*N*Me Amide as a Synthetic Surrogate for the Thioester Moiety in Thiocoraline

Judit Tulla-Puche,*[,†] Eleonora Marcucci,[†] Elisabet Prats-Alfonso,[†] Núria Bayó-Puxan,[†] and Fernando Albericio*[,†,‡,§]

Institute for Research in Biomedicine, Barcelona Science Park, 08028 Barcelona, Spain, Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain, CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, 08028 Barcelona, Spain

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Bridged *N*-methyl amides are used as isosteres for depsi and thiodepsi bonds in thiocoraline. The introduction of *N*Me-amides in bridges mimics the thioester bonds without imposing steric hindrance and allows conservation of the hydrogen bonding map of the natural product. *N*Me-azathiocoraline was constructed by solid-phase *N*-methylation of the side chain of diaminopropionic acid (Dap). The three consecutive *N*-methyl amino acids could be coupled in good yields by using HATU/HOAt/DIEA in DMF, and the final octapeptide was also obtained on solid phase following a 4 + 4 fragment coupling approach. *N*Me-azathiocoraline (NMA) displayed nanomolar activity in the same order as the natural product and the same mode of action. In fact, modeling of *N*Me-azathiocoraline bonded to a TCGA sequence showed how the methyl groups remained further away from the DNA strand without changing the recognition pattern of thiocoraline. Moreover, *N*Me-azathiocoraline displayed an increased stability in human serum as compared to the parent natural product. This approach could be used in other depsipeptides and side chain to side chain cyclic peptides.

Introduction

Many depsipeptides of natural origin show biological activities of relevance.[1] However, only a few of these compounds have entered clinical trials because of problems of bioavailability as well as low stability in plasma favored by the presence of the ester bonds. Thus, the thiodepsipeptide thiocoraline,[2] a potent marine antitumoral compound that bisintercalates to DNA, is highly unstable[3] and requires the aid of delivery systems for its administration. Another important feature of natural peptides and depsipeptides is the presence of *N*-methylated residues in their sequence. The immunosuppressive cyclic peptide cyclosporin, for instance, bears seven *N*-methyl amino acids.[4] Because the presence of *N*-methyl groups confers resistance to proteolytic cleavage,[5] the introduction of these groups has been widely used to prevent enzymatic degradation. Moreover, *N*-methylation favors the cis conformation of the peptide bond[6−8] and also introduces conformational restraints that may enhance the population of single conformations that are crucial for biological activity (bioactive conformation).[9] Therefore, *N*-methylation is increasingly used as a strategy to enhance potency,[10] permeability,[11] and receptor selectivity.[12] A way to systematically explore these changes is to perform an *N*-methyl scan, where a library of analogues is constructed by *N*-methylating at each single site. This method has been applied successfully to endothelins,[13] somatostatin,[14] and RGD peptides.[15] Recently, multiple *N*-methylation has also been introduced and proved to be advantageous.[16] The introduction of *N*-methyl amino acids in peptides has generally been performed chemically.[17] Although not all *N*-methyl amino acids are commercially available, *N*-methylated peptides can be obtained by previous methylation of the amino acid in solution or by carrying out in situ *N*-methylations on the solid support[18−20] during peptide elongation. Powerful coupling reagents are required in order to obtain acceptable purities of the *N*-methylated peptides.[21,22]

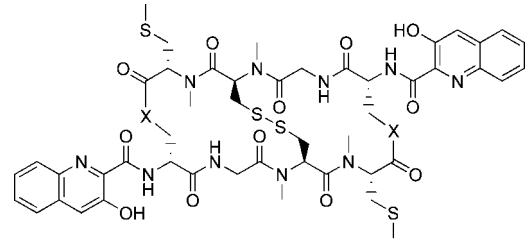

Figure 1. Structures of thiocoraline and analogues with replacements at the thioester moieties.

Up to now, *N*-methylation has been limited mostly to the backbone, but to the best of our knowledge, it has not been broadly reported as the replacement of the ester bond into a depsipeptide neither in side chain to side chain cyclic peptides.[23]

In our search for thiocoraline analogues with increased metabolic properties, we focused our attention on finding a replacement for the thioester bridges, thought to be responsible for the low stability. First of all, thioesters were substituted by amides, azathiocoraline,[24,25] and later by ester bonds, oxathiocoraline[26] (Figure 1). Nevertheless, both analogues showed a considerable loss of activity with respect to the natural product. In the case of oxathiocoraline, this loss of activity was due to the unexpected instability of the ester bonds compared to the natural thioester bridges. As for azathiocoraline, the loss of activity was attributed to the presence of the amide hydrogens, which changed the hydrogen bonding map of the molecule and therefore its binding to DNA. We reasoned that a good option to both recover activity and to increase the stability of the molecule would be to block

* To whom correspondence should be addressed. Phone: +34 93 4037088. Fax: +34 93 4037126. E-mail: albericio@pcb.ub.es (F.A.); judit.tulla@irbbarcelona.org (J.T.-P.).
† Institute for Research in Biomedicine, Barcelona Science Park.
‡ Department of Organic Chemistry, University of Barcelona.
§ CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park.

