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J. Am. Chem. Soc., **2005**, 127 (49), 17286-17295• DOI: 10.1021/ja0542946 • Publication Date (Web): 12 November 2005

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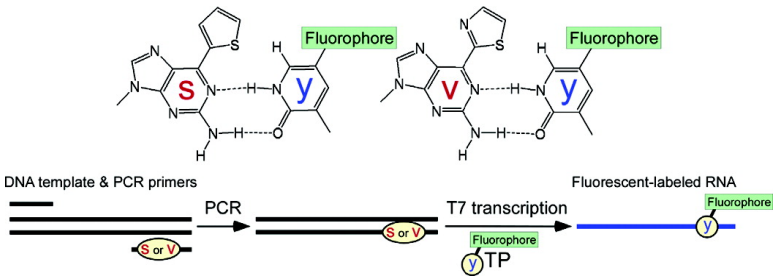

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Site-Specific Fluorescent Labeling of RNA Molecules by Specific Transcription Using Unnatural Base Pairs

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Abstract: Site-specific fluorescent labeling of RNA molecules was achieved by specific transcription using an unnatural base pair system. The unnatural base pairs between 2-amino-6-(2-thienyl)purine (**s**) and 2-oxo-(1H)pyridine (**y**), and 2-amino-6-(2-thiazolyl)purine (**v**) and **y** function in transcription, and the substrates of **y** and 5-modified **y** bases can be site-specifically incorporated into RNA, opposite **s** or **v** in DNA templates, by T7 RNA polymerase. Ribonucleoside 5′-triphosphates of 5-fluorophore-linked **y** bases were chemically synthesized from the nucleoside of **y**. These fluorescent substrates were site-specifically incorporated into RNA by transcription mediated by the **s**−**y** and **v**−**y** pairs. By using this fluorescent labeling method, specific positions of Raf-binding and theophylline-binding RNA aptamers were fluorescently labeled, and the specific binding to their target molecules was detected by their fluorescent intensities. This site-specific labeling method using an unnatural base pair system will be useful for analyzing conformational changes of RNA molecules and for detecting interactions between RNA and its binding species.

Introduction

Fluorescent labeling of RNA molecules is an important technique in both basic and applied sciences. Fluorescence-based detection offers flexible availability, not only for the replacement of radiolabeled detection, but also for a wide range of RNA-based biotechnologies,[1] such as single-molecule imaging techniques, probes for structural and functional analyses, and molecular beacons using fluorescence resonance energy transfer (FRET). For example, structural analyses of dynamic rearrangements of RNA molecules were performed by the site-specific attachment of fluorophores to RNA.[2] Thus, the site-specific incorporation of fluorophores into RNA at desired positions has become a powerful method to expand the utility of fluorescently labeled RNA molecules.

The current methods for the site-specific fluorescent labeling of RNA are still restrictive. One reliable method is the introduction of a fluorescent unit into RNA fragments during chemical RNA synthesis.[1a,2a,3] This method is useful for short RNA fragments but becomes cumbersome for RNAs consisting of more than ∼70 nucleotides. Thus, the direct incorporation of a fluorescent agent into RNA during transcription using DNA templates is a more useful method. The 5′-termini of RNA can be site-specifically labeled by conventional T7 transcription using fluorescent cap analogues, such as a coumarin-derivatized GTP,[3,4] or by T7 transcription under the T7 2.5 promoter using N^6- or 5′-fluorescein-derivatized AMP.[5] Modified UTP and CTP that link with fluorophores at position 5 are also available,[6] but these substrates are randomly incorporated into RNA and cannot be used extensively. Consequently, there have been only a few reports on fluorescent analogues introduced into large RNA molecules.

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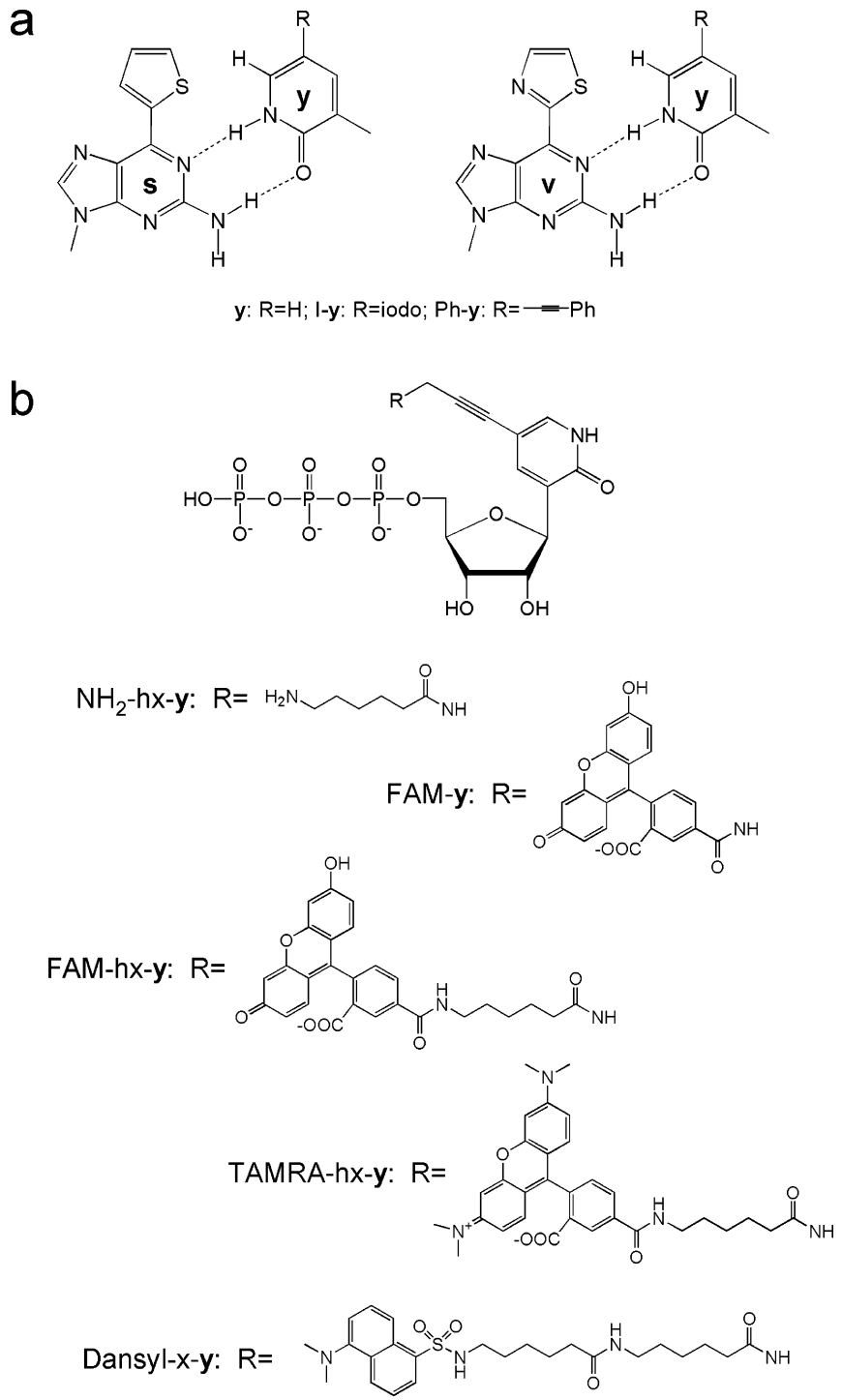

Figure 1. The unnatural **s**−**y** and **v**−**y** base pairs (a) and the nucleoside 5′-triphosphates of the 5-fluorophore-linked **y** bases (b).

