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Thioester Bonds of Thiocoraline Can Be Replaced with *N*Me-Amide Bridges without Affecting Its DNA-Binding Properties

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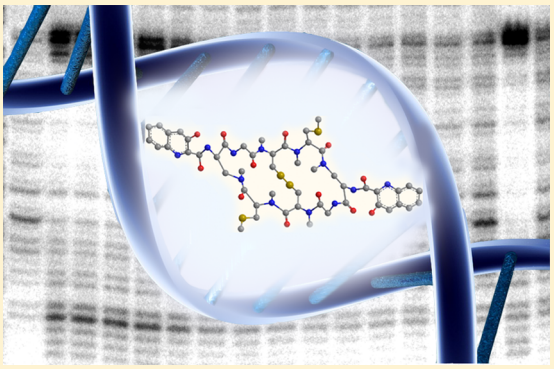
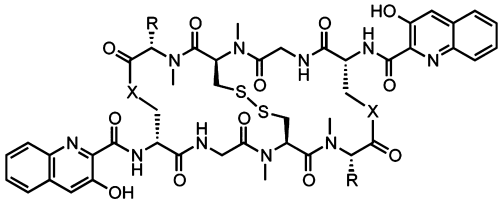
Supporting Information

ABSTRACT: In the search for new drug candidates for DNA recognition, affinity and sequence selectivity are two of the most important features. *N*Me-azathiocoraline, a synthetic antitumor bisintercalator derived from the natural marine product thiocoraline, shows similar potency to the parent compound, as well as possessing enhanced stability. Analysis of the DNA-binding selectivity of *N*Me-azathiocoraline by DNase I footprinting using universal substrates with all 136 tetranucleotides and all possible symmetrical hexanucleotide sequences revealed that, although this ligand binds to all CpG steps with lower affinities than thiocoraline, it displays additional binding to AT-rich sites. Moreover, fluorescence melting studies showed a strong interaction of the synthetic molecule with CACGTG and weaker binding to ACATGT and AGATCT. These findings demonstrate that *N*Me-azathiocoraline has the same mode of action as thiocoraline, mimicking its DNA-binding selectivity despite the substitution of its thioester bonds by *N*Me-amide bridges.



KEYWORDS: *Thiocoraline, bisintercalator, DNA binding, antitumor, DNase I footprinting, fluorescence melting*

DNA-interacting natural products and their derivatives account for a significant portion of anticancer drugs that have reached clinical trials.[1] The exceptional cytotoxic activity of these compounds at submicromolar concentrations and the development of new molecules with sequence-selective recognition have increased their relevance in biomedical research.[2] Most of these compounds have been isolated from various genera of bacteria and fungi, in which they are synthesized as defense metabolites, and drug-discovery programs have found them useful as novel pharmaceutical agents.



1) Thiocoraline	X = S	R = CH_2SMe
2) *N*Me-azathiocoraline	X = *N*Me	R = CH_2SMe
3) Oxathiocoraline	X = O	R = CH_2SMe
4) PEG-*N*Me-azathiocoraline	X = *N*Me	R = $CH_2NHCPEG$ (O)

Figure 1. Structures of thiocoraline and some of its synthetic analogues.

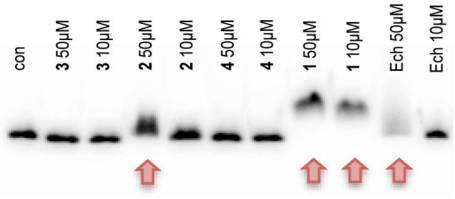


Figure 2. Band shift experiment with the *tyr*T DNA fragment for compounds **1**−**4** and echinomycin. Ligand concentrations are shown at the top of each gel lane. Track labeled "con" is the DNA incubated with the solvent conditions used for all the drugs. The arrows show the bands that migrated slower in the native gel due to ligand binding to the DNA.

