1280, 1260; δ_H 3.00 (3H, s, NMe), 5.3 (2H, s, NCH_2Cl), 5.6–5.80 (1H, m, CH=C), 6.00–6.25 (2H, m, $CH_2{=}C$); *m/z* (%) 135 (5), 133 (18) ($M^{+\cdot}$), 84 (40) ($M^{+\cdot} - CH_2Cl$), 55 (100), 27 (10).

N-(But-3-enyl)-*N*-methylbenzamide 14a

A 2 M THF solution of LDA (11 cm^3, 0.022 mol) was added under N_2 at 0 °C to *N*-(but-3-enyl)benzamide (0.02 mol, 3.5 g) in dry THF (50 cm^3). The solution was allowed to warm to room temperature and iodomethane (0.022 mol, 3.12 g) was added dropwise. After 1 h the solvent was removed and the residue was resuspended in DCM (50 cm^3), washed with water (2 × 20 cm^3), dried ($MgSO_4$) and concentrated. The crude product was purified by column chromatography using DCM–methanol (8 : 2) as eluant: ν_{max}/cm^{-1} 3010, 1650, 1510, 1500, 1480, 1380; δ_H 2.2 (2H, q, $J = 7$, $CH_2C{=}C$), 3.2 (3H, s, NMe), 3.4 (2H, t, $J = 7$, NCH_2), 5.1 (2H, m, $CH_2{=}C$), 5.7 (1H, m, CH=C), 7.4–7.8 (5H, m, Ar); *m/z* (%) 189 (8) ($M^{+\bullet}$), 148 (60), 135 (10), 105 (100), 77 (60), 39 (10). Found: C, 76.3; H, 8.0; N, 7.3%. $C_{12}H_{15}NO$ requires: C, 76.2; H, 7.9; N, 7.4%.

N-Benzyl-*N*-(but-3-enyl)benzamide 14b

The procedure for **14a** was followed substituting a dry THF solution (10 cm^3) containing benzyl bromide (0.022 mol, 3.76 g) and potassium iodide (2.4 mmol, 0.4 g) in place of iodomethane: ν_{max}/cm^{-1} 3010, 1700, 1660, 1600, 1500, 1480, 1440, 1370, 1300, 1240; δ_H 2.25 (2H, q, $J = 8$, $CH_2C{=}C$)), 3.3 (2H, t, $J = 8$, NCH_2), 4.45 (2H, s, NCH_2Ph), 5.0 (2H, m, $CH_2{=}C$), 5.7 (1H, m, CH=C), 7.2–7.8 (10H, m, 2 × Ar); *m/z* (%) 265 (10) ($M^{+\bullet}$), 224 (45), 210 (8), 174 (5), 105 (100), 91 (45), 77 (50). Found: C, 81.8; H, 7.2; N, 5.3%. $C_{18}H_{19}NO$ requires: C, 81.5; H, 7.2; N, 5.3%.

N-(But-3-ynyl)-*N*-methylbenzamide 15

The procedure for **14a** was followed substituting *N*-(but-3-ynyl)benzamide for *N*-(but-3-enyl)benzamide: ν_{max}/cm^{-1} 3050, 1650, 1580, 1550, 1520, 1500, 1480, 1400, 1350, 1300, 1280; δ_H 1.9 (1H, t, $J = 3$, HC≡C), 2.3 (2H, dt, $J = 3$, 5, $CH_2C{\equiv}C$), 2.92 (3H, s, NMe), 3.2 (2H, t, $J = 5$, NCH_2), 7.4–7.65 (5H, m, Ar); *m/z* (%) 187 (10) ($M^{+\bullet}$), 148 (55), 134 (20), 105 (100), 77 (70), 39 (12). Found: C, 77.2; H, 7.0; N, 7.4%. $C_{12}H_{13}NO$ requires: C, 77.0; H, 6.95; N, 7.5%.

