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ORIGINAL PAPER

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Modeling of the mechanism of nucleophilic aromatic substitution of fungicide chlorothalonil by glutathione

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Abstract Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile, TCIN, CAS 1897-45-6) is a broad range spectrum fungicide whose fungitoxic action has been associated with the rapid formation of conjugated chlorothalonil–cellular thiol derivatives, specifically with thiol-rich enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and with glutathione (GSH). The biotransformation reaction sequence between enzyme-activated glutathione (GSH) and chlorothalonil depletes cellular glutathione reserves. The conjugation of glutathione with chlorothalonil via nucleophilic aromatic substitution was modeled for an isolated reacting species using semiempirical self-consistent field molecular orbital (SCF-MO) theory at the PM3 level. The potential energy hypersurface at each of the three possible chlorinated attack sites on chlorothalonil was elaborated using a thiolate (CH_3S^-) anion as a model for an enzyme-activated glutathione molecule. Calculated free energies of activation for formation of mono-RSH conjugates suggest that the order of nucleophilic attack on chlorine positions in TCIN is 2>4, 6>5 although energy differences are small (on the order of 1–2 kcal mol^{-1}). Meisenheimer or σ-complexes have been isolated as true intermediates on the hypersurface for each reaction, suggesting that the mechanism follows a two-step pathway.

Keywords Chlorothalonil · Pesticide–glutathione conjugates · Nucleophilic aromatic substitution · Mechanism modeling

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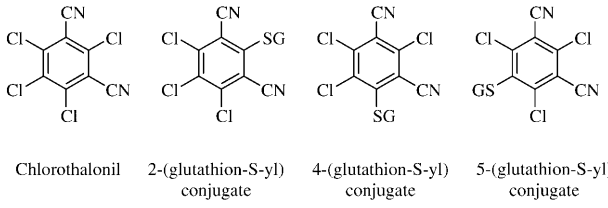
Abbreviations
TCIN 2,4,5,6-tetrachloroisophthalonitrile · *GST* glutathione-S-transferase · *GAPDH* glyceraldehyde-3-phosphate dehydrogenase · *AM1* Austin model 1 · *MNDO* modified neglect of differential overlap · S*CF-MO* self-consistent field molecular orbital · *PM3* parametric method 3 · *CDNB* 1-chloro-2,4-dinitrobenzene · *IM* ion–molecule · *TS* transition state · *INT* intermediate

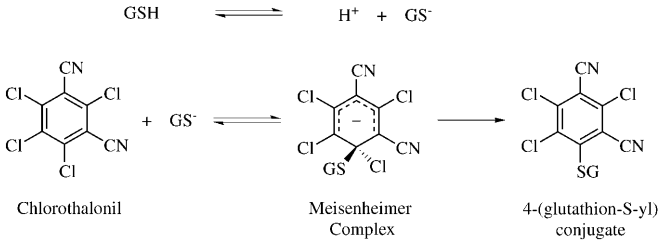
Introduction

Glutathione-S-transferases (GST) are a group of detoxifying enzymes found in most living organisms. GSTs catalyze the conjugation of a tripeptide glutathione (γ-Glu-Cys-Gly, GSH) to a variety of compounds with electrophilic groups. [1, 2] The detection of catalytic activity for the addition of GSH to 1,2-dichloro-4-nitrobenzene in cytosolic extracts of liver [3, 4] has escalated GST research in both terrestrial and marine organisms. [5] This class of enzymes is involved in the detoxification of known xenobiotics such as polynuclear aromatic hydrocarbons (PAHs), pesticides, carcinogens, chloroalkenes, polychlorinated biphenyls (PCBs), and other halogenated aromatics. [5, 6, 7, 8] Structures of the isoenzymes have been identified from five of the six principal families of this enzyme, differentiated by the type of binding sites and interactions between the protein and the thiolate formed from GSH. The active site of GST isoenzymes is characterized by the activation of the thiolate moiety of GSH by interaction with a hydroxyl group of serine or tyrosine. As a result, the thiolate formed by deprotonation of glutathione becomes a potent nucleophile interacting with a variety of electrophilic xenobiotic agents. [5, 7]

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile, TCIN) is an important broad range spectrum fungicide that is widely used in agriculture and other applications. [9] The fungicide is recognized as a skin and eye irritant



Scheme 1 Chlorothalonil and the 2-, 4-, and 5-conjugates with GSH



Scheme 2 Nucleophilic aromatic substitution of chlorothalonil with GS^-. The σ-complex as intermediate is also known as a Meisenheimer complex

with moderate acute and subchronic toxicity. The fungitoxic action of TCIN has been associated with its ability to form substituted chlorothalonil-glutathione derivatives rapidly, depleting cellular glutathione reserves. [10] In addition, TCIN is selectively toxic to fungi by binding to the thiol-rich enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). [11] Biotransformation reactions of chlorothalonil with GSH have been implicated in many higher animal systems. [12, 13, 14, 15] In vivo and in vitro studies of reactions between glutathione and TCIN show that nucleophilic replacement of chlorine at positions 2, 4, and 6 leads to the formation of mono-, di-, and tri-conjugation products. [10, 12, 16, 17, 18, 19]

In vivo, mono-conjugates are reabsorbed into the blood stream and arrive at the liver where further conjugation with GSH may lead to the formation of di-, tri-, and possibly tetra-GSH derivatives. In the liver, glutathione conjugates may then be secreted into the bile, before or after further GSH conjugation, for passage to the gastrointestinal tract, where they may be excreted or reabsorbed into the blood as intact GSH conjugates. [20] Reabsorbed GSH conjugates are returned to the liver for further conjugation and may reenter the bile or be directly transported to the kidney. Chlorothalonil metabolites finally arriving in the kidney consist of di- and tri-GSH conjugates, and cysteine S-conjugates. [12, 17] Biotransformation reactions of chlorothalonil in vivo or in vitro have not led to the isolation of mono-conjugates at sites 2 or 5 (Scheme 1). [10, 12, 13, 14, 15, 16, 17, 18, 19] However, the di- and tri-GSH conjugates, the 4,6-bis(glutathion-S-yl), 2,4-bis(glutathion-S-yl), and 2,3,6-tris(glutathion-S-yl) derivatives, have been observed from bile or liver subcellular fractions. [12, 13, 14, 15]

Vincent and Sisler [16] first suggested that chlorothalonil is an alkylating agent that reacts with functional cellular thiol groups by nucleophilic aromatic substitution, S_NAr, proceeding through a metastable σ-complex, also known as a Meisenheimer complex (Scheme 2). The first step of the reaction is usually the rate-determining step and involves the formation of the intermediate complex. In the second step, the intermediate breaks down to yield the thioether and chloride anion. Meisenheimer complexes are stabilized by strong electron-accepting groups *ortho* and/or *para* to the substitution site activating the ring to nucleophilic substitution. [21] Whether the Meisenheimer complex is a true intermediate or simply a transition state is open to debate. The reactivity of chlorothalonil at a particular site is facilitated by the electron-withdrawing properties of the nitrile and other chlorine atoms on *ortho* or *para* positions. According to a qualitative assessment of substituent effects, the *ortho*-positions, sites 2 and 4, to the nitrile groups would be more activated, hence reactive, than the chlorine located at site 5.

