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Structure–activity relationships in 3-isothiazolones

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The biological activity of a series of structurally diverse 3-isothiazolones (**1**) has been assessed by evaluating the minimum inhibitory concentration required to inhibit the growth of *E. Coli*. The structure and electronic properties of these derivatives have been calculated using both semi-empirical and *ab initio* molecular orbital methods. Multi-linear regression analysis shows no correlation between the experimental activity of the 3-isothiazolones and either the calculated geometries, electronic properties, or the frontier orbital energies of these derivatives, but a reasonable relationship is found with other parameters including their calculated solvation energies, suggesting that diffusion may play an important role in their mode of action.

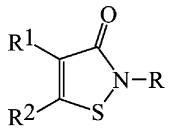
Introduction

It is well known that substituted 3-isothiazolones (**1**) are biologically active and some find commercial application as bactericides or fungicides.[1,2] The relative biological efficacy of individual isothiazolones, however, is highly dependent on the nature and position of the substituents attached to the heterocyclic ring. For example, while 4-methyl-*N*-methyl-3-isothiazolone (**1s**) and *N*-(2′-hydroxethyl)-3-isothiazolone (**1t**) have approximately the same biological activity against the bacteria *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and the fungus, *Aspergillus niger* (*A. niger*), 5-chloro-*N*-vinyl-3-isothiazolone (**1u**) is several orders of magnitude more active against all these organisms.[1] Historically, the first commercial biocide, 4,5-benzo-3-isothiazolone (**1g**), was synthesised in 1923,[3] but its anti-microbial properties were not disclosed until they were reported and patented in 1957 by Katz and Schroeder.[2] The synthesis of *N*-methyl-3-isothiazolone (**1m**) and the *N*-ethyl derivative were first described by Crow and Leonard in 1965[4] but their biological properties were not discussed. In 1973, the synthesis and biological properties of a large number of related 3-isothiazolones were described by Lewis, Miller and Law, with some showing a broad spectrum of antibacterial and antifungal activity at extremely low concentrations.[1] The commercial biocide, Kathon,[5] is a mixture of *N*-methyl-3-isothiazolone (**1m**) and the 5-chloro derivative (**1a**).

The biological activity of the 3-isothiazolones is thought to arise from their ability to initially diffuse through the membranes of bacteria or fungal cell walls and then react with important intracellular sulfur containing proteins, or simpler molecules inside the cell such as glutathione, causing the cell function to be impaired.[6,7] The mechanism of the intracellular reaction has not been elucidated fully, but in the simplest case, the mode of action is thought to involve nucleophilic attack by the sulfur atom of glutathione at the sulfur atom of the 3-isothiazolone, leading to the cleavage of the S–N bond to give a ring opened amidodisulfide (**2**) which can react further with the same nucleophile to give the β-mercaptoacrylamide (**4**) (Scheme 2).[8–12] This overall process *in vivo* can result in the death of the cell.

This reaction sequence is supported by the experimental isolation *in vitro* of the *N*-alkyl-amidodisulfide (**2**) in aqueous acid, and the *N*-alkyl-β-mercaptoacrylamide (**4**) in aqueous alkali.[9] However, the reaction of simple 3-isothiazolones with 2-methyl-2-propanethiol (*tert*-butyl mercaptan) always results in the formation of the disulfide (**2**) only[13,14] (Scheme 2, G = CMe_3) because further attack at the sulfur atom is sterically hindered by the size of the pendant tertiary butyl group. Furthermore, there appear to be no other competing reactions with this nucleophile even for 5-chloro-*N*-methyl-3-isothiazolone (**1a**) as no displacement of the ring chlorine is observed.

Recent kinetic studies on the reaction of the 3-isothiazolones (**1a**, **1f**, **1g**, **1m** and **1p**) with 2-methyl-2-propanethiol,[13] suggest that the mechanism of the reaction is more complex than that shown (Scheme 2). In these cases, the mechanism appears to be second order in 2-methyl-2-propanethiol and third order



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Substituent	MIC (10^{-6}M)
a R = Me; R^1 = H; R^2 = Cl	2.41
b R = 4-ClC_6H_4; R^1 = R^2 = Cl	3.90
c R = Me; R^1 = R^2 = Cl	9.76
d R = $CONH_2$; R^1 = R^2 = Cl	11.7
e R = n-C_6H_{13}; R^1 = R^2 = H	16.9
f R = Me; R^1 = R^2 = 4,5-cyclopropyl	39.0
g R = H; R^1 = R^2 = 4,5-benzo	55.8
h R = Me; R^1 = R^2 = 4,5-benzo	90.0
i R = Ph; R^1 = R^2 = Cl	93.8
j R = Ph; R^1 = R^2 = H	117.0
k R = $C_6H_5C_2H_4$; R^1 = R^2 = 4,5-benzo	188.0
l R = CH_2=CHCO; R^1 = R^2 = Cl	250.0
m R = Me; R^1 = R^2 = H	1230
n R = H; R^1 = Br; R^2 = H	1880
o R = H_2NCO; R^1 = R^2 = H	2500
p R = R^1 = R^2 = H	5000
q R = $SCCl_3$; R^1 = R^2 = H	1000
r R = $SCCl_3$; R^1 = R^2 = Cl	1.95
s R = R^1 = Me; R^2 = H	
t R = $HOCH_2CH_2$; R^1 = H; R^2 = H	
u R = CH_2:CH; R^1 = H; R^2 = Cl	
v R = PhCH:CH; R^1 = H; R^2 = Ph	

Scheme 1 Structures of the 3-isothiazolones and the experimental minimum inhibitory concentration found to inhibit the growth of *E. coli* (MIC).

