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Synthetic replacement of the methylamino group of neocarzinostatin chromophore with hydroxyl prohibits thiol activation in organic solvents and diminishes the rate and efficiency of thiol-promoted DNA cleavage in water

Andrew G. Myers,* Jun Liang and Marlys Hammond

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA

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Abstract

A synthesis of the neocarzinostatin chromophore analog **2**, in which the 2′-*N*-methylamino group of the natural product has been replaced by a hydroxyl group, is described. Thiol addition experiments in organic solvents and DNA-cleavage studies in aqueous solution are also described, and provide further support for the proposed participation of the carbohydrate methylamino group in neocarzinostatin activation.

Neocarzinostatin (neocarzinostatin protein–chromophore complex, holo-NCS) is the prototypical member of the chromoprotein enediyne antitumor agents and is also the first of the class to be characterized.[1,2] It consists of a highly unstable 'enediyne' chromophore component **1** noncovalently complexed to a carrier protein (apo-NCS). In vitro experiments have shown that the isolated chromophore component induces the cleavage of double-stranded DNA in the presence of a thiol co-factor.[3] The proposed mechanism of activation of the chromophore[4] involves thiol (thiolate) addition to C12 followed by biradical-forming cycloaromatization and hydrogen-atom abstraction from the deoxyribose backbone of DNA and is now supported by a large body of evidence.[5] It has been proposed that the 2′-*N*-methylamino group of the chromophore aminoglycoside residue facilitates the thiol addition reaction by functioning as an internal base.[6] This proposal is supported by thiol addition experiments with three chromophore analogs, *N*-nitroso neocarzinostatin chromophore **3**,[6] the synthetic analog **4**[6] and, most recently, neocarzinostatin chromophore aglycone **5**.[7] In addition, the three-dimensional structure of the neocarzinostatin protein–chromophore complex[8] shows the bound chromophore to adopt a conformation in which the *N*-methylamino group is favorably disposed for its proposed role in promoting nucleophilic addition to C12. Such a conformation had been proposed for **1** in solution,[2c] prior to the X-ray structure

* Corresponding author. Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA.

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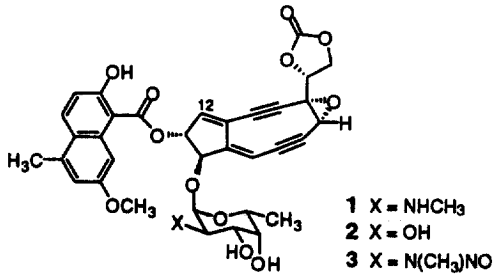
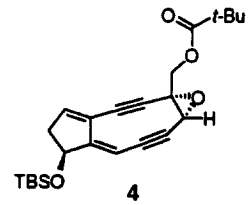
determination of the protein–chromophore complex, on the basis of well-established conformational features of carbohydrates (*exo*-anomeric effect).[9]





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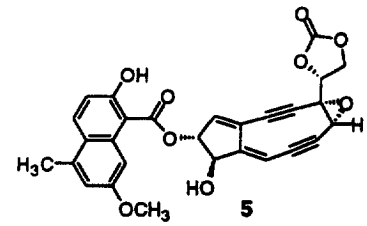

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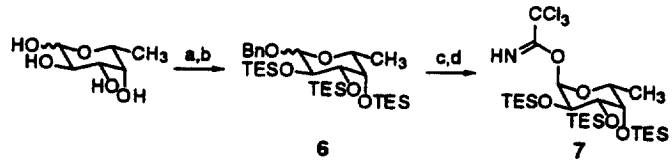
As part of a program to develop a convergent and enantioselective synthetic route to **1**[10] and analogs, we have undertaken, and report herein, glycosylation studies of neocarzinostatin chromophore aglycone **5** leading to the synthesis of the analog **2** in which the 2′-*N*-methylamino group of the natural product has been replaced by a hydroxyl group. Thiol addition experiments and DNA-cleavage studies with **2** are also described, providing the most compelling evidence to date for the proposed role of the carbohydrate amino group in neocarzinostatin activation.