A limited number of theoretical studies have been reported dealing with gas-phase nucleophilic aromatic substitution reactions. [22, 23, 24, 25] Several of these reports have utilized semiempirical self-consistent field molecular orbital (SCF-MO) calculations using the AM1 [26] and MNDO [27] formalisms to study reactions between halobenzenes and nucleophiles. According to these studies, a Meisenheimer complex was sometimes identified as a true intermediate, while in others it is a transition state. Theoretical treatments of nucleophilic substitution in halobenzenes with strong electron-withdrawing substituents, for example *p*-chloronitrobenzene [24] and fluorobenzene, [25] have identified Meisenheimer complexes. However, the lack of stabilizing substituents, for example chlorobenzene, di-, and tri- chlorinated benzenes, does not support intermediate complexes. [28] Recently, Zheng and Ornstein have used ab initio methods to model a similar biotransformation reaction successfully, specifically the S_NAr reaction of 1-chloro-2,4-dinitrobenzene (CDNB) by glutathione. [22] Their work showed that the modeled reaction of CDNB and a thiolate anion gives an effective comparison to the enzymatic reaction involving glutathione S-transferases.

In this study, we have investigated the reaction mechanism of nucleophilic aromatic substitution by an addition–elimination process involving the fungicide chlorothalonil and glutathione using semiempirical PM3 [29] molecular orbital theory. Although the reaction was modeled as an isolated system (gas phase), it has some parallels to the enzyme-catalyzed reaction. Both the enzyme system and the gas phase provide an environment unencumbered by solvent, which raises activation barriers. [22] Obviously the enzyme may place stereochemical and steric constraints on the reaction that are not present in the isolated reaction. In any case, our isolated model for the nucleophilic aromatic substitution does provide an understanding of the mechanism and energetics of the *intrinsic* reaction between chlorothalonil and activated glutathione. The minimum energy reaction hypersurface was modeled at three possible chlorinated attack sites. Identification of a Meisenheimer complex in

each case would imply that the mechanism follows a two-step bimolecular nucleophilic aromatic substitution reaction with similarity to the reaction with CDNB. Calculations on isolated reacting systems help to predict the most probable reaction sites and provide a better understanding of mono-, di-, and tri-GSH conjugate formation as described in the literature. [10, 12, 13, 14, 15, 16, 17, 18, 19, 20]

Computational methods

Semiempirical self-consistent field molecular orbital (SCF-MO) calculations were carried out using PM3 [29] methodology available in the software package GAUSSIAN 94. [30] PM3 molecular orbital theory was chosen over AM1 [26] for two reasons. First, PM3 is more efficient in dealing with hydrogen bonding. Second, AM1 gives a large error associated with the chloride ion. [29] Optimizations of geometry were accomplished with the Fletcher–Reeves [31, 32, 33, 34] method followed by a frequency calculation to ensure that the stationary points all have the proper local structure. Transition states were obtained using the Eigenvector Following [35] routine and characterized by calculating harmonic vibrational frequencies to verify the presence of only one imaginary force constant corresponding to the interconversion of reactants to products. [36] Although semiempirical molecular orbital methods are generally less reliable that high-level ab initio methods, PM3 calculations compare favorably with ab initio results and provide a semi-quantitatively correct estimate of energetics and structures. Other work [28] performed by our group dealing with chlorinated aromatic systems and thiolate (thiomethoxide) anion showed that PM3 results are very similar to the ab initio results with split-valence basis sets obtained at the HF level of theory.

Results and discussion

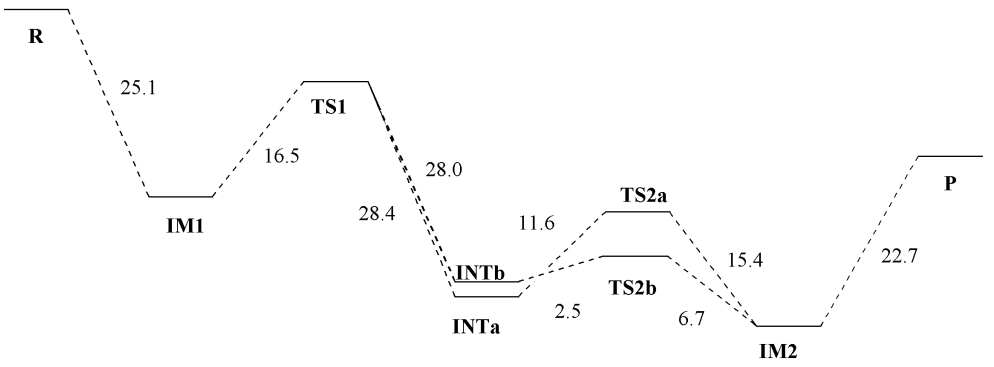
The potential energy hypersurface of the reactions between chlorothalonil and thiomethoxide ion, a model for enzyme-activated glutathione at the reactive sites 2, 4, and 5, was investigated. Table 1 lists the calculated heats of formation for each species involved in this study. Figure 1 presents the calculated potential energy surfaces for reactions of thiomethoxide at each unique site on the chlorothalonil molecule. In each reaction profile, the approach of the thiolate anion (CH_3S^-) to TCIN leads to an initial ion–molecule complex (IM1). Development of a bond between thiolate anion and chlorothalonil at a given reactive position on the aromatic ring results in a Meisenheimer complex as a true intermediate (INT) after passing through the first transition state (TS1). This Meisenheimer complex breaks down by passing through a second transition state (TS2) followed by a second ion–molecule complex (IM2). Further dissociation produces the isolated products, an aromatic thioether and chloride ion. In our studies, we have identified two different conformations of the intermediate (INTa and INTb) and two related conformations of the second transition state (TS2a and TS2b) complex corresponding to separate σ-complex breakdown pathways. The calculated PM3 optimized geometries of reactant and products are shown in Fig. 2.

