Inhibition of the Tetracycline Efflux Antiport Protein by 13-Thio-Substituted 5-Hydroxy-6-deoxytetracyclines

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A series of 13-(alkylthio) and 13-(arylthio) derivatives of 5-hydroxy-6-deoxytetracycline (tetracycline, Tc) were synthesized and compared to the clinically used antibiotics tetracycline, methacycline, and minocycline for their ability to inhibit tetracycline efflux in an everted membrane vesicle assay of bacterial resistance to tetracyclines. The assay screened for the ability of tetracycline analogues to inhibit [^{3}H]tetracycline uptake into everted membrane vesicles, thereby evaluating the molecular prerequisites for inhibition of an efflux-dependent resistant bacterial system. Thiol adducts attached at the exocyclic C13 carbon of methacycline led to an increase in inhibitor potency as compared to the reference antibiotics. The most potent inhibitors of [^{3}H]tetracycline uptake into everted vesicles among these analogues, particularly members of the alkyl series, revealed important structure–activity relationships between inhibitor potency, steric parameters, and lipophilicity at the C13 sulfur position.

In recent years, the clinical effectiveness of the tetracycline family of antibiotics has declined due to the emergence of tetracycline-resistant bacterial strains bearing acquired resistance determinants.[1,2] These resistance determinants, numbering more than a dozen, are spread in the microbial world via transposon and plasmid DNA vectors. Two major mechanisms for resistance have been described: active efflux and ribosomal protection.[1-4] The most prominent mechanism among Gram-negative bacteria is active efflux of tetracycline. In these resistant bacteria, resistance is usually not expressed, but becomes inducible by contact of the bacterium with subinhibitory amounts of tetracycline. Once resistance to tetracycline is expressed, customary therapeutic tetracycline concentrations are useless and bacterial growth and infection continues despite the presence of the antibiotic.

Tetracycline efflux is mediated by family of inner membrane proteins called the Tet proteins,[5] with a molecular weight of 42–43 kDa of slightly different amino acid composition specified by at least five different tetracycline-resistance determinants (clases A–E).[2,6] Tet is an integral membrane protein whose action reduces intracellular tetracycline concentrations, ensuring survival of the host bacterium. Active efflux is energized by protonmotive force and functions as a tetracycline–cation antiport system with protons.[3,7] Tet proteins of somewhat larger size, but encoded by other determinants (classes K–L) have been described among Gram-positive bacteria, in particular *Staphylococcus aureus* and *Streptococcus* species.[8-10]

The Model of Tetracycline Efflux and Resistance

In order to build a molecular view of the substrate requirements for Tet protein interaction, we tested various tetracycline analogues for their ability to inhibit uptake of [^{3}H]tetracycline (^{3}H-Tc) into everted vesicles derived from a tetracycline-resistant bacterium containing an antiport efflux protein (Nelson et al., in preparation). Among the compounds tested, we found that 13-(phenylthio)-5-hydroxy-6-deoxytetracycline (1) was a potent inhibitor of the efflux system with an IC_{50} of 6.9 μM. In an effort to develop a more potent inhibitor of Tc efflux and build a molecular view of the inhibitor–Tet protein interaction, we subsequently synthesized a series of 13-(alkylthio) and 13-(arylthio) derivatives of 5-hydroxy-6-deoxy-Tc.

The compounds were evaluated for their ability to inhibit Tc uptake in the everted membrane vesicle preparation prepared from Tc-resistant *Eschrichia coli* D1-209, possessing plasmid R222, which bears the class B determinant in Tn10. Class B is the most prevalent Tc-resistance determinant among *E. coli*, and is commonly found in many bacterial genera.[11] Moreover, everted membrane vesicles from class B containing resistant cells exhibit the highest transport activity of all the Gram-negative determinants so far studied. When everted membrane vesicles are energized by lithium lactate, ATP, or NADH, they accumulate ^{3}H-Tc, which is readily measured by nitrocellulose membrane filtration and scintillation counting.[3] The uptake of Tc into everted vesicles occurs by Michaelis–Menton applicable kinetics with K_m of 8–20 μM.[3] The K_m values for Tc uptake into everted membrane vesicles reported by Yamaguchi et al.[7] are between 22 and 50 μM, depending upon the dication species present for transport. Using Mg^{2+} as the dicationic species, our laboratory routinely gets K_m values between 8 and 20 μM. When a competitor for Tc transport is added to energized vesicles along with ^{3}H-Tc, the amount of inhibition can be measured as a decrease in accumulation of labelled drug at the 2.5-min point. This endpont reflects approximately the steady-state balance of intravesicular accumulation and extravesicular escape of ^{3}H-Tc. An example of intravesicular uptake of ^{3}H-Tc in the presence of 13-(phenylthio)-5-hydroxy-6-deoxy-Tc is presented in Figure 1. The 2.5-min time point was chosen because of its

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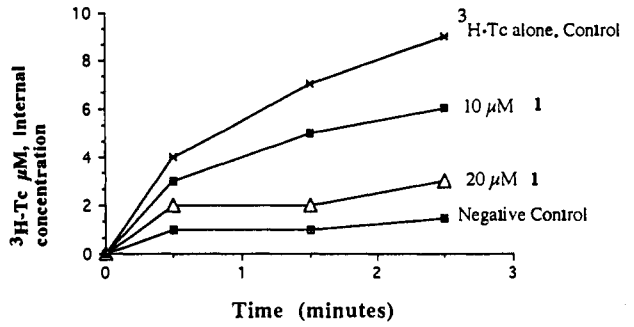


Figure 1. Inhibition of uptake of ^{3}H-Tc into lithium lactate energized *E. coli* D1-209 everted membrane vesicles by 13-(phenylthio)-5-hydroxy-6-deoxy-Tc (**1**). The curves represent those of a typical experiment; see the Experimental Section. Control = 2.76 μM ^{3}H-Tc. Negative control = vesicles in the presence of 2.76 μM ^{3}H-Tc with the protonophore CCCP.

reproducibility among vesicle preparations and compounds. Most compounds were tested multiple times (n = >3) using vesicles energized by lithium lactate as the positive control and vesicles treated with the protonophore CCCP [[(3-chlorophenyl)hydrazono]propanedinitrile] as the negative control. The IC_{50} values were calculated and averaged and are listed in Table I. In each assay methacycline was used as a reference compound. The standard deviation in the IC_{50} values for methacycline (2.5 μM) was 1.1 μM for over 25 determinations.

A major concern in the testing of potential inhibitors is that drug not interfere with the functioning of the vesicle membrane, by alteration of membrane energization or pH gradient formation. The quenching of acridine orange fluorescence has been shown to be an effective and general method for detecting the formation of pH gradients in everted membrane vesicles.[12] Vesicles energized in the presence of the acridine orange dye decrease fluorescence to a level 40–60% of the original. When the proton gradient is collapsed by the addition of a protonophore, such as CCCP, the fluorescence level returns to that before energization. Using Tc-sensitive cell vesicles this method identifies any compound that acts by membrane or energy disruption. All of the compounds reported herein were tested in this manner; none was found to interfere with sensitive vesicle uptake of acridine orange. The inhibition data for the compounds did not provide any insight into the nature of the inhibitory process, i.e. the competitive or noncompetitive interaction of the compounds with the Tet protein.

