J. Pharm. Pharmacol. 1989, 41: 373–378
Received September 1, 1988

Conformational Analysis of 9-Substituted Adenines in Relation to Their Microsomal N^1-Oxidation

SIU PING LAM, DAVID JOHN BARLOW AND JOHN WILLIAM GORROD

Chelsea Department of Pharmacy, King's College London, University of London, UK

Abstract—Metabolic *N*-oxidation of adenine, 9-methyladenine, 9-benzyladenine, 9-benzhydryladenine and 9-trityladenine has been investigated using hepatic microsomes from hamster, guinea-pig, rabbit, mouse, rat, and dog. N^1-Oxide formation occurs with 9-benzyladenine and 9-benzhydryladenine using liver preparations of all species examined, although to different extents. The *N*-oxidase activity was found, amongst rodents, in the order hamster > mouse > rabbit > rat > guinea-pig. Microsomal preparations from dog liver contained a small quantity of P-450 and yet produced a relatively large amount of the *N*-oxides, possibly indicating that other cytochromes in addition to P-450 may be involved in the *N*-oxidation of these compounds. The most favourable conformations of these 9-substituted analogues have been established using computer graphics modelling and ^{1}H NMR techniques. Results obtained confirmed the importance of the stereochemical properties of these compounds in relation to N^1-oxidation. These observations substantiate and extend our previous findings on the electronic, lipophilic, and stereochemical factors affecting the *N*-oxidation of adenine derivatives.

Many amino-azaheterocycles have been shown to be *N*-oxidized at the exocyclic amino group to mutagenic hydroxylamines or to innocuous *N*-oxides at the ring nitrogen (Gorrod 1985, 1987). These observations have led us to investigate possible factors controlling the site of biological *N*-oxidation of these compounds. In view of the importance of 6-aminopurine (adenine) and the potential toxicity of its hydroxylamine, we examined its metabolism in-vitro by hepatic microsomal systems. In an earlier communication, we reported the formation of the N^1-oxide of 9-benzyladenine (9BA) but not the *N*-oxygenation of adenine (Ad) or 9-methyladenine (9MA) during their microsomal metabolism (Lam et al 1987). In continuing our investigations into the structural, as well as physicochemical factors, that may influence metabolic *N*-oxidation of these 9-substituted adenine derivatives, we have studied N^1-oxide formation of 9-benzhydryladenine (9BHA) and 9-trityladenine (9TA) as substrates using hepatic microsomal preparations from hamster, guinea-pig, rabbit, mouse, rat and dog. Stereochemical properties of 9-substituted adenine analogues were established by computer graphic modelling and ^{1}H NMR techniques. Data obtained is discussed in relation to metabolic N^1-oxidation of these compounds.

Materials and Methods

Chemicals used for the preparations of substrates and their potential metabolites were purchased from Sigma UK Ltd., Lancaster Synthesis (Lancaster UK) and British Drug House (BDH) (Dorset, UK).

Ad, 9MA, 9BA and their corresponding N^1-oxides AdNO, 9MANO, and 9BANO and 6-hydroxylamines, i.e. 6-hydroxyaminopurine (6HP), 6-hydroxyamino-9-methylpurine (6H9MP) and 9-benzyl-6-hydroxyaminopurine (9B6HP) were obtained as described previously (Lam et al 1987).

Correspondence to: J. W. Gorrod, Chelsea Department of Pharmacy, King's College London, University of London, Manresa Road, London SW3 6LX, UK.

Preparation of 9BHA base was a modification of the method of Montgomery & Thomas (1964). The oily residue of 9BHA was triturated with diethyl-ether and the resulting solid washed with acetone. Recrystallization from methanol yielded 9BHA, melting point 209–212°C. (Found: C,71.46; H,5.02; N,23.41; $C_{18}H_{15}N_5$ requires C,71.74; H,5.02; N,23.24%.)

9BHANO was synthesized as follows: 9BHA (0·5 g) was dissolved in glacial acetic acid (2 mL). Hydrogen peroxide 30% w/v (1·9 mL) was added and the mixture was left at room temperature (20°C) for one week, after which, sodium bicarbonate solution (1 M) was added until the mixture was just alkaline to litmus. The solution was thoroughly shaken and extracted with 5 × 50 mL dichloromethane. The organic extracts were combined and evaporated to dryness using a rotary film evaporator (RFE), 9BHANO was recrystallized from methanol, melting point 246–248°C decomp., yield 53%. (Found: C,67.30; H,4.79; N,21.77; $C_{18}H_{15}N_5O$ requires C,68.12; H,4.76; N,22.07%.)

