Demethylation of theophylline (1,3-dimethylxanthine) to 1-methylxanthine: the first step of an antioxidising cascade

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The reaction of theophylline with HO[•] radical, produced by photolytic methods at pH 7, was studied in aqueous solution and the products characterised by HPLC and GC-MS. In addition to the expected 1,3-dimethyluric acid, the formation of 1-methylxanthine and, to a lesser extent, of 3-methylxanthine was observed. Theoretical calculations confirmed the preferred formation of the former compound. Both demethylated products were also observed upon reaction of theophylline with O^{•−} radical anion at pH~13, and 1-methylxanthine was consumed faster than 3-methylxanthine after its formation. Molecular oxygen had no significant effect on the formation of the mono-methylxanthine derivatives. A reaction mechanism for the demethylation of theophylline by oxidising radicals is proposed. This demethylation reaction can play an important role in the protection of biological targets against oxidative stress as the first step of an antioxidising cascade.

Keywords: methylated xanthines, demethylation, redox properties, free radicals

Introduction

Oxidative stress in living tissues has been well characterised as the result of chemical changes in biological molecules induced by endogenous or exogenous oxidising species. The main biological targets of oxidative stress are proteins, lipids and DNA.¹ The agents of oxidative stress are UV or ionising radiations (γ - and X-rays) or chemical agents such as metabolites, xenobiotics and, mainly, free radicals, in particular the so-called reactive oxygen species (ROS) and reactive nitrogen species (RNS).^{2–4} Therefore, there has been increasing interest in studying and developing antioxidants to protect biological molecules against oxidative stress. Among the several classes of antioxidants studied, xanthines play an important role. These

compounds have a significant antioxidant effect and can protect biological targets against damage by ROS.^{5,6} Xanthine (3,7-dihydropurine-2,6-dione) and its riboside xanthosine are metabolites of the DNA purine bases found in human tissues and fluids. *N*-methyl derivatives of xanthine (such as caffeine, theobromine and theophylline) are present in several beverages and foods like coffee, tea, chocolate and cola.

Although the role of methylxanthines has been well documented *in vivo*, through metabolic degradation by enzymes (as, for example, in the case of caffeine⁷), there is not much information about the degradation of these compounds by oxidising radicals *in vitro*.

In a recent study,⁸ it has been demonstrated that 1-methylxanthine, a non-naturally occurring xanthine derivative, has higher antioxidant power compared to that of some natural xanthines usually present in the human diet, such as theophylline. It was also suggested that the high antioxidant capacity of the latter could be attributed to its conversion to 1-methylxanthine during metabolism.

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$R_1 = R_3 = \text{CH}_3$	Theophylline
$R_1 = \text{CH}_3, R_3 = \text{H}$	1-Methylxanthine
$R_1 = \text{H}, R_3 = \text{CH}_3$	3-Methylxanthine

Figure 1 Structure of xanthine derivatives

In the present work, the partial conversion of theophylline into 1-methylxanthine (1-MeX) was studied under oxidation by the biologically relevant hydroxyl radical, as a model for the possible demethylation pathway. By using HPLC and GC-MS, 1-MeX was identified by comparison of its retention time, UV and mass spectra with those of an authentic sample used as standard. The results show that theophylline undergoes demethylation from *N*(3) yielding 1-MeX and also from *N*(1) yielding 3-MeX. The mechanism of demethylation was supported by computational calculations. The structures of the compounds studied are presented in Figure 1.

Materials and methods

Reagents

1-Methylxanthine (1-MeX), 3-methylxanthine (3-MeX), theophylline (1,3-DiMeX) and adenine (all from Sigma), hydrogen peroxide (30% w/v, Panreac) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA; Fluka) were used without further purification. 4-Mercaptopyridine-*N*-oxide (Py-4-SH) was prepared as described in the literature.^{9,10} All solutions were prepared in Millipore Milli-Q water, buffered with Na_2HPO_4 or KH_2PO_4 (both Aldrich) and the pH adjusted with perchloric acid (Aldrich) or potassium hydroxide (Merck).

