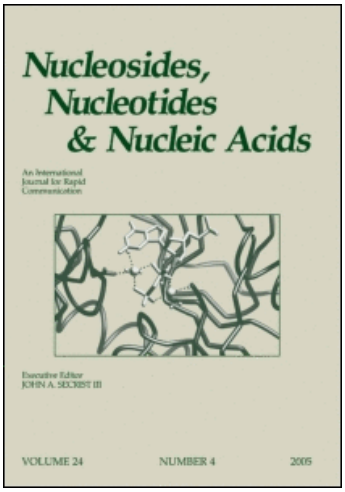

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The Synthesis, Fluorescence and Antiviral Studies of 3'-Amino-2',3'-Dideoxythymidine/substituted 10-Cyano-9-isothiocyanatoanthracene Adducts

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THE SYNTHESIS, FLUORESCENCE AND ANTIVIRAL STUDIES OF 3'-AMINO-2',3'-DIDEOXYTHYMIDINE/SUBSTITUTED 10-CYANO-9-ISOTHIOCYANATOANTHRACENE ADDUCTS

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ABSTRACT - Adducts of 3'-amino-2',3'-dideoxythymidine and various methoxy-substituted 10-cyano-9-isothiocyanatoanthracenes were prepared for use as fluorescent-tagged molecular probes. The thymidine/anthracene adducts were subjected to antiviral assays to determine if the adducts possessed antiviral activity.

There is increasing interest in oligonucleotides (ca. 20 to 30 mers) for use as oligonucleotide probes and antisense inhibitors useful in oligonucleotide therapeutics.[1,2,3,4,5,6] Fluorescent oligonucleotides have recently received wide attention as probes for nucleic acid-protein interactions[7], DNA sequencing[8,9], and nucleic acid hybridization.[10,11,12] Methods used to prepare these oligomers include automated synthesis of oligonucleotides containing additional reactive functions which are used to attach fluorescent markers. As many of the popular labeling agents are designed for reactions with an amino group, aminonucleotides or nucleotides containing a linker arm with a terminal amino group are most often used.[13,14,15,16] The amino group can then nucleophilically attack the reactive site of the fluorescent dye. One of the most commonly used reactive groups used to conjugate fluorescent tags to amino groups is the isothiocyanate group.

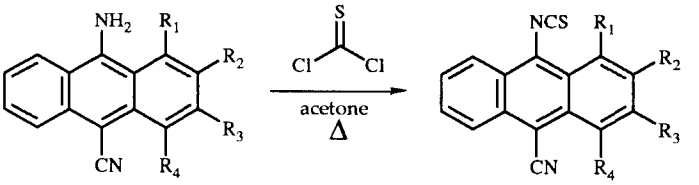
Our laboratory reported previously[17] the synthesis of some novel substituted aminoanthracenes resulting from the reaction of halomethoxybenzenes with α-cyano-o-

tolunitrile via aryne intermediates which had very high extinction coefficients and large fluorescent emission values. We then sought ways in which these compounds could be used to label molecules of biological importance. Since isothiocyanate groups are commonly used to conjugate reporter groups to biomolecules we decided to convert the aminoanthracenes into anthracene isothiocyanates. Thus aminoanthracenes **1a-d** were cleanly converted to anthracene isothiocyanates **2a-d** in good yield by refluxing with excess thiophosgene in acetone, as shown in **Figure 1**.[17]

The resulting isothiocyanates show fluorescence emission intensities which exceed those of dansyl chloride. This trend remained constant even after serial dilution of all solutions to 10^{-8}M.[18]

The major drawback faced when trying to find applications for these compounds was their insolubility in water, which precluded their use in standard protocols for labeling proteins and antibodies. However, since the synthesis of DNA oligomers is done in acetonitrile for 5'-tagged phosphoramidite monomers and the 3'-tagged monomers can be chemically converted to the triphosphate, we decided to show that the anthracene isothiocyanates could be coupled to aminonucleosides and that the fluorescence emission intensities would be comparable to both the free tag and to dansyl chloride. In our ongoing work to develop fluorescent-tagged antisense DNA, we have found it efficacious to prepare pre-tagged nucleoside monomers which can be site-specifically incorporated into DNA oligomers. We decided to attach the tag compounds to 3'-amino-3'-deoxythymidine, which could eventually be used to label the ends of oligonucleotides. The following sequence was used to prepare 3'-amino-3'-deoxythymidine: 1) 5'-Tritylthymidine was prepared by the method of Munson[19], recrystallized from toluene, and dried in a freeze drier. 2) 2,3'-Anhydro-5'-tritylthymidine was prepared by the method of Agyei-Aye et. al.[20] 3) 5'-Trityl-3'-azido-3'-deoxythymidine was prepared by the method of Glinski et. al.[21] 4) The trityl group was removed by refluxing the compound in 80% acetic acid for 30 minutes, removing the solvent under reduced pressure and triturating the solid residue with ethyl ether to give 3'-azido-3'-deoxythymidine. 5) 3'-Amino-3'-deoxythymidine was prepared by the method of Mungall et. al.[22] except that the product was recovered in pure form from the aqueous layer by freeze drying.

