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Incorporation of a fluorescent guanosine analog into oligonucleotides and its application to a real time assay for the HIV-1 integrase 3′-processing reaction

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ABSTRACT

We have synthesized a highly fluorescent (quantum yield 0.88) guanosine analog, (3-methyl-8-(2-deoxy-β-D-ribofuranosyl) isoxanthopterin (3-MI) in a dimethoxytrityl, phosphoramidite protected form, which can be site-specifically inserted into oligonucleotides through a 3′,5′-phosphodiester linkage using an automated DNA synthesizer. Fluorescence is partially quenched within an oligonucleotide and the degree of quench is a function of the fluorophore's proximity to purines and its position in the oligonucleotide. As an example of the potential utility of this class of fluorophores, we developed a continuous assay for HIV-1 integrase 3′-processing reaction by incorporating 3-MI at the cleavage site in a double-stranded oligonucleotide identical to the U5 terminal sequence of the HIV genome. Integrase cleaves the 3′-terminal dinucleotide containing the fluorophore, resulting in an increase in fluorescence which can be monitored on a spectrofluorometer. Substitution of the fluorophore for guanosine at the cleavage site does not inhibit integrase activity. This assay is specific for the 3′-processing reaction. The change in fluorescence intensity is linear over time and proportional to the rate of the reaction. This assay demonstrates the potential utility of this new class of fluorophore for continuous monitoring of protein/DNA interactions.

INTRODUCTION

Fluorescence is an important tool for studying nucleic acids and protein/DNA interactions. Fluorophores have a high specific activity, are sensitive to changes in their immediate environment and reflect these changes through measurable differences in fluorescence properties. Oligonucleotide probes containing fluorophores are increasingly used to detect gene expression *in situ* (1) and in quantitative analysis of DNA products from polymerase chain reaction assays (2). Protein/DNA interactions (3) and DNA hybridization (4) can also be studied with this technique. Commonly used fluorophores like fluorescein are often linked to the termini of an oligonucleotide, attached to nucleotides prior to incorporation into the oligonucleotide or attached to the internucleotidic phosphorus residues of oligonucleotides containing phosphorothioate diesters (5). Fluorescent nucleotide analogs have been described and their potential utility in studying protein/DNA interactions has been investigated (6–8). A lumazine (pteridine) fluorophore has been incorporated into DNA as a nucleotide analog and studied as part of an energy transfer system with a ruthenium complex as the energy acceptor (9–10).

We have developed a new highly fluorescent pteridine nucleotide analog which can be incorporated into an oligonucleotide strand through a 3′,5′-phosphodiester linkage and by synthesizing it as a phosphoramidite, it can be site-specifically inserted into an oligonucleotide using an automated DNA synthesizer. An assay for the HIV-1 integrase using this fluorescent guanosine analog was developed and serves to demonstrate the unique properties of this new class of fluorophores.

Integrase is a retroviral encoded protein responsible for the integration of viral DNA into the host cell's genome, a critical step in the life cycle of a retrovirus, like HIV. This protein functions in a step-wise manner. First, the termini of the linear HIV DNA are prepared for insertion by cleavage of a dinucleotide from both of the 3′-ends (3′-processing reaction). The host DNA is cleaved non-specifically and the recessed 3′-ends of the HIV DNA are ligated to the 5′-ends of the host DNA. The 3′-ends of the host DNA and the 5′-ends of the viral DNA are believed to be ligated and repaired by host mechanisms (11–13). Although the site of insertion into host DNA apparently is not sequence-dependent, integrase requires a specific base sequence in the termini of the HIV DNA for the 3′-processing and insertion steps to occur (14–16).

The integrase catalyzed 3′-processing and strand transfer reactions have been studied *in vitro* using recombinantly produced

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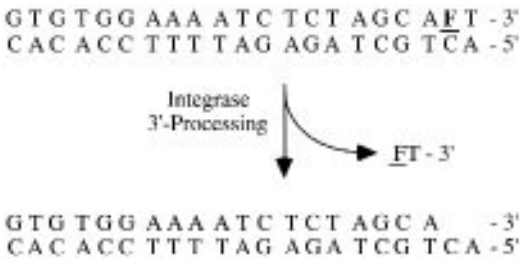


Figure 1. Integrase 3′-processing reaction with 3-MI-containing oligonucleotide substrate. The endonucleolytic action of integrase on the double-stranded oligonucleotide releases the 3-MI-containing 3′-terminal dinucleotide from the strand. 3-MI is denoted as F.

protein and short double-stranded oligonucleotides (21mers) with sequences identical to either the U5 or U3 terminus of HIV-1 DNA as substrates (17–22). Typically, the substrate is ^{32}P-labeled and the reaction products are subjected to polyacrylamide gel electrophoresis and autoradiography to detect cleavage (19mer) or strand transfer (≥30mer) products. This method does not allow continuous monitoring of the reaction in real time and only a limited number of discrete time points during the reaction can be analyzed. This method has been adapted for use in microtiter plates (19–22) which is potentially more useful for screening for inhibitors of the 3′-processing or the strand transfer reactions. However, because these techniques only provide data at single time points, they still are not as well suited as continuous assays for detailed mechanistic studies of 3′-processing.

This manuscript describes a new real time fluorescence assay for the HIV-1 integrase 3′-processing reaction, demonstrating the potential utility of this new class of fluorophore. The assay employs the fluorescent pteridine nucleoside analog of 3-methyl-8-(2-deoxy-β-D-ribofuranosyl) isoxanthopterin (3-MI) which is site-specifically inserted into the double stranded oligonucleotide substrate. Cleavage of the 3′-terminal dinucleotide containing 3-MI results in a measurable change in fluorescence intensity. Figure 1 shows a schematic of the integrase cleavage step with release of 3-MI (F) from the oligonucleotide.

MATERIALS AND METHODS

Synthesis of the phosphoramidite form of 3-MI is described in detail below and the final steps are outlined in Scheme 1.