9TA was synthesized by tritylation of adenine with trityl chloride. Adenine (3·1 g) was stirred as a suspension in dichloromethane (100 mL) containing triethylamine (7 mL). Tritylchloride (3·8 g) in dichloromethane (25 mL) was added and the mixture kept at room temperature (20°C) for 6 h, evaporated to dryness using RFE. The residue was washed with a small amount of water and treated with excess chloroform. Insoluble material was filtered off and the organic phase evaporated to dryness. Recrystallization from absolute alcohol gave 9TA, yield 33%, melting point 249–250°C decomp. (Found: C,76.28; H,5.12; N,18.50; $C_{24}H_{19}N_5$ requires C,76.73; H,5.07; N,18.56%.)

9TANO was synthesized by *m*-chloroperbenzoic acid oxidation in dichloromethane. 9TA (0·5 g) was dissolved in the minimum amount of dichloromethane. *m*-Chloroperbenzoic acid (0·3 g) in dichloromethane was added to the solution. After 6 h, the mixture was evaporated to dryness using RFE at 45°C, the residue washed with diethyl-ether and recrystallized from absolute ethanol to give 9TANO, yield 47%, melting point 240–241°C decomp. (Found:

C,73.36; H,4.92; N,17.69; $C_{24}H_{19}N_5O$ required C,73.26; H,4.87; N,17.80%.)

Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP were purchased from the Boehringer Corporation (London) Ltd. All other chemicals and solvents used were obtained from BDH.

^{1}H-NMR spectra of Ad, 9MA, 9BA, 9BHA & 9TA were determined as described previously (Lam et al 1987).

Computer-aided conformational analyses of the 9-substituted adenines were performed using the molecular mechanics and model-building facilities of the CHEMMOD package, implemented on a U-MAN 1000 microcomputer graphic systems.

The partition coefficients (P) of Ad, 9MA, 9BA and 9BHA in an octanol/phosphate buffer (0·2 M, pH 7·4) system were determined spectrophotometrically. Spectrophotometric analyses were carried out at appropriate wavelengths, i.e. 261 nm for Ad and 9MA, 261·5 nm for 9BA and 262·5 nm for 9BHA. The concentration of each compound in the aqueous phase after 12 h partitioning was measured using a Kontron Uvikon 860 UV-VIS spectrophotometer against a calibration curve previously constructed. All determinations were carried out at 20 ± 1°C. Log P of 9TA was calculated (Rekker 1977).

The HPLC chromatograph used was an isocratic system comprising a single Cecil pump, a Rheodyne model 7125 injector fitted with a 20 μL loop, a suitable guard column (ODS or cationic exchange) (length 5 cm, internal diameter 2 mm), and a 25 cm analytical column. The columns used were either 25 cm Nucleosil SA 5 μm cationic exchange column for all incubations or a 25 cm Spherisorb ODS 5 μm for the analysis of adenine which was derived from 9TA metabolism. These columns were connected to a Cecil UV variable wavelength detector. All columns were purchased from HPLC Technology Ltd., UK. The mobile phases and operation conditions used were as follows (all flow rates were at 1 mL min^{-1}):

(1) Ad and potential metabolites: mobile phase: $NH_4H_2PO_4$ (0·12 M), methanol (10% v/v), pH = 3·7.
(2) 9MA and potential metabolites: mobile phase: $NH_4H_2PO_4$ (0·22 M), methanol (15% v/v), pH = 3·4.
(3) 9BA and potential metabolites: mobile phase: $NH_4H_2PO_4$ (0·16 M), methanol (35% v/v), pH = 3·7.
(4) 9BHA and potential metabolites: mobile phase: $NH_4H_2PO_4$ (0·12 M), methanol (45% v/v), pH = 3·6.
(5) 9TA and potential metabolites (Nucleosil SA 5 μm): mobile phase: $NH_4H_2PO_4$ (0·1 M), methanol (50% v/v), pH = 4·0.
(6) 9TA and Ad (Spherisorb 5 μm): mobile phase: Phosphate buffer (0·01 M), acetonitrile (7% v/v), pH = 7.

