Studies on the biological activity of some nitrothiophenes

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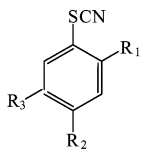
The biological activity of nineteen substituted thiophenes (**3**) have been assessed by evaluating the minimum inhibitory concentration required to inhibit the growth of *E. coli*, *M. luteus* and *A. niger*. The series displays a wide range of activities with 2-chloro-3,5-dinitrothiophene (**3a**) or 2-bromo-3,5-dinitrothiophene (**3c**) showing the highest activity against all three organisms, while the simplest compound of the series, 2-nitrothiophene (**3s**) shows the smallest activity in each case. The mode of action of **3a** and **3c** is thought to involve nucleophilic attack by intracellular thiols at the 2-position of the heterocyclic ring leading to displacement of halogen, but other active derivatives, such as 2,4-dinitrothiophene (**3h**) and 5-nitrothiophene-2-carbaldehyde (**3d**) which have no displaceable halogen or leaving group are thought to act by forming Meisenheimer complexes.

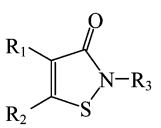
Introduction

A wide range of organosulfur compounds are biologically active and some find commercial application as fungicides and bactericides.[1–4] However, their biological activity is dependent not only on the presence of sulfur but often on the presence of additional activating groups. For example, the fungicidal activity of phenylthiocyanate (**1a**) is substantially enhanced by the presence of electron attracting substituents in the aromatic ring so that the 2,4-dinitro derivative (**1b**), which is highly effective against the fungus *Aspergillus niger*, has been patented as a potent antifungal agent.[1] In contrast, heterocyclic compounds such as 3-isothiazolones, are highly effective against the bacteria *Escherichia coli* and *Staphylococcus aureus* but again the relative biological efficacy of these molecules is highly dependent on the nature and position of the substituents attached to the heterocyclic ring.[2,3] Thus 5-chloro-*N*-methyl-3-isothiazolone (**2a**) is several orders of magnitude more active than the simpler *N*-methyl derivative (**2b**).[2,5]



(**1**)

a $R_1 = R_2 = R_3 = H$
b $R_1 = R_2 = NO_2$; $R_3 = H$
c $R_1 = H$; $R_2 = NO_2$; $R_3 = CO_2H$



(**2**)

a $R_1 = H$; $R_2 = Cl$; $R_3 = Me$
b $R_1 = R_2 = H$; $R_3 = Me$
c $R_1 = R_2 = H$; $R = Ph$
d $R_1 = R_2 = Cl$; $R_3 = Me$
e $R_1 = R_2 = H$; $R_3 = C_2H_4C_6H_5$

In both cases, the biological activity of these molecules 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 thiols inside the cell, causing the cell function to be impaired (see later).

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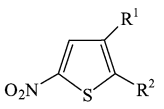
Substituted thiophenes (**3**) are also biologically active[6] and like the phenylthiocyanates they generally require electron attracting nitro groups to enhance activity.[4,6–13] For example, 2,4-dinitrothiophene (**3h**) and related derivatives are fungicides,[9] 2-methylamino-3,5-dinitrothiophene (**3t**) is an effective marine anti-fouling agent[10] and 2-acetyl-3,5-dinitrothiophene (**3u**) has pronounced antibiotic properties.[11,12] More recent studies have described the biocidal properties of 2,4-dinitro-5-thiomethoxythiophene (**3v**) and the related sulfoxide and sulfone derivatives,[13] but there is little systematic information on the mode of action of the nitrothiophenes.

In the present studies we have synthesised and experimentally assessed the biological activity of nineteen diverse nitrothiophenes (**3**), by identifying the minimum inhibitory concentrations required to inhibit actively growing cultures of the Gram negative bacterium, *Escherichia coli* (*E. coli*), the Gram positive bacterium, *Micrococcus luteus* (*M. luteus*), and a typical fungus, *Aspergillus niger* (*A. niger*), all using agar diffusion techniques. As previous studies have shown that electron attracting groups are necessary to enhance the biological activity of the thiophene against fungi and bacteria, the series selected all contained one nitro group in the 2-position of the thiophene ring.



(**3**)

Substituent

a $R^1 = NO_2$; $R^2 = Cl$
b $R^1 = NO_2$; $R^2 = OPh$
c $R^1 = NO_2$; $R^2 = Br$
d $R^1 = H$; $R^2 = CHO$
e $R^1 = NO_2$; $R^2 = OMe$
f $R^1 = NO_2$; $R^2 = NHCOCH_3$
g $R^1 = H$; $R^2 = CH{=}CHCHO$
h $R^1 = NO_2$; $R^2 = H$
i $R^1 = NO_2$; $R^2 = SPh$
j $R^1 = NO_2$; $R^2 = SC(CH_3)_3$
k $R^1 = NO_2$; $R^2 = SCN$
l $R^1 = NO_2$; $R^2 = SO_2C(CH_3)_3$
m $R^1 = NO_2$; $R^2 = NH_2$
n $R^1 = H$; $R^2 = Br$
o $R^1 = NO_2$; $R^2 = N{=}NC_6H_4N(C_2H_4CO_2CH_3)_2$
p $R^1 = NO_2$; $R^2 = NHCOPh$
q $R^1 = NO_2$; $R^2 = NHPh$
r $R^1 = H$; $R^2 = Cl$
s $R^1 = R^2 = H$
t $R^1 = NO_2$; $R^2 = NHMe$
u $R^1 = NO_2$; $R^2 = COCH_3$
v $R^1 = NO_2$; $R^2 = SO_2CH_3$

