

## SYNTHESIS OF PYRENE LABELED RNA FOR FLUORESCENCE MEASUREMENTS

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□ *The fluorophores 1-ethynylpyrene and 1-(p-ethynyl-phenylethynyl)-pyrene were attached to RNA through a Sonogashira cross-coupling with 5-iodocytidine either in solution through phosphoramidite synthesis or via on-column conjugation during solid-phase oligonucleotide synthesis. Six probes with the sequence 5'-CUU UUC UUU CUU-3' were derivatized with both fluorophores, whereby the position of the modified cytidine was varied. Fluorescence measurements showed sensitivity of the pyrene group to its environment in the single strands and corresponding duplexes.*

**Keywords** Pyrene; RNA; fluorescence

### INTRODUCTION

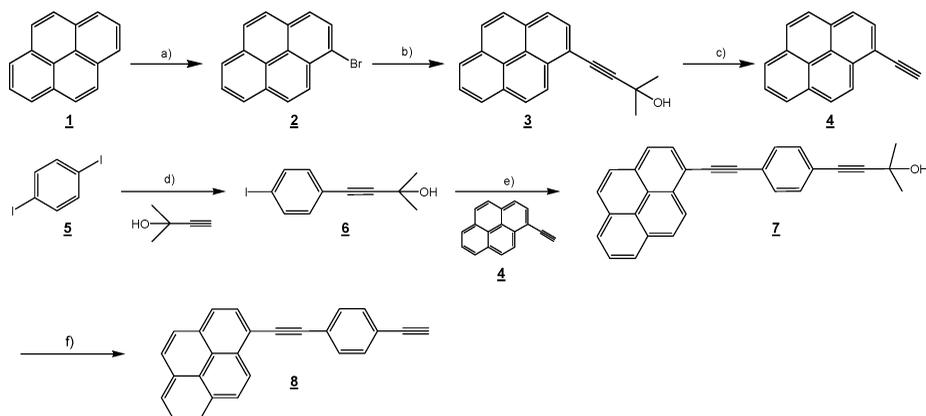
Fluorescence is a powerful tool for the detection and structural investigation of biomolecules like oligonucleotides.<sup>[1]</sup> Selected examples for the use of pyrene modified oligonucleotides include their application as RNA recognition probes,<sup>[2a]</sup> to detect the B-Z-DNA transition in twofold modified oligonucleotides<sup>[2b]</sup> and in the detection of single nucleotide polymorphism.<sup>[2c]</sup> The predictable structure of DNA and RNA can also serve as scaffold for the construction of fluorescent nano-architectures.<sup>[3]</sup> 1-Ethynylpyrene itself was first introduced to the 5-position of 2'-deoxyuridine by Pd-catalyzed cross-coupling, subsequent formation of corresponding phosphoramidite and incorporation into DNA.<sup>[4]</sup>

### SYNTHESIS

#### Fluorophores

1-Ethynylpyrene **4** was synthesized in three steps from pyrene according to procedures described in literature: selective mono-bromination,<sup>[5a]</sup>

