Bioorganic & Medicinal Chemistry 18 (2010) 4231–4237

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Semi-synthetic analogues of thiostrepton delimit the critical nature of tail region modifications in the control of protein biosynthesis and antibacterial activity

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ARTICLE INFO

Article history:
Received 14 February 2010
Revised 24 April 2010
Accepted 30 April 2010
Available online 8 May 2010

Keywords:
Polythiazole antibiotics
Translational inhibitor
Bacterial ribosome

ABSTRACT

We report the successful production of selectively-modified tail analogues of the natural product antibiotic thiostrepton, which have been used to evaluate the critical nature of this section of the antibiotic to its inhibition of protein synthesis. This work highlights the tail region as a critical area for future semi-synthetic or synthetically bioengineered thiostrepton derivatives.

1. Introduction

The clinical problem of bacterial multidrug resistance has fuelled renewed interest in the polythiazole peptide antibiotic class as potential drug molecules. Thiostrepton (**1**) is prototypical of this class, whose members invariably exhibit potent activity against Gram-positive bacteria.[1] Chemical analyses and X-ray and NMR studies have elucidated the molecular structure of thiostrepton (Fig. 1)[2–4] and its target site has been located to the binding domain of ribosomal protein L11 within the GTPase centre of the bacterial ribosome, where its primary binding interaction with rRNA is cooperatively enhanced by its interaction with the N-terminal domain (NTD) of L11.[5,6]

It is presently surmised that the binding of **1** to the bacterial ribosome disrupts the dynamic interactions between L11 and the rRNA required for the translocation step of protein synthesis[7], thereby halting translation at this stage.[8] While the ribosomes of both Gram-positive and Gram-negative bacteria are affected, the latter bacteria are insensitive to **1** as the antibiotic is thought to be unable to penetrate the Gram-negative cell wall. Eukaryotic ribosomes such as those from *Homo sapiens*, are insensitive to **1** due to absence of the cooperative interaction with the eukaryotic equivalent of L11[9], as well as rRNA sequence differences[10], a factor contributing to the current interest in this class of antibiotic as selective antibacterial agents. Additional to its antibacterial activity, **1** also exhibits interesting antagonistic activity to *Plasmodium falciparum*[11], *Mycobacterium tuberculosis* and various cancer cell lines[12,13] and two instances of gene regulation by **1** have been described.[14,15] Recently, the molecular structure of a 23S ribosomal methyltransferase from *Streptomyces cyaneus*, one of the microorganisms that biosynthesizes thiostrepton, has been determined.[16] This enzyme confers thiostrepton resistance to this antibiotic-producing organism by specifically methylating the 2'-OH moiety of

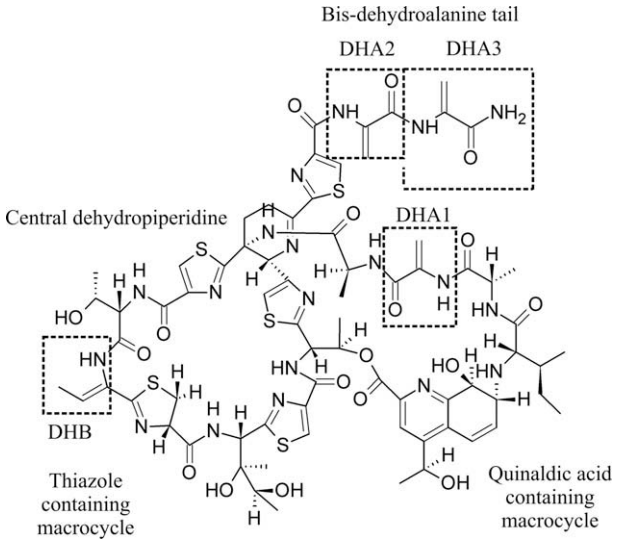

Figure 1. Thiostrepton (**1**).

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doi:10.1016/j.bmc.2010.04.098

adenine 1067 in bacterial 23S rRNA, a key residue in the ribosomal binding site for this antibiotic.[17]

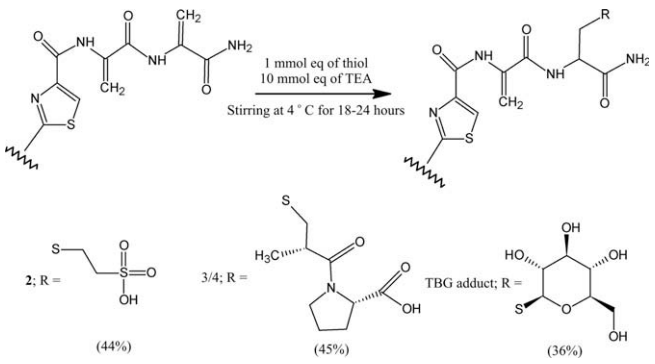
In spite of its diverse biological activities, the lipophilicity of **1** has prevented its clinical use. Available evidence suggests the tail region to be the least crucial to its biological properties[3,5,6] and this region features two DHA (dehydroalanine) residues, each of which possesses a functional group susceptible to chemical modification through Michael addition reactions. It has been demonstrated that heteroatom additions indeed could be accomplished to tail DHA residues on the related polythiazole antibiotic, nocathiacin[18,19] with a consequent improvement in aqueous solubility. However, the complexity of the thiostrepton molecule, which includes two other potentially reactive sites on its macrocycles (DHA1 and DHB (dehydrobutyrine)) along with an internal lactone susceptible to hydrolysis[2], makes a similar approach to its modification more challenging. Moreover, a previous report observed that random and extensive chemical addition of thiols to the four unsaturated sites in **1** render the antibiotic biologically inactive.[13]

Although modification of the tail region of **1** could presumably lead to several derivatives, the nature of the permissible binding interactions between these potential tail modified analogues and the bacterial ribosome was unknown. In light of the recent discovery of the gene cluster encoding the proteins necessary for biosynthesis of **1**,[20] we sought a rapid semi-synthetic approach towards its tail modification to assess the suitability of this site for alterations that would aim to improve the physicochemical profile of the antibiotic, ahead of extensive genetic manipulations and/or chemical modifications.