Chemistry

The 13-thio-substituted derivatives of 5-hydroxy-6-deoxy-Tc was synthesized using modifications of the methods described by Blackwood et al. and are outlined in Scheme I.[13,14] Starting with 6-methylene-5-hydroxy-Tc (methacycline, **I**), the substituted 13-thio compounds were produced in moderate yield by the anti-Markovnikov free-radical addition of mercaptans to the C6, C13 exocyclic double bond. Methacycline **I** was suspended in ethanol containing a catalytic amount of 2,2′-azobis(2-methylproprionitrile) (AIBN) and 2–10 equiv of reacting thiol and heated to give compounds **1–7**, **10–22**, and **24–27**. Cyclopentyl mercaptan reacted with 5-deoxymethacycline in a similar fashion forming 13-cyclopentylthio-5,6-dideoxy-Tc (**22**). Reaction progress was monitored via thin-layer chromatography using Na_2EDTA-impregnated silica gel plates and Fast Blue B salt as a visualization reagent and/or C18-reverse phase HPLC using UV detection at 280 nm. The presence of the cations Mg^{2+} and Ca^{2+} in chromatography silica gel combined with the potent metal-chelating properties of the tetracyclines makes chromatography difficult and neccessitates the use of a chelating agent in both the solid and mobile phases.[15]

The highly reactive thiophenol regents were found to cause gross degradation of methacycline to unknown products when using literature concentrations of reagent. Limiting the amount of thiol to 2 equiv with purification by column chromatography led to the substituted 13-(phenylthio)-5-hydroxy-6-deoxy-Tc series **1–4** in low yield.[14]

Oxidation of 13-(benzylthio)-5-hydroxy-6-deoxy-Tc (**5**) by Oxone (1:1 equiv of $2KHSO_5/KHSO_4/K_2SO_4$–sulfide) in methanol afforded 13-(benzylsulfinyl)-5-hydroxy-6-deoxy-Tc (**8**) in excellent yield. Oxidation of **5** by Oxone (2 equiv) led to the production of 13-(benzylsulfonyl)-5-hydroxy-6-deoxy-Tc (**9**) in excellent yield.[16,17]

Reaction of 13-(cyclopentylthio)-5-hydroxy-6-deoxy-Tc (**19**) with morpholine and formaldehyde in *tert*-butyl alcohol formed the water-soluble Mannich derivative 13-(cyclopentylthio)-5-hydroxy-6-deoxy-*N*-(morpholinomethyl)-Tc (**23**) in excellent yield.[18] FAB high-resolution mass spectral measurements correlated well within the experimental limits for these compounds.[19]

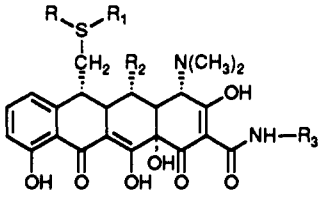
Biology: Results

Everted Membrane Vesicle Assay. The effects of compounds **1–27**, as well as the antibiotics methacycline, minocycline, and tetracycline, on the uptake of ^{3}H-Tc into everted membrane vesicles prepared from Tc-resistant *E. coli* D1-209 were examined (Table I). In these assays, methacycline and minocycline exhibited similar IC_{50} values (2.5 and 2.6 μM, respectively) whereas tetracycline was less active (13.2 μM; its approximate K_m). Methacycline was chosen as a standard in all the assays because it is used as the starting material for 13-thio-substituted derivatives, while minocycline was chosen as a comparison compound because of its enhanced antibacterial activity against Tc-resistant organisms.

Comparing the inhibitory activity of the 13-thio-substituted 5-hydroxy-6-deoxy-Tc series revealed that the introduction of a phenyl or benzyl group at the exocyclic position resulted in a substantial increase in potency that was dependent upon the extent of ring substitution. Phenyl substitution at the C13 sulfur (**1**) resulted in an approximate 2-fold increase in activity (6.9 μM) as compared to tetracycline (13.2 μM), with further potency increases occurring with 4-Cl or 4-Br substitution (**2**, 1.6 μM; **3**, 1.4 μM). The most active compound of the phenyl series was the 4-OCH_3 derivative (**4**, 1.2 μM); however, the potency increase was only slight. C13 sulfur benzyl derivatization resulted in a similar increase in inhibitory activity (**5**, 1.4 μM), whereas further ring subsitution by halogens (4-Cl, 3,4-Cl_2) resulted in concurrent decreases in potency (**6**, 2.1 μM; **7**, 5.2 μM). Oxidation of the 13-benzylthio group to the sulfoxide increased inhibitory potency 2-fold (**8**, 0.7 μM) while further oxidation to the 13-benzyl sulfone (**9**, 1.5 μM) returned activity to that of the benzyl sulfide. In this series of aromatic derivatives the rank order of potency was 13-(benzylsulfinyl) > 13-(benzylthio) ≥ 13-[(4-substituted-phenyl)thio] > 13-(phenylthio) ≫ tetracycline.

The alkyl-substituted series **10–27** exhibited inhibitory activity that was clearly dependent upon the size, lipo-

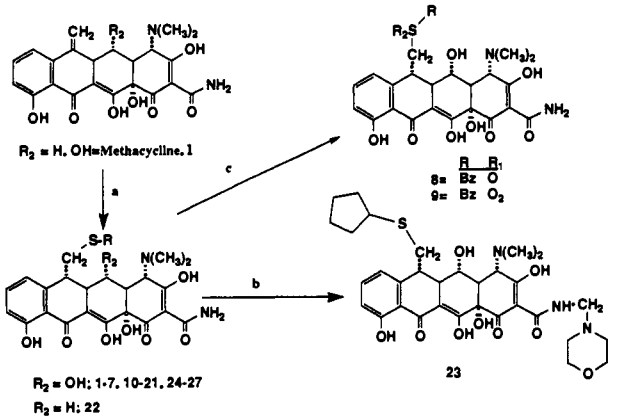
Table I. Chemical Structure, Bioactvity Data, and Substituent Physicochemial Parameters for 13-Thio-Substituted 5-Hydroxy Tetracyclines



no	R	R_1	R_2	R_3	IC_{50}, μM[a]	log (1/IC_{50})	L[c]	B_1[c]	B_4[c]	π[d]
1	C_6H_4		OH	H	6.9 ± 0.9	−0.838	6.28	1.71	3.11	2.13
2	4-Cl-C_6H_4		OH	H	1.6 ± 0.1	−0.204	7.74	1.80	3.11	2.83
3	4-Br-C_6H_4		OH	H	1.4 ± 0.2	−0.146	8.05	1.80	3.11	3.32
4	4-$CH_3OC_6H_4$		OH	H	1.2 ± 0.2	−0.079	8.20	1.80	3.11	2.09
5	C_7H_7		OH	H	1.4 ± 0.1	−0.146	3.63	1.52	6.02	2.63
6	4-Cl-C_7H_6		OH	H	2.1 ± 0.7	−0.322	4.42	1.52	7.44	3.33
7	3,4-Cl_2-C_7H_5		OH	H	5.2 ± 1.1	−0.716	4.42	1.52	7.44	4.03
8	C_7H_7	O	OH	H	0.7[f]	0.154	3.63	1.52	6.02	
9	C_7H_7	O_2	OH	H	1.5[e]	−0.176	3.63	1.52	6.02	
10	CH_3		OH	H	7.9 ± 2.3	−0.898	3.00	1.52	2.04	0.5
11	CH_2CH_3		OH	H	0.7 ± 0.2	0.222	4.11	1.52	2.97	1.0
12	$CH_2CH_2CH_3$		OH	H	0.7 ± 0.1	0.222	5.05	1.52	3.49	1.5
13	$CH(CH_3)_2$		OH	H	0.7 ± 0.1	0.222	4.11	2.04	3.16	1.3
14	$(CH_2)_3CH_3$		OH	H	0.8 ± 0.1	0.155	6.17	1.52	4.42	2.0
15	$CH_2CH(CH_3)_2$		OH	H	0.4 ± 0.1	0.255	5.05	1.90	3.49	1.8
16	$C(CH_3)_3$		OH	H	0.5[e]	0.301	4.11	2.59	2.97	2.0
17	$(CH_2)_5CH_3$		OH	H	4.7 ± 1.0	−0.643	8.22	1.52	5.87	2.5
18	cyclohexyl		OH	H	0.6 ± 0.2	0.222	6.17	2.04	3.49	2.5
19	$(CH_2)_2CH(CH_3)_2$		OH	H	0.3 ± 0.1	0.533	4.97	2.04	3.98	2.14
20	$(CH_2)_2CH(CH_3)_2$		OH	H	0.3 ± 0.9	0.533	6.17	1.52	4.42	2.3
21	$(CH_2)_9CH_3$		OH	H	11.2 ± 1.8	−1.017	12.32	1.52	8.80	5.5
22	cyclopentyl		H	H	0.4 ± 0.1	0.255	4.97	2.04	3.98	2.14
23	cyclopentyl		OH	b	1.0 ± 0.3	0.000	4.97	2.04	3.98	
24	CH_2CH_2OH		OH	H	4.7 ± 0.6	−0.672	4.79	1.52	3.38	0.39
25	$CH_2CHOHCH_2OH$		OH	H	5.7 ± 1.2	−0.755	5.73	1.52	3.38	0.29
26	$(CH_2)_2CO_2H$		OH	H	43.2 ± 9.3	−1.633	5.96	1.60	3.11	
27	$CH_2CH_2CH_2Cl$		OH	H	0.6 ± 0.2	0.222	6.82	1.52	3.49	2.2
	tetracycline				13.2 ± 4.2					
	methacycline				2.5 ± 1.1					
	minocycline				2.6 ± 0.9					