Reaction conditions and HPLC analysis

The reactions with $\text{HO}^\bullet$ radical at pH 7 were performed by irradiating aqueous solutions containing 1 mM 1,3-DiMeX, 20 mM H_2O_2 and 5 mM phosphate buffer. The reactions with $\text{O}^\bullet$ radical were performed by irradiating solutions containing 2 mM 1,3-DiMeX and 0.5 mM 4-mercaptopyridine-*N*-oxide

at pH ~13. The reaction times presented correspond to the higher global yields obtained for the demethylation products. In the case of the reaction with $\text{O}^\bullet$ radical, the higher reaction time reflects the lower reactivity with a given substrate as compared to the reaction with $\text{HO}^\bullet$ radical.¹¹ For the evaluation of the protection of adenine by antioxidants at pH 7, solutions containing 0.5 mM of both adenine and antioxidant, 20 mM H_2O_2 and 5 mM phosphate buffer were submitted to UV irradiation. The concentrations of both substrates in each binary mixture were monitored as a function of the irradiation time. Light was emitted from a 150 W Hanau high-pressure Hg lamp and UV irradiations were carried out in standard quartz cells placed at 5 cm from the lamp core. HPLC analysis of the irradiated solutions at room temperature (~20°C) were performed in a system consisting of a LC-10AS Shimadzu pump, a Reodhyne 7125 injector fitted with a 20 μl loop, a reversed phase analytical column (Purospher® RP18e column, 250-4 mm, 5 μm , Merck) and a Shimadzu SPD-M10A photodiode array detector. The eluent was a 5 mM KH_2PO_4 (pH 4) aqueous solution containing 20% (v/v) methanol (HPLC grade, Panreac) at 0.8 ml/min.

GC sample preparation

The reaction of theophylline with $\text{HO}^\bullet$ radical at pH 7 was carried out in an 800-ml irradiation vessel, using the same UV lamp placed in the centre of the reaction mixture. The formation of the reaction products was monitored by HPLC. After obtaining a maximal yield of demethylation products (corresponding to a total irradiation time of 18 min), the reaction mixture was concentrated, extracted with ethyl acetate and the extract evaporated. A sample of the dry residue was derivatised with 50 μl of MSTFA for 30 min at 70°C for GC-MS analysis.

GC-MS analysis

The separation and detection of the silylated derivatives was achieved using a GC Agilent 6850 interfaced to a MS Agilent 5975C VL MSD detector system. The gas chromatograph was equipped with a capillary column HP-5MS (5% phenylmethylsiloxane, model HP 19091S-433E, 30.0 m, 0.25 mm i.d. with 0.25 μm film thickness, Agilent Technologies Inc.). The experimental conditions for GC-MS were regulated as follows: carrier gas flow rate (helium, 1 ml/min), injector temperature 250°C, detector temperature 280°C, splitless mode, injection volume 1.0 μl , pressure 10.47 psi, purge flow 20 ml/min, average velocity 37 cm/s. The GC oven temperature

Figure 2 HPLC chromatogram after 4 min. UV irradiation of an aqueous solution of 1 mM theophylline, 20 mM H_2O_2 and 5 mM phosphate buffer (reaction with $\text{HO}^\bullet$ radical, pH 7)

was kept at 100°C for 3 min and programmed to 250°C at a rate of $6^\circ\text{C}/\text{min}$. The solvent delay was 6 min. The MS spectra were obtained by Electron Impact (EI) at 70 eV by using MSD ChemStation software (Agilent). The spectral m/z range used was 40–500 Da.

Computational methods

All species were drawn and pre-optimised using a Dreiding type molecular mechanics force field,¹² implemented in the Marvin software and the Calculator plug-ins.¹³ Calculations were performed using the Parametrized Model 6 (PM6)¹⁴ as implemented in MOPAC2009,¹⁵ within the restricted Hartree–Fock formalism and using a Conductor-like Screening Model (COSMO) method to approximate the effect of the solvent; water was used for the calculations. In all instances, geometry optimization was terminated when the gradient dropped below 0.01.

Results and discussion

Oxidation of theophylline by the hydroxyl radical

The reaction of theophylline with the hydroxyl radical, generated by UV photolysis of hydrogen peroxide, was performed under the conditions described above. After short irradiation times, the reaction products began to be detected. Figure 2 shows a chromatogram obtained after UV irradiation of an aqueous solution containing theophylline and H_2O_2 . In addition to the major peak of unreacted substrate (retention time ~ 15 min) two main products were detected – the corresponding uric acid of theophylline (1,3-dimethyluric acid) and 1-methylxanthine. For longer irradiation times, the concentration of these products decreased due to their reaction with $\text{HO}^\bullet$ radical.

