

This paper is dedicated to Professor Maciej Wiewiórowski

Acid promoted transformations of fluorescent luminarosine and its 2'-modified analogues^o

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The susceptibility of highly fluorescent luminarine nucleosides to acid promoted anomerization reactions has been studied in order to select a derivative with suitable properties for chemical synthesis of luminarine-labeled oligo(deoxy)ribonucleotides. Both O-acetylated derivatives Ia-c and parent luminarosine IIa, as well as 2'-O-methyluminarosine IIb, and 2'-deoxyluminarosine IIc undergo anomerization at pH = 4 however, at considerably different velocities. In the case of O-protected nucleosides (Ia-c), the anomerization leads to an equilibrium mixture of respective β and α furanosides, the rate and extent of anomerization decreasing in the following order: Ic >> Ia > Ib. Parent nucleosides (IIa-c) bearing free hydroxyls are generally more susceptible to anomerization than the O-acetylated derivatives but a similar order of reactivity (IIc >> IIa > IIb) is observed. In each case, a complex mixture containing both β and α ribopyranosyl and -furanosyl forms is formed. Their structure and anomeric configuration have been proved by ¹H and ¹³C NMR spectroscopy. The results point to 2'-O-methyluminarosine as the fluorophore of choice for further derivatization and chemical introduction into oligo(deoxy)ribonucleotides.

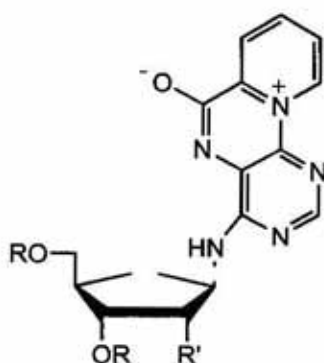
As we have previously described [1], light induced transformation of blue emitting *N*-[9-(2',3',5'-tri-*O*-acetyl- β -D-ribofuranosyl)]purin-

6-yl]pyridinium chloride [2] led to the formation of green-yellow emitting fluorophore 7-(2',3',5'-tri-*O*-acetyl- β -D-ribofuranosylamino)-

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Abbreviations: DMSO, dimethylsulfoxide; TMS, tetramethylsilane.



pyrido[2,1-*h*]pteridin-11-ium-5-olate, **Ia**, named 2',3',5'-tri-*O*-acetyluminarosine. Both the parent riboside (luminarosine) **IIa**, and the aglycone (luminarine) were subjected to detailed structural [1, 3] and photophysical [4] studies.

The attractive emission properties ($\lambda_{Ex} = 425$ nm, $\lambda_{Em} = 530$ nm, $\Phi_F = 0.65$, $\tau_F = 8$ ns) as well as chemical stability under the conditions of oligonucleotide synthesis, clearly indicated a great potential of luminarosine as a fluorescent probe. Sequence-specific introduction of the luminarine fluorophore into oligodeoxynucleotides has been already achieved [5].

Recently, considerably improved conditions for the photosensitized preparation of 2',3',5'-tri-*O*-acetyluminarosine, **Ia**, and its 2'-*O*-methyl-, **Ib**, and 2'-deoxy-, **Ic**, analogues, the key compounds in our ongoing studies on the synthesis of luminarine-labelled oligonucleotides, have been developed [6].

As recognized earlier [1, 4, 6] the NH-glycosidic bond of luminarosine exhibits a tendency to an undesired, in view of potential application in fluorescent labelling of nucleic acids, acid-catalysed $\beta \rightleftharpoons \alpha$ anomerization. Upon treatment with 80% aq. acetic acid (i.e. under conditions of removal of the 5'-*O*-dimethoxytrityl protective group on monomer level) a pure β -D-ribofuranosyl form of **Ia** undergoes substantial (45% yield) conversion to the respective α -furanoside [4]. This observation makes it necessary to examine the anomerization properties of the *O*-acetylated nucleosides **Ia-c** and *O*-deprotected luminarosine

	R	R'
Ia	Ac	OAc
Ib	Ac	OCH ₃
Ic	Ac	H
IIa	H	OH
IIb	H	OCH ₃
IIc	H	H

IIa as well as its 2'-deoxy (**IIc**), and 2'-*O*-methyl (**IIb**) analogues in order to select a derivative of suitable properties for chemical synthesis of the luminarine-labelled oligodeoxyribonucleotides. Results of this investigation are presented and discussed in this paper.