[a] The conditions are described in the Experimental Section. Data are the mean ± SEM. [b] *N*-morpholinomethyl. [c] References 20 and 21. [d] References 22 and 23. [e] Average of two determinations. [f] One determination.

Scheme I [a]



[a] (a) RSH, where R = substituted or unsubstituted alkyl, aryl, or benzyl, AIBN; (b) 37% formaldehyde, morpholine; (c) $2KHSO_5/KHSO_4/K_2SO_4$ 1 or 2 equiv/sulfide.

philicity, and polarity of the C13 sulfur substituent. Linear alkyl chains showed increased potency over the arylthio series. The inhibitory activity of the 13-methylthio derivative (**10**, 7.9 μM) was slightly greater than that of tetracycline (13.2 μM), but increasing the substituent size from one to two carbons, making the ethyl-substituted analogue, produced enhanced activity (**11**, 0.7 μM). Activity was maintained up to the propyl and butyl derivatives (**12**, 0.7 μM; **14**, 0.8 μM) whereby further increases in chain length to the hexyl derivative resulted in marked decreases in activity (**17**, 4.7 μM). The decyl derivative was the least potent of the linear alkyl series (**21**, 11.2 μM). Branched aliphatic derivatives isopropyl, isobutyl, and *tert*-butyl (**13**, 0.7 μM; **15**, 0.4 μM; **16**, 0.5 μM) showed increases in activity over the linear three- and four-carbon homologues. Further increases in potency were obtained, with the alicyclic cyclopentyl and isopentyl derivatives being the most potent of all the compounds tested (**19**, 0.3 μM; **20**, 0.3 μM). Of particular note, the cyclohexyl derivative was considerably more active than its linear-chain homologue (**18**, 0.6 μM). In fact, it closely resembled the cyclopentyl derivative. 13-(Cyclopentylthio)-5-deoxy-6-deoxy-Tc (**22**, 0.4 μM) showed similar activity to the 5-hydroxy analogue, whereas the Mannich base prodrug form of **19** exhibited a small decrease in potency (**23**, 1.0 μM).

Structure–Activity Relationships (SAR). All compounds which were tested for inhibitory potency were subjected to an SAR analysis. Correlations of the bioassay data with substituent size parameters suggest that the compounds most active in inhibiting the Tet protein in everted vesicles are those that contain an aliphatic moiety that is of optimal size at the C13 sulfur. For size attributes within the alkyl derivatives, a plot of log (1/IC_{50}) versus aliphatic substituent chain length or optimal width as chosen from Verloop–Hoogenstraaten STERIMOL val-

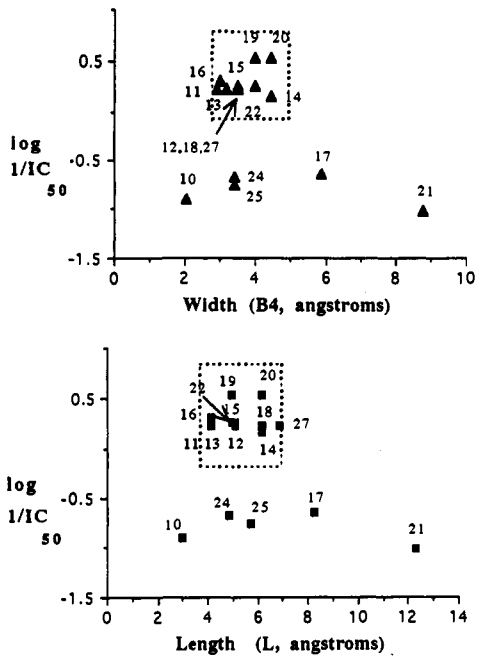


Figure 2. Plot of the log ($1/IC_{50}$) for uptake inhibition versus Veerloop–Hoogenstraaten STERIMOL parameters (L, B_4).[20,21] The boxed area represents compounds of favorable bioactivity.

ues[20,21] depicts the relationship between bioactivity and molecular size. The importance of substituent alkyl chain length and width on inhibitory activity is depicted in Figure 2. The more potent inhibitors of efflux had steric substituent L values between approximately 4.4–6.2 Å, reflecting particular length and bulk requirements along the L axis. The B_4 substituent parameter showed a similar correlation, where optimum width was between 3.0 and 4.2 Å. Other substituent width parameters (B_1, B_2, B_3) did not correlate well with inhibitory potency. Two compounds, **24** and **25**, polar analogues of ethyl and propyl derivatives **11** and **12**, exhibited poor potency although they fell within the range of favorable width and length. These findings suggested that other parameters affecting bioactivity were involved.

Effect of Lipophilicity on Inhibitory Potency. Introduction of polar alkyl derivatives containing hydroxyl or carboxylic acid groups resulted in a large decrease in potency as compared to the linear alkyl chain homologues. Hydroxyethyl analogue **24**, 2,3-dihydroxypropyl analogue **25**, and propyl 3-carboxylic acid derivative **26** exhibited decreasing activity as polarity increased (IC_{50} values of 4.7, 5.7, and 43.2 μM, respectively). An inspection of the plot of log ($1/IC_{50}$) vs π (calculated from the normalization of 13-thio-5-hydroxy-6-deoxymethacycline to 0.00;[22,23] Figure 3) graphically illustrates the relationship of substituent π values on the activity of 13-(alkylthio) derivatives, and that the more potent of the derivatives fall within a range of π values (approximately 1.00 and 2.50; see Table I). In order to establish this site as a hydrophobic area, a lipophilic analogue, 13-[(3-chloropropyl)thio]-5-hydroxy-6-deoxy-Tc (**27**), was synthesized and found to be a potent inhibitor of Tc efflux, demonstrating an IC_{50} of 0.6 μM. A plot of the log ($1/IC_{50}$) values for the substituted 13-(propylthio) and 13-(ethylthio) derivatives versus their substituent π values indicates that there is a linear correlation between activity and lipophilicity within molecules of comparable size (Figure 4). The lipophilic 3-chloro derivative was approximately 10 times more potent compared to its 2,3-dihydroxypropyl

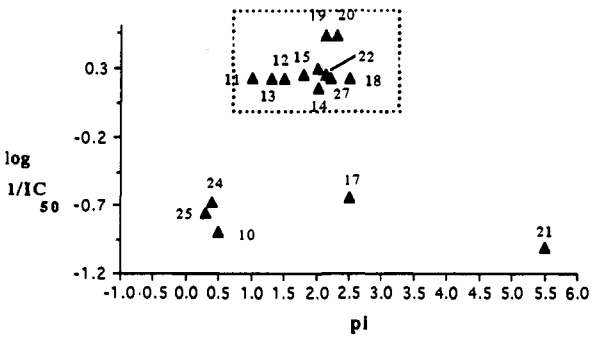


Figure 3. Plot of log ($1/IC_{50}$) for uptake inhibition versus substituent lipophilicity values.[22,23] The boxed area represents compounds of favorable bioactivity.