Table 1 Calculated PM3 heats of formation (kcal mol^{-1}) for species from the reaction of chlorothalonil with thiolate

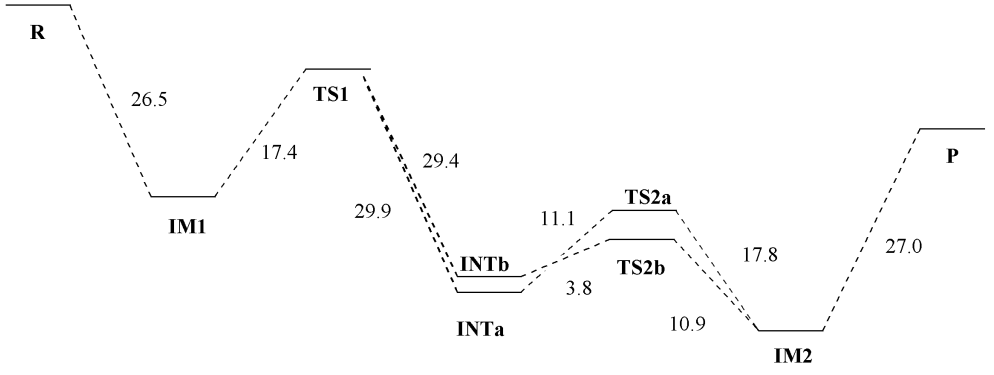
Reaction species	Reaction at site 2 $\Delta H°_f$	Reaction at site 4 $\Delta H°_f$	Reaction at site 5 $\Delta H°_f$
Thiolate anion	–22.2	–22.2	–22.2
Chlorothalonil	79.2	79.2	79.2
IM1	31.9	30.5	30.3
TS1	48.4	47.9	47.9
INTa	20.0	18.1	25.9
INTb	20.4	18.5	25.8
TS2a	31.6	29.2	29.8
TS2b	22.9	22.3	25.8
IM2	16.2	11.4	11.2
Chlorothalonil thioether	90.1	89.7	89.0
Chloride anion	–51.2	–51.2	–51.2
Activation energy	16.5	17.4	17.6
Heat of reaction	–18.1	–18.5	–19.2

For each reactive site, the overall reactions are exothermic, and the two transition state energies are lower than combined energies of the isolated reactants. However, calculations predict a modest energy barrier from the first ion–molecule complex to the corresponding transition state. The activation energies of the reactions at sites 2, 4, and 5 are 16.5, 17.4, and 17.6 kcal mol^{-1}, respectively. The calculated activation energies suggest that the order of nucleophilic attack on chlorine positions in TCIN would be 2>4, 6>5 although energy differences are insignificant. The activation energies suggest that the *ortho*-positions to the cyano group in TCIN are slightly more activated than site 5. However, reaction at site 5 is predicted to be feasible. Compared to the PM3 value for the reaction of CDNB with a thiolate anion (17.8 kcal mol^{-1}), the activation energy for TCIN at the reaction sites 2, 4, and 5 are about 1.3, 0.4, and 0.2 kcal mol^{-1}, respectively, lower in energy. The activation energy values suggest that in the gas phase TCIN and CDNB are of comparable reactivity with thiomethyl anion. It is useful to note that PM3 energy values may require adjustment to account for the general difficulty in treating anions at SCF levels. For instance, the proton affinity of the thiolate ion is predicted by PM3 to be 20–30 kcal mol^{-1} lower than that from experiment [37, 38] or higher levels of theory.[1] Our calculated ab initio proton affinities for thiolate ion, 363.9 kcal mol^{-1} (HF/3-21G*), 361.2 kcal mol^{-1} (HF/6-31G*), 359.0 kcal

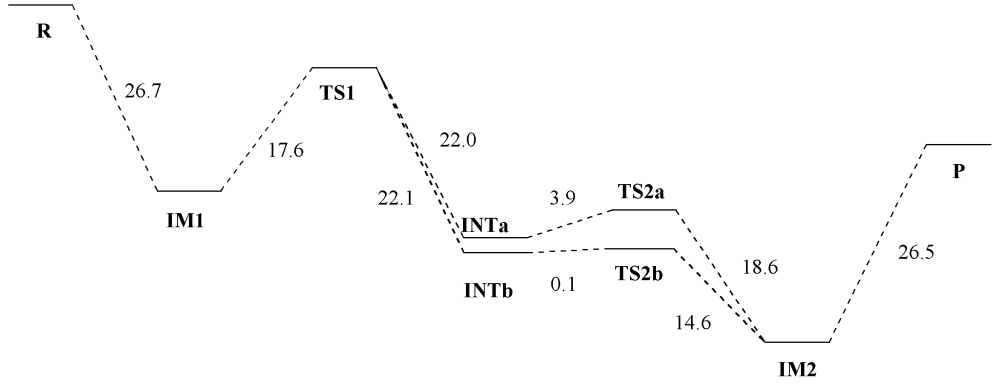
[1] The proton affinity of thiolate ion is predicted to be 333.9 kcal mol^{-1} by PM3 in comparison with experimental gas-phase proton affinities of 357.7, 362.5, and 359.0 kcal mol^{-1}, [37] and 395.3 and 391.5 kcal mol^{-1} [38].



(a) Hypersurface for the reaction between chlorothalonil and thiolate anion at reactive site 2



(b) Hypersurface for the reaction between chlorothalonil and thiolate anion at reactive site 4



(c) Hypersurface for the reaction between chlorothalonil and thiolate anion at reactive site 5

Fig. 1 The calculated potential energy surface of chlorothalonil at the PM3 level of theory. Values in kcal mol^{-1}

mol^{-1} (HF/6-311++G(2d,p)), and 359.7 kcal mol^{-1} (MP4(SDQ)/6-311++G(2d,p)), agree more closely with literature values from experiment.

In addition, the energy barrier for the formation of the intermediate (INTa or INTb) is considerably larger than its breakdown. The barrier height of formation for the second transition state (TS2a) is about half of the activation energy for the first step, except for the intermediate formed by the reaction at site 5. The energy barrier for the formation of TS2a or TS2b at site 5 is less than one half of that predicted at sites 2 and 4, indicating that the Meisenheimer intermediate complex for this reaction is only weakly stable. For sites 2 and 4, the second breakdown pathway leading from INTb to IM2 through TS2b is associated with an activation energy that is approximately one third that of the pathway leading from INTa to IM2 through TS2a. The corresponding barrier at site 5 is almost non-existent. The calculated PM3 optimized geometries for species involved in these reactions are shown in Figs. 3, 4, 5, and 6.

