# Design and Synthesis of a Nitrogen Mustard Derivative Stabilized by Apo-neocarzinostatin

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Neocarzinostatin (NCS) is an antitumor antibiotic comprising a 1:1 protein−chromophore complex and exhibits cytotoxic action through DNA cleavage via H-abstraction. Cytotoxic activity resides with the chromophore **1** alone, while the protein (apoNCS) protects and transports labile **1**. The naphthoate portion (**2**) of NCS chromophore (**1**) is important for binding to apoNCS and DNA intercalation. In this paper we describe our attempts to use apoNCS to improve the hydrolytic stability of novel bifunctional DNA alkylating agents. The nitrogen mustards, melphalan and chlorambucil, were both conjugated to **2**, and the biological activities of these conjugates were assessed. Chlorambucil did not benefit from conjugation. The melphalan conjugate (**6**) formed covalent DNA adducts at guanine bases and exhibited greater in vitro cytotoxic activity than unmodified melphalan. Fluorescence and NMR spectroscopy showed that **6** binds to apoNCS. Binding to apoNCS-protected **6** reduced the extent of hydrolysis of the conjugate. This novel approach demonstrates for the first time that an enediyne apoprotein can be used to improve the stability of substances that are of potential interest in cancer chemotherapy.

## Introduction

The nitrogen mustards, chlorambucil and melphalan, produce interstrand cross-links by DNA alkylation and are widely used in chemotherapy. However, the efficiency of these agents is limited by deactivation through reaction with nucleophiles such as water, proteins, and thiols and further through low affinity for DNA. Attempts to address the latter deficiency have met with some success: conjugation to DNA targeting moieties (binders and intercalators) produces agents with increased affinity for DNA in vitro, though the effectiveness of these species in vivo is varied.[1] To our knowledge, no attempts have been made to address the lability of these compounds. Herein, we describe a novel strategy for the improvement of the stability of this class of chemotherapeutic agent.

Neocarzinostatin (NCS), an antitumor antibiotic isolated from *Streptomyces carzinostaticus*, comprises a highly reactive enediyne chromophore **1** (Figure 1) noncovalently bound to an 11 kDa protein (apoNCS).[2−4] NCS has found clinical application in Japan against leukemia and cancers of the bladder, stomach, pancreas, liver, and brain.[5] The protein component provides essential protection for the labile chromophore from heat, UV light, and attack by nucleophiles and is reported to transport **1** into cells.[6] The protein alone is not cytotoxic. The cytotoxic activity results from cycloaromatization of the enediyne ring of **1**, producing a diradical species that targets DNA.[7] Minor groove binding and intercalation of the naphthoate moiety **2** positions the diradical species to facilitate hydrogen abstraction from the deoxyribose sugar backbone, leading to single and double stranded DNA cleavage.[8,9] Naphthoate **2** is also an important element in the binding of the chromophore to the apo-protein; we have demonstrated that simple NCS analogues that contain the naphthoate **2** bind within the apoNCS binding site.[10]

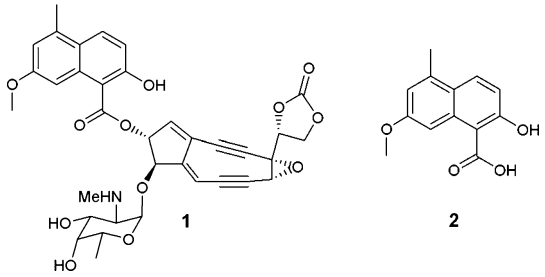


**Figure 1.**

Some of the deficiencies of the nitrogen mustards might be remedied by conjugation to **2.** First, noncovalent binding of such a conjugate to apoNCS has the potential to protect the reactive nitrogen mustard and to improve transport in vivo; second, the introduction of an intercalating motif may improve the affinity for DNA. The possible use of the protein as a stabilizer and transporter for reactive small molecules is significant, and while there are potential difficulties in using protein-based drug delivery systems, the clinical use of NCS indicates that such approaches may hold promise and warrant further investigation. We report the syn-