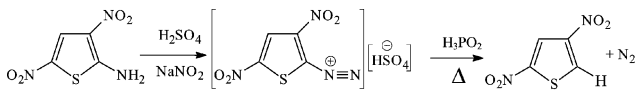
Results and discussion

Synthetic aspects

There were only a limited number of relevant nitrothiophene compounds commercially available at the time of biological evaluation, such as 5-nitrothiophene-2-carbaldehyde (**3d**) and 2-amino-3,5-dinitrothiophene (**3m**), which were used directly; these compounds were also used as suitable precursors for the preparation of other derivatives (see later). To ensure that the activity of the nitrothiophenes was thoroughly examined, a suitable reference set of active compounds were devised which (i) contained a varied set of substituents including both electron donating and electron attracting substituents; (ii) were stable both in air and water; and (iii) were suitably soluble both in water and organic solvents for biological test purposes. The following derivatives were synthesised:

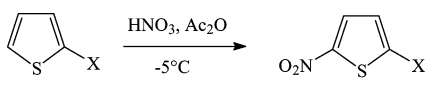
2-Nitrothiophene (3s). Thiophene was nitrated using a modified literature procedure with acetyl nitrate[14] taking into account the usual precautions for this reagent.[15] As the product was found to be light sensitive, the crude nitrothiophene mixture was extracted into petroleum spirit, cooled and isolated in the absence of light, to give the expected product mixture of 2-nitrothiophene (85%) and 3-nitrothiophene (15%) in an overall yield of 31%.

2,4-Dinitrothiophene (3h). The new route adopted was based on a method used for the synthesis of azothiophene dyes by diazotization of 2-amino-3,5-dinitrothiophene (**3m**) with sodium nitrite and sulfuric acid in a mixture of acetic and propionic acids.[16] Decomposition of the diazonium salt by heating with hypophosphorous acid[17,18] gave the required product, but isolation proved difficult. A troublesome extraction of the highly acidic reaction mixture with dichloromethane, followed by a washing step with a saturated solution of sodium hydrogen carbonate finally afforded (**3h**) in 22% yield.



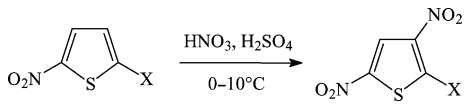
(**3m**) (**3h**)

2-Chloro-5-nitrothiophene (3r) and 2-bromo-5-nitrothiophene (3n). Nitration of the respective 2-halogenothiophene with acetyl nitrate[15,19] gave crude products which were decolourized using activated charcoal during recrystallization, and isolated in the absence of light, to give (**3r**) and (**3n**) in 51 and 31% yield, respectively.



(**3r**) X = Cl
(**3n**) X = Br

2-Chloro-3,5-dinitrothiophene (3a) and 2-bromo-3,5-dinitrothiophene (3c). These compounds were prepared by nitrating either (**3r**) or (**3n**) using a chilled mixture of fuming nitric and concentrated sulfuric acids[19] to give the products in 72 and 78% yield, respectively.



***N*-(3,5-Dinitrothien-2-yl)acetamide (3f) and *N*-(3,5-dinitrothien-2-yl)benzamide (3p).** These could not be prepared by standard acylation techniques from 2-amino-3,5-dinitrothiophene (**3m**) as the electron withdrawing properties of the two nitro groups reduces the nucleophilicity of the amino group to such an extent that it is unreactive. Successful reaction conditions were eventually found by adding a catalytic amount of concentrated sulfuric acid[20] to the acetylation reaction (using acetic anhydride) or benzoylation reaction (using benzoyl chloride) both at room temperature to give the desired products in 62 and 65% yield, respectively.

3,5-Dinitro-2-phenoxythiophene (3b). This compound was synthesised in 52% yield (Scheme 1) from a solid state reaction between freshly prepared potassium phenoxide and 2-chloro-3,5-dinitrothiophene (**3a**).[19]

(**3a**)

(**3b**) R = OPh (**3i**) R = SPh
(**3e**) R = OMe (**3q**) R = NHPh
(**3k**) R = SCN

Scheme 1 Nucleophilic substitution reactions of 2-chloro-3,5-dinitrothiophene (**3a**).