1,3-Dimethyluric acid is an expected product, since it is known that xanthine and its derivatives react with the hydroxyl radical to yield the corresponding uric acid, upon addition of $\text{HO}^\bullet$ to C(8) followed by oxidation of the transient reducing radical.¹⁶ However, 1-methylxanthine is a new product, usually not referred as an oxidation product of theophylline. The formation of this compound and of a smaller amount of 3-methylxanthine (3-MeX) was confirmed by comparison of the retention times and UV spectra with those of authentic samples, as well by MS. The GC-MS chromatogram of a sample obtained in the GC sample preparation, as described in the experimental section, showed small peaks at retention times 10.7 min and 11.4 min, consistent with those obtained for authentic samples (derivatised the same way) of 3-MeX and 1-MeX, respectively. The mass spectra (consistent also with those of derivatised authentic samples) showed a molecular ion at m/z 310 and a base peak at m/z 295, corresponding to a fragment obtained by loss of a methyl group (see Fig. 3). Both spectra show an intense peak at m/z 73, corresponding to the trimethylsilyl ion from MSTFA. These results show that the two new products, formed by $\text{HO}^\bullet$ radical

Figure 3 Mass spectra of derivatised demethylation products obtained after reaction of theophylline with $\text{HO}^\bullet$ radical (see conditions described in Fig. 2)

Figure 4 Dependence of concentration on irradiation time in the UV photolysis of binary mixtures 0.5 mM in both substrates (adenine and antioxidant) and 20 mM H_2O_2 at pH 7

oxidation of theophylline, include two trimethylsilyl groups upon derivatisation, demonstrating that a methyl group has been lost from theophylline.

The GC chromatogram of the derivatised reaction mixture showed other peaks, some of which could be attributed to known further oxidation products of xanthines (e.g. dimethylparabanic acid, dimethylurea and dimethylallantoin), by comparison with the spectra database. The possible reaction pathway for the formation of 1-MeX is presented and discussed below. It is known that $\text{HO}^\bullet$ reacts with heterocyclic aromatic compounds mainly by addition to a double bond and, to a lesser extent, by H atom abstraction.^{17,18} The formation of the demethylated product (1-MeX) implies the loss of a carbon atom consistent with this latter mechanism (see Scheme 1), while the formation of the uric acid results from the usual addition of $\text{HO}^\bullet$ to a double bond. A trace of another possible demethylation product, 3-methylxanthine, was also detected (see Fig. 2). These demethylated products were always observed in small concentrations (compared with that of theophylline),

even for long irradiation times, which suggests that they are also consumed by the oxidising radical. This behaviour was confirmed with more detail concerning the reaction with $\text{O}^{\bullet-}$ (see below). Therefore, the expected further oxidation products by $\text{HO}^\bullet$ radical (1-methyl and 3-methyluric acids and xanthine) would be formed in yields below both HPLC and GC-MS detection limits. Under physiological conditions (pH 7) the demethylation of theophylline to 1-MeX seems to be significant, since both compounds play an important role as antioxidants that can protect biological targets against oxidative stress. In the case of, for example, adenine, as shown in Figure 4, both these compounds can protect the DNA base against oxidation by the hydroxyl radical.

In a recent study,⁸ it was demonstrated that 1-MeX is a stronger antioxidant than theophylline. Therefore, the present results suggest that the partial conversion of theophylline to 1-MeX can have a synergistic effect, where the demethylation reaction plays an important role as the first step of an antioxidising cascade.

