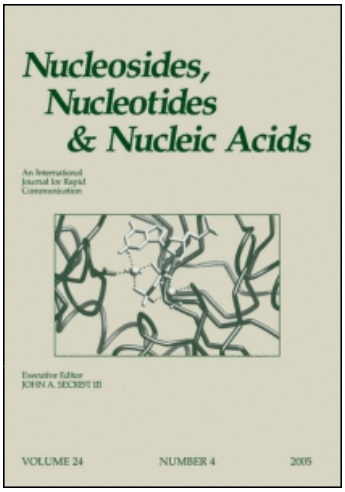

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PHOTOSENSITIZED PREPARATION OF FLUORESCENT LUMINAROSINE AND ANALOGUES[⊗]

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ABSTRACT: Optimized conditions for photosensitized preparation of green-yellow fluorescent (λ_{Em}=530 nm) 7-(β-D-ribofuranosylamino)pyrido[2,1-*h*]pteridin-11-ium-5-olate (luminarosine) and its 2'-deoxy- and 2'-*O*-methyl- analogues, the key compounds in our studies on the synthesis of fluorescence-labeled oligonucleotides, have been developed.

Fluorescent derivatives of nucleobases are desirable in view of the continued widespread use of fluorescence-based techniques in various studies of nucleic acids including non-isotopic detection, within scheme of hybridization and sequence analysis processes[1,2], as well as their structures and dynamics[3,4]. Two major goals remained to be of challenge in the area of nucleic acids stereodynamics: (i) design of the nucleoside fluorophores with appropriate photophysical properties and of non-disruptive character to the structure of interest[3,5] and (ii) sequence-specific introduction of the emitter/acceptor pair of nucleoside fluorophores to the interior of DNA or RNA sequence. Fluorophores absorbing and emitting in the visible region would be of particular interest.

As we have previously described[6], light induced transformation of blue emitting *N*-[9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)purin-6-yl]pyridinium chloride[7], **1a**, led to the

[⊗] article in memoriam of the late Professor Tsujiaki Hata

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formation of green-yellow emitting fluorophore 7-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosylamino)pyrido[2,1-*h*]pteridin-11-ium-5-olate, **3a**, named 2′,3′,5′-tri-*O*-acetylluminarosine. Both the parent riboside (luminarosine) and the aglycone (luminarine) were subjected to detailed structural[6,8] and photophysical[9] studies. The attractive emission properties (λ_{Exc} = 425 nm, λ_{Em} = 530 nm, Φ = 0.65, τ = 8 ns) as well as chemical stability under the conditions for oligonucleotide synthesis, clearly indicated a great potential of luminarosine as a nucleosidic fluorescent probe. Sequence-specific introduction of luminarine fluorophore into oligodeoxynucleotides has already been achieved[10]. Of particular importance with respect to the synthesis of luminarine labeled oligonucleotides are the 2′-deoxy-, **3b**, and 2′-*O*-methyl-, **3c**, analogues of luminarosine.

Although synthesis of 2′,3′,5′-tri-*O*-acetylluminarosine, **3a**, by near UV light irradiation of the corresponding pyridinium salt, **1a,** in aqueous solution (c ≈ 2 mM) at pH ≈ 7.5 under aerobic conditions proceeds reasonably well[6], synthesis of *O*-acetylated 2′-deoxyluminarosine[10], **3b**, and 2′-*O*-methylluminarosine, **3c**, derivatives has been less satisfactory. Irradiation of the pyridinium salts **1b**[10] and **1c** under the above conditions leads to **3b** and **3c** in yields not exceeding 30% and 15%, respectively.

For these purposes, in furthering our approach towards sequence specific introduction of luminarine fluorophore into oligonucleotides, we describe in this paper a new, general and highly efficient procedure for the sensitized photochemical preparation of 2′,3′,5′-tri-*O*-acetylluminarosine **3a** and its 2′-deoxy-, **3b**, and 2′-*O*-methyl-, **3c**, analogues.

