DOI: 10.1002/cmdc.200800371

Inhibition of Peroxidase-Catalyzed Iodination by Cephalosporins: Metallo-β-Lactamase-Induced Antithyroid Activity of Antibiotics

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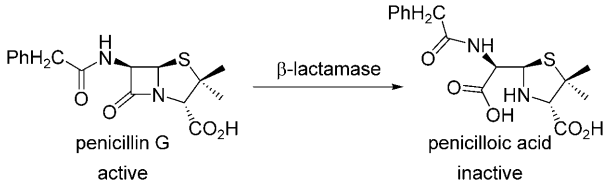
Antibiotics based on β-lactams such as penicillins, cephalosporins, and penems are the most commonly used drugs for bacterial infections.[1] However, the clinical applications of some of these antibiotics are highly limited due to β-lactamase activity, which inactivates the antibiotics by hydrolysis of the β-lactam ring (Scheme 1). The active site of these enzymes contains either a serine residue (serine β-lactamases) or zinc ions (metallo-β-lactamases, mβls).[2–7] The metalloenzymes are particularly efficient in hydrolyzing a wide variety of antibiotics, including the latest generation of cephalosporins, cephamycins, and imipenem.[5–7] As there is no effective and irreversible enzyme inhibitor available, the mβls are emerging as a major threat to effective clinical treatments.



Scheme 1. Hydrolysis of the β-lactam ring in penicillin G by β-lactamases, leading to the formation of penicilloic acid.

Although the β-lactams are among the safest classes of antibiotics, some of the commonly used antibiotics show side effects.[8,9] For example, some members of the cephalosporin family of antibiotics are involved in disulfiram-type reactions that lead to the inhibition of aldehyde dehydrogenase,[8] resulting in the accumulation of acetaldehyde. These antibiotics have also been associated with hypoprothrombinemia, in which a deficiency of prothrombin results in impaired blood clotting.[9] It has been shown that this blood disorder is due to the inhibition of β-carboxylation of glutamic acid, a vitamin-K-dependent reaction required for the formation of active clotting factors. Calesnick et al. reported that the growth-stimulating properties of antibiotics such as penicillin can be attributed to their antithyroid activity.[10] In contrast, Libby and Meites have shown that penicillin and related antibiotics do not have any negative effect on thyroid activity.[11] These findings were further supported by another study in which the results differed from that of Calesnick et al. not only in degree, but in direction.[12] However, it is unknown whether the antibiotics affect thyroid activity upon hydrolysis by β-lactamases. To test this hypothesis, we studied the hydrolysis of some commonly used antibiotics (Figure 1) by the mβl from *Bacillus cereus* and the antithyroid activity of the hydrolyzed products.