MATERIALS AND METHODS

Materials. Acetonitrile (HPLC grade) and all the other chemicals (reagent grade) were purchased from Aldrich. The preparation and spectral characteristics of **Ia-c** and **IIa, c** was described previously [1, 4-6].

7-(2'-*O*-Methyl- β -D-ribofuranosyl)luminarine, **IIb**. A sample of 7-(3',5'-di-*O*-acetyl-2'-*O*-methyl- β -D-ribofuranosyl)luminarine **Ib** (60 mg, 0.135 mmol) was dissolved in 2.5% aqueous ammonia (2 mL) and the resulting solution kept at room temperature. As revealed by HPLC, de-*O*-acetylation of **Ib** was completed within about 2 h. The crude product was purified by means of reversed phase column chromatography to give 43 mg (90% yield) of **IIb** in form of red powder.

UV(H₂O) λ_{max} , nm (ϵ): 265 (15700), 424 (11200), MS m/z 359; ¹H NMR (CD₃OD), 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, H1), 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, OCH₃); ¹³C NMR (CD₃OD), 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), 33.54 (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'), 1.19 (C-3'), 63.23 (C-5'), 58.84 (O-CH₃).

Methods. A Waters 600E instrument equipped with Waters 991 Photodiode Array UV and Waters 470 Scanning Fluorescence detectors were used for analytical and semi-preparative scale HPLC separations. ¹H and ¹³C NMR spectra were recorded on a Varian Unity 300 spectrometer in D₂O and DMSO-d₆ with dioxane and TMS as internal standards. All chemical shifts are converted to TMS scale. High resolution liquid secondary ion mass spectral analyses (HR-LSIMS) were performed on an AMD Intectra Model 604 double focusing, reversed geometry instrument fitted with cesium ion gun operating at ion energy of about 12 keV and accelerating voltage 8 kV. Samples were dissolved in glycerol and analysed in positive ion mode.

Anomerization reactions. Samples of pure β -D-ribofuranosyl forms (β_F) of each of the nucleosides, **Ia-c** and **IIa-c**, were dissolved in 0.1 M acetate buffer of pH 4.0 to give 1 mM solutions. These solutions were kept at room temperature and the anomerization reactions were followed by HPLC using the following columns: a Waters NovaPak C18 (8 mm \times 10 cm) in the case of compounds **Ia-c** and **IIa, c** and a Waters DeltaPak C4, 100 Å (4.9 mm \times 25 cm) in the case

of **IIb**. The columns were eluted isocratically ($V = 0.8$ mL/min) with the following solvent systems: A: 21% aqueous CH₃CN (compounds **Ia-c**), B: 8% aqueous CH₃CN (**IIa**), C: 7% aqueous CH₃CN (**IIc**) and 8.5% aqueous CH₃CN (**IIb**), each buffered with ammonium acetate at the concentration of 0.1 M. Analogous conditions were applied for isolation of single isomeric species for their NMR spectral characterization.

RESULTS AND DISCUSSION

As revealed by HPLC, under mild acidic conditions each of the acetylated β -D-ribofuranosyl forms (β_F) of **Ia-c** undergoes gradual transformation into the respective α -D-furanosyl anomer (α_F) (Fig. 2) until the equilibrium state is reached. Figure 1A shows an HPLC profile of the equilibrium mixture obtained in the case of **Ic**. Similar HPLC profiles with well separated peaks of the two anomers were also obtained in the case of the other two acetylated derivatives (**Ia, b**). The products of anomerization assignment to α_F and β_F forms of **Ia-c** has been accomplished previously with help of ¹H and ¹³C NMR spectroscopy [1, 5, 6].