Synthesis of 3-methyl-8-(2-deoxy-5-O-dimethoxytrityl-β-D-ribofuranosyl) isoxanthopterin-3′-O-(β-cyanoethyl, N-diisopropyl) phosphoramidite) (Scheme 1)

Step 1. 6-Amino-2-methylthio-4(3H)-pyrimidinone (36). Twenty-five grams of pulverized 6-amino-2-thioxodihydro-4(3H)-pyrimidinone (Aldrich, Milwaukee) was added to 100 ml of a 10% solution of NaOH and then 25 g of technical dimethylsulphate was added to this solution in small portions while stirring. Water was added as necessary when the resulting precipitate became too thick to permit thorough mixing. The mixture was left standing at room temperature for 15 min until it gave an acid reaction and the precipitate was then filtered by suction. This methylthiopyrimidine was placed in a flask while still moist and dissolved in 200 ml of 95% ethanol by heating to the boiling point of the ethanol. The flask was then cooled and allowed to stand at room temperature for 1 h and the precipitate was collected by filtering. Twenty to 25 g of product (75–90% yield) was obtained.

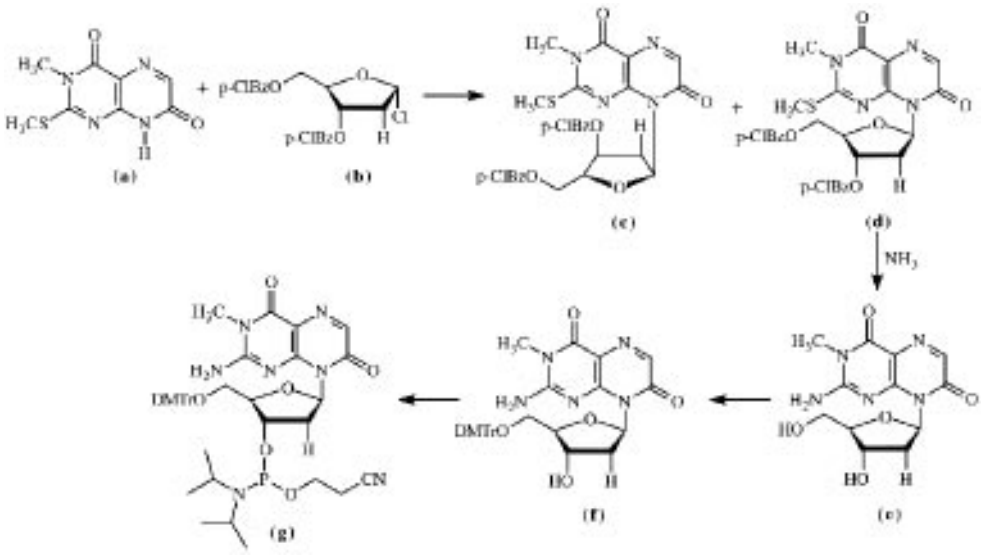
Step 2. 6-Amino-3-methyl-2-methylthio-4(3H)-pyrimidinone (37). Ten grams of the 6-amino-2-methylthio-4(3H)-pyrimidinone from step 1 was added to 65 ml of 1N KOH solution and then 9 g of dimethyl sulfate was added gradually while agitating by frequent shaking. The resulting colorless, crystalline precipitate was filtered off by suction as soon as the solution became acid to litmus. The filtrate was then neutralized with NaOH and evaporated to dryness. The residue was washed with cold water, the solid filtered out and added to the crystals already obtained. The combined solids were then treated with dilute ammonia to dissolve the small quantity of 6-amino-2-methylthio-4(3H)-pyrimidinone still found to be present. The residual crystalline precipitate which was not soluble in ammonia contained two compounds with markedly different melting points and solubility in ether. The compound with the lower melting point was very soluble in ether, whereas the compound with the higher melting point was nearly insoluble in this solvent. Ether, therefore, provided a means of separating these compounds. The ether soluble compound was 6-amino-2-methylthio-4-methoxypyrimidine which was extracted from the solid residue by repeated ether washings and filtering out the solid residue. The solid residue was then recrystallized from alcohol as slender prisms (60% yield, m.p. 255°C). Elemental analysis calculated for $C_6H_9ON_3S$: N, 24.57. Found N, 24.71

Step 3. 6-Amino-3-methyl-2-methylthio-5-nitroso-4(3H)-pyrimidinone (38). One hundred and eleven grams of 6-amino-3-methyl-2-methylthio-4(3H)-pyrimidinone from step 2 was suspended in 1 l of 30% acetic acid and 50 g of sodium nitrite in 100 ml of H_2O was added drop-wise. The mixture was stirred for an additional hour at room temperature and then cooled at 4°C overnight. The precipitate was collected and washed with H_2O, then acetone and dried at 100°C. This yielded 119.5 g (92% yield) of a chromatographically uniform crude product (m. p. 230°C decomp.) Recrystallization of 1 g of this material from 240 ml of H_2O gave 0.52 g of blue crystals (m.p. 234°C decomp.).

Step 4. 5,6-Diamino-3-methyl-2-methylthio-4(3H)-pyrimidinone. Forty milliliters of a 20% aqueous ammonium sulfide solution was added to 4.0 g (0.02 mol) of 6-amino-3-methyl-2-methylthio-5-nitroso-4(3H)-pyrimidinone from step 3. The mixture was heated under reflux for 30 min. After cooling, the precipitate was collected, washed with ethanol and dried in a desiccator to give 2.72 g (75% yield) of colorless crystals (m.p. 211–212°C).

Step 5. 6-Amino-3-methyl-2-methylthio-4-oxodihydropyrimidin-5-yl-azomethin-carbonic acid ethylester (39). Six grams of the 5,6-diamino-3-methyl-2-methylthio-4(3H)-pyrimidinone prepared in step 4 was dissolved in 200 ml of warm H_2O and the solution was then cooled to room temperature and combined with 6 g ethyl glyoxylate-hemiethylacetal. The thick precipitate that immediately resulted was drawn off after 1 h and recrystallized from ethanol producing 8 g of bright yellow crystals (m.p. 178°C). Analysis calculated for $C_{10}H_{14}N_4O_3S•H_2O$: C, 41.66; H, 5.59; N, 19.44. Found: C, 42.18; H, 5.57; N, 19.32.