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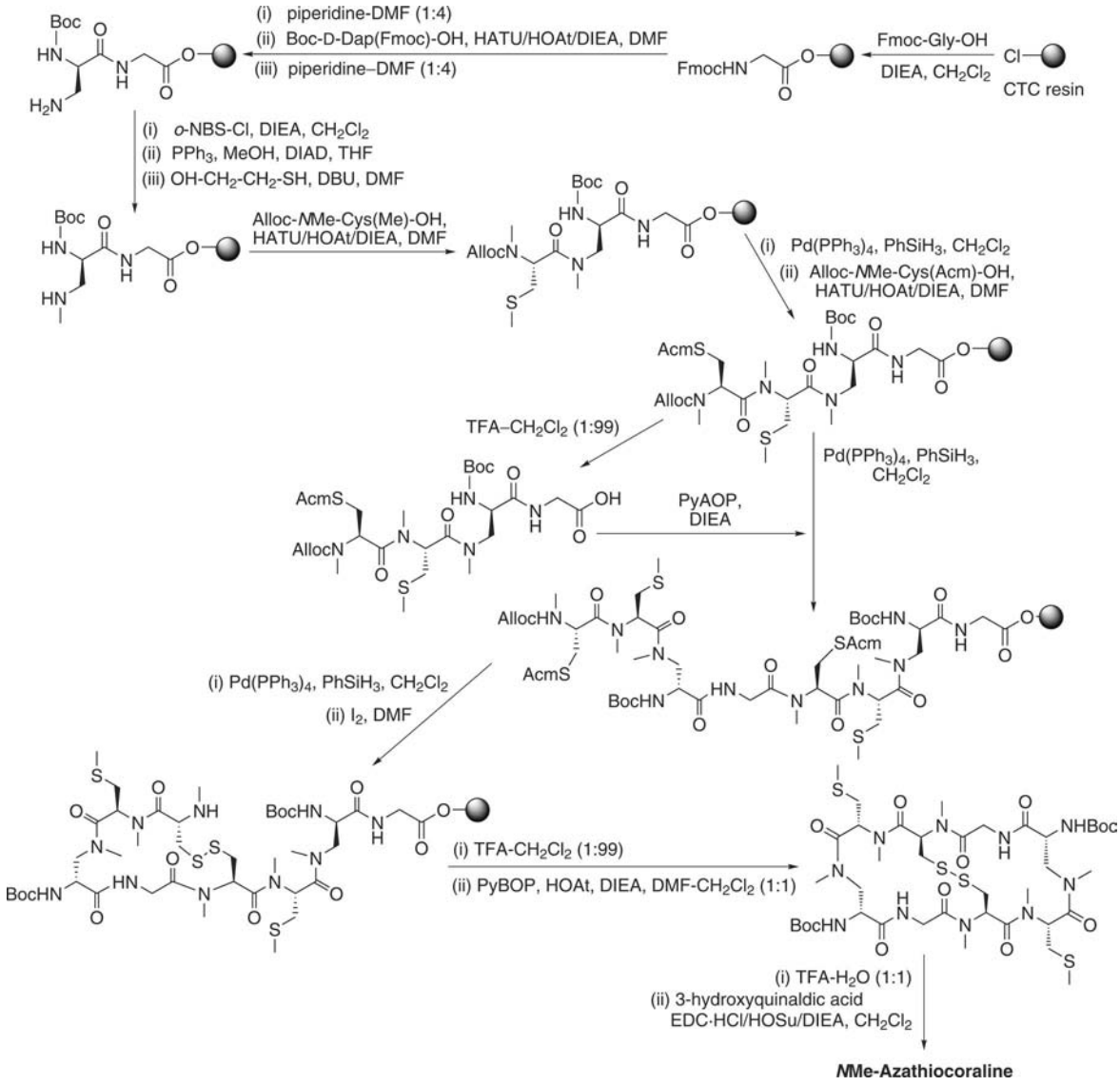
Scheme 1. Solid-Phase Synthesis of *N*Me-Azathiocoraline



hydrogen bonding by introducing methyl groups. To the best of our knowledge, this is the first time that bridged *N*-methyl amides have been used as isosteres for thiodepsi bonds.

The introduction of this extra *N*-methylation increases the synthetic challenge of this already difficult molecule because of the consecutive methylated residues,[21,27] two already on the backbone, and the third, the new *N*-methyl introduced in the side-chain of Dap[a] to form the bridge. Another concern was the rigidity of this chain upon the introduction of the *N*-methyl group on the bridge, which, together with Gly, were the only flexible points of the already highly constrained skeleton of thiocoraline. This rigidity could cause problems during cyclization.

Results and Discussion

Synthesis of *N*Me-azathiocoraline (NMA). The isostere of Cys, Dap, was used to construct the amide bridge. In order to *N*-methylate the side chain of Dap, it was thought that the best option would be on solid-phase under Mitsunobu conditions of the *o*-nosyl(*o*-NBS)-protected amine.[19,20,28] In this way, the protected α-amine would remain unaffected. One limitation of this approach is the presence of the *N*Me-Cys(Me) residue, which does not tolerate the methylation conditions (complex mixtures are obtained). Thus, the synthesis had to follow a [4 + 4] fragment coupling strategy to prevent the methylation of the second Dap by performing the solid-phase *N*-methylation before the incorporation of *N*Me-Cys(Me). In a first attempt, Boc-D-Dap(Fmoc)-OH was loaded on 2-clorotrityl chloride (2-CTC) resin[29,30] and the solid-phase *N*-methylation under Mitsunobu conditions was undertaken. After Fmoc removal with piperidine-DMF (1:4), the amino group was reprotected with the *o*-NBS group, and then the *N*-methylation was carried out