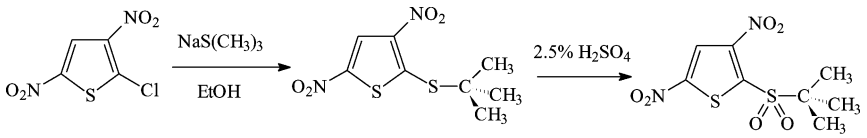
2-Methoxy-3,5-dinitrothiophene (3e). Although the existing synthetic method using sodium methoxide reported very small yields,[19] we have found that provided the reaction is carried out at 0 °C by combining ice cold solutions of sodium methoxide solution and (**3a**) in methanol, the reaction proceeds readily to give the product in a very respectable yield of 80% (Scheme 1).

3,5-Dinitro-2-phenylsulfanylthiophene (3i). This compound was prepared in 48% yield (Scheme 1) by adding a mixture of thiophenol and a small amount of a 10% aqueous sodium hydroxide to a methanolic solution of 2-chloro-3,5-dinitrothiophene (**3a**).

2-Anilino-3,5-dinitrothiophene (3q). This compound was prepared in 73% yield (Scheme 1) by treating 2-chloro-3,5-dinitrothiophene (**3a**) with aniline in methanol at room temperature.[19]

3,5-Dinitro-2-thiocyanatothiophene (3k). This, a new compound, was synthesized in 73% yield (Scheme 1) by reacting 2-chloro-3,5-dinitrothiophene (**3a**) with potassium thiocyanate in acetone.

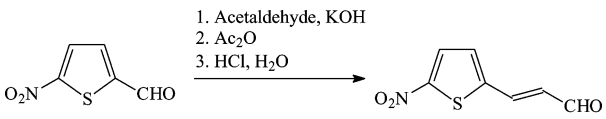
2-(2-Methyl-propane-2-sulfanyl)-3,5-dinitrothiophene (3j) and 2-(2-methyl-propane-2-sulfonyl)-3,5-dinitrothiophene (3l). Both new compounds, were synthesized by treating an ethanolic solution of 2-chloro-3,5-dinitrothiophene (**3a**) with sodium 2-methyl-2-propanethiolate and neutralizing the mixture with 2.5% aqueous sulfuric acid. The mixed isolated products were separated by column chromatography using toluene as an eluant.



(**3a**) (**3j**) (**3l**)

2-Methyl-2-propanethiolate was selected in preference to other alkylthiolates because once the product is formed (**3j**), the sulfur atom is sterically hindered and it does not undergo further reaction with the initial reagent to form disulfides.

β-(5-Nitro-2-thienyl)acraldehyde (3g). was prepared in 13% yield overall by the alkali catalysed condensation of 5-nitrothiophene-2-carbaldehyde (**3d**) with acetaldehyde using acetic anhydride in the dehydration step.[21]



(**3d**) (**3g**)

Experimental activity of the nitrothiophenes

The minimum inhibitory concentrations of all nineteen nitrothiophenes (**3a**–**s**) determined against *E. coli*, *M. luteus* and *A. niger* are shown in Table 1. Overall, fifteen of the nitrothiophenes inhibited the growth of the Gram negative bacteria, *E. coli*, and seventeen inhibited the growth of the Gram positive bacteria *M. luteus*. The series displays a wide range of activities with 2-chloro-3,5-dinitrothiophene (**3a**) and 2-bromo-3,5-dinitrothiophene (**3c**) showing the highest activity against *E. coli* and *M. luteus*, respectively, while the simplest compound of the series, 2-nitrothiophene (**3s**) shows the smallest activity against both organisms. While the high biological activity appears to be associated with the presence of two nitro groups in the heterocyclic ring, there are notable exceptions such as 5-nitrothiophene-2-carbaldehyde (**3d**) and 5-nitrothiophene-2-acrylaldehyde (**3g**) which are both more active against *E. coli* than 2,4-dinitro-2-thiocyanatothiophene (**3k**) or 2-amino-3,5-dinitrothiophene (**3m**) (Table 1). On balance, the activity of the nitrothiophenes does not seem to be attributable to any simple structural feature and other factors or properties must therefore contribute to their observed activity (see below). It is also true that there are sometimes wide discrepancies between the measured activities of the derivatives against *E. coli* and *M. luteus* suggesting that the mode of action is different between Gram negative and Gram positive bacteria respectively. For example, 5-nitrothiophene-2-carbaldehyde (**3d**) is highly active against *E. coli* but an order of magnitude less active against *M. luteus*, while 2,4-dinitro-5-thiocyanatothiophene (**3k**) is highly active against *M. luteus* but an order of magnitude less active against *E. coli* (Table 1). This difference in activity found between Gram positive and Gram negative bacteria probably reflects the different structure of their cell walls, and is not unusual as 5-chloro-*N*-methyl-3-isothiazolone (**2a**) has twice the activity against *E. coli* as it does against

Table 1 Minimum inhibitory concentration (MIC, μM) of the thiophene derivatives required to inhibit the growth of *E. coli*, *M. luteus*, and *A. niger*