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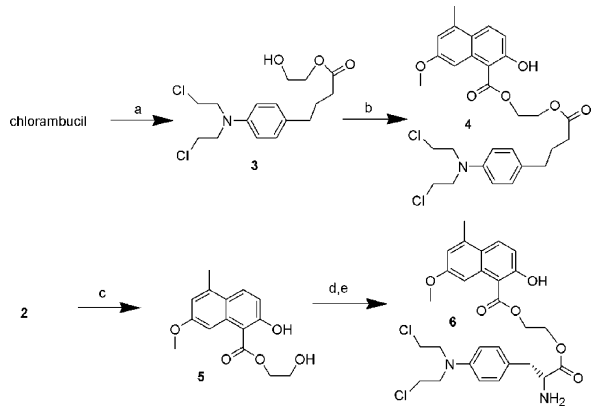
**Scheme 1.** Synthesis of Compounds **4** and **6**[a]




[a] Reagents and conditions: (a) chlorambucil (1.0 equiv), ethylene glycol (10 equiv), DCC (1.1 equiv), DMAP (0.1 equiv), $CH_2Cl_2$, 0 °C, 16 h, 96%; (b) **2** (1.0 equiv), DCC (1.1 equiv), $CH_2Cl_2$:THF (1:1), 0 °C, 16 h, 82%. (c) DCC (1.1 equiv), ethylene glycol (10 equiv), $CH_2Cl_2$:THF (1:1), 0 °C, 16 h, 91%. (d) DCC (1.1 equiv), *N*-Boc melphalan (1.0 equiv), $CH_2Cl_2$:THF (1:1), 0 °C, 16 h, 41%. (e) TFA:$CH_2Cl_2$ (1:1), 0 °C, 45 min, 99%.

thesis of naphthoate conjugates of chlorambucil and melphalan and demonstrate that the inherent in vitro cytotoxicity of the latter is improved. Furthermore, we evaluate their binding to apoNCS and demonstrate that such binding improves the stability toward hydrolysis.

## Results and Discussion

**Chemistry.** Conjugation of chlorambucil or melphalan to the NCS naphthoate **2** was achieved through sequential dicyclohexyl carbodiimide (DCC) coupling reactions utilizing ethylene glycol as a short flexible linker.[11] Coupling of chlorambucil gave **3** and the subsequent coupling of **2** produced **4** in high yield, Scheme 1. Prior to coupling the amino group of melphalan was protected as the *N*-*tert*-butyloxycarbonyl (*N*-Boc) derivative by treatment with di-*tert*-butyl dicarbonate $(Boc)_2O$.[12] Convergent coupling of *N*-Boc melphalan to the ester **5** was found to be higher yielding than the linear approach. Deprotection of the coupled product under acidic conditions gave the desired product **6** in high yield. After purification all compounds were protected from light and stored at −20 °C to prevent degradation.

**In Vitro Evaluation of Biological Activity.** Nitrogen mustards form covalent adducts with DNA via formation of a transient aziridinium species that attack the nucleophilic $N^7$ position of guanine nucleotides.[1] The ability of the naphthoate conjugates **4** and **6** to form covalent DNA adducts was measured using the *Taq* polymerase stop assay,[13] Figure 2. Both conjugates produced DNA adducts, with the level of damage **4** ≈ chlorambucil < melphalan < **6**. The position of DNA damage observed was similar for all compounds, strongly suggesting that DNA alkylation at the $N^7$ position of guanine nucleotides occurs.[13] No significant alkylation of adenine nucleotides was detected, demonstrating that, unlike some intercalators,[14] the naphthoate did not alter the intrinsic sequence selectivity of the nitrogen mustards. Conjugation of **2** increased the level of DNA alkylation exhibited by melphalan but did not alter that of chlorambucil, indicating that the presence of **2** alone is insufficient to produce effective intercalation. This observation is consistent with the work of Sartorius et al. who concluded that a positively charged side chain is required for intercalation of naphthalene derivatives;[15] which is present in **6** but not **4**.

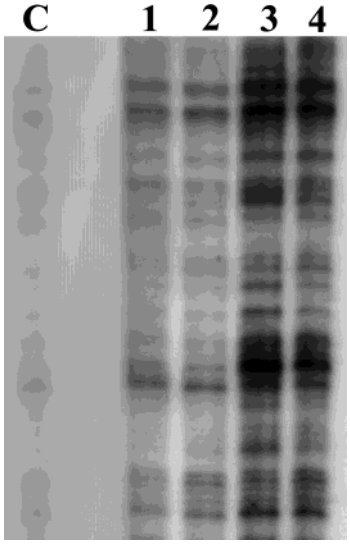



**Figure 2.** *Taq* polymerase stop assay. C: negative control; lane 1: **4** (2.5 $\mu$M); lane 2: chlorambucil (2.5 $\mu$M); lane 3: **6** (2.5 $\mu$M); lane 4: melphalan (2.5 $\mu$M).