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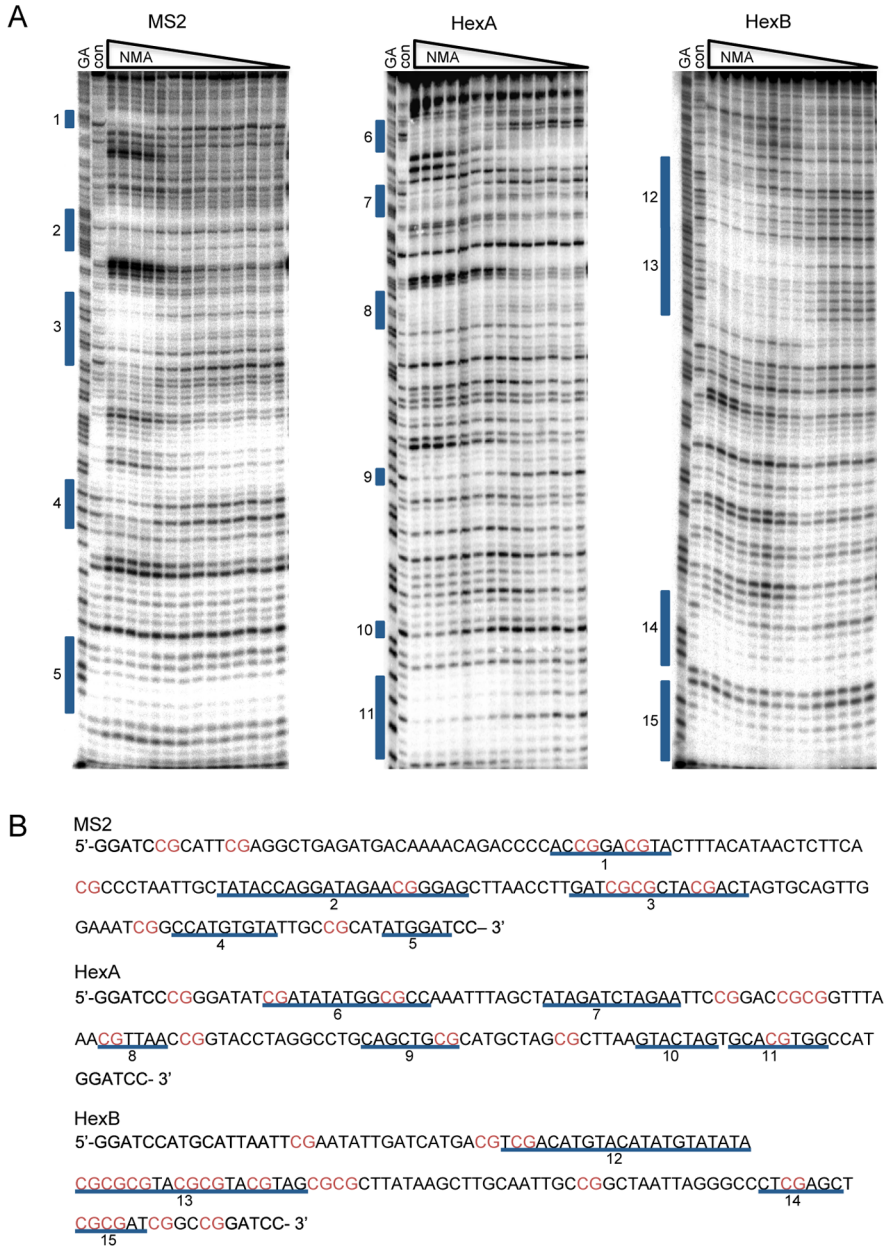

Figure 3. (A) DNase I footprints for *N*Me-azathiocoraline. The three panels show the interaction of NMA with the MS2, HexA, and HexB fragments. Ligand concentrations are 500, 400, 300, 200, 100, 80, 60, 40, 20, 10, 5, 1, 0.5, and 0.1 μM. Tracks labeled ″GA″ are markers specific for purines. Tracks labeled ″con″ are the DNA incubated with the solvent conditions used for the drug. The bars shown alongside the gels indicate the regions of attenuated cleavage. (B) Sequences of the footprinting substrates MS2, HexA, and HexB. The fragments were each labeled at the 3′-end and only the labeled strand is shown. The attenuated cleavage regions observed in panel A are underlined and numbered. Thiocoraline preferred binding site CpG appears in red for comparison purposes.

Bisintercalators comprise an important family of natural products that bind duplex DNA through the insertion of two planar chromophores between the bases.[3] Since the characterization of echinomycin,[4] the first bifunctional intercalating agent to be described, much research effort has been focused on the isolation, biological activity, mechanisms of action, and structure−activity relationships of other similar bisintercalators. These efforts were encouraged when echinomycin passed phase I clinical trials,[5] but after showing minimal to no activity in phase II trials[6] the necessity of finding a new candidate was prevailing.

