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New, ionic side-products in oligonucleotide synthesis: formation and reactivity of fluorescent N-/purin-6-yl/pyridinium salts[1]

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ABSTRACT

Fluorescent N-/purin-6-yl/pyridinium salts are formed in pyridine assisted phosphorylations and arenesulphonations of the hypoxanthine lactam system under various conditions including those used in oligonucleotide synthesis. The N^1-methyl-N^3-/purin-6-yl/imidazolium salt is generated in phosphorylation with TPSCl / 1-methylimidazole as a coupling system. Both salts are representatives of a new family of ionic side-products in oligonucleotide synthesis involving hypoxanthine residues. Their isolation procedure has been developed. High reactivity of N-/purin-6-yl/pyridinium salts towards some reagents used in oligonucleotide chemistry, e.g. pyridinium mediated conversion of hypoxanthine into 6-aminopurine, can result in point mutations in synthesized oligomer.

INTRODUCTION

Evaluation of the nature of side reactions in oligonucleotide synthesis is of considerable interest. In recent years much attention has been paid to the problem of protection of N-acylguanine /2-12/ and uracil or thymine /2,9, 10,11,13-15/ lactam systems during oligonucleotide synthesis. Reese and Ubasawa observed /16,17/ transformation of O-protected uridine and O,N-protected guanosine into the corresponding 4-/azol-1-yl/- and 6-/azol-1-yl/ derivatives in reactions with 1-/arene-sulphonyl/azoles solely or, more efficiently, in the presence of diphenylphosphate in pyridine. Formation of the same type of side-products should also be considered /12/ during 3'-OH component phosphorylations with $POCl_3$ derived agents containing azoles /12,18-22/.

Studies presented in this paper were initiated by an unexpected observation concerning phosphorylation of O-protected inosine with 4-chlorophenyl-phosphorodichloridate in the presence of 1,2,4-triazole in pyridine i.e. under conditions proposed by Sung /18,19/ for transformation of O-protected thymidine into the 4-/1,2,4-triazol-1-yl/ analogue. Instead of 6-/1,2,4-triazol-1-yl/ derivative, a quantitative formation of the fluorescent N-/purin-6-yl/pyridinium salt was observed.

We realized that such behaviour of the hypoxanthine lactam system and possibly other base residues in pyridine assisted phosphorylations used in oligonucleotide synthesis by the phosphotriester apporach, could generate a new, ionic side-products. In order to test this assumption and to evaluate possible further consequences of N-/purin-6-yl/pyridinium salt formation we conducted experiments on /i/ pyridine assisted phosphorylations and arenesulphonations of the hypoxanthine as the model of heteroaromatic lactam system and the /ii/ reactivity of the resulting pyridinium salts towards some reagents used in oligonucleotide synthesis and deprotection. Results of these studies are presented in this paper.