Metabolites were detected by monitoring at 265 nm and quantified using a LDC/Milton Roy CI-10B integrator. This system gave a linear detector response between 2 and 100 nmol of metabolites when they were added to incubation mixtures and carried through the extraction and concentration procedures. Information obtained from these calibrations using a range of concentration of standard metabolites was stored in the integrator for direct quantitative analysis.

The animals used were: female New Zealand white rabbits (2–3 kg), male albino Wistar rats (250–300 g), male Syrian hamsters (80–100 g), male albino Dunkin Hartley guinea-pigs (400–600 g), male L.A.C.A albino mice (40–60 g) and male and female Beagle dogs (Huntingdon Research Centre, UK).

Hepatic microsomes from different mammalian species were prepared at 0–4°C by the calcium precipitation method based on Gibson & Skett (1986). Animals were starved overnight before death but were given free access to drinking water. Animals were killed by cervical dislocation and exsanguination before the removal of the liver. Dogs were anaesthetized with pentobarbitone. The livers were removed immediately and immersed in ice-cold isotonic saline solution. The livers, after removing the gall bladder and connective tissue, were washed and perfused with saline to remove excess blood, blotted dry with filter paper, weighed and cut into small pieces and immersed in sucrose solution (0·25 M, 1 g in 3 mL). Livers were homogenized using a Sorvall Omni-Mix homogenizer or an Ultra-Turrax homogenizer for 5 × 5 s. The homogenate was centrifuged at 13 000 *g* (10 500 rev min^{-1}) for half an hour using a Sorvall Superspeed RC2-B refrigerated centrifuge. The supernatant was decanted, and calcium chloride solution (80 mM, 1 in 10 mL of supernatant) added and re-centrifuged at 29 000 *g* (15 500 rev min^{-1}) for 15 min to sediment microsomes. The resultant pinkish pellet of microsome was removed from the bottom of the tube, leaving any glycogen behind, and resuspended in an equal volume of potassium chloride solution (0·15 M). This suspension was recentrifuged at the same speed for a further 15 min to obtain washed microsomes. The pellet was re-suspended in phosphate buffer (0·2 M, pH 7·4) at a concentration equivalent to 0·5 g original liver per mL. Microsomes were usually used within 24 h of preparation but in some cases they were stored at −80°C, after 20% glycerol was incorporated in the final suspension media.

Microsomal protein concentration was determined by the method of Lowry et al (1951), following the modification of Miller (1959), and cytochrome P-450 content by the method of Omura & Sato (1962).

Substrates (2 μmol) were incubated at 37°C with hepatic microsomes from different species for 20 min as described previously (Lam et al 1987). Metabolites formed from 9BA, 9BHA and 9TA were extracted with 3 × 5 mL of 5% ethanol in dichloromethane, and 4-amino-2,6-dimethylpyrimidine (4A26DMPY) was used as internal standard. 4-Chloro-2,6-diaminopyrimidine (4C126DAPY) was also used as internal standard for the quantitation of adenine from 9TA incubations. In Ad and 9MA metabolism, ethanol was used for extraction as previously described. Caffeine was used as internal standard for Ad assay whereas 4A26DMPY was used for 9MA assay. The concentrated extracts were dissolved in about 200 μL ethanol for HPLC analysis.

Results and Discussion

In-vitro metabolism of Ad, 9MA, 9BA, 9BHA and 9TA using hepatic microsomes from hamster, mouse, rabbit, rat, guinea-pig and dog was carried out as described. Extracts from incubates were analysed for metabolites formed using the HPLC systems described. Ad, 9MA, 9BA, 9BHA, 9TA and their corresponding N^1-oxides and hydroxylamines were

Table 1. HPLC analysis of adenine, 9-substituted adenines and potential metabolites.

Compound	Retention time (min)					
	(B)1	2	3	4	5	6
Ad	18·29	10·75	6·90	7·91	—	6·02
AdNO	6·10	—	—	—	—	—
6HP	11·15	—	—	—	—	—
9MA	—	20·47	—	—	—	—
9MANO	—	8·82	—	—	—	—
6H9MP	—	13·38	—	—	—	—
9BA	—	—	16·08	—	—	—
9BANO	—	—	9·08	—	—	—
9B6HP	—	—	13·53	—	—	—
9BHA	—	—	—	18·33	—	—
9BHANO	—	—	—	9·56	—	—
9TA	—	—	—	—	22·84	—
9TANO	—	—	—	—	11·02	—
Caf.	13·03	—	—	—	—	—
4A26DMPY	—	17·34	11·11	12·29	15·23	—
4C126DAPY	—	—	—	—	6·53	7·99