N-Benzoyl-3-methylenepyrrolidine 21

N-Benzylpyrrolidin-3-one (0.02 mol, 3.5 g) in dry DCM (30 cm^3) was added to a stirred solution of 5,10,15,20-tetraphenylporphine iron(III) chloride (3.0 mmol, 2.1 g) and *tert*-butylhydroperoxide (0.2 mol, 18 g) in dichloromethane (10 cm^3). The reaction mixture was maintained at room temperature for 48 hours; every 4 hours a DCM solution (10 cm^3) containing *tert*-butylhydroperoxide (0.2 mol) was added. The solvent was removed and the solid residue subjected to chromatography on silica gel using diethyl ether–hexane 8 : 2 as the mobile phase to obtain *N*-benzoylpyrrolidin-3-one (ν_{max}/cm^{-1} 2980, 1750, 1720, 1500; δ_H 2.4 (2H, t, $J = 7$, CH_2CO), 3.5–3.95 (4H, m, $NCH_2CO + NCH_2$), 7.2–7.7 (5H, m, Ar)) which was used directly in the next step. Methyltriphenylphosphonium bromide (4.07 mmol, 1.45 g) was dissolved in dry THF (30 cm^3). The solution was cooled to −15 °C and, under N_2, a 2.5 M hexane solution of butyllithium was added dropwise (4.07 mmol, 1.53 cm^3). The mixture was maintained at −15 °C and a dry THF solution (20 cm^3) of *N*-benzoylpyrrolidin-3-one (3.9 mmol, 0.70 g) was added. The reaction mixture was maintained at −15 °C for 30 min, brought to room temperature and left for a further 2 h. Ammonium chloride (0.1 mol, 5.2 g) was added, the solvent removed, the solid residue resuspended in DCM (100 cm^3) and the organic phase washed with water (2 × 20 cm^3), dried ($MgSO_4$), concentrated and subjected to chromatography on silica gel using diethyl ether–hexane 7 : 3 as eluant: ν_{max}/cm^{-1} 3000, 1680, 1520, 1470; δ_H 2.0 (2H, t, $J = 5$, $CH_2C{=}C$), 3.2 (2H, m, NCH_2), 3.4 (2H, m, $NCH_2C{=}C$), 5.0 (2H, m, $CH_2{=}C$), 7.1 (5H, m); *m/z* (%) 187 (25) ($M^{+\bullet}$), 173 (35), 105 (100), 82 (40), 77 (70). Found: C, 77.6; H, 7.05; N, 7.6%. $C_{12}H_{13}NO$ requires: C, 77.0; H, 6.95; N, 7.5%.

N-(But-3-ynyl)benzamide 23

N-(But-3-ynyl)amine hydrochloride (0.10 mol, 10.55 g)[51] was dissolved in dry THF (200 cm^3) in the presence of a large excess of triethylamine. Benzoyl chloride (0.11 mol, 12.7 g) in THF (50 cm^3) was added dropwise. The reaction mixture was refluxed overnight, cooled, the white precipitate filtered off, the solvent removed and the solid residue purified by chromatography using diethyl ether as eluant: ν_{max}/cm^{-1} 3350, 3300, 3150, 1710, 1650, 1550, 1500, 1480; δ_H 1.8 (1H, t, $J = 3$, HC≡C), 2.1 (2H, dt, $J = 3$, 5, $CH_2C{\equiv}C$), 3.15 (2H, m, $J = 5$, NCH_2), 6.8 (1H, br s, NH), 7.4–7.85 (5H, m, Ar); *m/z* (%) 173 (20) ($M^{+\bullet}$), 149 (15), 134 (50), 105 (100), 77 (90), 68 (50), 39 (25). Found: C, 76.6; H, 6.4; N, 8.0%. $C_{11}H_{11}NO$ requires: C, 76.3; H, 6.4; N, 8.1%.

N-Benzoyl-3-formylpyrrolidine 25

A 2.5 M hexane solution of butyllithium (5.23 mmol, 2.1 cm^3) was added dropwise, at −15 °C and under N_2, to methoxymethyltriphenylphosphonium bromide (5.23 mmol, 2.23 g) in dry THF (30 cm^3). The mixture was maintained at −15 °C for 30 min then a dry THF solution (20 cm^3) of *N*-benzoylpyrrolidin-3-one (5.0 mmol, 0.90 g) (see the synthesis of **19** above) was added. The reaction mixture was maintained at −15 °C for 30 min, brought to room temperature and left for a further 2 h. Ammonium chloride (0.1 mol, 5.2 g) was added, the solution extracted with chloroform (2 × 50 cm^3), the organic phase evaporated, the solid residue resuspended in 0.1 M HCl (50 cm^3) and refluxed for 30 min. After cooling, the acid solution was extracted with chloroform (2 × 50 cm^3), the organic phase washed with water (2 × 20 cm^3), dried ($MgSO_4$) and, after concentration, subjected to chromatography on silica gel using diethyl ether–hexane 8 : 2 as eluant: ν_{max}/cm^{-1} 3020, 2800, 1725, 1700, 1610, 1500; δ_H 2.2 (2H, m, CH_2), 2.9 (1H, m, CHC=O), 3.4–3.7 (4H, m, 2 × NCH_2), 7.1 (5H, m, Ar), 9.6 (1H, d, $J = 1.5$, CHO); *m/z* (%): 203 (20) ($M^{+\bullet}$), 174 (30) (M − CHO), 105 (100), 98 (40), 77 (70). Found: C, 70.7; H, 6.15; N, 7.0%. $C_{12}H_{13}NO_2$ requires: C, 70.9; H, 6.4; N, 6.9%.

