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FLUORESCENT NUCLEOSIDE WITH A NEW HETEROCYCLIC BETAINE AS THE AGLYCONE PHOTOCHEMICAL PREPARATION AND PROPERTIES

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Abstract - An efficient, photochemical conversion of N-[9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purin-6-yl]pyridinium chloride into a highly fluorescent nucleoside, having a new heteroaromatic betaine as the aglycone, occurs when an aerated aqueous solution of the pyridinium salt is exposed to sunlight. The chemical deacetylation of the fluorescent nucleoside (named tri-O-acetyl-luminarosine) to parent riboside (luminarosine) and N-glycosidic bond cleavage to aglycone (luminarine) is also described.

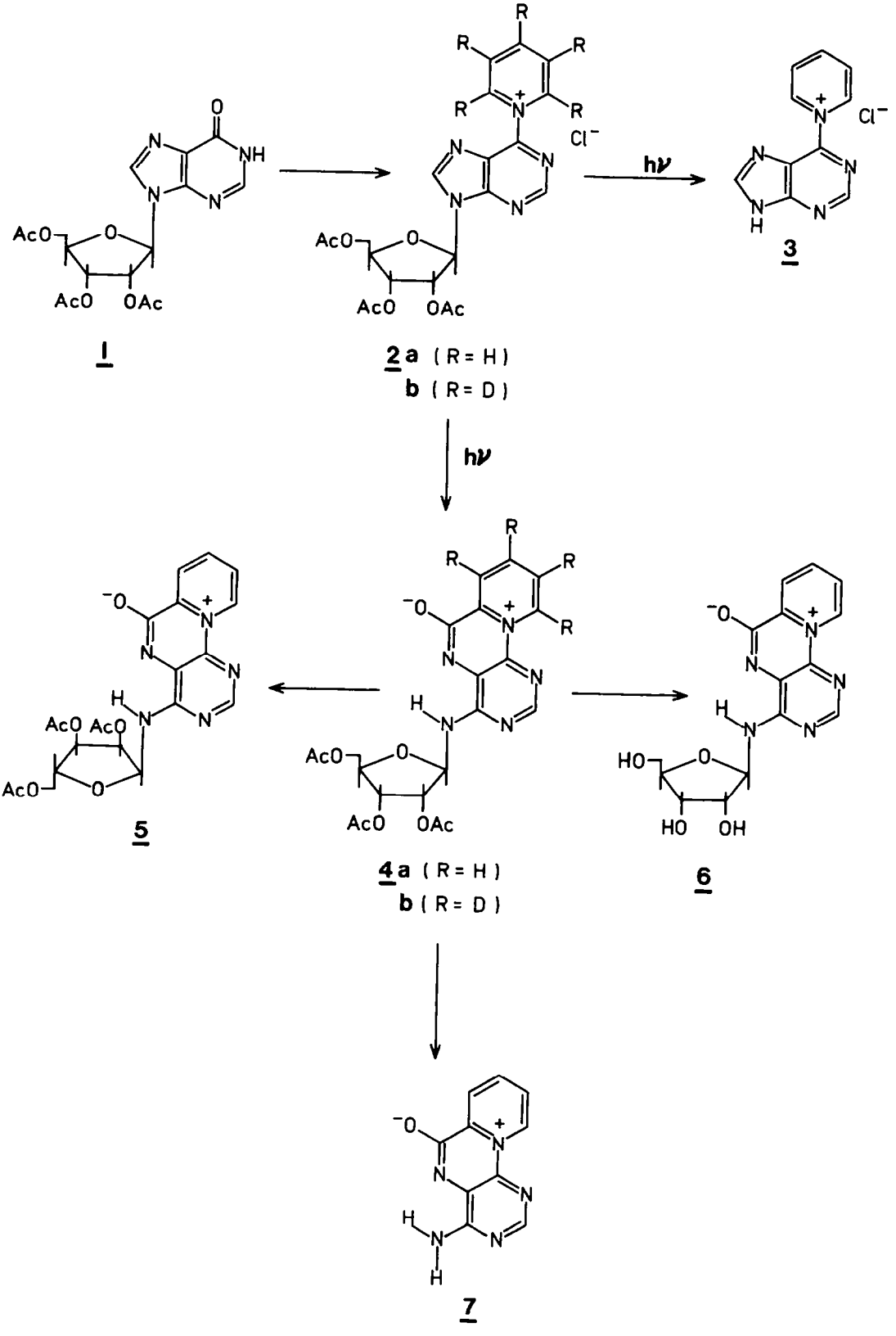
INTRODUCTION

We have reported recently[1] that 2',3',5'-tri-O-acetylinosine, **1**, treated with various phosphorylating agents in the presence of pyridine forms water soluble N-[9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purin-6-yl]pyridinium chloride **2a**. This and other pyridinium salts derived from guanine, thymine and uracil nucleobases have been considered as new ionic side-products in oligonucleotide synthesis by phosphotriester method[1-3]. Some of them have been offered as novel synthetic intermediates in nucleoside chemistry[4,5]. The crystal structure of N-(2-aminopurin-6-yl)pyridinium chloride dihydrate has been solved[6].

Here we would like to report a very efficient, visible light-induced transformation of **2a** into a new nucleoside fluorophore: 7-(2',3',5'-tri-O-acetyl-β-D-ribofuranosylamino)-pyrido[2,1-h]pteridin-11-ium-5-olate, **4a**, which we have termed 2',3',5'-tri-O-acetyl-luminarosine. Both the parent ribonucleoside **6** and the aglycone **7** named luminarosine and luminarine respectively, have been also obtained.

RESULTS and DISCUSSION

We have previously observed[7] that N-[9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purin-6-yl]pyridinium chloride, **2a**, when irradiated with UV-light (λ = 290 nm) in deoxygenated aqueous solution at pH 4.0 - 4.5, undergoes N-glycosidic bond cleavage to form N-(purin-6-yl)pyridinium chloride, **3**. However, when aerated solutions of **2a**, adjusted to pH 7.0 - 7.5 by addition of $NaHCO_3$ either are exposed to sunlight or subjected to irradiation with near UV light



1

2a (R = H)
b (R = D)

3

4a (R = H)
b (R = D)

5

6

7