Internal standards: Caf.: caffeine; 4A26DMPY: 4-amino-2,6-dimethylpyrimidine; 4C126DAPY: 4-chloro-2,6-diaminopyrimidine.
(B)1, 2, 3, 4, 5 and 6 are mobile phase systems (see text for conditions).

successfully separated and detected using these HPLC systems. The retention times of individual compounds in each system are summarized in Table 1. *N*-Oxides and hydroxylamines were eluted before their parent compounds. Different mobile phases were used for the six systems to minimize the analysis time. The chromatographic properties of each compound using these HPLC systems allowed simultaneous separation and identification of *N*-oxidation products obtained from metabolic studies of these substrates.

The metabolic results, summarized in Table 2, showed that adenine appeared to be resistant to biological *N*-oxidation when hepatic microsomes of several species were used as enzyme source: neither AdNO nor 6HP were detected. No 6-hydroxylamines of any substrate were detected in any of the experiments. This is probably a result of the delocalization of the lone pair electrons of the 6-NH_2 nitrogen due to conjugation with the ring system. These results are in good agreement with our previous observations (Lam et al 1987).

N-Dealkylation of 9-substituted analogues except 9TA to form adenine appears to be a common metabolic pathway with all substrates with most species. Demethylation of 9MA was not observed when microsomes from the mouse and dog were used. This may be due to a difference of cytochrome isozymes in different species (Kato 1979). No detritylation of 9TA was observed which was clearly due to the absence in the 9-trityl group of an α hydrogen adjacent to the 9-N nitrogen in this molecule (McMahon 1966; Gorrod & Temple 1976; Guengerich & Macdonald 1984). In cases when 9MA was the substrate, another metabolite in addition to adenine was apparent. This metabolite was most probably a carbon hydroxylation product with the 9-*N*-methyl group still intact as this metabolite was not produced from adenine and it was stable when treated with titanous chloride solution.

Table 2. Microsomal metabolism results of adenine and derivatives.

Species	Substrate	Metabolite nmol (mg protein)$^{-1}$/20 min (nmol (nmol P450)$^{-1}$/20 min)		
		Ad.	N^1-O	6-NHOH
1. Hamster (M)	Ad	—	N.D.	N.D.
	9MA	0·8 (0·8)	N.D.	N.D.
	9BA	2·2 (2·0)	12·3 (10·9)	N.D.
	9BHA	4·7 (3·7)	12·6 (10·0)	N.D.
	9TA	N.D.	N.D.	N.D.
2. Mouse (M)	Ad	—	N.D.	N.D.
	9MA	N.D.	N.D.	N.D.
	9BA	1·3 (1·3)	4·7 (4·8)	N.D.
	9BHA	0·6 (1·1)	2·8 (4·9)	N.D.
	9TA	N.D.	N.D.	N.D.
3. Rabbit (F)	Ad	—	N.D.	N.D.
	9MA	0·2 (0·1)	N.D.	N.D.
	9BA	1·3 (0·8)	2·5 (1·5)	N.D.
	9BHA	1·0 (0·6)	3·4 (2·1)	N.D.
	9TA	N.D.	N.D.	N.D.
4. Rat (M)	Ad	—	N.D.	N.D.
	9MA	0·9 (1·0)	N.D.	N.D.
	9BA	0·4 (0·4)	1·5 (1·5)	N.D.
	9BHA	0·4 (0·7)	0·9 (1·4)	N.D.
	9TA	N.D.	N.D.	N.D.
5. Guinea-pig (M)	Ad	—	N.D.	N.D.
	9MA	0·3 (0·2)	N.D.	N.D.
	9BA	0·8 (0·6)	1·2 (0·9)	N.D.
	9BHA	1·8 (1·2)	1·5 (1·1)	N.D.
	9TA	N.D.	N.D.	N.D.
6. Dog (M)	Ad	—	N.D.	N.D.
	9MA	N.D.	N.D.	N.D.
	9BA	2·5 (5·5)	5·8 (12·7)	N.D.
	9BHA	0·8 (1·8)	3·3 (7·3)	N.D.
	9TA	N.D.	N.D.	N.D.
7. Dog (F)	Ad	—	N.D.	N.D.
	9MA	N.D.	N.D.	N.D.
	9BA	1·5 (1·8)	14·6 (17·3)	N.D.
	9BHA	2·4 (5·3)	3·5 (7·6)	N.D.
	9TA	N.D.	N.D.	N.D.