		E. coli		*M. luteus*		*A. niger*
Compound	Solvent	MIC	log (1/*C*)[a]	MIC	log (1/*C*)[a]	MIC
3a	DMF	106	3.97	134	3.87	—
	MeOH	152	3.82	115	3.94	76
	Acetone	174	3.76	—	—	—
3b	DMF	166	3.78	157	3.80	—
3c	Acetone	205	3.69	78	4.11	—
3d	MeOH	289	3.54	2546	2.59	743
3e	DMF			1082		
	MeOH	336	3.47	1711	2.77	411
3f	DMF	435	3.36	659	3.18	—
3g	MeOH	603	3.22	1350	2.87	671
3h	MeOH	633	3.20	2658	2.58	520
3i	DMF	1250	2.90	816	3.09	—
3j	Acetone	1650	2.78	5001	2.30	—
3k	MeOH	1985	2.70	200	3.70	—
3l	Acetone	3763	2.42	321	3.49	—
3m	MeOH	4352	2.36	2241	2.65	>10 000
3n	MeOH	14 966	1.82	17 151	1.77	—
3o	MeOH	>10 000	N/A	2315	2.64	—
3p	DMF	>100 000	N/A	—	—	—
3q	DMF	>100 000	N/A	>10 000	N/A	—
	Acetone	>100 000	N/A	>10 000	N/A	
3r	MeOH	>100 000	N/A	>100 000	N/A	>10 000
3s	MeOH	>100 000	N/A	>100 000	N/A	>10 000

[a] The MIC values are also given in the form log 1/*C* where *C* is the minimum inhibitory concentration in moles per litre.

S. aureus (Gram positive), while *N*-phenyl-3-isothiazolone (**2c**) is highly active against *S. aureus* but an order of magnitude less active against *E. coli*.[2]

The activity of the derivatives tested against *A. niger* appears to follow a similar trend to the results found for *E. coli* with 2-chloro-3,5-dinitrothiophene (**3a**) showing the highest activity while 2-nitrothiophene (**3s**) shows the smallest (Table 1).

However, these results suggest that the activity of the nitrothiophenes against *A. niger* are less sensitive to structural changes than those results obtained using bacteria. Thus 2,4-dinitrothiophene (**3h**) shows a similar activity to 5-nitrothiophene-2-carbaldehyde (**3d**), 2-methoxy-3,5-dinitrothiophene (**3e**) and β-(5-nitro-2-thienyl)acraldehyde (**3g**) but all are an order of magnitude less than that found for (**3a**) (Table 1).

Mode of action

The precise mechanism of how organosulfur compounds disable the cells of bacteria and fungi is not known, but is well established that low molecular weight thiols, such as glutathione (GSH), and reactive protein sulfhydryls such as cysteine are primary participants in cellular anti-oxidant processes.[22–26] Glutathione is abundant in cytoplasm, nuclei, and mitochondria (3–10 mM), while reactive protein sulfhydryls are abundant in both soluble proteins and in membrane-bound proteins. The sulfur atom in these species easily accommodates the loss of a single electron and the thiol groups can also partially ionize to produce the more reactive and strongly nucleophilic thiolate anion, *e.g.*

$$\mathrm{G{-}SH} \rightleftharpoons \mathrm{G{-}S^- + H^+}$$

. While the sulfur atom in cysteine partially ionizes at neutral or cellular pH, the high pK_a of glutathione at 9.3, means there is very little of the anion at pH = 7. Despite this, the thiolate anion appears to be responsible for the reactivity of cellular thiols during xenobiotic metabolism where for example, glutathione transferases bind to glutathione in such a way that the sulfur is induced to ionize more completely and then react with xenobiotic materials such as carcinogens, mutagens, toxins and drugs.[22–26]

The mode of action of organosulfur compounds against bacteria or fungi will clearly vary depending on the structural and electronic features of the biocide. For example, phenylthiocyanates (**1**) are attacked by a variety of nucleophiles (X^-) either at the cyano carbon resulting in displacement of the phenylthiolate ion [eqn (1)], or at the sulfur atom resulting in the displacement of the cyanide ion [eqn (2)], or at the aromatic ring resulting in the displacement of the thiocyanate ion [eqn (3)] as shown[27–32]

$$\mathrm{RSCN + X^- \rightarrow RS^- + XCN} \quad (1)$$

$$\mathrm{RSCN + X^- \rightarrow RSX + CN^-} \quad (2)$$

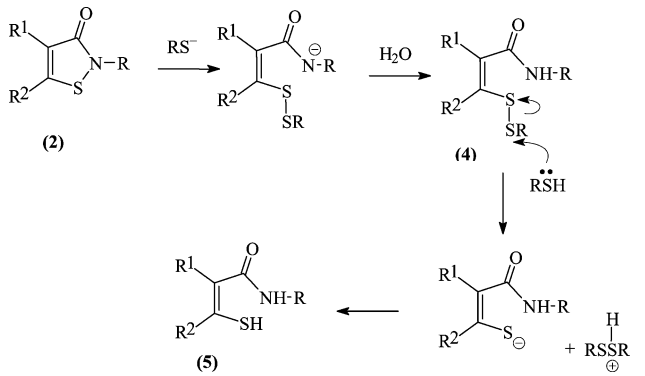
$$\mathrm{RSCN + X^- \rightarrow RX + SCN^-} \quad (3)$$