(λ > 300 nm), an efficient transformation into a new lypophilic photoproduct having an intense yellow-green fluorescence occurs. The UV-VIS monitoring of this transformation showed the gradual decrease of the absorption band at λ = 280 nm, characteristic of **2a**[1], and the concomitant formation of a new band at λ = 420 nm. Similarly, in the fluorescence spectra the decrease in the emission of **2a** at λ = 424 nm[1] was accompanied by the appearance of a new fluorescence band at λ = 527 nm. The major photoproduct was isolated after the preparative scale irradiation of **2a** (see experimental) by extracting the irradiated solution with chloroform. Careful TLC analysis of the crude extract revealed the presence of a small amount of a second product (partially overlapping spots) of an analogous, intense fluorescence. The two photoproducts were separated by silica gel column chromatography and their structures established mainly on the basis of ^{1}H and ^{13}C NMR spectroscopy. The relevant data are summarized in Tables 1 and 2.

Initially, the ^{1}H NMR spectrum of **4a** (Fig.1a) was analysed by means of a series of homonuclear decoupling experiments and was then checked by spectrum simulation (Fig.1b). A comparison was also made with a spectrum of the deuterated derivative, **4b**, (Fig.1c) which is the photoproduct of **2b** synthesized from **1** by using pyridine-d_5 as a solvent in phosphorylation reaction[1]. The low field region (δ > 8.0 ppm) of the ^{1}H NMR spectrum of **4a** consists of 4 spin systems, characteristic of an α-substituted pyridinium ring. As can be seen in Fig.1c, these signals are absent in the spectrum of **4b**. The only signal present in this region, the singlet at 8.33 ppm, was assigned to pyrimidine C(9)-H of **4a,b** (see Fig.1 inset, for numeration). When D_2O was added to the solutions of both **4a** and **4b** the doublet at 7.66 ppm disappeared and the 6.17 ppm doublet of doublets collapsed into a doublet, indicating the presence of a C(1')H-NH glycosidic linkage. The signal pattern within the range of 6.17-2.11 ppm for both **4a** and **4b** revealed that the 2',3',5'-tri-O-acetyl-β-D-ribofuranosyl moiety was preserved within the photoproduct structure. This was further supported by the FAB mass spectrum of **4a** in which the fragments corresponding to the loss of tri-O-acetylribose and tri-O-acetylribosylamine from the molecular ion are present at 214 and 198 mass units respectively.