Scheme 1 Reaction mechanism of demethylation of theophylline to 1-methylxanthine

Oxidation of theophylline under highly basic conditions ($\text{O}^{\bullet-}$)

To confirm the reaction pathway by H atom abstraction, theophylline was reacted at highly basic conditions, to study the reaction products resulting from oxidation by the conjugate base of the $\text{HO}^\bullet$ radical, which is known to be a much better H atom abstractor than $\text{HO}^\bullet$.¹⁹ Figure 5 shows a typical chromatogram obtained after UV irradiation of an aqueous solution containing theophylline and 4-mercaptopyridine-*N*-oxide, a good precursor of the $\text{HO}^\bullet$ radical,²⁰ that can be used at very high pH values, enabling the study of reaction products resulting from oxidation by the $\text{O}^{\bullet-}$ radical. At pH ~13 (where $\text{O}^{\bullet-}$ is the major radical species, due to the pKa value of $\text{HO}^\bullet$

Figure 5 HPLC chromatogram after 25 min. UV irradiation of an aqueous solution of 2 mM theophylline, 0.5 mM 4-mercaptopyridine-*N*-oxide (reaction with O_2^- , pH ~13)

= 11.9²¹), no uric acid was detected in the reaction mixture, as expected, but the formation of 1-MeX is evident. This is consistent with the ability of O^- to react by H abstraction, as discussed above. Moreover, in addition to 1-MeX, the other demethylation product 3-MeX is observed. This means that, under oxidation by O^- , only demethylation occurs, either from *N*(3) to yield 1-MeX or from *N*(1) to yield 3-MeX. In Figure 5, the peaks with retention times lower than that of 3-MeX correspond to the disulphide compounds well known as degradation products of 4-mercaptopyridine-*N*-oxide.²⁰

Another interesting observation is that demethylation at pH 13 occurring from *N*(1)-CH₃ was observed at a higher extent for all UV irradiation times (0–25 min), 3-MeX being formed in a larger amount than 1-MeX. The predominance of 3-MeX, as compared to 1-MeX, in solution after a long irradiation time can be due to the higher reactivity of this latter with O^- radical anion. To confirm this hypothesis, 1 mM solutions of 1-MeX and 3-MeX were reacted with O^- radical anion produced by UV photolysis of 4-mercaptopyridine-*N*-oxide at short irradiation times. By plotting ln of the concentration of the substrate as a function of irradiation time, the pseudo-first-order rate constants were determined as $(9.0 \times 10^{-4})^{-s}$ and $(1.6 \times 10^{-3})^{-s}$ for 3-MeX and 1-MeX, respectively, showing that 1-MeX is consumed faster than 3-MeX after their formation under the reaction conditions.

A possible reaction mechanism for the demethylation reaction (illustrated for demethylation

from *N*(3) to yield 1-MeX) is proposed in Scheme 1. This reaction mechanism pathway, leading to the loss of a methyl group attached to a nitrogen atom, seems to be a common one, as it was already observed by radical oxidation of caffeine²² or enzymatic metabolism of xanthines²³ and oxidative degradation of aminopyrine.^{24–26}

Effect of molecular oxygen

The results reported above were obtained with aqueous solutions of the substrates prepared under normal atmospheric conditions (air saturated). To elucidate a possible effect of molecular oxygen, the same experiments (reaction with $HO^\bullet$ and O^- radicals) were performed with argon de-aerated solutions, maintaining all other parameters constant. In both cases, very similar results were obtained (data not shown). In the case of reaction with $HO^\bullet$, the yield of 1-MeX formed after 4 min of irradiation of H_2O_2 -containing solution was exactly the same with or without oxygen, although the amount of 1,3-dimethyluric acid was larger in the presence of oxygen, which is an expected result. In fact, it has been observed in several purinic systems (*e.g.* adenine,^{27,28} guanosine^{29,30} and xanthines¹⁶) that the formation of an hydroxylation product at C(8), as is the case of uric acid, is enhanced in the presence of molecular oxygen (or another external oxidant) due to the necessary oxidation of the radical precursor, the C(8)-OH adduct. Concerning the reaction with O^- , the formation of 1-MeX and 3-MeX was also observed in the absence of oxygen without significant differences as compared to the results obtained with aerated aqueous solutions. These results show that molecular oxygen has no significant effect on the demethylation reaction under study, suggesting that molecular oxygen is not responsible for the second oxidation step proposed in Scheme 1, even in aerated aqueous media.