Once the aminothymidine was at hand, it was conjugated to the anthracene isothiocyanates **2a-c** by combining the two in absolute ethanol and heating to gentle boil for a brief period of time, allowing the solution to cool and stand overnight at room temperature, and precipitating the adducts by addition of an equal volume of water. This gave the conjugate adducts **MF1**, **MF2**, and **MF3** shown in **Figure 2** in fairly good yield.



1 2

1,2	substituent	yield
a	$R_1 = OCH_3$; R_2, R_3, R_4 = H	81%
b	R_1, $R_4 = OCH_3$; R_2, R_3 = H	81%
c	R_1, $R_2 = OCH_3$; R_3, R_4 = H	85%
d	R_1, R_2, $R_3 = OCH_3$; R_4 = H	82%

Figure 1. Synthesis of anthracene isothiocyanates from the corresponding 9-amino compounds.

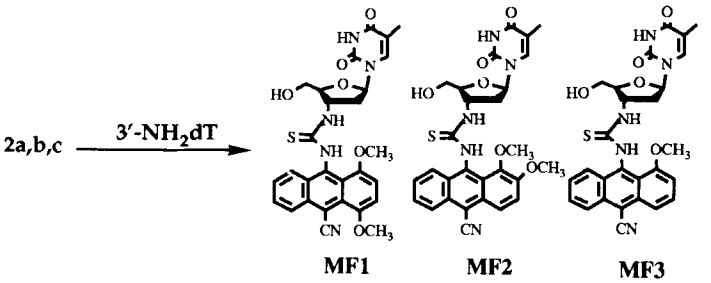


Figure 2. Labeling of 3'-aminothymidine with anthracene isothiocyanates

The conjugation of the anthracene isothiocyanates to the aminothymidines results in fluorescence intensities comparable to the free isothiocyanates and higher than that of dansyl chloride, as shown in **Table 1.**

Since there are several examples of 3'-substituted thymidines that show moderate to appreciable antiviral activity, we decided to subject compounds MF1-3 to several antiviral assays. The compounds were evaluated for activity against HIV-1, HIV-2, vesicular stomatitis virus, Coxsackie virus B4, polio virus-1, parainfluenza-3 virus, reovirus-1, Sinbis virus, Semliki forest virus, herpes simplex virus-1 (KOS, TK$^-$ B2006, TK$^-$ VMW1837), herpes simplex virus-2 (G), and vaccinia virus; they showed no appreciable activity. The cytotoxicity of the compounds in human T-lymphocyte (MT4 and CEM), HeLa, Vero and E_6SM cell cultures is shown in **Table 2.**

Table 1. Fluorescence data for compounds MF1-3 relative to dansyl chloride.[a]

Compound	Emission λ_{max}	Intensity
MF1	565 nm	51.2
MF2	564 nm	89.1
MF3	514 nm	84.6
dansyl chloride	515 nm	2.8

a- all solutions 10^{-7}M in dichloromethane. Exitation wavelength 366 nm.

Table 2. Cytotoxicity of compounds MF1-3 in various cell lines.

Cmpnd	CC_{50}[a] (μg/mL)		Minimum Cytotoxic Concentration[b] (μg/mL)		
	MT-4	CEM/0	HeLa	Vero	E_6SM
MF1	7.10	7.63	≥ 40	≥ 10	40
MF2	5.12	13.9	≥ 200	≥ 40	40
MF3	52.5	38.5	≥ 200	≥ 40	≥10

a- 50% cytotoxic concentration or concentration required to reduce MT-4 or CEM cell viability by 50%.
b- required to cause a microscopically detectable alteration of normal cell morphology.

The conversion of the 3'-tagged thymidines to the respective triphosphates, their incorporation into model anti-sense oligomers and *in vivo* fluorescence studies will be reported in a later paper.

Experimental:

General methods: Melting points were obtained on a Melt-Temp apparatus and are uncorrected. Fluorescence data was obtained using a Perkin-Elmer LS-5B luminescence spectrometer. ^{1}H and ^{13}C NMR data were obtained at 200.132 and 50.327 MHz, respectively, on a Bruker 200 MHz NMR equipped with an Aspect 2000 computer and using d6-DMSO as the solvent.; all chemical shifts reported relative to TMS. Mass spectral data (FAB) were obtained by the Midwest Center for Mass Spectrometry using a 3-NBA matrix. Solvents were obtained from commercial sources and used without further purification.