However, the degree of attack at a given atomic centre is highly dependent on the number and nature of substituents present in the phenyl ring. For example, nucleophiles such as phenylthiolate or azide ion attack phenylthiocyanate (**1a**) at the cyano carbon displacing the phenylthiolate ion[27] in line with eqn (1), while 2,4-dinitrophenylthiocyanate (**1d**) is attacked at the aryl carbon resulting in the displacement of the thiocyanate anion[27–29] in line with eqn (3). The ultimate products obtained from these reactions are not straightforward because the initial products formed are able to react further either with the original nucleophile or the displaced anion. For example, 2-nitro-5-thiocyanobenzoic acid (**1c**) reacts with alkanethiols in water to form 2-nitro-5-sulfidobenzoic acid *via* eqn (1), which then reacts with the initial thiocyanate to form a symmetrical disulfide *via* eqn (4)[31,32]

$$\mathrm{RSCN + RS^- \rightarrow RSSR + CN^-} \quad (4)$$

Theoretical studies on the reaction between phenylthiocyanate (**1a**) and methanethiol in water also indicate that the initial nucleophilic attack would be expected to occur at the cyano carbon in preference to the sulfur atom.[33,34]

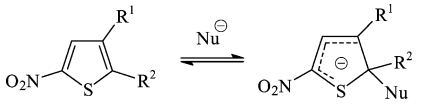
In contrast, the heterocyclic isothiazolones (**2**), which in theory could be attacked at either the carbonyl carbon or the ring sulfur atom, are attacked exclusively by a variety of alkyl thiols, such as cysteine and glutathione, at the sulfur only to give a ring opened alkylamidodisulfides (**4**) which react further with the same nucleophile to give β-mercaptoacrylamides (**5**),[2,3,5,35–42] *i.e.*



The corresponding reactions of the 2-nitrothiophenes (**3**) are different to those of the phenylthiocyanates (**1**) or the isothiazolones (**2**) but equally complex. The most active derivatives found in these studies against *E. coli* and *M. luteus* are 2-chloro-(**3a**) and 2-bromo-3,5-dinitrothiophene (**3c**), respectively (Table 1), suggesting that their mode of action simply involves nucleophilic attack by intracellular thiols such as glutathione at the activated 2-position of the ring, causing displacement of the halogen atom. Indeed, it is well established that activated halothiophenes react with a variety of nucleophiles *via* a two step S_NAr mechanism which involves the displacement of the halogen,[43–48] and we have used these reactions to prepare (**3b**), (**3e**), (**3k**), (**3i**) and (**3q**) from 2-chloro-3,5-dinitrothiophene (**3a**) (see Scheme 1). The higher activity of 2-phenoxy-3,5-dinitrothiophene (**3b**) over the 2-methoxy derivative (**3e**) also supports this mechanism of nucleophilic displacement by cellular thiols at the 2-position of the thiophene ring as the phenoxy goup is a better leaving group than the methoxy group.

However, this mode of action cannot apply to 5-nitrothiophene-2-carbaldehyde (**3d**) nor to 2,4-dinitrothiophene (**3h**), which have good to moderate activity against *E. coli* (Table 1), because neither has a displaceable leaving group. Historically, when the first nitrothiophenes were prepared it was noted that they formed brightly coloured solutions on the addition of alkali.[43] These were due to the formation of Meisenheimer complexes such as (**6e**), (**6h**) and (**6s**) which are, in some cases are remarkably stable in solution and more stable than the related 1,3,5-trinitrobenzene complexes.[49]

In general, these complexes (Scheme 2) are formed by attack of the nucleophile at the 2- (or 5-) position of the thiophene ring and the negative charge is thought to be delocalised both by the nitro group(s) and the vacant *d* orbitals on the sulfur atom.



(**1**) (**6**)

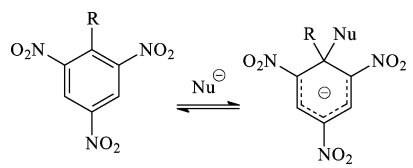
e $R^1 = NO_2$; R^2 = OMe; Nu = MeO^-

h $R^1 = NO_2$; R^2 = H; Nu = MeO^- or H^-

s $R^1 = R^2$ = H; Nu = MeO^-

Scheme 2 Formation of Meisenheimer complexes between 2-nitrothiophenes and nucleophiles.

In principle, many of the nitrothiophenes could behave the same way *in vivo* and react with intracellular thiols to form Meisenheimer complexes but there are no reports of this type of reaction occurring though the related trinitrobenzenes (**7**) readily form Meisenheimer complexes with thiolate ions *in vitro* (Scheme 3).[50–51] For example, kinetic and equilibrium data have been reported[50] for the formation of Meisenheimer complexes (**8a**–**c**) between either 1,3,5-trinitrobenzene (**7a**), 2,4,6-trinitrophenetole (**7b**) or 1-chloro-2,4,6-trinitrobenzene (**7c**) and ethanethiolate, and it is known that glutathione reacts with 1,3,5-trinitrobenzene (**7d**) to form the complex (**8d**).[51] It is possible therefore that the nitrothiophenes (**3e**) and (**3h**), as well as many of the other derivatives with no displaceable halogen, are biologically active because they form stable complexes within the cell with glutathione or related sulfhydryls.