Ad: Adenine; N^1-O: N^1-oxide; 6-NHOH: 6-hydroxylamine; M: Male; F: Female; N.D.: Not detected.

N^1-Oxide formation was detected when 9BA and 9BHA were used as substrates with all species under study. However, the formation of an N^1-oxide of Ad, 9MA or 9TA was not observed.

In continuation of our investigation into the mechanism of enzymatic N^1-oxide formation mediated via microsomal cytochromes and the possible correlation of the electron density on a particular nitrogen and its susceptibility towards chemical *N*-oxidation (Gorrod 1987), we utilized the chemical shift value (δ) of the 2-H proton of the 9-substituted adenine derivatives. This value established by ^{1}H NMR spectroscopy was used to obtain an indication of changes in electron density. The spectral characteristics of adenine and 9-substituted derivatives are summarized in Table 3. These results provide information allowing characterization of the substrates. 9-Substitution on the adenine molecule causes electron redistribution within the molecule. This can be seen from the chemical shift values of the two purine protons of the 9-substituted derivatives which are different from those of adenine. 9-Methyl ($\delta = 8{\cdot}31$ ppm), 9-benzyl ($\delta = 8{\cdot}42$ ppm), and 9-benzhydryl ($\delta = 8{\cdot}34$ ppm) substitutions have a

Table 3. ^{1}H NMR characteristics and partition coefficients of adenine and 9-substituted derivatives.

Compound	Chemical shift δ (ppm) 2-H	8-H	9-CHR$_1$R$_2$	$-C_6H_5$	Partition coefficient log P
Ad	8·28	8·31	—	—	−0·051
9MA	8·31	8·17	3·87	—	−0·027
9BA	8·42	8·26	5·49	7·34	1·696
9BHA	8·34	7·95	7·31m		3·191
9TA	8·05	8·10	—	7·31	5·079*

The H^1 NMR spectra were determined in CD_3COOD at 90MHz using a Perkin-Elmer R32 NMR spectrometer, with TMS as an internal standard. Solute concentration was about 0·04M. 9MA: R_1; R_2 = H. 9BA: R_1 = H; $R_2 = C_6H_5$. 9BHA: R_1; $R_2 = C_6H_5$. m = multiplet; * = see Rekker (1977).

deshielding effect whereas 9-trityl substitution (δ = 8·05 ppm) has a shielding effect on the 2-H protons when compared with adenine (δ = 8·28 ppm). 9MA, 9BA, 9BHA and 9TA all cause upfield shifts of the 8-H protons compared with adenine ($\Delta\delta = -0{\cdot}05$ to $-0{\cdot}36$ ppm). The small chemical shift changes of 9MA observed offer a possible explanation as to the lack of *N*-oxide formation as has been discussed previously. The introduction of a 9-benzhydryl group into adenine has an overall electron withdrawing effect on the 2-H proton (2H: $\Delta\delta$(9BA-Ad) = +0·14 ppm; $\Delta\delta$(9BHA-Ad) = +0·06 ppm) similar to 9BA. This observation supports our belief that these changes may eventually lead to the higher conjugative overlap of the lone pair electrons of the 6-amino group with the ring electrons, resulting in a higher availability of electrons at the N^1-nitrogen thereby allowing N^1-oxide formation. Indeed, the relevance of this change in chemical shift to *N*-oxide formation is supported by the metabolic results from 9TA. This analogue did not form N^1-oxide with any of the species examined; its 2-H chemical shift was higher than that of adenine (2H: $\Delta\delta$(9TA-Ad) = −0·23 ppm) and was opposite to those changes observed with 9BA and 9BHA.