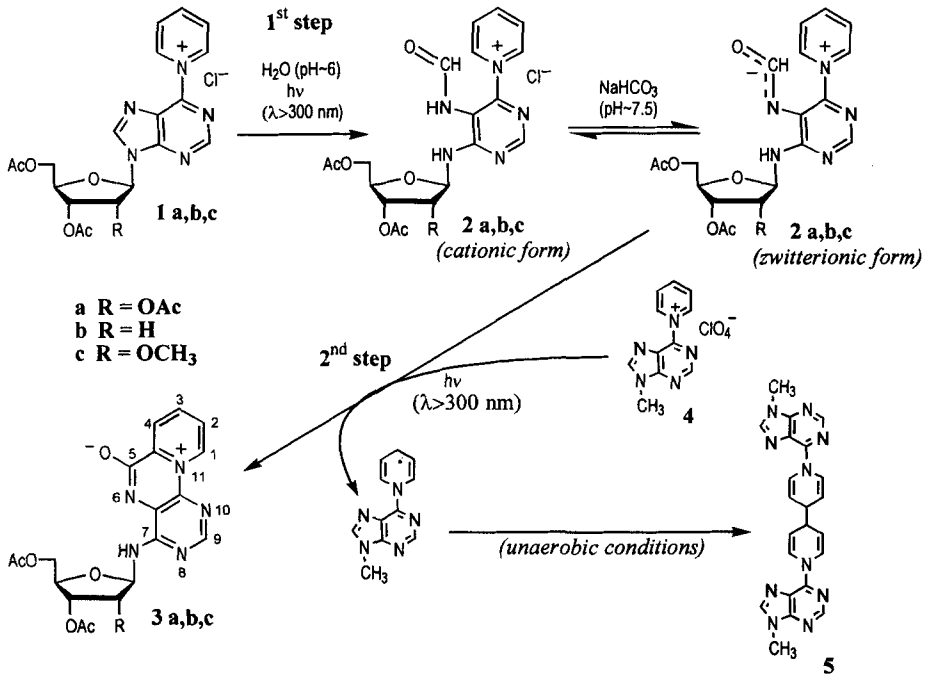
RESULTS AND DISCUSSION

Preparative photochemical synthesis of luminarosine and analogues.

As we have recently shown[11], the phototransformation of **1a** into **3a** occurs in a multistep, pH-dependent process. In the first step, **1a** undergoes light-induced, hydrolytic ring opening in the imidazole portion of the purine ring to form *N*-[5-formamido-6-[(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)amino]pyrimidin-4-yl]pyridinium chloride, **2a**, as a thermally stable intermediate. At pH > 7, this compound exists partly in the electrically neutral, zwitterionic form (pK_a = 7.8), which undergoes electron-transfer-induced ring closure to give luminarosine **3a**. This process is sensitized by the excited triplet state of

1a. Thus in this unique phototransformation **1a** serves both as a substrate and a sensitizer.

Based on those observations, the following improved and general two-step, one-pot procedure for the photosensitized preparative scale synthesis of luminarosine and analogues (**3a-c**) was designed.



In the first step, **1a** was quantitatively converted into **2a** by irradiation of its deoxygenated aqueous solution (c = 0.2 mM) at pH ≈ 6-6.3 (the natural pH of the solution) with near UV light (λ > 300 nm, see experimental for details) as revealed by the UV-spectral and HPLC analyses. Our attempts to increase the concentration of the solution of **1a** to be irradiated resulted in elongation of the irradiation times required for the photoconversion with concomitant, substantial decrease in the yield of **2a**. Similar effects were observed with lowering of the pH of the solution of **1a** from pH 6.2 to 5.0. Thus the above conditions for the transformation of **1a** into **2a** are optimal with respect to both the concentration and the pH of the solution. Once the conversion of **1a** to **2a** was completed, the pH of the irradiated solution was raised to 7.5 with saturated aq. $NaHCO_3$, then, the *N*-(9-methylpurin-6-yl)pyridinium perchlorate, **4** (1.5 molar

TABLE 1. Chemical yields of the first and second steps* of the irradiation of pyridinium compounds **1a-c** and the overall yields of the formation of **3a-c**.

substrate	1st step (**1a-c**→**2a-c**)	2nd step (**2a-c**→**3a-c**)	Overall yields of **3a-c**
1a	>95	>95	90
1b	>95	85	80
1c	60	85	51

*As determined by HPLC

equivalent with respect to **1a**) was added as a sensitizer, and irradiation was continued under continuous flow of nitrogen. We have previously shown[12] that in contrast to light-sensitive *N*-(purin-6-yl)pyridinium compounds in the nucleoside series, the 9-methyl derivative **4**, is photochemically stable under the above conditions of irradiation. However, similarly as **1a**, it undergoes intersystem crossing to the excited triplet state of strong electron accepting abilities. The electron-transfer based sensitizing properties of **4**, have already been demonstrated by us[11] and others[13].

