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Fluorescent Guanosine-Nucleotide Analogs Suitable for Photoaffinity-Labeling Experiments

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The synthesis and properties of strongly fluorescent derivative of inosine and its nucleotides are described. Reaction of 2-chloro-inosinic acid with sodium azide leads to a product bearing the tetrazole ring between position 2 and 3. Methylation at N^1 was effected with dimethyl sulfate. The corresponding nucleosides and their 5′-triphosphates were also prepared. Only the non-methylated series is at equilibrium with small concentrations of their azido forms, and can be photolyzed by wavelengths above 300 nm. Both series are strongly fluorescent, their emission and excitation fluorescence spectra, as well as their quantum yields were measured. The nucleoside 5′-triphosphates are able to initiate and sustain polymerization of porcine brain tubulin.

Fluorescent nucleotide analogs have found useful application in the evaluation of their binding to enzymes or membranes. Usually, changes in hydrophobicity of their surroundings, or energy transfer with suitable acceptor molecules with concomitant enhancement or quenching of the fluorescence have been studied [1,2]. During the past few years, the etheno derivatives of adenine and cytosine, easily obtained from physiological nucleotides by reaction with chloroacetaldehyde, in particular have been applied to this end [3]. Since the deviation from the parent structure is small, the derivative is accepted at the binding sites for adenosine nucleosides in some cases [4,5]. To our knowledge has been proposed for this purpose; in fact, the number of guanosine analogs in general appears to be quite small.

In this communication, the synthesis and some properties of a small family of analogs of guanosine and their 5′-mono, di- and triphosphates are described, derived from the parent base tetrazolo [5,1-*b*]6-oxopurine. Since, in some cases, they are in equilibrium with appreciable amounts of their 2-azido-6-hydroxypurine counterparts, these nucleotides may also be applied for photoaffinity labeling during their decay by irradiation.

MATERIALS AND METHODS

Methods

Fluorescence excitation of Fig.3 and emission spectra of Fig.2 were taken in a Schoeffel fluorescence spectrometer. Lamp characteristics were corrected for by automatic comparison, the correction of the emission part was achieved by a thermoelement. Samples were measured at 25 °C. The fluorescence in the plots shown in Fig.4 and 5 were taken in a Hitachi-Perkin-Elmer spectrometer MPF 2A, and are not corrected in the emission part.

Irradiation experiments were carried out with the aid of a mercury lamp (Q 700, Hanau). The nucleotide solutions were positioned at a distance of 7 cm from the light source in a quartz reagent tube (internal diameter 0.8 cm) within a double-walled quartz tube kept at 14 °C with the aid of a thermostat. A WG 320 filter (Schott Co.) cut off all light below a wavelength of 313 nm.

Electrophoreses were run on paper in 0.1 M ammonium citrate buffer, pH 3.5 (system A) or on Polygram CEL 300 poly(ethylenimine) thin-layer plates plates (Machery & Nagel Co.) in 0.05 M sodium citrate buffer, pH 6.3 (system B). Thin-layer chromatography was performed on silica gel in chloroform/methanol, 7/3 (system C), 3/7 (system D), or 8/2 (system E) (v/v). The organic phosphates were detected by the method of Hanes *et al.* [6].

The R_F values of some nucleotides and nucleosides normalized at 1 for compound IIa are as follows in system A: IIa = 1, IIIa = 0.85, IMP = 0.72, 2-chloro-inosinic acid = 0.72, XMP = 0.61, IIc = 0.55, GMP = 0.52, AMP = 0.3; normalized at 1 for compound IIIa in system B: IIIa = 1, IMP = 0.82, AMP = 0.65, GMP = 0.54, XMP = 0.52, 2-chloro-inosinic acid = 0.49, IIa = 0.38.

The R_F values of nucleosides in thin-layer chromatography in system C are: IIc = 0.11, IIIc = 0.35,

Enzyme. Acid phosphatase (EC 3.1.3.2).

IV = 0.75; system D: IIc = 0.68, IIIc = 0.59, IV = 0.78; system E: 2-amino-6-chloro-purine riboside = 0.37, 2-amino-6-methoxy-purine riboside = 0.41, 2-fluoro-6-methoxy-purine riboside = 0.47, 2-hydrazino-6-methoxy-purine riboside = 0.26, 2-azido-6-methoxy-purine riboside (compound IV) = 0.53.