The importance of lipophilicity of 9-substituted adenines in their N^1-oxidation has been previously emphasized. The partition coefficients of Ad, 9MA, 9BA and 9BHA are shown in Table 3. Adenine and 9MA which did not form N^1-oxides are hydrophilic (log P(Ad) = −0·051, log P(9MA) = −0·027) whereas the lipophilic 9BA (log P = 1·696) and 9BHA (log P = 3·191) formed N^1-oxide as the main metabolite. These results strongly supported the requirement of lipophilicity and although the partition coefficient of 9TA was not determined experimentally as a result of its insolubility in the aqueous system, its extreme lipophilic characteristics was estimated as 5·079 using the free energy-related log P terms and 9MA as parent structure (Rekker 1977). However, this highly lipophilic adenine derivative did not form N^1-oxide with any species. This could be a result of the insolubility of the substrate in aqueous medium, leaving only a small amount of dissolved compound available for metabolism. Alternatively, the high lipophilicity of 9TA may hinder its penetration through lipophilic membranes into the enzyme active site. In other studies (Devinsky & Gorrod 1987), it has been shown that lipophilicity may not be the only determining factor for *N*-oxidation as rates of *N*-oxidation decreased beyond certain log P values.

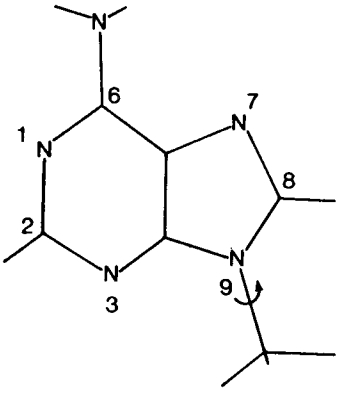
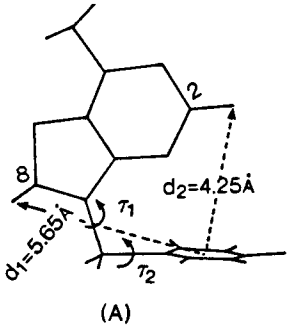
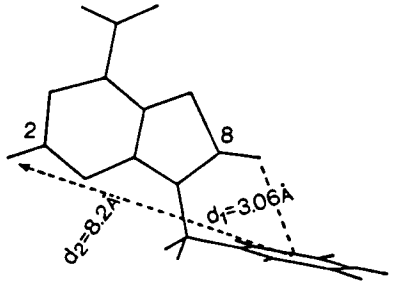


FIG. 1. Graphic display of 9MA.

The stereochemical properties of these 9-substituted adenine derivatives were therefore investigated: computer graphic modelling of the compounds was used to determine the most probable conformations, and these results were then correlated with the observed ^{1}H NMR data. For each compound (except 9TA) the potential energies were calculated as a function of the bond rotations (torsion angles τ) relating the 9-substituents and the adenine ring systems. The low energy conformers observed were equated with those most likely to occur in solution. The structures of the compounds and details of the analysis are shown in Figs 1–4. For 9BA, 9BHA and 9TA there are essentially two preferred conformations, which may loosely be described as *cis*- and *trans*- forms. To establish which of these conformations was likely to predominate in solution, the distances between the phenyl ring centres and 2-H/8-H protons were calculated—



(A)



(B)

FIG. 2a. Graphic displays of *cis* [A] and *trans* [B] conformations of 9BA.

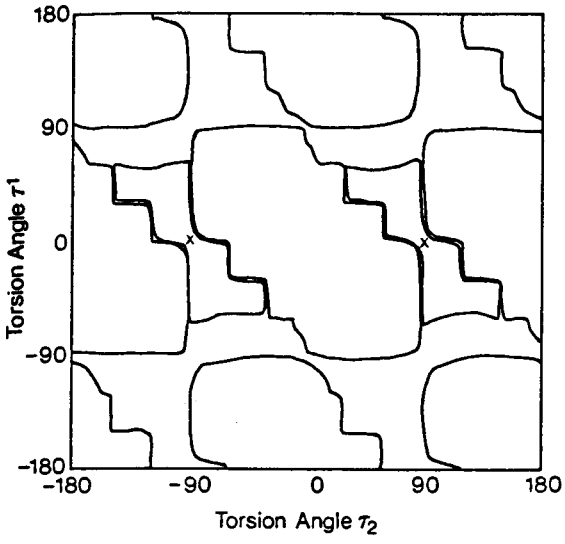


FIG. 2b. Variation of the potential energy of 9BA as a function of the torsion angles τ_1 & τ_2. Contours are shown at intervals of 1 kcal mol^{-1} with the lowest energy level ($-17{\cdot}6$ kcal mol^{-1}) indicated by x.