Irradiation of **2a** in the presence of **4**, results in almost complete transformation of the former into desired luminarosine, **3a**, whereas the latter undergoes reductive dimerization typical for pyridinium salts and related charged *N*-heteroaromatic compouds participating in excited state electron transfer processes[14]. The dimeric product **5**[11] which is formed as a result of this process is insoluble in water and can be removed by filtration of the irradiated solution through a membrane filter. Compound **3a** can be isolated in more than 90% overall yield by extraction of the filtrate with chloroform.

This procedure was applied for the phototransformation of **1b** and **1c,** leading to desired *O*-acetylated 2'-deoxyluminarosine, **3b**, and 2'-*O*-methylluminarosine, **3c**, in 80% and 51% overall yields, respectively. The yields of the individual steps, *i.e.* the formation of ring-opened intermediates **2a-c** (1st step), and their subsequent transformation to luminarosine analogues **3a-c** (2nd step) are summarized in Table 1 (see above). As can be seen from Table 1, the lowest yield of **3c** is due to the less efficient transformation of the pyridinium salt **1c** into the ring-opened intermediate **2c**.

Acetylated forms **3a** and **3b** as well as unprotected luminarosine[6] and 2′-deoxyluminarosine[10] were characterized previously. Here spectral data for 2′-*O*-methylluminarosine are presented.

Synthesis of photosensizer, *N*-(9-methylpurin-6-yl)pyridinium perchlorate, 4.

Simple and efficient synthesis of **4,** developed in the course of this work, was based on quantitative N(9)-methylation of the *N*-(purin-6-yl)pyridinium chloride[11], with dimethyl sulfate in 80% aqueous acetonitrile in the presence of sodium bicarbonate, followed by $Cl^- \rightarrow ClO_4^-$ exchange. This approach appeared to be superior to the previously used less efficient, three step procedure involving the 9-methylation of adenine, its deamination to 9-methylhypoxanthine and conversion of the latter to the pyridinium salt **4** (ca. 50 % overall yield). Salts such as **4** are useful intermediates for the preparation of 9-alkyl-6-substituted purines[15].

EXPERIMENTAL

General.

^1H and ^{13}C NMR spectra were recorded on a Varian Unity$^+$ 300 MHz spectrometer. Chemical shifts, δ are reported relative to TMS as internal standard. The UV absorption was measured on a Perkin-Elmer Lambda-17 spectrophotometer. Fluorescence spectra were collected on a Perkin-Elmer MPF 66 spectrofluorometer. FAB-MS spectra were recorded on AMD 604 instrument using *m*-nitrobenzyl alcohol as the matrix. Short column chromatography was performed on silica gel 60H and RP-silica gel (Merck). High-performance liquid chromatography (HPLC) was carried out with a Waters 600E instrument, using a multisolvent delivery system, equipped with 991 photodiode array and 470 fluorescence detectors. Reversed-phase columns, NovaPak C18 and DeltaPak C-4 (Waters) were used with isocratic and gradient modes (acetonitrile in 0.1 M aq. ammonium acetate).

Materials.

Pyridinium salts, *N*-[9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)purin-6-yl]pyridinium chloride[7], **1a;** *N*-[9-(3,5-di-*O*-acetyl-β-D-2-deoxyribofuranosyl)purin-6-yl]-pyridinium chloride[10],**1b**, were synthesized as reported. HPLC grade solvents (Merck) and all the other chemicals (Aldrich) were used as received. Water used in the preparation of aqueous solutions for photochemical experiments was triply distilled.