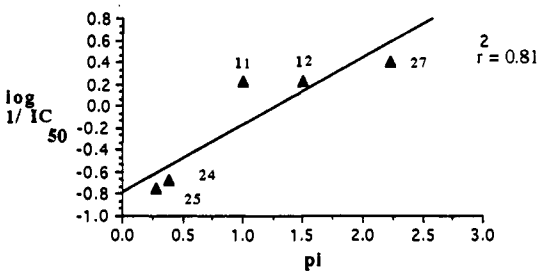


Figure 4. Plot of log ($1/IC_{50}$) for uptake inhibition versus substituent lipophilicity values for ethyl and propyl derivatives **11**, **12**, **24**, **25**, and **27**.[22,23]

analogue and slightly more potent than the unsubstituted propyl parent. Similarly, the ethyl derivative was 8–10 times more potent than the hydroxyethyl compound, indicating more favorable uptake inhibition by lipophilic substituents. While the hydroxyethyl and 2,3-dihydroxypropyl derivatives were of approximately molecular size for efflux inhibition, the polarity of the hydroxyl groups led to a substantial decrease in potency.

Discussion

A number of Tc analogues were quite potent inhibitors of tetracycline uptake into everted vesicles derived from tetracycline-resistant *E. coli*. The increased activity of the lipophilic and sterically accommodating 13-(alkylthio) analogues as compared to the arylthio and alkylthio polar derivatives and the discrete size constraints within the 13-(alkylthio) series strongly suggest that a lipophilic area or "pocket" exists within the domains of the Tet protein and that, for optimal inhibition of efflux by 13-substituted tetracyclines in everted vesicles, certain criteria of lipophilicity and size must be met. The width and length of the alkyl substituent, as well as the polarity of attached functional groups influence the inhibitory activity. Therefore, it seems likely that the superior activity exhibited by 13-(cyclopentylthio)-5-hydroxy-6-deoxy-Tc (**19**) versus the arylthio series and reference antibiotics is presumably due to the space-filling feature and concurrent lipophilicity of the 13-cyclopentyl substituent. The putative lipophilic region or pocket is further substantiated by the linear correlation of activity between the lipophilic derivatives and their polar analogues. On the contrary, increases in aliphatic substituent width, length, and polarity would be detrimental to maximal inhibition because of one, a combination, or all three of these parameters describing the lipophilic region being exceeded.

In summary, to our knowledge this is the first detailed structure–activity study of inhibitors of the Tet protein efflux system. These studies should lead to a further

understanding of tetracycline efflux mechanisms and to the potential development of compounds effective against tetracycline-resistant bacteria. The antibacterial properties of these compounds in tetracycline-sensitive and -resistant bacteria will be reported in a forthcoming paper.

Experimental Section

Chemistry. All compounds, even those purified by preparative HPLC, did not furnish satisfactory elemental analysis. This problem has been seen by other investigators, where carbon values differed by as much as 0.8% while hydrogen and nitrogen values were within the 0.4% limit.[13] Because of the observed nature of these compounds, we chose to rely on HPLC, high-resolution FAB-MS, and NMR spectroscopy for the determination of purity, all of which indicated compound homogeneity. Melting points were taken on a Mel-Temp capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 300 MHz on a Bruker AM 3000 spectrometer. The chemical shift values are expressed in δ values (ppm) relative to tetramethylsilane or 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt, as either an internal or external standard using $CDCl_3$, DMSO-d_6, or MeOH-d_4 as the solvent. Column chromatography was performed according to the method of Still[24] using Baker "flash" grade silica gel (40 μm) that was treated with a saturated solution of Na_2EDTA, washed with water, filtered, and dried in an oven at 130 °C for 3 h prior to use. Analytical TLC separations employed the use of 0.25-mm silica gel plates with fluorescent indicator obtained from J. T. Baker Chemical Co., Philipsburg, NJ, that were pretreated by immersion into a saturated solution of Na_2EDTA for 5 min and reactivated at 130 °C for 3 h. Solvent systems used were as follows: 50:50:5, $CHCl_3$/MeOH/5% Na_2EDTA (lower phase) (I), 65:20:5, $CHCl_3$/MeOH/5% Na_2EDTA (lower phase) (II). Visualization of TLC was accomplished by 0.5% aqueous Fast Blue BB salt and heating at 130 °C for 5 min. Analytical HPLC was performed on a Waters Bondapack C18 reverse-phase column by using two Waters 501 HPLC pumps at a 1.6 mL/min flow rate controlled by Maxima Chromatography software. Detection was by UV absorption with a Model 441 absorbance detector operating at 280 nm. Mobile phases used followed a linear gradient from 30% to 100% MeOH over 30 min at 1.6 mL/min flow rate followed by isocratic elution with MeOH; solvent system A, 0.02 M Na_2HPO4 + 0.001 M Na_2-EDTA adjusted to pH 4.5 with H_3PO_3; solvent system B, 100% MeOH. Semipreparative HPLC separations used a Waters semipreparative C18 reverse-phase column at a flow rate of 6.4 mL/min. High-resolution FAB mas spectrometry was performed on a MAT CH-5-DF spectrometer by James Nielson of the Physical Analytical Chemistry Unit at the Upjohn Co., Kalamazoo, MI.

General Procedure for the Synthesis of 13-[(Substituted-phenyl)thio]-5-hydroxy-6-deoxytetracyclines. 13-(Phenylthio)-5-hydroxy-6-α-deoxytetracycline (1). A mixture of methacycline hydrochloride (3.0 g, 6.2 mmol), AIBN (250 mg), and thiophenol (1.32 g, 12.4 mmol) in EtOH (50 mL) was heated at reflux for 6 h while under N_2. The reaction mixture was cooled, filtered to remove insolubles, and concentrated to one-fifth volume under reduced pressure. Precipitation of the resultant solution in cold Et_2O led to isolation of crude product (2.17 g). The solid was dissolved in hot H_2O, and extracted into $CHCl_3$ at pH 5.0. Removal of the solvent and treatment with activated charcoal in MeOH led to isolation of the product (0.958 g, 27.1%): mp = 164–171 °C dec; TLC Rf = 0.67 (I); HPLC t_R = 14.45 min; ^{1}H NMR ($CDCl_3$) δ 11.9 (br, s), 9.3 (br s), 7.35 (m, 6 H), 6.83 (d, 1 H), 6.74 (d, 1 H), 5.95 (br s, 1 H), 4.10 (br s, 1 H), 3.82 (s, 2 H), 3.60 (br s, 1 H), 3.10 (m, 2 H), 2.60 (m, 1 H), 2.48 (s, 6 H); MS (FAB) m/z 552, 553 ([M + H]+), 445 (M – C_6H_5 – S + H).

13-[(4-Chlorophenyl)thio]-5-hydroxy-6-α-deoxytetracycline (2). This compound was prepared in low yield (25.3%, 874 mg) as a dark yellow solid as described for 1: mp = 167–178 °C dec; TLC R_f = 0.74 (I); HPLC t_R = 16.45 min; ^{1}H NMR ($CDCl_3$) δ 11.9 (br, s), 9.3 (br s), 7.2–7.60 (br m, 5 H), 6.81 (d, 1 H), 6.79 (d, 1 H), 5.93 (br s, 1 H), 4.11 (br s, 1 H), 3.82 (s, 2 H), 3.60 (br s, 1 H), 3.10 (m, 2 H), 2.60 (m, 1 H), 2.50 (s, 6 H); MS (FAB) m/z 586, 587 ([M + H]$^+$), 445 (M – (4-ClC_6H_4 – S + H)).