2. Results and discussion

Using the conditions detailed in Scheme 1, polar low molecular weight thiols were used to successfully effect a single site modification at the tail of **1**. These molecules were selected as modifying groups since their dissimilar structures would enable a preliminary assessment of the structural importance of this region for antibiotic activity and its suitability as modification site, while their polar properties would potentially improve upon the aqueous solubility of **1**. Recently, diastereomeric mixtures of tail modified Michael adducts of **1** from a truncated version of the molecule have been prepared.[21] However, a mono-addition to the tail of the *intact* thiostrepton molecule was achieved here.

The complete, unambiguous ^{1}H chemical shift assignment for **1** has been previously accomplished using one and two dimensional NMR experiments[4,22]; therefore 2D TOCSY experiments were employed to determine the site of Michael addition. These experiments verified the addition site as DHA3 and additionally showed **2**, **3** and **4** to be diastereomerically pure, with **3** and **4** representing a diastereomeric pair (Fig. 3). The corresponding diastereomer for MENSA adduct (**2a**) was located in the HPLC fraction that eluted after 29.2 min (Fig. 2); confirmed by TOCSY (Supplementary data). As is evident from the HPLC chromatogram, **2a** was produced in smaller quantities relative to **2** and as a result, sufficient amounts of this diastereomer could not be isolated to carry out detailed biological testing. Screening of the remaining HPLC fractions by mass spectrometry did not detect additional single addition products in either reaction and furthermore, no evidence of the loss of the thiol appendage(s) was observed throughout the extensive chromatography and complementary physical characterisation conducted in the purification of the Michael adducts. Despite repeated attempts, diastereomerically pure samples of the single-site Michael adducts with TBG could not be obtained; however, mass spectrometry and ^{1}H NMR analyses are entirely consistent with the presence of the desired single addition product (Supplementary Fig. 3).



Scheme 1.

Inhibition of bacterial growth was assessed against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*, in accordance with the methods prescribed by the National Committee for Clinical Laboratory Standards[23] (Table 1). Overall, activity was lessened with respect to the parent compound; however, the activity displayed by **2** compared favourably with that which has been reported for conventional antibiotics.[24] The activities of **3** and **4** were further diminished with respect to **1** and no activity was detected at the highest concentrations of compounds **1**–**4** against *E. coli*, indicating no extension of antibacterial activity to Gram-negative bacteria. Preliminary screens for antibacterial activity by the suspected TBG adduct using disc diffusion[25] found no activity up to the highest concentration tested (50 μg/ml).

As mentioned earlier, thiostrepton exerts its antibacterial activity through the inhibition of protein synthesis. This in vivo antibacterial activity is restricted to Gram-positive bacteria, however protein translation on the ribosomes of both Gram-positive and Gram-negative species is inhibited by the antibiotic.[5,26,27] The effect of the Michael adducts on bacterial protein synthesis was therefore assessed with in vitro translation reactions by *E. coli* -ribosomes in the presence of **2**, **3** and **4** and the general decrease in protein expression observed in the presence of increasing concentrations of these compounds indicates that the observed antibacterial activities can be attributed to their inhibitory effects on protein synthesis. This effect was more evident in the presence of **2**, where a marked reduction in protein expression occurred in a dose-dependent fashion similar to the effect seen with **1** (Fig. 4). While this trend was apparent over a comparable concentration range of **3** or **4**, it was observed at a much diminished extent. In addition, the biological activity assessment of the captopril analogues seems to suggest that stereochemistry at this site does not affect activity (exemplified by preliminary comparisons of protein translation inhibition for compounds **3** versus **4** presented in Fig. 4). It was interesting to find that the inhibition of luciferase expression by compound **2** under the experimental conditions was to an extent comparable to that of **1**. As such, the finding of activity for these Michael adducts is promising, since this compound was observed to have increased aqueous solubility relative to **1** (Fig. 5, Table 2).