Stability considerations governed the choice of conditions for the glycosylation reaction as well as the selection of protective groups for the carbohydrate residue. The Schmidt, or trichloroacetimidate, method[11] was chosen for the coupling reaction and triethylsilyl ethers were chosen to protect the hydroxyl groups of the D-fucose residue, after control experiments confirmed that **1** was stable under conditions that would lead to their removal. The tris-triethylsilyl-protected α-fucosyl trichloroacetimidate **7** was prepared from D-fucose by a four-step sequence (Scheme 1). Thus, treatment of D-fucose with acetyl chloride (0.5 equiv.) in benzyl alcohol[12] at 23°C afforded a mixture (inconsequential) of α- and β-glycosides (2.6:1, respectively, 81% yield after chromatography) which was silylated with triethylsilyl triflate (6 equiv.) in pyridine at 0°C (97% yield). Attempts to remove the anomeric benzyl ether by hydrogenolysis in protic solvents such as methanol or ethanol led to competitive desilylation.[13] By conducting the hydrogenolysis in freshly distilled ethyl acetate as solvent desilylation was suppressed, and the desired protected fucose derivative bearing a free anomeric hydroxyl group was obtained (94% yield). Trichloroacetimidate formation with trichloroacetonitrile (20 equiv.) and sodium hydride (1 equiv.) in dichloromethane at 0°C then provided the α-imidate **7** exclusively, in 86% yield after purification by flash column chromatography on silica gel deactivated with triethylamine.



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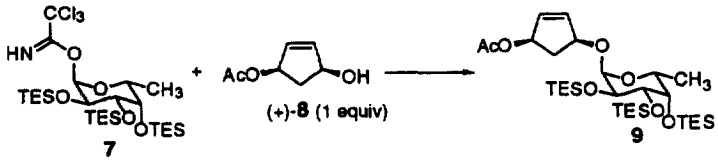
Scheme 1. [a](a) AcCl, BnOH, 23°C, 81%; (b) TESOTf, pyridine, CH_2Cl_2, 0°C, 97%; (c) H_2, 20% $Pd(OH)_2/C$, EtOAc, 94%; (d) Cl_3CCN, NaH, CH_2Cl_2, 0°C, 86%

Model glycosylation experiments with the cyclopentenol **8** as substrate established that the trichloroacetimidate **7** was a highly reactive glycosyl donor. For example, glycosylation of **8** with **7** (1 equiv.) in toluene at –20°C employing triflic acid as a catalyst (0.04 equiv.) led to complete glycosylation within 10 min, albeit with poor α-selectivity (~1:1, Table 1, entry 1). When an excess of the imidate (3 equiv.) was employed in the coupling reaction, the ratio of α:β anomers was improved (Table 1, entry 2). Using trimethylsilyl triflate (0.01 equiv.) to promote the coupling reaction and ether as the solvent, the ratio was

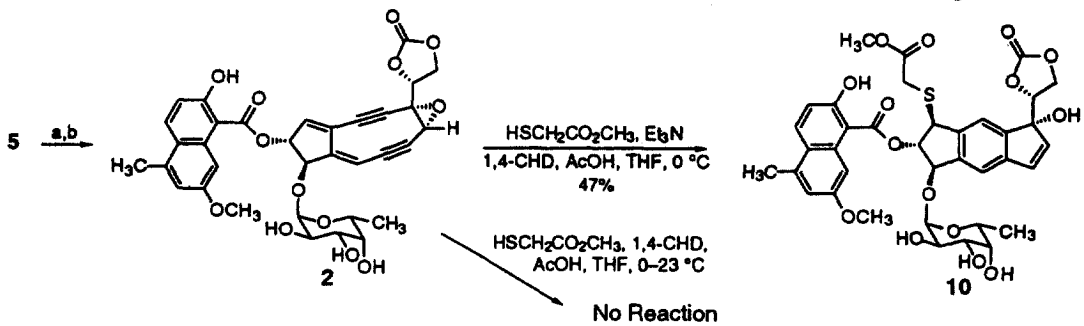
Table 1
Glycosylation of the model compound (+)-8



Entry	7	Conditions	Ratio α : β	Yield (%)
1	1.0 equiv	TfOH (0.04 equiv), toluene, –20 °C	1.1	72
2	3.0 equiv	TfOH (0.01 equiv), toluene, –20 °C	2.3	89
3	3.0 equiv	TMSOTf (0.01 equiv), Et_2O, –20 °C	7.3	88
4	3.0 equiv	TMSOTf (0.01 equiv), Et_2O, –30 °C	11.0	95

further improved (entry 3). When the latter coupling was conducted at a lower temperature (–30°C, entry 4) optimum results were obtained, providing an α:β ratio of 11:1 with 95% efficiency.

Application of the conditions found optimal above to the coupling of **7** and neocarzinostatin chromophore aglycone **5** provided the desired α-glycoside ($J_{1',2'}$=3.6 Hz) as the sole product in 63% yield after purification by flash column chromatography. The coupled product proved to be more stable than the starting aglycone, but decomposition nevertheless occurred, even when the product was stored at –80°C in deoxygenated solvents. Typically, this intermediate was not stored, but was immediately subjected to desilylation with 5% HF in acetonitrile at –30°C. Complete deprotection occurred within 1–1.5 h. The analog **2** was obtained in 67% yield after purification by flash column chromatography on silica gel. The stability of the analog **2** was found to be comparable to that of neocarzinostatin chromophore **1**.