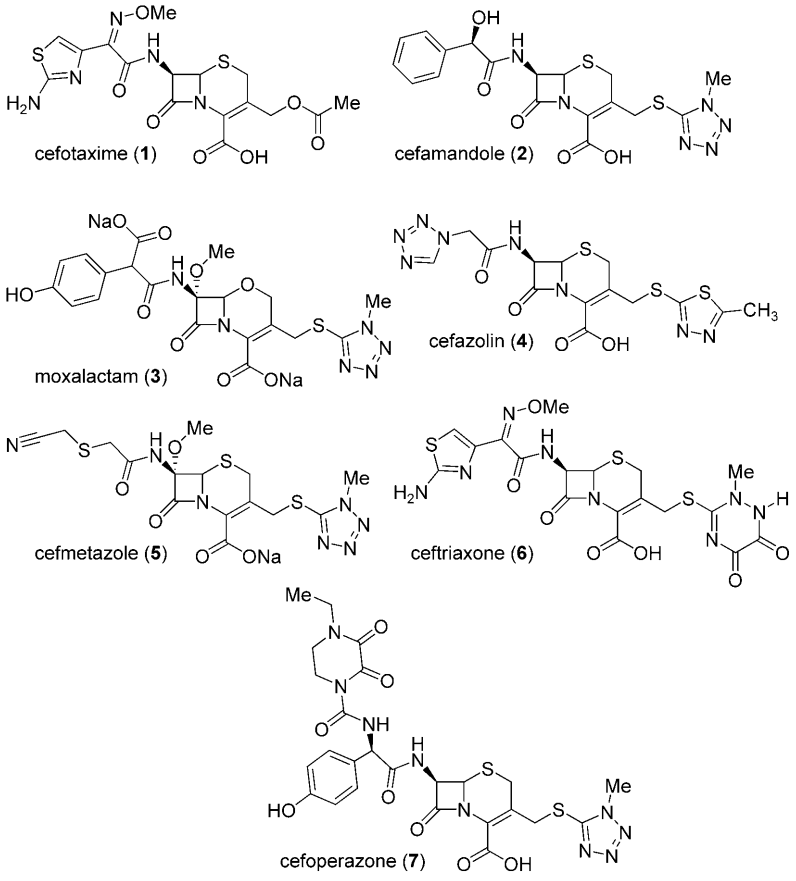


Figure 1. Chemical structures of some commonly used antibiotics employed in the present study.

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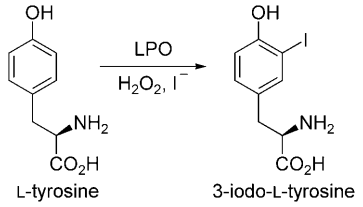
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.200800371.

It is known that thyroxine (T4), the main secretory hormone of the thyroid gland, is produced via thyroglobulin by the thyroid peroxidase (TPO)/hydrogen peroxide/iodide system. The synthesis of T4 by TPO involves two independent steps: iodination of tyrosine and phenolic coupling of the resulting iodotyrosine residues.[13] Therefore, the inhibition of TPO-catalyzed iodination has been used extensively to study the effect of antithyroid drugs on thyroid hormone synthesis.[14] We used lactoperoxidase (LPO) in the present study, as this enzyme is readily available in purified form, and LPO has been shown to behave very similarly to TPO with respect to iodination of thyroglobulin, the natural substrate, and other iodide acceptors.[15] The iodination of L-tyrosine was studied by using an LPO/H_2O_2/I^- assay, and the conversion of L-tyrosine to 3-iodo-L-tyrosine (Scheme 2) was determined by reversed-phase HPLC.[16] The inhibition of LPO-catalyzed iodination was studied by following the decrease in the formation of 3-iodo-L-tyrosine in the presence of various concentrations of antibiotic compounds.



Scheme 2. Iodination of L-tyrosine by the LPO/H_2O_2/I^- system.

When tested as an inhibitor of LPO-catalyzed iodination, penicillin G did not show any noticeable activity up to a concentration of 200 μM. This is in agreement with the report of Libby and Meites that penicillin G does not possess antithyroid activity.[11] Similarly, the cephalosporins (Figure 1) also did not show any significant inhibition of LPO activity. For example, when iodination was carried out in the presence of cefazolin (**4**), essentially no inhibition was observed (Figure 2 A, line a). To determine whether the hydrolyzed products of these β-lactams can inhibit LPO activity, compounds **1**–**7** were treated with mβl and then added to the LPO reaction mixture. The hydrolyzed products from penicillin G did not affect the iodination reaction. Interestingly, when the cephalosporins **2**–**7**, with a heterocyclic side chain (Figure 1), were treated with mβl, these compounds showed a dramatic effect on LPO-catalyzed iodination (Table 1) which can be ascribed to the hydrolysis of the β-lactam ring by mβl. Furthermore, the IC_{50} values indicate that the β-lactams with a five-membered tetrazole (compounds **2**, **3**, **5**, and **7**) or thiadiazole moiety (compound **4**) inhibit the LPO reaction more efficiently than compound **6**, which contains a triaxone group. On the other hand, cefotaxime (**1**), a cephalosporin antibiotic that lacks a heterocyclic thiol moiety, did not inhibit LPO activity even after a complete hydrolysis by mβl (Figure 2 B). These observations suggest that the heterocyclic thiol side chains play an important role in inhibition.