Although similar inhibitory effects on protein translation were found for **1** and **2** by cell free translation assays, the minimum inhibitory concentration for **2** was found to be much greater than **1**. Likewise, the inhibitory effect of **3** and **4** on cell free protein translation was not reflected in whole cell assays. Taken together, these results imply that compound transport may be a limiting factor to antibacterial activity. Nevertheless, the relative effect on protein translation of **2** with respect to **3**/**4** is indicative of the importance of the structure of the tail region for antibacterial activity. The non-covalent interaction between L11-NTD and **1** seems crucial for the tight binding of **1** to its target site and indeed, biochemical and structural data show that the tail region contributes significantly to this interaction.[5,8,9,28,29] It can therefore be

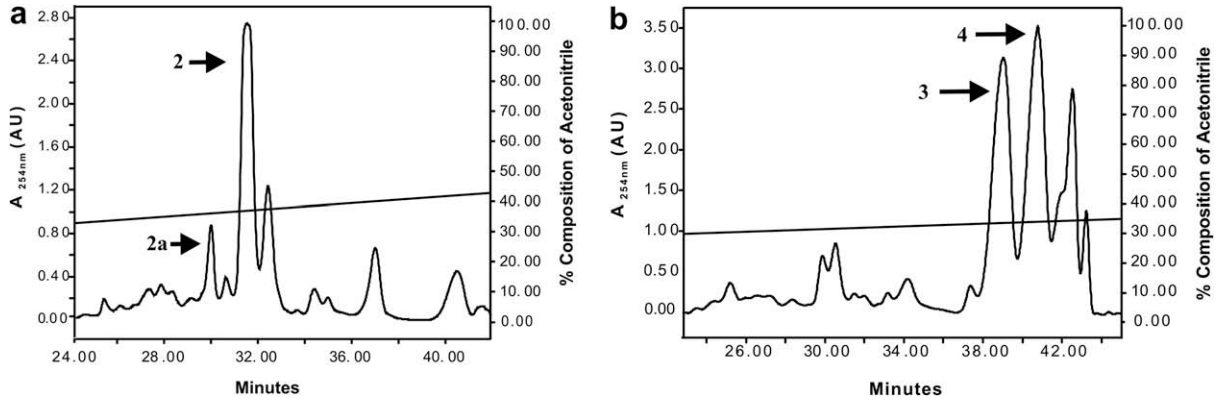


Figure 2. RP-HPLC Purification of Michael adducts. (a) MENSA reaction. Compound **2a**, t_R: 29.2 min; Compound **2,** t_R: 31 min. (b) Captopril reaction. Compound **3**, t_R: 37.2 min; Compound **4**, t_R: 39.4 min.

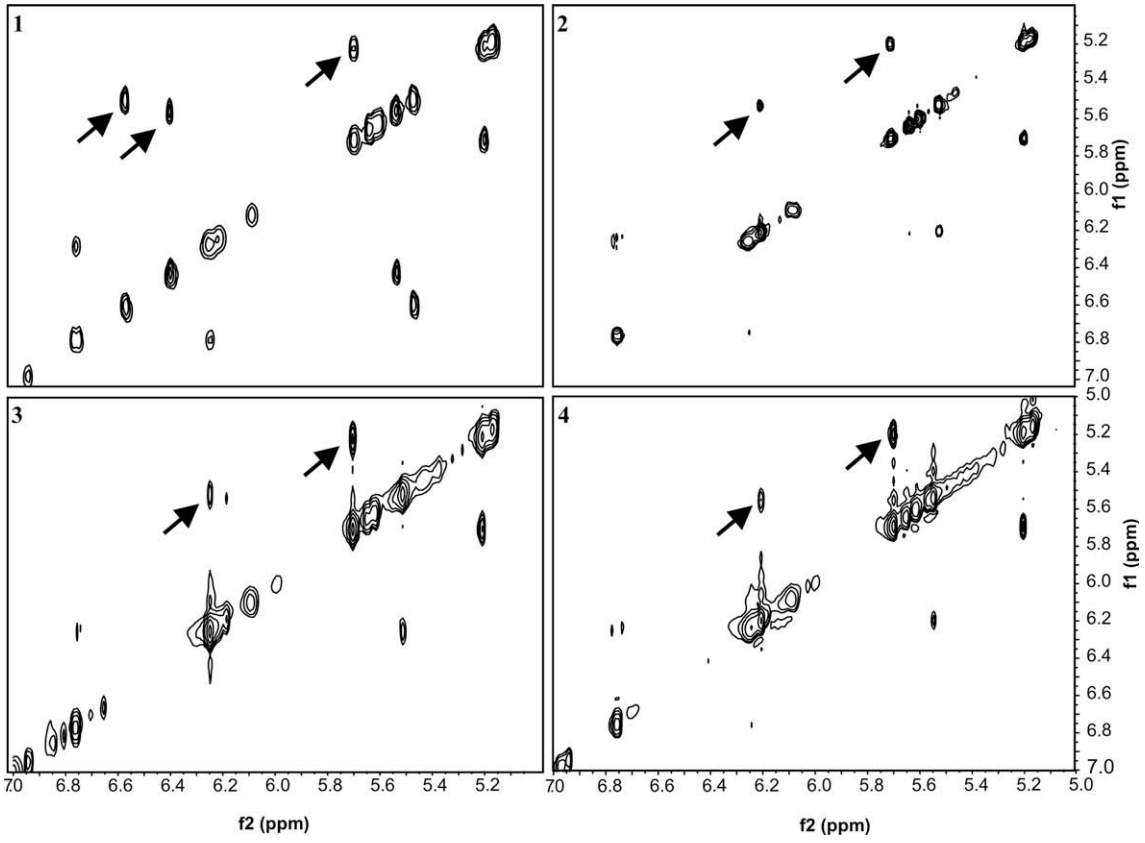


Figure 3. Confirmation of the Michael addition site in **2**, **3** and **4** by TOCSY. Analyses of spectra exhibit ^{1}H correlations in the olefin region for 1, 2, 3 and 4. Cross peaks arising from olefin protons of DHA1 (5.38 ppm, 5.84 ppm), DHA2 (5.76 ppm, 6.77 ppm) and DHA3 (5.66 ppm, 6.59 ppm) are indicated by arrows in the spectrum from **1**. The DHA3 olefin proton is absent in the spectra from the analogues and cross-peaks at 5.76, 6.40 ppm (2 and 3) and 5.76, 6.44 ppm (4) assign these chemical shifts to DHA2 olefin protons, thus confirming Michael addition to DHA3. 3 and 4 represent a diastereomeric pair which accounts for the difference between their DHA2 ^{1}H chemical shifts.

