The thioester bonds of thiocoraline can be replaced with *N*Me-amide bridges without affecting its DNA-binding properties

Rubí Zamudio-Vázquez, Fernando Albericio, Judit Tulla-Puche, and Keith R. Fox

Institute for Research in Biomedicine, Barcelona Science Park, 08028 Barcelona, Spain.

CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, 08028 Barcelona, Spain.

Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain.

School of Chemistry, University of KwaZulu-Natal, 4001-Durban, South Africa.

Centre for Biological Sciences, University of Southampton, Southampton SO17 1BJ, United Kingdom.

SUPPORTING INFORMATION

1. DRUGS

Thiocoraline A was isolated and purified by PharmaMar, S.A. (Colmenar Viejo, Madrid, Spain), as described previously.[1] Echinomycin and Actinomycin D were purchased from Sigma-Aldrich. *N*Me-azathiocoraline, oxathiocoraline and PEG-NMe-azathiocoraline were synthesized as previously described.[2] TANDEM was provided by Mark Searcey (School of Chemical Sciences and Pharmacy, University of East Anglia) and was prepared as described previously.[3]

Compound	Study	Breast MDA-MB-231	NSCLC (Lung) A549	Colon HT-29
Thiocoraline **1**	TGI	9.50E-9	2.42E-8	2.68E-8
	LC_{50}	3.46E-8	9.50E-8	1.38E-7
	GI_{50}	2.07E-9	6.22E-9	4.15E-8
*N*Me-Azathiocoraline **2**	TGI	4.26E-8	2.00E-8	1.13E-8
	LC_{50}	3.73E-7	1.65E-7	7.47E-7
	GI_{50}	4.08E-9	3.39E-9	2.08E-8
Oxathiocoraline **3**	TGI	2.75E-6	2.75E-6	3.55E-6
	LC_{50}	6.40E-6	7.91E-6	7.55E-6
	GI_{50}	4.62E-7	3.11E-7	4.00E-7
PEG-*N*Me-Azathiocoraline **4**	TGI	>8.95E-6	>8.95E-6	>8.95E-6
	LC_{50}	>8.95E-6	>8.95E-6	>8.95E-6
	GI_{50}	>7.09E-6	>7.09E-6	>7.09E-6

Table S1. Cytotoxic activity of thiocoraline and some of its synthetic analogues on three different human cancer cell lines. TGI: drug concentration causing total growth inhibition. LC_{50}: drug concentration causing 50% net cell killing. GI_{50}: drug concentration causing 50% growth inhibition. Data adapted from supplementary references 2 and 4.

2. EXPERIMENTAL PROCEDURES

2.1. DNA fragments

Radiolabeled DNA fragments of the MS2 clone (which contains all 136 tetranucleotide sequences) were prepared by digesting the plasmids with HindIII and SacI and filling the 3'-end of the HindIII site with [α-^{32}P]dATP using reverse transcriptase.
The sequences of HexA and HexB were designed to contain all 64 symmetrical hexanucleotide sequences between them. HexA contains the sites for EcoRI (GAATTC) and PstI (CTGCAG) but not HindIII (AAGCTT) and SacI (GAGCTC), whereas HexB contains the sites for HindIII and SacI but not EcoRI and PstI. Plasmids containing the HexA and HexB sequences were cut with HindIII/SacI and EcoRI/PstI, respectively, and labeled at the 3'-end of the EcoRI or HindIII sites with [α-^{32}P]dATP using reverse transcriptase.
*tyr*T 3'-end labeled fragment was prepared by filling in the sticky-ends with [α-^{32}P]dATP after a double digestion with EcoRI and AvaI.
The radiolabeled DNA fragments were separated from the remainder of the plasmid DNA on 6% polyaclylamide gels. The DNA was eluted from the gel and dissolved in 10mM Tris-HCl, pH 7.5, containing 0.1mM EDTA at a concentration of 10-20 cps/µl as determined with a handheld Geiger counter. This produces a fragment concentration of approximately 10 nM, which is much lower than the dissociation constant of the ligand.

2.2. Band shift experiment

3'-end radiolabeled *tyr*T fragment (1.5 µl) was mixed with two concentrations of the tested compounds (dissolved in 10 mM Tris–HCl, pH 7.5, containing 10 mM NaCl) and left to equilibrate for 30 minutes at room temperature. A native 6% polyacrylamide gel was run at 400 V for about 45 minutes. The gel was then fixed in 10% (v/v) acetic acid, transferred to Whatman 3MM paper, and dried under vacuum at 80°C. Dried gel was exposed to a Kodak Phosphor storage screen, which was scanned using a Molecular Dynamics Storm 860 phosphorimager.