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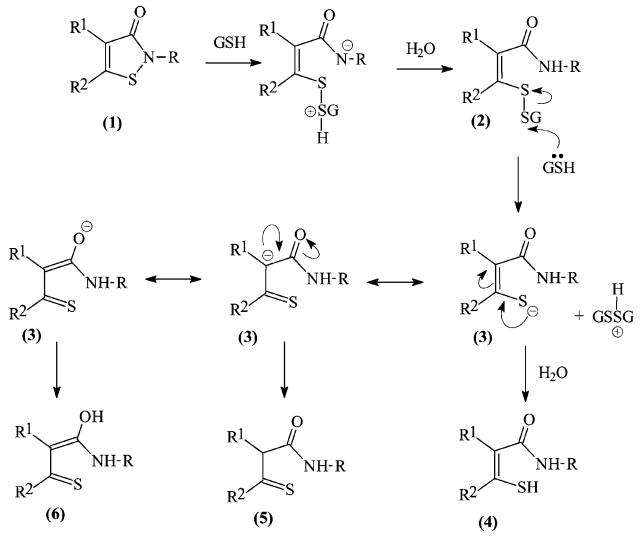


Scheme 2 Mechanistic path for the ring-opening reaction of 3-isothiazolones with sulfur containing nucleophiles (GSH = glutathione).

overall,[13] implying that the product resulting from the initial attack requires a second thiol molecule to generate the disulfide (**2**).

Recent experimental and theoretical studies have suggested that the extremely high biocidal activity of 5-chloro-*N*-methyl-3-isothiazolone (**1a**) may arise in part because delocalization of the charge on the intermediate β-mercaptoacrylamide anion (**3**) can lead to the formation of a highly reactive β-thioacyl chloride (**5**) in addition to the β-mercaptoacrylamide (**4**).[11,15] If formed *in vivo*, the thioacyl chloride (**5**) would be expected to react rapidly with intracellular species to disable the cell. Previously we have calculated the conformations, structure and properties of a limited series of 3-isothiazolones and shown that although structural effects do not appear to play an important role in the inhibition mechanism, the electronic properties calculated in terms of the atomic charge at sulfur and the LUMO energy appeared to show an approximate correlation with their reported biological activity.[16]

In the present studies we have experimentally assessed the biological activity *in vitro* of eighteen structurally diverse 3-isothiazolones, supplied by Zeneca Specialties (now trading as Avecia Ltd.), by identifying the minimum inhibitory concentrations required to inhibit an actively growing *E. Coli* culture *via* agar diffusion techniques. In tandem, we have calculated the structures and properties of all these molecules in an attempt to develop a quantitative structure–activity relationship (QSAR) which could be used to interpret the known activity and which would be useful for predictive purposes.

Experimental

Escherichia coli 8277 was obtained from the UK National Collection of Industrial and Marine bacteria (NCIMB).[13] All media and solutions were maintained at pH 7.6 using a 0.1 M buffer of tris(hydroxymethyl)aminomethane ("Trizma", Sigma-Aldrich), and the *E. coli* cultures were incubated using bacterial lawns prepared from a mixture of nutrient broth E (2 ml at 13 g l^{-1})[17] and nutrient agar (10 ml at 23 g l^{-1})[17] using the standard agar diffusion method.[18] For each of the eighteen isothiazolones (**1**), a series of concentrations were prepared by dilution of a 0.01 M stock solution (1 ml) with an equal volume of Trizma base solution at pH 7.6 and the process repeated with the diluted solution to give a set of concentrations ranging from 10^{-2} M to 1.5×10^{-7} M. The isothiazolone solutions were then applied to standard antibiotic assay disks (6 mm diameter),[18] and the impregnated disks were placed at the centre of the prepared bacterial lawns set on Petri dishes,[18] and incubated at 37 °C for twenty four hours to allow the growth of the *E. coli* lawn to fully develop. At concentrations of the isothiazolones which inhibited growth, a clear zone was apparent around the antibiotic assay disk, and this was considered to be the approximate minimum inhibitory concentration (MIC). The MICs were then refined by repeating the above procedure using a narrower concentration range for each of the isothiazolones tested. The refined minimum inhibitory concentrations obtained for each of the eighteen compounds are shown in Scheme 1.