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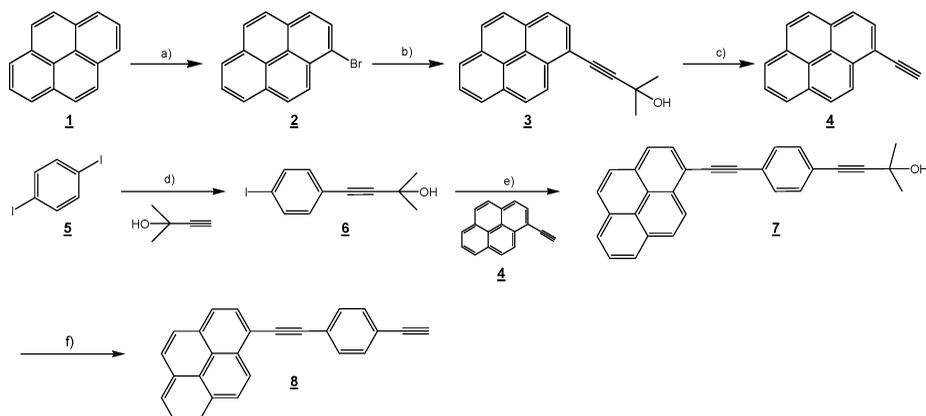
**SCHEME 1** Synthesis of 1-ethynylpyrene **4** and 1-(*p*-ethynyl-phenylethynyl)-pyrene **8**, reaction conditions: a) HBr/H<sub>2</sub>O<sub>2</sub>, MeOH/Et<sub>2</sub>O, 16 hours, rt, 96%; b) 2-Methyl-3-butyn-2-ol, morpholine, 10 hours, 110°C, 72%; c) NaH, toluene, 15 minutes, reflux, 86%; d) (PPh<sub>3</sub>)<sub>2</sub>PdCl<sub>2</sub>, CuI, TEA, toluene, 3 hours, 40°C, 47%; e) (PPh<sub>3</sub>)<sub>2</sub>PdCl<sub>2</sub>, CuI, TEA, THF, 20 hours, rt, 97%; f) NaH, toluene, 15 minutes, reflux, 86%.

Sonogashira coupling with 2-methyl-3-butyn-2-ol<sup>[5b]</sup> and final deprotection of the *tert*-butanol group. 1-(*p*-ethynyl-phenylethynyl)-pyrene **8** was obtained in three steps from diiodobenzene through two successive cross-couplings as described in Scheme 1.

## Phosphoramidites

To introduce these fluorophores into RNA, we used two different methods: A labeled cytidine-phosphoramidite was prepared and incorporated into the oligonucleotide. The multi-step reaction affords the product in an overall yield of 11% (R = **4**) and 13% (R = **8**) starting from protected cytidine. Critical step is the Pd(0) catalyzed cross-coupling between the protected 5-iodocytidine and the corresponding fluorophore (Scheme 2). Indeed, using the usual but bulkier *tert*-dimethylsilyl group as 2'-OH protecting group instead of the cyanoethoxymethyl (CEM) groups<sup>[6]</sup> decreases the yield drastically. The coupling yield in RNA is comparable to the natural bases but the final deprotection of the CEM group with TBAF is only partial, which demands a further separation of both products with anion-exchange HPLC.

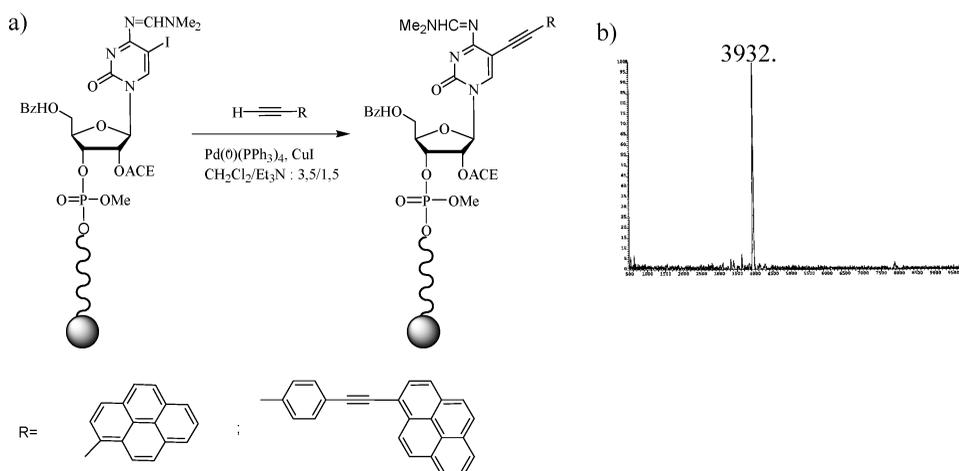
5-iodocytidine phosphoramidite **13** was easily synthesized using the ACE chemistry recently put forward by Dharmacon<sup>[7]</sup> and incorporated into RNA. The fluorophores were post-synthetically introduced during the oligonucleotide solid-phase synthesis, as shown in Figure 1 with the same procedure already used in our laboratory for nitroxides.<sup>[8]</sup> In this case Pd(0) was preferred to Pd(II) and DCM to DMF.<sup>[9]</sup> This method presents several advantages in comparison to the derivatization in solution and was favored:



**SCHEME 2** Synthesis of modified phosphoramidite **12**, reaction conditions: a) abs. pyridine, DMTrCl, rt, 5 hours, 52%; b) 1,2-dichloroethane, cyanoethoxymethylchloride (CEMCl), Bu<sub>2</sub>SnCl<sub>2</sub>, EtN(Pr)<sub>2</sub>, 90 minutes rt, 30 minutes 70°C, 49%; c) **4** or **8**, abs. DMF, 10 mol% Pd(PPh<sub>3</sub>)<sub>4</sub>, 20 mol% CuI, Et<sub>3</sub>N, rt, 24 hours, 90% (**4**), 85% (**8**); d) abs. DCM, DIPEA, 0°C → rt, 3 hours, 47% (**4**), 58% (**8**).

the amount of fluorophore necessary is smaller and the yields are quantitative. Furthermore, the synthesis of only one phosphoramidite is necessary to introduce different and various fluorophores what represents a large gain of time.

Six modified RNAs were synthesized by the method on-column with high purity (Figure 1b). Melting curve measurements showed a noticeable destabilization up to 6°C of the labeled RNAs in comparison to the unmodified duplexes (Table 1) due to the orientation of the bulky pyrene group into the narrow major groove. Yet, due to a dangling end effect<sup>[10]</sup> a slight stabilization of 3.7°C and 2.0°C was observed for RNA 3 and RNA



**FIGURE 1** a) Sonogashira cross-coupling during the solid-phase synthesis (ACE chemistry); b) MALDI of RNA 5, calc. mass: 3933.5 g/mol.

**TABLE 1** Synthesized RNAs with the corresponding masses and  $T_m$ -values. C': cytidine labeled with 1-ethynylpyrene, C\*: cytidine labeled with 1-(*p*-ethynyl-phenylethynyl)-pyrene

RNA	Sequence	Calc. mass	Found mass (MALDI) [m/z]	$T_m$ [°C]	$\Delta T_m$
1	5'-CUU UUC UUU C'UU-3' 3'-GAA AAG AAA GAA-5'	3834.4	3833.0	$38.3 \pm 0.2$	-3.4
2	5'-CUU UUC' UUU CUU-3' 3'-GAA AAG AAA GAA-5'	3834.4	3835.4	$36.1 \pm 0.1$	-5.3
3	5'-C'UU UUC UUU CUU-3' 3'-GAA AAG AAA GAA-5'	3834.4	3837.1	$45.1 \pm 0.2$	+3.7
4	5'-CUU UUC UUU C*UU-3' 3'-GAA AAG AAA GAA-5'	3933.5	3941.0	$35.4 \pm 0.2$	-6.0
5	5'-CUU UUC* UUU CUU-3' 3'-GAA AAG AAA GAA-5'	3933.5	3932.6	$38.2 \pm 0.2$	-3.2
6	5'-C*UU UUC UUU CUU-3' 3'-GAA AAG AAA GAA-5'	3933.5	3933.5	$43.4 \pm 0.2$	+2.0
7	5'-CUU UUC UUU CUU-3' 3'-GAA AAG AAA GAA-5'	3609.1 3936.6	3607.2 3936.0	$41.4 \pm 0.2$	/