Table 1 Partial charges calculated for the most stable tautomer of theophylline (*N*(7)-H)

	Position	Atom	Partial charge (esu)
	2	N(1)	−0.7296
	4	N(3)	−1.2106
	13	C(1)	−0.7897
	14	C(3)	0.2638
	15	H(3)	0.0300
	16	H(3)	−0.0229
	17	H(3)	0.1172
	18	H(1)	0.2781
	19	H(1)	0.3921
	20	H(1)	0.2572

Figure 6 Reaction profiles for H abstraction of theophylline from *N*(1) and *N*(3) methyl groups, by reaction with HO $\cdot$ radical

Theoretical calculations

Theoretical calculations obtained with the PM6 model were performed to confirm the preferred demethylation position of theophylline by HO $\cdot$ radical. Since the *N*(7)-H tautomer is 6.12 kJ/mol more stable than the *N*(9)-H tautomer in aqueous solution, corresponding to more than 92% of the former in the equilibrium, only this one was considered. Table 1 presents the partial charges of the atoms involved in both possible demethylation reactions derived from the electrostatic surface potential. According to the values presented, the global charges of the *N*(1)-CH $_3$ and *N*(3)-CH $_3$ moieties are -0.1184 and -0.1645 (electrostatic units), respectively, showing that oxidation by H abstraction from *N*(3) is more favourable. These calculations are in good agreement with the experimental results obtained at pH 7 discussed above.

The same model was used to calculate the activation energies (E_a) for both demethylation reactions. As shown in Figure 6, the transition state of the demethylation reaction from *N*(1) (TS $_{N1}$) is 78 kJ/mol higher than TS $_{N3}$. These results suggest that the preferred demethylation from *N*(3) is kinetically controlled.

Conclusions

On reaction with oxidising radicals, theophylline undergoes demethylation from *N*(3) to yield 1-methylxanthine (and at a lesser extent from *N*(1) to

yield 3-methylxanthine). Both theophylline and 1-methylxanthine protect effectively biological targets against damage by oxidising radicals. Considering this reaction and assuming that the mono-methylated xanthine derivative can also demethylate to yield xanthine, which can also react with the oxidising radical, one equivalent of theophylline may scavenge a larger equivalent amount of the oxidant. Therefore, the demethylation reaction disclosed in this work can play an important biological role as the first step of an antioxidising cascade.

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References

1. von Sonntag C. *The Chemical Basis of Radiation Biology*. London: Taylor and Francis, 1987.
2. Cadet J, Berger M. Radiation-induced decomposition of the purine-bases within DNA and related model compounds. *Int J Radiat Biol* 1985; **47**: 127–143.
3. Steenken S. Purine bases, nucleosides, and nucleotides: aqueous solution redox chemistry and transformation reactions of their radical cations and e $^-$ and OH adducts. *Chem Rev* 1989; **89**: 503–520.
4. Myers LS. In: Pryor WA. (ed) *Free Radicals in Biology*. New York: Academic Press, 1990.
5. Langman SR, Shohoji MCB, Telo JP, Vieira AJSC, Novais HM. EPR spectroscopy study of the radical oxidation of hydroxypurines