Anomerization of 5'-O-deprotected nucleosides **IIa-c** of the luminarosine family, under analogous conditions (pH 4.0) appeared to be

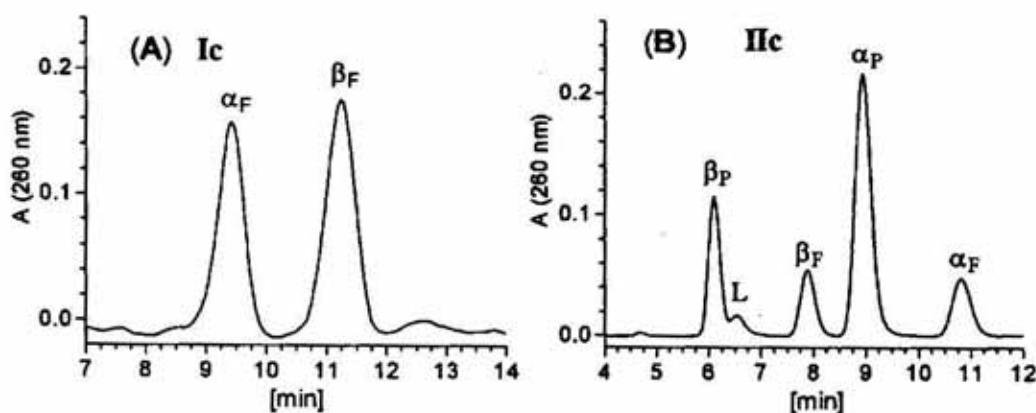


Figure 1. HPLC analyses of the anomerization equilibrium mixtures obtained in the case of A, **Ic** and B, **IIc**.

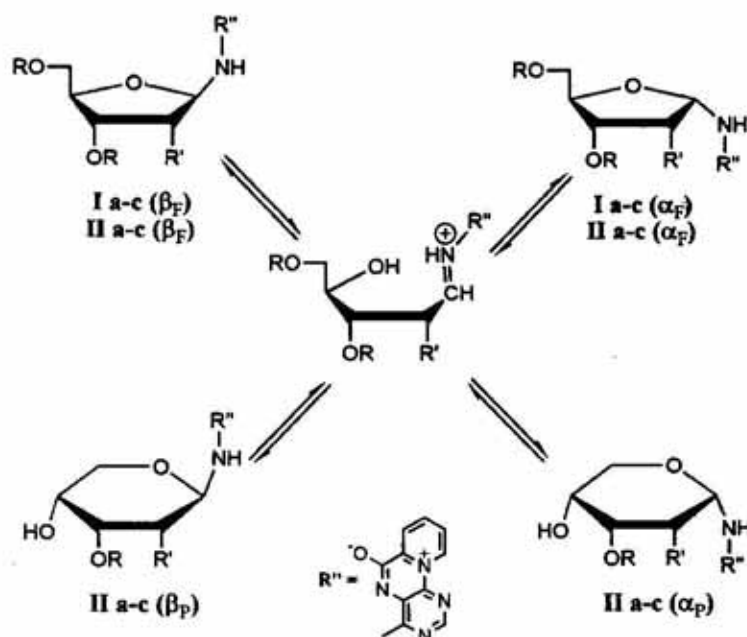


Figure 2. Mutarotation of the luminarine nucleosides Ia-c and IIa-c.

Symbols (β_F) and (α_F) denote the β - and α -D-ribofuranosyl forms, whereas (β_P) and (α_P) refer to the pyranosyl anomers.

more complex. The HPLC analysis of the reaction mixture in the case of 2'-deoxy- β -D-ribofuranosylluminarine, **IIc** (Fig. 2) revealed the formation of the respective α -anomer and three other products of the same absorption and emission properties characteristic for luminarosine [4].