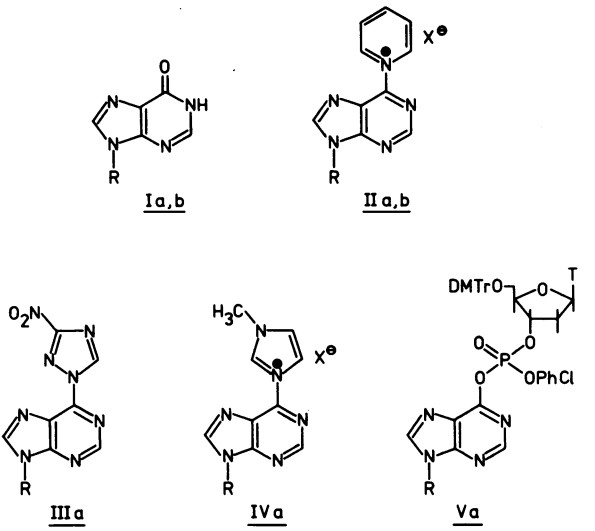
RESULTS AND DISCUSSION

When pyridine solution of 2',3',5'-tri-O-acetylinosine 1a was treated overnight with 4-ClPhOPOCl$_2$ /1.5 eqv./ and 1,2,4-triazole /3 eqv./ quantitative formation /tlc/ of a polar, fluorescent product was observed. To isolate the product, the phosphorylation mixture was evaporated, dissolved in cold water, treated with Dowex 1 resin /HCO_3^-/ to neutralize pyridine hydrochloride, the filtrate was concentrated and passed through silica gel in ethanol-water-formic acid to remove 1,2,4-triazole, and subsequently through Dowex 1 /Cl^-/. ^{1}H NMR spectrum /D_2O/ indicated three coupled multiplets at low field, centred at δ 10.14, 8.98 and 8.46 ppm, which strongly suggested the presence of the N-arylpyridinium cation. In the spectrum of the product synthesized in pyridine-d_5, these multiplets vanished making both purine ring proton signals clearly visible at 9.19 and 8.94 ppm. This suggested that the isolated fluorescent product is N-/9-/2',3',5'-tri-O-acetyl-β-D-ribofuranosyl/-purin-6-yl/ pyridinium chloride IIa /X=Cl, positive silver nitrate test, fluorescence /H_2O/λ_{max} 438 nm, λ Exc 313 nm/. It appeared, see below, that transformation of Ia into IIa /X=Cl/ can also be achieved without 1,2,4-triazole. In that case isolation procedure described above without the silica gel chromatography step, led to pure IIa /X=Cl, 87% yield/ which could be stored in dark as concentrated aqueous solution /0.2M, oxygen free/ or as a powder after lyophilization. Pure IIa /X=Cl/ is stable within the pH range of 1.5 - 6.5.

The 9-methylhypoxanthine Ib was transformed into crystalline N-/9-methylpurin-6-yl/pyridinium chloride IIb /X=Cl, fluorescence /H_2O/λ_{max} 450 nm, λ Exc 313 nm/ with 70% yield.

Pyridine assisted phosphorylations of the hypoxanthine lactam system

Table 1 summarizes the results of pyridine assisted phosphorylations of 2',3',5'-tri-O-acetylinosine Ia with two sets of phosphorylating agents under



Scheme 1. a: R= 2',3',5'-tri-O-acetyl-β-D-ribofuranosyl
b: R= CH_3

conditions which in most cases are closely related, except for reaction time, to those applied during oligonucleotide synthesis by the phosphotriester method.

Phosphorylations with $POCl_3$ derivatives. Ia treated in pyridine with $POCl_3$ /2 eqv./ formed IIa /X=Cl/ as a sole product. Course of this reaction clearly explains the reasons why pyridine cannot be used as a base in reactions of hypoxanthine residues with $POCl_3$ in order to obtain 6-chloropurines.

Analogous results have been obtained in pyridine assisted reactions with: $POCl_3$ /2 eqv./ and 1,2,4-triazole /6 eqv./; dioxane solution phosphorotri-/1,2,4-triazolide/ /2 eqv./ /23/; 4-ClPhOPOCl_2 /1.5 eqv./; 4-ClPhOPOCl_2 /1.5 eqv./ and 1,2,4-triazole /3 eqv./ and with dioxane solution of 4-chlorophenyl-phosphorodi-/1,2,4-triazolide/ /24/ /2 eqv./. In all above described cases the quantitative formation of N-/purin-6-yl/pyridinium salts was reached after 17 hrs at room temperature. We found that reaction with 4-ClPhOPOCl_2 /entry 4/ is most suitable for the preparation of IIa /X=Cl/. The formation of N-/purin-6-yl/pyridinium salts must be considered when applying conditions given under entries 5 and 6 for the phosphorylation of inosine and most likely guanosine derived 3'-OH component for oligonucleotide synthesis /25,26/; drying of 3'-OH component by concentration of its pyridine solution should be avoided.

Table 1. Course of pyridine assisted phosphorylations of hypoxanthine lactam system Ia.

Phosphorylation with:	Composition of the mixture [a]				
	Ia	IIa	IIIa	IVa	Va
1. $POCl_3$	-	S	-	-	-
2. $POCl_3$, 1,2,4-triazole	-	S	-	-	-
3. PO/1,2,4-triazolide/$_3$	-	S	-	-	-
4. 4-ClPhOPOCl_2	-	S	-	-	-
5. 4-ClPhOPOCl_2, 1,2,4-triazole	-	S	-	-	-
6. 4-ClPhOPO/1,2,4-triazolide/$_2$	-	S	-	-	-
7. 4-ClPhOPOCl_2,3-nitro-1,2,4-triazole	m	M	m	-	-
8. 4-ClPhOPOCl_2, tetrazole [aa]	m	m	-	-	-
9. TDE, TPSCl	-	S	-	-	-
10. TDE, TPSCl, 1-methylimidazole	-	m	-	M	-
11. TDE, TPSCl, tetrazole	m	m	-	-	m
12. TDE, TPSTe	m	m	-	-	m
13. TDE, TPSNT	-	m	M	-	-
14. TDE, TPST	M	m	-	-	-

[a] Composition of the phosphorylation mixtures was estimated by tlc /systems A, B and D, see experimental/. S- sole, M- major, m- minor component. Here and within all discussion the nature of anion X is specified for isolated salts only

[aa] mixture contains mostly other unindentified products.