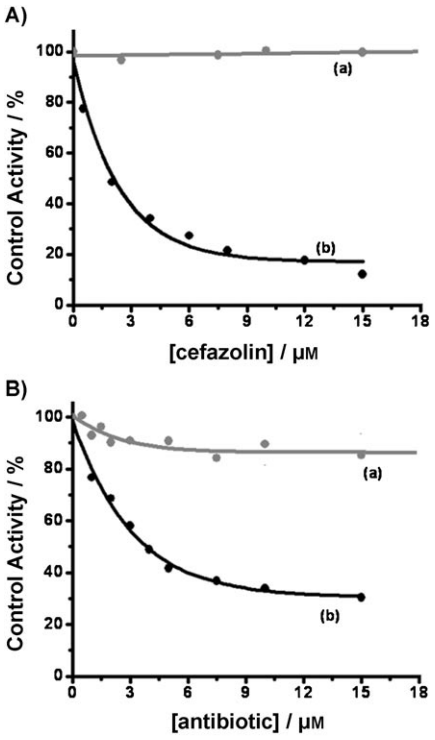


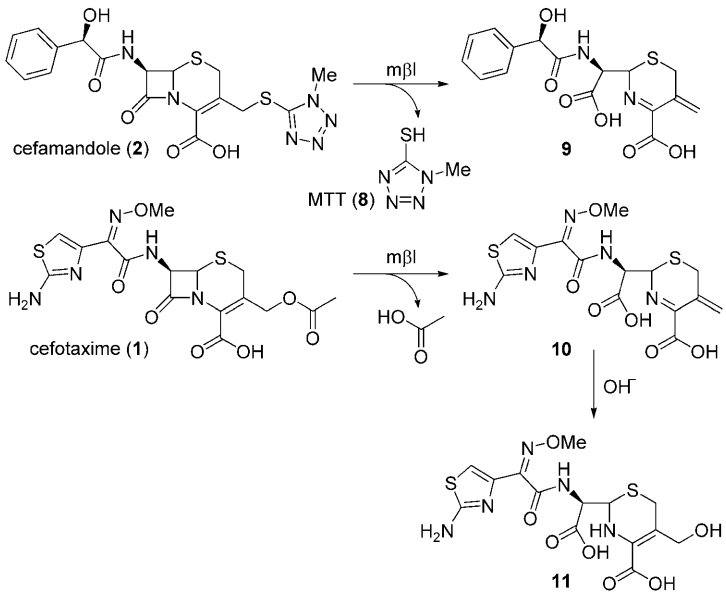
Figure 2. A) Inhibition of LPO-catalyzed iodination by cefazolin in the absence (line a) and presence (line b) of mβl. B) Inhibition by cefotaxime (line a) and cefoperazone (line b) in the presence of mβl.

Table 1. Inhibition of LPO-catalyzed iodination by some antibiotics in the presence of metallo-β-lactamase.

Compound	IC_{50} [μM][a]	Compound	IC_{50} [μM][a]
1	no inhibition	**5**	7.87 ± 0.14
2	4.11 ± 0.04	**6**	35.06 ± 0.42
3	4.14 ± 0.28	**7**	1.75 ± 0.08
4	2.31 ± 0.04		

[a] Assay conditions: L-tyrosine (0.3 mM), KI (0.3 mM), lactoperoxidase (20 nM), and H_2O_2 (0.8 mM) in phosphate buffer (100 mM, pH 7.5) at 25 °C. The antibiotics were allowed to undergo complete hydrolysis by the metallo-β-lactamase. IC_{50} values were determined by plotting the percent of control activity against inhibitor concentration.

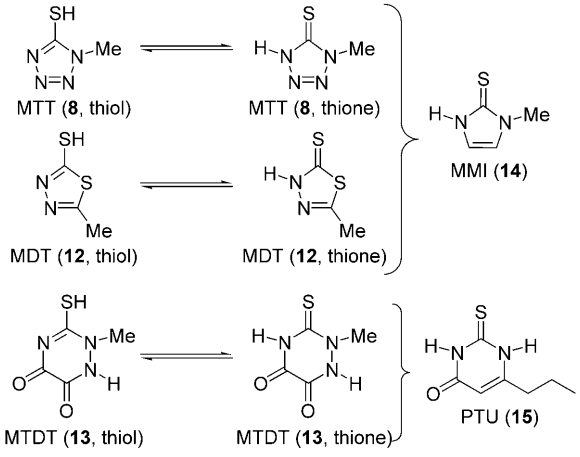
The isolation and characterization of products from the reactions of cephalosporins with mβl indicate that the heterocyclic side chain is eliminated by cleavage of the β-lactam ring. For example, the hydrolysis of cefamandole (**2**) produces 1-methyl-1*H*-tetrazole-5-thiol (MTT, **8**) in nearly quantitative yield (Scheme 3). In contrast to cefamandole (**2**), which produces a heterocyclic thiol, the hydrolysis of cefotaxime eliminates acetic acid and the *exo*-methylene compound **10**. The latter compound undergoes further attack by an external nucleophile, OH^-, to produce the 3′-hydroxymethyl compound **11** (Scheme 3). Our attempts to isolate the *exo*-methylene compound **10** were unsuccessful, and ^{1}H NMR investigations clearly indicate the absence of any such species in the products. The third-generation antibiotics moxalactam (**3**) and cefmetazole (**5**) also generate MTT (**8**) during hydrolysis. Similarly, the mβl-