[a] Abbreviations: Dap, diaminopropionic acid; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide; HOAt, 1-hydroxy-7-azabenzotriazole(3-hydroxy-3*H*-1,2,3-triazolo-[4,5-*b*]pyridine); DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; NMA, *N*Me-azathiocoraline; *o*-NBS, *o*-nosyl; 2-CTC, chlorotrityl chloride (Barlos) resin; DIAD, diisopropyl azodicarboxylate; THF, tetrahydrofuran; PyAOP, 7-azabenzotriazol-1-yl-oxytris-(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-oxytris-(pyrrolidino) phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; EDC·HCl, 1-[3-(dimethylaminopropyl]-3-ethylcarbodiimide; HOSu, *N*-hydroxysuccinimide; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; HR-ESMS, high resolution electrospray mass spectrometry; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; ACN, acetonitrile; TCA, trichloroacetic acid.

Table 1. In Vitro Results[a]

		breast MDA-MB-231	NSCLC A-549	colon HT29
thiocoraline	GI50	3.11×10^{-9}	5.57×10^{-9}	1.21×10^{-8}
	TGI	2.07×10^{-8}	2.49×10^{-8}	2.25×10^{-8}
	LC50	2.25×10^{-7}	2.09×10^{-7}	nd
*N*Me-azathiocoraline	GI50	4.08×10^{-9}	3.39×10^{-9}	2.08×10^{-8}
	TGI	4.26×10^{-8}	2.00×10^{-8}	1.13×10^{-8}
	LC50	3.73×10^{-7}	1.65×10^{-7}	7.47×10^{-7}
oxathiocoraline	GI50	4.62×10^{-7}	3.11×10^{-7}	4.00×10^{-7}
	TGI	2.75×10^{-6}	2.75×10^{-6}	3.55×10^{-6}
	LC50	6.40×10^{-6}	7.91×10^{-6}	7.55×10^{-6}
azathiocoraline	GI50	2.14×10^{-6}	3.74×10^{-6}	3.12×10^{-6}
	TGI	$>8.90 \times 10^{-6}$	$>8.90 \times 10^{-6}$	$>8.90 \times 10^{-6}$
	LC50	$>8.90 \times 10^{-6}$	$>8.90 \times 10^{-6}$	$>8.90 \times 10^{-6}$

[a] In vitro results are reported in molar units.

using PPh_3, MeOH, and diisopropyl azodicarboxylate (DIAD) in THF.[19,20] Finally, the *o*-NBS group was removed with β-mercaptoethanol and DBU in DMF. However, the methylation of this first amino acid was accompanied by a significant loss of peptide from the resin, thereby indicating that the 2-ClTrt ester of a N^{α}-*o*-NBS-amino acid was not totally stable to the nucleophilic conditions involved in these reactions due to the electron withdrawing effect of the *o*-NBS group. However, the same ester bond was completely stable later in the synthetic process when the α-amino group was protected as an acyl group.

To overcome this problem, another starting point for the elongation was chosen. Thus, Fmoc-Gly-OH was loaded as a first amino acid, followed by Fmoc removal and coupling of Boc-D-Dap(Fmoc)-OH (Scheme 1). After performing the *N*-methylation under the same conditions as explained above, the tetrapeptide could be obtained in good purity. Coupling between consecutive *N*Me amino acids was accomplished by using HATU/HOAt/DIEA in DMF.[21]

At this point, the resin was split in two portions: in 1/3 of the resin, the Alloc group was removed, whereas protected peptide from the remaining 2/3 of the resin was cleaved, lyophilized, and coupled to the unprotected tetrapeptidyl resin using PyAOP and DIEA.[31−33] After obtaining the octapeptide, the disulfide bridge was accomplished on the resin with I_2 in DMF, and the peptide was cleaved and cyclized in solution by using PyBOP, HOAt, and DIEA, in CH_2Cl_2-DMF.[32] Finally, the Boc groups were removed using TFA-CH_2Cl_2 (1:1), and the 3-hydroxyquinaldic units introduced with the aid of EDC·HCl and HOSu to prevent overacylation of the quinaldic alcohol. The peptide was purified by reversed-phase HPLC, characterized by NMR and HR-ESMS and subjected to biological activity assays.

In Vitro Studies. The pure peptide was subjected to in vitro assays in breast, nonsmall cell lung (NSCLC), and colon cell lines. In all cases, the peptide showed nanomolar activity (Table 1) in the same order of magnitude as the natural product. Compared to the amide (azathiocoraline) and the ester (oxathiocoraline) analogues, *N*Me-azathiocoraline was 3 and 2 orders of magnitude more active, respectively. This result shows how the methyl groups effectively blocked hydrogen bonding