The position of the methyl group of compound IIIc was determinated with mass spectra in a Varian MAT CH 5 spectrometer and of IIIa with ^{13}C-nuclear magnetic resonance spectra in a Bruker HX 90 spectrometer. Infrared spectra was measured with a Perkin-Elmer 457 spectrometer.

Porcine brain tubulin was isolated as previously described [7]. Measurements of the polymerization reaction were taken in an Ubbelohde-type microviscosimeter Ic (Schott Co.). The apparatus was kept at 37 °C. The protein was depolymerized by incubation in 0.1 M morpholine ethane sulfonate buffer, pH 6.94, 1 mM ethyleneglycol bis(2-aminoethyl)-*N*, *N'*-tetracetic acid without addition of nucleotide for 30 min at 0 °C. After centrifugation in the cold for 1 h at 35000 rev./min, the protein solution was preincubated in the viscosimeter for 10 min and polymerization was started by the addition of nucleotide solution to a final concentration of 1 mM.

2-Chloro-inosinic Acid (I)

1.2 mmol of $CuCl_2$ were suspended in 20 ml of dried acetonitrile, and 1.4 mmol of nitrosyltetrafluoroborate were added. After a clear solution was obtained, 2 mmol of dry $CaCO_3$ were added, and finally 1 mmol of guanosine 5'-phosphate. The mixture was stirred for 30 min at room temperature, then the same amount of the fluoroborate was dissolved, and stirring was continued for 2 h. After filtration, the addition of 30 ml of a 5% sodium bicarbonate solution resulted in a green precipitate, which was dissolved by addition of glacial acetic acid. The solution was concentrated in the vacuum to 10 ml, and applied to a 2.5 × 10-cm column of Chelex 100 in the Tris form. The nucleotide was eluted with 500 ml of water, the solution was concentrated to a small volume and chromatographed over a 4 × 20-cm column of DEAE-Sephadex A-25 in the formate form, and eluted with 0.2 M ammonium formate buffer, pH 3.5. The fractions containing the nucleotide, monitored by its characteristic absorbance spectrum, were combined. The solution was freed from cations by passage over an appropriate Dowex 50 column, and formic acid was removed by aerotropic distillation with toluene in the vacuum. Yield: 66%. λ_{max} (pH 1) for 2-chloroinosine = 252 nm, ε = 12000 M^{-1} cm^{-1}. Analysis: $C_{10}H_{10}N_4O_8Cl_1P_1Na_2 \cdot 7\,H_2O$, M_r 552.52, (over saturated NaCl solution): calculated: C 21.74%, H 4.34%, N 10.14%, Cl 6.42%, P 5.61%, found: C 21.60%, H 4.39%, N 10.04%, Cl 6.35%, P 5.62%.

2-Azido-inosine 5'-Monophosphate (IIa)

1 mmol of cl^2IMPNa_2 (I) were dissolved in 10 ml of dimethylsulfoxide at 70 °C. 1.2 mmol of sodium azide were added, and the mixture was kept at 70 °C for 12 h. After cooling, 20 ml of water were added. The solution was applied to a 2.5 × 7-cm column of DEAE-Sephadex in its Cl^- form, washed with water, and eluted with a linear gradient to 0.4 M lithium chloride, containing 0.05 M formic acid. 10-ml fractions were collected. Usually, the nucleotide appeared in fractions 90–140. After concentration of the combined fractions to a few milliliters, 2 mmol of barium acetate were added, and the barium salt of the nucleotide was precipitated by addition of 2 vol. of ethanol. After thorough washing with 70% ethanol with centrifugation the white powder was dried in the vacuum. Purity of the compound was checked by electrophoresis in the systems A and B. Yield: 76%.