***N*-[9-(3,5-di-*O*-acetyl-2-*O*-methyl-β-D-ribofuranosyl)purin-6-yl]pyridinium chloride (1c).** A solution of 3′,5′-di-*O*-acetyl-2′-*O*-methylinosine[16] (552 mg, 1.7 mmol) in dry pyridine (5 mL) was treated for 18 h in dark with 4-chlorophenyl phosphorodichloridate (468 μL, 2.88 mmol). Ice-cold water (30 mL) was added, and the reaction mixture was concentrated under reduced pressure to 25% of the initial volume. Dilution with water and evaporation was repeated until all pyridine was removed. Aqueous solution was then neutralized (pH 6.1 - 6.5) with Dowex-1 resin (HCO_3^- form). The filtrate was concentrated to a small volume (3 mL) and passed through Dowex-1 resin (Cl^- form). Subsequent concentration under reduced pressure gave aqueous solution of **1c** (75% yield, determined by UV analysis) stable when stored at 5°C. Solid analytical sample of **1c** was obtained upon lyophilization. Pure by the HPLC analysis (Table 2). Anal. calcd. for $C_{20}H_{22}N_5O_6Cl$: C 51.79; H 4.78; N 15.10. Found: C 51.95; H 4.90; N 15.45. UV (H_2O), λ_{max}, nm (ε): 273 (8600), 300 (7300). Fluorescence: λ_{Exc} = 313 nm, λ_{Em} = 438 nm (uncorrected). ^1H NMR (D_2O), δ:10.09 (d, 2 ,J = 7.1 Hz, Hα), 9.18 (s, 1, H2), 8.90 (t, 1, J = 7.8 Hz, Hγ), 8.89 (s, 1, H8), 8.40 (t, 2, J = 7.8 Hz, Hβ), 6.38 (d, 1, J = 4.8 Hz, H1′), 4.30 (t, 1, J = 4.7 Hz, H3′), 3.96 (t, 1, J = 5.1 Hz, H2′), 3.74 (m, 3, H4′, H5′, 5″), 3.51 (s, 3, OCH_3), 2.19, 2.12 (s, 6, Ac). ^{13}C NMR (D_2O), δ: 172.2, 171.1 (CO-Ac), 155.41 (C-4), 153.19 (C-2), 151.40 (C-γ) 149.42 (C-8), 147.21 (C-6), 144.30 (C-α), 129.50 (C-β), 126.12 (C-5), 85.86 (C-1′), 82.89 (C-2′), 81.11 (C-4′), 69.49 (C-3′), 63.55 (C-5′), 59.14 (OCH_3), 21.08, 20.89 (CH_3-Ac).

Preparation of (9-methylpurin-6-yl)pyridinium perchlorate (4). *N*-(purin-6-yl)pyridinium chloride[11] (1.63 g, 7 mmol) was dissolved in 80% aq. CH_3CN (50 mL) and methylated with $(CH_3)_2SO_4$ (1.76 g, 14 mmol) in the presence of sodium bicarbonate (14 mmol). The mixture was stirred for 2 h at room temperature and then extracted with chloroform (2 × 50 mL). The aqueous layer was concentrated to a volume of *ca.* 2 mL and chromatographed on a reversed phase silica gel column eluted with water containing 0-15% of acetonitrile. Fractions containing the desired product were combined, concentrated to a small volume under reduced pressure and passed through a Dowex (ClO_4^- form) column. The eluate was concentrated under vacuum and finally freeze-dried to give **4** (2.15 g, 95% yield) of spectral data as reported previously[7].

Photosensitized preparation of 2′,3′,5′-tri-O-acetylluminarosine (3a) and analogues (3b,c). General procedure. An aqueous solution of the respective pyridinium salts, **1a-c** (0.3 mM, 1.7 L aliquots, pH 5.8-6.2) was irradiated at $\lambda > 300$ nm in a 2 L photochemical reaction vessel equipped with a quartz immersion well and a water cooled, Pyrex shielded, high pressure mercury lamp TQ 150 (Original Hanau) for *ca.* 2 h. Prior to irradiation, the solution was deoxygenated by bubbling argon for 30 min through the magnetically stirred solution. Argon bubbling was continued throughout the irradiation. Once the conversion of the pyridinium salts **1a-c**, into the respective ring-opened products, **2a-c**, was completed (confirmed by HPLC analysis, Table 2), a concentrated aqueous solution of the sensitizer **4** (0.15 M, 6 mL) was added to the irradiated solution, and its pH was adjusted to 7.5 with saturated aq. $NaHCO_3$. Then, the irradiation was continued under the flow of argon until HPLC analysis revealed almost complete transformation of the intermediate ring-opened products **2a-c** into desired luminarine nucleosides, **3a-c** (Table 2). The reaction mixture was filtered through a 0.45 μ membrane filter to remove the water-insoluble photoproduct **5**, and the filtrate extracted with chloroform (3 times 200 mL). The chloroform layer was dried over $MgSO_4$ and concentrated to a small volume. The crude products were purified on a silica gel column eluted with chloroform containing 0-20% methanol to give **3a-c**.