Another attractive approach is to incorporate fluorescent residues into RNA at desired positions during transcription mediated by extra base pairs. Recently, we developed unnatural base pairs between 2-amino-6-(2-thienyl)purine (**s**) and 2-oxo-(1H)pyridine (**y**),[7] and 2-amino-6-(2-thiazolyl)purine (**v**) and **y**[8] (Figure 1a), which exhibit high specificity in transcription. The bulky 6-(2-thienyl) group of **s** and the 6-(2-thiazolyl) group of **v** prevent noncognate pairing with C or T, but the relatively small hydrogen at position 6 of **y** maintains the shape complementarity of the **s**−**y** and **v**−**y** pairings. Thus, the substrates of **y** and 5-modified **y** bases can be site-specifically incorporated into RNA, opposite **s** or **v** in DNA templates, by T7 transcription.[9] In particular, the **v**−**y** pair exhibits high efficiency and selectivity and is suitable for the incorporation of modified **y** substrates linked with a large functional group at position 5.[9c]

Here, we report the syntheses of ribonucleoside 5′-triphosphates of 5-fluorophore-linked **y** bases (Figure 1b) and their

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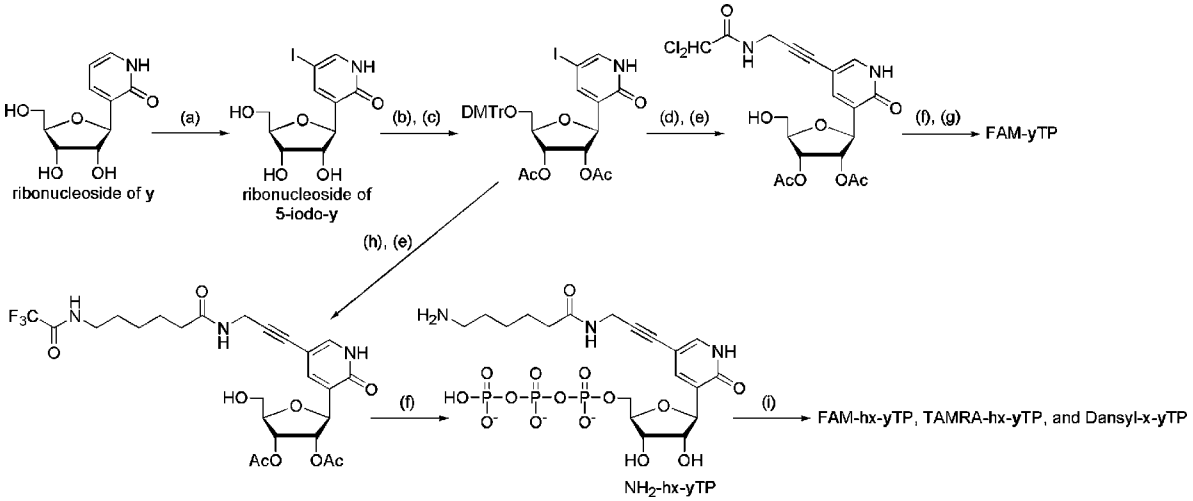
Scheme 1. Synthesis of the Nucleoside 5′-Triphosphates of 5-Fluorophore-Linked **y** Bases[a]



[a] Conditions: (a) *N*-iodosuccinimide, CH_3CN, DMF, 80 °C, 2 h; (b) DMTr-Cl, pyridine, room temperature; (c) (i) $(CH_3CO)_2O$, pyridine, room temperature, (ii) C_2H_5OH, reflux; (d) CuI, $Pd[P(C_6H_5)_3]_4$, DMF, triethylamine, room temperature, then 2,2-dichloro-*N*-prop-2-ynyl-acetamide; (e) $CHCl_2COOH$, CH_2Cl_2, 0 °C; (f) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one/dioxane, tri-*n*-butylamine, bis(tri-*n*-butylammonium)pyrophosphate, I_2/H_2O/pyridine, dioxane, pyridine, room temperature, then NH_4OH; (g) FAM-*N*-hydroxysuccinimidyl ester/DMF, 0.1 M $NaHCO_3-Na_2CO_3$ buffer (pH 8.5), room temperature, then NH_4OH; (h) CuI, $Pd[P(C_6H_5)_3]_4$, DMF, triethylamine, room temperature, then *N*-(2-propynyl)-6-trifluoroacetamidohexanamide; (i) R-*N*-hydroxysuccinimidyl ester (R = FAM or TAMRA)/DMF, 0.1 M $NaHCO_3-Na_2CO_3$ buffer (pH 8.6), room temperature, 3.5 h, then NH_4OH or Dansyl-x-*N*-hydroxysuccinimidyl ester/DMF, 0.1 M sodium borate buffer (pH 8.5), room temperature, 48 h, then NH_4OH.

site-specific incorporation into RNA by T7 transcription, using DNA templates containing **s** or **v**. We also describe the application of this method to the fluorescent labeling of two different RNA aptamers.

Results and Discussion

Ribonucleosides of 5-fluorophore-linked **y** bases were synthesized via the nucleoside of 5-iodo-**y** (Scheme 1). Iodination of the nucleoside of **y** was performed by using *N*-iodosuccinimide, and the yield (75%) was improved as compared to our previously reported yield (34%), which was obtained by using iodine and potassium iodide.[9a] Then, the 5′- and 2′,3′-hydroxyl groups of the nucleoside of 5-iodo-**y** were protected with a dimethoxytrityl group and acetyl groups, respectively. The fully protected nucleoside was reacted with 2,2-dichloro-*N*-prop-2-ynyl-acetamide or *N*-(2-propynyl)-6-trifluoroacetamidohexamide by palladium-catalyzed coupling, using copper(I) iodide and triethylamine.[10] After deprotection of the 5′-dimethoxytrityl group, both nucleoside derivatives of 5-[3-(2,2-dichloroacetamido)-1-propynyl] **y** and 5-[3-(6-trifluoroacetamidohexanamido)-1-propynyl] **y** were converted to 5′-triphosphates by a conventional method.[11] The acyl protecting groups in these compounds were fully removed with concentrated ammonia, and the triphosphate of 5-(3-amino-1-propynyl) **y** or 5-[3-(6-aminohexanamido)-1-propynyl] **y** (NH_2-hx-**y**TP) was obtained. The triphosphates were treated with the *N*-hydroxysuccinimidyl esters of fluorescent derivatives, 5-carboxyfluorescein (FAM), 5-carboxytetramethylrhodamine (TAMRA), and 6-[(5-dimethylaminonaphthalene-1-sulfonyl)amino]hexanoic acid (Dansyl-x). The products were purified by DEAE Sephadex column chromatography and C18 reversed-phase HPLC, and the triphosphates of 5-(3-amino-1-propynyl) **y** linked with FAM (FAM-**y**TP) or 5-[3-(6-aminohexanamido)-1-propynyl] **y** linked with FAM (FAM-hx-**y**TP), TAMRA (TAMRA-hx-**y**TP), or Dansyl-x (Dansyl-x-**y**TP) were each obtained. The structures of the products were confirmed by ^1H and ^{31}P NMR and mass spectrometry (see the Supporting Information). The molar absorption coefficients (ϵ) determined by quantitative analysis of the phosphorus in the compound,[12] the emission maxima, and the quantum yields of each triphosphate are shown in Table 1 and Figure 1 in the Supporting Information.

Table 1. Absorption and Emission Maxima and Quantum Yields of Nucleoside 5′-Triphosphates of 5-Fluorophore-Linked **y** Bases[a]

compounds	absorption maxima (nm)	emission maxima (nm)	quantum yield
FAM-hx-**y**TP	493 (ϵ 62 000)	522 (excited at 493 nm)	0.55
TAMRA-hx-**y**TP	553 (ϵ 85 000)	578 (excited at 553 nm)	0.50
FAM-**y**TP	493 (ϵ 70 000)	521 (excited at 493 nm)	0.67
Dansyl-x-**y**TP	252 (ϵ 31 400), 319 (ϵ 10 600)	387, 541 (excited at 320 nm)	0.014

[a] The data were measured using a 1 μM concentration of the compound in 10 mM sodium phosphate buffer (pH 7.0) at 25 °C.

The incorporation efficiency and selectivity of these triphosphates into RNA were examined by gel electrophoresis of ^{32}P-labeled transcripts (17-mer) of DNA templates (35-mer) containing **v**, **s**, or A at position +13 (Figure 2 and Figure 2 in the Supporting Information). T7 transcription was performed using 1 mM natural NTPs and fluorophore-linked **y**TP with [γ-^{32}P] GTP, at 37 °C for 3 h (Figure 2a). With the incorporation of the fluorophore-linked **y**, the full-length transcripts (17-mer) exhibited large mobility shifts on the gel (Figure 2b, lanes 1, 3, 5, and 7). Furthermore, in the transcription using the template containing **v**, no band corresponding to the native 17-mer was observed (Figure 2b, lane 9), and in the transcription using the native template with fluorescent-linked **y**TP, there was no band corresponding to the transcripts containing fluorophore-linked **y** bases (Figure 2b, lanes 2, 4, 6, and 8). These results strongly suggest that these fluorophore-linked **y**TPs were not incorporated