Step 6. 3-Methyl-2-methylthio-4,7(3H,8H)-pteridinedione sodium salt. 33.8 grams of 6-amino-3-methyl-2-methylthio-4-oxodihydro-



Scheme 1. Synthesis of the 3-MI phosphoramidite.

pyrimidin-5-yl-azomethin-carbonic acid ethylester crystals from step 5 were suspended in 100 ml of EtOH. Six hundred millilitres of 0.5 N $NaHCO_3$ were added and the solution was refluxed for 30 min. The clear solution was treated with charcoal and then cooled overnight. The precipitate which formed was collected, washed with MeOH and dried at 100°C under vacuum yielding 21.5 g (70%) of faint yellow powder (m.p. >300°C).

*Step 7. 2-Deoxy-3,5-di-O-(4-chlorobenzoyl)-α-D-ribofuranosyl chloride (*40*).* This describes the synthesis of a similar compound, 2-deoxy-3,5-di-*O*-*p*-toluyl-α-D-ribofuranosyl chloride. 13.6 g (0.1 mol) of 2-deoxy-D-ribose (Aldrich, Milwaukee) and 27 ml of 1% methanolic HCl was added to 243 ml of methanol and the mixture was allowed to stand sealed for 12–15 min to form methylglycoside. Afterwards, 3–5 g silver carbonate was added to immediately bind all HCl. The clear, filtered solution was evaporated under vacuum to a syrup-like consistency and the remaining methanol was separated out by repeated evaporation under vacuum after adding small amounts of dry pyridine. Finally the mixture was dissolved in 80 ml pyridine and acylated with 34 g (0.22 mol) 4-chlorobenzoyl chloride while cooling. The mixture was then heated for 2 h at 40–50°C or allowed to stand overnight at room temperature. Water was added and the mixture was extracted with 200 ml of ether. The ether solution was then washed free of pyridine using water followed by dilute sulfuric acid and then by $KHCO_3$ solution. The ether solution was then evaporated under vacuum to form a honey-yellow syrup. From this syrup, it was possible to obtain crystallized 1-*O*-methyl 3,5-di-*O*(4-chlorobenzoyl)-2-deoxy-D-ribofuranoside by seeding. This was converted to the 1-chloro sugar by dissolving the syrup in 20–50 ml of glacial acetic acid and mixing with 80 ml of glacial acetic acid saturated with HCl gas. The solution was maintained at 10°C and more gaseous HCl was bubbled through the solution until the mixture hardened into a thick crystalline paste (~10 min). After not more than 30 min, the crystalline substance was washed twice on a filter under low vacuum with absolute ether. The substance was then dried in a vacuum desiccator over soda lime and phosphorous pentaoxide. In this form, the 2-deoxy-3,5-di-*O*-(4-chlorobenzoyl)-α-D-ribofuranosyl chloride is stable for weeks. Prior to the next step, the compound is recrystallized from toluene or carbon tetrachloride to give 34.8 g (81% yield).

Step 8. 3-Methyl-2-methylthio-8-[2-deoxy-3,5-di-O-(4-chlorobenzoyl)-β-D-ribofuranosyl]-4,7(3H,8H)pteridinedione (d in Scheme 1). 9.85 g of 3-methyl-2-methylthio-4,7(3H,8H) pteridinedione sodium salt from step 6 (**a** in Scheme 1) were suspended in 400 ml of anhydrous CH_2Cl_2 and 17.2 g of 2-deoxy-3,5-di-*O*-(4-chlorobenzoyl)-D-ribofuranosyl chloride from step 7 (**b** in Scheme 1) were added and the suspension was stirred at room temperature for 2 h. The reaction mixture was treated twice with 200 ml of 5% $NaHCO_3$. The two layers were separated and after the aqueous phase was extracted with CH_2Cl_2, organic layers were combined, dried over Na_2SO_4 and evaporated.The residue was recrystallized five times from MeOH/ethyl acetate, yielding the α-anomer. The anomeric β-nucleoside remains in the mother liquid and was purified by silica gel flash chromatography (4×20 cm) using 4 l of CH_2Cl_2 and then 5 l CH_2Cl_2:MeOH, 200:1. Evaporation yields 14.2 g (57%) of a yellowish solid foam, which may be recrystallized from ethyl acetate:ether, 1:1 giving chromatographically pure crystals of the β-nucleoside (**d** in Scheme 1) (m.p. 130–133°C) Analysis calculated for $C_{27}H_{22}Cl_2N_4O_7S$: C, 52.52; H, 3.59; N, 9.07. Found: C, 52.45; H, 3.61; N, 8.90.

*Step 9. 3-Methyl-8-(2-deoxy-β-D-ribofuranosyl) isoxanthopterin also called 2-amino-3-methyl-8-(2-deoxy-β-D-ribofuranosyl)-4,7 (3H,8H)-pteridinedione (**e** in Scheme 1).* A solution of 3.3 g (4 mmol) of 3-methyl-2-methylthio-8-[2-deoxy-3,5-di-*O*-(4-chlorobenzoyl)-β-D-ribofuranosyl]-4,7(3H,8H)-pteridinedione (from step 8; **d** in Scheme 1) in 100 ml of dry acetonitrile was added to 100 ml of ammonia saturated methanol at room temperature. The mixture was allowed to stand for 24 h. A small amount of insoluble material was filtered off and the filtrate evaporated to dryness. After two co-evaporations with methanol, the precipitate was suspended in 20 ml of warm methanol. Fifty millilitres of

ethyl ether was added and the mixture was chilled at 4°C for 3 days. The resulting precipitate was collected and dried at 60°C under vacuum yielding 1.46 g (88% yield) of colorless crystals (m.p. >250°C decomp). Analysis calculated for $C_{12}H_{15}N_5O_5$•1/2 H_2O: C, 45.28; H, 5.07; N, 22.00. Found: C, 45.55; H, 5.07; N, 21.92.