13-[(4-Bromophenyl)thio]-5-hydroxy-6-α-deoxytetracycline (3). This compound was prepared in low yield (21.0%, 437 mg) as a dark yellow solid as described for 1: mp = 166–172 °C dec; TLC R_f = 0.75 (I); HPLC t_R = 16.58 min; ^{1}H NMR ($CDCl_3$) δ 11.7 (br, s), 9.2 (br s), 7.19–7.63 (br m, 5 H), 6.78 (d, 1 H), 6.28 (d, 1 H), 5.94 (br s, 1 H), 4.13 (br s, 1 H), 3.84 (s, 2 H), 3.62 (br s, 1 H), 3.10 (m, 2 H), 2.60 (m, 1 H), 2.49 (s, 6 H); MS (FAB) m/z 630, 631 ([M + H]$^+$), 445 (M – (4-BrC_6H_4 –S + H)).

13-[(4-Methoxyphenyl)thio]-5-hydroxy-6-α-deoxytetracycline (4). This compound was prepared in low yield (18.9%, 651 mg) as a yellow solid as described for 1: mp = 164–170 °C dec; TLC R_f = 0.64 (I); HPLC t_R = 15.48 min; ^{1}H NMR ($CDCl_3$) δ 11.8 (br, s), 9.3 (br s), 7.2–7.63 (br m, 5 H), 6.77 (d, 1 H), 6.27 (d, 1 H), 5.92 (br s, 1 H), 4.11 (br s, 1 H), 3.84 (s, 2 H), 3.62 (br s, 1 H), 3.3 (m, 3 H), 3.13 (m, 2 H), 2.61 (m, 1 H) 2.49 (s, 6 H); MS (FAB) m/z 582, 583 ([M + H]$^+$), 445 (M – (4-$CH_3C_6H_4$ – S + H)).

13-(Benzylthio)-5-hydroxy-6-α-deoxytetracycline (5). Methacycline hydrochloride (1.0 g, 2.1 mmol) was placed in a round-bottom flask and suspended in 20 mL of EtOH. Benzyl mercaptan (0.5 mL, 6.3 mmol) and AIBN (200 mg) were added and the reaction mixture refluxed with stirring for 2 h while under N_2. This solution was added dropwise to 200 mL of rapidly stirred cold Et_2O and the resulting precipitate collected by filtration. The solid was dissolved in 20 mL of H_2O and brought to pH 4.5 by 1.0 N KOH. Filtration produced a red-orange solid that was dissolved in 200 mL of hot CH_2Cl_2 and filtered. Benzene (25 mL) was added and the solution concentrated to 25 mL on a steam bath. On cooling, a dark brown solid was removed by filtration. Concentration of the filtrate gave 310 mg of the product in 26.0% yield: TLC R_f = 0.25 (II); HPLC t_R = 17.9; ^{1}H NMR ($CDCl_3$) δ 11.9 (br s), 9.3 (br s), 7.35 (m, 6 H), 6.83 (d, J = 8.3 Hz, 1 H), 6.74 (d, J = 8 Hz, 6 H), 5.95 (br s, 1 H), 4.10 (br s, 1 H), 3.82 (s, 2 H), 3.60 (br s, 1 H), 3.3 (m, 3 H), 3.10 (m, 2 H), 2.60 (m, 1 H), 2.51 (s, 6 H); HRMS (FAB) m/z 567 ([M + H]$^+$), 550, 91.

This material was converted to HCl salt by dissolving in MeOH, adding water, and adjusting to pH 1.5 followed by lyophilization.

13-[(4-Chlorobenzyl)thio]-5-hydroxy-6-α-deoxytetracycline (6). This compound was prepared in low yield (35.2%, 632 mg) as a yellow solid as described for 5: mp = 183–191 °C dec; TLC R_f = 0.53 (II); HPLC t_R = 22.25 min; ^{1}H NMR (MeOH-d_4) δ 7.2–7.4 (br m 5 H), 6.95 (d, 2 H), 6.80 (m, 1 H), 3.75 (s, 2 H), 2.65 (s, 6 H); HRMS (FAB) calcd for $C_{29}H_{28}N_2O_8SCl$ 601.1411 (M + 1), found 601.1424 (M + 1).

13-[(3,4-Dichlorobenzyl)thio]-5-hydroxy-6-α-deoxytetracycline (7). This compound was prepared in low yield (29.2%, 146 mg) as a brown-yellow solid as described for compound 5: mp 193–196 °C dec; TLC R_f = 0.55 (I); HPLC t_R = 24.61 min; ^{1}H NMR (MeOH-d_4) δ 7.41 (m, 5 H), 7.10 (d, 1 H), 6.65 (m, 1 H), 4.35 (s, 1 H), 3.70 (s, 2 H), 2.68 (br s, 6 H); HRMS (FAB) m/z 635 ([M + H]$^+$), 637, 159, 160.

13-(Benzylsulfinyl)-5-hydroxy-6-α-deoxytetracycline (8). To a solution of 100 mg (0.177 mmol) of sulfide 5 in 10 mL of EtOH was added 1.8 mL (0.18 mmol) of a 0.1 M aqueous Oxone solution (Aldrich Chemical, Milwaukee, WI). The mixture was stirred for 25 min at room temperature and evaporated to dryness. Water was added to bring the volume to 5.0 mL. The mixture was sonicated and filtered giving 22 mg of impure product as a brown solid. The pH of the filtrate was adjusted to 4.5 and the solution extracted 10 times with $CHCl_3$. Concentration of $CHCl_3$ extract gave 36 mg (35%) of the compound as a yellow solid. The solid was dissolved in 10 mL of 1:2 MeOH/H_2O and adjusted to pH 1.5 with 10% HCl. Removal of the solvent in vacuo and lyophilization gave the hydrochloride salt: TLC R_f = 0.20 (I); ^{1}H NMR (MeOH-d_4) δ 7.4 (m, 5 H), 7.25 (m, 1 H), 6.65 (m, 1 H), 6.41 and 6.20 (d, J = 8 Hz, 8 H), 4.3 (m, 1 H), 2.5 (br s, 6 H); MS (FAB) m/z 583 ([M + H]$^+$), 566.

13-(Benzylsulfonyl)-5-hydroxy-6-α-deoxytetracycline (9). To a solution of 100 mg (0.177 mmol) of sulfide 5 in 10 mL of EtOH was added 3.9 mL (0.39 mmol) of a 0.1 M aqueous Oxone solution. The mixture was stirred for 1 h at room temperature and filtered. Removal of the solvent in vacuo gave 158 mg of white solid, which was chromatographed on EDTA silica and eluted with solvent system II. Fractions corresponding to the compound were pooled and concentrated to give 50 mg of 19 as a tan solid. The solid was dissolved in 10 mL of 1:2 MeOH/H_2O

and adjusted to pH 1.5 with 10% HCl. Removal of the solvent in vacuo and lyophilization gave the hydrochloride salt. TLC R_f = 0.20 (I); MS (FAB) m/z 599 ([M + H]$^+$), 582.