*tyr*T

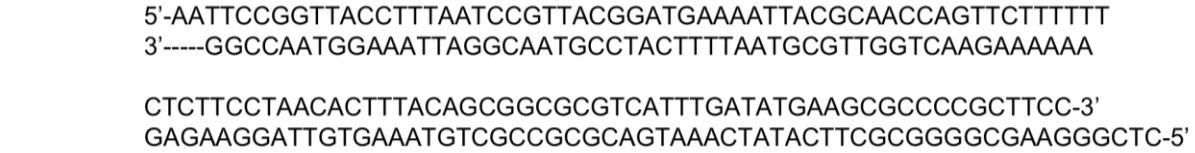

Figure S1. Sequence of the *tyr*T fragment used for the band shift experiment.

2.3. DNase I footprinting

Radiolabeled DNA (1.5 µl) was mixed with various concentrations of the tested compounds (dissolved in 10 mM Tris–HCl, pH 7.5, containing 10 mM NaCl). The mixture was left to equilibrate overnight at room temperature, before adding 2 µl DNase I (approximately 0.01 units/ml) dissolved in 20 mM NaCl, containing 2 mM $MgCl_2$ and 2 mM $MnCl_2$. The digestion was stopped after 1 min by adding 4 µl of formamide containing 10 mM EDTA, 1 mM NaOH, and 0.1% (w/v) bromophenol blue. The digestion products were boiled for 3 min and resolved on 8% polyacrylamide gels containing 8 M urea, which were run at 1500 V for about 2 h. Gels were then fixed in 10% (v/v) acetic acid, transferred to Whatman 3MM paper, and dried under vacuum at 80°C. Dried gels were exposed to a Kodak Phosphor storage screen, which was scanned using a Molecular Dynamics Storm 860 phosphorimager.