*Step 10. Methyl-8-(2-deoxy-5-O-dimethoxytrityl-β-D-ribofuranosyl) isoxanthopterin (**f** in Scheme 1).* Fifty millilitres of dry pyridine was added to 3.1 g (10 mmol) of 3-methyl-8-(2-deoxy-β-D-ribofuranosyl) isoxanthopterin from step 9 (**e** in Scheme 1), and the solution was evaporated. The evaporation was repeated three times with 50 ml of dry pyridine each time, and the residue was then suspended in 50 ml of dry pyridine. 5.1 g (15 mmol) of dimethoxytrityl chloride was added to this solution and the mixture was stirred at room temperature. After 10 min a clear solution was obtained and after 3 h the reaction was stopped by addition of 10 ml of methanol. The solution was evaporated, the residue dissolved in CH_2Cl_2 and then extracted twice with a 5% aqueous solution of $NaHCO_3$. The organic layer was dried over $MgSO_4$ and the filtrate evaporated again. The residue was dissolved in CH_2Cl_2:methanol, 2:1, put on a silica-gel column (3 × 20 cm, packed with toluene:ethyl acetate, 2:1) for flash chromatography. A gradient was used to achieve purification: 500 ml toluene/ethyl acetate 1:1, 2.5 l of ethyl acetate, 1 l of ethyl acetate/methanol 99:1 and 2 l of ethyl acetate/methanol 98:2. The substance from the last two fractions in ethyl acetate/methanol-was evaporated and dried under vacuum to give 3.9 g (63% yield) of colorless amorphous solid. Analysis calculated for $C_{33}H_{33}N_5O_7$•1/2 H_2O: C, 63.86; H, 5.52 ; N, 11.28. Found: C, 63.90; H, 5.82; N, 10.86.

*Step 11. 3-Methyl-8-(2-deoxy-5-O-dimethoxytrityl-β-D-ribofuranosyl)isoxanthopterin-3′-O-(bβ-cyanoethyl, N-diisopropyl)phosphoramidite (**g** in Scheme 1).* A suspension of 3.06 g (4.9 mmol) of 3-methyl-8-(2-deoxy-5-*O*-dimethoxytrityl-β-D-ribofuranosyl) isoxanthopterin from step 10 (**f** in Scheme 1) and 0.18 g (25 mmol) of tetrazole was stirred under argon with 2.2 g (7.3 mmol) of β-cyanoethoxy-bis-diisopropylaminophosphane. The suspension became clear after 30 min and the reaction was stopped after 4 h. The reaction solution was extracted once with a 5% aqueous solution of $NaHCO_3$, the organic layer was dried over $MgSO_4$ and the filtrate evaporated to dryness. Purification was achieved through flash-chromatography on a silica-gel column (3× 20 cm) in 200 ml of hexane/ethyl acetate 2:1 followed by 2 l of hexane/ethyl acetate 1:1. The last fraction containing the product was collected, evaporated to dryness and dried under vacuum to give 2.38 g (59% yield) of a colorless amorphous solid. Analysis calculated for $C_{42}H_{50}N_7O_8P$•H_2O (820.8):C, 61.45; H, 6.26; N, 11.94; Found C, 61.56; H, 6.47; N 11.51.

Synthesis and purification of oligonucleotides

3-Methyl-8-(2-deoxy-β-D-ribofuranosyl) isoxanthopterin was incorporated directly into oligonucleotides with an Applied Biosystems Model 392 (Foster City, CA) automated DNA synthesizer following standard protocols recommended by the manufacturer. The 3-MI phosphoramidite was placed in bottle position 5 on the synthesizer and treated in the same way as the standard phosphoramidites. Table 1 lists the sequences of the oligonucleotides (21mers) synthesized and the sites of substitution of 3-MI for guanosine.

Table 1. Base sequence of single-stranded oligonucleotides without (LTR) and with (PTR) 3-MI (**F**) substituted for G at various positions

Name	Oligonucleotide sequence	Relative quantum yield[a]
Complementary strands identical to U5 terminus of HIV-1 genome		
LTR5	5′- GTG TGG AAA ATC TCT AGC AGT -3′	
LTR4	[b]3′- CAC ACC TTT TAG AGA TCG TCA -5′	
3-MI containing oligonucleotides		
PTR5	[b]5′- GTG TGG AAA ATC TCT AGC A**F**T -3′	0.14
PTR1	5′- GT**F** TGG AAA ATC TCT AGC AGT -3′	0.13
PTR2	5′- GTG T**F**G AAA ATC TCT AGC AGT -3′	0.10
PTR3	5′- GTG TG**F** AAA ATC TCT AGC AGT -3′	0.03
PTR4	5′- GTG TGG AAA ATC TCT A**F**C AGT -3′	0.06
PTR7	3′- CAC ACC TTT TA**F** AGA TCG TCA -5′	0.04
PTR8	3′- CAC ACC TTT TAG A**F**A TCG TCA -5′	0.05
PTR9	3′- CAC ACC TTT TAG AGA TC**F** TCA -5′	0.29

Oligonucleotides have sequences identical to the U5 terminus of the HIV genome. Fluorescence intensity of the 3-MI-containing oligonucleotides is referenced to quinine sulfate at an optical density of 0.04 (350 nm) in 0.1 N H_2SO_4. The fluorescence intensity of 3-MI is partially quenched with incorporation into an oligonucleotide (the quantum yield of unincorporated 3-MI is 0.88). [a]Quantum yields are for the single-stranded oligonucleotide. [b]Substrate for integrase 3′-processing assay.

The synthetic oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis (19:1 acrylamide:bis). Bands were detected by UV shadowing, excised and extracted into 0.3 M NaAcetate pH 5.2 at 37°C over 12 h, followed by ethanol precipitation. (23). UV-shadowing revealed a single band as the final product of the synthesis. 3-MI and 3-MI-containing oligonucleotides were protected from light during synthesis and purification and stored at –80°C. Complementary oligonucleotide strands were annealed to form the double-stranded substrate by mixing equimolar amounts of each strand in 0.1 M NaCl, heated to 85°C and allowed to cool slowly to room temperature. The concentration of double stranded oligonucleotide was estimated from the absorbance at 260 nm using an extinction coefficient of 409.14 (μM) (24).