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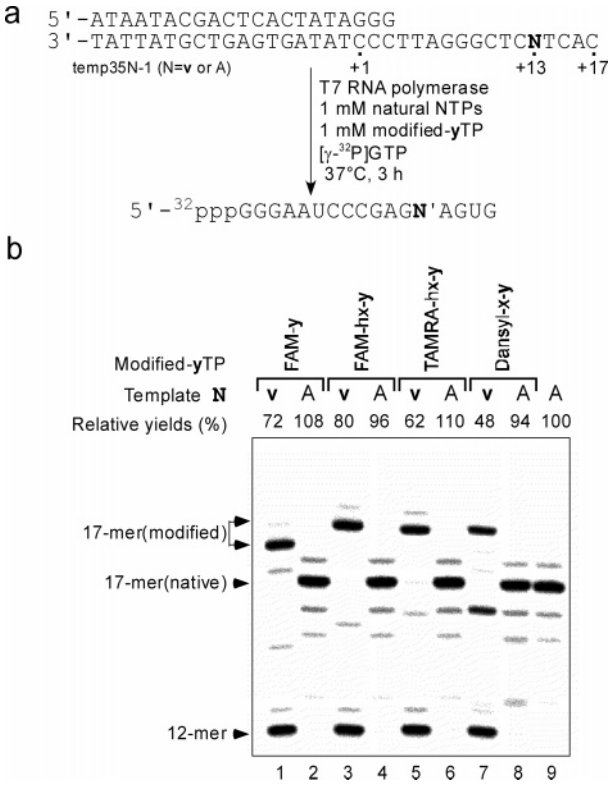

Figure 2. T7 transcription mediated by the **v**−**y** pair. (a) Schemes of the experiments. (b) Gel electrophoresis of transcripts using the templates (N = **v** or A) with the natural NTPs (1 mM) and each of the substrates of 5-fluorophore-linked **y** (1 mM). Transcripts were labeled with [γ-^{32}P]GTP. The relative yields (lanes 1−8) of each transcript were determined by comparison to the yield of the native transcript from the template consisting of the natural bases (lane 9), and each yield was averaged from three data sets.

into RNA opposite the natural bases and were site-specifically incorporated opposite **v** in the template. In addition, since the site-specific incorporation of **y**TP opposite **v** or **s** was confirmed by a nucleotide-composition analysis of the transcripts,[8] the fluorophore-linked **y**TPs might also be incorporated at the desired position. Although truncated transcripts (12-mer) were observed, the full-length products (17-mer) containing the fluorophore-linked **y** transcribed from the **v** template were obtained with relatively high yields (48−80% relative to that of the transcript with natural bases only; Figure 2, lane 9). The yields of the transcription mediated by the **v**−**y** pair were higher than those mediated by the **s**−**y** pair (Figure 2 in the Supporting Information).

The triphosphate of 3-(6-aminohexanamido)-1-propynyl **y** (NH_2-hx-**y**TP) was also site-specifically incorporated into RNA with a good yield (Figure 2 in the Supporting Information). The high selectivity of the NH_2-hx-**y** incorporation was confirmed by the mobility shift of the transcript on the gel (Figure 2 in the Supporting Information) and by the nucleotide-composition analysis (Figure 3 in the Supporting Information). This substrate is useful for posttranscriptional modifications at its incorporation sites in RNA molecules. To test the modification of the transcripts, the 5′-^{32}P-labeled 17-mer fragment, containing NH_2-hx-**y** at position 13, was treated with excess amounts of 5-carboxyfluorescein *N*-hydroxysuccinimidyl ester (FAM-SE) or 5-carboxytetramethylrhodamine *N*-hydroxysuccinimidyl ester (TAMRA-SE) (0−20 mM) in a buffer (pH 8.3) at 37 °C for 12 h, and the products were analyzed on a gel (Figure 3). Although

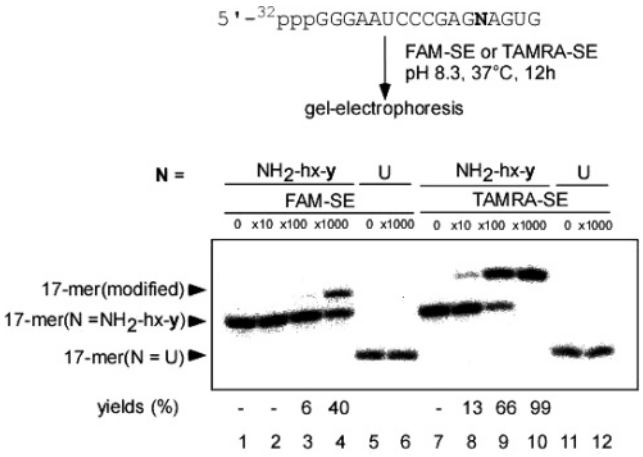

Figure 3. Posttranscriptional fluorophore labeling of an RNA fragment containing NH_2-hx-**y**. A 5′-^{32}P-labeled RNA fragment (17-mer) containing NH_2-hx-**y** (20 μM) was labeled with 5-carboxyfluorescein *N*-hydroxysuccinimidyl ester (FAM-SE) or 5-carboxytetramethylrhodamine *N*-hydroxysuccinimidyl ester (TAMRA-SE) in 0.1 M sodium tetraborate−HCl buffer (pH 8.3). The efficiency (%) of the modification was calculated from the intensities of the bands corresponding to the modified 17-mer and the original 17-mer.

the efficiency of this internal modification of the fragment was relatively lower than that of a terminal modification, such as the N^6 modification of a 5′-terminal adenosine of an RNA fragment,[5] the NH_2-hx-**y** site in the 17-mer was completely modified in the presence of 1000 molar equivalents of TAMRA-SE (Figure 3, lane 10). The native RNA fragment was not modified under the same conditions (Figure 3, lanes 6 and 12), indicating that the NH_2-hx-**y** site was selectively modified with these reagents.

The site-specific fluorescent-labeling of RNA was applied to the detection of RNA−protein interactions using an anti-(Raf-1) RNA aptamer, which specifically binds to the human Raf-1 protein and inhibits the interaction between Raf-1 and Ras.[13] From a vector encoding the original anti-(Raf-1) RNA aptamer, the template containing **v** was prepared and amplified by PCR, using a 3′-primer containing **v** (Figure 4a). During T7 transcription, FAM-hx-**y**TP was incorporated at position 90 of the aptamer (100-mer), and the transcript was purified by gel electrophoresis. To monitor the binding ability of the aptamer to the protein, we performed a filter-binding assay with the fluorescently labeled aptamer and the Ras-binding domain of Raf-1 (Raf-1 RBD) (Figure 4b). The RNA aptamer (10 nM) was incubated with various concentrations (47−975 nM) of the Raf-1 RBD at room temperature for 30 min. The RNA−protein complex was isolated by filtration on modified nitrocellulose filters, and the fluorescent intensity on the filter was measured with a Bio-Rad Molecular Imager FX Pro system (Figure 4b). Even in the binding with 50 nM Raf-1 protein (2.5 pmol in 50 μL), the FAM-labeled aptamer could be detected, and the dissociation constant (K_d = 184 ± 46 nM) was determined without any activity loss of the aptamer (Figure 4c). Thus, this site-specific fluorescent labeling of RNA will be useful as a nonradiolabeling method for the detection of target molecules bound with fluorescently labeled RNA aptamers.

Next, to examine the potential of this site-specific fluorescent labeling, we introduced the fluorophore-linked **y** into another

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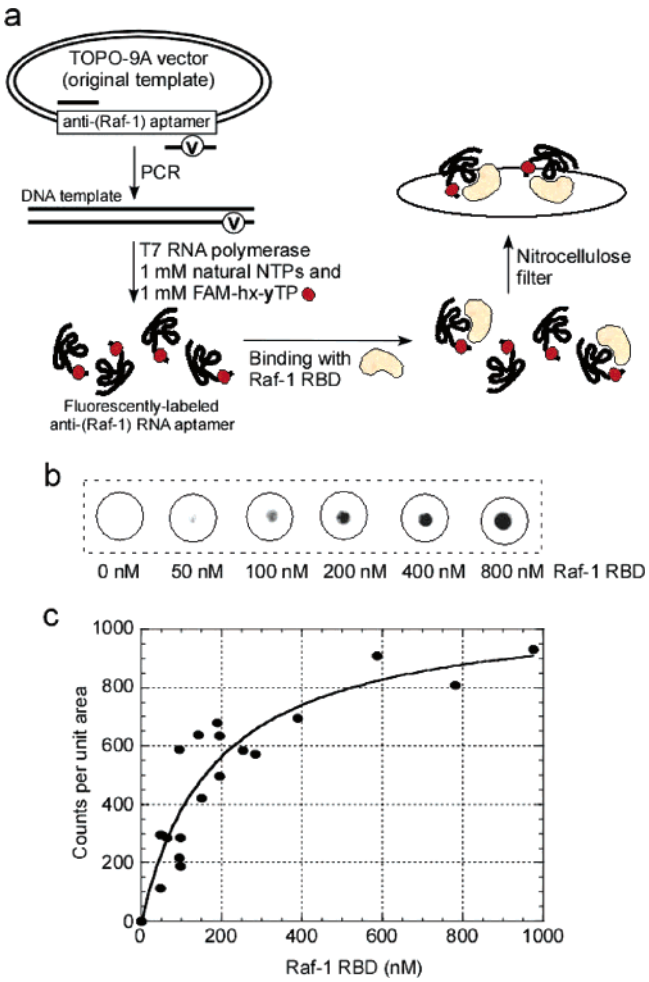

Figure 4. Site-specific FAM-hx-**y** labeling of the anti-(Raf-1) RNA aptamer and the binding of the aptamer to the Raf-1 RBD. (a) Scheme of the site-specific fluorescent-labeling of the anti-(Raf-1) RNA aptamer and its filter-binding assay with the Raf-1 RBD. (b) The fluorescent intensity of the RNA aptamer bound to the Raf-1 RBD on the filters. A Bio-Rad Molecular Imager FX Pro system was used to excite the samples at 488 nm and to detect the fluorescence at 530 nm. (c) Binding curves for the RNA aptamer with the Raf-1 RBD.