Scheme 3. The elimination of 1-methyl-1*H*-tetrazole-5-thiol (MTT, **8**) from cefamandole (**2**) and acetic acid from cefotaxime (**1**) upon hydrolysis by mβl, and the conversion of *exo*-methylene compound **10** into 3′-hydroxymethyl compound **11** upon attack by hydroxide.

catalyzed hydrolysis of cefazolin and ceftriaxone produces the corresponding thiols, 2-methyl-1,3,4-thiadiazole-2-thiol (MDT, **12**) and 2-methyl-1,2,4-triazine-5,6-dione-3-thiol (MTDT, **13**), respectively. It should be noted that such elimination may also occur during the hydrolysis of some of the antibiotics by the serine β-lactamases and also by the intrinsic mode of action of the cephalosporins, that is, by inhibition of transpeptidases.

Interestingly, further characterization of the products indicates that the thiols generated by hydrolysis of the β-lactam ring undergo tautomerism to produce the corresponding thiones (Scheme 4). Structural analysis of the thiones reveals that the thione moiety in these compounds is very similar to the pharmacophores present in the commonly used antithyroid drugs methylimidazole (MMI, **14**) and 6-*n*-propyl-2-thiouracil (PTU, **15**), which exhibit their antithyroid activity by inhibiting



Scheme 4. Thiol⇌thione tautomerism of thiols eliminated from antibiotic side chains by the hydrolysis of β-lactam.

TPO-catalyzed iodination.[14] In fact, antithyroid agents and some of these thiones can be considered as bioisosteres according to Grimm's hydride displacement law.[17] For example, a C–H group in a compound can be replaced by nitrogen without altering its biological activity. Therefore, MMI and MTT can be considered as bioisosteres. Because of the structural similarities between the antithyroid drugs and the thiols/thiones eliminated from the antibiotics, MTT, MDT, and MTDT must inhibit peroxidase-catalyzed iodination reactions in their isolated forms.

To understand the effect of various thiones on iodination reactions, we carried out the inhibition of LPO-catalyzed iodination of L-tyrosine and compared the activities of these compounds with those of MMI (**14**) and PTU (**15**) under identical experimental conditions (Table 2). As expected, MTT (**8**) and MDT (**12**) inhibited the LPO activity with IC_{50} values of 7.24 ± 0.44 μM and 3.10 ± 0.65 μM, respectively, which are similar to that of MMI (**14**) (4.11 ± 0.41 μM). Furthermore, the inhibition of LPO by MTT (**8**) and MDT (**12**) was found to be irreversible, which indicates that the mechanism of inhibition is similar to that of

Table 2. Inhibition of LPO-catalyzed iodination by commonly used antithyroid drugs and other thiones.

Compound	IC_{50} [μM][a]	K_i [μM]
MTT (**8**)	7.24 ± 0.44	0.73 ± 0.10
MDT (**12**)	3.10 ± 0.65	0.16 ± 0.03
MTDT (**13**)	16.31 ± 0.35	1.10 ± 0.06
MMI (**14**)	4.11 ± 0.41	0.54 ± 0.13
PTU (**15**)	12.05 ± 0.87	0.67 ± 0.01
DMETT (**16**)	7.73 ± 0.11	0.80 ± 0.06
MMA (**18**)	25.30 ± 1.03	3.14 ± 0.49

[a] Assay conditions: L-tyrosine (0.3 mM), KI (0.3 mM), lactoperoxidase (20 nM) and H_2O_2 (0.8 mM) in phosphate buffer (100 mM, pH 7.5) at 25 °C. IC_{50} values were determined by plotting the percent of control activity against inhibitor concentration.