Abbreviations: TDE - 5'-O-dimethoxytritylthymidine-3'-/4-chlorophenyl/-phosphate triethylammonium salt; TPSCl - 2,4,6-triisopropylbenzenesulphonyl chloride; TPST, TPSNT and TPSTe - 1/2,4,6-triisopropylbenzenesulphonyl/ -1,2,4-triazole, 3-nitro-1,2,4-triazole and -tetrazole respectively.

An addition of 3-nitro-1,2,4-triazole /4 eqv./ to the reaction mixture containing Ia and 4-ClPhOPOCl_2 /2 eqv./ led to the formation of IIa /X=Cl/ as the major product and 6-/3-nitro-1,2,4-triazol-1-yl/-9-/2',3',5'-tri-O-acetyl-β-D-ribofuranosyl/purine IIIa with 10% yield /see ref. 22/.

Phosphorylations with species formed from nucleoside-3'-phosphodiester and TPSCl or its azole derivatives. 5'-O-dimethoxytritylthymidine-3'-/4-chlorophenyl/phosphate triethylammonium salt /TDE/ was used instead of diphenylphosphate /17/ as phosphodiester component in order to get the phosphorylating system used in oligonucleotide synthesis. Although TPSCl alone is no longer in

use as the coupling agent in oligonucleotide synthesis we investigated the reactivity of TPSCl /2.5 eqv./ combined with TDE /1.1 eqv./ towards Ia in pyridine /Table 1, entry 9/. It appeared that IIa, isolated as chloride, is formed as the sole nucleoside product in this strongly coloured mixture.

TPSCl in combination with 1-methylimidazole /27,28/ has been proposed as the coupling system for oligonucleotide synthesis. We found /entry 10/ that pyridine solution of Ia treated with TDE /1.1 eqv./, TPSCl /2.5 eqv./ and 1-methylimidazole /5 eqv./ gave a new fluorescent salt N^1-methyl-N^3-/9-/2',3',5'--tri-O-acetyl-β-D-ribofuranosyl/purin-6-yl/imidazolium chloride IVa /X=Cl/ /δ 10.09 ppm imidazolium, 8.94 and 8.75 ppm purine protons, fluorescence /H_2O/ λ_{max} 400 nm, λ Exc 298 nm/. A small amount of IIa was also found. A possible formation of IVa in coupling reactions with 1-methylimidazole must be considered especially when solid support techniques are used /28/. Chemistry of IVa and related N-protected guanine derivatives is under investigation /30/.

Mixture of Ia, TDE /1.1 eqv./ and TPSCl /3.5 eqv./ in the presence of tetrazole /9 eqv./ in pyridine - the system proposed /29/ as more efficient than TPSTe solely, led to an equal formation of the O^6-phosphotriester intermediate Va which was isolated in a low yield /^{31}P NMR, $CDCl_3$, -14.59 ppm, diastereoisomers not resolved/ and IIa. The observation of slow decomposition of Va in chloroform to Ia and TDE, and the formation of IIa when treated overnight with pyridine gave additional support to the structure proposed for Va. Analogous distribution of products was observed when Ia was treated with TDE /1.1 eqv./ and TPSTe /3 eqv./ in pyridine /entry 12/. The expected /16,17/ 6-/tetrazol-1-yl/derivative was not observed. However, when Ia was treated with TDE /1.1 eqv./ and TPSNT /3 eqv./ in pyridine /entry 13/, 6-/3-nitro-1,2,4-triazol-1-yl/derivative IIIa was formed in 65% yield. Only a small amount of IIa was found. This reaction is certainly of preparative value and results obtained can be compared with those in the case of 2',3',5'-tri-O-acetyl-N^2-benzoylguanosine /16,17/. Reaction with the much less active TPST /3 eqv./ and TDE /1.1 eqv./ /entry 14/ led only to a small amount of IIa.

At present we are working on analytical test based on Zincke reaction /see below/ to quantify Ia → IIa transformation. At least 5% of such conversion is expected after 10-45 min. for most of reactions studied.

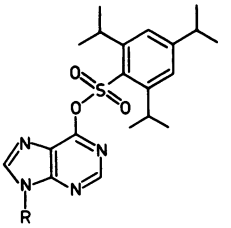
Mechanism of IIa formation - ^{31}P NMR studies. The isolation of Va /entry 11, 12, Table 1/ and some previously reported observations concerning phosphorylation of guanine residues /3,31-33/ allow us to suggest that formation of N-/purin-6-yl/pyridinium salts consists of two steps: O^6-phosphorylation of the hypoxanthine ring followed by nucleophilic displacement at C^6 with pyridine.

We tried to confirm this assumption by ^{31}P NMR experiments for a two simplest and most representative cases.