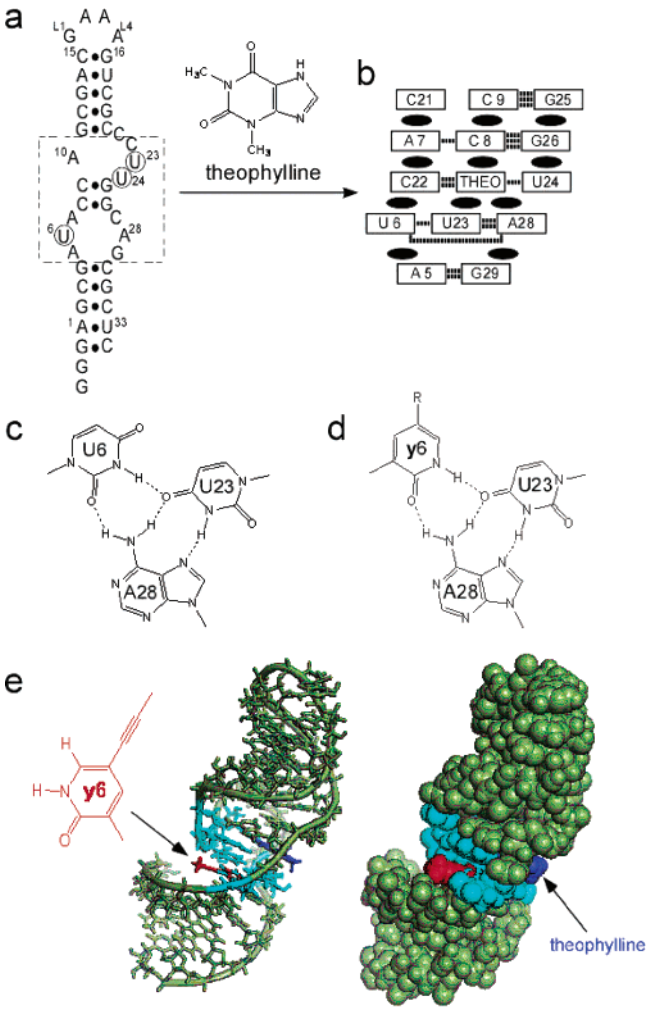

Figure 5. Structure of the theophylline-binding RNA aptamer: the secondary structure (a), the structural interactions in the binding site (b), the U6-U23-A28 base triplet (c), the **y**6-U23-A28 base triplet (d), and the 5-propynyl-**y** base superposition at the U6 position in the aptamer−theophylline complex (PDB 1O15) (ref 14f) (e). The 5-propynyl-**y** base is shown in red, the theophylline is in blue, and the U6, C22, U23, U24, and A28 nucleotides are in cyan.

RNA aptamer that specifically binds to the bronchodilator theophylline[2c,14] (Figure 5a). Fluorophores at a specific position in RNA molecules are useful for detecting conformational changes by measuring the alterations in the fluorescent intensity, as well as for sensing target molecules. Theophylline binding causes a unique structural motif (Figure 5b) to appear in the RNA aptamer. In the complex, two base triplets, U6-U23-A28 (Figure 5c) and A7-C8-G26, and two independent bases, C22 and U24, surround the theophylline and form a highly specific ligand-binding site, but this site is not stably formed in the absence of the ligand.[2c,14] Jucker et al. introduced a fluorescent adenine analogue, 2-aminopurine, into position 27 of the aptamer to analyze the theophylline binding by stopped-flow fluorescence spectroscopy.[2c] Thus, we sought an appropriate position that could be replaced with fluorophore-linked **y**, and the theophylline binding was monitored by the change in the fluorescence intensity.

To determine the appropriate site in the theophylline-binding aptamer for the introduction of fluorophore-linked **y**, we first examined the effects of an unmodified **y** substitution for U at position 6, 23, or 24 in the binding motif of the aptamer. Each aptamer (41-mer) containing **y** was prepared by T7 transcription using DNA templates containing **s** and **y**TP (Figure 4 in the Supporting Information). The site-specific incorporation of **y** into the aptamers was confirmed by a nucleotide-composition analysis using 2D TLC (Figure 4 in the Supporting Information). The dissociation constants (K_d) of the aptamer binding to theophylline (Table 2 and Figure 5 in the Supporting Information) were determined by a published equilibrium method,[14a] using ^{3}H-labeled theophylline in a buffer containing 100 mM HEPES (pH 7.3), 50 mM NaCl, and 5 mM $MgCl_2$. The **y** substitution at U23 or U24 significantly reduced the binding abilities of the aptamers (K_d > 1500 nM), as compared with that of the original aptamer (K_d = 331 ± 16 nM). As for the replacement of U23, the 4-keto group of U23 forms hydrogen bonds with both U6 and A28 in the base triplet. Since the

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Table 2. Theophylline Binding of RNA Aptamers Containing Modified **y** Bases

unnatural base	inserted positon	K_d [nM][a]	K_f [nM][b]
FAM-**y**	U6	206 ± 16	510 ± 64
FAM-hx-**y**	U6	192 ± 11	566 ± 115
TAMRA-hx-**y**	U6	~1000	488 ± 107
Ph-**y**	U6	536 ± 42	
I-**y**	U6	551 ± 68	
y	U6	217 ± 13	
y	U23	>1500	
y	U24	>1500	
unmodified		331 ± 16	

[a] The dissociation constant values for theophylline binding were measured with equilibrium filtration assays using ^{3}H-labeled theophylline (20 nM) and various RNA concentrations (25−1500 nM). [b] The equilibrium constant values for theophylline binding were determined from the equilibrium fluorescence of 100 nM RNA containing fluorophore-linked **y**, obtained at various theophylline concentrations (0−20 μM).

unnatural **y** base lacks the 4-keto group of U, the replacement of U23 with **y** might be unfavorable for the binding. However, no interaction of the 4-keto group of U24 was detected in the NMR analysis,[14b,c] but the 3-imino group of U24 directly interacted with the theophylline. This suggests that the 4-keto group of U24 has an important role in forming the binding motif, and U24 actually takes part in the metal binding and the U-turn formation in the aptamer structure.[14b,d] On the other hand, the aptamer in which U6 was replaced with **y** efficiently bound to theophylline, with a dissociation constant of 217 ± 13 nM. This indicates that the removal of the U6's 4-keto group does not adversely affect the binding ability of the aptamer. Actually, this 4-keto group is not involved in the U6-U23-A28 base triplet in the aptamer.[14b,c] In addition, the introduction of 5-modified **y** bases, such as 5-phenylethynyl-2-oxo(1H)pyridine (Ph-**y**)[9b] and 5-iodo-2-oxo(1H)pyridine (I-**y**),[9a] into the aptamer in place of U6 still maintained the aptamer's binding ability (K_d = 536 ± 42 nM for the Ph-**y** replacement and K_d = 551 ± 68 nM for the I-**y** replacement, Table 2 and Figure 5 in the Supporting Information). Interestingly, the K_d value of the aptamer modified with **y** at position 6 (K_d = 217 ± 13 nM) was slightly lower than that of the unmodified aptamer (K_d = 331 ± 16 nM). Thus, the substitution of U6 with **y** allows the formation of the base triplet to capture the theophylline (Figure 5d), and the U6 position might be a suitable site for the introduction of a fluorophore-linked **y**.

We prepared aptamers containing FAM-**y**, FAM-hx-**y**, or TAMRA-hx-**y** at the U6 position by T7 transcription using the DNA template containing **s** and examined the binding of theophylline. The K_d values of the binding of aptamers containing FAM-**y** or FAM-hx-**y** to ^{3}H-labeled theophylline were approximately 200 nM (Table 2), which is the same as that of the unmodified and U6-modified aptamers. In addition, the modeling of the aptamer[14f] with U6 replaced by 5-propynyl-**y** showed that the propynyl group protrudes from the major groove of the structure (Figure 5e). These results suggest that the introduction of the large group at the U6 position did not affect the binding ability. In contrast, the binding ability of the aptamer containing TAMRA-hx-**y** to ^{3}H-labeled theophylline was significantly reduced (Table 2 and Figure 5 in the Supporting Information).