reasonably expected that modifications to this region would affect its interaction with the L11-NTD. This further implies that there are conformational and/ or spatial limitations to modifications of the tail region that do not compromise activity, which are not immediately obvious from the current crystallographic information on the ribosome–thiostrepton complex that suggests a clear space available for tail-modified analogues.

To date, there is no existing structure of the *E. coli* ribosome bound with **1**, however a crystal structure of the 50S ribosomal subunit from *Deinococcus radiodurans* bound with **1** is available.[8] The 50S subunit from *D. radiodurans* is virtually identical to that of *E. coli*[30]; therefore, its interactions with **1** can be expected to represent what occurs with *E. coli* ribosomes that were used in the in vitro translation assays. An examination of this structure in the region of the interaction between the thiostrepton tail and the L11-NTD suggests that an appendage to the thiostrepton tail would potentially induce disruptions to this interaction, to a degree dependent on the nature of the appendage. This is depicted in Figure 6c, which points toward more extensive disruptions being induced by captopril. Given the importance of the interaction between L11-NTD and the tail region for binding and activity, this is not inconsistent with our findings of similar inhibition of translation by **1** and **2**, compared to the reduced inhibition by **3** and **4**. It should be noted that although Figure 6 shows the bound position

Table 1
Antibacterial activity of **1** and its Michael adducts

Compound	Minimum inhibitory concentrations[a] (μg/ml)		
	S. aureus (ATCC 6338)	*B. subtilis* (ATCC 6633)	*E. coli*
1	⩽0.0625	⩽0.25	⩾32
2	⩽2	⩽8	⩾128
3	⩽32	⩽32	⩾128
4	⩽32	⩽32	⩾128
TBG adduct[b]	⩾50	⩾50	⩾50

[a] Minimum inhibitory concentrations are reported as less than or equal to lowest compound concentration that inhibited visible bacterial growth. Test concentrations of compounds **1–4** against *S. aureus* and *B. subtilis* and for **1** against *E. coli* were 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 μg/ml. Test concentrations for **2–4** against *E. coli* were 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 μg/ml. Antimicrobial susceptibility tests were performed in triplicate, with DMSO present at 5% (v/v).
[b] Activity was assessed by disc diffusion.

for the tail of **1**, the tail is believed to exhibit conformational flexibility in solution in the unbound state.[31] Moreover, the current structural knowledge of the region of the thiostrepton binding site on the 50S subunit allows for alternative positions of the tail that could result in diminished or eliminated interactions with the L11-NTD.

3. Conclusions

Through the use of facile semi-synthesis, novel, regio-specific derivatives of the intact thiostrepton molecule have been prepared. These tail modified analogues highlight an important contribution made by the tail region of the molecule to antibacterial activity, which can be drastically altered depending on the nature of modifications made to this region. While a multitude of regio-specific tail analogues could be generated by the approach described here, we submit that future biosynthetic and semi-synthetic studies that focus on this region take into account the potential steric and spatial limitations to the practical modification of thiostrepton in this region, which have become evident from our examination of structurally dissimilar modifying groups. Further approaches to utilise this region to improve the water solubility of **1** and extend its spectrum of activity should be the focus of future research with this antibiotic.

4. Experimental

4.1. General methods

Syntheses of Michael adducts were performed under an inert atmosphere. Liquid chromatography was performed with a Zorbax Eclipse XBD C-8 column (4.6 mm × 150 mm), using the Waters 625 Liquid Chromatography System. The polar and non-polar mobile phases were 0.1% trifluoroacetic acid and acetonitrile (Caledon Laboratory Chemicals; Georgetown, Ontario), respectively. HRMS was recorded with a Micromass Q-TOF Ultima Global at a mass resolution of 7000 FWHM, with the doubly charged molecular ion of **1** as the reference. NMR spectra were acquired on a Bruker Advance 600 MHz spectrometer. ^{1}H chemical shifts are in ppm relative to residual solvent peaks (7.24 ppm for $CDCl_3$). Typical NMR samples were prepared by dissolving 2–4 mg of analyte in 0.6–0.7 ml of mixture of deuterated chloroform/deuterated methanol (4:1) (Cambridge Isotopes Laboratories, Andover, MA).TOCSY spectra were acquired using the pulse sequence for homonuclear Hartman–Hahn transfer[32], with 80 ms mixing time. Luminescence was recorded in white 96-well microplates with a SpectraMax M5 multiplate reader (Molecular Devices). All compounds tested were >95% pure as determined by analytical HPLC.