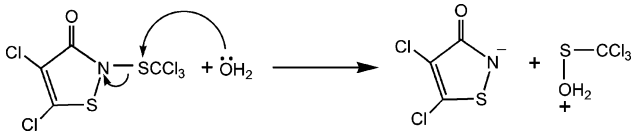
Methods of calculation

Molecular orbital calculations were carried out on empirical structures for the 3-isothiazolones using the MNDO,[19] AM1[20] and PM3[21] methods of the MOPAC93 Program[22] with full optimisation of all bond lengths, angles, and torsion angles in cartesian space (keywords: prec mndo xyz ef nomm). The effect of water on the structures and energies was assessed using the COSMO method[23] incorporated in the MOPAC 93 program (keywords: prec mndo xyz ef eps = 78.4). In this solvation model, the solute molecule is embedded in a cavity constructed from the intersecting van der Waals spheres of the component atoms surrounded by a continuum which is modelled as a conductor. The surface between the continuum and the solute is then partitioned into a large number of segments and the interaction between the charge density at each segment polarises the surrounding medium and produces a reaction field which in turn acts on the solute. A series of reference calculations were carried out also at the *ab initio* RHF level using the 6-31G** basis set of the GAMESS program[24] to check the validity of the semi-empirical results (directives: runtype optxyz, scftype direct rhf). Molecules and crystal structures were displayed and analysed using the SYBYL Molecular Modelling package.[25] Multi-linear regression analysis between the calculated properties and the experimental activity was carried out using the SPSS package.[26]

Results and discussion

Experimental activity of the 3-isothiazolones

The minimum inhibitory concentrations of all eighteen 3-isothiazolones (**1a–r**) determined using the agar diffusion method are shown in Scheme 1. The series displays a wide range of activities with 4,5-dichloro-*N*-thio(trichloro)methoxy-3-isothiazolone (**1r**) showing the highest activity and the parent compound of the series, 3-isothiazolone (**1p**) showing the lowest. However, it has been brought to our attention that the former (**1r**) may be susceptible to hydrolysis under the test conditions leading to the formation of two products[14] (Scheme 3).



Scheme 3 Postulated hydrolysis products of 4,5-dichloro-*N*-thio(trichloro)methoxy-3-isothiazolone (**1r**).

It follows that the high activity of (**1r**) and to a lesser extent, the related 2-thio(trichloro)methoxy-*N*-isothiazolone (**1q**), may arise because of the combined activity of all three species, including the highly reactive trichloromethyl thiol. Consequently, as the mode of action of these derivatives (**1r**) and (**1q**) is likely to be markedly different from the other stable 3-isothiazolones, it was decided to omit these molecules from the subsequent theoretical treatment. Of the remaining sixteen derivatives, 5-chloro-*N*-methyl-3-isothiazolone (**1a**) is the most active compound, followed by 4,5-dichloro-*N*-(4-chlorophenyl)-3-isothiazolone (**1b**), 4,5-dichloro-*N*-methyl-3-isothiazolone (**1c**), and 4,5-dichloro-*N*-acetylamino-3-isothiazolone (**1d**) with minimum inhibitory concentrations, *C*, of 2.41, 3.90, 9.76, and 11.7×10^{-6} M respectively against *E. coli* (Scheme 1) giving log $1/C$ values

Table 1 Energies, electronic and geometric properties of the 3-isothiazolones (**1**) calculated at the MNDO level in the gas phase *versus* experimental activity against *E. coli*[a]

	Log1/*C*	E_{HOMO}	E_{LUMO}	Q_S	q_S	θ_S	μ	MV	LogMW	Cl_4	Cl_5	$\delta H°_S$
la	5.62	−9.44	−0.78	0.43	0.97	93.7	2.05	97.40	2.18	0	1	−10.19
lb	5.41	−9.70	−1.23	0.45	0.94	93.5	2.80	168.9	2.45	1	1	−12.04
lc	5.01	−9.60	−1.14	0.45	0.93	94.0	3.00	109.0	2.27	1	1	−10.53
ld	4.93	−9.87	−1.42	0.51	1.01	94.3	0.53	119.3	2.33	1	1	−20.62
le	4.77	−9.15	−0.32	0.38	0.97	94.4	2.83	167.3	2.27	0	0	−10.32
lf	4.41	−9.07	−0.49	0.43	0.88	93.5	3.18	123.7	2.19	0	0	−10.04
lg	4.25	−8.88	−0.66	0.40	0.94	93.9	3.24	106.8	2.18	0	0	−11.58
lh	4.05	−8.82	−0.65	0.39	0.91	95.0	3.02	123.7	2.22	0	0	−9.94
li	4.03	−9.54	−1.09	0.45	0.94	93.6	3.15	156.4	2.29	1	1	−10.55
lj	3.93	−9.14	−0.32	0.39	0.99	93.9	2.85	133.9	2.25	0	0	−11.24
lk	3.73	−8.80	−0.64	0.39	0.89	95.1	2.72	206.5	2.41	0	0	−9.86
ll	3.60	−9.77	−1.32	0.48	1.13	94.3	0.92	133.4	2.35	1	1	−16.45
lm	2.91	−9.17	−0.34	0.38	0.97	94.3	3.01	85.10	2.06	0	0	−10.96
ln	2.73	−9.41	−0.73	0.41	0.95	93.3	3.50	85.30	2.25	0	0	−13.90
lo	2.60	−9.49	−0.69	0.44	1.09	94.6	2.42	95.70	2.15	0	0	−23.80
lp	2.30	−9.25	−0.36	0.38	0.87	93.3	3.16	68.40	2.00	0	0	−12.69