We then measured the fluorescent intensities of the aptamers in the presence or absence of theophylline, using an FP-6500 spectrofluorimeter, by monitoring the fluorescent emission. The fluorescent intensities of the aptamers containing FAM-**y**, FAM-hx-**y**, or TAMRA-hx-**y** increased depending on the amount of theophylline and doubled with the addition of saturating concentrations of theophylline (5−20 μM) (Figure 6). Although the K_d value of the TAMRA-hx-**y** aptamer to ^{3}H-labeled theophylline was very high, an increase in the fluorescent intensity of the aptamer was observed (Figure 6d). These fluorescent intensity alterations might be caused by a conformational rearrangement of the aptamer upon binding to the target theophylline. In the absence of theophylline, these fluorescent residues might reside inside the aptamer and stack with the neighboring bases, and thus, the stacking would reduce the fluorescent intensity. Upon theophylline binding, the fluorescent residues might move outside and become exposed to the solvent, increasing the fluorescent intensity.

The equilibrium constant values obtained from the fluorescent intensity alterations of the aptamers (designated by K_f) (Figure 6b and Table 2) were somewhat different from the dissociation constant values (K_d) obtained by using ^{3}H-labeled theophylline. In the case of the aptamers containing FAM-**y** and FAM-hx-**y**, the K_f values were larger than their K_d values. This suggests that when theophylline, at concentrations around 0.2−0.5 μM, binds to the aptamer, the fluorophores still stack with the neighboring bases inside the structure. In contrast, the K_f value of the aptamer containing TAMRA-hx-**y** was one-half of its K_d value. This might be because the sterically hindered dimethylamino group of TAMRA facilitates the movement of the TAMRA residue to the outside, by the weak theophylline binding, but also reduces the binding ability. In either case, the K_f values of the aptamers depend on the environmental changes of the fluorophore residues induced by a certain concentration of theophylline, and thus, the values are practically useful for estimating the detection sensitivity of the aptamer as a sensor.

The fluorescently labeled aptamers retained their high specificity for theophylline. The fluorescent intensities of the aptamers were not enhanced in the presence of an excess amount (20 μM) of caffeine, which is structurally similar to theophylline (Figure 6, parts a, c, and d). In particular, no significant intensity alteration of the aptamer labeled with FAM-**y** was observed with the addition of 20 μM caffeine (Figure 6a). The sensitivity of this method is much higher than those of modular aptamer sensors and fluorescing molecular switches.[15] Although the introduction of another fluorophore, 2-aminopurine, at position 27 of the theophylline-binding aptamer also produced a useful probe of the binding,[2c] the preparation of the aptamer containing 2-aminopurine requires chemical synthesis. Thus, the transcription system using the unnatural base pairs is the simplest approach for preparing RNA molecules containing fluorescent probes at desired positions, and the aptamers site-specifically labeled with the fluorophore-linked **y** bases could selectively and efficiently detect their target molecules.

In this study, we have described the site-specific fluorescent labeling of RNA molecules by transcription using unnatural base pairs. Several fluorophore-linked **y**TPs were chemically synthesized from the nucleoside of **y**. These fluorophore-linked **y**TPs can be site-specifically incorporated into RNA molecules by T7 RNA polymerase, using DNA templates containing **s** or

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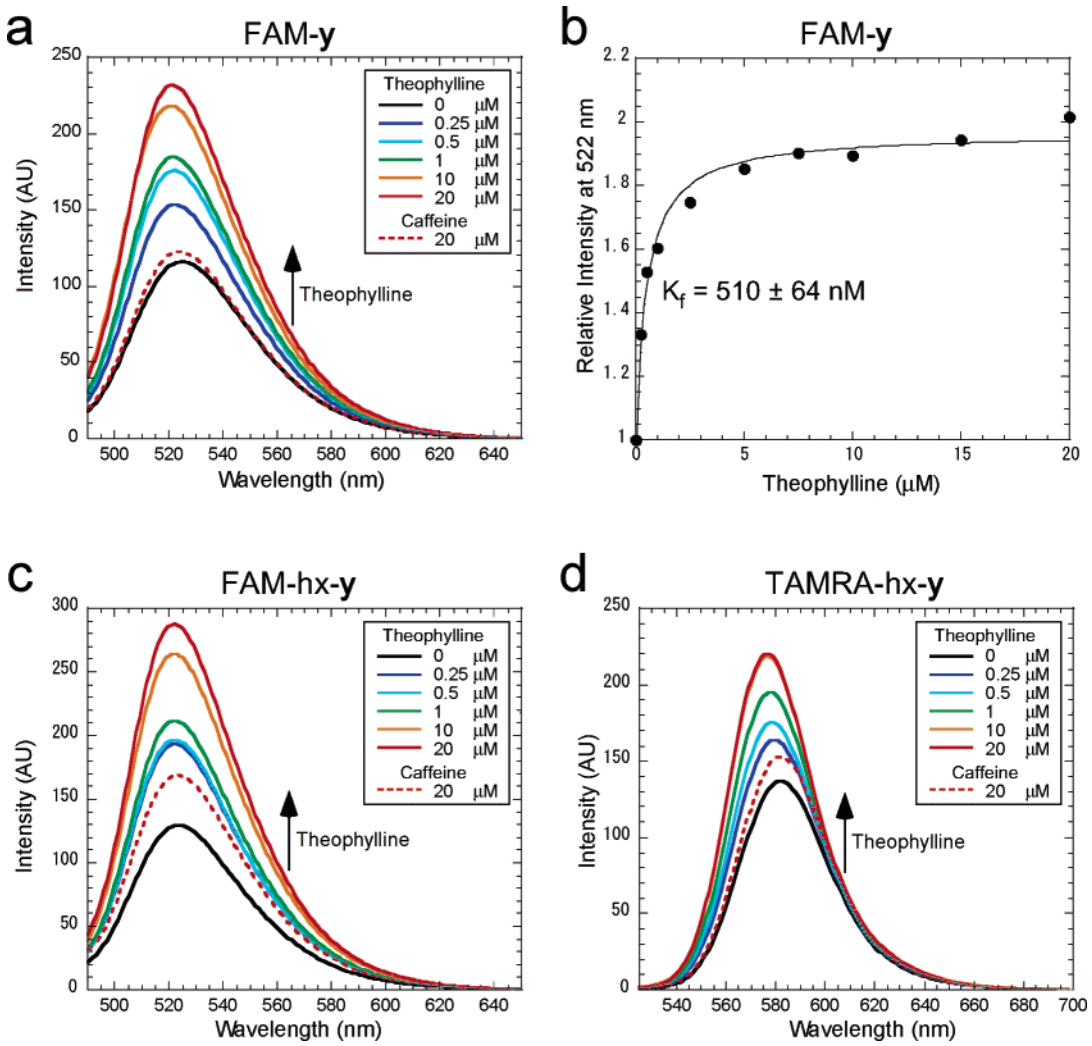

Figure 6. Fluorescence spectra of the theophylline-binding RNA aptamers site-specifically labeled with FAM-**y** (a), FAM-hx-**y** (c), or TAMRA-hx-**y** (d), in the presence of increasing amounts of theophylline (0−20 μM) or caffeine (20 μM). Fluorescence intensity at 522 nm vs concentration curves for theophylline binding with the FAM-**y**-labeled RNA aptamer (b).

v. The RNA aptamers labeled with the fluorophore-linked **y** bases at specific positions retained the aptamer's binding ability, and the binding to their target molecules was detected by the fluorescent intensity.

As shown in the labeling of the anti-(Raf-1) RNA aptamer, the 3′-regions of RNA aptamers are suitable as labeling sites with the fluorophore-linked **y**TPs for the detection of the aptamers. This is because in the in vitro selection, the 3′-regions of RNA pools are hybridized with primers during reverse transcription and PCR, and thus, the 3′-regions of the RNA aptamers generally do not participate in the binding to their target molecules. In addition, DNA templates containing the unnatural **s** or **v** bases can be easily prepared from the original templates by PCR, using a 3′-primer containing **v** or **s** (Figure 4a). In this regard, this 3′-labeling method might be effective in a recently developed in vitro selection method using capillary electrophoresis and an RNA pool that is labeled with FAM at the 5′-end.[16] This is because the tertiary structures of RNA aptamers often involve the 5′ region, and thus for the fluorescent labeling of RNA aptamers, their 3′ regions may be better than the 5′ regions.