λ_{max} (pH 7): 301 nm, ε = 12500 M^{-1} cm^{-1}; 268 nm, ε = 6300 M^{-1} cm^{-1}. The infrared absorption spectra showed for the free acid in KBr disc: $\tilde{\nu}$ = 2160 cm^{-1} (weak) (N=N=N), $\tilde{\nu}$ = 1710–1740 cm^{-1} (broad) (C=O), $\tilde{\nu}$ = 1570 (broad) (C=C, C=N); for the barium salt in KBr disc: $\tilde{\nu}$ = 1680 cm^{-1} (C=O), $\tilde{\nu}$ = 1520–1540 cm^{-1} (C=C, C=N), no peak for (N=N=N).

Analysis:
$(C_{10}H_9N_7O_8P_1Ba)_2Ba$, M_r = 1184.24.
Calculated:
C 20.28%, H 1.52%, N 16.55%, P 5.23%, Ba 34.80%;
found:
C 20.28%, H 1.61%, N 16.72%, P 5.08%, Ba 34.66%.

2-Azido-inosine (IIc)

For the preparation of the nucleoside, 1 mmol of this barium salt was dissolved in 4 ml of 0.1 M acetic acid, the pH value was adjusted to 5.6. A solution of 20 mg of acid phosphatase in 2 ml 0.1 M acetic acid/NaOH buffer, pH 5.6, was added. After 3 h at room temperature, the barium phosphate was filtered off, and the solution was passed through a Sephadex G-10 column (3 × 120 cm) in water. The fractions containing the nucleoside were identified by ultraviolet spectroscopy and thin-layer chromatography in the system C and D and lyophilized. Yield: 94%.

The infrared absorption spectrum measured in KBr disc showed $\tilde{\nu}$ = 1680 cm^{-1} (C=O), $\tilde{\nu}$ = 1520, 1530 cm^{-1} (C=C, C=N).

Analysis: $C_{10}H_{10}N_7O_5Na$; M_r = 331.09; calculated:

C 36.27%, H 3.02%, N 29.60%; found: C 36.65%, H 3.19%, N 26.46%.

2-Azido-inosine 5'-Triphosphate (IIb)

0.5 mmol of the free 2-azido-inosine monophosphoric acid, prepared from the barium salt over a Dowex 50 column in the H^+ form, was suspended in 4 ml of dry dimethylformamide, containing 0.5 mmol of trioctylamine. After the nucleotide was dissolved, the solution was evaporated to dryness in the vacuum several times with addition of fresh solvent. Finally, to a solution in 3 ml of dimethylformamide, 0.2 ml of diphenyl chlorophosphate and 0.2 ml of *tert.*-butylamine were added. After 4 h at room temperature 1 mmol of *tert.*-butylammonium pyrophosphate [8] in 2 ml of dry dimethylformamide was added. After 12 h the solution was diluted with 10 ml of 80% methanol, and applied to a 2.5 × 9-cm column of DEAE-Sephadex A-25 in the chloride form, equilibrated with methanol/water (1/1). The column was washed with 50 ml of this solvent, then with 50 ml of water, finally it was eluted with a gradient to 0.4 M LiCl containing 0.05 M formic acid. The fractions were monitored by system A and B. The solution of the triphosphate was evaporated to a small volume under vacuum, and the barium salt was precipitated, as described above. Yield: 22%. Identified as the triphosphate by determination of inorganic phosphate after alkaline hydrolysis.

N^1-Methylated Compounds (IIIa, IIIb, IIIc, see Scheme 1)

1 mmol of 2-azido-inosine 5'-monophosphoric acid, dissolved in 50 ml of water and at a pH value of 3.5 after addition of sodium bicarbonate, was mixed with 1.5 ml of dimethyl sulfate, and the pH was kept at 3.5–3.8 by addition of saturated sodium bicarbonate solution. After 2–3 h, the mixture was chromatographed on DEAE-Sephadex A-25 as described above. The fractions were evaluated by electrophoresis in system A and B, and worked up as described for compound IIa. Yield: 40.5%.