(**7**) (**8**)

a R = H; Nu = EtS^-

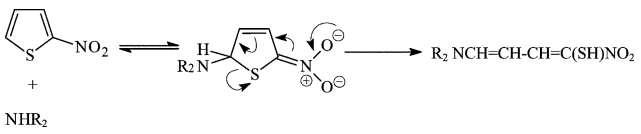
b R = OEt; Nu = EtS^-

c R = Cl; Nu = EtS^-

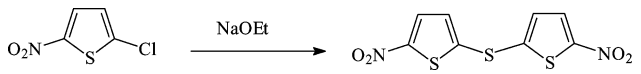
d R = H; Nu = Glutathione

Scheme 3 Formation of Meisenheimer complexes between 1,3,5-trinitrobenzene and nucleophilic reagents.

In addition to these two possible modes of action there is a further possibility which involves the opening of the heterocyclic ring. Although these reactions are almost certainly possible when nitrothiophenes are attacked by *hard* nucleophiles, it is not known whether *soft* nucleophiles such as thiolates can act in the same way as the products have rarely been identified.[43,44] 2-Nitrothiophene (**3s**) and 3,4-dinitrothiophene readily react with secondary amines under mild conditions to yield ring opened products *via* an intermediate disulfide,[43,44] *e.g.*:



Furthermore, 2-chloro-5-nitrothiophene (**3r**) reacts with sodium allyloxide or ethoxide to form bis(5-nitro-2-thienyl)sulfide[52] probably by a similar ring opening process to generate an intermediate reactive thiol which presumably reacts further with the initial thiophene, *i.e.*



While it is possible that the mode of action of the nitrothiophenes may involve a ring opening process, this seems to be unlikely as there are no strong nucleophilic species present at physiological pH within the cell. On balance, it seems more likely that the mode of action involves attack by intracellular thiols, such as glutathione, either to form stable Meisenheimer complexes or to undergo nucleophilic displacement to liberate for example, the chloride, bromide, phenoxide or methoxide anion. In both these cases, the cell properties would be affected by the formation of intracellular thiophene-containing species which would be expected to markedly interfere with the physical properties of the cell and its ability to replicate.

Conclusion

The mode of action of the nineteen substituted thiophenes (**3**) assessed against *E. coli*, *M. luteus* and *A. niger* probably arises by at least two different mechanisms within the respective cell. Thus 2-chloro-3,5-dinitrothiophene (**3a**) and 2-bromo-3,5-dinitrothiophene (**3c**), which show the highest activity against all three organisms, probably react with by intracellular thiols by displacement of halogen at the 2-position of the heterocyclic ring, while other active derivatives, such as 2,4-dinitrothiophene (**3h**) and 5-nitrothiophene-2-carbaldehyde (**3d**) which have no displaceable halogen or leaving group are thought to react by forming Meisenheimer complexes.

Experimental

Instrumentation

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC spectrometer operating at either 400 or 100 MHz, respectively. In order to see the heteroaromatic ^{13}C nuclei bonded to nitro groups, which require a longer relaxation time, a 6 s delay was enforced between each scan. All chemical shifts are recorded in parts per million (δ) relative to tetramethylsilane and were recorded in deuterated chloroform unless stated otherwise. The mass spectra were recorded by the EPSRC Mass Spectrometry Centre at Swansea using a VG analytical quattro II triple quadrupole mass spectrometer for the low resolution spectra. The parent ion and the five most abundant peaks (with a mass to charge ratio of greater than 37) have been included. Accurate mass measurements were performed using a Finnigan MAT 900 XL mass spectrometer with perfluorotributylamine as the reference compound for electronic ionisation and polyethylenimine for chemical ionisation measurements. Infra-red spectra were recorded on a Mattson Satelitte

FTIR spectrometer, while ultra-violet and visible spectra were recorded on a Unicam UV 300 UV/VIS spectrometer. Melting points were recorded on a electrothermal IA9100 digital melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out on Whatman 250 μm layer silica gel fluorescent plates. Flash column chromatography was performed with matrix silica 60 (particle size 35–70 μm). The compounds were loaded on to the column after first preabsorbing on to identical silica using acetone as the solvent. Any crystals synthesized were dried under vacuum in the presence of phosphorous pentoxide.

Reagents

Thiophene, 2-chlorothiophene, 2-bromothiophene and 5-nitrothiophene-2-carbaldehyde were supplied by Sigma-Aldrich Chemicals. All other reagents used were purchased from Lancaster, Sigma-Aldrich, or Fisher chemical companies and were used as supplied without further purification. 2-Amino-3,5-dinitrothiophene and 4-(*N*,*N*-bis(2-acetoxyethyl)aminophenylazo)-3,5-dinitrothiophene were supplied by Zeneca Specialities (now trading as Avecia Ltd).

Synthetic products

Most of the known products were synthesized using literature procedures which are described in the text (above). However, where the experimental procedure differed significantly from that published, or the derivatives have not previously been described (**3j**, **3l** and **3k**) full details have been included.