[a] *C* is the minimum inhibitory concentration of the respective isothiazolone (10^{-6} M); E_{HOMO} and E_{LUMO} are the energies of the highest occupied and lowest unoccupied molecular orbitals respectively in eV; Q_S is the total atomic charge at sulfur; q_S is the number of electrons at sulfur in the HOMO; θ_S is the angle at the sulfur atom of the heterocyclic ring; μ is the molecular dipole moment in D; MV is the molecular volume in $Å^3$; logMW is the log of the molecular weight; Cl_4 and Cl_5 are indicator variables for the presence or absence of a chlorine atom at the 4- or 5-position of the heterocyclic ring; $\delta H°_S$ is the difference between the calculated heats of formation in water and the gas phase (see text).

of 5.62, 5.41, 5.01 and 4.93 respectively (Table 1). While the high biological activity appears to be associated with the presence of chlorine in the heterocyclic ring, the activity of the remaining isothiazolones cannot be attributed to any other simple structural feature and other factors must be responsible for their activity. However, in the case of *N*-hexyl-3-isothiazolone (**1e**), which exhibits the fifth highest activity, the long aliphatic chain is clearly beneficial as the closely related *N*-methyl-3-isothiazolone (**1m**) has only a relatively low activity (Table 1). There is no overall obvious pattern to the other results and accordingly we have related the biological activity of these molecules to a combination of physical and/or chemical properties in an attempt to develop a QSAR model.

In most QSAR treatments, the biological response of a molecule is related to its distribution or partition between the aqueous and membrane phases of the cell which is represented by water and octanol.[27] In the simplest case,[27] the biological activity of a series of compounds is related to a hydrophobic substituent coefficient π defined as:

$$\pi = \log P_X - \log P_H \quad (1)$$

where P_H is the partition coefficient of the parent species and P_X is the partition coefficient of a derivative in the two solvents. For many aromatic systems, the overall biological response has additionally been attributed to electronic interactions[27] such as the Hammett substituent constant, σ,[28,29] and steric factors such as the Taft steric substituent, E_s,[29,30] so that the biological response is related to at least three parameters.[31–33] Here, the minimum inhibitory concentration, *C*, of an active aromatic molecule is related to experimental data by the equation:[31,32]

$$\log 1/C = a\pi + b\sigma + cE_s + \text{constant} \quad (2)$$

where π, σ, E_s are the hydrophobic, electronic and steric substituent parameters or properties respectively and *a*, *b* and *c* are coefficients fitted by regression analysis. In other cases, it has been shown that log 1/*C* is often parabolically related to log *P*, leading to the inclusion of a further term, π^2, in the general equation.[27,31]

Theoretically, there have been numerous studies which have attempted to relate the calculated properties of a range of organic molecules to their biological activity.[34] Quantum chemistry has played an important role in this approach, and here the structures and electronic properties of the molecules have been calculated using a variety of different molecular orbital methods.[34] Errors arising from the approximate nature of semi-empirical methods and the absence of solvation effects in the calculations appear to be transferable within structurally related series so that the calculated properties can be meaningful.[34,35] Good correlations have been reported between calculated properties and biological properties such as enzyme inhibition activity and hallucinogenic activity among others.[36–40] A large number of quantum chemical properties have been described in the literature including the use of atomic charges, HOMO and LUMO energies, orbital electron densities, superdelocalizabilities, atom–atom and molecular polarizabilities, dipole moments and polarity indices, and molecular energies including heats of formation, ionization potentials and electron affinities.[34]

Calculated properties of the 3-isothiazolones

In the present studies, we have selected a number of calculated parameters which we considered relevant, in an attempt to develop a model which would describe the biological properties of the 3-isothiazolones. Initially however it was necessary to find a suitable method to calculate the structures of all the isothiazolones discussed here before the properties were evaluated. Preliminary calculations were carried out using the MNDO, AM1, and PM3 methods of the MOPAC 93 package on 4,5-benzo-3-isothiazolone (**1g**) and the structural results compared with the crystal structure of the same molecule[41] in the Cambridge Structural Database.[42] The atom numbering system used is shown in Fig. 1 and the calculated bond lengths and angles are shown in Table 2.

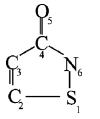


Fig. 1 Atom numbering convention used for the isothiazolones.