MMI (**14**). The IC_{50} value for MTDT (**13**) (12.05 ± 0.87 μM) was found to be slightly higher than that of MTT (**8**) and MDT (**12**), but this value is similar to that of PTU (**15**) (16.31 ± 0.35 μM). Although MTDT (**13**) was found to be slightly less active than MTT (**8**) and MDT (**12**), a complete inhibition of LPO activity was observed with MTDT (**13**) (see figure S12 a–c, Supporting Information).

Further experiments with other related compounds suggest that 1-(2-(dimethylamino)ethyl)-1*H*-tetrazole-5-thiol (DMETT, **16**) and 2-(2-mercapto-4-methylthiazol-5-yl)acetic acid (MMA, **18**), which are present in the extended-spectrum third-generation antibiotics cefotiam (**17**)[18] and cefodizime (**19**)[19] respectively (Figure 3), are also potent inhibitors of the LPO-catalyzed iodination reaction. The IC_{50} value of 7.73 ± 0.11 μM obtained for DMETT (**16**) is almost identical to that of MTT (**8**) (7.24 ± 0.44 μM), indicating that the replacement of the N–Me group in MTT (**8**) by an N–$CH_2CH_2NMe_2$ group does not alter the in-

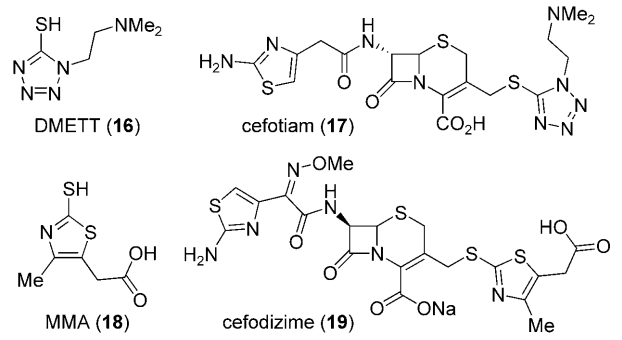


Figure 3. Structures of β-lactams with 2-(2-mercapto-4-methylthiazol-5-yl)-acetic acid (MMA) and 1-(2-(dimethylamino)ethyl)-1*H*-tetrazole-5-thiol (DMETT) side chains.

hibition properties. In contrast, the activity of MMA (**18**), with a thiazole moiety, is much lower than that of MDT (**12**), which contains a thiadiazole ring. These observations indicate that the relative contributions of thiol and thione tautomers determine the inhibitory properties. Therefore, it is important to understand the effect of various substituents on thiol⇌thione tautomerism. To this end, we are currently performing a detailed theoretical study to determine the energy required for the conversion of thiols to the corresponding thione tautomers.[20]

In summary, we have shown for the first time that the heterocyclic thiol side chains present in some commonly used antibiotics possess strong antithyroid activity.[21] The enzymatic hydrolysis of the β-lactam ring in such antibiotics leads to the formation of thiols, which undergo tautomerism to produce the corresponding thiones. The efficient and irreversible inhibition of peroxidase-catalyzed iodination by the thiones suggests that the production of β-lactamases and subsequent hydrolysis of antibiotics would affect thyroid activity. These studies indicate that the antithyroid activity of heterocyclic side chains must be taken into account in the design of new antibiotics based on cephalosporins.

Experimental Section

Materials and methods: Lactoperoxidase from bovine milk was purchased from Fluka; L-tyrosine, 3,5-diiodo-L-tyrosine, the sodium salt of cefotaxime, cefamandole, moxalactam, cefazolin, cefmetazole, ceftriaxone, cefoperazone, 2-(2-mercapto-4-methylthiazol-5-yl)acetic acid (MMA), and deuterated solvents (D_2O and $CDCl_3$) were obtained from Aldrich. Penicillinase (β-lactamase II, BcII) from *Bacillus cereus* was obtained from Sigma. The antithyroid drugs (2-mercapto-1-methylimidazole (MMI) and 6-*n*-propyl-2-thiouracil (PTU)) were obtained from TCI (Tokyo Kasei, Japan). 1-methyl-1*H*-tetrazole-5-thiol (MTT), 2-methyl-1,3,4-thiadiazole-2-thiol (MDT), 2-methyl-1,2,4-triazine-5,6-dione-3-thiol (MTDT) and 1-methyl-5-(methylthio)-1*H*-tetrazole were purchased from Alfa Aesar. HPLC solvents were obtained from Merck. All other chemicals were of the highest purity available.