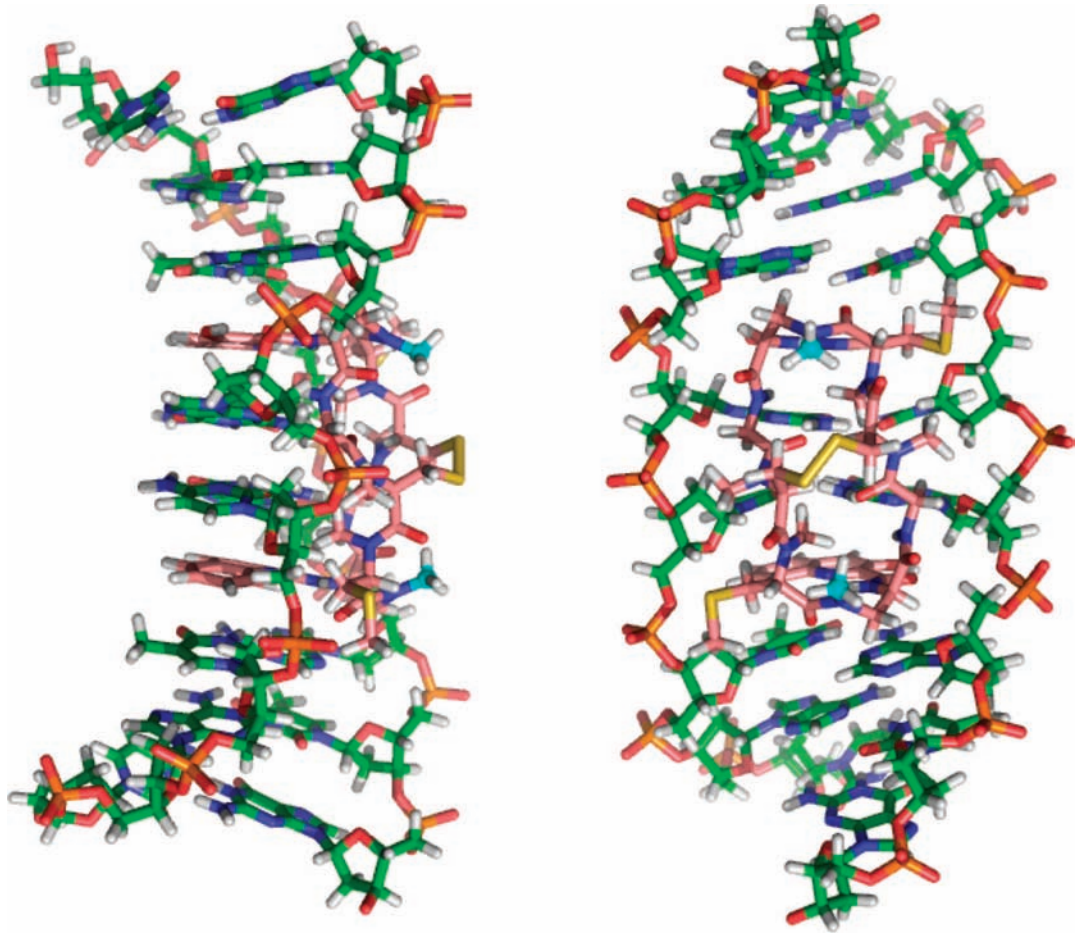


Figure 2. Molecular model of *N*Me-azathiocoraline bound to a self-complementary $(CATCGATG)_2$ octanucleotide. Side (left) and front (right) views show the notable unwinding of the double helix brought about by bis-intercalation of the hydroxyquinoline rings on both sides of the central CpG step. Carbon atoms in DNA are colored in green, whereas carbon atoms in *N*Me-azathiocoraline are colored in pink with the exception of the *N*-methyl groups (cyan), which protrude from the minor groove into the solvent.

without interfering with DNA binding. In fact, modeling of *N*Me-azathiocoraline bonded to a TCGA sequence shows how the methyl groups remained further away from the DNA strand without changing the recognition pattern of thiocoraline. There was only a slight increase in rigidity as compared to related bisintercalation complex structures, where the flexible ester bonds adopt two conformations.[34] In this case, and due to the intrinsic rigidity of the *N*Me amide bond, only one conformation is possible (Figure 2).

Stability Assays. Stability in human serum was studied for *N*Me-azathiocoraline and thiocoraline for a period of 120 h. Comparative results (see Supporting Information) showed that *N*Me-azathiocoraline is more resistant to degradation, with a half-life of 23.1 h, whereas for thiocoraline, a half-life of 14.4 h was obtained. This finding represents a major advantage of *N*Me-azathiocoraline over the natural product.

Mode of Action. To study whether *N*Me-azathiocoraline acted by bisintercalation, in the same manner as the natural product, a DNA binding assay was perfomed (see Supporting Information).[35] Comparison with thiocoraline at 1:200, 1:400, 1:800, and 1:1600 ratios showed the same pattern for both compounds: an initial decrease in mobility of the supercoiled DNA (unwinding), and a saturation point followed by a recovery in mobility at higher concentrations, which results from the rewinding of DNA. This finding indicates how the introduction of bridged *N*Me-amides does not change the mode of action of the natural product.

Conclusion

In conclusion, we have demonstrated that the introduction of *N*Me-amides in bridges mimics the thioester bonds without imposing steric hindrance. Bridged-*N*Me amides allow conservation of the hydrogen bonding map of the natural product. *N*Me-azathiocoraline displays in vitro activity in the same order as the natural product, similar behavior when interacting with DNA in the agarose gel electrophoresis and, finally, it is significantly more stable. This approach could be used to enhance stability in other depsipeptides and side chain to side chain cyclic peptides with similar problems.