General Procedure for the Preparation of Compounds MF1, MF2 and MF3: Into a 50 mL Erlenmeyer flask were placed 3'-amino-3'-deoxythymidine (70 mg; 0.3 mmol) and absolute ethanol (20 mL). The flask was warmed slightly to facilitate dissolution. To the resulting solution was added the anthracene isothiocyanate (75 mg; 0.23 mmol). A capillary tube was placed into the flask to prevent bumping and the dark red suspension heated to a gentle boil for 20 minutes. Ethanol (20 mL) was added and heating continued until the volume of the solution was reduced by half. The total heating time was 45 minutes. During this time the deep red suspension changed to a deep orange solution. The solution was then cooled to room temperature and allowed to stand overnight in the hood. Water (20 mL) was then added to precipitate out the product. The suspension was then filtered and the product allowed to air-dry.

3'-N(1,4-Dimethoxy-10-cyano-9-aminoanthracene-9-N-thiocarbonyl)-amino-3'-deoxythymidine (MF1) rust red powder (yield 73%), mp 172-175°C. Mass spec (FAB): expected, 562.1760 (M+H); found, 562.1751 (M+H). ^{1}H NMR: 7.35-7.82 (m, 4H, Ar and H-6), 6.74 (d, 1H, J = 7.1 Hz, Ar), 6.53 (d, 1H, J = 7.1 Hz, Ar), 6.13 (t, 1H, J = 6.8 Hz, H-1'), 5.11 (s, 1H, OH-5'), 4.18 (m, 1H, H-3'), 3.99 (s, 3H, OCH_3),3.97 (s, 3H, OCH_3), 3.72 (m, 1H, H-4'), 3.61 (dd, 1H, H-5'), 3.50 (dd, 1H, H-5'), 2.13 (m, 2H, H2'), 1.77 (s, 3H, CH_3).

^{13}C NMR: 163.61, 150.72, 150.34, 147.30, 135.95, 133.57, 129.58, 129.30, 127.14, 126.70, 126.59, 125.83, 124.29, 123.57, 117.89, 109.35, 107.30, 105.21, 105.15, 100.16, 87.57, 83.64, 75.13, 61.66, 56.81, 56.26, 56.11, 37.13, 12.18 ppm.

3'-N(1,2-Dimethoxy-10-cyano-9-aminoanthracene-9-N-thiocarbonyl)-amino-3'-deoxythymidine (MF2) dark red powder (yield 79%), mp 158-160° C. Mass spec (FAB): expected, 562.1760 (M+H); found, 562.1756 (M+H). ^{1}H NMR: 7.24-7.90 (m, 7H, Ar and H-6), 6.13 (t, 1H, J = 6.8 Hz, H-1'), 5.10 (s, 1H, OH-5'), 4.17 (m, 1H, H-3'), 4.03 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 3.74 (m, 1H, H-4'), 3.62 (dd, 1H, H-5'), 3.58 (dd, 1H, H-5'), 2.15 (m, 2H, H2'), 1.77 (s, 3H, CH_3).

^{13}C NMR: 163.58, 155.48, 150.96, 150.31, 149.52, 138.69, 133.92, 133.64, 131.64, 129.34, 123.59, 123.52, 122.35, 117.95, 115.71, 109.25, 105.84, 98.63, 85.25, 84.79, 83.54, 61.19, 56.89, 12.13 ppm.

3'-N(1-Methoxy-10-cyano-9-aminoanthracene-9-N-thiocarbonyl)-amino-3'-deoxythymidine (MF3) rust orange solid (yield 75%), mp 177-179° C. Mass spec (FAB): expected, 532.1655 (M+H); found, 532.1658 (M+H). ^{1}H NMR: 7.4-7.9 (m, 8H, Ar and H-6), 6.[illegible]1 (t, 1H, J = 6.8 Hz, H-1'), 5.07 (s, 1H, OH-5'), 4.15 (m, 1H, H-3'), 4.05 (s, 3H, OCH_3), 3.71 (m, 1H, H-4'), 3.64 (dd, 1H, H-5'), 3.53 (dd, 1H, H-5'), 2.12 (m, 2H, H2'), 1.78 (s, 3H, CH_3).

^{13}C NMR: 163.64, 150.71, 150.37, 147.29, 135.97, 133.56, 129.61, 129.29, 127.16, 126.69, 126.62, 125.81, 124.33, 123.55, 117.91, 109.39, 107.33, 105.24, 105.12, 100.19, 87.58, 83.62, 75.12, 61.63, 56.87, 56.11, 37.33, 12.19 ppm.

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