Furthermore, as shown in the site-specific labeling of the theophylline-binding RNA aptamer, the site-specific incorporation of the fluorophore-linked **y** bases into RNA would be a powerful tool for the detection of interactions between RNA molecules and their ligands. Conformational changes are important for many RNA molecules that bind ligands with high affinity and specificity, causing alterations of the environments of some nucleotides at specific sites in the RNA molecules. These alterations can be detected by the intensity changes of the fluorophores at the specific sites. In addition, by combining this method with other 5′- or 3′-labeling methods,[3−5] different fluorophores can be incorporated into the same strand of a RNA molecule, which would be useful for FRET applications. Thus, this site-specific labeling method using an unnatural base pair system will be useful for analyzing the local conformational changes of RNA molecules and for detecting the interactions between RNA molecules and binding species.

Materials and Methods

General. Reagents and solvents were purchased from standard suppliers and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) using 0.25 mm silica gel 60 plates impregnated with 254 nm fluorescent indicator (Merck).

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Nucleosides and triphosphates were purified with a Gilson HPLC system using an analytical column (Synchropak RPP, 250 mm × 4.6 mm, Eichron Technologies) or a preparative C18 column (Waters Microbond Sphere, 150 mm × 19 mm). ^{1}H (270 and 300 MHz), ^{13}C (68 MHz), and ^{31}P NMR (109 MHz) spectra were recorded on JEOL EX270 and Bruker AV300 magnetic resonance spectrometers. High-resolution mass spectra (HRMS) were recorded on a JEOL HX-110 or JM-700 mass spectrometer.

Oligodeoxynucleotide Synthesis. The phosphoramidite derivatives of **v** and **s** were prepared as described.[7,8] DNA fragments were synthesized on an Applied Biosystems 392 DNA synthesizer using standard β-cyanoethyl phosphoramidite chemistry. For the synthesis of DNA fragments containing **v**, base-labile protection, using phenoxyacetyl for A, *p*-isopropylphenoxyacetyl for G, and acetyl groups for C, was used,[17] and these phosphoramidites of the natural bases were purchased from Glen Research (Virginia). In addition, the acetic anhydride for the capping step was replaced by phenoxyacetic anhydride in the reagent.[18] DNA fragments were purified by gel electrophoresis.

3-(β-D-Ribofuranosyl)-5-iodo-2-oxo(1H)pyridine. 3-(β-D-Ribofuranosyl)-2-oxo(1H)pyridine (378 mg, 1.66 mmol) was dissolved in CH_3CN and evaporated to dryness in vacuo twice. The residue was dissolved in DMF (3.3 mL) and CH_3CN (3.3 mL) with *N*-iodosuccinimide (748 mg, 3.32 mmol), and the solution was stirred at 80 °C for 2 h. After cooling, 1 M $NaHCO_3$ (3.3 mL) was added to the reaction mixture, and it was evaporated in vacuo. The residue was dissolved in water (17 mL), and then acetic acid (189 μL) and a 5% sodium hydrogen sulfite solution (5 mL) were added. The product (441 mg, 75%) was purified by reversed-phase HPLC (Waters Microbond Sphere model C18, with gradients of 4−15% (6 min) and 15−30% (6 min) CH_3CN in water). The NMR spectra of the product were identical to the published data.[9a]

3-(5-O-Dimethoxytrityl-β-D-ribofuranosyl)-5-iodo-2-oxo(1H)-pyridine. 3-(β-D-Ribofuranosyl)-5-iodo-2-oxo(1*H*)pyridine (178 mg, 0.51 mmol) was coevaporated with dry pyridine three times. The residue was dissolved in pyridine (5 mL) with 4,4′-dimethoxytrityl chloride (183 mg, 0.54 mmol), and the solution was stirred at room temperature for 1.5 h. The solution was partitioned with EtOAc and H_2O. The organic layer was washed with a saturated $NaHCO_3$ solution twice, dried with $MgSO_4$, and evaporated in vacuo. The residue was purified by silica gel column chromatography (0−2% CH_3OH in CH_2Cl_2) to obtain the dimethoxytrityl derivative (272 mg, 82%).

3-(2,3-Di-O-acetyl-5-O-dimethoxytrityl-β-D-ribofuranosyl)-5-iodo-2-oxo(1H)pyridine. A quantity of 3-(5-O-dimethoxytrityl-β-D-ribofuranosyl)-5-iodo-2-oxo(1*H*)pyridine (266 mg, 0.41 mmol) was co-evaporated with dry pyridine three times. The residue was dissolved in pyridine (4 mL) with acetic anhydride (110 μL, 1.17 mmol), and the solution was stirred overnight at room temperature. The solution was partitioned with EtOAc and H_2O. The organic layer was washed with a saturated $NaHCO_3$ solution and brine, dried with $MgSO_4$, and evaporated in vacuo. The residue was dissolved in ethanol (50 mL) and refluxed for 1.5 h. After evaporation, the crude product was purified by silica gel column chromatography (1−4% CH_3OH in CH_2Cl_2) to yield the acetylated derivative (253 mg, 84%).

3-(2,3-Di-O-acetyl-β-D-ribofuranosyl)-5-[3-(2,2-dichloroacetamido)-1-propynyl]-2-oxo(1H)pyridine. 3-(2,3-Di-O-acetyl-5-O-dimethoxytrityl-β-D-ribofuranosyl)-5-iodo-2-oxo(1*H*)pyridine (268 mg, 362 μmol) was dissolved in CH_3CN and evaporated to dryness in vacuo twice. The residue was dissolved in DMF (2 mL) with Cu(I)I (12.4 mg, 65.2 μmol) and $Pd[P(C_6H_5)_3]_4$ (37.7 mg, 32.6 μmol), and after the addition of triethylamine (91 μL, 652 μmol), it was stirred in the dark at room temperature. To the solution, 2,2-dichloro-*N*-prop-2-ynyl-acetamide (162 mg, 978 μmol) in DMF (1.5 mL) was added dropwise, and the solution was stirred at room temperature for 3.5 h. The reaction mixture was diluted with EtOAc. The solution was washed with H_2O and brine, dried with $MgSO_4$, and evaporated in vacuo. 3-(2,3-Di-O-acetyl-5-O-dimethoxytrityl-β-D-ribofuranosyl)-5-[3-(2,2-dichloroacetamido)-1-propynyl]-2-oxo(1*H*)pyridine (251 mg, 89%) was purified from the residue by silica gel column chromatography (0.5−3% CH_3OH in CH_2Cl_2). To a solution of 3-(2,3-di-O-acetyl-5-O-dimethoxytrityl-β-D-ribofuranosyl)-5-[3-(2,2-dichloroacetamido)-1-propynyl]-2-oxo(1*H*)pyridine (251 mg, 323 μmol) in dichloromethane (32 mL) was added dichloroacetic acid (323 μL, 3.92 mmol), and the mixture was stirred at 0 °C for 15 min. The reaction mixture was poured into a saturated $NaHCO_3$ solution and was stirred vigorously. The aqueous layer was extracted with dichloromethane five times. The combined organic layer was dried with $MgSO_4$ and evaporated in vacuo. The product (136 mg, 88%) was purified from the residue by silica gel column chromatography (2−10% CH_3OH in CH_2Cl_2).

3-(β-D-Ribofuranosyl)-5-[3-(fluorescein-5-carboxamido)-1-propynyl]-2-oxo(1H)pyridine 5′-triphosphoric acid (FAM-yTP). 3-(2,3-Di-O-acetyl-β-D-ribofuranosyl)-5-[3-(2,2-dichloroacetamido)-1-propynyl]-2-oxo(1*H*)pyridine (47.5 mg, 0.1 mmol) was dissolved in pyridine and evaporated to dryness in vacuo. The residue was dissolved in pyridine (100 μL) and dioxane (300 μL). Then, a 1 M solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one in dioxane (110 μL, 0.11 mmol) was added. After 10 min, tri-*n*-butylamine (100 μL) and 0.5 M bis(tri-*n*-butylammonium)pyrophosphate in DMF (300 μL, 0.15 mmol) were quickly added to the mixture. The reaction mixture was stirred at room temperature for 10 min. A solution of 1% iodine in pyridine/water (2 mL, 98/2, v/v) was then added. After 15 min, the oxidation was quenched by the addition of a 5% sodium hydrogen sulfite solution (150 μL). The reaction mixture was evaporated in vacuo, and the residue was dissolved in water (10 mL). After 30 min, concentrated ammonia (20 mL) was added to the solution, and the reaction mixture was stirred at room temperature for 5 h and lyophilized. The residue was treated with concentrated ammonia (3 mL) at 55 °C for 3 h to remove the protecting groups and was concentrated. The residue was purified by DEAE Sephadex A-25 column chromatography (1.5 cm × 30 cm, eluted by a linear gradient from 50 mM to 1 M TEAB) to give 3-(β-D-ribofuranosyl)-5-(3-amino-1-propynyl)-2-oxo(1*H*)pyridine 5′-triphosphoric acid. This was dissolved in 0.1 M $NaHCO_3$−Na_2CO_3 (pH 8.6, 4 mL) and was reacted with 5-carboxyfluorescein *N*-hydroxysuccinimidyl ester (FAM-SE) (14.5 mg, 30.6 μmol) in DMF (1 mL) in the dark at room temperature. After 12 h, the reaction mixture was treated with concentrated ammonia (1 mL) for 30 min. The product (10 μmol, 10%) was purified by DEAE Sephadex A-25 column chromatography (1.5 cm × 30 cm, eluted by a linear gradient from 50 mM to 1 M TEAB) and by C18 HPLC (eluted by a linear gradient of 0%−50% CH_3CN in 100 mM TEAA, pH 7.0).