Thiocoraline (**1**), a 2-fold-symmetric bicyclic nonribosomal octathiodepsipeptide, is produced by two strains of marine actinomycetes, *Micromonospora sp.* L-13-ACM2-092, isolated from a soft coral,[7] and *Micromonospora sp*. ML1, isolated from a marine mollusk.[8,9] This compound shows potent antimicrobial activity against Gram-positive bacteria, in vitro cytotoxic activity at low nanomolar concentrations against a variety of cancer cell lines, and in vivo activity against human carcinoma xenografts.[10] It inhibits DNA elongation by polymerase α, thus causing cell cycle arrest in the G1 phase and a decrease in the rate of S phase progression toward G2/M phases.[10] This biologically active peptide binds to double-stranded DNA adopting a staple form that facilitates intercalation of the two 3-hydroxyquinaldic acid moieties from the minor groove and shows selectivity for CpG.[11]

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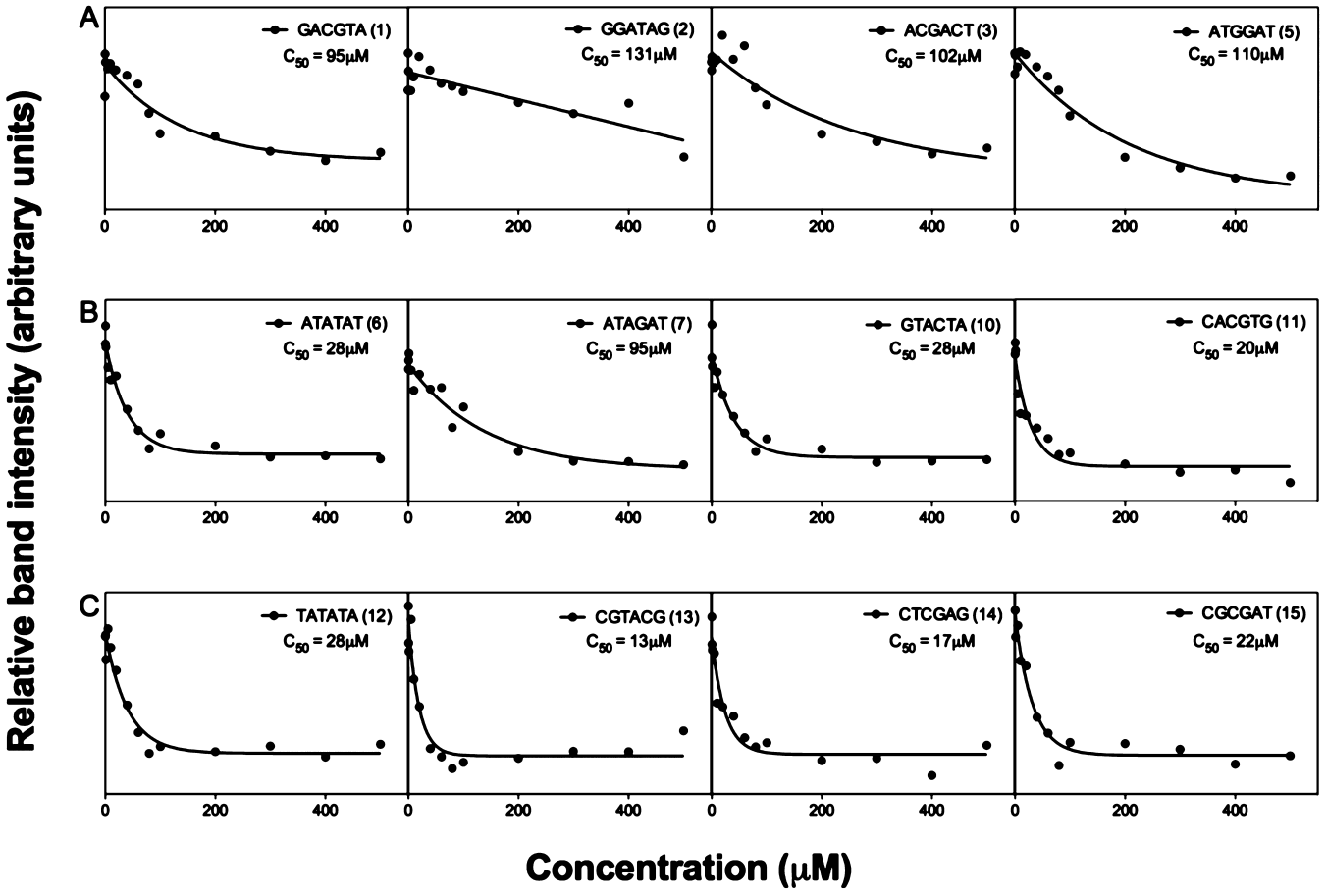