The results of the NMR experiments did not exclude the possibility that the tricyclic heteroaromatic structure bearing a positively charged nitrogen atom (N-11) could be a quaternary salt and therefore the photoproduct was tested for the presence of the counterion. However, the negative result of this test ruled out such a possibility and allowed us to conclude unequivocally that the aglycone of the phototoproduct was the mesomeric betaine. The final structural proof was provided by the accurate mass measurement of the parent ion in the FD mass spectrum of **4a**, which gave the molecular composition ($C_{21}H_{21}N_5O_8$) in agreement with the proposed structure and the elemental analysis.

The assignment of the ^{13}C NMR spectrum of **4a** was made by measuring (i) the proton coupled spectra using the gated decoupling technique[8], (ii) partially relaxed FT spectra[9], (iii) long range selective ^{1}H decoupled spectra with low power ^{1}H irradiation[10,11], and by comparison with the spectra of **4b**. The ^{13}C chemical shifts and the $^{1}J_{C,H}$ coupling constants are summarized in Table 2. The assigned $^{2}J_{C,H}$ and $^{3}J_{C,H}$ coupling constants are shown in Fig.1 (inset).

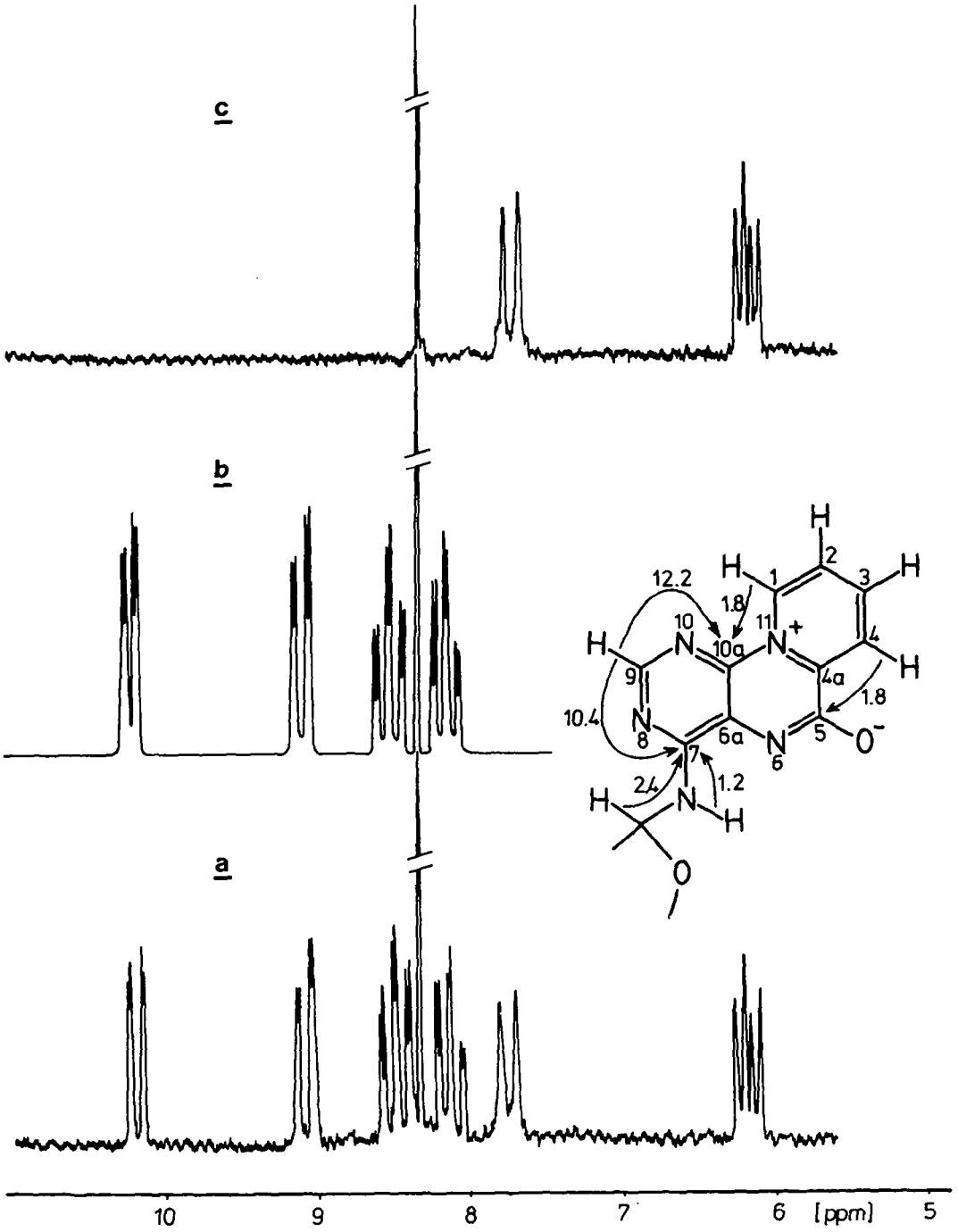


Fig.1. The observed ^{1}H NMR spectra of **4a** (trace a) and **4b** (trace c). Trace (b) shows the simulated spectrum of **4a**. Inset: the numbering of the heterocyclic skeleton of **4a**. The arrows show the assigned long range ^{1}H-^{13}C couplings (±0.1 Hz).

Table 1. ^{1}H NMR data for compounds **4a**, **4b**, **5**, **6** and **7**
a) Proton chemical shifts (ppm)(internal TMS)

Proton	**4a***	**4b***	**5***	**6****	**7*****
H-1	10.19 (d)	-	10.18 (d)	10.10 (d)	10.39 (d)
H-4	9.08 (d)	-	9.08 (d)	8.83-8.85 (m)	9.05 (d)
H-3	8.51 (t)	-	8.51 (t)	-	8.85 (t)