Experimental Section

Boc-D-Dap(Me)-Gly-*O*-CTC-PS. CTC resin (400 mg, 1.6 mmol/g) was placed in a 10 mL polypropylene syringe fitted with 2 polyethylene filter discs. The resin was washed with DMF (5 × 1 min) and CH_2Cl_2 (3 × 1 min), and a solution of Fmoc-Gly-OH (118.8 mg, 0.4 mmol) and DIEA (474 μL, 2.66 mmol) in CH_2Cl_2 was added. After 10 min, more DIEA (237 μL, 1.33 mmol) was added and the mixture was stirred for 50 min at room temperature. The reaction was quenched by the addition of MeOH (320 μL), and the mixture was stirred for a further 10 min. After filtration, the peptide resin was washed with CH_2Cl_2 (3 × 1 min), DMF (3 × 1 min), and piperidine-DMF (1:4; 2 × 1 min, 2 × 5 min). A loading of 0.93 mmol/g was obtained, as calculated by Fmoc quantitation. Next Boc-D-Dap(Fmoc)-OH (682 mg, 1.6 mmol) was introduced with HATU (456 mg, 1.6 mmol), HOAt (218 mg, 1.6 mmol), and DIEA (570 μL, 3.2 mmol) as coupling reagents in DMF. After stirring for 35 min and filtration, the peptide resin was washed with DMF (3 × 0.5 min), CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), and the Fmoc group cleaved with piperidine-DMF (1:4; 1 × 1 min; 3 × 5 min; 1 × 10 min), piperidine-DBU-toluene-DMF (1:1:4:14; 2 × 5 min), and finally the resin was washed again with DMF (5 × 0.5 min) and CH_2Cl_2 (3 × 0.5 min). A solution of *o*-NBS-Cl (354 mg, 1.6 mmol) and DIEA (0.726 μL, 4 mmol) in CH_2Cl_2 was added to the resin and the mixture stirred for 90 min. After filtration and washings with CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), CH_2Cl_2 (3 × 0.5 min), and THF (3 × 0.5 min), a solution of PPh_3 (524 mg, 2 mmol) and MeOH (160 μL, 4 mmol) in THF, and a solution of DIAD (404 μL, 2 mmol) in THF were mixed and added to the peptide resin. After stirring the resin for 1 h, it was washed with THF (3 × 0.5 min), CH_2Cl_2 (3 × 0.5 min), and DMF (3 × 0.5 min). An aliquot was cleaved and analyzed by analytical HPLC (gradient: 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 10.0 min; 90% purity) and HPLC-ESMS (gradient: 0:100 to 100:0 (ACN/H_2O) in 15 min); *m*/*z* calculated for $C_{17}H_{24}N_4O_9S$, 460.13; found, 460.10 $[M + H]^+$. After treatments (2 × 15 min) with DBU (300 μL, 2 mmol) and 2-mercaptoethanol (280 μL, 4 mmol) in DMF, the resin was washed with DMF (3 × 0.5 min), CH_2Cl_2 (3 × 0.5 min), and DMF (3 × 0.5 min). An aliquot was cleaved and analyzed by analytical HPLC (gradient: 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 4.23 min) and HPLC-ESMS (gradient: 5:100 to 100:0 in 15 min) (ACN/H_2O); t_R = 3.87 min; *m*/*z* calculated for $C_{11}H_{21}N_3O_5$, 275.15; found, 276.73 $[M + H]^+$.

{[Alloc-*N*Me-Cys(Acm)-*N*Me-Cys(Me)&][Boc-D-Dap(Me&)-Gly-*O*-CTC-PS]}-Protected Tetrapeptide. Elongation of the peptide chain was carried out by adding Alloc-*N*Me-AA-OH in the presence of HATU (456 mg, 1.6 mmol), HOAt (218 mg, 1.6 mmol), and DIEA (570 μL, 3.2 mmol) in DMF for 35 min and, after filtration, the resin was washed with DMF (3 × 0.5 min) and CH_2Cl_2 (3 × 0.5 min). The De Clercq test was used to indicate completion of the couplings. After introducing Alloc-*N*Me-Cys(Me)-OH (373 mg 1.6 mmol), an aliquot was cleaved and analyzed by analytical HPLC (gradient: 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 10.3 min; 92% purity) and HPLC-ESMS (gradient: 5:100 to 100:0 in 15 min) (ACN/H_2O); t_R = 9.65 min; *m*/*z* calculated for $C_{20}H_{34}N_4O_8S$, 490.21; found, 491.91 $[M + H]^+$. Next, the peptide resin was treated with $Pd(PPh_3)_4$ (46 mg, 0.04 mmol) and $PhSiH_3$ (492 μL, 4 mmol) in CH_2Cl_2 (3 × 15 min) to remove the Alloc group and then washed with CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), CH_2Cl_2 (3 × 0.5 min), and DMF (3 × 0.5 min). Introduction of Alloc-*N*Me-Cys(Acm)-OH (464 mg, 1.6 mmol) in the conditions described above required a second coupling. An aliquot was cleaved and analyzed by analytical HPLC [gradient: 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 9.3 min (minor), 9.7 min (major); 80% purity] and HPLC-ESMS (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 9.7 min; *m*/*z* calculated for $C_{27}H_{46}N_6O_{10}S_2$, 678.00; found, 677.91 $[M + H^+]$).