**Table 1.** Cytotoxicity and Binding Affinity of **4**, **6**, Melphalan, and Chlorambucil

| compds | cytotoxicity,[a] $IC_{50}$, $\mu$M | $K_d$ (apoNCS) |
|---|---|---|
| melphalan | 30 ± 10 | —[c] |
| **6** | 7 ± 4 | 47 ± 5 $\mu$M |
| chlorambucil | 95 ± 25 | —[c] |
| **4** | 200 ± 50[b] | >1 mM[d] |

[a] Average of three experiments. [b] Estimated by extrapolation. [c] Insufficient change in fluorescence. [d] Binding too weak for accurate measurement.

The in vitro cytotoxic activity of the compounds against the human leukemia cell line K562 was measured using the MTT assay, Table 1.[16] The ability of the compounds to inhibit cell growth correlated well with their DNA damaging properties. The potency of **4** was reduced compared to chlorambucil, while **6** had increased potency compared to the parent compound melphalan. The reduced potency of **4** may reflect the increased hydrophobicity of the compound.

**Binding to apoNCS.** The ability of the naphthoate conjugates to bind to apoNCS was examined using fluorescence quenching titration experiments.[10] The binding of the chlorambucil derivative **4** was weak and did not allow a dissociation binding constant ($K_d$) to be determined accurately. However, the melphalan derivative **6** showed significant binding to apoNCS, Table 1. The lack of binding for **4** was surprising given that we have previously demonstrated that conjugation of **2** is sufficient to sequester other small molecules within the apoNCS binding site.[10] The alkyl chain of **4** is one carbon longer than that of **6**, which may in part account for the difference in binding through increased flexibility of the linker region or unfavorable steric hindrance in the binding site. In addition, **6** possesses an amino group six bonds from the naphthoate carbonyl; curiously, the corresponding carbonyl and methlyamino groups of **1** have the same separation. In **6**, but not **4**, this may allow favorable interaction between the positively charged

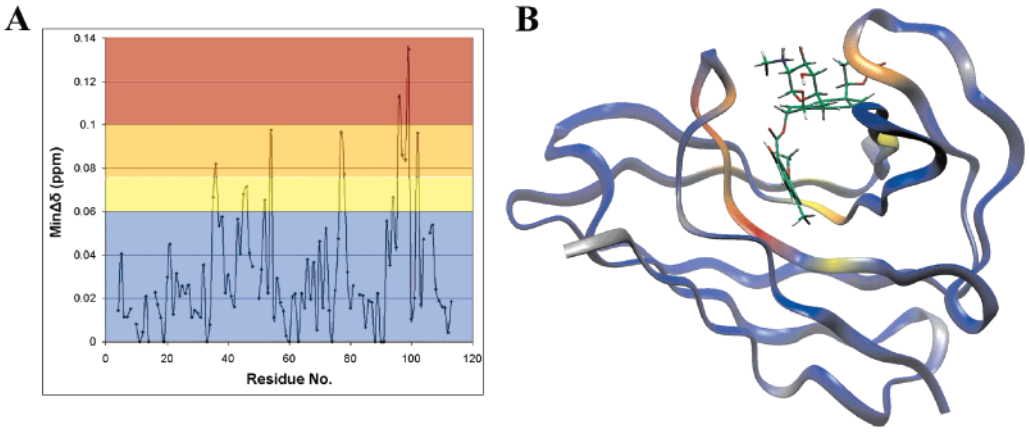



**Figure 3.** (A) Backbone NH Min$\Delta\delta$ analysis versus residue number; (B) Backbone NH Min$\Delta\delta$ mapped onto apoNCS.[17] The coloring corresponds to four groups: >0.1 ppm: 'highly shifted' as red, 0.1−0.075 ppm: 'moderately shifted' as orange, 0.075−0.06 ppm: 'slightly shifted: as yellow, <0.06 ppm: 'unshifted' as blue, and unassigned residues in gray The position of NCS chromophore is shown to aid identification of the binding site.

amino group and the aromatic ring of Phe 78 analogous to an interaction observed in the crystal structure of holo-NCS.[17] The nitrogen mustards alone, which were anticipated not to bind apoNCS, showed negligible change in fluorescence in the presence of apoNCS.