Both ion–molecule complexes (IM1 and IM2) are stationary states corresponding to minima on the potential

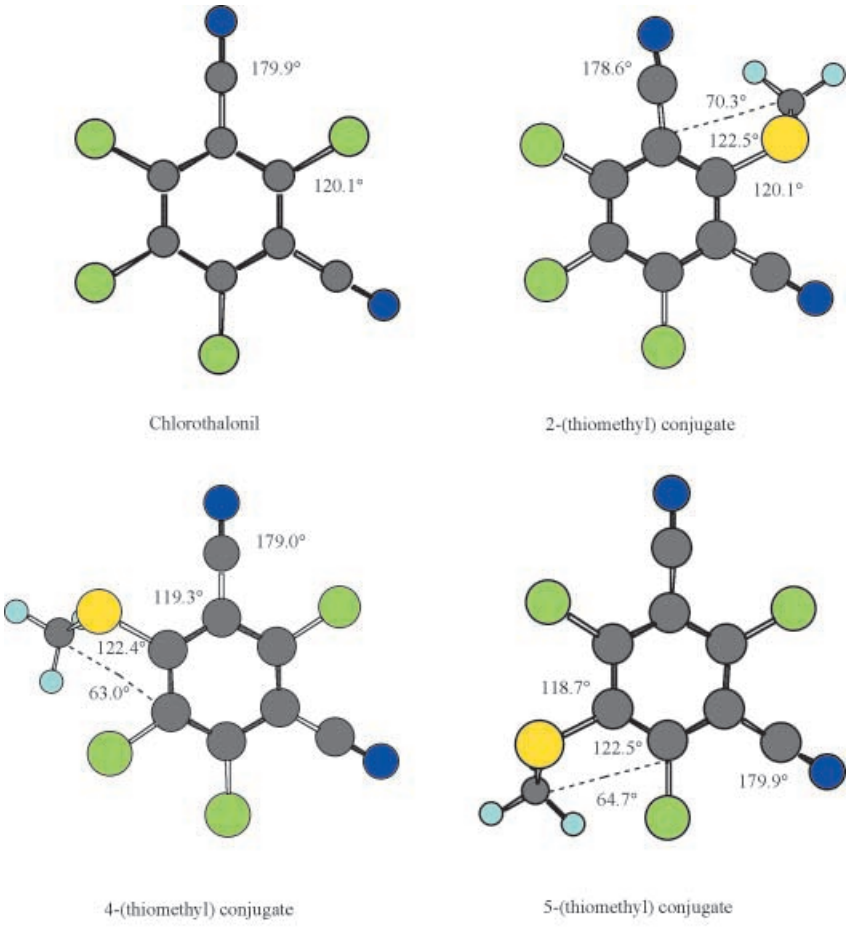


Fig. 2 Calculated geometries of chlorothalonil and thioether products of reactions at sites 2, 4, and 5 using the PM3 level of theory

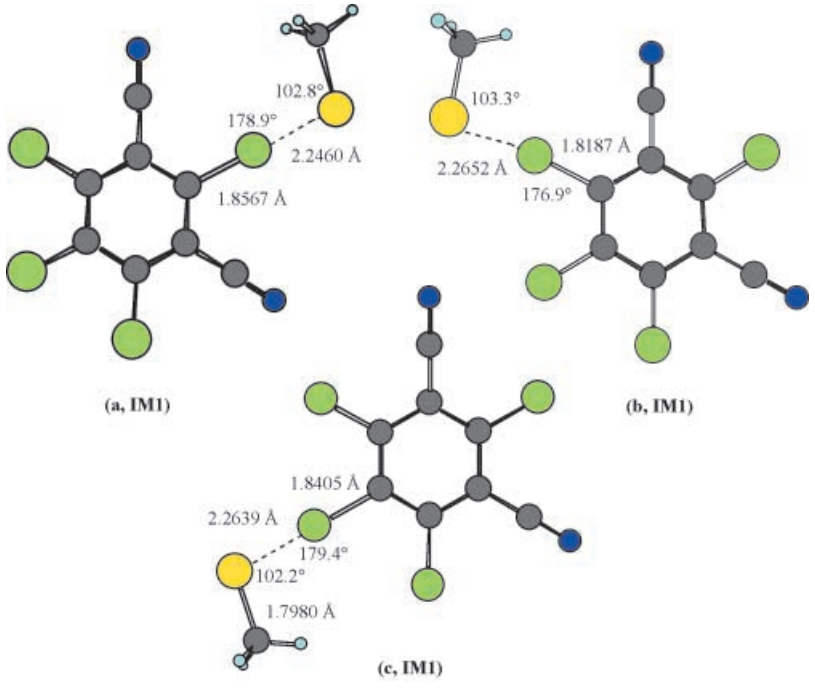


Fig. 3a–c Calculated geometries of the first ion–molecule (IM1) complexes involving chlorothalonil at reactive site 2 (**a**), 4 (**b**), and 5 (**c**) using the PM3 level of theory