Two of those products that appeared to be the major components of the equilibrium mix-

ture, had mass spectra identical with that of the starting 2'-deoxyluminarosine, **IIc**(β_F) with molecular ion at $m/z = 329$. Both components when separated and left at pH 4 for about 5 h gave the same equilibrium mixture of products (Fig. 1B) as obtained with the **IIc**(β_F) as starting anomer. These observations strongly indicate that the two major, unidentified products are the anomeric pair

Table 1. Selected ^1H and ^{13}C NMR data for the furanosyl and pyranosyl anomers of IIa, c.

	IIa (β_F)	IIc (β_F)	IIa (α_P)	IIc (α_P)	IIa (β_P)	IIc (β_P)
C1'	84.89	82.23	77.03	74.03	77.48	73.88
C2'	74.06	35.59	67.77	34.00	69.46	36.62
C3'	70.54	72.43	70.69	66.63	71.08	66.68
C4'	84.08	86.86	67.00	66.55	66.99	66.26
C5'	61.76	62.87	61.04	61.77	64.18	61.61
NH	8.10	-	8.75	8.70	7.80	7.88
H1'	5.82	5.86	5.67	5.73	5.48	5.7
H2'	-	-	3.72	2.07	3.62	2.14
H2''	-	-	-	1.96	-	1.88
$J_{\text{H1}'\text{-H2}'}$	-	-	3.91	4.33	9.15	8.8
$J_{\text{H1}'\text{-H2}''}$	-	-	-	4.33	-	small

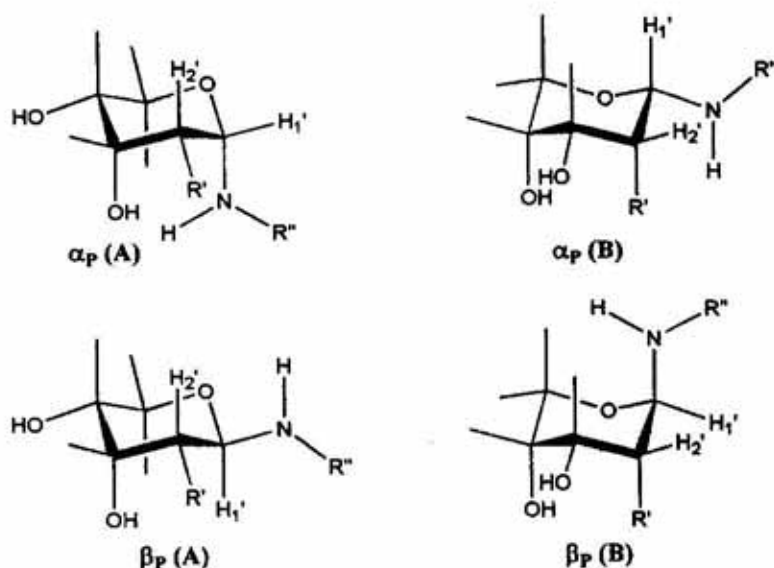


Figure 3. Ideal chair conformations for the D-ribopyranosyl luminarines IIa-c

of pyranosides (β_P and α_P) formed as a result of an acid catalyzed mutarotation of the aminoriboside IIc bearing a free 5' OH group [7] (Fig. 2).