All chemical reactions were carried out in open atmosphere in phosphate-buffered medium. Buffers of desired pH were freshly prepared prior to use. The HPLC experiments were performed on an analytical HPLC system equipped with a PDA detector controlled by EMPOWER software (Waters, Milford MA, USA). The reaction products were isolated and purified by using reversed-phase flash chromatography (Biotage) and preparative HPLC (Waters) systems. The final products were obtained from the column fractions by freeze-drying the samples on a lyophilizer. ^{1}H NMR spectra (400 MHz) were obtained on a Bruker Avance 400 instrument. Chemical shifts are reported in ppm with respect to $SiMe_4$ as internal standard.

Isolation and purification of hydrolyzed products and the heterocyclic compound: The starting β-lactam substrate (100 mg) was allowed to react with BcII (1 mg) in 5 mL phosphate buffer (1 M, pH 7.5). The reactions were monitored by HPLC using a C_{18} column. When 40–50% of the starting materials were hydrolyzed, the solvent was evaporated under reduced pressure, and most of the buffer salts were removed by dissolving the residues in CH_3OH. The methanolic solution was evaporated to dryness, and the residues were subjected to reversed-phase flash chromatography to obtain the pure hydrolyzed products.

Elimination of side group during the hydrolysis of cefotaxime (**1**): The reaction mixture was injected and the reaction progress was monitored by HPLC using an isocratic solvent eluent system (CH_3CN/H_2O, 15:85 *v/v*). The two peaks were observed in the chromatogram. The last peak was characterized as cefotaxime: ^{1}H NMR (D_2O): $\delta = 2.06$ (s, 3H), 3.38 (d, $J = 18.0$ Hz, 1H), 3.66 (d, $J = 18.0$ Hz, 1H), 3.96 (s, 3H), 4.66 (s, 1H), 4.70 (s, 1H), 5.23 (d, $J = 4.4$ Hz, 1H), 5.80 (d, $J = 4.4$ Hz, 1H), 6.99 ppm (s, 1H). The polar hydrolyzed compound, which eluted ahead of the starting material, was characterized as compound **11**: ^{1}H NMR (D_2O): $\delta = 3.36$ (d, $J = 17.6$ Hz, 1H), 3.58 (d, $J = 17.6$ Hz, 1H), 3.89 (s, 3H), 4.17 (s, 2H), 5.11 (d, $J = 4.0$ Hz, 1H), 5.70 (d, $J = 4.4$ Hz, 1H), 6.93 ppm (s, 1H); ^{13}C NMR (D_2O): $\delta = 24.4$, 56.0, 57.5, 59.8, 61.5, 112.3, 120.0, 128.3, 139.2, 146.9, 162.7, 163.8, 167.9, 169.9 ppm; MS (ESI): 451.9 [*M*+Na−H].

Elimination of side group during the hydrolysis of moxalactam (**3**): The hydrolyzed product of the moxalactam (**3**) was characterized by MS, and the peak corresponds to $t_R = 2.0$ min in HPLC; this species was found to have an exocyclic methylene group, resulting from the elimination of 5-methylterazole-2-thiol (MTT, **8**). The eliminated group was also isolated by reversed-phase flash chromatography. ^{1}H NMR ($CDCl_3$): $\delta = 3.94$ ppm (s, 3H); ^{13}C NMR ($CDCl_3$): $\delta =$ 34.6, 164.6 ppm.

Acknowledgements

This study was supported by the Department of Science and Technology (DST), New Delhi, India. G.M. acknowledges the DST for the award of a Ramanna Fellowship, and A.T. thanks the University Grants Commission (UGC), New Delhi, for a research fellowship.

Keywords: antibiotics · antithyroid drugs · drug resistance · metallo-β-lactamase · peroxidase

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[20] Our preliminary computational data indicate that the thiones are more stable than the thiol tautomers; A. Tamilselvi, G. Mugesh, unpublished results.
[21] There are numerous examples of antibiotics that contain MTT, MDT, and related heterocyclic side chains. Some of these compounds are summarized in figure S24 (Supporting Information).

Received: November 7, 2008
Published online on December 23, 2008