Spectrofluoroscopic analysis

Fluorescence intensity was monitored with an SLM model 8000 (SLM Instruments, Urbana, IL) spectrofluorometer interfaced with an SPEX 386 computer (SPEX Industries, Edison, NJ). The light source was a 450 W xenon arc lamp cooled by a chilled water system. Samples were maintained at selected temperatures with a NESLAB water bath temperature controller (NESLAB Instruments Inc., Portsmouth, NH). Relative quantum yield determinations were measured on samples with optical densities at 360 nm of ≤0.12 in 0.1 M Tris-Cl, pH 7.6 at room temperature using 1 × 1 cm^2 square cuvettes. Excitation was at 360 nm and emission was measured at 460 nm. Corrected emission spectra were integrated and referenced to quinine sulfate at an optical density of 0.04

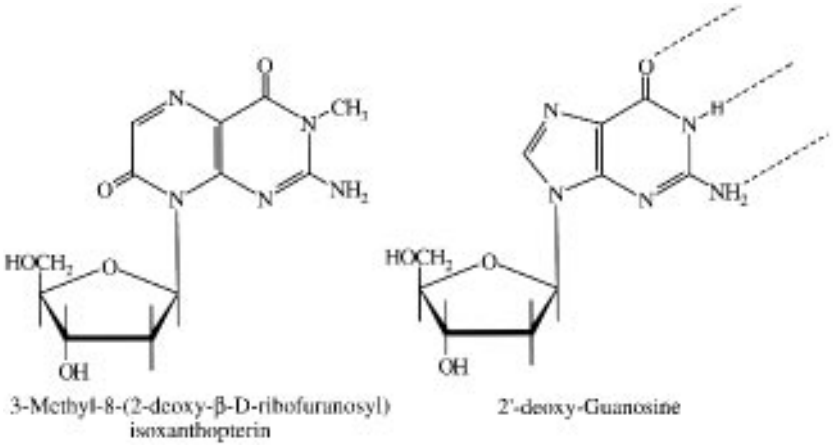


Figure 2. Chemical structures of 3-MI, a fluorescent pteridine which is structural analog of guanosine and 2′-deoxyguanosine.

(360 nm) in 0.1 N H_2SO_4. Absorbance measurements were made on a Hewlett-Packard Model 8452a spectrophotometer (Palo Alto, CA).

Integrase assay

Purified recombinant HIV-1 integrase was obtained from R. Craigie (Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD). The reaction mixture for the integrase assay contained 25 mM MOPS, pH 7.2, 50 mM NaCl, 7.5 mM $MnCl_2$, 10 mM 2-mercaptoethanol, 100 µg/ml BSA, 10% (w/v) glycerol, 3-MI-containing oligonucleotide substrate (PTR5:LTR4 from Table 1) and 520 nM integrase (monomer) in a total volume of 60 µl. The reaction mixture was incubated at 37°C and was monitored by observing the increase in fluorescence intensity of the reaction mixture from release of the 3-MI-containing dinucleotide during 3′-processing. The reaction was carried out in cuvettes measuring 3×3 mm^2. A brass adapter was used to accommodate these small cuvettes in the spectrofluorometer. The reaction rate (rate of increase in fluorescence intensity) was determined by regression analysis of the linear portion of the fluorescence intensity versus time plot.

Comparison of fluorescence assay to standard gel technique

The 3-MI-containing oligonucleotide substrate and the standard oligonucleotide with guanosine in place of 3-MI were radiolabeled at the 5′-end using T4 kinase and [^{32}P]ATP. The kinase was heat inactivated and an equimolar amount of complementary strand was added to each oligonucleotide. The complementary strands were annealed by heating to 95°C and then cooling slowly to room temperature. The solution was then passed through a G-25 Sephadex quick-spin column (Boehringer-Mannheim) to separate double-stranded oligonucleotide from unincorporated radiolabel. Separate integrase reactions with standard ^{32}P-radiolabeled substrate or 3-MI-containing ^{32}P-radiolabeled substrate were carried out as described above with fluorescence monitoring of the 3-MI-containing substrate. Reactions were stopped after 20 min by the addition of EDTA to a final concentration of 8.1 mM. Samples were added to Maxam–Gilbert loading dye and aliquots were electrophoresed on a denaturing 20% polyacrylamide gel. Gels were dried and exposed in a Molecular Dynamics PhosphorImager or a Betascope 603 blot analyzer (Betagen, Waltham, MA). The % of the oligonucleotide cleaved was calculated as previously reported (25).

RESULTS

Fluorescence of 3-MI

3-MI, an analog of guanosine (Fig. 2), is highly fluorescent. The relative quantum yield of 3-MI referenced to quinine sulfate was 0.88. The µM extinction coefficients of 3-MI measured in methanol are 3.69 at 254 nm and 4.13 at 350 nm. Insertion of this fluorophore into an oligonucleotide partially quenches its fluorescence intensity and the degree of quench appears to be related to the fluorophore's proximity to purines and position within the oligonucleotide (Table 1). Fluorescence intensity is maximally quenched when the fluorophore is surrounded by purines and least quenched when the fluorophore is surrounded by pyrimidines and near the end of the strand.