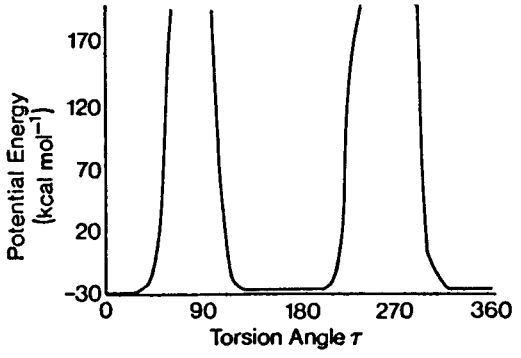
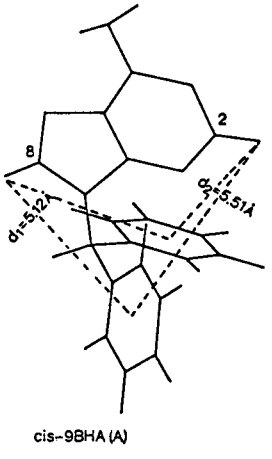


FIG. 3b. Variation of potential energy for 9BHA as a function of the torsion angle τ.



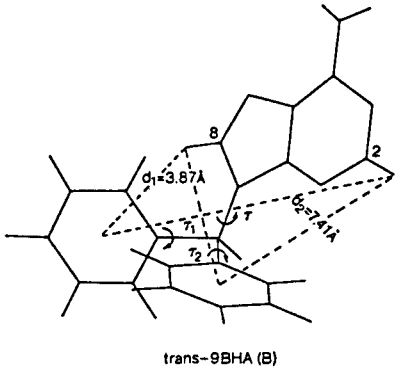
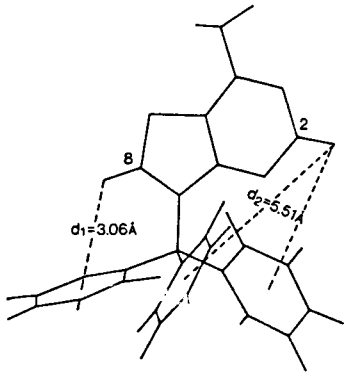


FIG. 3a. Graphic displays of *cis* [A] and *trans* [B] conformations of 9BHA.



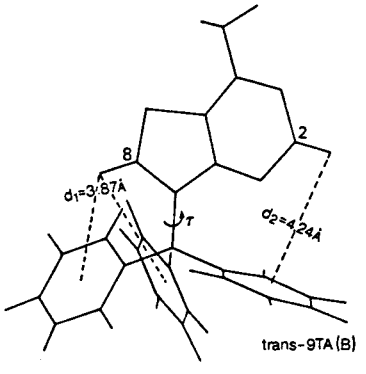


FIG. 4. Graphic displays of *cis* [A] and *trans* [B] conformations of 9TA.

giving a crude measure of the likely ring-current effects on these protons, which could then be compared with the experimentally determined chemical shifts. The greater shielding effects of the phenyl rings on the 8-H protons of 9BA and 9BHA when compared to those of adenine (see Table 3) suggested that the *trans* conformers were predominant; the distance from the centre of the phenyl ring being closer to the 8-H than to the 2-H protons (Figs 2a, 3a). In the case of 9TA, the model-building studies indicated that there would be pronounced ring current effects for both 2-H and 8-H protons with all proton-ring distances being in the range 3–5·5 Å. Since the NMR data showed that there was a marginally greater effect on the 2-H proton, we predict that it is the *trans*-conformation of 9TA which predominates in solution. While the *trans* conformers of these 9-substituted adenines were predominant in solution, only N^1-oxides of 9BA and 9BHA but not of 9TA were metabolically formed.

When these structures were further examined, it could be seen that the phenyl rings of the *trans* conformers of 9BA and 9BHA were close to the 8-H protons, exposing the N^1 nitrogen atom for oxidation. In *trans* 9TA, however, the proximity of the third phenyl ring to the 2-H proton may obstruct direct access of oxygen transfer to the molecule. The extra phenyl ring in that position may alternatively disturb the conformation of microsomal protein preventing binding of substrate to enzyme and hence N^1-oxide formation. These observations suggested that both 9BA and 9BHA had similar structural conformations which were not probable for the other analogues and may be related to the observed *N*-oxidation results. This analysis further substantiates our previous suggestion as to the importance of stereochemistry in controlling the *N*-oxidation of these compounds (Lam et al 1987).