Figure 4. Footprinting plots for the interaction of NMA. Numbers in parentheses correspond to the footprints in Figure 3. The ordinate shows the ligand concentration (micromolar), and the abscissa corresponds to the relative band intensity (arbitrary units) in the presence of the ligand. C_{50} values represent the ligand concentration that reduced the intensity of bands in the footprint by 50%. (A) Sequences found in MS2. (B) Sequences found in HexA. (C) Sequences found in HexB.

Table 1. Effect of Ligands on the Melting Temperature of the Fluorescently Labeled Oligonucleotides

intermolecular duplexes[a]	name	ΔT_m (°C)[b] NMe-azathiocoraline	ΔT_m (°C)[b] thiocoraline
5′-F-AAAACACGTGTTTT 3′-Q-TTTTGTGCAAAA	CACGTG	7.6	0.1 and 11.7[c]
5′-F-AAAAACATGTTTTT 3′-Q-TTTTTGTACAAAAA	ACATGT	4.5	0.5 and 6.6[c]
5′-F-AAAAAGATCTTTTT 3′-Q-TTTTTCTAGAAAAA	ACATCT	2.2	0
5′-F-AAAAAATATTTTTT 3′-Q-TTTTTTATAAAAAA	AATATT	1.4	0
5′-F-AAAAGTGCACTTTT 3′-Q-TTTTCACGTGAAAA	GTGCAC	0.7	1

[a]In each case the fluorophore (F) is FAM and the quencher (Q) is Dabcyl. [b]The increase in melting temperature (ΔT_m, °C) is produced by the addition of 100 μM of the ligands. [c]These showed biphasic melting curves.

Thiocoraline shares several common motifs with echinomycin since both molecules are bicyclic structures with C_2 symmetry that bisintercalate their chromophore moieties into DNA, and their peptidic scaffolds are rich in non-natural amino acids such as *N*-methylated residues and amino acids of D-configuration. These extremely complex molecules represent a challenge for chemical synthesis, which is mandatory when the amount of material obtained from natural sources is not enough for therapeutic applications. Recently, we reported the first solid-phase synthesis of thiocoraline by a combined approach involving chemical and enzymatic methods.[12] This synthetic scheme could be applied to obtain a library of analogues and study the structural features that are important for the DNA binding selectivity with the aim of developing a drug that interacts specifically with unique DNA sequences and alters the expression of targeted individual genes.

Our previous work has addressed some aspect of the synthesis of thiocoraline analogues with improved pharmacokinetic properties. We first reported the solid-phase synthesis of oxathiocoraline (**3**) (Figure 1), in which the thioester bridges were replaced by ester bonds; however, this approach caused a considerable loss of cytotoxic activity because of instability.[13] We later synthesized *N*Me-azathiocoraline (**2**) (NMA), in which the thiodepsi bonds, responsible for the low stability of thiocoraline in human serum, were replaced with bridged *N*-methyl amide isosteres.[14] NMA displayed similar nanomolar antitumor activity to thiocoraline and an increased stability in human serum compared to the natural parent molecule. NMA conserves the hydrogen bonding pattern of the natural product, as a result of the intrinsic rigidity of the *N*Me-amide bonds, in such a way that only one conformation is possible, as demonstrated by modeling studies. Finally, in order to overcome the low solubility of this compound, we prepared a PEGylated analogue of NMA (**4**); however, this displayed no