- in aqueous solution: acid-base properties of the derived radicals. *J Chem Soc Perkin Trans 2* 1996; 1461–1465.
6. Vieira AJSC, Telo JP, Pereira HF, Patrocínio PF, Dias RMB. Antioxidant effect of naturally occurring xanthines on the oxidative damage of DNA bases. *J Chem Phys* 1999; **96**: 116–123.
 7. Dash SS, Gummadi SN. Catabolic pathways and biotechnological applications of microbial caffeine degradation. *Biotechnol Lett* 2006; **28**: 1993–2002.
 8. Santos PMP, Telo JP, Vieira AJSC. Structure and redox properties of radicals derived from one-electron oxidised methylxanthines. *Redox Rep* 2008; **13**: 123–133.
 9. Ochiai E. Recent Japanese work on the chemistry of pyridine 1-oxide and related compounds. *J Org Chem* 1953; **18**: 534–551.
 10. Barton DHR, Crich D, Kretzschmar G. The invention of new radical chain-reactions. 9. Further radical chemistry of thiohydroxamic esters – formation of carbon carbon bonds. *J Chem Soc Perkin Trans 1* 1986; 39–53.
 11. Armstrong DA, Asmus KD, Bonifacic M. Oxide radical anion reactivity with aliphatic amino compounds in aqueous solution: comparison of H-atom abstraction from C–H and N–H groups by $O^{\cdot -}$ and $HO^{\cdot}$ radicals. *J Phys Chem A* 2004; **108**: 2238–2246.
 12. Mayo SL, Olafson BD, Goddard III WA. Dreiding: a generic force field for molecular simulations. *J Phys Chem* 1990; **94**: 8897–8909.
 13. Marvin version 5.00 and Calculator plugins for Marvin 5.00, ChemAxon, Budapest, Hungary. 2008. Available from: <<http://www.chemaxon.com>>.
 14. Stewart JJP. Optimization of parameters for semiempirical methods V: modification of NDDO approximations and application to 70 elements. *J Mol Modeling* 2007; **13**: 1173–1213.
 15. Stewart JJP. MOPAC2009. Stewart Computational Chemistry, Colorado Springs, CO. 2008. Available from: <<http://openmopac.net>>.
 16. Vieira AJSC, Steenken S. Radical oxidation of xanthines: a reaction pathway to uric acid? *J Chem Phys* 1996; **93**: 235–243.
 17. Ferradini C, Pucheault J, Biologie de l'action des rayonnements ionisants. Paris: Masson, 1983; 98–99.
 18. von Sonntag C. *Free-Radical-Induced DNA Damage and Its Repair – A Chemical Perspective*. Berlin: Springer, 2006; 49–57.
 19. Scholes ML, Schuchmann MN, von Sonntag C. Enhancement of radiation-induced base release from nucleosides in alkaline solution: essential role of the $O^{\cdot -}$ radical. *Int J Radiat Biol* 1992; **61**: 443–449.
 20. Vieira AJSC, Telo JP, Dias RMB. Generation of hydroxyl radical by photolysis of mercaptopurine *N*-oxides: application to redox chemistry of purines. *Methods Enzymol* 1999; **300**: 194–201.
 21. Rabani J, Matheson MS. Pulse radiolytic determination of pK for hydroxyl ionic dissociation in water. *J Am Chem Soc* 1964; **86**: 3175–3176.
 22. Dalmazio I, Santos LS, Lopes RP, Eberlin MN, Augusti R. Advanced oxidation of caffeine in water: on-line and real-time monitoring by electrospray ionization mass spectrometry. *Environ Sci Technol* 2005; **39**: 5982–5988.
 23. Kanazawa H, Atsumi R, Matsushima Y, Kizu J. Determination of theophylline and its metabolites in biological samples by liquid chromatography–mass spectrometry. *J Chromatogr A* 2000; **870**: 87–96.
 24. Griffin BW, Ting PL. Mechanism of *N*-demethylation of aminopyrine by hydrogen peroxide catalyzed by horseradish peroxidase, metmyoglobin, and protohemin. *Biochemistry* 1978; **17**: 2206–2211.
 25. Lasker JM, Sivarajah K, Mason RP, Kalyanaram B, Abou-Donia MB, Eling TE. A free radical mechanism of prostaglandin synthase-dependent aminopyrine demethylation. *J Biol Chem* 1981; **256**: 7764–7767.
 26. Pérez-Gilbert M, Sanchez-Ferrer A, Garcia-Carmona F. Oxidation of aminopyrine by the hydroperoxidase activity of lipoxigenase: a new proposed mechanism of *N*-demethylation. *Free Radic Biol Med* 1997; **23**: 548–555.
 27. Vieira AJSC, Steenken S. Pattern of OH radical reaction with adenine and its nucleosides and nucleotides. Characterization of two types of isomeric OH adduct and their unimolecular transformation reactions. *J Am Chem Soc* 1990; **112**: 6986–6994.
 28. Dias RMB, Vieira AJSC. Substituent effect on superoxide elimination from peroxy radicals of adenine and methylated derivatives. *J Photochem Photobiol A: Chem* 1998; **117**: 217–222.
 29. Candeias LP, Steenken S. Reaction of HO center dot with guanine derivatives in aqueous solution: formation of two different redox-active OH-adduct radicals and their unimolecular transformation reactions. Properties of G(-H)(center dot). *Chem Eur J* 2000; **6**: 475–484.
 30. Vieira AJSC, Candeias LP, Steenken S. Hydroxyl radical induced damage to the purine bases of DNA: *in vitro* studies. *J Chem Phys* 1993; **90**: 881–897.