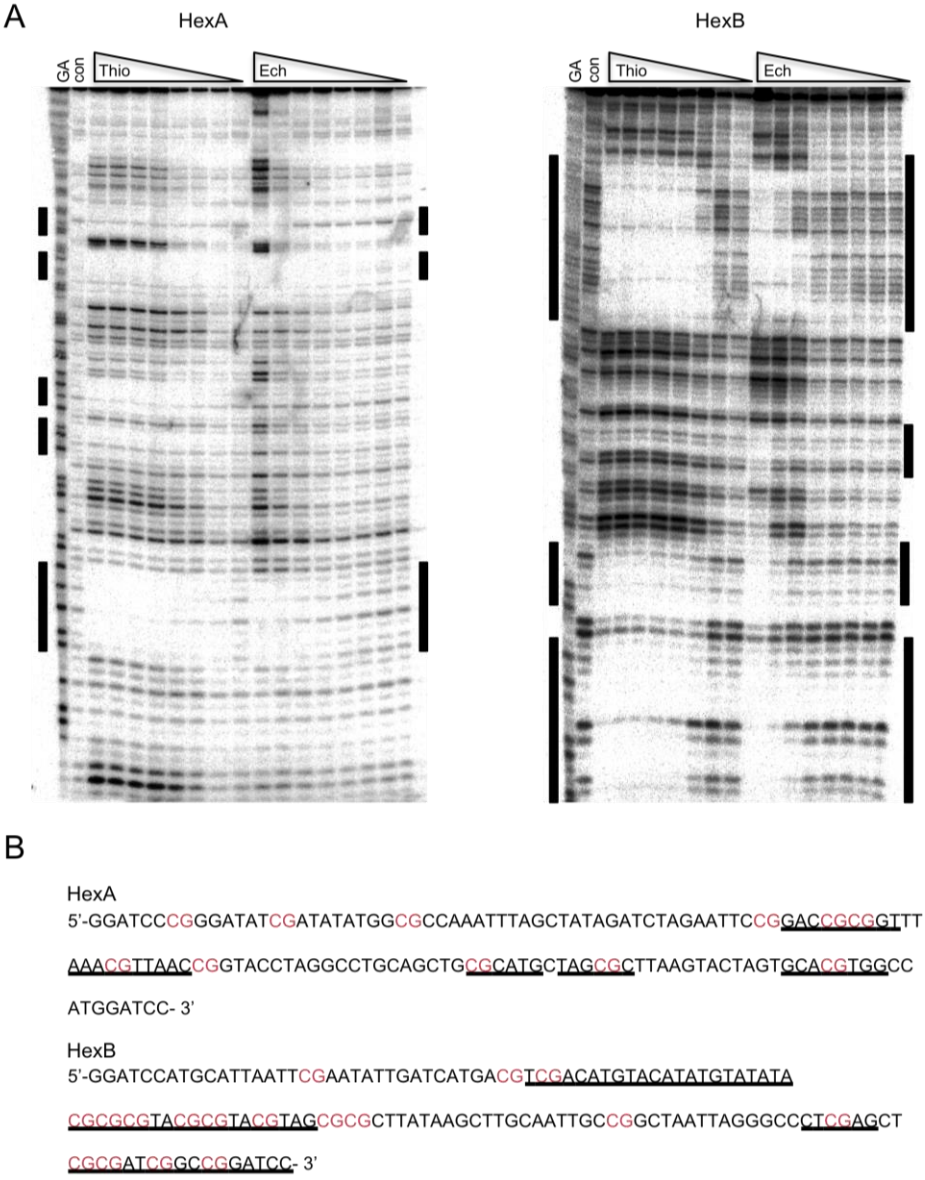

Figure S2. (A) DNase I footprints for thiocoraline (Thio) and echinomycin (Ech). The panels show the interaction of both natural drugs with HexA and HexB fragments. Ligand concentrations are 200, 100, 50, 20, 10, 5, 1 and 0.1 μM. Tracks labeled "GA" are markers specific for purines. The bars shown on the left side of each one of the gels indicate the regions of attenuated cleavage by thiocoraline and the bars shown on the right side indicate the footprints by echinomycin. (B) Sequences of the footprinting substrates HexA and HexB. The fragments were each labeled at the 3'-end and only the labeled strand is shown. The attenuated cleavege regions observed in the presence of thiocoraline are underlined. Thiocoraline preferred binding site CpG appears in red.

2.4. Quantitative analysis

The intensity of each of the bands within the footprints was estimated using ImageQuant software and this was normalized relative to the total intensity of all the cleavage products in the same lane. C_{50} values representing the ligand concentration that reduced the intensity of bands in the footprint by 50% were estimated from this by fitting the data with a simple binding curve. Since the DNA concentration (approximately nanomolar) is much lower than the ligand dissociation constants (typically micromolar), the C_{50} values approximate the ligand dissociation constants.

Site	Sequence	Fragment	C_{50} (μM)
1	GACGTA	MS2	95 ± 10
2	GGATAG	MS2	131 ± 17
3	ACGACT	MS2	102 ± 12
5	ATGGAT	MS2	110 ± 15
6	ATATAT	HexA	28 ± 6
7	ATAGAT	HexA	95 ± 7
10	GTACTA	HexA	28 ± 3
11	CACGTG	HexA	20 ± 2
12	TATATA	HexB	28 ± 2
13	CGTACG	HexB	13 ± 3
14	CTCGAG	HexB	17 ± 4
15	CGCGAT	HexB	22 ± 5

Table S2. C_{50} values (μM) for the interaction of NMA with various binding sites on MS2, HexA and HexB, determined from the footprinting plots presented in Figure 3.

2.5. Fluorescence melting

Fluorescence melting curves were determined in a Roche LightCycler, using a total reaction volume of 20 μl. The melting profiles for up to 32 samples could be recorded simultaneously. For each reaction the final oligonucleotide concentration was 0.25 μM, diluted in an appropriate buffer. In a typical experiment the samples were first denatured by heating to 95°C at a rate of 0.1°C s^{-1}. The samples were then maintained at 95°C for 5 min before annealing by cooling to 25°C at 0.1°C s^{-1} (this is the slowest heating and cooling rate for the LightCycler). They were held at 25°C for a further 5 min and then melted by heating to 95°C at 0.1°C s^{-1}. Recordings were taken during both the melting steps as well as during annealing. The LightCycler has one excitation source (488 nm) and three channels for recording fluorescence emission at 520, 640 and 705 nm. For the studies in this work we measured the changes in fluorescence at 520 nm. The data were normalized to show the fractional change in fluorescence for each sample between the starting and maximal values. T_m values were determined from the first derivatives of the melting profiles using the Roche LightCycler software.