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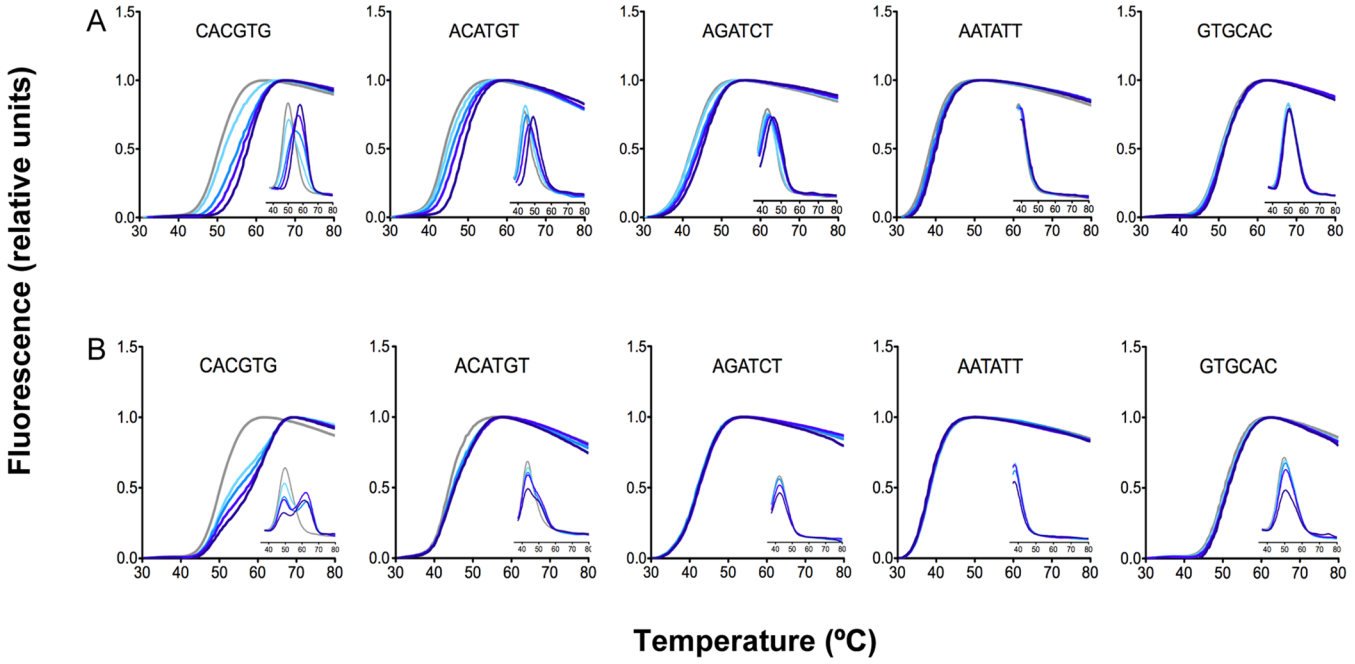

Figure 5. Fluorescence melting curves for the fluorescently labeled intermolecular duplexes. The experiments were performed in 10 mM sodium phosphate pH 7.4, containing 100 mM NaCl and 0.25 μM duplex DNA. The insets show the first derivatives of the melting profiles. The ordinate shows the relative fluorescence of the samples, which has been normalized to the same initial value. The abscissa shows the temperature in °C. (A) The curves correspond to NMA concentrations of 0, 25, 50, 100, and 200 μM increasing from left to right. (B) The curves correspond to thiocoraline concentrations of 0, 10, 25, 50, and 100 μM increasing from left to right.

biological activity,[15] most probably because the PEG groups prevent the molecule from adopting the correct conformation for bisintercalation and alter the ligand's hydrophobicity, which also contributes to the binding strength.[16]

Since *N*Me-amides proved to be excellent surrogates for thioester bonds, we examined the DNA-binding sequence selectivity of NMA using DNase I footprinting and fluorescence melting studies in order to determine whether this chemical substitution altered the binding properties when compared to thiocoraline. To demonstrate that DNA-binding properties, such as binding strength, correlate with the biological activity of the compounds, a native gel was run using *tyr*T plasmid DNA in the presence of two concentrations of the natural compound **1** and the synthetic analogues **2**, **3**, and **4**, as well as the bisintercalator echinomycin (Figure 2). A clear band shift was observed with both natural compounds and with NMA (**2**), whereas compounds **3** and **4** showed no appreciable binding to DNA under the conditions tested. Since ligands that are in rapid exchange do not always produce band shifts, footprinting experiments were performed with these compounds. Again, these experiments also showed no DNA-binding of compounds **3** or **4** (data not shown). This observation correlates with the finding that NMA was the only thiocoraline analogue among those tested that shows equal cytotoxic activity to that of the parent compound.