The extent of N^1-oxidation of 9BA and 9BHA varied with different species. The *N*-oxidase activity when expressed as nmol product formed per mg microsomal protein was found amongst rodents in the order hamster > mouse > rabbit > rat > guinea-pig (Table 2). This may be due to different amounts of the enzyme responsible for the process in various species. When these results were compared, based on P-450 content of the microsomal preparation, it was interesting to observe the similarities between the amount of 9BANO and 9BHANO formed in each species. These findings strongly indicated the possible involvement of cytochrome P-450 in N^1-oxide formation as has been reported for the *N*-oxide formation of 5- and 6-substituted 2,4-diaminopyrimidines (Watkins & Gorrod 1987; El-Ghomari & Gorrod 1987). Nevertheless, microsomal preparations from dog liver contained only a small quantity of P-450 and yet produced relatively large amounts of *N*-oxides, possibly indicating that other cytochromes in addition to P-450 were involved in the *N*-oxidation process or that dog cytochrome P-450 possesses a very high specific activity for these substrates. Other enzyme systems such as the flavin containing mono-oxygenases which are responsible for the biological *N*-oxidation of a wide range of aliphatic and tertiary aromatic amines could also be involved in the observed *N*-oxidation. However, these enzymes have not been reported to participate in the *N*-oxidation of nitrogen containing aromatic heterocycles (Gorrod 1987; Zeigler 1988).

Acknowledgements

SPL wishes to thank the Croucher Foundation for a Scholarship and the ORS scheme for an award. DJB acknowledges the support of the London University Central Research Fund. This work was supported in part by the Medical Research Council.

References

Devinsky, F., Gorrod, J. W. (1987) Microsomal N-oxidation of long chain N,N-dimethylalkylamines: Quantitative structure-activity relationships. Europ. J. Drug Metab. Pharmacokin. 12: 267–273

El-Ghomari, K., Gorrod, J. W. (1987) Metabolic N-oxygenation of 2,4-diamino-6-substituted pyrimidines. Ibid. 12: 253–258

Gibson, G. G., Skett, P. (1986) Introduction to drug metabolism. Chapman and Hall, London. pp 241.

Gorrod, J. W. (1985) Amine-imine tautomerism as a determinant of the site of biological N-oxidation. In: Gorrod, J. W., Damani, L. A. (eds) Biological Oxidation of Nitrogen in Organic Molecules. Ellis Horword Ltd., Chichester, U.K. pp 219–230.

Gorrod, J. W. (1987) The *in vitro* metabolism of amino azaheterocycles. In: Benford, D. J., Bridges, J. W., Gibson, G. G. (eds) Drug Metabolism—from Molecules to Man. Taylor & Francis, London. pp 456–461

Gorrod, J. W., Temple, D. J. (1976) The formation of an N-hydroxymethyl intermediate in the N-demethylation of N-methylcarbazole *in vivo* and *in vitro*. Xenobiotica 6: 265–274

Guengerich, F. P., Macdonald, T. L. (1984) Chemical mechanism of catalysis by cytochromes P-450. Accts. Chem. Res. 17: 9–16

Kato, R. (1979) Characteristics and differences in the hepatic mixed function oxidases of different species. Pharmacol. Ther. 6: 41–98

Lam, S. P., Devinsky, F., Gorrod, J. W. (1987) Biological N-oxidation of adenine and 9-alkyl derivatives. Europ. J. Drug Metab. Pharmacokin. 12: 239–243

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall. R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265–275

McMahon, R. E. (1966) Microsomal dealkylation of drugs: Substrate specificity and mechanism. J. Pharm. Sci. 55: 941–946

Miller, G. L. (1959) Protein determination for large number of samples. Anal. Chem. 31: 964

Montgomery, J. A., Thomas, H. J. (1964) On the alkylation of adenine. J. Heterocyl. Chem. 1: 115–120

Omura, T., Sato, R. (1962) A new cytochrome in liver microsomes. J. Biol. Chem. 237: 1375–1376

Rekker, R. F. (1977) The hydrophobic fragmental constant: its derivation and application: a means of characterizing membrane systems. Pharmacochemistry Library Vol. 1. Elsevier Scientific Publishing Co, Amsterdam

Watkins, P. J., Gorrod, J. W. (1987) Studies on the *in vitro* biological N-oxidation of trimethoprim. Europ. J. Drug Metab. Pharmacokin., 12: 245–251

Zeigler D. M. (1988) Flavin-containing monooxygenases: catalytic mechanism and substrate specificities. Drug Metab. Rev., 19: 1–32