λ_{max} (pH 7): 307.5 nm, $\varepsilon = 8850\ M^{-1}\ cm^{-1}$; 264.5 nm, $\varepsilon = 4850\ M^{-1}\ cm^{-1}$. The infrared absorption spectra showed for the free acid in KBr disc: $\tilde{\nu} = 2160\ cm^{-1}$ (very weak) (N=N=N), $\tilde{\nu} = 1710$–$1740\ cm^{-1}$ (broad) (C=O), $\tilde{\nu} = 1550$–1580 (broad) (C=C, C=N) and for the barium salt in KBr disc $\tilde{\nu} = 1720\ cm^{-1}$ (C=O), $\tilde{\nu} = 1550, 1580\ cm^{-1}$ (C=C, C=N), no peak for (N=N=N).
Analysis: $C_{11}H_{12}N_7O_8PBa$, $M_r = 538.49$; calculated: 24.54%, 2.23%, 18.20%, 5.75%, 25.51%; found: 24.35%, 2.73%, 18.14%, 5.54%, 25.55%.

The corresponding nucleoside (IIIc) was prepared by enzymic hydrolysis, as described above. Yield: 95%. The infrared absorption spectrum measured in KBr disc showed: $\tilde{\nu} = 1720\ cm^{-1}$ (C=O), $\tilde{\nu} = 1560, 1580\ cm^{-1}$ (C=C, C=N).
Analysis: $C_{11}H_{13}N_7O_5$, $M_r = 323.18$; calculated: C 40.85%, H 4.02%, N 30.33%; found: C 40.75%, H 4.22%, N 29.58%.

The N^1-methyl-nucleoside 5'-triphosphate (IIIb) of this family was prepared from the monophosphate as described for 2-azido-inosine 5'-triphosphate. Fractions from DEAE-Sephadex chromatography were identified by electrophoresis in systems A and B. The yield was 32% in this preparation.

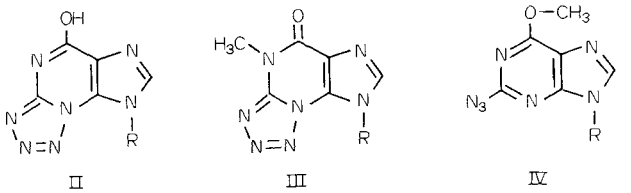
2-Azido-6-methoxy-purine Riboside (IV)

This synthesis was carried out in analogy to the procedures of Gerster *et al.* [9], to the stage of 2-fluoro-6-methoxypurine riboside. This in turn could be transformed to the 2-hydrazino compound by reaction with hydrazine in methanol. The reaction mixture was brought to dryness below 40 °C and the residue was diazotized in the stoichiometric amount of 1 N HCl with sodium nitrite. 2-Azido-6-methoxy-purine riboside appeared as a white precipitate, and was recrystallized from ethanol/water (4/1).

λ_{max} (pH 7): 269 nm, $\varepsilon = 15300\ M^{-1}\ cm^{-1}$; 223 nm, $\varepsilon = 12100\ M^{-1}\ cm^{-1}$. The infrared absorption spectrum showed $\tilde{\nu} = 2140\ cm^{-1}$ (strong), $\tilde{\nu} = 1600\ cm^{-1}$ (C=C, C=N), no peak for (C=O).
Analysis: $C_{11}H_{13}N_7O_5$, $M_r = 323.18$; calculated: C 40.85%, H 4.02%, N 30.33%; found: C 40.73%, H 3.97%, N 29.73%.

RESULTS AND DISCUSSION

2-Chloro-inosinic acid was obtained from GMP in a novel reaction with nitrosyl tetrafluoroborate in acetonitrile using $CuCl_2$ as a catalyst. Preparations of the other compounds described here (Scheme 1) followed modifications of standard procedures, except the formation of nucleosides from the corresponding



II III IV

II, III a: R = ribosyl 5' - monophosphate
b: R = ribosyl 5' - triphosphate
c: R = ribose
IV : R = ribose

Scheme 1. *Formulae of compounds II, III and IV.* The bases of the nucleotide analogs are in (II) tetrazolo[5,1-*b*]6-oxo-purine or 2-azido-6-oxo-purine and in (III) tetrazolol[5,1-*b*]1-methyl-6-oxo-purine or 1-methyl-2-azido-6-oxo-purine