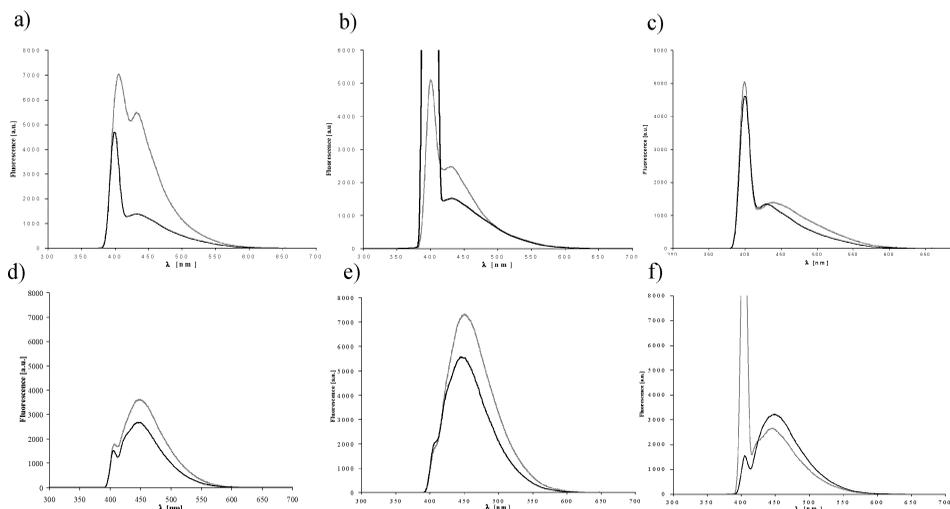
6, respectively, where the fluorophore is situated at the end of the strand and can interact through  $\pi$ - $\pi$  stacking with the neighboring nucleobase of the complementary strand. The CD spectrum of unmodified RNA7 shows maxima at 270 nm (global) and 225 nm (local) and minima at 245 nm (local) and 210 nm (global) typical for A-form RNA oligonucleotides.<sup>[11]</sup> The pyrene labeled RNAs exhibit extrema at the same wavelengths with only slight decrease in intensity.

## FLUORESCENCE SPECTRA

Emission spectra of the single stranded (ss) and double stranded (ds) RNAs were measured in phosphate buffer (140 mM NaCl, 10 mM phosphate, 2.5  $\mu$ M oligonucleotide concentration) to study the influence of the pyrene residues **4** and **8** depending on the position in the strand (Figure 2).

Emission spectra are broadened and red-shifted compared to pyrene monomer Fluorescence (Bande at 400 nm is an artefact and results from scattered excitation). This can be explained with an electronic coupling through the triple bond in the conjugated  $\pi$ -system.<sup>[12]</sup>

Upon hybridisation the fluorescence intensity largely increases especially in the case of 1-ethynylpyrene. Indeed, in the ssRNA the emission is partially quenched through electron transfer between pyrene and the neighboring pyrimidines<sup>[13]</sup> what is not valid anymore after formation of the duplex. Due to the longer phenyl linker this quenching effect is not so pronounced for 1-(*p*-ethynyl-phenylethynyl)-pyrene, in particular when the fluorophore is situated in the middle of the strand (Figure 2e). In the case of dangling ends (RNA3 and RNA6) the fluorescence intensity does not change or even slightly decreases. Indeed the positive effect of hybridisation



**FIGURE 2** Fluorescence spectra of a) RNA1, b) RNA2, c) RNA3, d) RNA4, e) RNA5 and f) RNA6; ssRNA (solid), dsRNA (dashed);  $\lambda_{exc}$  = 395 nm.

is compensated by the electron transfer due to the stacking interaction with the neighboring nucleobase.

## OUTLOOK

The pyrene residues in the presented oligonucleotides are placed in the less accessible major groove of RNA explaining the thermal destabilization of modified oligonucleotides. Using the adenosine 2-position it could be possible to address the more accessible minor groove in the case of RNA. Interactions in twofold pyrene modified oligonucleotides could be used to investigate the kinetics of RNA hybridization and folding.

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