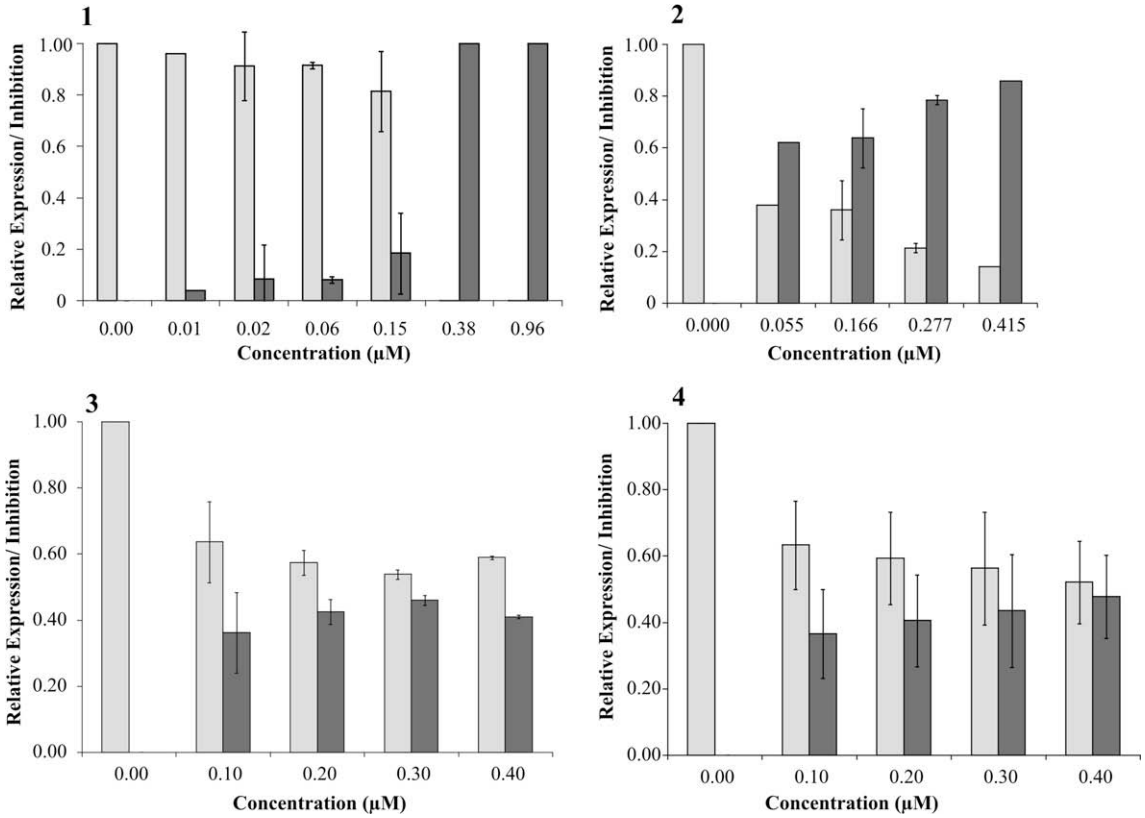


Figure 4. Effect of **1** and analogues on in vitro protein synthesis. Expression □/■ inhibition are expressed relative to control reactions where thiostrepton is absent. Values reflect the means of at least two experiments. Error bars represent one standard deviation of the mean.

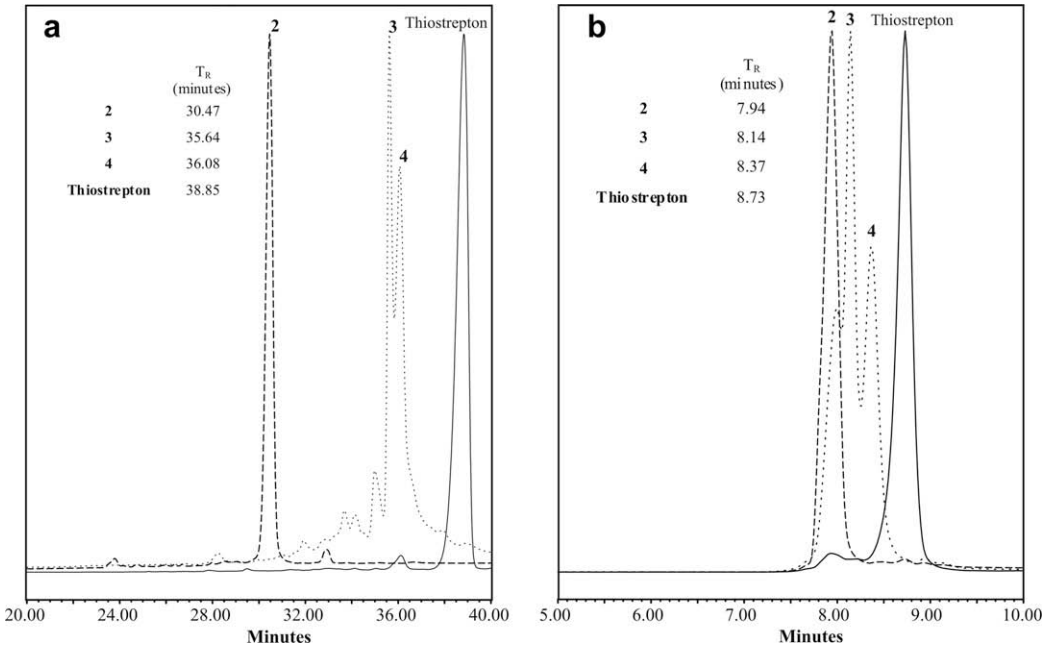


Figure 5. Relative polarity of Michael adducts. RP-HPLC chromatograms ($A_{254\ nm}$) of thiostrepton and Michael adducts using (a) gradient and (b) isocratic elution. See Section 4 for details.