N-(3-Pyridylmethyl)acrylamide

A solution of 3-(aminomethyl)pyridine (0.1 mol, 10.8 g) and triethylamine (0.11 mol, 11.2 g) in dry diethyl ether (50 cm^3) was added dropwise to an ice-cold solution of acryloyl chloride (0.1 mol, 9.05 g) in dry diethyl ether (100 cm^3). The solution was allowed to attain room temperature, the solid filtered off and the solvent removed to give a residue that was resuspended in DCM (50 cm^3), washed with brine (2 × 10 cm^3), water (2 × 10

cm^3), and dried ($MgSO_4$). The solvent was evaporated and the residue purified by chromatography on silica using DCM–methanol (9:1) as eluant: ν_{max}/cm^{-1} 3400, 3080, 1690, 1600; δ_H 4.45 (2H, d, $J = 7$, NCH_2Ar), 5.2 (1H, m, =CHCO), 5.6 (1H, m, CH_2=C), 6.0 (1H, m, CH_2=C), 6.7 (1H, br s, NH), 7.2 (1H, dd, $J = 4$, 6, 5-H), 7.6 (1H, d, $J = 6$, 4-H), 8.4 (1H, d, $J = 4$, 6-H), 8.65 (1H, br s, 2-H); *m/z* (%) 162 (30) ($M^{+\bullet}$), 135 (45) ($M^{+\bullet} - CH_2$=CH), 107 (80) ($NHCH_2Pyr$), 55 (100) ($M^{+\bullet} - NHCH_2Pyr$). Found: C, 66.9; H, 6.0; N, 17.0%. $C_9H_{10}N_2O$ requires: C, 66.7; H, 6.2; N, 17.3%.

N-Methyl-*N*-(3-pyridylmethyl)acrylamide 27

The method used for the synthesis of **14a** was followed, replacing *N*-(but-3-enyl)benzamide with *N*-(3-pyridylmethyl)-acrylamide and using DCM–methanol (9:1) as the eluant for chromatography: ν_{max}/cm^{-1} 3010, 1690, 1600; δ_H 3.0 (3H, s, NMe), 4.66 (2H, s, NCH_2), 5.7–5.77 (1H, m, =CHCO), 6.4–6.71 (2H, m, CH_2=C), 7.3 (1H, dd, $J = 5$, 7, 5-H), 7.5 (1H, d, $J = 7$, 4-H), 8.0 (1H, d, $J = 5$, 6-H), 8.3 (1H, br s, 2-H), 7.2–7.8 (4H, m, Ar); *m/z* (%): 176 (25) ($M^{+\bullet}$), 148 (60) ($M^{+\bullet} - CH_2$=CH), 121 (50) ($M^{+\bullet} - CH_2$=CHCO), 79 (15) (Pyr), 55 (100) (CH_2=CHCO). Found: C, 68.4; H, 7.0; N, 16.2%. $C_{10}H_{12}N_2O$ requires: C, 68.2; H, 6.8; N, 15.9%.

(±)-*N*-Methyl-3-hydroxypyrrolidin-2-one 33

The method of Jacob *et al.* [32] for the synthesis of (*5S*)-3-hydroxycotinine was followed on an 11 mmol scale using *N*-methyl-2-pyrrolidone in place of (*S*)-cotinine: bp 106–110 °C/2 mmHg; ν_{max}/cm^{-1} 3400, 1720, 1640, 1520; δ_H 1.95 (1H, m, CH), 2.25 (1H, m, CH), 2.85 (3H, s, NMe), 3.1 (2H, t, $J = 7.5$, NCH_2), 4.3 (1H, t, $J = 7.5$, CHO), 4.85 (1H, br s, OH); *m/z* (%) 115 (20) ($M^{+\bullet}$), 100 (25), 98 (100), 47 (60). Found: C, 52.3; H, 8.0; N, 12.3%. $C_5H_9NO_2$ requires: C, 52.2; H, 7.8; N: 12.2%.