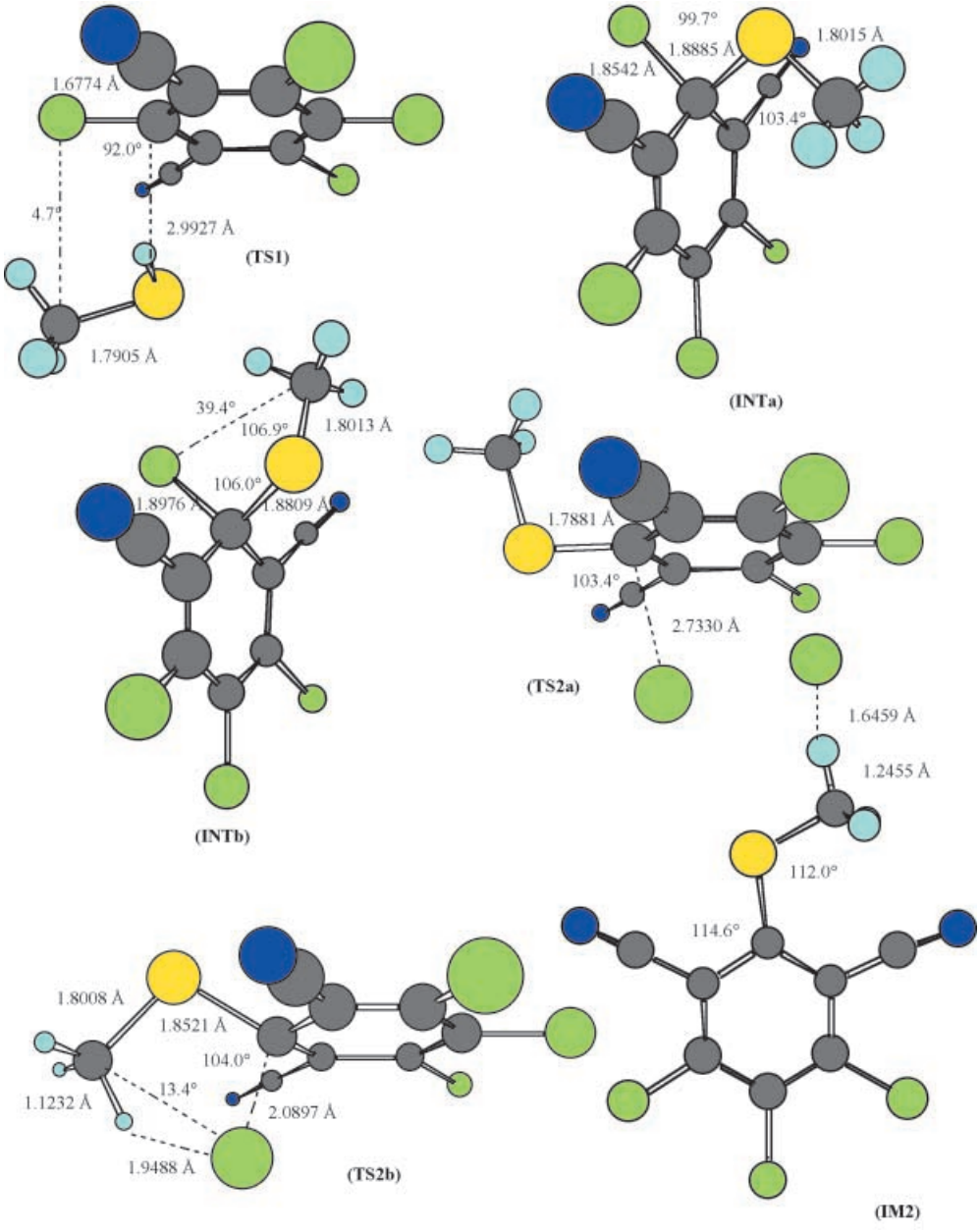


Fig. 4 Calculated geometries for compounds involving chlorothalonil at reactive site 2 using the PM3 level of theory

energy surface (Figs. 3, 4, 5, and 6). In each IM1 formed, the negatively charged nucleophilic group lies in the aromatic plane, which is consistent with favorable charge–dipole interactions. The geometry of the IM1 complexes agrees with previous studies performed at the semiempirical [25] and the ab initio [22, 28] levels of theory. Previous semiempirical studies have located charge-transfer complexes in which the nucleophile lies above the aromatic ring. [23, 24] All attempts to locate such charge-transfer complexes with the anion above the aromatic ring consistently lead to our reported structures, indicating that this type of charge-transfer complex does not exist as a minimum on the potential energy hypersurface. Further attempts to find lower energy ion–molecule complexes, such as complexes where the negatively charged sulfur atom is placed between two aromatic chlorines, have always produced our reported structures. In the ion–molecule complex formed between TCIN and thiolate anion, the negatively charged sulfur atom lies in a direct line to the bound chlorine at a distance of about 2.2–2.3 Å. The aromatic C–Cl bond opposite the negatively charged sulfur is increased about 10%. Although such ion–molecule complexes are common in the gas phase, they probably have little importance in the enzymatic reaction.

The first transition state (TS1) was identified involving partial bond formation between the aromatic carbon and sulfur atom of the thiolate anion in each reaction scheme (Figs. 4, 5, and 6). The nucleophile is located above the ring plane at a C–S bond distance of about 2.9–3.2 Å. In the transition state structure, the aromatic ring remains essentially planar consistent with the relat-

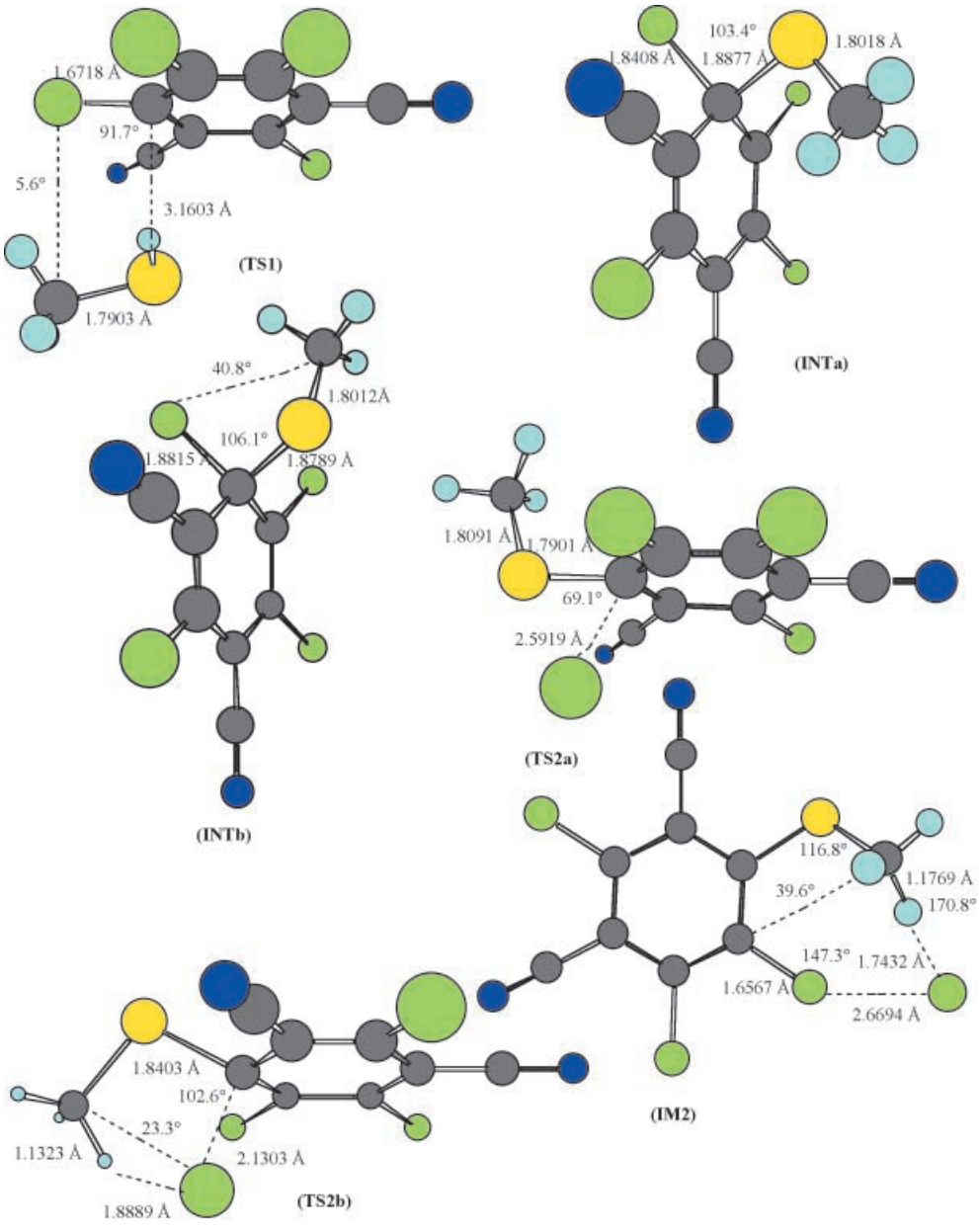


Fig. 5 Calculated geometries for compounds involving chlorothalonil at reactive site 4 using the PM3 level of theory

ed structures for chlorinated aromatic systems [28] and CDNB. [22] Both Mulliken and CHELP charges substantiate that only a small portion of the negative charge from the thiolate ion (9% – Mulliken – and 15% – CHELP) is transferred to the aromatic ring at the transition state (TS1). The lowest unoccupied molecular orbital (LUMO), routinely related to reactivity of electrophilic reagents, is a π MO in the electrophile chlorothalonil, which becomes the LUMO of the transition state (TS1) (Fig. 7). In contrast, the highest occupied molecular orbital (HOMO) of the nucleophile (thiolate) becomes the HOMO of the transition state, localized almost exclusively in the thiolate ion.