13-(Methylthio)-5-hydroxy-6-α-deoxytetracycline (10). Methacycline hydrochloride (1.0 g, 2.1 mmol) was placed in a stainless steel bomb apparatus and suspended in 150 mL of EtOH. Cold methanethiol (10.0 mL, 195 mmol) was added via cold syringe to the mixture. The radical initiator AIBN (250 mg) was added and the apparatus sealed. The bomb was heated for 4 h in an oil bath at 70 °C with occasional shaking. The apparatus was cooled and flushed of methanethiol with a gentle stream of nitrogen for 2 h. The solution was filtered and concentrated in vacuo, yielding a yellow amorphous solid. The mixture was dissolved in 30 mL of H_2O, the pH brought to 4.5 with 1.0 M NaOH, and the reaction mixture extracted into 30-mL portions of CH_2Cl_2. Concentration of the solvent resulted in a mixture with starting methacycline and product. Removal of impurities was facilitated by dissolving the solid in MeOH and treatment with activated charcoal (0.4 g) yielded 206 mg (18.4%) of the product as a yellow-brown solid: mp = 143–146 °C dec; TLC R_f = 0.59 (I); HPLC t_R = 12.45 min; ^{1}H NMR (MeOH-d_4) δ 7.36 (t, 1 H), 6.94 (d, J = 9.0 Hz, 1 H), 6.71 (d, J = 9.0 Hz, 1 H), 3.65 (m, 2 H), 2.62 (s, 6 H), 1.92 (s, 3 H); HRMS (FAB) calcd for $C_{23}H_{26}N_2O_8S$ 491.1488 (M + 1), found 491.1481 (M + 1).

General Procedure for the Synthesis of 13-(Alkylthio)-substituted 5-Hydroxy-6-deoxytetracyclines. 13-(Ethylthio)-5-hydroxy-6-α-deoxytetracycline (11). Methacycline hydrochloride (5.0 g, 10.4 mmol) was placed in a round-bottom flask and suspended in 100 mL of EtOH. Twenty milliliters of ethanethiol (16.8 g, 0.270 mol) and AIBN (250 mg) were added, and the reaction mixture was refluxed with stirring for 12 h while under N_2. The mixture was reduced to one-fifth volume by distillation and filtered. The filtrate was dripped slowly into cold Et_2O with stirring, resulting in the formation of a yellow precipitate. The precipitate was filtered, dissolved in H_2O, and brought to pH 4.5 with 1.0 M NaOH. This solution was filtered, and extracted with CH_2Cl_2, yielding a dark yellow solid (620 mg). The solid was dissolved in MeOH and treated with charcoal, yielding a yellow solid in 22.9% yield (1.16 g): mp = 136–141 °C dec; TLC R_f = 0.68 (I); HPLC t_R = 13.64 min; ^{1}H NMR (MeOH-d_4) δ 7.38 (t, 1 H), 6.98 (d, 1 H), 3.65 (m, 2 H), 2.58 (s, 6 H), 2.48 (m, 2 H), 1.18 (m, 3 H); HRMS (FAB) calcd for $C_{24}H_{28}N_2O_8S$ 505.1644 (M + 1), found 505.1630 (M + 1).

13-(Propylthio)-5-hydroxy-6-α-deoxytetracycline (12). This compound was prepared in low yield (25%, 256 mg) as a yellow solid as described for 11: mp = 130–140 °C dec; TLC R_f = 0.70 (I); HPLC t_R = 20.18 min; ^{1}H NMR (DMSO-d_6) δ 7.50 (t, 1 H), 7.05 (d, 1 H), 6.85 (d, 1 H), 4.32 (d, 2 H), 3.15 (s, 1 H), 2.65 (s, 6 H) 2.32–2.52 (m, 2 H), 1.51–1.80 (m, 2 H), 0.9–1.22 (m, 3 H); HRMS (FAB) calcd for $C_{25}H_{30}N_2O_8S$ 519.1801 (M + 1), found 519.1815 (M + 1).

13-(Isopropylthio)-5-hydroxy-6-α-deoxytetracycline (13). This compound was prepared as described for 11, but isolated as follows. After volume reduction, the ethanol was filtered into cold Et_2O and the solid filtered, dried, and chromatographed on EDTA-impregnated silica (EDTA–silica). Fractions led to isolation of the product in low yield (32.4% yield): mp = 114–119 °C dec; TLC R_f = 0.63 (I); HPLC t_R = 19.31 min; ^{1}H NMR (MeOH-d_4) δ 7.56 (m, 1 H), 7.04 (d, 1 H), 4.40 (d, 1 H), 4.32 (s, 1 H), 3.15 (s, 1 H), 2.68 (s, 6 H), 1.21–1.30 (m, 1 H), 1.00–1.10 (m, 6 H); HRMS (FAB) calcd for $C_{25}H_{30}N_2O_8S$ 519.1801 (M + 1), found 519.1815 (M + 1).

13-(Butylthio)-5-hydroxy-6-α-deoxytetracycline (14). This compound was prepared in low yield (23.0%, 147 mg) as a amorphous yellow solid as described for 11: mp = 117–126 °C dec; TLC R_f = 0.65 (I); HPLC t_R = 20.20 min; ^{1}H NMR ($CDCl_3$) δ 7.52 (t, 1 H), 7.08 (d, 1 H), 6.88 (d, 1 H), 4.30–4.32 (m, 2 H), 2.65–2.22 (m, 10 H), 1.20–1.70 (m, 4 H) 0.95–1.15 (m, 4 H), 0.95–1.15 (m, 3 H); HRMS (FAB) calcd for $C_{26}H_{32}N_2O_8S$ 533.1957 (M + 1), found 533.1963 (M + 1).

13-(Isobutylthio)-5-hydroxy-6-α-deoxytetracycline (15). This compound was prepared in low yield (29.5%, 198 mg) as a yellow solid as described for 11: mp = 110–115 °C dec; TLC R_f = 0.72 (I); HPLC t_R = 20.28 min; ^{1}H NMR (MeOH-d_4) δ 7.40 (d, 1 H), 4.32 (s, 2 H), 2.68–2.93 (m, 8 H), 1.60–1.73 (m, 2 H), 0.88 (d, 6 H); HRMS (FAB) calcd for $C_{26}H_{32}N_2O_8S$ 533.1981 (M + 1), found 533.1957 (M + 1).

13-(*tert*-Butylthio)-5-hydroxy-6-α-deoxytetracycline (16). This compound was prepared as described for 11, except for the following modifications. Methacycline hydrochloride (1.5 g, 3.0 mmol) was added to a solution of 10 mL of *tert*-butyl mercaptan in 200 mL of absolute EtOH. Radical initiator AIBN (350 mg) and *tert*-butyl mercaptan (25 mL) were added over 10 h at reflux until the product was seen by TLC. Reflux was continued for 6 h. The dark, oil residue was chromatographed on EDTA–silica; the fractions were combined and extracted into CH_2Cl_2, affording crude product as a dark solid in low yield (183 mg, 15.2%). Treatment with activated charcoal in MeOH and Et_2O trituration of the dried product gave a yellow solid: mp = 155–156 °C dec; TLC R_f = 0.86 (II); HPLC t_R = 16.84 min; ^{1}H NMR (MeOH-d_4) δ 7.61 (m, 1 H), 7.00–7.22 (m, 1 H), 6.88 (m, 1 H), 4.30–4.32 (m, 2 H) 2.65–2.22 (m, 10 H), 1.2 (m, 9 H); HRMS (FAB) calcd for $C_{26}H_{32}N_2O_8S$ 533.1957 (M + 1), found 533.1937 (M + 1).

13-(Hexylthio)-5-hydroxy-6-α-deoxytetracycline (17). This compound was prepared in low yield (31.5%, 318 mg) as a brown-yellow solid as described for 11: mp = 140–143 °C dec; TLC R_f = 0.69 (I); HPLC t_R = 24.52 min; ^{1}H NMR (MeOH-d_4) δ 7.56 (m, 1 H), 7.06 (m, 1 H), 6.84 (m, 1 H), 4.40 (m, 2 H), 2.68–2.93 (m, 10 H), 1.30–1.58 (br m, 6 H), 0.90 (br m, 4 H); HRMS (FAB) calcd for $C_{28}H_{36}N_2O_8S$ 561.2270 (M + 1), found 561.2259 (M + 1).