2-Amino-3,5-dinitrothiophene (3m). This compound was purified for biological testing *via* flash chromatography using an eluant of toluene–acetone (90 : 10); mp 178.9–180.8 °C (lit.[53] 179–180 °C); δ_H (d^6-acetone) 8.90 (2H, broad s, NH_2), 8.16 (1H, s, thio-*H*); δ_C 163.15 (*C*–NH_2), 131.95, 126.69 (*C*–NO_2), 126.52 (*C*–H); *m*/*z* (EI) 189 (7), 69 (32), 52 (68), 44 (100), 43 (35), 40 (64%); [M$^+$] 188.9845, $C_4H_3N_3O_4S$ requires 188.9844.

2,4-Dinitrothiophene (3h). Nitrosylsulfuric acid was first prepared by the addition of sodium nitrite (0.76 g, 11 mmol) to concentrated sulfuric acid (4.9 ml, $d = 1.84$). During the addition the mixture was continuously stirred and the temperature allowed to rise to 30 °C. The solution was next cooled to 5 °C before a mixture of acetic acid (29 ml) and propionic acid (5 ml) was added followed by the portionwise addition of 2-amino-3,5-dinitrothiophene (1.89 g, 10 mmol) over 30 min. During the additions and for a further 45 min the mixture was continuously stirred and the temperature kept below 5 °C. A solution of hypophosphorous acid (50 wt. solution in water, 11.12 g, 15 equiv.) was slowly added and the mixture stirred for 15 min at 0–5 °C before being allowed to return to room temperature during which time nitrogen was evolved as the diazonium salt decomposed. The acidic solution was stirred overnight at room temperature overnight to ensure that the reaction had gone to completion before being carefully extracted with dichloromethane (3 × 50 ml). The extracts were combined and washed with a saturated solution of $NaHCO_3$ to remove acetic and propionic acid residues. The organic layer was washed with water (50 ml), dried over $MgSO_4$ and evaporated to dryness to leave a dark brown coloured solid (0.39 g, 22% yield) which was purified using flash column chromatography using a 85 : 15 mixture of petroleum ether (30–40 °C) and acetone to give (**3h**); mp 55.3–56 °C (lit.[54] 56 °C); δ_H ($CDCl_3$) 8.48 (1H, d, *J* 1.98 Hz, Ar–*H*), 8.42 (1H, d, *J* 1.98 Hz, Ar–*H*); δ_C 151.29, 146.04 (*C*–NO_2), 130.90, 122.40 (*C*–H); IR (Nujol) 1548 (NO_2 asym. str.), 1336 cm^{-1} (NO_2 sym. str.); *m*/*z* (EI) 174 (11), 82 (66), 69 (69), 44 (78), 40 (100), 38 (56%); [M$^+$] 173.9736, $C_4H_2N_2O_4S$ requires 173.9735.

2-(2-Methylpropane-2-sulfanyl)-3,5-dinitrothiophene (3j) and 2-(2-methylpropane-2-sulfonyl)-3,5-dinitrothiophene (3l). 2-Chloro-3,5-dinitrothiophene (0.25 g, 1.2 mmol) was dissolved in ethanol (50 ml) and mixed with a solution of sodium 2-methyl-2-propanethiolate (0.4 g, 3.6 mmol, 3 equiv.) in ethanol (25 ml). The solution which turned dark red was left overnight and then poured into aqueous 2.5% H_2SO_4. The resulting yellow precipitate was filtered off, washed with water, and dried (0.22 g). ^{1}H NMR and TLC analysis using toluene as the eluant showed that the crude material was a mixture of two compounds (R_f 0.65, 0.43) present in equal amounts. The two components were separated using flash column chromatography with toluene as the eluant to give 2-(2-methylpropane-2-sulfanyl)-3,5-dinitrothiophene (**3j**) mp 99.6–102.2 °C; R_f [toluene] 0.65; δ_H ($CDCl_3$) 8.32 (1H, s, Ar–*H*), 1.62 (9H, s, CH_3); δ_C 150.95, 141.67 (*C*–NO_2), 129.00 (*C*–S–C(CH_3)$_3$), 124.26 (*C*–H), 51.14 (C–S*C*(CH_3)$_3$), 30.04 (C–SC(*C*H_3)$_3$); IR (Nujol) 1543 (NO_2), 1308 cm^{-1} (NO_2); *m*/*z* (EI) 262 (1), 69 (32), 58 (15), 57 (100), 41 (63), 39 (17%); [M$^+$] 262.0081, $C_8H_{10}N_2O_4S_2$ requires 262.0082; and 2-(2-methylpropane-2-sulfonyl)-3,5-dinitrothiophene (**3l**) mp 161.9–164.3 °C; R_f [toluene] 0.43; δ_H ($CDCl_3$) 8.20 (1H, s, Ar–*H*), 1.48 (9H, s, CH_3); δ_C 153.50, 145.85 (*C*–NO_2), 140.37 (*C*–SO_2C(CH_3)$_3$), 124.20 (*C*–H), 65.39 (C–SO_2*C*(CH_3)$_3$), 24.19 (C–SO_2C(*C*H_3)$_3$); IR (Nujol) 1556 (NO_2), 1343 (NO_2), 1314 (S=O), 1126 cm^{-1} (S=O); *m*/*z* (EI) 81 (40), 69 (58), 57 (100), 41 (78), 39 (45%); (CI) 312 (30), 155 (21), 140 (12), 117 (11), 100 (100%); [M + NH_4]$^+$ 312.0323, $C_8H_{14}N_3O_6S_2$ requires 312.0324.