4 + 4 Approach: {[Boc-D-Dap(Me&^{1})-Gly-*N*Me-Cys(Acm)-*N*Me-Cys(Me)&^{2}][Alloc-*N*Me-Cys(Acm)-*N*Me-Cys(Me)&^{1}][Boc-D-Dap(Me&^{2})-Gly-*O*-CTC-PS]}—Linear Protected Octapeptide. The tetrapeptidyl resin was split into 2 fractions: 1/3 of the resin was treated with $Pd(PPh_3)_4$ and $PhSiH_3$ in CH_2Cl_2 as described above in order to remove the Alloc group and analyzed by analytical HPLC (gradient: 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 6.7 min (major), 6.9 min (minor). The remaining 2/3 of the resin were cleaved with TFA-CH_2Cl_2 (1:98, 5 × 1 min), and the filtrates were collected in presence of H_2O (12 mL, 60 mL per g of resin), dried, and lyophilized.

The lyophilized tetrapeptide was coupled to the peptide resin with PyAOP (94 mg, 0.18 mmol, 2 equiv calculated on loaded peptide) and DIEA (94 μL, 0.54 mmol) in DMF. The pH was adjusted to 8 with DIEA. The mixture was stirred overnight at 25 °C. Next, an aliquot of the resin mixture was taken, washed with MeOH, and the De Clercq test was used to indicate completion of the reaction. After a positive test, the same amount of PyAOP and DIEA was added, and the mixture was stirred for a further 3 h. After a positive test, more PyAOP and DIEA were added. After 2 h, the test was negative and, after filtration, the peptide resin was washed with DMF (3 × 0.5 min), CH_2Cl_2 (3 × 0.5 min), and DMF (3 × 0.5 min). An aliquot of the resin was cleaved and analyzed by analytical HPLC (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 10.3 min) and HPLC-ESMS (*m*/*z* calculated for $C_{50}H_{86}N_{12}O_{17}S_4$, 1254.5; found, 1254.32 $[M + H^+]$).

{[Boc-D-Dap(Me&^{1})-Gly-*N*Me-Cys(&^{2})-*N*Me-Cys(Me)&^{3}][*N*Me-Cys(&^{2})-*N*Me-Cys(Me)&^{1}][Boc-D-Dap(Me&^{3})-Gly-*O*-CTC-PS]}—Disulfide Bridge Formation. The Alloc group was cleaved by treatment (3 × 15 min) with $Pd(PPh_3)_4$ (46 mg, 0.04 mmol, 0.1 equiv) and $PhSiH_3$ (292 μL, 4 mmol, 10 equiv) in CH_2Cl_2 and

washed with CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), CH_2Cl_2 (3 × 0.5 min), and DMF (3 × 0.5 min). To make the disulfide bridge, a solution of I_2 (127 mg, 0.5 mmol, 5 equiv, 2.5 equiv × Acm) in DMF (0.01 M) was added to the peptide resin. The mixture was stirred for 10 min at room temperature and, after filtration, the treatment was repeated. Next the resin was washed with DMF (3 × 0.5 min), CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), and CH_2Cl_2 (3 × 0.5 min). HPLC-ESMS analysis of a cleaved peptide aliquot indicated the completion of the reaction. The peptide cleavage was achieved by treatment with a TFA-CH_2Cl_2 solution (2:98, 5 × 1 min), and the filtrates were collected in the presence of H_2O (6 mL, 60 mL per g of resin), dried, and lyophilized. The peptide was analyzed by analytical HPLC (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 9.0 min) and HPLC-ESMS (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 7.5 min; *m*/*z* calculated for $C_{40}H_{70}N_{10}O_{13}S_4$, 1026.40; found, 1026.45 $[M + H]^+$.

{[Boc-D-Dap(Me&^{1})-Gly-*N*Me-Cys(&^{2})-*N*Me-Cys(Me)&^{3}][Boc-D-Dap(Me&^{3})-Gly-*N*Me-Cys(&^{2})-*N*Me-Cys(Me)&^{1}]—Cyclization in Solution. The cyclic peptide (0.1 mmol), dissolved in CH_2Cl_2-DMF (9:1, 100 mL, 1 mM), was added to a solution of HOAt (54 mg, 0.4 mmol) in the minimum amount possible of DMF. DIEA was added until neutral pH, and when EDC (77 mg, 0.2 mmol) was added, the cyclization reaction started. The mixture was stirred for 5 h, and HPLC-MS analysis indicated the completion of the reaction. The organic layer was washed with saturated NH_4Cl (2 × 50 mL) and brine (2 × 50 mL), dried with $MgSO_4$, and evaporated under vacuum. The peptide was analyzed by analytical HPLC (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 12.3 min) and HPLC-ESMS (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 12.2 min; *m*/*z* calculated for $C_{40}H_{68}N_{10}O_{12}S_4$, 1008.4; found, 908.49 $[M + H^+ - Boc]$, 807.45 $[M + H^+ - 2$ Boc].

{[3-HQA-D-Dap(Me&^{1})-Gly-*N*Me-Cys(&^{2})-*N*Me-Cys(Me)&^{3}][3-HQA-D-Dap(Me&^{3})-Gly-*N*Me-Cys(&^{2})-*N*Me-Cys(Me)&^{1}]–*N*Me-azathiocoraline. The bicyclical peptide was dissolved in TFA-CH_2Cl_2 (1:1, 2 mL), and the mixture was stirred for 1 h at room temperature. The solvent was evaporated under reduced pressure, and the residual acid was removed by coevaporations of toluene. H_2O was added and the product lyophilized. It was dissolved in HCl (0.001 M) and lyophilized again. The unprotected bicyclical peptide was dissolved in CH_2Cl_2 (300 μL) and DIEA until neutral pH. 3-Hydroxyquinoline-2-carboxylic acid (37 mg, 0.2 mmol) was preactivated with EDC (38 mg, 0.2 mmol) and HOSu (22 mg, 0.2 mmol) in CH_2Cl_2 (1 mL) and, after 15 min, this solution was added to the previously prepared peptide solution. The mixture was stirred for 20 h and HPLC-MS analysis indicated completion of the reaction. The organic layer was washed with saturated NH_4Cl (2 × 50 mL) and brine (2 × 50 mL), dried with $MgSO_4$, and evaporated under vacuum. The peptide was analyzed by analytical HPLC (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R= 13.2 min) and HPLC-ESMS (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 13.3 min; *m*/*z* calculated for $C_{50}H_{62}N_{12}O_{12}S_4$, found, 1150.4; $[M + H^+]$ 1149.64.