Table 2
Theoretical Log *P* values for thiostrepton and adducts

Compound	*mi* Log *P*[a]	Viswanadhan et al.[33,b]	Klopman et al.[34,b]
Thiostrepton	−1.567	−1.01	−0.97
2 (neutral)	−4.151	−4.41	−1.91
2 (negative)	−4.796	−6.67	−4.38
3/**4** (neutral)	−2.969	−3.03	−0.85
3/**4** (negative)	−4.205	−6.71	−4.35
TBG adduct	−3.569	−5.89	−3.45
Erythromycin	2.281	1.22	4.20
Tetracycline	−0.978	−0.16	0.39

[a] *mi* Log *P*: Molinspiration Cheminformatics (www.molinspiration.com) calculated Log *P* using online Molinspiration Property Engine v2007.04.

[b] Log *P* values were also calculated by the methods of Viswanadhan et al.[33] and Klopman et al.[34] with electrolyte concentration: Cl^- = 0.1 (mol/dm^3) and Na^+/K^+ = 0.1 (mol/dm^3) as implemented in the structure property prediction and calculation plugins for Marvin 5.3.01, 2010, ChemAxon (http://www.chemaxon.com). See Section 4 for details.

4.2. Preparation of Michael adducts

To 50 mg (0.03 mmol) of thiostrepton (Sigma; Oakville Ontario) dissolved in 1 ml of dimethylformamide (Caledon Laboratory Chemicals; Georgetown, Ontario), were added 10 mmol equiv (42 μl) of triethylamine and 1 mmol equiv of aqueous 2-mercaptoethanesulfonic acid, captopril or 1-thio-2-β-D-glucose (all from Sigma; Oakville, Ontario), each prepared as 150 mM stock solutions. Reactions were mixed on a platform shaker for 18–24 h at 4 °C under an argon atmosphere and then dried under vacuum, leaving an orange-yellow solid. Michael adducts were isolated by RP-HPLC utilizing different elution conditions: *Method 1; purification of* **2**. The solid reaction product obtained after vacuum drying was dissolved in deionised H_2O (1.5 mg/ml), then separated over a linear increasing gradient of acetonitrile (0.5%/min), with flow rates of 0.5 or 1 ml/min. *Method 2; purification of* **3** *and* **4**. Vacuum dried reaction product was dissolved in 50% DMSO (1.4 mg/ml) and then separated over a linear increasing acetonitrile gradient of 0.25%/min, at a flow rate of 0.5 ml/min.

4.2.1. 2-Mercaptoethanesulfonic acid adduct (2)

HPLC (Method 1): t_R = 31 min. ^{1}H NMR (600 MHz, $CDCl_3$: CD_3OD (4:1)): δ 8.15, (s, 1H), 8.12 (s, 1H), 8.04 (s, 1H), 7.43 (d, *J* = 9.65 Hz, 1H), 7.40 (s, 1H), 7.18 (s, 1H), 7.06 (s, 1H), 6.77 (d, *J* = 9.84 Hz, 1H), 6.27, (dd, *J* = 5.92, 10.02 Hz, 1H), 6.23 (m, 1H),6.21 (s, 1H), 6.10 (q, *J* = 6.562 Hz, 1H), 5.71 (s, 1H), 5.64 (d, *J* = 3.45 Hz, 1H), 5.60 (s,1H), 5.52 (s, 1H), 5.20 (s, 1H), 5.19 (s, 1H), 5.17 (s, 1H), 4.86 (dd, *J* = 9.19, 12.74 Hz, 1H), 4.82–4.78 (m, 1H), 4.60 (m, 1H), 4.29 (m, 1H), 4.09–4.03 (m, 4H), 3.95 (m, 1H), 3.68 (dq, *J* = 6.20,12.47 Hz, 1H), 3.53 (m, 1H), 2.84 (d, *J* = 3.98 Hz, 1H), 2.50 (s, 1H), 2.19–2.15 (m, 1H), 1.78 (s, 1H), 1.59 (d, *J* = 6.57 Hz, 3H),1.48 (d, *J* = 7.08 Hz, 3H), 1.43 (m, 1H), 1.32 (dd, *J* = 3.65, 6.21 Hz, 3H), 1.26 (d, *J* = 6.61, Hz, 3H), 1.16 (d, *J* = 6.30 Hz, 3H),1.10 (s, 1H), 1.04 (d, *J* = 6.67 Hz, 3H), 1.02 (s, 3H), 0.99 (m, 2H), 0.88, (d, *J* = 6.88 Hz, 3H), 0.79 (m, 1H), 0.67 (d, *J* = 6.21 Hz, 3H). HRMS (ESI) calcd for $C_{74}H_{91}N_{19}O_{21}S_7$ (M+H$^+$) *m/z* 1807.4682; found: 1807.4820.