Reaction of *N*-chloromethylamides with Bu_3SnH

The *N*-chloromethylamide (0.005 mol), tributyltin hydride (0.05 mol) and 1,1′-azobis(cyclohexanecarbonitrile) (0.05 mol) were refluxed in dry acetonitrile (80 cm^3) for 1.5 h. The reaction mixture was cooled, the solvent removed, the residue resuspended in DCM and the solution analysed by GC and GCMS by comparison with authentic standards.

Semi-empirical calculations

Structure determinations and ΔH_f values were calculated using the AM1 SCF MO program within the MOPAC 4 package.[52] Radical structures were determined using unrestricted Hartree–Fock calculations. All structures were geometry optimised using the Broyden–Fletcher–Goldfarb–Shanno procedure and were performed on a Silicon Graphics Indy workstation.

Microsomes and microsomal incubations

Phenobarbital-induced rat liver microsomes [P450 content 1.95 nmol (mg microsomal protein)$^{-1}$] were obtained from the Institute of Cancer Research, Cancer Research Campaign Laboratories, Sutton, Surrey, UK. Microsomal protein content was determined by the method of Lowry *et al.* [53] using bovine serum albumin as standard. Cytochrome P450 content was determined using the method of Omura and Sato.[54] Microsomal incubations were performed in 0.1 M pH 7.4 phosphate buffer using a microsomal concentration of 1.5 (mg protein) cm^{-3} in the presence of an NADPH generating system comprising glucose-6-phosphate (6.25 nmol cm^{-3}), NADP (1.25 mmol cm^{-3}), $MgCl_2$ (6 mmol cm^{-3}) and glucose-6-phosphate dehydrogenase (2.5 U cm^{-3}). Reactions were performed at 37 °C and were initiated by addition of the substrate dissolved in 0.1 M phosphate buffer (pH 7.4). Aliquots (100 µl) were withdrawn at timed intervals and added to a 10% (w/v) solution of trichloroacetic acid (200 µl) to precipitate protein. The supernatant was then analysed directly, or, after centrifugation (5 min, 4500 rpm) a portion (200 µl) of the supernatant was treated with 1 M NaOH (200 µl) for 10 min, acidified with 1 M HCl (200 µl) and then analysed. For all reactions, separate control experiments were performed using two conditions (i) in the absence of microsomes and (ii) in the absence of the NADPH generating system. In neither case was transformation observed for any substrate, confirming the requirement for the full metabolic machinery.

GCMS analysis

Samples of the reaction mixture were saturated by the addition of ammonium acetate and extracted using ethyl acetate (2 cm^3). The organic phase was separated, dried ($MgSO_4$) and directly injected (0.5 µl) into the GC instrument without dilution. Thermal gradient programmes were used as follows. For *N*,*N*-dimethylaniline, *N*,*N*-dimethylbenzamide and *N*-cyanomethyl-*N*-methylaniline: 2 min at 110 °C, from 110 to 190 °C at 10 °C min^{-1}, isothermal at 190 °C for 10 min; for *N*,*N*-dimethyl-2-hydroxybenzamide: 1 min at 85 from 85 °C to 270 °C at 10 °C min^{-1}, isothermal at 270 °C for 20 min; for the *N*-(but-3-ynyl)- and *N*-(but-3-enyl) substrates: 1 min at 110 °C, from 110 to 270 °C at 10 °C min^{-1}, isothermal at 270 °C for 10 min. Quantitation involved calibration with authentic standards.

HPLC analysis

Separation of the amides was achieved using a Jones Chromatography C18 25 cm × 5 mm column, a flow rate of 1 cm^3 min^{-1}, a detection wavelength of 254 nm, and an eluant comprising solvent A [5% acetonitrile in 0.05 M phosphate buffer (pH 2)] and solvent B [60% acetonitrile in 0.05 M phosphate buffer (pH 2)]. A solvent gradient was used as follows: $t = 0$–15 min, 40 to 0% solvent A; $t = 15$–18 min, 100% solvent B; $t = 18$–19 min, 0 to 40% solvent A. Product identification and quantitation was achieved by comparison of retention times peak areas and diode array UV/vis spectra with those of synthetic standards. Separate injections were reproducible to ±1.5% and product concentrations were accurate to ±2%.

Determination of the kinetic parameters of the P450-mediated oxidations

Microsomal oxidations were monitored over two hours. Plots of metabolite formation *versus* time exhibited an initial, linear phase followed by a tailing-off in the reaction progress. The linear phase was used to determine the initial rate (v_i) and the kinetic parameters (V_{max} and V_{max}/K_M) were determined in the usual way from the Michaelis–Menten equation.

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