Intermediates observed in reactions at sites 2, 4, and 5 may assume two types of geometries each with a different energy. Complexes formed at sites 2 and 4 have lower energy when the methyl group of the nucleophile is located directly above the ring (INTa). A higher energy conformation (INTb) has the methyl group located away from the aromatic ring above the leaving group. Following the Intrinsic Reaction Coordinate (IRC) [39] from TS1 leads to this intermediate (INTb). On the reactant side of the reaction coordinate, the IRC proceeds to IM1. For intermediate complexes formed at reactive site 5, the INTb conformation is lower in energy than INTa. The energy differences between INTa and INTb conformations at all sites are minimal and are about 0.1–0.4 kcal mol^{-1}. The geometry of the INTa complex has not been previously reported in the literature. [22, 23, 24, 25, 28] The ring structure of both types of intermediates is still planar, indicating the presence of strong hyperconjugation between the π-orbital and the σ^*-antibond-

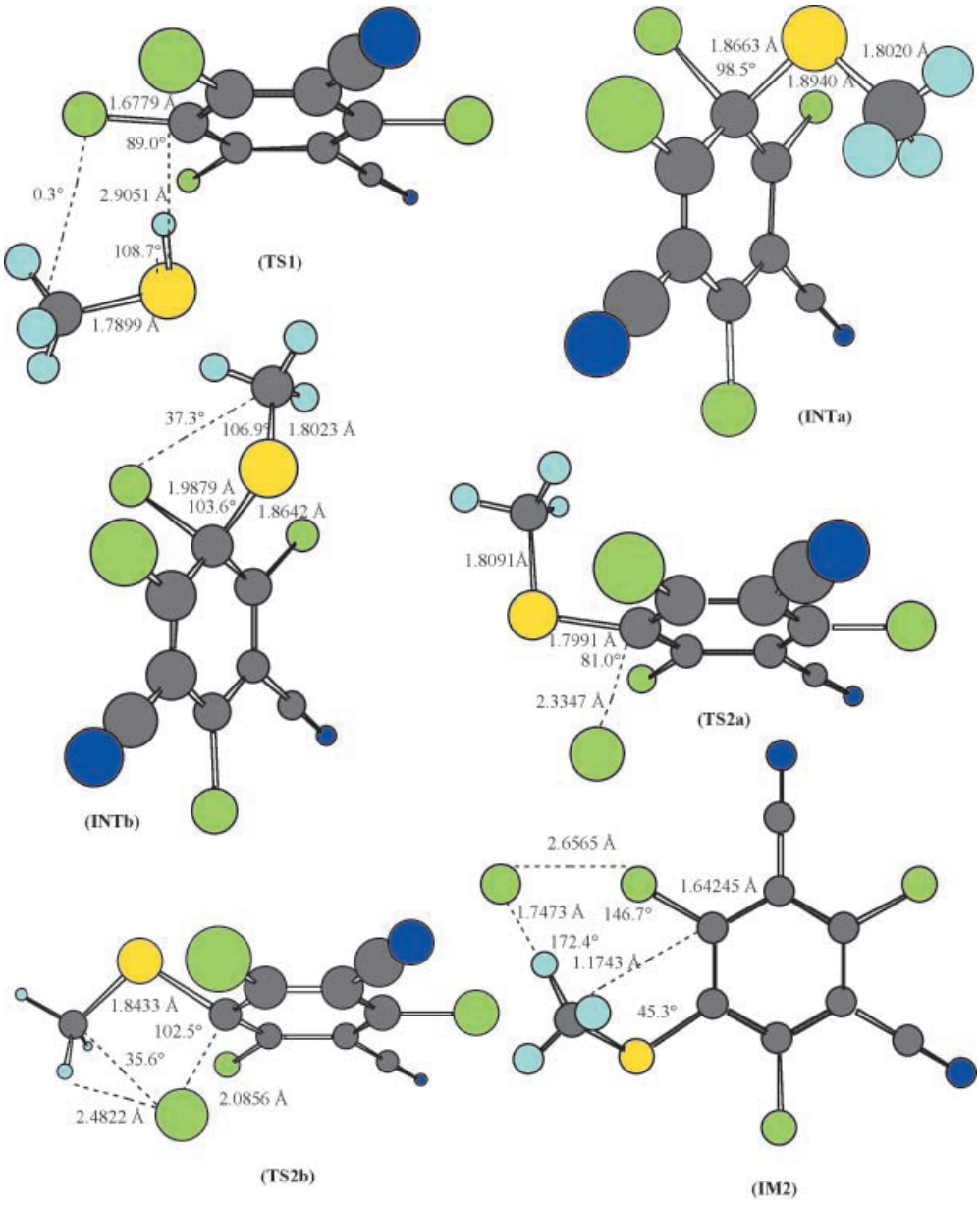


Fig. 6 Calculated geometries for compounds involving chlorothalonil at reactive site 5 using the PM3 level of theory

ing orbitals of the C–Cl and C–S bonds. The presence of strong hyperconjugation is evident in each intermediate by the long C–Cl and C–S bonds. The C–Cl and C–S bond lengths are about 1.8–1.9 Å and 1.9 Å, respectively. Although only relatively little negative charge is transferred from thiolate at the transition state, at the point that the intermediate, INTb, is reached almost all of the charge has been transferred to the aromatic ring as revealed by Mulliken and CHELP charges (90% and 72% charge transferred, respectively).

Each conformation of the intermediate complex breaks down into an ion–molecule complex by passing through a separate second transition state complex (TS2a and TS2b). The geometries of the two TS2 complexes are each related to one of the intermediate complexes (INTa or INTb). The geometry of the TS2a complex is similar to INTa except for the presence of a partially dissociated C–Cl bond of about 2.3–2.8 Å. In contrast, the TS2b complex has a geometry similar to INTb. The partially dissociated C–Cl bond length is between 2.0 and 2.2 Å.