4.2.2. Captopril adduct (3)

HPLC (Method 2): t_R = 37.2 min. ^{1}H NMR (600 MHz, $CDCl_3$: CD_3OD (4:1)): δ 9.76 (s, 1H), 8.67 (d, *J* = 8.90 Hz, 1H), 8.50 (s, 1H), 8.15 (s, 1H), 8.13 (d, 1.21 Hz, 1H), 8.03 (s, 1H), 7.86 (s, 1H), 7.66 (s, 1H), 7.46 (d, *J* = 10.05 Hz, 1H), 7.43 (s, 1H), 7.41 (d, *J* = 1.23 Hz, 1H), 7.17 (s, 1H), 7.06 (d, *J* = 1.23 Hz, 1H), 7.01 (d, *J* = 7.93 Hz, 1H), 6.94 (d, *J* = 7.07 Hz, 1H), 6.77 (d, *J* = 10.17 Hz, 1H), 6.26 (dd, obscure), 6.25 (s, 1H), 6.23–6.18 (m, 1H), 6.11 (q, *J* = 7.01, Hz), 5.70 (s, 1H), 5.65 (d, *J* = 8.61 Hz, 1H), 5.62 (d, *J* = 10.01 Hz, 1H), 5.51 (s, 1H), 5.21 (s, 1H), 5.20 (d, *J* = 9.89 Hz, 1H), 5.16 (s, 1H), 4.86 (dd, *J* = 9.27,12.53, 1H), 4.70–4.62 (m, 2H), 4.38–4.36 (m, 2H), 3.94–3.91 (m, 1H), 3.69 (dq, *J* = 5.68, 6.46 Hz,1H), 3.54 (m, 1H), 3.51 (m, 1H), 2.95–2.86 (m, 1H), 2.77–2.74 (m, 1H), 2.51 (d, *J* = 6.06, 1.38 Hz, 1H), 2.19 (m, 1H), 2.14 (m, 2H), 2.08 (m, 2H), 1.95–1.90 (m, 2H), 1.60 (d, *J* = 6.44 Hz, 3H), 1.49 (d, *J* = 7.03 Hz, 3H), 1.31 (d, *J* = 6.14 Hz, 3H), 1.25 (d, *J* = 6.57 Hz, 3H),1.18 (d, *J* = 6.38 Hz, 3H), 1.09 (d, *J* = 6.73 Hz, 3H), 1.06 (d, *J* = 6.61 Hz, 3H), 1.02 (s, 3H) 1.00–0.95 (m, 2H), 0.87 (d, *J* = 6.81 Hz, 3H), 0.77 (d, *J* = 6.67 Hz, 3H), 0.71 (d, *J* = 6.15 Hz, 3H). HRMS (ESI) calcd for $C_{81}H_{100}N_{20}O_{21}S_6$ (M+H$^+$) *m/z* 1881.5696; found: 1881.5864.

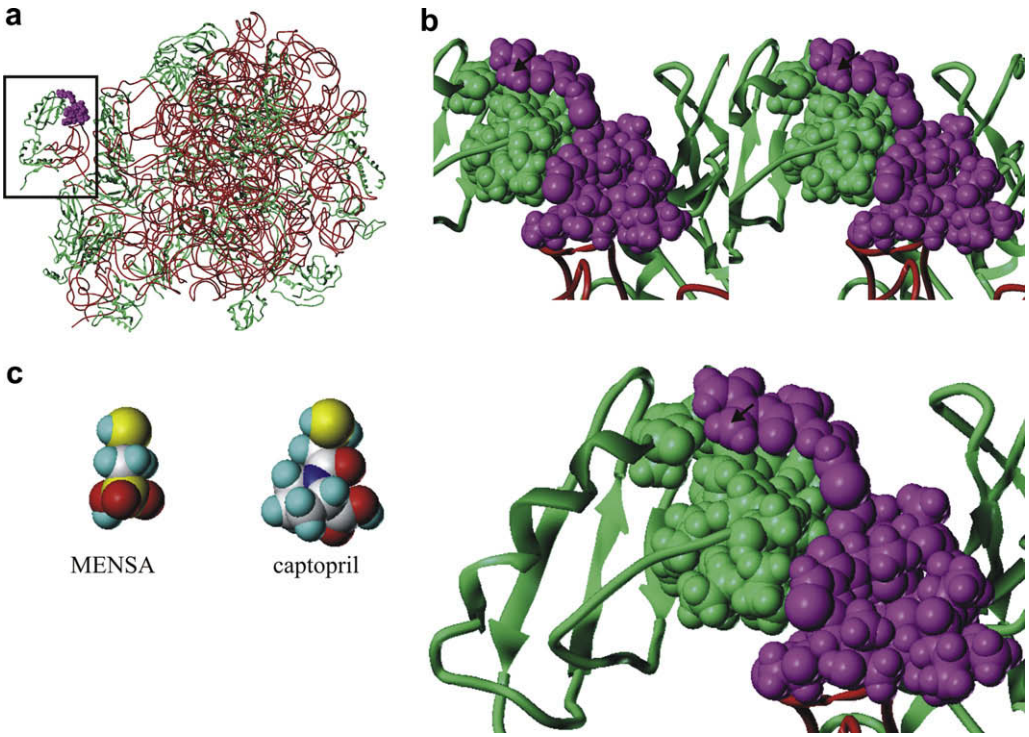


Figure 6. (a) Thiostrepton (magenta) bound to the 50S ribosomal subunit (rRNA in red; ribosomal proteins in green) from *D. radiodurans*. (b) The binding site of thiostrepton and Michael adducts (magnified view of the boxed region from a); the site of Michael addition is indicated by arrow. (c) MENSA and captopril juxtaposed with a region of the thiostrepton-50S complex highlighting the interaction between thiostrepton and the L11-NTD.