3,5-Dinitro-2-thiocyanatothiophene (3k). 2-Chloro-3,5-dinitrothiophene (0.33 g, 1.6 mmol) was dissolved in 20 ml of acetone and potassium thiocyanate (0.165 g, 1.7 mmol, 1.1 equiv.) added and the mixture was stirred for 5 min and then poured on to 70 ml of an ice cold aqueous 2.5% HCl. The resulting precipitate was filtered off, washed with water, and dried under vacuum in the presence on P_2O_5 to give a light brown coloured solid, 0.27 g (73% yield), which was purified by dissolving in hot toluene to remove insoluble impurities, and then recrystallized from aqueous ethanol to give glistening yellow coloured crystals of (**3k**); mp 108.0–109.7 °C; δ_H (d^6-acetone) 8.61 (1H, s, Ar–*H*); δ_C 141.53 (*C*–SCN), 124.97 (*C*–H), 108.784 (S–*C*≡N); IR (Nujol) 1553 (NO_2), 1335 (NO_2); *m*/*z* (EI) 231 (9), 69 (100), 51 (26), 49 (68), 46 (43), 44 (30%); [M$^+$] 230.9410, $C_5HN_3O_4S_2$ requires 230.9408.

Biological testing

Samples of *Escherichia coli*, *Micrococcus luteus* and *Aspergillus niger* were provided by the School of Biological Sciences at Swansea. *E. coli* and *M. luteus* cultures were incubated on lawns prepared from a mixture of nutrient broth E (13 g l^{-1}) and nutrient agar (28 g l^{-1})[55] using the standard agar diffusion method.[55–58] *A. niger* was cultivated using a mixture of soft nutrient agar (17 g l^{-1}) and potato dextrose agar (39 g l^{-1})[55] for distribution and growth of the spores.[55–58] Stock solutions (0.01 M) of each of

the nineteen nitrothiophenes (**3**), were prepared in an appropriate solvent (usually methanol) and diluted with more solvent to give a set of concentrations ranging from 10^{-2} M to 1.5×10^{-7} M. The nitrothiophene solutions were then applied to standard paper assay disks (6 mm diameter) and the impregnated disks were placed at the centre of the prepared lawns set on Petri dishes,[55–58] and incubated for 18 h at 37 °C for the bacteria and 28 °C for the fungus. At concentrations which inhibited growth, a clear zone was apparent around the antibiotic assay disk. A graphical technique was then used to determine the minimum inhibitory concentration (MIC)[59] of the respective nitrothiophene by plotting the diameter of the clear zone against the concentration and extrapolating the line of best fit to the origin. The assay was carried out twice for each compound; the results were plotted on the same graph and the graphical regression trendline fitted by averaging the results of the two assays. No obvious discrepancies were apparent between the two sets of results. In order to check the validity of the technique, two reference compounds with known MIC values were tested. 4,5-Dichloro-*N*-methyl-3-isothiazolone (**2d**) and *N*-(2-phenylethyl)-3-isothiazolone (**2e**) gave MIC values of 14 and 203 μM which were judged to be acceptable *versus* the literature values[5] of 9.8 and 188 μM, respectively.

The refined minimum inhibitory concentrations obtained for each of the nineteen compounds are shown in Table 1. All of the nitrothiophenes were evaluated against the two bacteria *E. coli* and *M. luteus* and eight selected derivatives were tested against the fungus *A. niger*. Because some of nitrothiophenes were difficult to dissolve in methanol, either acetone or DMF were used as alternative solvents. To check that these had no large effects, three derivatives with high, moderate and low activity (**3a**, **3e** and **3q**) were tested in at least two solvents (Table 1). While the change from methanol to DMF appears to increase the activity of (**3a**) against *E. coli*, the reverse is true for *M. luteus*, where the activities of both (**3a**) and (**3e**) decrease (Table 1). However, no changes were found for (**3q**) because of its very low activity (Table 1). Significantly, the magnitude of the solvent-induced variation in the MIC values observed shows only small changes which range from $1–2 \times 10^2$ for (**3a**) against both *E. coli* and *M. luteus*, and $1–2 \times 10^3$ for (**3e**) against *M. luteus*. These variations, therefore, are relatively small by comparison with the magnitude of the variation found in the MIC values of the nineteen derivatives which range 10^2 up to 10^6 (Table 1). For the few compounds that displayed no activity against the bacteria at the 10 000 μM level, the experiments were repeated using a more concentrated 0.1 M stock solution (where solubility would allow).

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