Dimer Strategy: {[*N*Me-Cys(&^{1})-*N*Me-Cys(Me)&^{2})][Boc-D-Dap-(Me&^{2})-Gly-OH]}$_2$ —Dimer. The tetrapeptide resin was treated with $Pd(PPh_3)_4$ and $PhSiH_3$ in CH_2Cl_2 in order to remove the Alloc group, as described above. The dimer formation was achieved by treatments (2 × 10 min) with a solution of I_2 (126.9 mg, 0.5 mmol) in DMF (10 mL), followed by washings with DMF (3 × 0.5 min), CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), and CH_2Cl_2 (3 × 0.5 min). HPLC-MS analysis of a cleaved peptide aliquot indicated the completion of the reaction. Next the peptide was cleaved by treatment with a TFA-CH_2Cl_2 solution (2:98, 5 × 1 min), and the filtrates were collected in presence of H_2O (6 mL, 60 mL per g of resin), dried, and lyophilized. The peptide was analyzed by analytical HPLC (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R= 7.1 min) and HPLC-ESMS (from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 6.1 min; *m*/*z* calculated for $C_{40}H_{68}N_{10}O_{12}S_4$, 1044.4; found, 1043.49 $[M + H]^+$, 522.49 $[M/2 + H]^+$.

{[Boc-D-Dap(Me&^{1})-Gly-*N*Me-Cys(&^{2})-*N*Me-Cys(Me)&^{3}][Boc-D-Dap(Me&^{3})-Gly-*N*Me-Cys(&^{2})-*N*Me-Cys(Me)&^{1}]—Cyclization Reaction. The peptide (0.05 mmol) was dissolved in CH_2Cl_2-DMF (9:1) and added to a solution of HOAt (54 mg, 0.4 mmol) in CH_2Cl_2-DMF (9:1, 50 mL, 1 mM). The addition of DIEA until pH 8 and PyBOP (208 mg, 0.4 mmol) started the reaction. The mixture was stirred for 12 h and HPLC-MS analysis indicated the completion of the reaction. The organic layer was washed with saturated NH_4Cl (2 × 50 mL) and brine (2 × 50 mL), dried with $MgSO_4$, and evaporated under vacuum. The bicyclic peptide was analyzed by analytical HPLC (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R= 12.1) and HPLC-ESMS (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 12.1 min; *m*/*z* calculated for $C_{40}H_{68}N_{10}O_{12}S_4$, 1008.40; found, 1008.89 $[M + H^+]$.

{[3-HQA-D-Dap(Me&^{1})-Gly-*N*Me-Cys(&^{2})-*N*Me-Cys(Me)&^{3}][3-HQA-D-Dap(Me&^{3})-Gly-*N*Me-Cys(&^{2})-*N*Me-Cys(Me)&^{1}]—*N*Me-azathiocoraline. Removal of the Boc groups and introduction of the heterocyclic units was performed as described earlier. Analytical HPLC (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min); t_R= 13.2) and HPLC-ESMS (gradient from 0:100 to 100:0 (ACN/H_2O) in 15 min; t_R = 13.3 min; *m*/*z* calculated for $C_{50}H_{62}N_{12}O_{12}S_4$, 1150.40; found, 1151.53 $[M + H^+]$.