(a) **7** (10 equiv), TMSOTf (0.1 equiv), Et_2O, 3A MS, –30 °C, 63%. (b) 5% HF, CH_3CN, –30 °C, 67%.

With substrate **2** in hand, thiol addition experiments in the mixed organic solvent AcOH:THF (1:9) were conducted, employing a large excess of methyl thioglycolate (MTG, 30 equiv.) and 1,4-cyclohexadiene as a hydrogen-atom donor. These conditions were identical to those of earlier experiments[5a] where the chromophore **1** was shown to react with MTG at –70°C. The analog **2** was found to be unreactive in this medium, even after 30 min at 23°C (^{1}H NMR analysis); however, addition of triethylamine (30 equiv.) led to rapid formation of the thiol adduct **10** at 0°C (<2 min, 47% yield, calcd for $C_{37}H_{38}NaO_{15}S$ $[M+Na]^+$, 777.1829; found, 777.1826). The stereochemistry of the adduct **10** was assigned on the basis of ^{1}H–^{1}H coupling constants, which closely matched those of C12 adducts previously characterized.[2c,6,7] This data fully conforms with prior thiol addition experiments using the analogs **3–5** and provides a compelling case for the participation of the glycosyl amino group in thiol activation of **1**.

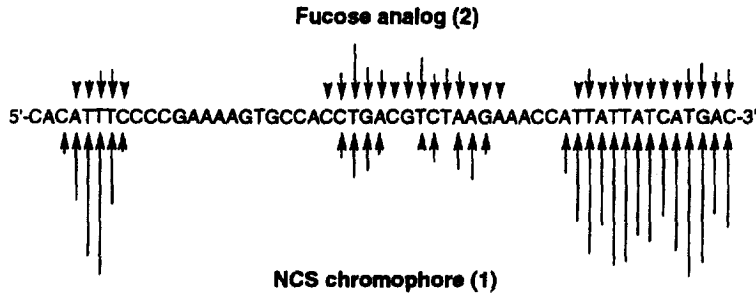


Figure 1. Histogram of DNA cleavage, upper: **2** (0.05 mM), calf thymus DNA (1 mM), 3′-^{32}P labeled DNA (see text, 50 kcpm), NaCl (20 mM), Tris–HCl (50 mM, final pH 7.5), MTG (3 mM), at 2°C; lower: **1** at 2.5-fold lower concentration (0.02 mM). DNA cleavage is normalized to the concentration of cleaving agent. After 30 min, each reaction was quenched; DNA cleavage products were assayed by denaturing 8% polyacrylamide gel electrophoresis using storage phosphor autoradiography for quantitative analysis

Thiol activation of the analog **2** was also examined in aqueous solution, in the presence of 3′-^{32}P-labeled double-stranded DNA (193-bp *Eco*RI/*Ssp*I restriction fragment from plasmid pBR322) using MTG or glutathione (GSH) as the nucleophile. DNA cleavage was observed with both thiols, but was greatly attenuated relative to cleavage by **1** (Fig. 1). Kinetic profiles for DNA cleavage by **1** and **2** were also obtained by gel analysis at various time-points over the course of reaction and showed that the rate of DNA cleavage by **2** with both thiols was slower than the corresponding reactions with **1** (~4.6-fold and 3.3-fold slower with MTG and GSH, respectively, under the pseudo-first-order conditions of 60-fold excess of thiol).

Analysis of the DNA damage pattern produced by **2** showed that damage occurred primarily at thymine and adenine residues, but no clear-cut sequence selectivity was evident. These observations parallel results with **1**[14] and **5**,[7] lending further support to our previous conclusion that the carbohydrate residue and, more specifically, the carbohydrate amino group, is not a primary determinant of the selectivity of DNA cleavage by NCS chromophore. It is also noteworthy that the addition of 1.8 equiv. of purified apoprotein prior to the thiol activation reaction effectively abolished DNA cleavage at 2°C by the analog **2**.[15] We believe that this is a consequence of tight binding of apo-NCS with **2** resulting in protection of **2** from thiol activation. Further evidence for the binding of apo-NCS with **2** was obtained by electrospray mass spectrometry, where an ion corresponding to the mass for the 1:1 complex was observed (calcd for [apo-NCS+**2**], 11743; found, 11743.9).

Acknowledgements

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15. Similar results are observed with neocarzinostatin chromophore **1**; cleavage in the presence of apo-NCS requires extended reaction ($\geq$12 h) at 37°C due to tight binding to apo-NCS.