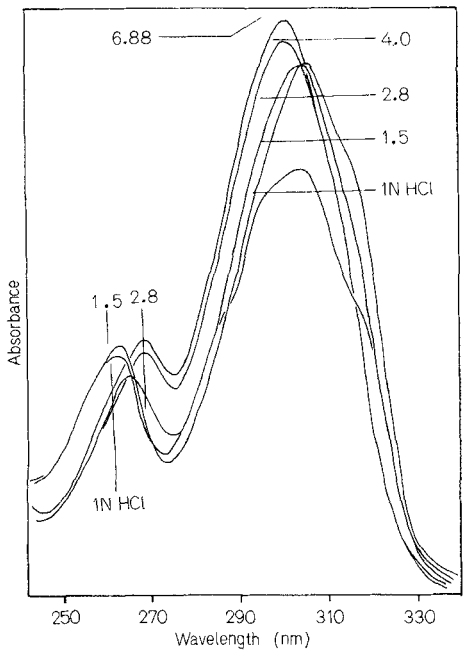


Fig. 1. *Variation in ultraviolet absorption spectrum of compound IIa with pH*

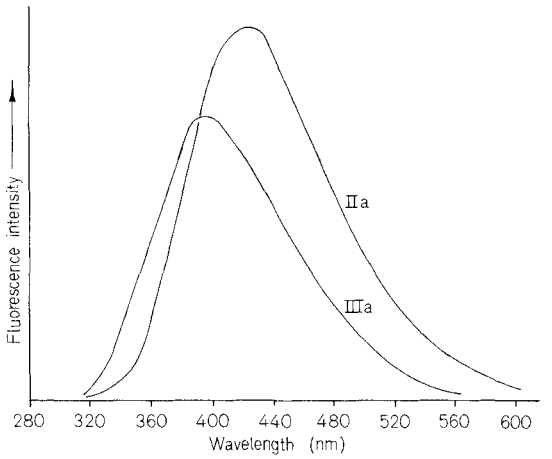


Fig. 2. *Corrected molecular fluorescence spectra of the compounds IIa and IIIa as the sodium salt in water, 10 μM, excitation: 313 nm*

5′-monophosphates, which was carried out by aid of the appropriate enzyme.

Since the methylation procedure with dimethyl sulfate might have led to a mixture of the N^1-methylated and 6-methoxy derivatives, we took car to prove the exclusive position of the methyl group at N^1. Finally, 2-azido-6-methoxy-purine riboside was synthesized, and compared to the N^1-methylated, as well as the nonmethylated compound by ^{13}C-nuclear magnetic resonance spectroscopy, which unequivocally proved the proposed structures.

The λ_{max} values and the molar absorption coefficients of the ultraviolet spectra of IIa, IIIa, and IV are shown in Materials and Methods. Only the nonmethylated series showed a dependency of λ_{max} values upon the pH value of the medium between 1.5 and 6.88. No measurements were carried out above pH 8, as both series were found not to be stable in that region. The changes in the spectrum of compound IIa at various pH values are demonstrated in Fig. 1. As may be seen, the spectrum at lower pH approaches the curve of the N^1-methylated series (Fig. 1). This may be attributed to the shift in the equilibrium of the lactam-lactim form of the base. A further indication for this comes from the data of infrared spectra (Materials and Methods), especially from the shifts in the C=O and C=N bands.

These spectra also permitted the estimate of the amount of the azido forms in equilibrium with the tetrazolo structures of the series. Investigations on azido-purines yield the same results [10]. We concluded that the N^1-methylated series (IIIa, IIIb, IIIc) exists practically completely in the form of the tetrazoles, while the nonmethylated series does form the azides, although probably not in excess of about 5% of the total. Tetrazolo[5,1-*b*]inosine (IIc) is a distinctly more acidic nucleoside than its N^1-methylated counterpart (IIIc). This should be an expression of the different distribution between the lactam and lactim form of the base, and can easily be demonstrated by electrophoresis of the nucleosides and nucleotides (Materials and Methods).