Purification and Characterization of *N*Me-azathiocoraline. The crude peptides were purified by semipreparative HPLC (linear gradient from 45:55 to 60:40 (ACN/ H_2O) in 30 min; flow rate 3 mL/min) to afford the pure *N*Me-azathiocoraline in 4.5% yield, t_R = 13.6 min; 99% purity). Characterization by MALDI-TOF (*m*/*z* calculated for $C_{50}H_{62}N_{12}O_{12}S_4$, 1150.4; found, 1151.5 $[M + H]^+$; 1173.8 $[M+ Na]^+$) and HRMS *(m*/*z* calculated for $C_{50}H_{63}N_{12}O_{12}S_4$, 1151.3566 $[M + H]^+$; found, 1151.3573. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 10.29 (s, 1H, OH), 7.82 (d, 1H, J = 8.4 Hz, ar. CH), 7.62 (d, 1H, J = 7.8 Hz, ar. CH), 7.54 (s, 1H, ar. CH), 7.48 (m, 2H, ar. CH), 6.58 (d, 1H, J = 3.0 Hz, J = 9.6 Hz, NH Gly), 6.13 (dd, 1H, J = 3.0 Hz, J = 11.4 Hz, CH$^\alpha$ Cys-S), 5.66 [dd, 1H, J = 5.4 Hz, J = 9.6 Hz, CH$^\alpha$ Cys(Me)], 4.74 (dd, 1H, J = 9.6 Hz, J = 16.8 Hz CH$^\alpha$ Gly), 4.70 (m, 1H, CH$^\alpha$ Dap), 4.62 (dd, 1H, J = 3.0 Hz, J = 16.8 Hz, CH$^\beta$ Dap), 3.70 (dm, 2H, CH$^\beta$ Cys-S), 3.65 (dd, 1H, J = 3.0 Hz, J = 14.4 Hz, CH$^\beta$ Dap), 3.45 (dd, 1H, J = 3.0, J = 16.8 Hz, CH$^\alpha$ Gly), 3.04 [(m, 1H, CH$^\beta$ Cys(Me)], 3.02 (s, 3H, *N*Me Cys-S), 3.00 [(m, 1H, CH$^\beta$ Cys(Me)], 2.97 (s, 6H, *N*Me Cys(Me) and *N*Me Dap), 2.17 (s, 3H, *S*Me). ^{13}C NMR ($CDCl_3$): δ 172.6 (C═O), 171.6 (C═O), 168.0 (C═O), 167.8 (C═O), 153.3 (ar. C), 138.5 (ar. C), 138.1 (ar. C), 130.8 (ar. C), 129.8 (ar. CH), 129.4 (ar. CH), 129.4 (ar. CH), 126.7 (ar. CH), 120.9 (ar. CH), 58.0 (CH$^\alpha$ Cys), 56.4 (CH$^\alpha$ Dap), 53.1 (CH$^\alpha$ Cys), 51.7 (CH$^\beta$ Dap), 44.3 (CH$^\beta$ Cys-S), 40.4 (CH Gly), 38.1 (*N*Me Dap), 33.4 [CH$^\beta$ Cys(Me], 30.9 (*N*Me Cys-S), 29.5, [*N*Me Cys(Me)], 15.7 (*S*Me).

Cell Growth Inhibition Assay. A colorimetric assay using sulforhodamine B (SRB) was adapted to perform a quantitative measurement of cell growth and viability by following a previously described method. The cells were seeded in 96-well microtiter plates at 5 × 10^3 cells/well in aliquots of 195 μL of RPMI medium and were left to grow in drug-free medium for 18 h to allow attachment to the plate surface. Samples were then added in aliquots of 5 μL (dissolved in DMSO-H_2O, 3:7). After 72 h of exposure, the antitumor effect was measured by the SRB methodology: cells were fixed by adding 50 μL of cold 50% (wt/vol) trichloroacetic acid (TCA) and were incubated for 60 min at 4 °C. Plates were washed with deionized H_2O and dried, and 100 μL of SRB solution (0.4 wt %/vol in 1% acetic acid) was added to each microtiter well and incubated for 10 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, and the bound stain was dissolved with Tris buffer. Optical densities were read on an automated spectrophotometer plate reader at a single wavelength of 490 nm. Data analyses were automatically generated by LIMS implementation. Assays were done in a dose−response manner at 10 concentrations (from 10 μg/mL with 1:2.5 dilutions to 0.0026 μg/mL). Although concentrations were adjusted in mg/mL, GI_{50} values were calculated in molarity. All assays were run in triplicate, and the curves were automatically

adjusted with 30 points by nonlinear regression using "Prism 3.03" (GraphPad) software.

Stability Assays. Stability in human serum (from human male AB, Aldrich, Milwaukee, WI) was carried out by incubation at 37 °C of thiocoraline or *N*Me-azathiocoraline with the serum (diluted 9:1 in HBSS buffer). The peptides were used at a final concentration of 20 μM. Aliquots (50 μL) were periodically taken at 0 to 120 h, and then ACN (200 μL) was added in order to precipitate the proteins and cooled to 4 °C. After 30 min, the sample was centrifuged at 10000 rpm for 15 min, and the supernatant concentrated and analyzed by HPLC (linear gradient from 50:50 to 100:0 (ACN/H_2O) in 8 min). For the blank sample, the procedure used was the same described above, except that H_2O was used instead of human serum. The kinetics analysis was performed by plotting the ln %A from the HPLC peak versus time using the least-squares method.

Agarose Gel Electrophoresis. Stock solutions of both *N*Me-azathiocoraline (NMA) at 0.1 mg/mL and thiocoraline at 1 mg/mL were prepared in DMSO and kept at −4 °C. Subsequent solutions were prepared by diluting with Tris·HCl 10 mM pH 7.0 to working concentrations. A solution of the plasmid pBR322 (8 μL) was further diluted with Tris·HCl 10 mM at pH 7.0 (72 μL). Incubation reactions were prepared by mixing the DNA solution (10 μL) with the compounds solutions (5 μL) at 1:200, 1:400, 1:800, and 1:1600 ratios, respectively. A DNA control sample was prepared by mixing DMSO (1 μL), plasmid solution (1 μL), and Tris·HCl 10 mM at pH 7.0 (13 μL). The reactions were incubated for 1 h at 25 °C and then quenched by addition of a bromophenol blue solution (3 μL). They were injected in a 0.5% agarose gel and run at 80 V. Finally, the gel was stained with ethinium bromide and visualized on a UV transilluminator.

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Supporting Information Available: General procedures and HPLC, HPLC-ESI, MALDI-TOF, HR-ESI, agarose gel electrophoresis, and stability in human serum. This material is available free of charge via the Internet at http://pubs.acs.org.

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