Integrase assay

Our assay for the integrase 3′-processing reaction is depicted in Figure 3. It is based on an *in vitro* cell-free and virus-free system in which purified recombinantly produced HIV-1 integrase is incubated with a short duplex oligonucleotide (21mer) with a sequence identical to the U5 terminus of viral DNA (17–19,26). 3-MI is substituted for guanosine at the cleavage site of the oligonucleotide substrate. As part of the 3′-processing reaction, the terminal 3′-dinucleotide, containing 3-MI, is cleaved from the oligonucleotide and the quenching effect of the neighboring adenosine is abrogated resulting in a marked increase in the fluorescence intensity of the reaction mixture (Fig. 3). The increase in fluorescence intensity was linear for up to 20 min. The rate of the increase in fluorescence intensity is proportional to the rate of the integrase 3′-processing reaction and, because the change in fluorescence intensity is dependent on release of the 3-MI-containing dinucleotide, this assay is specific for the integrase 3′-processing reaction. The potential increase in fluorescence intensity from

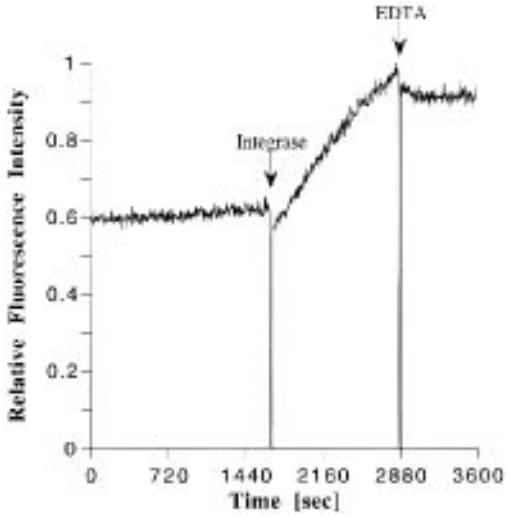


Figure 3. Change in fluorescence intensity over time during incubation of HIV-1 integrase with the 3-MI-containing oligonucleotide substrate. The slow time kinetic trace of fluorescence intensity depicts the blank rate prior to addition of integrase to the reaction mixture (0–1700 s), the increase in fluorescence intensity following addition of integrase to the reaction mixture (1700–2880 s) and cessation of the reaction following the addition of EDTA (>2880 s) which chelates manganese, a required co-factor. The fluorescence emission was measured at 460 nm following the excitation at 360 nm.

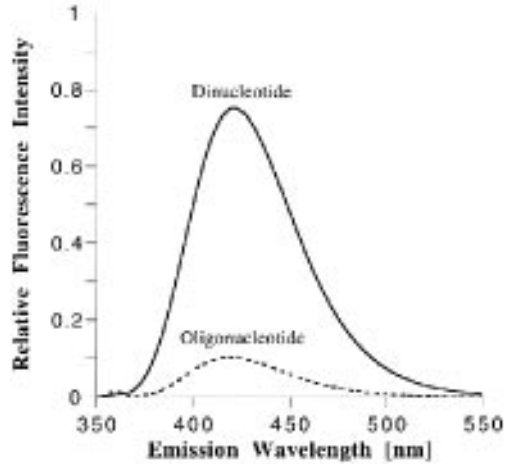


Figure 4. Comparison of the fluorescence emission spectra from 355 to 500 nm for the 3-MI-containing oligonucleotide and the 3-MI-containing dinucleotide cleavage product. Emission was at 460 nm following excitation at 360 nm. The oligonucleotide and dinucleotide solutions were analyzed at equimolar concentrations as measured by absorbance of the solution at 360 nm.

release of the 3-MI-containing dinucleotide is also illustrated by the marked difference in the fluorescence emission profiles of the 3-MI-containing oligonucleotide and the 3-MI-containing dinucleotide shown in Figure 4.

We demonstrated the comparability of our assay to the standard method of monitoring integrase using the identical 21-base oligonucleotide containing 3-MI at the cleavage site and also labeled at its 5′-end with ^{32}P. The reaction was analyzed both with real time fluorescence monitoring (Fig. 3) and by exposure in a phosphorimager cassette after separation of the reaction products on a 20% polyacrylamide gel (data not shown). The observed increase in fluorescence intensity after a 20 min incubation corresponded to cleavage of dinucleotide from 12% of the oligonucleotide substrate as measured by phosphorimager analysis of the resulting 19mer in the gel. Using a ^{32}P-radiolabeled oligonucleotide without 3-MI, 16% of oligonucleotide substrate is cleaved under the same conditions. This 4% difference is within experimental error. Substitution of 3-MI for guanosine at the cleavage site did not interfere with the 3′-processing reaction.

The specificity of the assay was tested by monitoring the fluorescence intensity of a reaction mixture containing no integrase, a reaction mixture with an integrase preparation which was boiled for 10 min to inactivate the protein prior to addition to the reaction mixture and a reaction mixture containing no Mn^{2+} (a required co-factor). The reaction mixture without integrase was allowed to incubate for 2 h. In each case there was no significant increase in fluorescence intensity, indicating that non-specific degradation of the 3-MI-containing oligonucleotide did not account for the observed increase in fluorescence intensity.

To test the specificity of the endonucleolytic activity of the integrase preparation, an oligonucleotide containing the fluorophore in a position other than the cleavage site was tested as a substrate (PTR4:LTR4 in Table 1). There was no increase in fluorescence intensity with this substrate suggesting that the endonucleolytic release of the fluorophore from the oligonucleotide is specific for the 3′-processing reaction and only occurs when the fluorophore is positioned at the integrase cleavage site.

We used our integrase assay to investigate the effect of varying the concentration of the critical components of the reaction mixture on the rate of the 3′-processing reaction (Fig. 5). The reaction increased with increasing substrate concentration up to 164 nM, but substrate concentrations above 164 nM appeared to be inhibitory (Fig. 5A). Because annealing of the double-stranded oligonucleotide substrate is performed in the presence of 0.1 M NaCl, the reaction rate was also measured in the presence of a fixed substrate concentration and increasing concentrations of NaCl (data not shown) which demonstrated that the inhibition observed at higher substrate concentrations was from the substrate and not from higher NaCl concentrations.

The reaction rate was also dependent on protein concentration up to a concentration of 16.7 μg/ml. The reaction rate was lower when the integrase concentration was increased to 20.7 μg/ml (Fig. 5B).