H-9	8.33 (s)	8.33 (s)	8.32 (s)	8.24 (s)	8.32 (s)
H-2	8.15 (t)	-	8.15 (t)	8.29 (t)	8.47 (t)
H-1	6.17 (dd)	6.18 (dd)	6.52 (dd)	5.82 (dd)	-
H-2,H-3'	5.44 (brs)	5.42 (brs)	5.57-5.34 (m)	4.24-3.87 (m)	-
H-4'				3.79 (m)	
H-5'	4.30 (brs)	4.29 (brs)	4.40-4.22 (m)	-	
-NH	7.86 (d)	7.74 (d)	8.08 (d)	8.10 (d)	-
others	(i)	(i)	(i)	(ii)	

*$CDCl_3$, **DMSO-d_6, ***CD_3OD+CD_3COOD
(i) -OCH_3: **4a**,**4b**: 2.22, 2.13 and 2.11; **5**: 2.31, 2.17 and 2.12
(ii) **6**: 2'-OH: 5.12 (d), 3'-OH and 5'-OH: 4.98-4.86 (m)

b) Values of proton-vicinal coupling constants [± 0.2 Hz]

Compound	H1-H2	H2-H3	H3-H4	NH-H1'	H1'-H2'
4a	6.6	7.3	8.1	9.0	5.1
4b	-	-	-	9.0	5.1
5	6.6	7.5	8.1	9.8	5.1
6	6.6	6.8	(i)	9.0	5.3
7	6.8	7.0	8.1	-	-

(i) unresolved

Table 2. ^{13}C NMR data for compounds **4a**, **4b**, **5**, **6** and **7**.
Chemical shifts, ppm and ($^1J_{C,H}$ coupling constants, ±0.6 Hz)

Carbon	**4a***	**4b***	**5***	**6****	**7*****
C-5	160.26	160.21	160.43	159.66	159.61
C-7	158.09	150.04	158.42	158.09	159.99
C-9	148.67 (205.7)	148.56 (205.7)	149.04 (205.7)	147.64 (205.8)	151.32 (205.1)
C-3	141.89 (172.1)	-	141.09 (171.8)	142.60 (171.9)	145.90 (174.6)
C-4a	138.32	138.32	138.59	132.27	139.89
C-10a	131.82	131.76	131.88	131.60	135.61
C-1	131.17 (194.7)	-	130.90 (194.1)	131.60 (194.6)	134.63 (196.5)
C-4	127.48 (177.0)	-	127.76 (177.6)	126.89 (177.2)	127.97 (178.2)
C-2	126.56 (173.4)	-	126.08 (172.1)	126.18 (172.8)	129.43 (176.4)
C-6a	125.05	125.05	125.75	124.45	120.00
C-1'	83.49 (167.2)	83.54 (167.2)	79.91 (170.0)	84.89 (165.4)	-
C-4'	78.99 (150.8)	78.93 (150.2)	78.78 (152.6)	84.08 (149.7)	-
C-2'	73.73 (153.8)	73.73 (153.3)	71.90 (156.9)	74.06 (150.3)	-
C-3'	70.92 (157.5)	70.92 (157.5)	69.95 (156.2)	70.54 (152.1)	-
C-5'	63.44 (149.5)	63.50 (148.9)	63.50 (148.9)	61.76 (142.5)	-
others	(i)	(i)	(i)		