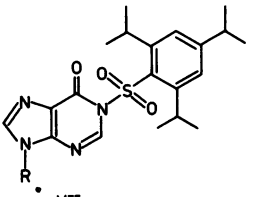
Table 1, entry 4. When a solution of Ia in pyridine was added to a nmr tube containing 4-ClPhOPOCl$_2$ in dioxane /+1.71 ppm/ two additional signals appeared immediately, the major at -8.81, decreased in time, the minor at -11.22 ppm, increased in time. To assign these two peaks we monitored the phosphorylation step separately. Ia dissolved in dioxane was treated with 4-ClPhOPOCl$_2$ /2 eqv./ and after 30 min. the reaction mixture was filtered directly into the nmr tube; the ^{31}P NMR spectrum showed the presence of a minor signal of unreacted 4-ClPhOPOCl$_2$ at +1.79 ppm and a main peak at -8.99 ppm assigned to the O^6-phosphorylated intermediate. The addition of pyridine at that stage led to the formation of two new signals, a minor at -5.19 and a major at -11.38 ppm. The signal at -8.99 ppm disappeared after 24 hrs while the one at -11.38 increased considerably. Addition of water at this point led to the appearance of only two signals: at -4.33 and a minor at -16.77 ppm which we assigned to 4-chlorophenylphosphate and symmetrical di/4-chlorophenyl/pyrophosphate /27/. The observations allowed us to assigne the signal at -11.38 ppm in dioxane-pyridine to 4-ClPhOPO/O^-/$N^+C_5H_5$ /-10.8 ppm in pyridine, ref. 27/ which is presumably formed from 4-chlorophenylchlorophosphate anion, an initial leaving group from C^6 upon nucleophilic attack by pyridine. To some extent, 4-ClPhOPO/O^-/$N^+C_5H_5$ is also formed by hydrolysis of 4-ClPhOPOCl$_2$ with traces of water /-11.18 ppm in dioxane-pyridine/.

Table 1, entry 9. When TPSCl /3 eqv./ and Ia were simultaneously added to TDE /1.1 eqv./ in pyridine, ^{31}P NMR spectra revealed besides the TDE signal /-6.17 ppm/ three characteristic signals at -19.81, -20.16 and -20.35 ppm, corresponding most probably /34,35/ to diastereoisomers of TDE-derived tetrasubstituted pyrophosphate. After 10 min. all phosphodiester reacted and after 1 hr two close signals at -14.56 and -14.75 ppm appeared which we assigned to two diastereoisomers of O^6-phosphorylated intermediate of a phosphotriester nature. It corresponds to the ^{31}P NMR data obtained for intermediate Va and those reported for diarylalkylphosphotriesters /36/. The intensity of the signal at -5.92 ppm increased considerably within 17 hrs with the concomitant disappearance of the signals at -14.56 and -14.75 ppm. We attributed signal at -5.92 ppm to TDE anion released from C^6 upon pyridine attack.

Results of ^{31}P NMR experiments prove /entry 4/ or strongly support /entry 9/ the proposed two step mechanism of Ia → IIa transformation via O^6-phosphorylated intermediates.



VIa



VIIa

Pyridine assisted arenesulphonations of the hypoxanthine lactam system

Although, both ^{31}P NMR data for the reaction under entry 9 /Table 1/ and the isolation of Va indicate that O^6-phosphorylation of Ia takes place readily, one cannot exclude that under conditions typical for the formation of mixed anhydride of phosphoric and sulphonic acid /35/ O^6-arenesulphonyl derivatives can be formed and serve as intermediates for N-/purin-6-yl/pyridinium salts. Reported by Sekine et al /3/ slow conversion of 2',3',5'-tri-O-acetyl-O^6-/2,4,6-triisopropylbenzenesulphonyl/-N^2-tritylguanosine in pyridine into related pyridinium salt strongly supports this suggestion. In order to check it, the reactivity of Ia towards TPSCl, TPST, TPSNT and TPSTe /5 eqv./ in pyridine was tested /Table 2/. All reactions were terminated after 17 hrs. To facilitate their analysis the preparation of O^6-/2,4,6-triisopropylbenzenesulphonyl/derivative VIa was attempted under conditions described for O^6-mesylation of N-benzoylguanine residues /37/. However, when Ia was treated overnight with TPSCl /2.6 eqv./ in the presence of triethylamine /3 eqv./ in methylene chloride crystalline N^1-/2,4,6-triisopropylbenzenesulphonyl/derivative VIIa was obtained /65% yield/; the desired O^6-isomer VIa was formed with ca 15% yield. The structure of VIIa was proved by its conversion to pyrimidine ring-open product: 1-/β-D-ribofuranosyl/-5-aminoimidazol-4-/N-2,4,6-triisopropylbenzenesulphonyl/carboxamide in reaction with sodium hydroxide in ethanol /38/ /data not shown/. From both isomers only O^6-derivative VIa reacts with pyridine to form N-/purin-6-yl/pyridinium salt IIa /X=$ArSO_3$/ /80%, 48 hrs/.