None of the three methods gives completely satisfactory results though the PM3 method gives the best S1–C2 and C3–C4 bond lengths at 1.756 and 1.482 Å *versus* the crystallographic data but both the charge at sulfur and the dipole moment appear to be far too small (Table 2). While the AM1 method gives the best C4–O5 and C4–N6 bond lengths at 1.239 and 1.398 Å, the MNDO method gives the best C2–C3–C4 and C4–N6–S1 angles at 111.6 and 115.9°, both compared with the experimental data

Table 2 Geometries, electronic properties and frontier orbital energies of the 3-isothiazolones (**1**) calculated at the semi-empirical and 6-31G** levels *versus* X-ray data and experimental activity against *E. coli*[a]

	1g					**1a**	**1l**	**1p**
	AM1	PM3	MNDO	6-31G**	X-Ray[b]	6-31G**	6-31G**	6-31G**
S1–C2	1.706	1.756	1.689	1.758	1.744	1.742	1.743	1.740
C2–C3	1.418	1.409	1.431	1.383	1.384	1.323	1.321	1.326
C3–C4	1.486	1.482	1.494	1.480	1.464	1.475	1.487	1.475
C4–O5	1.239	1.214	1.223	1.197	1.233	1.199	1.179	1.197
C4–N6	1.398	1.443	1.412	1.364	1.360	1.366	1.409	1.370
S1–N6	1.660	1.771	1.651	1.710	1.702	1.702	1.730	1.696
C2–Cl						1.712	1.704	
N6–CR						1.451	1.416	
S1–C2–C3	109.7	111.9	111.2	111.8	112.1	114.1	113.9	113.7
C2–C3–C4	112.4	113.4	111.6	113.5	111.8	112.2	113.8	113.2
C3–C4–N6	109.1	110.0	107.3	107.8	109.8	108.9	108.2	107.4
C4–N6–S1	114.0	111.7	115.9	117.7	115.8	115.6	113.9	116.7
O5–C4–N6	122.8	119.9	121.6	124.8	123.3	123.8	126.7	124.6
N6–S1–C2	94.98	92.17	93.94	89.54	90.55	89.18	90.31	89.01
E_{HOMO}	−8.296	−8.829	−8.876	−8.479		−9.145	−9.638	−8.955
E_{LUMO}	−0.493	−0.870	−0.663	2.623		2.805	1.910	3.069
Q_S	0.453	0.149	0.396	0.443		0.495	0.538	0.439
μ	3.109	2.756	3.235	3.639		2.792	5.873	3.915
Log1/*C*	4.25	4.25	4.25	4.25	4.25	5.62	3.60	2.30

[a] Bond lengths are given in angstroms, angles in degrees; *C* is the minimum inhibitory concentration of the respective isothiazolone (10^{-6} M); E_{HOMO} and E_{LUMO} are the energies of the highest occupied and lowest unoccupied molecular orbitals respectively in eV; Q_S is the total atomic charge at sulfur; μ is the molecular dipole moment in D. [b] Average geometries from the two molecules in the unit cell (ref. 41).

(Table 2). However, the geometry around the sulfur atom is likely to be more important than other parts of the structure because it is the site of nucleophilic attack leading to the cleavage of the S1–N6 bond (Scheme 2). On balance there is little to choose between the AM1 and MNDO methods on this criteria with both giving similar results for the S1–C2 and S1–N6 bond lengths compared with the experimental values (Table 2). However as the MNDO method is marginally superior to the AM1 method for the key C2–S1–N6 bond angle (subsequently used as a variable), it was decided to use the MNDO method to calculate the structures and electronic properties of all the other isothiazolones both in the gas phase and in water. A series of reference calculations was also carried out at the 6-31G** level (see later) using the same numbering convention (Fig. 1).

Eleven calculated properties were employed to model the activity of each isothiazolone; these were as follows:

(A) The energies of the frontier molecular orbitals (E_{LUMO} and E_{HOMO}). When the isothiazolone reacts with a thiol in the way shown (Scheme 2), frontier orbital interactions between the HOMO of the nucleophile and the LUMO of the substrate are thought to be important in the formation of a transition state.[43] In the mechanism shown, the lone pair of electrons at the sulfur atom of the nucleophile would be expected initially to donate into the LUMO of the isothiazolone and it follows that the lower the LUMO energy (E_{LUMO}) the more readily it will be attacked. In contrast, the HOMO energy (E_{HOMO}) is the energy required to remove an electron from the molecule, and the higher the value, the less likely it is that the molecule will wish to acquire electrons from an attacking nucleophile. Both these quantities are directly related to the electron affinity and ionization potential of the molecules in question, though they have positive rather than negative values.

(B) Total atomic charge on the sulfur atom of the isothiazolone ring (Q_S). The main activity of the isothiazolones is thought to arise from the nucleophilic attack of cellular thiol species on the electron deficient ring sulfur atom ultimately causing the S–N bond to cleave to form a disulfide bond (Scheme 2). The calculated results show that the valence electrons at the sulfur atom of the heterocyclic ring are extensively delocalized over the molecule resulting in an overall positive charge at sulfur (Table 1). It follows that the greater the positive charge on the sulfur of the isothiazolone ring the more easily it will undergo reaction with the nucleophile, *assuming* that the transition state for the reaction is an early one.

(C) Electron density on the sulfur atom in the HOMO of the isothiazolone (q_S). The HOMO of the isothiazolone is a π-orbital which contains two electrons which are distributed over the conjugated atoms of the ring system and the calculations indicate that the sulfur atom accommodates around one of these. As the lone pair of electrons at the sulfur atom lie in the molecular plane (*xy* axis) to form an approximately trigonal arrangement with the S1–N6 and S1–C2 bonds of the heterocyclic ring, nucleophilic attack at sulfur is therefore likely to occur either above or below the molecular plane to form a transition state which may involve the formation of an initial π-complex between the reactants. The electron density, or number of electrons, found in the π-orbital of the sulfur atom, may therefore play a role in the attack by a nucleophile at that centre.