*$CDCl_3$, **DMSO-d_6, ***CD_3OD+CD_3COOD
(i)-$COCH_3$: **4a**, **4b**: 169.69, 169.53, 20.96 and 20.53; **5**: 170.34, 169.91, 169.53, 20.80, 20.70 and 20.59.

The minor photoproduct 5 was isolated in chromatographically pure form, with 3% yield from the first few fractions eluted during the purification of 4a. The identity of its absorption, fluorescence and mass spectra with those of 4a, pointed to an isomeric structure with an identical chromophore. Based on the ^{1}H and ^{13}C NMR spectra (Tables 1,2) the isomeric photoproduct was identified as 7-(2',3',5'-tri-O-acetyl-α-D-ribofuranosylamino)-pyrido[2,1-h]pteridin-11-ium-5-olate, 5.

It was also found, after extraction of the irradiated solution with chloroform, that the amount of the α-anomer 5 in the chloroform layer, increased up to 10% when the extract was left for a few hours without washing it carefully with water. This indicates that the process of $\beta \rightarrow \alpha$ anomerization occurs after phototransformation.

The chemical properties of 4a have also been studied. The preliminary results showed that the heterocyclic part of the molecule is exceptionally resistant toward acids but is susceptible to attack by bases. This led initially to some difficulties in its de-O-acetylation. However, the treatment of 4a with an anhydrous solution of triethylamine in methanol (3.5%, v/v) gave the desired, deacetylated nucleoside 6 (luminarosine) which crystallized from the reaction mixture as a chromatographically pure product in 60% yield. An additonal amount (ca. 17%) of 6 was recovered from the filtrate by reverse-phase chromatography in water-methanol.

When treated with a mixture of 3.5 N aq. HCl/dioxane/isopropanol (1:1:1, v/v) 4a slowly undergoes (70°C, 6 days) N-glycosidic bond cleavage with formation of the aglycone 7-amino-pyrido[2,1-h]pteridin-11-ium-5-olate, 7 (luminarine). Small amounts of partially deacetylated 4a have been also detected in the reaction mixture by TLC. Pure 7 was obtained as small, dark-orange crystals, with 50% yield after reversed-phase chromatography with a gradient of acetone in water.

The tricyclic, heteroaromatic system of 4-7 should be considered as a new, to our knowledge, member of the heterocyclic mesomeric betaines[12]. We hope that this new class of nucleoside fluorophores[13], with remarkable fluorescein-like emission properties[14] capable of laser action, will find interesting applications, especially as molecular probes in nucleic acids and other systems.

EXPERIMENTAL

Thin layer chromatography was performed on Merck silica plates in the following solvent systems: A -chloroform/methanol 9:1 (v/v), B -chloroform/methanol 8:2 (v/v), and C -ethanol/1M ammonium acetate 7:3 (v/v). Short column chromatography[15] was performed on silica gel (Merck H60) in chloroform containing methanol or on Merck reverse-phase silica gel 60 (70-230 mesh). Melting points were taken on a Boetius apparatus and are uncorrected. UV-VIS absorption and fluorescence emission spectra were measured in water (pH 6.5) on a Cary 118 spectrophotometer and a Spex Fluorolog spectrofluorometer. Mass spectra were taken on Jeol JMS-D-100 (FD) and VG 7070 HE (FAB) spectrometers. NMR spectra were run on Jeol 90FX Fourier transform NMR spectrometer. Elemental analyses were made on a Perkin Elmer 240 analyser.

Photochemical preparation of 4a and 5.