Those two products were isolated by preparative HPLC and subjected to ^1H and ^{13}C NMR analysis. Some selected ^1H and ^{13}C NMR data are summarized in Table 1. The analysis of ^{13}C chemical shifts provides a clear proof that the isolated isomeric products are indeed pyranosides. In agreement with the literature data for the most common aldo- and keto-pyranoses and furanoses [8], the signals of C4' of the two products exhibit a significant upfield shift characteristic for the pyranosyl forms, compared with those of the furanosyl isomers. The anomeric carbon atoms C1' are also shifted up, though to a lesser extent. The analysis of ^1H NMR spectra of the two isolated ribopyranosyl forms of IIc permits their anomeric assignment. For this purpose, the chemical shifts of the ribosylamine hydrogen, NH as well as the coupling constants $J_{\text{H1}',\text{H2}'}$ and $J_{\text{H1}',\text{H2}''}$ were analysed. According to the vicinal Karplus correlation [9], small vicinal coupling constants are observed for diequatorial and equatorial-axial orientations of protons. Visual inspection of the four ideal chair conformations which can be envisaged for pyranoside forms of IIa-c (Fig. 3) clearly shows that two conformers,

$\alpha_P(\text{A})$ and $\beta_P(\text{B})$ possess vicinal diequatorial and equatorial-axial arrangement of the anomeric and H2' and H2'' protons. Furthermore, in the case of the $\alpha_P(\text{A})$ anomer the luminarine group is axially oriented with the glycosylamine proton NH pointing towards the 3' OH group, whereas in the case of the $\beta_P(\text{B})$ anomer that proton lies above the sugar ring, away from the 3' OH.

It can be expected that the interaction of the 3' hydroxyl with the NH proton in the case of the $\alpha_P(\text{A})$ conformer should result in a substantial downfield shift of the resonance of the latter compared with that of the $\beta_P(\text{B})$ isomer. Therefore, based on the fact that no similar interaction of the 3' hydroxyl with the NH proton is possible in the case of the other two conformers, $\alpha_P(\text{B})$ and $\beta_P(\text{A})$ (cf. Fig. 3), the α -pyranoside form was assigned to that of the two isomeric products for which the two coupling constants, $J_{\text{H1}',\text{H2}'}$ and $J_{\text{H1}',\text{H2}''}$, are small (about 4 Hz) and the resonance of the NH proton is shifted downfield as compared with the other isomer. The large and small values of coupling constants, $J_{\text{H1}',\text{H2}'}$ and $J_{\text{H1}',\text{H2}''}$, respectively, observed for the other pyranoside form point to the presence of $\beta_P(\text{A})$ conformer in which the anomeric proton is axially oriented with the bulky luminarine group adopting the equatorial position.

A similar mixture of anomeric furanosyl and pyranosyl forms was also obtained in the case of **IIa**. Respective ^{13}C and ^1H NMR data for the pyranosyl anomers **IIa** (α_{F}) and **IIb** (β_{F}) are included in Table 1.

The third product, whose formation in small amounts was observed both in the case of **IIc** (Fig. 1B) and **IIa** was identified as the aglycone, luminarine, by comparison (HPLC, UV) with an authentic sample of the latter obtained previously [1, 4]. The formation of luminarine indicates that, under mild acidic conditions, partial hydrolysis of NH-glycosidic bond in **IIa, c** occurs in addition to anomerization reactions.

One should also note that both in the case of **IIc** and **IIa**, the α -pyranosyl isomers are favoured (68.8% and 66.0%, respectively) at equilibrium (see Table 2). This can be rationalized in terms of possible stabilization of those forms by intramolecular hydrogen bonding between suitably oriented NH hydrogen and 3' OH in the $\alpha_{\text{P}}(\text{A})$ conformers (cf. Fig. 3).

The anomerization of 2'-O-methyl luminarine, **IIb**, substantially differs from that of **IIa** and **IIc**. In this case, the HPLC analysis of the equilibrium mixture revealed a partial conversion of the starting β -furanosyl anomer into the α -furanosyl form as the main reaction pathway. Contrary to **IIa, c**, only small amounts of one of the two pyranosyl anomers could be detected in the mixture. Unfortunately, the small amounts of the pyranosyl product precluded its NMR spectral analysis and anomeric assignment.