In the absence of Mn^{2+} there is no change in fluorescence intensity confirming that Mn^{2+} is a required co-factor. The optimal Mn^{2+} concentration in our assay system was 7.5 mM (Fig. 5C). In addition, the reaction did not proceed unless the Mn^{2+} was added to the reaction mixture (containing the oligonucleotide substrate) before integrase, suggesting that integrase must interact with Mn^{2+} before binding to the oligonucleotide substrate. Pre-incubation of the integrase with the chelating agent, EDTA, prior to its addition to the reaction mixture completely inhibits the reaction, but pre-incubation of the reactants with EDTA prior to the addition of integrase did not completely inhibit the reaction, presumably because of the presence of Mg^{2+} bound to integrase in the protein preparation. The minimum EDTA concentration necessary to stop an ongoing reaction was 8.0 mM in the presence of 7.5 mM Mn^{2+} (Fig. 5D). However, this amount of EDTA added to an ongoing reaction did not stop the reaction immediately. The optimal pH for the reaction appeared to be 7.2 (Fig. 5E).

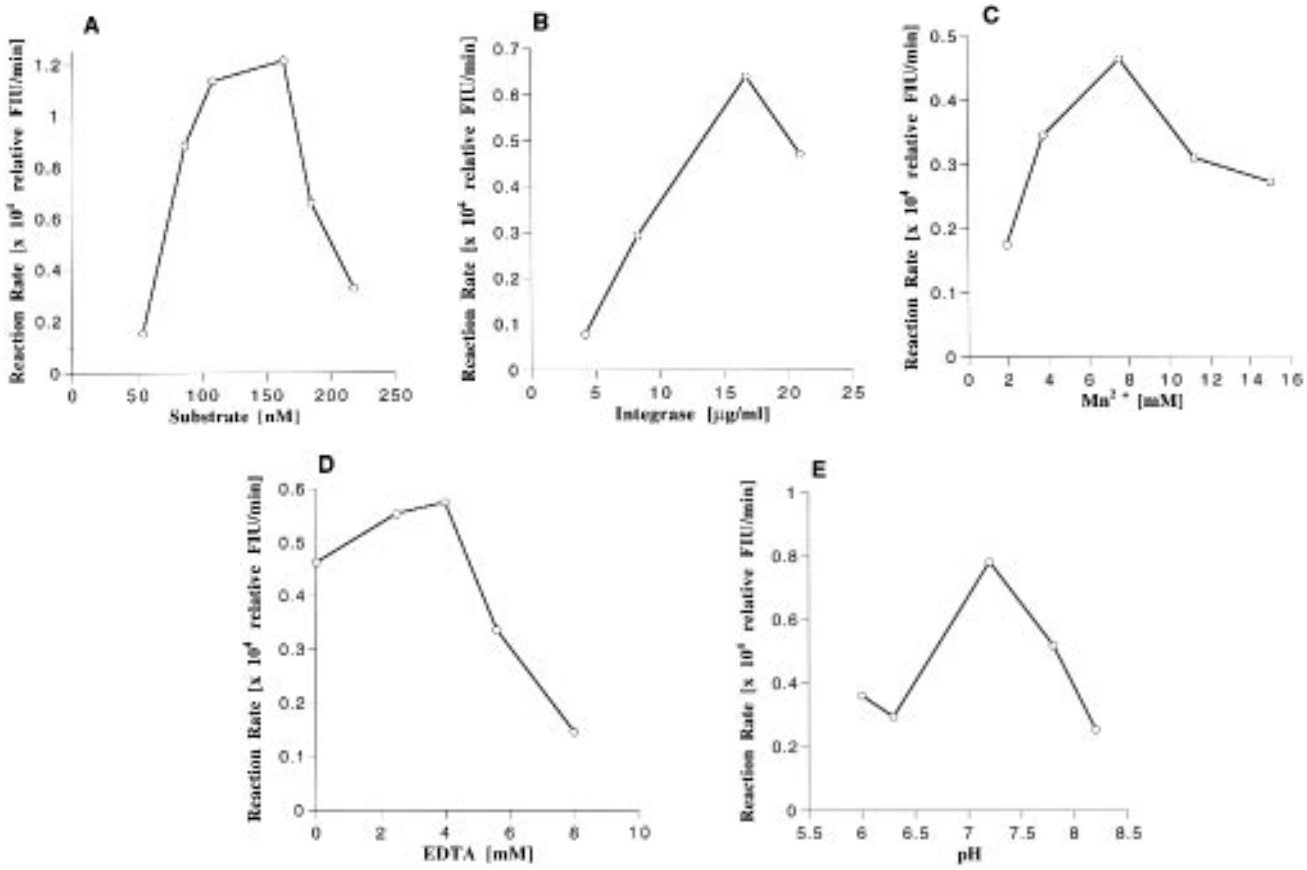


Figure 5. Definition of optimal assay conditions. (**A**) Effect of substrate concentration on the rate of reaction with a fixed protein concentration of 16.7µg/ml. Substrate concentrations exceeding 164 nM were inhibitory. (**B**) Effect of integrase concentration on the rate of reaction with a fixed substrate concentration of 164 nM. (**C**) Effect of Mn^{2+} concentration on the rate of the reaction at an integrase concentration of 16.7 µg/ml and a substrate concentration of 164 nM. (**D**) Effect of EDTA concentration on the reaction rate in the presence of 7.5 mM Mn^{2+}. (**E**) Effect of pH on the reaction rate.