The location at which **6** bound to apoNCS was examined by following the perturbation of chemical shift of backbone amides upon complex formation using $^{1}H,^{15}N$ HSQC NMR spectroscopy.[18,19] In the absence of large change in the tertiary conformation of the protein, residues that display perturbed chemical shifts upon binding can be interpreted as being close to the position at which binding occurs. The location of chemical shift perturbations was visualized by using the minimum change in chemical shift (Min$\Delta\delta$) values of backbone amide resonances to color a ribbon representation of NCS, Figure 3. The interactions were clearly localized to the apoNCS binding cleft, consistent with those naphthoate conjugates that we have previously shown to occupy the binding site.[10] Residues with high and moderate shifts were found in the $\beta$-strands that form the base and two sides of the binding site, which are in close contact with the naphthoate moiety in the natural holo-complex.[17] Residues of the loops surrounding the apoNCS binding site also showed slight to moderate shifts, including the Phe 78 resonance, which showed a moderate shift. The perturbation of Phe 78 may reflect the postulated charged-aromatic interaction, as the benzyl side chain is known to alter conformation upon binding of the natural chromophore.[20]

Binding to apoNCS provides essential protection for its natural and labile chromophore **1** from inactivation by heat, UV light, and attack by nucleophiles.[21] We postulated that the labile nitrogen mustard conjugate **6** might be afforded similar protection from hydrolysis when bound to apoNCS. Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to follow the hydrolysis of **6** in the presence and absence of apoNCS, Figure 4. The half-life ($t_{1/2}$) of **6** changed from 3.5 ± 0.5 h to 8.1 ± 1.5 h in the presence of 1 equiv of apoNCS, a greater than 2-fold reduction in the extent of hydrolysis. No effect on the extent of hydrolysis of **4** was observed in the presence or absence of apoNCS (data not shown). These data demonstrate that **6** binds apoNCS in an orientation and with a lifetime sufficient

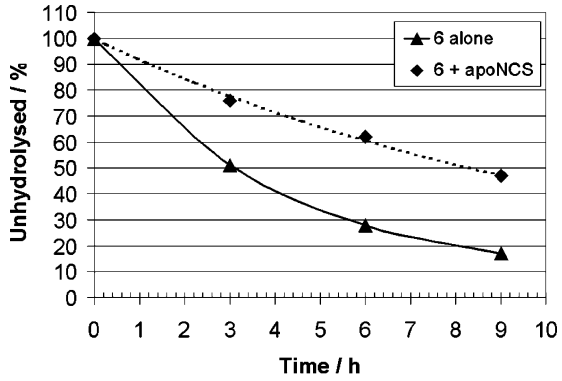



**Figure 4.** Hydrolysis of **6** in the presence and absence of apoNCS. Representative time course experiment shown.

to protect it from solvent, producing measurable benefit on the longevity of the small molecule. The level of protection achieved will depend on the dissociation constant $K_d$ of the complex; as $K_d$ decreases, the amount of time that the drug is in free solution, and hence unprotected, will decrease. However, the location, specific orientation and hence the burial of the labile portion of the drug within the apoNCS binding site will also strongly affect the level of protection.

We tested the ability of apoNCS to improve the in vitro cytotoxicity of **4** and **6**. Coincubation of **4** or **6** with apoNCS did not alter their $IC_{50}$ against the cultured human leukemia cell line K562 (data not shown). The former result reflects the lack of interaction between **4** and apoNCS. The latter result is consistent with the results of Goldberg and co-workers,[21,22] who have extensively studied the mechanism of action of NCS. They demonstrated that, despite the extreme lability of the chromophore,[21] the chromophore alone is as active as the holo-complex against cultured HeLa cells.[22] However, the holo-complex is essential for in vivo activity.[5]

## Conclusions

Previously, we have shown that simple NCS analogues that contain the naphthoate **2** bind within the apoNCS binding site.[10] Here, we have demonstrated that drug−naphthoate conjugates may retain the ability to bind within the apoNCS binding site, and that such binding provides protection from hydrolysis. We have

also demonstrated that reactive small molecule drugs conjugated to **2** can retain their inherent cytotoxic activity. Indeed, for the nitrogen mustard melphalan the conjugation of **2**, a known DNA intercalator, improved the level of DNA damage and in vitro cytotoxicity. However, for chlorambucil the inverse was observed, supporting suggestions that the naphthoate **2** is a poor intercalator in the absence of a positively charged side chain.[8,15]