Figure 4 shows the plots of concentration of the starting β -furanosyl anomers and the isomeric products formed *versus* time for all six derivatives, **Ia-c** and **IIa-c**. The rate and equilibrium constants for $\beta_{\text{F}} \xrightleftharpoons[k_{-1}]{k_1} \alpha_{\text{F}}$ anomerization reactions in the case of the O-acetylated nucleosides **Ia-c** were extracted from the kinetic data using the expressions:

$$\ln \frac{[\beta_{\text{F}}] - [\beta_{\text{F}}]^{eq}}{[\beta_{\text{F}}]_0 - [\beta_{\text{F}}]^{eq}} = -(k_1 + k_{-1})t$$

and

$$\frac{k_1}{k_{-1}} = \frac{[\alpha_{\text{F}}]^{eq}}{[\beta_{\text{F}}]^{eq}} = K$$

where $[\beta_{\text{F}}]_0$ is the initial concentration of starting β -furanoside, $[\beta_{\text{F}}]$ the concentration converted into α -furanoside at time $t(\text{h})$ and $[\beta_{\text{F}}]^{eq}$ the concentration converted at equilibrium, whereas in the case of **IIa-c** the initial rate constants for the disappearance of the β -furanosyl anomers were calculated. All those rate constants are listed in Table 2 together with the distribution of individual anomeric forms at equilibrium. As can be seen, both the rate and extent of the anomerization of the luminarine β -D-ribofuranosides strongly depend on the substitution within the sugar moiety. The O-acetylated derivatives **Ia-c** are generally less susceptible to the anomerization than the parent nucleosides **IIa-c** bearing free sugar hydroxyl groups, however, in both series, the same order of reactivity with respect to the substituent at the C2' position in the sugar is observed: **Ic** \gg **Ia** $>$ **Ib** and **IIc** \gg **IIa** $>$ **IIb**. Such a dependence could be explained by considering the generally accepted mechanism of the anomerization of glycosylamines which assumes protonation of the sugar ring oxygen in the first step with the subsequent formation of the cationic iminium intermediate shown in Fig. 2 [10]. Thus, electron withdrawing substituents should make the sugar ring oxygen less susceptible to the protonation and, in consequence, result in lower anomerization rates. The observed decreasing order of reactivity correlates well with increasing electron-withdrawing ($-I$) inductive effect of the substituents: **a** ($\text{R} = \text{H}$) $<$ **b** ($\text{R} = \text{OH}$) $<$ **c** ($\text{R} = \text{OC}$ -