The sequence selectivity of NMA was examined through the use of footprinting, which is one of the most powerful techniques for the study of ligand−DNA interactions.[17] The success of this technique depends on using an appropriate DNA substrate since the binding preferred sequence of a highly selective ligand may not be present in the chosen fragment. In order to partly overcome this problem, we have previously prepared DNA fragments that contain all the 136 possible tetranucleotide sequences (MS1 and MS2)[18] and a series of fragments (HexA and HexB) that together contain all possible symmetrical hexanucleotide sequences.[19] These universal footprinting substrates were used to study the sequence selectivity of *N*Me-azathiocoraline.

Figure 3 shows the footprints produced upon digestion of the radiolabeled MS2, HexA, and HexB fragments with DNase I following incubation with 14 different concentrations of NMA, ranging from 500 to 0.1 μM. The footprints produced by this molecule were very similar, but not identical, to those of thiocoraline and echinomycin (Figure S2, Supporting Information), which means that the synthetic analogue binds selectively to CpG as well. Nonetheless, in some cases, other regions close to AT-rich sequences were also protected by NMA. It was also observed that the natural compounds bind with higher affinity than the synthetic one since footprints appeared with lower concentrations.

Quantitative analysis of the protection patterns produced by varying ligand concentrations can also provide an estimate of the binding strength and the relative affinities for different binding sites.[20] In Figure 4, we report the C_{50} values for some of the sequences that were protected by NMA from DNase I cleavage. The results confirm that NMA displays higher affinities for CpG-containing sequences, as does thiocoraline. However, several TpA steps also generated good binding sites. The best binding affinity was obtained with CGTACG to which NMA could either bind to the central TpA step or to the two outer CpG steps.[21,22]

On the basis of the footprinting results and to further explore the interaction of NMA with AT-rich sequences as well as with CpG sites, we designed five fluorescently labeled oligodeoxynucleotides (Table 1) for fluorescence melting studies.[23] These oligos contain the site of interest in the center of their sequences, one containing a CpG step, two others a central ApT with different adjacent bases, and one with TpA and GpC

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sites for comparison. The footprinting results showed that both molecules bind with high affinity to the sequence CACGTG and the best duplex stabilization for both compounds was obtained with this sequence in the melting experiments (Figure 5), producing monophasic melting curves with NMA and biphasic melting curves with the natural compound thiocoraline, thus suggesting a difference in binding kinetics. To the best of our knowledge, this is the first time that a biphasic transition has been observed with this molecule, and this suggests that the ligand is in very slow exchange with the free DNA, effectively producing noninterconverting free and bound DNA species that melt at different temperatures.[24] A biphasic curve was also observed with ACATGT bound to thiocoraline, even though it does not contain a CpG binding site. In contrast, NMA produced a simple monophasic profile with a melting temperature (T_m) of 43 °C that changed upon addition of increasing concentrations of the drug. The stabilization by NMA was much weaker for AGATCT and AATATT, for which no stabilization was observed with thiocoraline, thereby confirming that NMA can bind to AT-rich sequences, albeit with lower affinity than to CpG. The sequence GTGCAC was designed to confirm that the two drugs do not bind GpC. To validate these results, we also tested other sequence-selective intercalators, such as echinomycin, TANDEM, and actinomycin D, as controls (see Supporting Information). As expected, echinomycin showed the best binding to CACGTG sequence, while TANDEM stabilized the AATATT duplex, and actinomycin D showed the best stabilization with GTGCAC.

In summary, we have demonstrated that the mode of action of NMA is identical to that of the natural bisintercalator thiocoraline, although the DNA-binding affinity of NMA is weaker than the parent compound, probably as a consequence of the rigidification brought about by the *N*Me-amide. However, these two compounds share the same sequence selectivity toward CpG. Surprisingly, both molecules display the same low nanomolar biological activity, which could be explained by compensation of the lower affinity for CpG sites by means of additional binding to AT-rich tracts. Substitution of the thioester bonds by *N*Me-amide bridges enhances serum stability without compromising the DNA binding specificity. We conclude that this approach might be useful for the synthesis of other depsipeptide analogues.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

[Detailed experimental procedures, materials and methods, biological activity of thiocoraline analogues, and footprinting experiments with thiocoraline and echinomycin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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