These data represent the first evidence that binding to apoNCS may confer the similar benefits for small-molecule conjugates as reported for binding of the natural chromophore **1**. However, as binding is only moderate (micromolar) in comparison to the natural ligand (nanomolar), an improvement in $K_d$ for the drug−naphthoate complexes can be expected to produce an improvement in the beneficial effects obtained by such binding. Protein engineering offers the potential to modulate the affinity of binding of a given small molecule to the protein by altering residues within the apoNCS binding site. Indeed, Heyd et al. recently selected proteins from a phage display library of apoNCS binding site variants that showed improved binding to an unnatural ligand (testosterone).[23]

In the natural holo-complex, apoNCS is believed to provide a transport function for the natural chromophore; both apo- and holo-NCS enter mammalian cells and penetrate at least as far as the nuclear membrane.[6] Furthermore, conjugation of poly(styrene-*co*-maleic acid anhydride) to apoNCS produces the pharmaceutical SMANCS, which displays improved tumor uptake and toxicological profile.[24] Thus, other small molecules bound to apoNCS, such as **6**, may also benefit from improved targeting in vivo.

## Experimental Section

**General.** Glassware was oven dried at 160 °C. Solvents quoted as dry were freshly distilled; all other chemicals used were obtained from commercial sources and used without further purification. Identical samples of 2-hydroxy-5-methyl-7-methoxy-naphthoic acid **2** were obtained following the procedures of Takahashi et al.[25,26] or Rucker et al.[27] *N*-Boc-melphalan was synthesized as described previously.[12] Neocarzinostatin apo-protein (apoNCS) was expressed from the pCANTAB−(NCS) vector in *E. coli* (HB2151), with uniform $^{15}$N-labeling achieved using M9 minimal media containing $^{15}NH_4Cl$ as the sole nitrogen source.[10]

Nuclear magnetic resonance NMR spectra for small molecules were recorded on a Bruker DMX 300 MHz spectrometer at ambient temperature and are reported as chemical shifts in parts per million referenced against the undeuterated solvent peak. $^{13}$C NMR and DEPT were run on the same machine at 75 MHz and referenced against the solvent peak. IR samples were recorded on a Perkin-Elmer FT-IR 298 spectrophotometer as thin films on NaCl plates. Accurate mass values are within 5 ppm of the calculated value.

Reversed phase high performance liquid chromatography (RP-HPLC) was conducted on a Jasco system composed of two PU-980 pumps, a UV-970M multiwavelength detector, and a Hypersil HyPurity Elite C18 column (150 × 4.6 mm, 3 μm, ThermoQuest). A solvent gradient was produced by varying the proportion of two buffers; buffer A (5.0% v/v aqueous MeCN, 0.1% v/v $CF_3CO_2H$) and buffer B (95% v/v MeCN, 5.0% v/v $H_2O$, 0.085% v/v $CF_3CO_2H$). Separations were performed using a flow rate of 1.0 mL/min with a solvent gradient of 20% B for 2.5 min, 20−80% B linear gradient in 30 min, 80−100% B linear gradient in 2.5 min and 100−20% B linear gradient in 2.5 min. The eluant was monitored simultaneously at wavelengths of 210, 280, and 330 nm.

**Chemistry. 4-{4-[Bis-(2-chloro-ethyl)-amino]-phenyl}-butyric Acid 2-Hydroxy-ethyl Ester (3).** Chlorambucil (100 mg, 0.33 mmol, 1.0 equiv) was added to a stirred solution of ethylene glycol (184 μL, 3.3 mmol, 10 equiv), DMAP (20 mg, 0.165 mmol, 0.5 equiv), and DCC (75 mg, 0.36 mmol, 1.1 equiv) in dry $CH_2Cl_2$ (25 mL) under $N_2$ at 0 °C, and the resulting solution was stirred for 16 h. The reaction mixture was diluted with hexane (20 mL) and dried over $Na_2SO_4$ and the solvent removed in vacuo. Purification by column chromatography (40:1 $CH_2Cl_2$:MeOH) gave **3** as a viscous oil (0.11 g, 0.32 mmol, 96%).