(D) Angle at the sulfur atom (θ_S). While the unstrained sulfur bond angle C–S–C in dimethyl sulfide[44] is 99.0°, the value is much smaller in five membered rings such as thiophene[45] at 92.1°. In the isothiazolone ring, the corresponding C–S–N angle is even smaller with crystallographic values of 90.7° for both 4,5-benzo-3-isothiazolone (**1g**)[41] and 5-phenyl-(*E*)-2-styryl-3-isothiazolone (**1v**),[46] though the calculated results are somewhat larger (Table 1), as we have noted previously using semi-empirical methods.[16] In this approach, it is assumed that the insertion of substituents into the ring may cause changes in the C–S–N angle, which in turn, may result in increased ring strain. If the ring opening step is an essential part of the mechanism of the mode of action, smaller angles at the sulfur atom will result in more strain and higher reactivity.

(E) Dipole moment (μ). The calculated dipole moment of the isothiazolones is the vector sum of all the charges at each atomic centre. Molecules with large dipole moments are often referred to as "polar" and are generally soluble in polar solvents, such as water, and more likely to diffuse through the water filled porin channels in the outer membrane of the gram negative bacteria. In contrast, those with small dipole moments have a less polar character, are less soluble in water, and more likely to diffuse though the lipophilic diffusion channels of the membrane itself.

Table 3 Energies, electronic and geometric properties of the 3-isothiazolones (**1**) calculated at the MNDO level in water *versus* experimental activity against *E. coli*[a]

	Log1/*C*	E_{HOMO}	E_{LUMO}	Q_S	q_S	θ_S	μ	MV	LogMW	Cl_4	Cl_5	$\delta H°_S$
1a	5.62	−9.50	−0.75	0.45	1.03	93.6	3.73	97.4	2.18	0	1	−10.19
1b	5.41	−9.63	−1.13	0.52	0.96	93.5	5.22	169.6	2.45	1	1	−12.04
1c	5.01	−9.59	−1.09	0.51	0.99	93.9	5.43	108.3	2.27	1	1	−10.53
1d	4.93	−9.79	−1.24	0.51	1.02	94.5	9.54	117.9	2.33	1	1	−20.62
1e	4.77	−9.34	−0.32	0.39	1.02	94.4	5.19	167.3	2.27	0	0	−10.32
1f	4.41	−9.29	−0.61	0.47	0.89	93.5	5.67	123.7	2.19	0	0	−10.04
1g	4.25	−8.97	−0.68	0.41	0.86	93.9	5.41	107	2.18	0	0	−11.58
1h	4.05	−8.98	−0.70	0.39	0.84	95.0	5.09	123.8	2.22	0	0	−9.94
1i	4.03	−9.61	−1.12	0.51	0.89	93.5	5.48	158.4	2.29	1	1	−10.55
1j	3.93	−9.35	−0.34	0.40	1.01	93.9	5.18	134.4	2.25	0	0	−11.24
1k	3.73	−8.97	−0.70	0.39	0.83	95.0	4.80	207.1	2.41	0	0	−9.86
1l	3.60	−9.77	−1.24	0.51	1.02	94.4	8.63	133.4	2.35	1	1	−16.45
1m	2.91	−9.33	−0.32	0.40	1.02	94.3	5.38	85.2	2.06	0	0	−10.96
1n	2.73	−9.40	−0.58	0.47	0.98	93.5	6.63	85.3	2.25	0	0	−13.90
1o	2.60	−9.53	−0.55	0.42	1.06	94.7	9.26	95.5	2.15	0	0	−23.80
1p	2.30	−9.33	−0.29	0.41	1.05	93.4	5.70	68.5	2.00	0	0	−12.69

[a] See Table 1 for key.

(F) Molecular volume (MV) and molecular weight (MW). The molecular volume of the molecule (Å^3), based on the three dimensional van der Waals surface area, is a direct measure of the overall dimensions of the reactive surface of the molecule. Size may play a key role in the way hydrophilic molecules gain entry into the cell, for example by diffusion through the outer membrane proteins of *E. coli*, with small molecules showing faster diffusion rates than large ones. However, for lipophilic molecules, size may prove to be an advantage as the size of the invading species may disrupt the ordered membrane, and allow quicker diffusion into the cell and increased cellular damage. The effect of molecular weight on activity would be expected to be similar to the role described for molecular volume but here the substitution of the isothiazolones with additional atoms such as halogens or sulfur with "heavy" atomic masses might cause greater disruption of the cell membranes.

(G) Indicator variables (Cl_4 and Cl_5). These studies have suggested that the presence of chlorine in the isothiazolone ring appears to be associated with enhanced activity. Consequently, indicator variables (0 or 1) were used to indicate the absence or presence of chlorine at the both the 4- and/or 5 position of the isothiazolone heterocyclic ring (Cl_4 and Cl_5).