In the second ion–molecule complexes formed by reaction at sites 4 and 5, the chloride ion is directly adjacent to one of the methyl hydrogens of the thioether at a distance of about 1.7 Å. The distance between the chloride and the nearest bound chlorine atom is between 2.6 and 2.7 Å. A Cl–-Cl–C angle of about 146–147° is formed. The aromatic C–Cl bond has slightly decreased due to interactions with the leaving group. The Cl–-H distance on the thioether and the Cl–-H–Cl angle are between 1.1 and 1.2 Å and between 170 and 172°, respectively. In addition, the methyl of the thiolate group is lo-

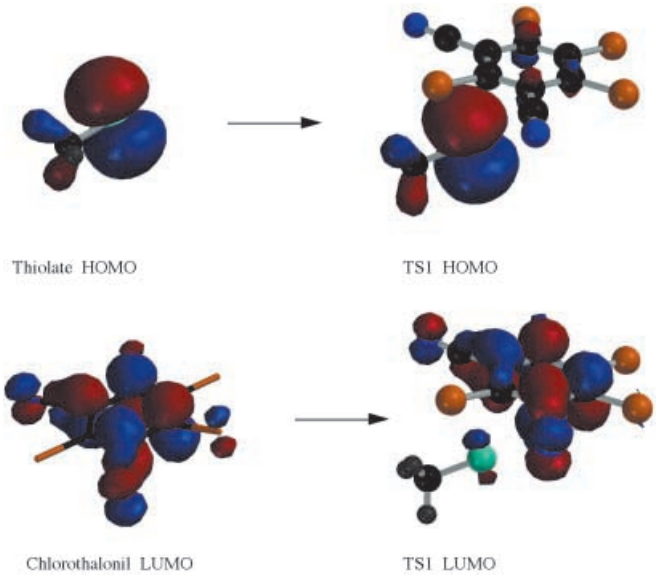


Fig. 7 Graphical maps of HOMO and LUMO of the transition state (TS1) and their origin in MOs of chlorothalonil and thiolate

cated slightly above the ring plane at a dihedral angle of around 40–45°, which is similar to the geometry of the thioether product.

The overall reaction of TCIN and thiolate to thioether and chloride ion is calculated to occur at sites 2, 4, and 5 with standard enthalpies of reaction of –18.1, –18.5, and –19.2 kcal mol^{-1}, respectively. Compared to the PM3 standard enthalpy of reaction for CDNB with a thiolate anion (–20.0 kcal mol^{-1}), the standard enthalpies of reaction for TCIN at all sites are close in energy although the reaction with TCIN is slightly less exothermic. It appears that the formation of the thioether at site 5 is slightly more energetically favorable compared to product formation at sites 4 and 2. The overall reaction enthalpies do not correspond to the observed product distribution and reactivity. In contrast, the product distributions are more consistent with the calculated activation energies. This suggests that this reaction process is kinetically, rather than thermodynamically, controlled. However, energy differences among the reaction sites are too small to draw definitive conclusions about relative reactivity of the various sites.

Free energies and entropies along with activation parameters were calculated for all reaction species using the calculated harmonic vibrational frequencies and standard thermodynamic equations (Table 2). Gas phase free energy surfaces (PM3) were constructed showing profiles of the reactions of thiolate anion and chlorothalonil at each reactive site (Fig. 8). The formation of the ion–molecule complex, IM1, formed by the reaction at site 5 appears to be entropically disfavored compared to the formation of IM1 at site 2 and 4 by over 5 cal mol^{-1} K. The minimum for this ion–molecule complex is lower in energy on the free energy surface, whereas the minima of the ion–molecule complexes formed by the reaction at sites 2 and 4 are greater in energy on the free energy surface. This results in a free energy of activation at site 5 that is 1.5–2.5 kcal mol^{-1} greater than that at sites 2 and 4. The difference in free energies of activation between sites 2 and 4 is negligible making these reaction sites virtually indistinguishable in reactivity.

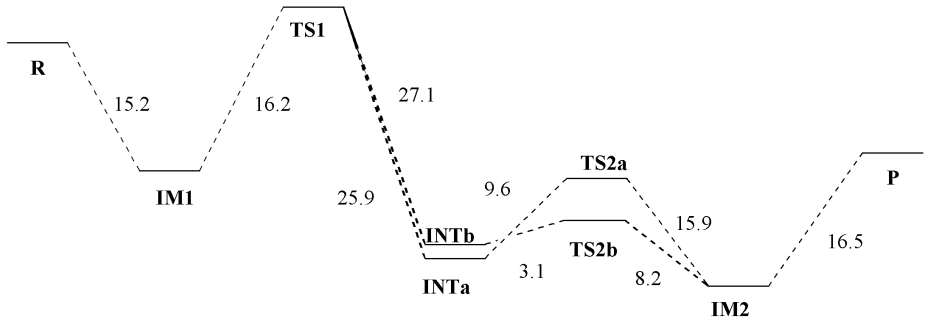
Conclusions

Our calculations provide an understanding of the intrinsic reactivity of chlorothalonil with a glutathione model with some implications for the enzyme system. In summary, we have demonstrated that the reactions of chlorothalonil (TCIN) with enzyme-activated glutathione (modeled by thiomethoxide ion) are predicted to take place by a two-step process through an intermediate Meisenheimer complex. The minimum energy reaction pathway for each substitution consists of an initial formation of an ion–molecule complex, IM1, movement through a first transition state, TS1, to form the

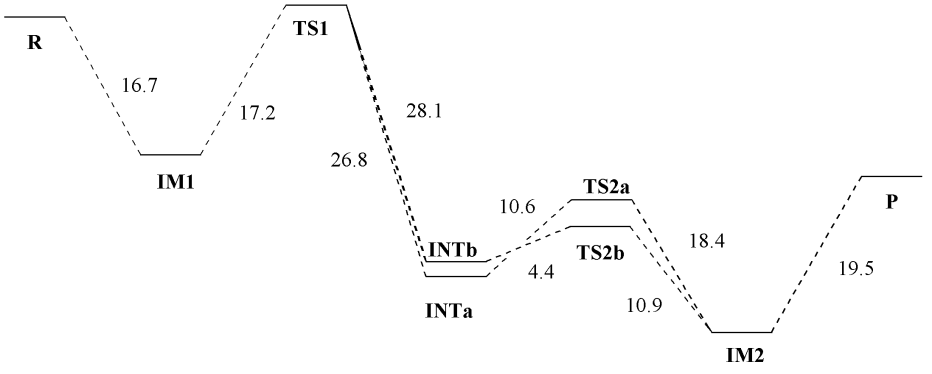
Table 2 Calculated PM3 free energies and entropies for species involved in the reactions of chlorothalonil with thiolate (298 K and 1 atm)

Reaction species	Reaction at site 2		Reaction at site 4		Reaction at site 5	
	$G°$ (kcal mol^{-1})	$S°$ (cal mol^{-1} K^{-1})	$G°$ kcal mol^{-1}	$S°$ cal mol^{-1} K^{-1})	$G°$ (kcal mol^{-1})	$S°$ (cal mol^{-1} K^{-1})
Thiolate anion	–13.9	57.9	–13.9	57.9	–13.9	57.9
Chlorothalonil	92.1	115.9	92.1	115.9	92.1	115.9
IM1	63.0	141.7	61.5	141.8	60.0	148.3
TS1	79.2	142.6	78.7	142.9	78.7	142.6
INTa	53.3	137.1	51.9	136.1	58.4	138.5
INTb	52.1	140.0	50.6	139.0	57.5	141.9
TS2a	62.9	141.6	62.5	134.6	61.9	138.2
TS2b	55.2	134.9	55.0	133.4	59.6	132.4
IM2	47.0	139.6	44.1	136.0	43.9	136.3
Chlorothalonil thioether	124.2	128.5	124.3	126.8	123.6	127.0
Chloride anion	–60.7	36.6	–60.7	36.6	–60.7	36.6
$\Delta G^{‡}$	16.2		17.2		18.7	
$\Delta S^{‡}$		0.8		1.1		–5.6