13-(Cyclohexylthio)-5-hydroxy-6-α-deoxytetracycline (18). This compound was prepared as described for 11. Isolation of product was accomplished by column chromatography on EDTA–silica gel or by semipreparative HPLC. The HPLC fractions corresponding to the product were combined, dried, dissolved in H_2O, extracted into butanol, dried in vacuo, and suspended in a minimal amount of H_2O (10 mL). The solution was brought to pH 4.5 by 0.1 M NaOH and extracted three times with 30-mL portions of CH_2Cl_2. The combined extracts were concentrated and dried to yield a brownish yellow solid in low yield (15%): mp = 134–136 °C dec; TLC R_f = 0.68 (I); HPLC t_R = 24.43 min; ^{1}H NMR (MeOH-d_4) δ 7.40 (t, 1 H), 7.18 (d, 1 H), 6.68 (d, 1 H), 4.02 (s, 2 H), 2.58 (s, 6 H), 1.40–1.70 (br m, 2 H), 1.00–1.15 (br m, 6 H), 0.75–0.85 (br m, 2 H); HRMS (FAB) calcd for $C_{28}H_{34}N_2O_8S$ 559.2114 (M + 1), found 559.2138 (M + 1).

13-(Cyclopentylthio)-5-hydroxy-6-α-deoxytetracycline (19). This compound was prepared as described for 11. Purification was either by column chromatography on EDTA–silica, by extraction at pH 4.5 into CH_2Cl_2, or by HPLC chromatography. An analytical sample was produced by HPLC as a yellow solid in moderate yield (28.3%). Higher yields were obtained by the extraction method and treatment with activated charcoal in MeOH (32.1%): mp = 132–139 °C dec; TLC R_f = 0.80 (I); HPLC t_R = 21.19 min; ^{1}H NMR (MeOH-d_4) δ 7.38 (t, 1 H), 7.02 (d, 1 H), 6.72 (d, 1 H), 4.10 (s, 2 H), 2.70 (br s, 6 H), 1.81–2.01 (br m, 2 H), 1.28–1.75 (br m, 6 H), (br m, 2 H); HRMS (FAB) calcd for $C_{27}H_{32}N_2O_8S$ 545.1957 (M + 1), found 545.1960 (M + 1).

13-(Isopentylthio)-5-hydroxy-6-α-deoxytetracycline (20). This compound was prepared as described for 11. Purification was by filtering a MeOH extract (10 mL) of the dried reaction mixture and precipitation with cold Et_2O (900 mL). The solid was filtered, dried, and dissolved in H_2O. The solution was brought to pH 4.5 and extracted with CH_2Cl_2 as before. The dried organic layer was dried over Na_2SO_4 and concentrated in vacuo to yield the product as a yellow solid in low yield (11.3%, 94 mg): mp = 119–127 °C dec; TLC R_f = 0.79 (I); HPLC t_R = 23.17 min; ^{1}H NMR (MeOH-d_4) δ 7.39 (t, 1 H), 6.95 (d, 1 H), 6.75 (d, 1 H), 4.31 (s, 2 H), 2.62 (s, 6 H), 2.35–2.45 (br m, 2 H), 1.25–1.50 (br m, 2 H), 0.75–0.80 (br d, 8 H); HRMS (FAB) calcd for $C_{27}H_{35}N_2O_8S$ 547.2114 (M + 1), found 547.2097 (M + 1).

13-(*n*-Decylthio)-5-hydroxy-6-α-deoxytetracycline (21). This compound was prepared as described for 11 except for the following modifications. The reaction mixture was reduced in volume by half and triturated with cold Et_2O (1 L). The product was collected to yield 3.49 g of an oily, dark residue that was not amenable to purification by column chromatography. The mass was extracted in a Soxhlet extraction apparatus in $CHCl_3$ overnight and treated with activated carbon to yield the desired compound in low yield (14.3%, 44 mg); mp = 110–119 °C dec. Semipreparative HPLC afforded a compound with the following

characteristics and spectra: TLC R_f = 0.85 (I); HPCL t_R = 27.29 min; ^{1}H NMR (MeOH-d_4) δ 7.39 (t, 1 H), 7.00 (d, 1 H), 6.74 (d, 1 H), 4.31 (br s, 2 H), 2.78 (s, 6 H), 2.35–2.45 (br m, 2 H), 0.88–1.65 (br m 20 H, decyl); HRMS (FAB) calcd for $C_{32}H_{44}N_2O_8S$ 617.2896 (M + 1), found 617.2893 (M + 1).

13-(Cyclopentylthio)-5-deoxy-6-α-deoxytetracycline (22). 5-Deoxymethacycline hydrochloride (1.0 g, 2.1 mmol) was placed in a round-bottom flask and suspended in 30 mL of EtOH. Three milliliters of cyclopentyl mercaptan (2.68 g, 28 mmol) and AIPN (150 mg) were added, and the reaction mixure was refluxed with stirring for 1.5 h while under N_2. The solution was filtered and dripped slowly into cold Et_2O. The resultant precipitate was filtered, dissolved in H_2O, and brought to pH 4.5 with 1.0 M NaOH. This solution was filtered and extracted multiple times with CH_2Cl_2, yielding a dark yellow solid. Further treatment with activated charcoal led to isolation of the product in low yield (15.0%, 124 mg): mp = 161–164 °C dec; TLC R_f = 0.73 (I); HPLC t_R = 22.62 min; ^{1}H NMR (MeOH-d_4) δ 7.38 (t, 1 H), 6.90 (m, 1 H), 6.60 (d, 1 H), 3.90 (s, 1 H), 2.85 (s, 6 H), 2.20–2.35 (br m, 2 H), 1.87–2.05 (br m, 4 H), 1.25–2.0 (br m, 4 H); MS (FAB) m/z 529, 530 ([M + H]$^+$), 429 (M + H − (cyclopentyl − S + H)).

13-(Cyclopentylthio)-5-hydroxy-6-α-deoxy-*N*-(morpholinomethyl)tetracycline (23). To a solution of 71.2 mg (0.130 mmol) of sulfide **19** in 10 mL of *tert*-butyl alcohol was added 11.3 μL of distilled morpholine (11.3 mg, 0.130 mmol) and 10.3 μL of 37% formaldehyde (0.127 mmol). The mixture was stirred for 30 min at room temperature and heated to reflux for 15 min. The solution was filtered hot, cooled, and filtered again. Concentration resulted in 62.5 mg (77.8%) of the product as a yellow solid. TLC and HPLC analysis led to identification of the starting material as well as the product due to reversion by H_2O or acids: TLC R_f = 0.12 (I); HPLC t_R = 8.7 min; ^{1}H NMR (MeOH-d_4) δ 7.40 (t, 1 H), 7.05 (d, 1 H), 6.78 (d, 1 H), 4.10 (s, 1 H), 3.6–3.8 (br m, 4 H), 2.8–3.0 (br m, 4 H), 2.20–2.35 (br m, 2 H), 2.63 (s, 6 H); HRMS (FAB) calcd for $C_{32}H_{41}N_3O_9S$ 644.2642 (M + 1), found 644.2659 (M + 1) **Rolitetracycline:** TLC R_f = 0.10 (I), HRMS (FAB) calcd for $C_{27}H_{33}N_3O_9$ 544.2295 (M + 1), found 544.2300 (M + 1).