Data presented in the Table 2 show the following order of reactivity of the condensing agents towards the hypoxanthine lactam system in pyridine solution: TPSTe > TPSNT ≫ TPSCl /TPST not reactive/ and confirm that N-/purin-6-yl/pyridinium salts can be formed via O^6-arenesulphnated intermediates which seem to be more reactive than related N-tritylguanine derivatives /3/. At present it is difficult to compare our results with those reported for mesitilenesulphonation of guanine /39/ and N-benzylguanine /17/ residues; we are reinvestigating those reactions in view of pyridinium salt formation and

Table 2. Course of pyridine assisted arenesulphonations of hypoxanthine lactam system of Ia.

Arenesulphonation with:	Composition of the mixture [a]				
	Ia	IIa	IIIa	VIa	VIIa
1. as ref. TPSCl, Et_3N, CH_2Cl_2	-	-	-	m	M
2. TPSCl	M	m	-	m	m
3. TPST	S	-	-	-	-
4. TPSNT	-	M	m	m	m
5. TPSTe	-	M	-	-	m

[a] Composition of the arenesulphonation mixtures was estimated by tlc /systems A, C and D/. S- sole, M- major, m- minor component.

differences in the reactivity of both parent lactam systems.

Reactivity of N-/purin-6-yl/pyridinium salts.

N-arylpyridinium salts, especially those with aryl groups with electron withdrawing properties, undergo a nucleophilic attack by amines or hydroxyl ion at the α -carbon of pyridinium ring which often results in ring opening and cleavage with the formation of the appropriate triene and release of arylamine. These transformations are referred to as Zincke reaction /40-42/.

We found that IIa /X=Cl/ and IIb /X=Cl/ react with an excess of dimethylamine and conc. aqueous ammonia or /data not shown/ with aqueous sodium hydroxide, morpholine, benzylamine and aniline in dioxane with quantitative /tlc/ formation of partially deacetylated adenosine or 9-methyladenine, respectively. Deep yellow colour, due to formation of the ring-open intermediates, which is characteristic for all these reactions, enables fast UV-VIS detection of IIa. A high efficiency of the ring opening process allows mediated by IIa conversion of Ia into 2',3',5'-tri-O-acetyladenosine in a one-flask fashion with 75% yield. As far as we know this is the first transformation of this kind performed with such high efficiency.

Presented above results forced us to inspect the reactivity of N-/purin-6-yl/pyridinium salts /IIa,b X=Cl/ towards selected reagents commonly used in oligonucleotide synthesis by phosphotriester approach.

IIa and IIb treated overnight with aqueous solutions containing HCO_3^- anions /sat. $NaHCO_3$, 1M TEAB buffer pH 7.5/ were to small extent reversed to parent Ia and Ib. IIa resists short passage through layer of RP-2 silica gel in aceton-water, however, it is extensively decomposed in the presence of silica gel in chloroform-methanol mixtures; in the case of IIb, formation of 6-

methoxy-9-methylpurine was proved. Both IIa and IIb are decomposed with the formation of numerous coloured compounds when treated with anhydrous triethylamine in pyridine /43/; partial formation of 6-aminopurine residues was observed when triethylamine-pyridine-water medium /see ref. 44/ was applied. Dual reactivity of N-/purin-6-yl/pyridinium salts is also expressed during oxime ion treatment /45,46/. Reaction of IIa and IIb with less than 5 eqv. of 0.5M solution of N^1,N^1,N^3,N^3-tetramethylguanidine salt of 2-pyridinealdoxime in dioxane-water 1:1 v/v leads to related hypoxanthine and adenine residues in a 1:1 ratio. However, under conditions usually applied during removal of internucleotide arylphosphotriesters /above 5 eqv. of oxime ion/ both IIa and IIb are quantitatively transformed into partially deacetylated adenosine and 9-methyladenine respectively. Such behaviour of IIa and IIb is in contrast to that of 4-/azol-1-yl/- or 6-/azol-1-yl/derivatives of uracil and thymine or N-acylguanine residues respectively which can be reversed to parent heteroaromatic lactam systems by reaction with oxime ion /16,17,19,20/. Although survival of N-/purin-6-yl/pyridinium salts up to subsequent conc. aqueous ammonia treatment is rather doubtful it should be stressed that this reagent transforms both IIa and IIb quantitatively /room temp., 1 hr/ into the respective 6-aminopurine residues.