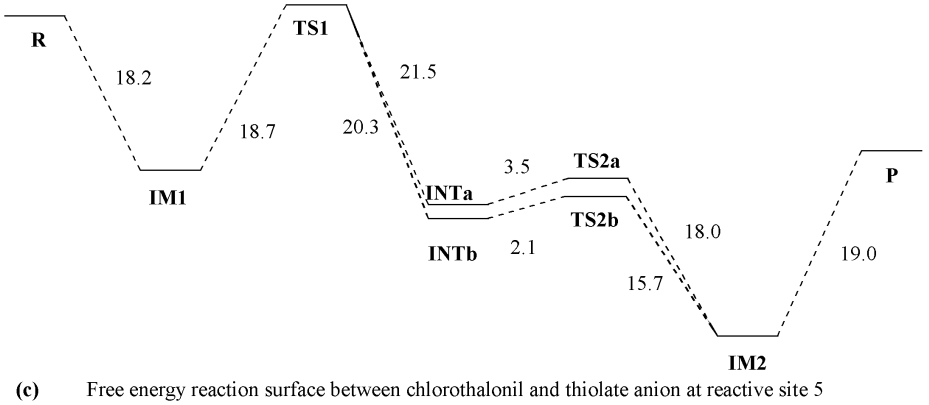
Fig. 8 The calculated free energy surface of chlorothalonil at the PM3 level of theory. Values in kcal mol^{-1}



(a) Free energy reaction surface between chlorothalonil and thiolate anion at reactive site 2



(b) Free energy reaction surface between chlorothalonil and thiolate anion at reactive site 4



(c) Free energy reaction surface between chlorothalonil and thiolate anion at reactive site 5

Meisenheimer complex, INTa or INTb. Decomposition of the intermediate through a second transition state, TS2a or TS2b, yields a second ion–molecule complex, IM2, which then dissociates to form the isolated products. Calculated activation energies suggest that the relative rates of conjugate formation with TCIN would be 2>4, 6>5, but energy differences are negligible and must be viewed with caution.

Consistent with our calculations, experimental product distributions [10, 12, 13, 14, 15, 16, 17, 18, 19] from in vivo and in vitro studies show conjugation with glutathione at sites 2, 4, and 6. In contrast, we predict the formation of the 5-(glutathion-S-yl) conjugate to be disfavored over other sites in TCIN by kinetic factors. Although the activation energy difference for conjugation at site 5 and at site 4 is computed to be small, the difference in free energy of activation for these sites is somewhat greater, suggesting that entropy factors are important in disfavoring formation of the 5-(glutathion-S-yl) derivative.

Thus, conjugation by glutathione at sites 2, 4, and 6 is predicted to predominate for TCIN in our study. It is difficult to predict which site, 2 or 4 (6), would be favored. Although the calculated activation energy or free energy of activation is less for the formation of the 2-thiomethyl conjugate by about 1 kcal mol^{-1}, formation of the 4-thiomethyl conjugate is thermodynamically favored by about 0.5 kcal mol^{-1}. Again the energy differences are virtually insignificant for sites 2 and 4 (6).

Qualitatively considering known substitution and steric effects, the most probable nucleophilic attack site would be site 4. This reactive site is both *ortho* and *para* to electron-withdrawing nitrile groups. The 4 or 6 positions are more accessible to nucleophilic attack than the 2 position mainly due to steric interactions. In addition, the attacking nucleophile has two equivalent sites from which to choose, thus the sites 4 or 6 are statistically favored by a factor of two. In vivo and in vitro studies have implicated the 4-(glutathion-S-yl) derivative [12, 13, 14, 15, 16, 17, 18] as a key product. The 4-(glutathion-S-yl) conjugates have been attributed to nephrotoxicity in rats and toxicity to the kidney. [12, 17] Monoglutathione conjugates are known to elicit the same nephrotoxic effects as those observed with chlorothalonil when administered at equimolar doses for 90 days to rats. [17] It has been suggested that this is related to the further conjugation with GSH leading to the formation of di- and tri-GSH conjugates. Although Hamersak et al. [18] have isolated and spectroscopically characterized primarily the 4-(glutathion-S-yl) conjugate when TCIN was treated with less than equimolar amounts of glutathione, considerable amounts of the 2,4-bis and 4,6-bis derivatives have been isolated when greater amounts of glutathione are present. [12, 13, 14, 15, 16] The 2,4,6-tris (glutathion-S-yl) derivative [12, 13, 14, 15, 16, 18] conjugate has been observed when high quantities of glutathione are present.

The further reaction with additional molecules of glutathione makes it difficult to assess the relative reactivity of sites 2, 4, and 6. The absence of the 2-mono(glutathion-S-yl) conjugate is puzzling. It is conceivable that, when the 2-(glutathion-S-yl) conjugate is formed, it may be more easily reabsorbed and further reacted than the 4-(glutathion-S-yl) derivative. Alternatively the 2-(glutathion-S-yl) compound may simply be more reactive than 4-(glutathion-S-yl), accounting for the appearance of 2-conjugation only in the di- or tri-GSH conjugates. A further consideration is that the enzyme may place stereochemical and steric constraints on the approach of the substrate, which result in reactivity differences for the three reaction sites, which are not reflected in the isolated reaction. In any case, it is clear that the similar predicted reactivity at sites 2 and 4 is borne out by the isolated products in the in vivo and in vitro experimental studies.

Finally and perhaps most importantly, this investigation confirms that the reaction between chlorothalonil and enzyme-activated glutathione as modeled by thiomethoxide follows a mechanism which is very similar to that for 2,4-dinitrochlorobenzene (CDNB), a known and well-studied substrate of glutathione-S-transferases. It is consistent with a two-step process through a Meisenheimer intermediate, which was also predicted for the reaction of glutathione with CDNB at both the PM3 [40] and ab initio [22] levels. The activation energies, free energies of activation, and overall reaction enthalpies for chlorothalonil are very comparable with those calculated for CDNB. Both theory and experiment strongly indicate that these nuclear aromatic substitution reactions are not only feasible but also important pathways by which cellular glutathione is depleted by chlorothalonil.

Supplementary material. Geometries of reactants, products, ion–molecule complexes, intermediates, and transition states for the compounds studied are available as PDB files.

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