(H) Solvation energy terms (δH_S and δH_S^2). The Hansch partition coefficient is often critical in assessing the efficacy of a biocide as it mirrors the ability of the molecule to permeate into the cell. As a theoretical alternative to measuring the Hansch partition coefficient, the hydrophilicity or hydrophobicity of a compound can be evaluated by assessing the degree to which it is stabilized in water. By calculating the difference in the heat of formation ($\Delta H°_f$) of the isothiazolone in the gas phase on the one hand, which represents the hydrophobic environment of the cell membrane, and the modified value in water on the other, which represents the aqueous phase of the cell, a measure of the solvation energy, $\delta H°_S$, can be evaluated, *i.e.*

$$\delta H°_S = \Delta H°_f(H_2O) - \Delta H°_f(\text{gas}) \quad (3)$$

Isothiazolones with large solvation energies would be expected to be more soluble in the aqueous phase of the cell but less soluble in the hydrophobic phase and *vice versa*. A further term, $\delta H°_S{}^2$, was also evaluated in these studies to reflect the parabolic dependence of log 1/*C* on log *P* (see above).

In addition to assessing the solvation energies ($\delta H°_S$), some of the other properties (A–E) have been calculated in both the gas phase and water to generate two possible QSAR models. Theoretically, the variations produced by changing the dielectric constant of the solvent from 1 (gas) to 78.4 (water) have relatively little effect on the C2–S1–N6 angle, but other properties such as the frontier orbital energies, atomic charges, and dipole moments show much larger differences (Tables 1 and 3). The possibility that the MNDO results show the wrong trends because of the approximations inherent in the method, was carefully checked by calculating four molecules with widely different activity at the ab initio 6-31G** level[24] and comparing the two sets of results (Table 2).

Structure–activity relationships

The results obtained were subjected to multi-linear regression with the SPSS package[26] and analysed using (1) the *t*-statistic, which measures the significance of each individual independent variable in the regression equation, (2) the *F*-statistic, which assesses the overall significance of the model, (3) the square of the multiple correlation coefficient, R^2, which indicates how well the regression line fits the data, and finally (4) the predictive residual sum of the squares, *PRSS*, which reflects the difference between actual activity and predicted activity.[32,33,40] Three methods were used in the SPSS regression analysis, including forward stepping, where each independent variable is added into the regression equation one at a time, backward stepping, where all the independent variables are used to describe the dependant variable and are removed one at a time, and a third method which involves the generation of all possible combinations of independent variables giving rise to 2^{n-1} equations, with the best selected on the basis of statistical significance and chemical theory.[32,33,40]

A cursory examination of the calculated results either in the gas phase (Table 1) or in water (Table 3) before analysis, however, shows no obvious correlations between most of the properties and the experimental data. Thus the positive charge at the sulfur atom of the most active isothiazolone (**1a**) is smaller than that found on the less active derivatives (**1b–d**, **1i**, **1l**, and **1o**) while the electron density at the same atom in the HOMO is larger, excepting (**1l**), implying that the sulfur atom of the former is less likely to be attacked by a nucleophilic species. Furthermore, the C–S–N bond angle is larger in (**1a**) than the less active derivatives such as (**1b**, **1f**, **1i**, **1n** and **1o**) suggesting that ring strain is also unimportant. More refined calculations at the 6-31G** level on four representative isothiazolones (**1a**), (**1g**), (**1l**) and (**1p**) which show high, moderate and low activity show *exactly the same trends* as the MNDO results though the molecular geometries are more reliable as shown by the results on 3,4-benzo-isothiazolone when compared to crystallographic data (Table 2). At the 6-31G** level, the C–S–N bond angle in (**1p**) is smaller than that found in either (**1a**), (**1g**), or (**1l**) even though

this derivative is the least active (Table 2). The LUMO energies are also ambiguous at this level of calculation with (**1l**) showing a lower value than more active derivatives such as (**1a**) and (**1g**). The charge at sulfur is largest for (**1l**) followed by (**1a**) with (**1g**) and (**1p**) showing similar values, while the dipole moments run in the order (**1l**) > (**1p**) > (**1g**) > (**1a**). Significantly, all these trends in the angles, LUMO energies, charge at sulfur, and dipole moments at the 6-31G** level are reflected in the MNDO results (Tables 1 and 3).

Not surprisingly, the inclusion of these variables in the multilinear regression analysis either in the gas phase or in water, did not produce a satisfactory correlation coefficient or *F*-statistic. After careful analysis, however, it was found that the best equation obtained to describe the activity of the 3-isothiazolones contained only four parameters, but it was necessary to exclude three results (**1d**, **1i** and **1k**) from the data set (see later) to achieve a statistically significant correlation, *i.e.*

$$\log 1/C = 1.28\delta H^\circ_S + 0.034\delta H^\circ_S{}^2 + 4.39\log \mathrm{MW} + 0.93\mathrm{Cl}_5 + 4.20 \quad (4)$$

The four term eqn. (4) based on 13 observations is significant at the 5% confidence level and it explains 92% of the variance in the activity of the isothiazolones. The equation is robust as the correlation matrix shows no extreme collinearity between pairs of properties, excepting δH°_S and $\delta H^\circ_S{}^2$ *i.e.*