CONCLUSIONS

Our results allow to conclude that a new family of side-products of ionic nature such as IIa and IVa can be formed during oligonucleotide synthesis involving hypoxanthine residues; participation of readily generated O^6-phosphorylated intermediates is most likely. It is certain that N-/purin-6-yl/pyridinium salts formed will not endure the standard conditions of oligonucleotide deprotection and this will result in point mutations.
The conversion to 6-aminopurine residues is most likely. In order to avoid this,hypoxanthine lactam system must be protected. We expect analogous situation for the currently studied reactivity of T, U and G heteroaromatic lactam systems.

EXPERIMENTAL PART

Pyridine and dioxane were refluxed over calcium hydride, distilled and stored over molecular sieves 4A. Dowex resins were prewashed until UV-pure eluates were obtained. Thin layer chromatography was performed on Merck F_{254} silica gel plates in the following systems: A - chloroform-methanol 9:1 v/v, B - chloroform-methanol 5:1 v/v, C - chloroform-methanol 25:1 v/v, D - ethanol -1M ammonium acetate 7:3 v/v. Short column chromatography /47/ was performed on silica gel Merck H60 in chloroform containing methanol. Melting points were

taken on Boetius apparatus and are uncorrected. Spectra were measured on: Carl Zeiss Jena UV-VIS spectrophotometer, Perkin Elmer MPF 3 spectrofluorometer, Jeol JMS-D-100 mass spectrometer and Jeol 90FX Fourier transform NMR instrument for ^{1}H, ^{13}C and ^{31}P nuclei /90, 22.5, 34.6 MHz respectively/. TMS and dioxane /both as internal standards/ and 85% H_3PO_4 /external/ were used for δ_H, δ_C and δ_P chemical shifts measurements respectively. Elemental analyses were made on Perkin Elmer 240 analyser.

Pyridine assisted phosphorylations of Ia. Analytical procedure /Table 1/

2',3',5'-Tri-O-acetylinosine Ia /48/ /0.5 mmol/ was dissolved in pyridine /2.5 ml/ and in order given treated with specified reagents in dark. Dry conditions throughout; for entries 1-8 initial temp. +5°C /10 min./

1. $POCl_3$ /1 mmol/
2. 1,2,4-triazole /3 mmol/, $POCl_3$ /1 mmol/
3. dioxane solution /10 ml/ of PO/1,2,4-triazolide/$_3$ made /23/ from $POCl_3$ /1 mmol/; final mixture concentrated to 5 ml
4. 4-ClPhOPOCl_2 /0.75 mmol/
5. 1,2,4-triazole /1.5 mmol/, 4-ClPhOPOCl_2 /0.75 mmol/
6. dioxane solution /8 ml/ of 4-ClPhOPO/1,2,4-triazolide/$_2$ made /24/ from 4-ClPhOPOCl_2 /1 mmol/
7. 3-nitro-1,2,4-triazole /2 mmol/, 4-ClPhOPOCl_2 /1 mmol/
8. tetrazole /2 mmol/, 4-ClPhOPOCl_2 /1 mmol/
9. TDE /24/ /0.55 mmol/, TPSCl /1.25 mmol/
10. TDE /0.55 mmol/, 1-methylimidazole /2.5 mmol/, TPSCl /1.25 mmol/
11. TDE /0.55 mmol/, tetrazole /4.5 mmol/, TPSCl /1.75 mmol/
12. TDE /0.55 mmol/, TPSTe /49/ /1.5 mmol/
13. TDE /0.55 mmol/, TPSNT /45/ /1.5 mmol/
14. TDE /0.55 mmol/, TPST /49/ /1.5 mmol/

Tlc analysis /system A/ revealed total consumption of Ia for all reactions after 17-24 hrs /with exception of case under entry 14 in which most of Ia remained unreacted after 48 hrs/. Pyridine was evaporated /temp. 30-35°C/ and residue obtained treated with ice-cold water /2.5 ml/.

For entries 1-8: Aqueous solution was treated with charcoal, filtered and neutralized with Dowex 1 resin /HCO_3^-/ up to pH 6.5 under continuous flow of argon. Resin was filtered and solution concentrated to one third of the volume to remove pyridine. Aqueous solution was extracted with chloroform /2 x 5 ml/ and both layers were analysed by tlc /systems A, B and D; see Table 1/ and by UV-VIS spectra. For entries 9-14: Reaction products were partitioned between water originally added and chloroform /2 x 5 ml/. Both layers were analysed by tlc /system A and D; cationic structures enter chloroform layer in the presence of lipophilic anionic species/ and aqueous layer inspected by UV-VIS spectra.