**2-Hydroxy-7-methoxy-5-methyl-naphthalene-1-carboxylic Acid 2-(4-{4-[Bis-(2-chloro-ethyl)-amino]-phenyl}-butyryloxy)-ethyl Ester (4).** DCC (50 mg, 0.24 mmol, 1.1 equiv) was added to a stirred solution of **2** (51 mg, 0.22 mmol, 1.0 equiv) and **3** (75 mg, 0.22 mmol, 1.0 equiv) in dry $CH_2Cl_2$:THF (1:1, 10 mL) under $N_2$ at 0 °C and the resulting solution was stirred for 16 h. The reaction mixture was washed with brine (3 × 50 mL), extracted with $CH_2Cl_2$ (3 × 100 mL), dried over $MgSO_4$ and the solvent removed in vacuo. Purification by column chromatography (3:1 PE:EtOAc) gave **4** as a viscous oil (0.10 g, 0.18 mmol, 82%). RP-HPLC >95% unhydrolyzed.

**2-Hydroxy-7-methoxy-5-methyl-naphthalene-1-carboxylic Acid 2-Hydroxy-ethyl Ester (5).** DCC (73 mg, 0.36 mmol, 1.1 equiv) was added to a stirred solution of **2** (75 mg, 0.32 mmol, 1.0 equiv) and ethylene glycol (0.2 mL, 3.6 mmol, 11 equiv) in dry $CH_2Cl_2$:THF (1:1, 15 mL) under $N_2$ at 0 °C, and the resulting solution was stirred for 16 h. The reaction mixture was warmed to room temperature, and the solvent was removed in vacuo. The residue was dissolved in ether (20 mL), passed though a pad of Celite, and dried over $MgSO_4$ and the solvent removed in vacuo. Purification by column chromatography (4:1 PE:EtOAc) gave **5** as white solid (79 mg, 0.29 mmol, 91%).

**Preparation of 2-Hydroxy-7-methoxy-5-methyl-naphthalene-1-carboxylic Acid 2-(3-{4-[Bis-(2-chloro-ethyl)-amino]-phenyl}-2-*tert*-butoxycarbonylamino-propionyloxy)-ethyl Ester (5′).** DCC (62 mg, 0.29 mmol, 1.1 equiv) was added to a stirred solution of **5** (75 mg, 0.27 mmol, 1.0 equiv) and *N*-Boc melphalan (106 mg, 0.27 mmol, 1.0 equiv) in dry $CH_2Cl_2$ (15 mL) under $N_2$ at 0 °C, and the resulting solution was stirred for 16 h. The reaction mixture was warmed to room temperature, hexane (50 mL) added, and the suspension filtered to remove excess urea. The resulting solution washed with brine and dried over $MgSO_4$ and the solvent removed in vacuo. Purification by column chromatography (4:1 P:EtOAc) gave **5′** as an oil (79 mg, 0.11 mmol, 41%).

**Preparation of 2-Hydroxy-7-methoxy-5-methyl-naphthalene-1-carboxylic Acid 2-(2-Amino-3-{4-[bis-(2-chloro-ethyl)-amino]-phenyl}-propionyloxy)-ethyl Ester (6).** TFA (10 mL, excess) was added to a solution of **5′** (59 mg, 89 μmol) in dry $CH_2Cl_2$ (10 mL) at 0 °C under $N_2$ and the reaction stirred for 1 h. The TFA was quenched with $NaHCO_3$, extracted with $CH_2Cl_2$, and dried over $MgSO_4$ and the solvent removed in vacuo. Purification by column chromatography (40:1 $CH_2Cl_2$:MeOH) gave **6** as an oil (50 mg, 89 μmol, 99%). RP-HPLC >95% unhydrolyzed.

***Taq* Polymerase Stop Assay.** *Taq* polymerase stop assays were conducted in a manner analogous to that described previously.[13] Template DNA (pUC18 *Hind III* digest, 0.5 μg) in FP buffer (50 mM KCl, 20 mM HEPES, 1 mM DTT, 1 mM $MgCl_2$, 0.5 mM EDTA) and 10% v/v DMSO was reacted with test compound for 2.5 h at room temperature and then precipitated twice with ethanol and vacuum-dried. The synthetic oligonucleotide primer PUC1 (5′-CTC ACT CAA AGG CGG TAA TAC-3′) was 5′-end labeled with [$\gamma$-$^{32}$P] ATP using T4 polynucleotide kinase and purified using a sephadex column. Linear PCR (94 °C 3′, 30 cycles of {94 °C 1′, 60 °C 1′,72 °C 1′}, 72 °C 4′, 4 °C 5′) of the damaged template (0.5 μg) with labeled PUC1 primer (0.5 ng) was performed in a thermal cycler using *Taq* DNA polymerase (10 units) in a final volume of 100 μL of reaction buffer (200 μM each dNTP, 50 mM KCl, 10 mM Tris·HCl pH 9.0, 1.5 mM $MgCl_2$, 1% Triton X-100). The PCR product was precipitated twice with ethanol