DISCUSSION

The chemical synthesis of pteridine nucleosides (27) is commonly performed via the silyl method first described by Birkofer (28). There are a series of modifications of this approach depending upon the heterocycle and sugar derivative used in the glycosylation reaction. Attempts to react 3-methyl-isoxanthopterin directly with 3,5-di-*O*-*p*-chlorobenzoyl-2-deoxyribofuranosyl chloride (**b** in scheme 1) using standard techniques failed because of the low solubility of the silylated heterocyclic base in aprotic solvents. 3-Methyl-2-methylthio-4,7-(3H, 8H)pteridinedione (**a** in scheme 1) was then chosen as starting material for the glycosylation reaction since the analogous ribosylation with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose worked well under BF_3-catalysis (29). The more sensitive halo-sugar (**b** in scheme 1) however, required special reaction conditions to achieve the desired glycosylation. The substituted pteridinedione (**a** in scheme 1) was suspended in acetonitrile and the halo-sugar (**b** in scheme 1) was subsequently added. Hexamethyldisilazane and trimethylsilyl chloride were then added for mild silylation at room temperature. After 30 min the glycosylation was initiated by drop-wise addition of $SnCl_4$ and after exactly 20 min the reaction mixture was quenched by a cold sodium bicarbonate solution. Extraction of the pteridine nucleoside led to the anomeric mixture of α- (**c** in scheme 1) and β-2′-deoxyribonucleoside (**d** in scheme 1) which was separated into the components by fractional crystallization. Ammonia treatment resulted in nucleophilic displacement of the methylthio group and cleavage of the sugar protecting groups to give (**e** in scheme 1). Dimethoxytritylation was performed in pyridine at room temperature and gave (**f** in scheme 1) in 63% yield after chromatographic purification on a silica-gel column. The conversion of (**f** in scheme 1) into the final product, 3-methyl-8(2-deoxy-5-*O*-dimethoxytrityl-β-D-ribofuranosyl) isoxanthopterin-3′-*O*-(β-cyanoethyl, N-diisopropyl) phosphoramidite (**g** in scheme 1) was also problematic for solubility reasons and was best achieved in CH_2Cl_2 with β-cyanoethoxy-bis-diisopropylaminophosphane under tetrazole catalysis. This resulted in a moderate 59% yield after flash chromatography. Synthesis of this fluorescent nucleotide analog in its phosphoramidite form provided a means of incorporating 3-MI directly into an oligonucleotide using an automated DNA synthesizer.

As a fluorescent probe, 3-MI has several unique features. It is highly fluorescent and the degree of fluorescence intensity is very sensitive to its immediate molecular environment. Fluorescence intensity is quenched by inserting 3-MI into an oligonucleotide and the degree of quench is a function of its position in the strand and the structure of the neighboring bases. The most unique features of this fluorophore are its structural similarity to

guanosine and its ability to be site specifically inserted into an oligonucleotide through a 3′,5′-phosphodiester linkage. Because 3-MI is actually incorporated into the DNA, it may be more likely to reflect subtle changes in the tertiary structure of the DNA than externally linked fluorophores. We have compared the melting temperatures (T_m) of 3-MI-containing double-stranded oligonucleotides with the Tm of double-stranded oligonucleotides containing mismatched base pairs at equivalent positions and have found that 3-MI incorporation depresses the T_m to the same degree as a single base pair mismatch (to be reported elsewhere).

Our assay for the integrase 3′-processing reaction takes advantage of these fluorescence properties of 3-MI. A 21-base pair oligonucleotide with a sequence identical to the U5 terminus of the HIV genome was constructed with 3-MI in place of guanosine at the integrase cleavage site. This 3-MI-containing oligonucleotide was accepted by integrase as a substrate for the 3′-processing reaction, indicating that the presence of 3-MI in place of guanosine at the cleavage site did not substantially interfere with the integrase-catalyzed cleavage of the 3′-terminal dinucleotide containing 3-MI. As shown in Figure 3, fluorescence intensity increases linearly only after the addition of integrase to the reaction mixture and the increase in fluorescence intensity ceases with the addition of EDTA which chelates the required divalent cation co-factor, Mn^{2+}, indicating that the change in fluorescence intensity is specific for the integrase-catalyzed cleavage of the 3′-terminal dinucleotide.

This continuous assay for the integrase 3′-processing reaction offers significant advantages over the standard fixed-time techniques. Our fluorescence technique measures a true reaction rate (60 measurements per min), whereas the fixed-time methods may require many separate experiments to determine a rate. Measuring the reaction rate rather than the reaction end-products reduces the chance for error which is a potential drawback of fixed-time procedures. Performing kinetic studies with fixed-time methods is less practical and less quantitative. The continuous fluorescence assay yields immediate results which allows the investigator to alter experimental conditions based on the results of the previous experiment. The fluorescence assays also avoid the use of radioisotopes.

Optimal assay conditions were defined as shown in Figure 5. At substrate concentrations below 164 nM, the reaction rate increased in proportion to the increase in the substrate concentration. A reduction in the reaction rate was observed at substrate concentrations exceeding 164 nM and the amount of the reduction in the rate was a function of the concentration of substrate. This inhibitory effect has been observed not only with specific LTR DNA but also with non-specific DNA (14,30,31). The reaction rate was also dependent on integrase concentration up to 16.5 µg/ml. The reduction in rate at higher integrase concentrations may be the result of aggregation. The optimal $MnCl_2$ concentration (7.5 mM) falls within the wide range of concentrations used by others of 1–25 mM (32–33). The requirement for the protein to be exposed to the Mn^{2+} before, or at the same time, it is exposed to the DNA suggests that the Mn^{2+} is necessary to achieve productive binding of integrase to its substrate. The optimal pH of 7.2 is also in agreement with the findings of others.

With this assay the optimal reaction rate is observed with a 4:1 ratio of protein (monomer):substrate. The fact that the substrate is only partially consumed (as determined by the gel electrophoresis/phosphorimager analysis done concurrently with the fluorescence assay) with this high protein:substrate ratio is suggestive that integrase does not turnover. This incomplete processing of the substrate could be a result of the necessarily artifactual nature of the *in vitro* assay systems. If the protein is active as a tetramer or dimer and the majority of it is not in the appropriate form under the reaction conditions, this could account for the lack of complete processing. Although others have observed turnover in the reverse reaction (disintegration) (34) or with Rous sarcoma virus (35) we have not found evidence for turnover with the 3′-processing reaction of the HIV-1 integrase.

This assay demonstrates the potential utility of this new class of fluorophore for continuous monitoring of protein/DNA interactions. Monitoring fluorescence intensity or fluorescence anisotropy of oligonucleotides containing this fluorophore can detect subtle changes in the immediate molecular environment. This compound could prove useful in studies of other protein/DNA interactions, as a GTP analog or as a marker for DNA degradation. The HIV integrase assay described here may prove useful in mechanistic studies and in screening for inhibitors which could eventually prove useful in treating HIV infection and AIDS.

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