Both series are strongly fluorescent compounds, showing a single emission band as shown in Fig. 2 for the nucleoside monophosphates. Both show relatively high Stoke's shifts, amounting to 10.5×10^3 cm^{-1} or 30 kcal (125.5 kJ) in the case of the nonmethylated substance, and 8.6×10^3 cm^{-1}, or 22.8 kcal (95.4 kJ) for the N^1-methylated series. The corrected fluorescence excitation spectra are shown in Fig. 3. When compared to the ultraviolet-absorbance spectra, they indicated that the longer-wavelength absorbance band is primarily responsible for fluorescence excitation. The relative fluorescence intensity at the maximum in all cases is dependent on the pH value of the medium, although the effect is quite small in the case of the N^1-methylated series (Fig. 4). In the nonmethylated series, the wavelength of maximum fluorescence is decreased from 425 nm at pH 7 to 400 nm in 1 N hydrochloric acid.

From this, a dependence of fluorescence intensity upon hydrophobicity of the medium was to be expected. It is shown for the nucleoside monophosphates (IIa and IIIa) in Fig. 5. The increase of fluorescence for the N^1-methylated compound might be ascribed to a hypochromic shift of a n-π^* promotion (Fig. 5C), while the slight increase of fluorescence energy at high concentrations of ethanol is probably due to polarity changes in the solvent [11].

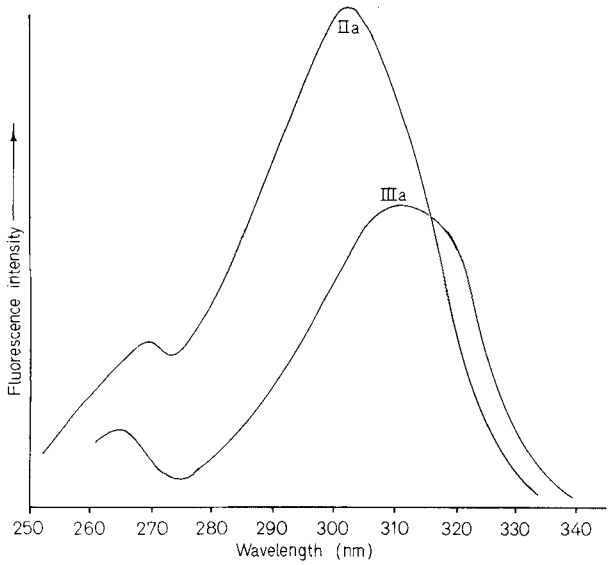


Fig. 3. *Corrected fluorescence exitation spectra of the compounds IIa and IIIa as the sodium salts in water, 10 μM, emission: IIa 425 nm, IIIa 396 nm*

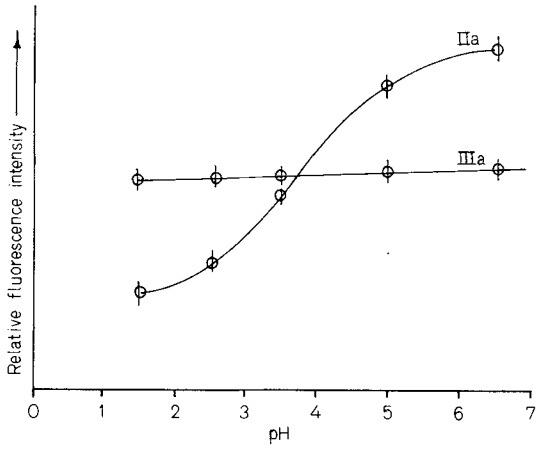


Fig. 4. *Variation in relative fluorescence intensity of compounds IIa and IIIa with pH*