3-(2,3-Di-O-acetyl-5-O-dimethoxytrityl-β-D-ribofuranosyl)-5-[3-(6-trifluoroacetamidohexanamido)-1-propynyl]-2-oxo(1H)pyridine. 3-(2,3-Di-O-acetyl-5-O-dimethoxytrityl-β-D-ribofuranosyl)-5-iodo-2-oxo(1*H*)pyridine (244 mg, 330 μmol) was dissolved in CH_3CN and evaporated to dryness in vacuo twice. The residue was dissolved in DMF (2 mL) with Cu(I)I (13.3 mg, 69.8 μmol) and $Pd[P(C_6H_5)_3]_4$ (36.4 mg, 31.5 μmol), followed by triethylamine (85 μL, 610 μmol), and was stirred in the dark at room temperature. To the solution, *N*-(2-propynyl)-6-trifluoroacetamidohexanamide (240 mg, 908 μmol) in DMF (1.5 mL) was added dropwise, and the solution was stirred at room temperature for 3.5 h. The reaction mixture was diluted with hexane/EtOAc (1:1). The combined solution was washed with H_2O and brine, dried with $MgSO_4$, and evaporated in vacuo. The product (255 mg, 88%) was purified from the residue by silica gel column chromatography (0.5−3% CH_3OH in CH_2Cl_2).

3-(2,3-Di-O-acetyl-β-D-ribofuranosyl)-5-[3-(6-trifluoroacetamidohexanamido)-1-propynyl]-2-oxo(1H)pyridine. To a solution of 3-(2,3-di-O-acetyl-5-O-dimethoxytrityl-β-D-ribofuranosyl)-5-[3-(6-trifluoroacetamidohexanamido)-1-propynyl]-2-oxo(1*H*)pyridine (245 mg,

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279 μmol) in dichloromethane (46 mL) was added dichloroacetic acid (470 μL, 5.69 mmol), and the mixture was stirred at 0 °C for 15 min. The reaction mixture was poured into a saturated $NaHCO_3$ solution and was stirred vigorously. The aqueous layer was extracted with dichloromethane 20 times. The combined organic layer was dried with $MgSO_4$ and evaporated in vacuo. The product (119 mg, 74%) was purified from the residue by silica gel column chromatography (0.5−4% CH_3OH in CH_2Cl_2).

3-(β-D-Ribofuranosyl)-5-[3-(6-aminohexanamido)-1-propynyl]-2-oxo(1H)pyridine 5′-triphosphoric acid (NH_2-hx-yTP). 3-(2,3-Di-O-acetyl-β-D-ribofuranosyl)-5-[3-(6-trifluoroacetamidohexanamido)-1-propynyl]-2-oxo(1*H*)pyridine (58.3 mg, 102 μmol) was coevaporated with dry pyridine three times. The residue was dissolved in pyridine (100 μL) and dioxane (300 μL). A 1 M solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one in dioxane (110 μL, 110 μmol) was added to the solution, and then the reaction mixture was stirred at room temperature for 10 min. Tri-*n*-butylamine (100 μL) was added to the reaction mixture, followed by a 0.5 M solution of bis(tri-*n*-butylammonium)pyrophosphate in DMF (300 μL). After 10 min, a 1% solution of iodine/H_2O/pyridine (2 mL) was added to the reaction mixture. After stirring for 15 min, the oxidation was quenched by the addition of a 5% sodium hydrogen sulfite solution (150 μL). Then the reaction mixture was evaporated in vacuo, and the residue was dissolved in H_2O (10 mL). After 30 min, concentrated ammonia (12 mL) was added to the solution, and the reaction mixture was stirred at room temperature for 3 h and lyophilized. The residue was treated with concentrated ammonia (4 mL) at room temperature for 3 h to remove the protecting groups and was lyophilized. The residue was purified by DEAE Sephadex A-25 column chromatography (1.5 cm × 30 cm, eluted by a linear gradient from 50 mM to 1 M TEAB) and by C18 HPLC (eluted by a linear gradient of 0−15% CH_3CN in 100 mM TEAA, pH 7.0).

3-(β-D-Ribofuranosyl)-5-[3-[6-(fluorescein-5-carboxamido)hexanamido]-1-propynyl]-2-oxo(1H)pyridine 5′-triphosphoric acid (FAM-hx-yTP). A 0.1 M $NaHCO_3$−Na_2CO_3 buffer solution (pH 8.6, 1.5 mL) of NH_2-hx-**y**TP (10 μmol) was reacted with FAM-SE (5.5 mg, 11.6 μmol) in DMF (200 μL) in the dark at room temperature. After 3.5 h, the reaction mixture was treated with concentrated ammonia (1 mL) for 2 h. The product (3.0 μmol, 30%) was purified by DEAE Sephadex A-25 column chromatography (1.5 cm × 30 cm, eluted by a linear gradient from 50 mM to 1 M TEAB) and by C18 HPLC (eluted by a linear gradient of 0−50% CH_3CN in 100 mM TEAA, pH 7.0).

3-(β-D-Ribofuranosyl)-5-[3-[6-(tetramethylrhodamine-5-carboxamido)hexanamido]-1-propynyl]-2-oxo(1H)pyridine 5′-triphosphoric acid (TAMRA-hx-yTP). A 0.1 M $NaHCO_3$−Na_2CO_3 buffer solution (pH 8.6, 1.2 mL) of NH_2-hx-**y**TP (8 μmol) was reacted with 5-carboxytetramethylrhodamine *N*-hydroxysuccinimidyl ester (TAMRA-SE) (5 mg, 9.5 μmol) in a DMF/H_2O mixture (150/150 μL), in the dark at room temperature. After 3.5 h, the reaction mixture was treated with concentrated ammonia (1 mL) for 2 h. The product (4.0 μmol, 50%) was purified by DEAE Sephadex A-25 column chromatography (1.5 cm × 30 cm, eluted by a linear gradient from 50 mM to 1 M TEAB) and by C18 HPLC (eluted by a linear gradient of 0%−50% CH_3CN in 100 mM TEAA, pH 7.0).

3-(β-D-Ribofuranosyl)-5-[3-[6-[6-[(5-dimethylaminonaphthalene-1-sulfonyl)amino]hexanamido]hexanamido]-1-propynyl]-2-oxo(1H)-pyridine 5′-triphosphoric acid (Dansyl-x-yTP). A solution of NH_2-hx-**y**TP (9 μmol) in a 0.1 M sodium borate buffer solution (pH 8.5, 1.8 mL) was reacted with 6-[(5-dimethylaminonaphthalene-1-sulfonyl)-amino]hexanoic acid *N*-hydroxysuccinimidyl ester (20 mg, 44 μmol) in DMF (1.8 mL), in the dark at room temperature. After 48 h, the reaction mixture was treated with concentrated ammonia (1 mL) for 1 h. The product (6.2 μmol, 68%) was purified by DEAE Sephadex A-25 column chromatography (1.5 cm × 30 cm, eluted by a linear gradient from 50 mM to 1 M TEAB) and by C18 HPLC (eluted by a linear gradient of 0−90% CH_3CN in 100 mM TEAA, pH 7.0).

Emission Spectra and Quantum Yields. Fluorescence spectra of 1 μM triphosphate solutions in 10 mM sodium phosphate buffer (pH 7.0) were measured on a JASCO FP-6500ST spectrofluorimeter at 25 °C. The spectra were collected for the instrument response (1 s), and all slits were set to 3 nm. Fluorescence quantum yields (Φ_f) were calculated by the equation $\Phi_f = (FA_s\eta^2F_s)/(AF_s\eta_0^2)$, where the subscript s refers to the standard and Φ is the quantum yield, F is the corrected, integrated fluorescence, A is the absorption at the excitation wavelength, η is the refractive index of phosphate buffer (1.33), and η_0 is the refractive index of H_2O (1.33). Quantum yield standards were fluorescein (1.0 μM in 0.1 N NaOH, $\Phi_s = 0.90$) and quinine sulfate (10 μM in 1 N H_2SO_4, $\Phi_s = 0.55$).