and vacuum-dried, and fragments were separated on a 6% polyacrylamide gel (7 M urea, Tris-boric acid-EDTA buffer) for approximately 3 h at 3000 V, 55 °C. Gels were transferred to filter paper and dried and autoradiographs obtained using film.

**Cytotoxicity Studies.** Human leukemia cell line K562 was grown in RPMI 1640 supplemented with 10% heat-inactivated foetal bovine serum and 2 mM L-glutamine without antibiotic at 37 °C, 5% $CO_2$, 95% air, 100% relative humidity. 200 $\mu$L of K562 cells ($5 \times 10^4$ cells/mL) were aliquoted into a 96-well microtiter plate and incubated for 1 h with the test compound (100 $\mu$M to 30 nM). After being washed with culture medium, the cells were incubated for a further 4 days before the number of viable cells was counted using the MTT assay.[16]

**Fluorescence Spectroscopy.** Fluorescence spectra were recorded on a Varian Cary Eclipse Fluorescence spectrophotometer with a Peltier temperature control device set to maintain the sample temperature at 25 ± 0.5 °C. Fluorescence quenching titration experiments were conducted using the constant dilution method described by Kondo et al.,[28] using 5 $\mu$M of naphthoate conjugates **4** or **6** with 0−150 $\mu$M of apoNCS or 10 $\mu$M apoNCS with 0−300 $\mu$M of melphalan or chlorambucil in 100 mM sodium acetate buffer pH 5.0 with 10% v/v MeOH. Dissociation constants ($K_d$) were calculated as the average value of three separate titration experiments analyzed using both a linear (eq 1) and a nonlinear method (eq 2).[28,29]

$$\frac{1}{\Delta F} = \frac{K_d}{\alpha[S_T]} = \frac{1}{[L_T]} + \frac{1}{\alpha[S_T]} \quad (1)$$

$$\Delta F = \Delta F_{max.} \frac{[L_T] - [S_T]\cdot{}^{\Delta F}/_{\Delta F_{Max}}}{K_d + [L_T] - [S_T]\cdot{}^{\Delta F}/_{\Delta F_{Max}}} \quad (2)$$

where $[S_T]$ and $[L_T]$ are the total concentration of substrate and ligand respectively, $\alpha$ is a constant, $\Delta F$ is the change in fluorescence, and $\Delta F_{max.}$ is the change in fluorescence at saturation.

**Protein Nuclear Magnetic Resonance Spectroscopy.** Protein nuclear magnetic resonance (NMR) spectroscopy was performed as described previously.[10] The perturbation of backbone amide chemical shifts was calculated relative to apoNCS recorded in 10% v/v MeOH-$d_4$ using the minimum chemical shift procedure, eq 3.[18,19]

$$\min\Delta\delta = \min\{({}^{HN}\Delta_{ppm})^2 + ({}^{N}\Delta_{ppm}{*}\alpha_N)^2\}^{1/2} \quad (3)$$

where ${}^{HN}\Delta_{ppm}$ and ${}^{N}\Delta_{ppm}$ are the $^1H_{HN}$ and $^{15}N_{NH}$ chemical shift changes and $\alpha_N$ is a scaling factor to account for the difference in spectral width of backbone $^{15}N$ relative to $^1H$. A value of $\alpha_N = 1/7$ was used for all residues except glycine ($\alpha_N = 1/5$).

**Hydrolysis Experiments.** Hydrolysis of the nitrogen mustard derivative **6** was conducted at 35 °C using 500 $\mu$M of **6** in 50 mM phosphate pH 5.0 with 10% v/v THF in the presence or absence of 500 $\mu$M apoNCS. Aliquots were withdrawn at appropriate times and analyzed immediately by RP-HPLC. The percentage unhydrolyzed **6** was calculated as the ratio of the unhydrolyzed peak to all species present, normalized to give 100% at the start of the experiment.

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**Supporting Information Available:** Spectral data for compounds **2**−**5**, **5′**, **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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