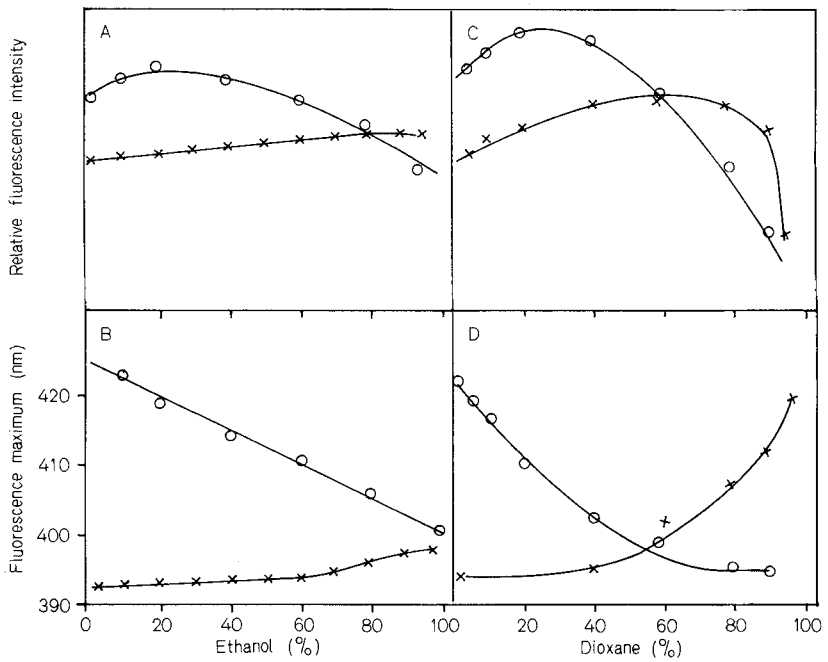


Fig. 5. *Variation in relative fluorescence intensity (A, C) and shift of fluorescence maximum (B, D) of compound IIa sodium salt (○) and IIIa sodium salt (×) with concentration of ethanol (A, B) or dioxane (C, D) in water*

For the determination of quantum yields [12], 2-aminopyridine was chosen as fluorescence standard. To start from an equal intensity of excitation, all measurements were taken with excitation at 313 nm. Our measurements indicated that 2-aminopyridine then shows the same quantum yield, 0.6, that has been determined with excitation at 285 nm [13]. Concentrations of the fluorescent dyes did not exceed 10 μM. The measurement of fluorescence intensity had been calibrated with the aid of a thermoelement. The readings of fluorescence intensities at the various wavelengths were recalculated for quantum yields [12]:

$$\frac{dE}{d\lambda}\,\lambda^3 = \frac{dQ}{d\nu}.$$

During the past few years, 1:N^6-ethenoadenosine and its derivatives have found wide application as

strongly fluorescent analogs of adenosine derivatives [3]. In Table 1, the quantum yields of the monophosphate and of the nucleotides IIa and IIIa of our series are compared. In the literature, a value of 0.59 is usually employed for 1:N^6-ethenoadenosine, while in our measurements a yield of 0.4 was obtained. This discrepancy is explained by the use of quinine sulfate as a standard, with a quantum yield of 0.7 [3] for determination of the value of 0.59. Our reference quantum yield value of 0.6 for 2-aminopyridine, however, in turn originated in comparison with quinine sulfate, its yield in this case was taken as a standard with 0.46 [13]. When this difference between the two quantum yield values of quinine sulfate is taken into consideration, a good agreement with literature values is reached. Roughly, our analogs then show half of the fluorescence intensity of 1:N^6-ethenoadenosine.

Photolysis was investigated for the substances IIa, IIIa, and IV. As shown in Fig. 6, the methylated series is much more stable against photolysis, in agreement with the very small content of the azido form in these cases. As our irradiation conditions used only wavelengths above 313 nm, a selective photolysis of the nonmethylated analogs IIa, IIb, IIc, and IV is possible in the presence of proteins, and without detriment to their enzymic activity.

As a test for the acceptance of our substances by at least some biological systems instead of guanosine nucleotides, the influence of IIb and IIIb upon the polymerization of tubulin was investigated. After depolymerization without addition of GTP, polymerization was induced under the influence of elevated temperature and 1 mM GTP, IIIb or IIb, as described in Methods. Control experiments without

Table 1. *Quantum yield values*
ε-AMP = 1:N^6-ethenoadenosine 5′-monophosphate

Compound	Solvent	Value
IIa Na salt	H_2O	0.26
IIIa Na salt	H_2O	0.17
ε-AMP Na salt	H_2O	0.4
ε-AMP	buffer pH 7	0.59 [3]
2-Aminopyridine	0.1 N H_2SO_4	0.6 [13]