T7 Transcription. Templates (10 μM) were annealed in 10 mM Tris−HCl buffer (pH 7.6) containing 10 mM NaCl, by heating at 95 °C for 3 min and slow cooling to 4 °C. Transcription was carried out in 40 mM Tris−HCl buffer (pH 8.0), 24 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 0.01% TritonX-100, 1 mM natural NTPs, 1 mM modified **y**TP, 2 μCi [γ-^{32}P]GTP, 2 μM template, and 50 units of T7 RNA polymerase (Takara, Kyoto). After an incubation at 37 °C for 3 h, the reaction was quenched by the addition of a dye solution (20 μL) containing 10 M urea and 0.05% BPB. The mixture was heated at 75 °C for 3 min and was loaded onto a 20% polyacrylamide-7 M urea gel. The products on the gels were analyzed with a Bio-imaging analyzer (BAS2500).

Posttranscriptional Fluorophore Labeling of an RNA Fragment Containing NH_2-hx-y. An RNA fragment (17-mer) containing NH_2-hx-**y** (20 μM, 2.5 μL) was labeled with a DMSO solution of FAM-SE or TAMRA-SE (0 or 0.2−20 mM, 2.5 μL) in 0.1 M sodium tetraborate−HCl buffer (pH 8.3) (15 μL). Reactions were incubated at 37 °C for 12 h. The reaction was quenched by the addition of the dye solution (20 μL). The mixture was heated at 75 °C for 3 min and was loaded onto a 20% polyacrylamide-7 M urea gel. The products on the gels were analyzed with the Bio-imaging analyzer.

Preparation of a Fluorescently Labeled RNA Aptamer. The DNA template for the fluorescently labeled anti-(Raf-1) RNA aptamer (RNA 9A)[13] at position 90 was amplified from the TOPO-9A vector,[13] by PCR using a 5′-end primer (39.45) and a 3′-end primer containing **v** (29.45**v**90) [39.45, 5′-GGTAATACGACTCACTATAGGGAGTG-GAGGAATTCATCG; 29.45**v**90, 5′-GCAGAAGCTT**v**CTGTCGCT-AAGGCATATG]. PCR was carried out in 10 mM Tris−HCl buffer (pH 8.3), with 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 μM each primer, 1 ng/μL TOPO-9A, and 0.025 U/μL Taq DNA polymerase (Takara, Kyoto), on a PTC-100 program thermal controller (MJ Research, Inc., Waltham, MA). The conditions were 15 cycles of denaturation at 94 °C for 15 s, primer annealing at 50 °C for 30 s, and primer extension at 72 °C for 1 min, followed by an incubation at 72 °C for 5 min. After a treatment with Micropure-EZ enzyme removers (Millipore, Billerica, MA), the PCR product was precipitated with ethanol, and then resuspended in water. Transcription was carried out in a buffer (100 μL) containing 40 mM Tris−HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 0.01% Triton X-100, 1 mM natural NTPs, 1 mM FAM-**y**TP, 2.5 μg of the template DNA, and 250 units of T7 RNA polymerase (Takara, Kyoto). After an incubation at 37 °C for 6 h, the reaction was quenched by the addition of the dye solution (100 μL). The mixture was heated at 75 °C for 3 min, and then was loaded onto a 10% polyacrylamide-7 M urea gel. The full-length, fluorescently labeled product was eluted from the gel with water and was precipitated with ethanol.

Filter-Binding Assay. The FAM-labeled RNA aptamer (10 nM final concentration) was incubated with various concentrations (47−975 nM) of the target protein, the Ras-binding domain (RBD) of Raf-1 fused with glutathione S-transferase (Raf-1 RBD), in 60 μL of binding buffer (PBS with 5 mM $MgCl_2$, buffer A), for 30 min at room temperature (22 °C). The solution (50 μL) was then gently vacuum-filtered over a HAWP filter (HAWP01300, Millipore, Billerica, MA) and was washed with 200 μL of buffer A. The filters were exposed and the blots were

quantified using a Bio-Rad Molecular Imager FX Pro system and the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA). The dissociation constant (K_d) was calculated with Kaleidagraph (Abelbeck Software, Reading, PA), by using the general curve fit for $y = A_0A_1/(A_0 + A_2)$, where y is the counts per unit area (mm^2) on the filter, A_0 is the protein concentration, A_1 is the expected counts per unit area (mm^2) on the filter when a fully saturated concentration of the protein is used, and A_2 is the K_d.

Site-Specific Incorporation of y or Its 5-Modified Bases into the Theophylline-Binding Aptamer by T7 Transcription. The chemically synthesized double-stranded DNA templates containing **s** were used for the introduction of **y** or its modified bases (Ph-**y**, I-**y**, and fluorophore-linked **y**) in place of the uridine at position 6, 23, or 24. The sequences of the templates containing **s** are listed in the Supporting Information. The numbering of the RNA aptamer (41-mer) corresponds to that for the construct (33-mer).[13] Templates (10 μM of a 59-mer coding DNA and a 59-mer noncoding DNA) were annealed in a buffer containing 10 mM Tris−HCl (pH 7.6) and 10 mM NaCl, by heating at 95 °C and slow cooling to 4 °C. Transcription was carried out in a buffer containing 40 mM Tris−HCl (pH 8.0), 24 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 0.01% Triton X-100, 1 mM natural NTPs, 1 mM unmodified **y**TP, or 1 mM Ph-**y**TP, or 0.25 mM I-**y**TP, or 1 mM fluorophore-linked **y**TP (FAM-**y**TP, or FAM-hx-**y**TP, or TAMRA-hx-**y**TP), 2 μM template, and 2.5 units/μL of T7 RNA polymerase (Takara, Kyoto). After an incubation at 37 °C for 6 h, the reaction was quenched by adding an equivalent volume of the dye solution. The reaction mixture was heated at 75 °C for 3 min, and the 41-mer transcripts were purified by gel electrophoresis.

Equilibrium Filtration Assays. Theophylline binding by the RNA aptamers containing **y** or its 5-modified bases at specific positions was assessed by the equilibrium filtration assay.[14a] The various concentrations of aptamers (54 μL; final concentration, 25−1500 nM in 100 mM Hepes (pH 7.3), 50 mM NaCl, and 5 mM $MgCl_2$) were mixed with 6 μL of 200 nM ^{3}H-labeled theophylline (Moravek), and the mixtures were incubated at 25 °C for 5 min. The mixture was then placed in a Microcon YM-10 filtration device (Amicon) and was centrifuged. A 10-μL sample of the filtrate was removed, and the radioactivity was determined by scintillation counting. The fraction of the theophylline binding to each RNA aptamer was determined by the difference between the theophylline concentration in the filtrates obtained in the presence and absence of the RNA. The dissociation constant (K_d) for theophylline binding was determined with the program Kaleidagraph using the following formula: $y = M_0M_1/(M_0 + M_2)$, where y is the fraction of theophylline binding, M_0 is the RNA concentration, M_1 is the binding capacity of theophylline, and M_2 is the K_d.

Fluorescent Measurements. Fluorescent profiles of each theophylline-binding aptamer containing FAM-**y** or FAM-hx-**y** or TAMRA-hx-**y** at the U6 position, in the presence of theophylline (0−20 μM) or caffeine (20 μM), were measured using an FP-6500 spectrofluorimeter (JASCO) at 25 °C. For the emission spectra, a 100 nM concentration of the aptamer was used, and the excitation wavelength was set at 434 nm (the aptamer containing FAM-**y** or FAM-hx-**y**) or at 500 nm (the aptamer containing TAMRA-hx-**y**) with a 5-nm spectral bandwidth. The K_f values, determined from the equilibrium fluorescence of each modified aptamer, were obtained with the program Kaleidagraph, using the following formula: $y = 1 + C_0C_1/(C_0 + C_2)$, where y is the relative fluorescent intensity at 522 nm for FAM and at 578 nm for TAMRA (the fluorescent intensity in the absence of theophylline was set to 1), C_0 is the theophylline concentration, C_1 corresponds to the relative increment value at the saturating concentration of theophylline, and C_2 is the K_f.

Acknowledgment. This work was supported by the RIKEN Structural Genomics/Proteomics Initiative (RSGI), the National Project on Protein Structural and Functional Analyses, Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant-in-Aid for Scientific Research (KAKENHI 15350097) from the Ministry of Education, Culture, Sports, Science, and Technology. We are grateful to Dr. Makoto Komiyama, The University of Tokyo, for his support and helpful discussions.

Supporting Information Available: NMR and MS data for the nucleoside derivatives of modified **y** bases; absorption and emission spectra of each nucleoside 5′-triphosphate of the 5-fluorophore-linked **y** base; incorporation experiments of 5-modified **y**TPs by T7 transcription and the nucleotide-composition analyses of the transcripts; binding curves for the modified RNA aptamers; complete list of authors for ref 7. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0542946