An aqueous stock solution (0.2 M) of N-[9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purin-6-yl]pyridinium chloride, 2a[1] (R_f=0.05 and 0.37 in systems A and C respectively) was diluted with twice distilled water to make 10 L of 5 x 10^{-4}M solution. The pH was adjusted to 7.0-7.5 by addition of sodium hydrogen carbonate (ca.5 eq.) and the aerated solution subjected (i) to irradiation with a halogen lamp (λ>340 nm) in a Pyrex, continuous flow, water-cooled photoreactor or (ii) exposed to sunlight in a glass vessel, under magnetic stirring. In both cases the progress of the reaction was monitored by measuring the UV-VIS absorption and/or fluorescence spectra of the samples taken from the irradiated solutions. After all 2a had reacted, the bright-

yellow solution was extracted with chloroform (3 x 500 ml). The chloroform layer was washed with water (500 ml), dried with Na_2SO_4 and evaporated under reduced pressure. TLC analysis (system A) of the residue revealed the presence of two overlapping, yellow spots (R_f=0.43 and 0.46) in a ratio of ca. 95:5. The mixture was partially separated on silca gel column eluted with 7% solution of methanol in chloroform (v/v). The fractions containing pure photoproduct (R_f=0.43) were evaporated, dissolved in chloroform and precipitated with hexane (500 ml) to yield **4a**, 825 mg (yield 35%).

Mp.90-95°C; UV-VIS (H_2O), λ nm (ϵ): 265 (16100,max), 300 (1650,min), 344 (3700,max), 360 (3600,min), 422 (11000,max); Fluorescence λ_{max} = 527 nm; FAB MS, mass (% rel. int.): 472 (61), 214 (100), 200 (2.1), 198 (6.3); FD MS 470 (100); Anal. calc. $C_{21}H_{21}N_5O_8$: C, 53.48; H, 4.49; N, 14.86. Found: C, 53.10; H, 4.44; N, 14.12.

The first few fractions from above separation (containing both photoproducts) were rechromatographed twice under identical conditions to give, after precipitation with hexane, 80 mg (3% yield) of α-anomer, **5**. The analytical data, except for the NMR (see Tables 1 and 2) were identical to those of **4a**.

The deuterated derivative **4b** was synthesized on a 0.25 mmol scale following exactly the above procedure.

De-O-acetylation of **4a** to **6**.

2',3',5'-tri-O-acetyl-luminarosine **4a** (470 mg, 1 mmol) was dissolved in anhydrous methanol (164 ml) and treated with triethylamine (6 ml). The solution was left at room temp. until all of **4** was transformed into **6** (R_f = 0.17 in system B); usually after 72 hrs. The product crystallized directly from the reaction mixture. Crystals were collected and washed with cold methanol to give 210 mg of pure luminarosine, **6**. An additional 60 mg of the product was obtained from the filtrate after it was concentrated and chromatographed on a reverse-phase silica gel column with a gradient of methanol in water. The overall yield was 77%.

Mp. 200°C, dec.; UV-VIS (H_2O), λ nm (ϵ): 265 (15800,max), 301 (1860,min), 347 (4100,max), 360 (4000,min), 424 (11100,max); Fluorescence λ_{max} = 530 nm; FAB MS, mass (% rel. int.): 346 (45.8), 214 (27.3), 202 (33.2), 201 (100), 199 (24.4), 181 (43.3); Anal. calc. $C_{15}H_{15}N_5O_5$: C, 52.17; H, 4.37; N, 20.28. Found: C, 51.95; H, 4.20; N, 19.98.

Cleavage of **4a** to **7**.

4a (470 mg, 1 mmol) was dissolved in a mixture of 3.5 N aq. HCl/dioxane/iso-propanol 1:1:1 (v/v, 80 ml) and heated at 70°C in a capped flask. After 6 days TLC analysis showed quantitative transformation of **4a** into another, more polar product (R_f = 0.15 in system A) having a characteristic intense, yellow-green fluorescence. The reaction mixture containing dark-coloured material was concentrated to ca. 3 ml and applied directly to the top of the short, reverse-phase silica gel column. The column was eluted with a gradient of acetone in water to give, after evaporation of the appropriate fractions, 100 mg of pure **7** as dark-orange crystals (yield 47%).

Mp.>300°C, dec.; UV-VIS (H_2O), λ nm (ϵ): 267 (9500,max), 303 (1300,min), 348 (2900,max), 362 (2800,min), 425 (6800,max); Fluorescence: λ_{max} = 534 nm; FAB MS, mass (% rel. int.): 214 (100), 213 (8.3), 201 (38.5), 199 (10.3), 181 (33.8), 175 (18.8); Anal. calc. $C_{10}H_7N_5O$: C, 56.34; H, 3.29, N, 32.86. Found: C, 55.92; H, 3.13; N, 32.36.

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