4.2.3. Captopril adduct (4)

HPLC (Method 2): t_R = 39.4 min. ^{1}H NMR (600 MHz, $CDCl_3$:CD_3OD (4:1)): δ 9.76 (s, 1H), 8.15 (s, 1H), 8.12 (s, 1H), 8.03 (s, 1H), 7.87 (s, 1H), 7.45 (d, J = 10.01 Hz, 1H), 7.43 (d, J = 10.29 Hz, 1H), 7.41, 7.17 (s, 1H), 7.06, 6.98 (d, J = 7.61 Hz, 1H), 6.95 (d, J = 7.78 Hz,1H), 6.77 (d, J = 9.87 Hz, 1H), 6.26 (dd, J = 5.58, 9.99 Hz, 1H), 6.23 (d, J = 6.23 Hz, 1H), 6.21 (s, 1H), 6.11 (q, J = 7.03 Hz, 1H), 5.70 (s, 1H), 5.66 (d, J = 8.65, 1H), 5.62 (d, J = 10.06 Hz, 1H), 5.55 (s, 1H), 5.21 (s, 1H), 5.19 (d, J = 6.62 Hz, 1H), 5.17 (d, J = 2.73 Hz, 1H), 4.86 (dd, J = 8.98, 12.59 Hz,1H), 4.76–4.73 (m, 2H), 4.34–4.30 (m, 2H), 3.95–3.93 (m, 1H), 3.69 (dq, J = 6.06, 6.42 Hz, 1H), 3.54 (m, 1H), 3.53–3.48 (m, 1H), 2.95–2.87 (m,1H), 2.79–2.75 (m, 1H), 2.51 (d, J = 6.63, 1H), 2.18–2.16 (m, 1H), 1.60 (d, J = 6.48 Hz, 3H), 1.49 (d, J = 6.98 Hz, 3H), (d, J = 6.28 Hz, 3H), 1.26 (d, J = 6.44 Hz, 3H), 1.18 (d, J = 6.37 Hz, 3H), 1.09 (d, J = 6.07, 3H), 1.05 (d, J = 6.70 Hz, 3H), 1.02 (s, 3H), 0.99–0.94 (m, 2H), 0.87 (d, J = 6.81 Hz, 3H), 0.77 (d, J = 7.21 Hz, 3H), 0.70 (d, J = 6.12 Hz, 3H). HRMS (ESI) calcd for $C_{81}H_{100}N_{20}O_{21}S_6$ ($M+H^+$) m/z 1881.5696; found: 1881.5860.

4.3. Relative polarity of thiostrepton and Michael adducts

4.3.1. RP-HPLC

Polar and non-polar mobile phases were 0.1% TFA and acetonitrile, respectively. Gradient elution was achieved over a linear, increasing gradient of acetonitrile of 1%/ min. Isocratic elution was performed with a 1:1 ratio of the polar and non-polar mobile phases.

4.3.2. Theoretical solubility calculations for thiostrepton and Michael adducts

Theoretical octanol/water partition coefficient, Log *P*, values were calculated using three different approaches (Table 2). *mi*-Log *P*: Molinspiration Cheminformatics (www.molinspiration.com) calculated Log *P* using online Molinspiration Property Engine v2007.04. Log *P* values were also calculated by the methods of Viswanadhan et al.[33] and Klopman et al.[34] with electrolyte concentration: Cl^- = 0.1 (mol/dm^3) and Na^+/K^+ = 0.1 (mol/dm^3) as implemented in the structure property prediction and calculation plugins for Marvin 5.3.01, 2010, ChemAxon (http://www.chemaxon.com). Chemical structures of thiostrepton and corresponding adducts were drawn in ChemBioDraw Ultra (version 11.0.1; CambridgeSoft, USA) and exported as SMILES structure format files. These files were then imported into the various Log *P* calculation software programs. Structures of tetracycline and erythromycin utilised for Log *P* comparison purposes were from PubChem (http://pubchem.ncbi.nlm.nih.gov).

4.4. Inhibition of protein synthesis

Inhibition of in vitro prokaryotic translation was quantified using the *E. Coli* S30 Extract System for Circular DNA (Promega) with the pBESTluc plasmid (Promega) according to the manufacturer's protocol. Individual translation reactions (25 µl) contained 10 µl of Premix without amino acids, 7.5 µl of S30 extract, 2.5 µl of complete amino acid mix, 2.5 µl of pBEST*luc* (700 ng/µl) and 2.5 µl of test compound. Control reactions received 2.5 µl of 50% DMSO. Test compounds were initially dissolved in DMSO and then diluted to appropriate working concentrations with deionised water. The final concentration of DMSO in the reactions was 2.5% (v/v). Control reactions received 2.5 µl of 50% DMSO in place of test compound. Reactions were incubated for 60 min at 37 °C and then placed on ice for 5 min, after which the luciferase assay reagent was added. Luminescence was recorded 5 min after the addition of 25 µl of SteadyGlo Luciferase assay reagent (Promega).

Acknowledgements

The authors gratefully acknowledge funding by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the University of Waterloo.

Supplementary data

Supplementary data (1D and 2D NMR spectra for thiostrepton and its analogues, purification and characterization of the TBG adduct) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.098.

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