N-/9-/2',3',5'-tri-O-acetyl-/β-D-ribofuranosyl/purin-6-yl/pyridinium chloride, IIa /X=Cl/. See analytical procedure - entry 4, scale 5 mmol. Aqueous solution obtained after initial work-up was passed through Dowex 1 /Cl^-/ column /10 ml/. Eluate was evaporated /35°C/ to get ca 0.2M stock aqueous solution of pure IIa /X=Cl/. Alternatively, lyophilization led to IIa /X=Cl/ as a powder, 2.138 g, 87% yield. Found: C 49.8, H 4.6, N 14.1, $C_{21}H_{22}N_5O_7Cl$ requires: C 51.2, H 4.5, N 14.2; λ_{max}/H_2O/ 273 nm /ε 8600/, 299 nm /7300/ sh, λ_{min} 244 nm /3700/; fluorescence /H_2O/λ_{max} 438 nm / λExc 313 nm/; δ_H/D_2O/ 10.14 /2H, m/, 9.19 /1H, s/, 8.98 /1H, m/, 8.94 /1H, s/, 8.46 /2H, m/, 6.58 /1H, d, J=4.6 Hz/, 6.01 /1H, t/, 4.70 /1H, t/, 2.20, 2.13, 2.10, /9H, 3s/; R_f 0.05 /A/, 0.37 /D/.

N-/9-methylpurin-6-yl/pyridinium chloride IIb /X=Cl/. Dry 9-methylhypoxanthine Ib /1.5 g, 10 mmol/ and 1,2,4-triazole /2.07 g, 30 mmol/ were suspended in dry pyridine /50 ml/ and treated with 4-ClPhOPOCl_2 /2.8 ml, 15 mmol/ initially at +5°C /10 min./ and then at room temp. in dark. Reaction was completed

after 20 hrs /leaving it up to 48 hrs facilitated crystallization of the fluorescent product. Crystals /in two crops/ containing co-crystallizying pyridine hydrochloride were washed with pyridine and dissolved in water /50 ml/, treated with Dowex 1 /HCO_3^-/ as for preparation of IIa /X=Cl/ and subsequently with Dowex 1 /Cl^-/. Aqueous eluate was co-evaporated with pyridine under argon until crystals of pure IIb /X=Cl/ appeared. After filtration and drying 1.73 g, 70% yield. Fine needles of IIb /X=Cl/ were also obtained by crystallization from isopropanol-hexane /oxygen free/ mp. 200°C decomp. Found: C 53.1, H3.8, N 28.1, $C_{11}H_{10}N_5Cl$ requires: C 53.3, H 4.0, N 28.3%; λ_{max} /H_2O/ 274 nm/ε 7000/ 298 nm /5900/, λ_{min} 247 nm /2200/, 290 nm /5800/; fluorescence /H_2O/ λ_{max} 450 nm / λ Exc 313 nm/; FDMS /m/z$^+$ 212/; δ_H /D_2O/ 10.03 /2H, q/, 9.12 /1H, s/, 9.03-8.83 /2H, m/, 8.73 /1H, s/, 8.42 /2H, m/, 4.03 /3H, s/; δ_C /D_2O/ 156.73, 152,39, 152.05, 151.01, 147.06, 144.20, 129.25, 125.56, 31.52; R_f 0.20 /D/.

6-/3-nitro-1,2,4-triazol-1-yl/-9-/2',3',5'-tri-O-acetyl-β-D-ribofuranosyl/purine IIIa. See analytical procedure - entry 13, scale 2 mmol. Organic layer obtained after initial work-up was dried /Na_2SO_4/, concentrated and the residue fractionated by short column chromatography in chloroform containing methanol /0-5%/. Appropriate fractions were combined and evaporated to give IIIa as a glass which treated with methanol give semicrystalline solid 589 mg, 60% yield, mp. 74-76°C. Found: C 33.9, H 3.6, N 22.6, $C_{18}H_{18}N_8O_9$ requires: C 44.1, H 3.7, N 22.9; λ_{max} /MeOH/ 289 nm /ε 13700/ λ_{min} 244 nm /6200/, 259 nm /8000/ infl.; δ_H /$CDCl_3$/ 9.80 /1H, s/, 9.00 /1H, s/, 8.57 /1H, s/, 6.35 /1H, d, J=5.1 Hz/, 5.98 /1H, t/, 5.68 /1H, t/, 3.68 /3H, m/, 2.18, 2.14, 2.10 /9H, 3s/; R_f 0.36 /C/.