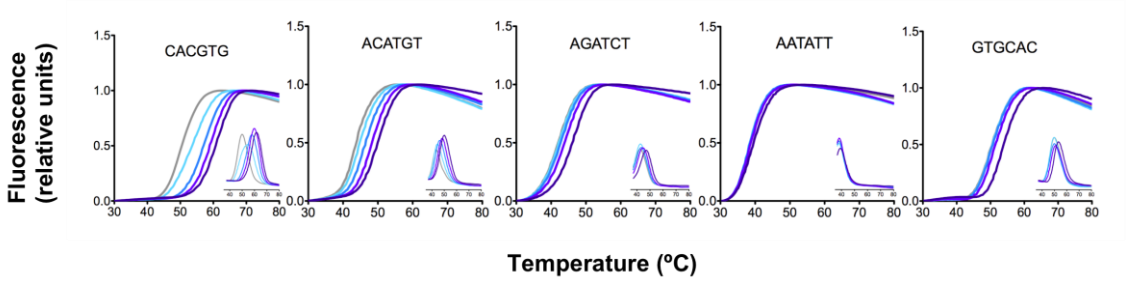

Figure S3. Melting profiles for the fluorescently labeled double-stranded oligodeoxynucleotides in the absence and presence of increasing concentrations of echinomycin. The inserts show the first derivatives of the melting profiles. The ordinate shows the relative fluorescence of the samples, which has been normalized to the maximum fluorescence change for that sample. The abscissa shows the temperature in °C. The curves correspond to echinomycin concentrations of 0, 10, 25, 50 and 100 μM increasing from left to right.

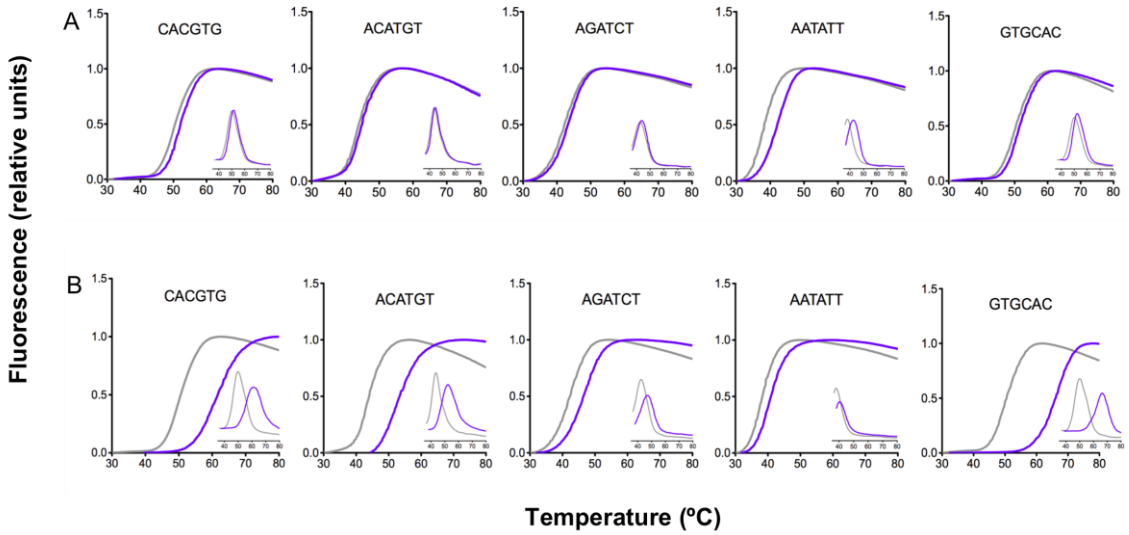

Figure S4. Melting profiles for the fluorescently labeled double-stranded oligodeoxynucleotides in the absence and presence of ligand. The inserts show the first derivatives of the melting profiles. The ordinate shows the relative fluorescence of the samples, which has been normalized to the maximum fluorescence change for that sample. The abscissa shows the temperature in °C. (A) The curves correspond to TANDEM at 0μM (grey) and 50 μM (blue). (B) The curves correspond to Actinomycin D at 0μM (grey) and 50 μM (blue).

3. SUPPLEMENTARY REFERENCES

(1) Ramos-Castro, A.; Fernández-Braña, A.; Lombó-Brugos, F.; Méndez-Fernández, C.; Salas-Fernández, J. A.; Velasco-Iglesias, A.; Schleissner-Sánchez, C. Genes involved in the biosynthesis of thiocoraline and heterologous production of same. WO Patent 2007/014971, May 10, **2007**.

(2) Tulla-Puche, J.; Auriemma, S.; Falciani, C.; Albericio, F. Orthogonal chemistry for the synthesis of thiocoraline-triostin hybrids. Exploring their Structure-Activity Relationship *J. Med. Chem.* **2013**, *56*, 5587-5600.

(3) Malkinson, J. P.; Anim, M. K.; Zloh, M.; Searcey, M.; Hampshire, A. J.; Fox, K. R. Efficient solid-phase-based total synthesis of the bisintercalator TANDEM. *J .Org. Chem.* **2005**, *70*, 7654–7661.

(4) Tulla-Puche, J.; Góngora-Benítez, M.; Bayó-Puxan, N.; Francesch, A. M.; Cuevas, C.; Albericio, F. Enzyme-labile protecting groups add a new orthogonal dimension to the synthesis of natural products: solid-phase synthesis of thiocoraline. *Angew. Chem. Int. Ed.* **2013**, *52*, 5726-5730.