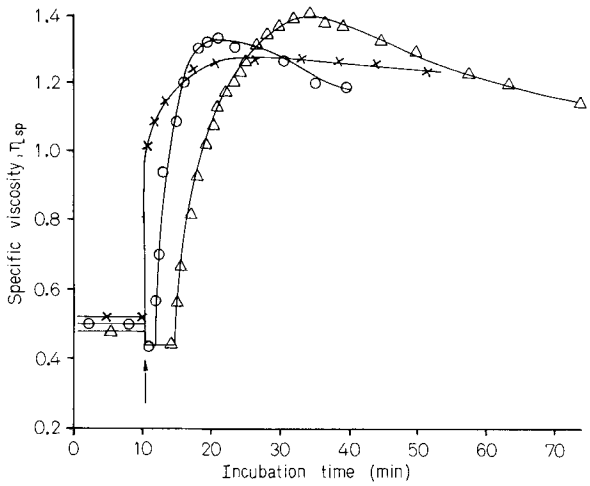


Fig. 7. *Effect of GTP (×), compound IIb (△) and compound IIIb (○) on tubulin polymerization.* Protein concentration was 5 mg/ml in nucleotide-free 2-morpholinoethane sulfonate buffer. After incubation for 10 min (indicated by the arrow), GTP or analogs were added to a final concentration of 1 mM

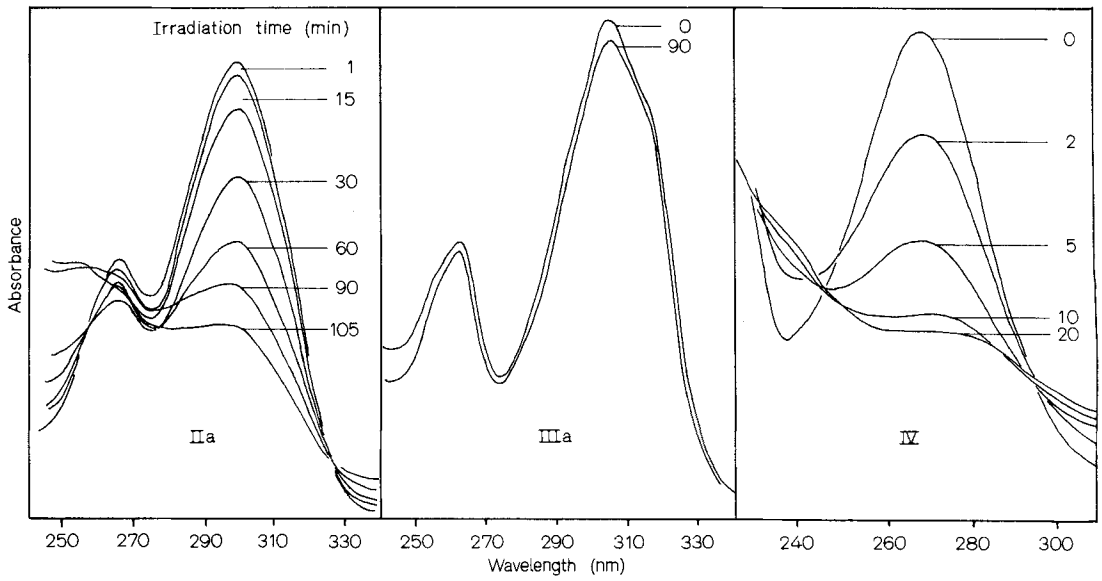


Fig. 6. *Variation in ultraviolet absorption spectra of compounds IIa, IIIa, and IV in water with irradiation time of light above 313 nm (see Materials and Methods).* Concentrations: IIa 75 μM, IIIa 100 μM, IV 50 μM

addition of nucleotide, or addition of GTP after preincubation for 15 min verified the role of the nucleotides in polymerization. In both cases, as shown in Fig. 7, the protein reached the same end value of viscosity as the controls, although were was a slight delay during the initial phase of polymerization, more pronounced in the case of IIb. In view of the alternating role of the two molecules of nucleotide bound per dimer of molecular weight 110000, we now want to initiate further experiments with ^{32}P-labeled analogs. These should allow us to ascertain the amount of nucleotide exchanged per monomer, and might permit photoaffinity labeling, as well as measurement of fluorescence changes during the polymerization process.

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