3a (216 mg. 90% yield, data as reported[6]); **3b** (170 mg, 81% yield, data as reported[10]) and **3c**, (115 mg, 51% yield). Analytical data for **3c**: Pure by the HPLC analysis (Table 2). Anal. calcd. for $C_{20}H_{21}N_5O_7$: C 54.17; H 4.77; N 19.79. Found: C 54.30; H 4.60; N 19.60. UV (H_2O), λ_{max}, nm (ε): 265 (16000), 422 (11000). Fluorescence: $\lambda_{Exc} = 420$ nm, $\lambda_{Em} = 528$ nm (uncorrected). ^1H NMR ($CDCl_3$), δ :10.21 (d, 1, J = 6.8 Hz, H1), 9.10 (d, 1, J = 9.1 Hz, H4), 8.47 (t, 1, J = 7.8 Hz, H3), 8.36 (s, 1, H9), 8.11 (t, 1, J = 6.8 Hz, H2), 7.74 (d, 1, $J_{NH-H1'} = 9.2$ Hz, NH), 6.09 (dd, 1, J = 6.1 Hz, $J_{NH-H1'} = 9.2$ Hz, H1′), 3.99 (dd, 1, J = 6.1 Hz, J = 4.8 Hz, H-2′), 5.29 (dd, 1, J = 5.4 Hz, J = 4.8 Hz, H3′), 4.32 (m, 1, H4′), 4.32 (m, 2, H5′, 5″), 3.48 (s, 3, OCH_3), 2.19, 2.16 (s, 6, CH_3-Ac). ^{13}C NMR ($CDCl_3$), δ: 170.85, 170.29 (CO-Ac), 160.57 (C-5), 158.54 (C-7), 149.22 (C-9), 141.31 (C-3), 138.35 (C-4a), 131.81 (C-10a), 130.98 (C-1), 127.75 (C-4), 126.25 (C-2), 125.34 (C-6a), 83.54 (C-1′), 82.18(C-4′), 79.08 (C-2′), 71.10 (C-3′), 63.67 (C-5′), 59.16 (O-CH_3), 21.07, 20.86 (CH_3-Ac).

TABLE 2. HPLC retention times* for the pyridinium salts **1a-c**, and the corresponding photoproducts **2a-c** and **3a-c**.

Solvent system	Retention time [min]								
	1			**2**			**3**		
	a	**b**	**c**	**a**	**b**	**c**	**a**	**b**	**c**
A	11.3			9			15.5		
B		11.2			8.8			14.5	
C			9			6.5			10

* Analyses were performed on a Waters DeltaPak C4, 300 Å column eluted isocratically with 0.1 M aq. CH_3COONH_4 containing 20% (system A); 12% (system B) and 22% of acetonitrile (system C). The flow rate was 0.8 mL / min.

2'-O-Methylluminarosine. De-*O*-acetylation of **3c** (44 mg, 0.1 mmol) was performed in 2.5% aqueous ammonia (20 mL) at room temperature. After 2.5 h, the HPLC analysis revealed quantitative transformation of **3c** into a single product. The reaction mixture was concentrated under reduced pressure, and resulted solid purified on reversed phase silica gel chromatography (methanol 0-10% gradient in water) to give amorphous 2'-*O*-methylluminarosine (33 mg, 92% yield). Pure by the HPLC analysis (Waters DeltaPak C4, 100Å column eluted isocratically with 0.1M aq. CH_3COONH_4 containing 8% of acetonitrile, flow rate 0.8 mL / min, retention time 9 min.). Anal. calcd. for $C_{16}H_{17}N_5O_5$: C 53.48; H 4.77; N 19.49. Found: C 53.55; H 4.90; N 19.05. m/z (FAB^+) 360 M+1. UV (H_2O), λ_{max}, nm (ε) : 265 (15700), 424 (11200). Fluorescence: λ_{Exc} = 420 nm, λ_{Em} = 527 nm (uncorrected). ^1H NMR (CD_3OD) δ: 10.28 (d, 1, J = 6.8 Hz, H1), 8.89 (d, 1, J = 7.1 Hz, H4), 8.70 (t, 1, J = 8.0 Hz, H3), 8.32 (t, 1, J = 6.8 Hz, H2), 8.31 (s, 1, H9), 5.99 (d, 1, J = 4.4 Hz, H1'), 4.36 (t, 1, J = 4.6 Hz, H3'), 3.98 (t, 1, J = 4.9 Hz, H2'), 3.71 (m, 3, H4', H5', 5''), 3.55 (s, 3, OCH_3). ^{13}C NMR (CD_3OD) δ : 161.3 (C-5), 158.42 (C-7), 150.77 (C-9), 144.44 (C-3), 139.51 (C-4a), 132.17 (C-10a), 133.54 (C-1), 128.40 (C-4), 127.65 (C-2), 126.2 (C-6a), 85.98 (C-1'), 85.50 (C-4'), 85.39 (C-2'), 71.19 (C-3'), 63.23 (C-5'), 58.84 (O-CH_3).

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