13-[(2′-Hydroxyethyl)thio]-5-hydroxy-6-α-deoxytetracycline (24). Methacycline hydrochloride (2.0 g, 4.2 mmol) was added to 2-mercaptoethanol (20 mL, 285 mmol) and heated on a steam bath for 30 min. The reaction mixture was poured into 200 mL of stirred Et_2O, and the precipitate was filtered. Upon standing the solid turned into a viscous gum. The mass was repeatedly triturated with Et_2O and yielded a yellow powder upon filtration. The powder was chromatographed on EDTA silica using solvent system II, yielding the desired compound in low yield (19.3% 236 mg): TLC R_f = 0.42 (I); HPLC t_R = 16.39 min; ^{1}H NMR (MeOH-d_4) δ 7.41 (m, 5 H), 7.10 (d, 1 H), 6.65 (d, 1 H), 4.35 (s, 1 H), 3.70 (s, 2 H), 2.68 (br s, 6 H); MS (FAB) m/z 635, 636 ([M + H]$^+$), 159, 161.

13-[(2,3-Dihydroxypropyl)thio]-5-hydroxy-6-α-deoxytetracycline (25). This compound was prepared as described for 11 in low yield (19.3%, 236 mg): 160–165 °C dec; TLC R_f = 0.25 (I); HPLC t_R = 15.18; ^{1}H NMR (MeOH-d_4) δ 7.45 (m, 1 H), 7.02 (m, 1 H), 6.78 (m, 1 H), 4.15 (m, 2 H), 2.65–2.95 (m, 10 H); HRMS (FAB) calcd for $C_{25}H_{30}N_2O_{10}S$ 551.1699 (M + 1), found 551.1729 (M + 1).

13-[(2-Carboxyethyl)thio]-5-hydroxy-6-α-deoxytetracycline (26). This compound was prepared as described for 11. Purification was by suspension of the reaction mixture in cold Et_2O (1 L). The precipitate obtained was filtered to facilitate removal of 3-mercaptopropionic acid. The solid was collected, dissolved in 30 mL of H_2O, and brought to pH 4.5. Extraction with $CHCl_3$ (50 mL) removed residual mercaptan. Further extraction with CH_2Cl_2 (300 mL) and concentration in vacuo led to isolation of the product as a brownish-yellow solid in low yield (17.3%): mp = 100–106 °c dec; TLC R_f = 0.79 (I); HPLC t_R = 14.68 min; ^{1}H NMR (DMSO-d_6) δ 7.43–7.51 (t, 1 H), 6.82–6.85 (d, 1 H), 6.59–6.83 (d, 1 H), 4.55 (s, 1 H), 3.79–3.90 (s, 1 H), 3.38–3.87 (m, 6 H), 2.51 (s, 6 H), 1.87–2.20 (m, 4 H); HRMS (FAB) calcd for $C_{25}H_{28}N_2O_{10}S$ 550.1531 (M + 1), found 550.1547 (M + 1).

13-(3-Chloropropylthio)-5-hydroxy-6-α-deoxytetracycline (27). This compound was prepared as described for 11 in low yield (21.4%): mp = 104–106 °C dec; TLC R_f = 0.80 (I); HPLC t_R = 19.67; ^{1}H NMR (DMSO-d_6) δ 7.53 (d, 1 H), 7.09 (d, 1 H), 6.85 (d, 1 H), 4.13 (s, 1 H), 3.54 (br m, 2 H), 2.5–2.6 (br m, 4 H), 2.20–2.35 (br m, 2 H) 2.47 (s, 6 H), 1.86–1.95 (br m, 2 H); HRMS (FAB) calcd for $C_{25}H_{29}N_2O_8SCl$ 554.6257 (M + 1), found 554.6264 (M + 1).

Methacycline: mp = 143–151 °C dec; TLC R_f = 0.52 (I); HPLC t_R = 10.79 min; ^{1}H NMR (MeOH-d_4) δ 7.45 (t, 1 H), 7.05 (d, 1 H), 5.44–5.55 (d of d's, 2 H, vinyl H), 4.55 (s, 1 H), 3.8 (m, 1 H), 3.65 (m, 1 H), 2.90 (s, 6 H).

Tetracycline Efflux Inhibition Assay. Preparation of Everted Membrane Vesicles. Everted membrane vesicles were prepared according to the method of McMurry.[3] Starter cultures of 100 mL (medium A) each were inoculated with *E. coli* D1-209 (containing plasmid R222) and grown overnight at 37 °C. These cultures were then used as inoculum into 650 mL of medium A. The new cultures were then grown to a A_{530} of approximately 0.8. The medium was centrifuged at 9400*g* for 10 min at 4 °C and the pellet resuspended in 500 mL of cell-wash buffer and centrifuged at 9400*g* for 10 min at 4 °C. The pellet was suspended in 10 mL of cell-wash buffer and subjected to cell lysis by a French press (5000 K; 350 psi, high). The resulting viscous solution was vortexed briskly for 5 min and centrifuged in Corex tubes (SS-34) at 21000*g*, for 10 min at 4 °C. The supernatant fraction was separated and ultracentrifuged at 150000*g* for 1 h at 4 °C in a SW 50.1 rotor. The supernatant layer was decanted and the pellet resuspended in vesicle-storage buffer. Protein determinations were made according to the method of Lowry[25] and aliquots stored at −70 °C until use; medium A, 0.25% glucose, 0.0001% vitamin B_1, 1 μg/mL tetracycline; cell-wash buffer, 100 mM K_2HPO_4–KH_2PO_4, 10 mM EDTA, pH 6.6; vesicle-storage buffer, 50 mM K_2HPO_4–KH_2PO_4, pH 6.6.

Vesicle Assay Procedure. Tetracycline efflux inhibition was measured as the inhibition of uptake of ^{3}H-Tc by everted membrane vesicles. Dilutions of the test compounds were made in EtOH and added to small test tubes (0.2, 0.5, 2.0, 5.0 μg/mL) and dried down under N_2. The standard (methaycline hydrochloride) was prepared in a similar fashion. The negative control consisted of a protonophore, CCCP (8 μM), added to disrupt proton-motive force, whereas the positive control received only lithium lactate (12 mM) to establish proton gradients. Thawed vesicles were diluted to 0.5 mg of protein/mL in vesicle assay buffer and stored in 300-μL aliquots on ice prior to use. At t = 0, lithium lactate (12 μL, 1.0 M) was added to a vesicle aliquot and incubated in a water bath at 30 °C for 2.5 min with gentle shaking (30 rpm). At this time 250 μL of energized vesicles were added to those tubes containing the drug concentrates and placed in the water bath. At time = 3.0 min ^{3}H-Tc was added to a concentration of 3 mM (10^6 cpm). After 30 s, 50 μL of the mixture was removed, diluted into 10 mL of filter-wash buffer, vortexed and filtered through presoaked (H_2O) Gellman Nitrocellulose filters (0.45 μm). An additional 5 mL of wash buffer was used as a rinse. Assayed time points were then repeated at 1.5 and 2.5 min after addition of the radiolabeled Tc; vesicle-assay buffer, 50 mM K_2HPO_4–KH_2PO_4, 10 mM $MgSO_4$, pH 7.5; vesicle-wash buffer, 100 mM K_2HPO_4–KH_2PO_4, 100 mM LiCl, pH 7.5.

Fluorescence Assay. Everted membrane vesicles from tetracycline-sensitive *E. coli* were prepared according to the method of McMurry.[3] Two milliliters of vesicle assay buffer was added to a cuvette equipped with a stir bar. Acridine orange (2.0 μM) was added (Sigma Chemical Co., St. Louis, MO) along with 20 μL of 6 mg/mL tetracycline-sensitive vesicles. After equilibration for 2 min lithium lactate was added to 20 μM and traced with an excitation wavelength at 490 nm while the emission wavelength at 530 nm was observed. After 3 min, analogue was added at the IC_{50} concentration and further observed for 1 min. The pH gradient was subsequently collapsed using CCCP (20 μM).

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