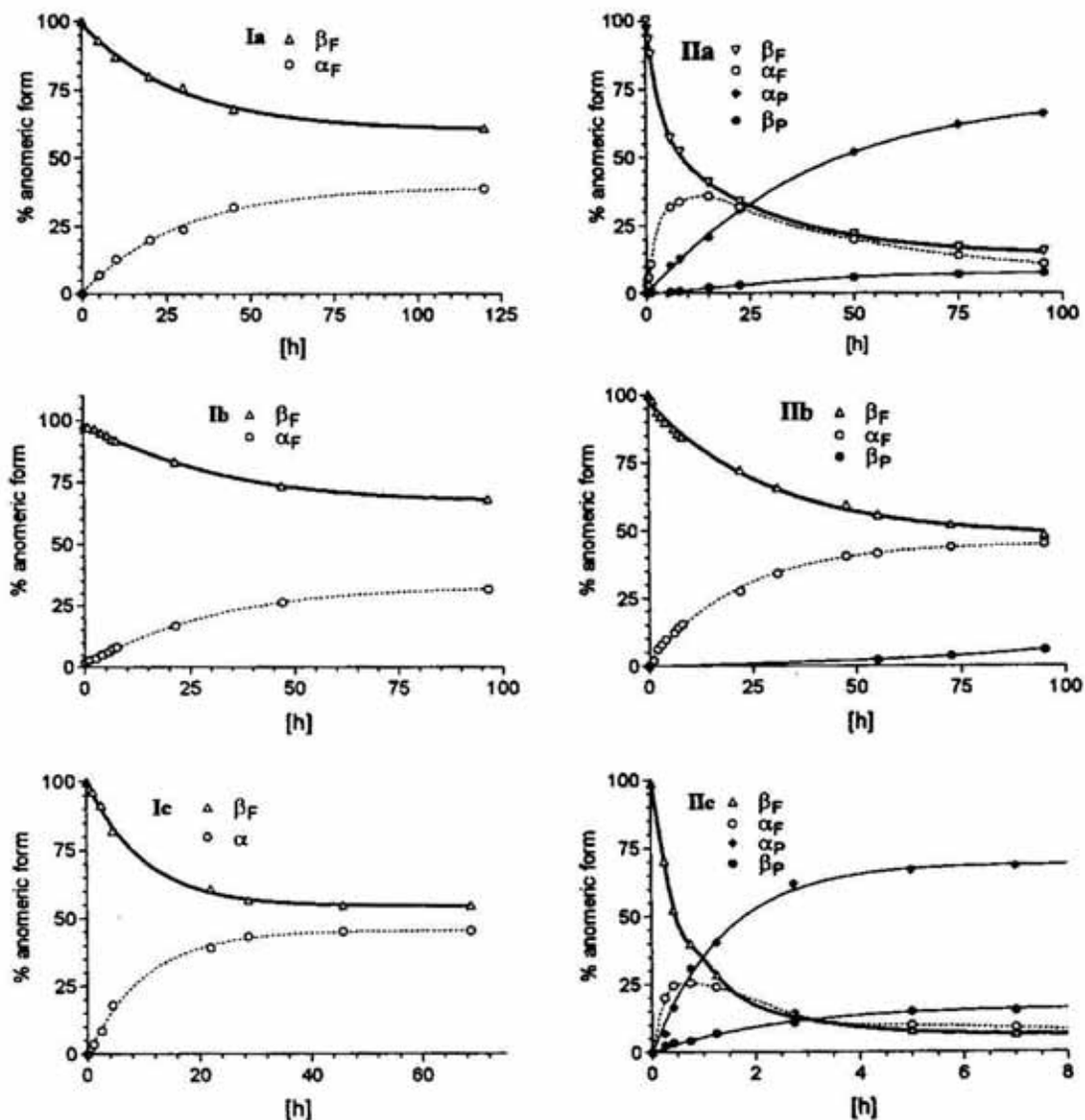


Figure 4. Kinetic plots for the acid catalysed anomerization reactions of the ribofuranosyl forms of the luminarine nucleosides Ia-c and IIa-c.

Table 2. Rate constants k and the distribution of products of the acid catalysed anomerisation reactions of the ribofuranosyl forms (β_F) of the luminarine nucleosides Ia-c and IIa-c

Compound	k [h^{-1}]	Furanosyl isomers (%)		Pyranosyl isomers (%)	
		α	β	α	β
Ia	0.0142	39.0	61.0	-	-
Ib	0.0120	31.7	68.3	-	-
Ic	0.0475	45.4	54.6	-	-
IIa	0.137	11.0	15.5	66.0	7.5
IIb	0.0275	45.0	48.5	6.5	-
IIc	1.497	8.7	5.2	68.8	17.3

H₃) [10]. Similarly, the greater reactivity of the **Ia-c** derivatives could be rationalized in terms of electron withdrawing effect of the O-acetyl groups in these compounds.

The results of kinetic studies described in this investigation clearly indicate that, in both series of luminarine nucleosides, **Ia-c** and **IIa-c**, the 2'-O-methyl derivatives (**Ib** and **IIb**) are least susceptible to the acid catalyzed mutarotation reaction and, therefore, most suitable for fluorescent labelling of oligo(deoxy)ribonucleotides *via* chemical synthesis.

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