	Cl_5	δH°_S	Log MW	$\delta H^\circ_S{}^2$
Cl_5	1.0000	-0.1068	0.5006	0.0757
δH°_S	-0.1068	1.0000	-0.0149	-0.9936
Log MW	0.5006	-0.0149	1.0000	-0.0050
$\delta H^\circ_S{}^2$	0.0757	-0.9936	-0.0050	1.0000

In this model, the square of the correlation coefficient, $R^2 = 0.92$, the *F*-statistic = 21.52, the *t*-statistic values lie in the range: 3.19 (Cl_5), 5.06 (δH°_S), 3.55 (Log MW), and 4.51 ($\delta H^\circ_S{}^2$), and the predictive residual sum of the squares, $PRSS = 4.77$ suggesting that the equation gives a good account of the experimental activity. The value for the heat of solvation, δH°_S, is always negative (Table 1), but when this is multiplied by the positive regression coefficient its combined effect becomes even larger, suggesting that the greater the degree of stabilisation the isothiazolone experiences in a polar environment, the *lower* the biological activity, *i.e.* log $1/C$ decreases as δH°_S becomes more negative and *vice versa*. Hence, it appears that the more hydrophobic the isothiazolones are, the greater the activity, suggesting that the mode of transport into the cell occurs by diffusion through the lipophilic membrane. This helps to explain why *N*-hexyl-3-isothiazolone (**1e**) is ten times more active than *N*-phenyl-3-isothiazolone (**1j**), which is ten times more active than *N*-methyl-3-isothiazolone (**1m**), which in turn, is four times more active than the parent isothiazolone (**1p**) (Scheme 1). These conclusions are supported by the work of Collier who showed that the entry of radio-labelled isothiazolones into *E. coli* appeared to occur by passive diffusion.[9] Furthermore, he found that there was very little difference in the rate of uptake by porin deficient *E. coli versus* the non-mutant strain, again suggesting that the mode of entry of isothiazolones into the Gram-negative cell arises by passive diffusion through the lipid bilayer.[9–12] The observation that *N*-aryl and *N*-aryloxyalkanoic acid derivatives of 4,5-benzo-3-isothiazolone (**1g**) are not effective against gram negative bacteria is also supportive as these highly polar molecules are unable to diffuse through the lipophilic environment of the outer membrane.[47]

The log of the molecular weight is also found to be a significant parameter and suggests that as the molecular weight of the isothiazolone increases with substitution, the greater the activity of the compound, but perversely molecular volume does not appear to be significant in the regression analysis. However, it has been established in several other studies that the larger the molecule is, the less likely it is to penetrate the channels in the lattice of the lipid bilayer.[48] It is therefore more probable that the significance of the molecular weight property is due to the high molecular weight associated with those isothiazolones which contain chlorine atoms which are likely to assist solubilization of the molecules in the hydrocarbon chains of the bilayer and facilitate diffusion. This effect is illustrated by the large cross correlation coefficient of 0.50 between the properties Cl_5 and log MW shown in the Table above.

Almost all of the most active 3-isothiazolones contain a chlorine atom in the 5-position of the ring and this property (Cl_5) appears to be highly significant in the regression model. The high activity of these 3-isothiazolones may be due to the formation of reactive β-thioacyl chlorides (Scheme 2), such as (**5**), by delocalization of the charge on the initially formed β-mercaptoacrylamide (**3**), as originally suggested for (**1a**).[11,15]

It is not clear why 4,5-dichloro-*N*-acetylamino-3-isothiazolone (**1d**), which is the fourth most active derivative of the series, has a much lower predicted activity than expected ($\log 1/C = 3.36$) and does not fit eqn. (4), but one likely explanation is that the MNDO method is poor at representing hydrogen bonding in amides resulting in lower than expected values for the solvation energies (Table 1). In contrast, 4,5-dichloro-*N*-phenyl-3-isothiazolone (**1i**) has a much larger predicted activity than expected ($\log 1/C = 5.46$) but there is no obvious explanation for this anomaly. However, in the case of 4,5-dibenzo-*N*-(1-ethylphenyl)-3-isothiazolone (**1k**), which also has a much larger predicted value ($\log 1/C = 5.47$) than the experimental activity (Table 1), the explanation for the deviation here may lie with the size of this molecule (the largest of the series), which is less able to enter the channels of the lipid bilayer as discussed above. Overall however, the application of eqn. (4) leads to satisfactory results in the other cases with predicted values for $\log 1/C$ of 5.40 and 3.59 for (**1b**) and (**1l**), which contain chlorine in the heterocyclic ring, and 4.39 and 2.21 for (**1f**) and (**1p**) which do not, *versus* the experimental values of 5.41 and 3.60, and 4.41 and 2.30 respectively (Table 1).

Conclusion

Neither the calculated geometries, electronic properties, nor the frontier orbital energies of the 3-isothiazolones (**1a–p**) appear to correlate with their measured biological activity in terms of the minimum inhibitory concentration required to inhibit the growth of *E. Coli*. However, a satisfactory relationship is found between the observed activity and other factors including the calculated solvation energies suggesting that diffusion plays an important role in their mode of action.

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