N^1-methyl-N^3-/9-/2',3',5'-tri-O-acetyl-β-D-ribofuranosyl/-purin-6-yl/imidazolium chloride IVa /X=Cl/. See analytical procedure - entry 10, scale 1 mmol. Obtained after initial work-up chloroform layer was extracted with 1M phosphate buffer pH 4.7 /2 x 5 ml/, concentrated and applied on short silica gel column. The elution with ethanol containing up to 10% of 1M aq. ammonium acetate gave fractions containing pure product R_f 0.26, system D. To obtain salt free aqueous solution of the product, fractions were saturated with NaCl, extracted with chloroform /5 x 5 ml/ and organic layer reextracted with water /5 ml/. Resulted aqueous solution was passed through layer of Dowex 1 /Cl^-/ /2 ml/, eluate concentrated and finally lyophilized to give IVa /X=Cl/ as a powder, 92 mg, 20% yield. Found: C 48.1, H 4.6, N 17.1, $C_{20}H_{23}N_6O_7Cl$ requires: C 48.5, H 4.7, N 17.0%; λ_{max} /H_2O/ 280 nm /ε 7300/, λ_{min} 232 nm /2100/, 250 nm /3700/ infl.; fluorescence /H_2O/ λ_{max} 400 nm / λ Exc 298 nm/; δ_H /D_2O/ 10.09 /exchangeable/, 8.94 /1H, s/, 8.75 /1H, s/, 8.63 /1H, d, J=2.20 Hz/, 7.77 /1H, d, 2.20 Hz/, 6.49 /1H, d, J=4.6 Hz/, 6.01 /1H, t/, 5.75 /1H, t/, 4.11 /3H, s/, 2.19, 2.11, 2.09 /9H, 3s/.

5'-O-Dimethoxytritylthymidine-3'-/4-chlorophenyl-9-/2',3',5'-tri-O-acetyl-β-D-ribofuranosyl/-purin-6-yl/phosphate Va. See analytical procedure - entry 12, scale 1 mmol. Organic layer obtained after initial work-up was dried /Na_2SO_4/, concentrated and the residue fractionated by short column chromatography in chloroform containing methanol /0-3%/. Appropriate fractions were combined and evaporated to give Va as a glass, 111 mg, 10% yield. δ_H /$CDCl_3$/ 8.66 /1H, s/, 8.28 /1H, s/, 7.56 /1H, bs/, 7.37-6.74 /18H, m/, 6.43 /1H, m/, 6.25 /1H, t/, 6.95 /1H, t/, 4.43 /6H, m/, 3.77 /6H, s/, 2.34, 2.15, 2.09 /12H, 3s/; δ_P /$CDCl_3$/ -14.59. R_f 0.8 /A/. Tlc analysis /system A/ showed that Va when standing in chloroform decomposes to Ia R_f 0.4 and TDE, R_f 0.1. When Va /10 mg/ was treated with pyridine /0.1 ml/ overnight, partial conversion to IIa, R_f 0.37 and TDE R_f 0.9 /in system D/ was observed.

O^6-/2,4,6-triisopropylbenzenesulphonyl/-2',3',5'-tri-O-acetylinosine VIa and N^1-/2,4,6-triisopropylbenzenesulphonyl/-2',3',5'-tri-O-acetylinosine VIIa. Dry 2',3',5'-tri-O-acetylinosine Ia /1.97 g, 5 mmol/ was dissolved in methylene chloride /40 ml/ containing triethylamine /2.08 ml, 15 mmol/ and treated

with TPSCl /3.94 g, 13 mmol/ overnight at room temp. Tlc analysis /system C/ revealed conversion of Ia into two products, major R_f 0.45 and minor, R_f 0.54. Mixture was extracted with 1M phosphate buffer, pH 7.5 /25 ml/, organic layer evaporated and the residue fractionated on silica gel in chloroform containing methanol /0-2%/ to yield:

VIa /R_f 0.54/ 0.528 g as a powder after precipitation from hexane /16% yield/. Found: C 56.0, H 6.0, N 8.2, $C_{31}H_{40}N_4O_{10}S$ requires: C 56.4, H 6.1, N 8.5%; λ_{max}/MeOH/ 240 nm /ε 16670/, 257 nm /11800/ sh, 282 nm /2300/ sh; δ_H/$CDCl_3$/ 8.59 /1H, s/, 8.20 /1H, s/, 7.22 /2H, s/, 6.22 /1H, d, J=5.2 Hz/, 5.93 /1H,t/, 5.65 /1H, t/, 4.41 /5H, m/, 2.92 /1H, m/, 2.14, 2.10, 2.08 /9H, 3s/, 1.31-1.22 /18H, 6s/.

VIIa /R_f 0.45/ 2.145 g of crystals from chloroform-hexane /65% yield/, mp.84-88°C, Found: C 55.9, H 6.1, N 8.3, $C_{31}H_{40}N_4O_{10}S$ requires: C 56.4, H 6.1, N 8.5%; λ_{max}/MeOH/ 243 nm /ε 22400/, 284 nm /6600/, λ_{min} 227 nm /14600/, 268 nm /5300/; δ_H /$CDCl_3$/ 8.87 /1H, s/, 7.90 /1H, s/, 7.18 /2H, s/, 6.12 /1H, d, J= 5.1 Hz/, 5.80 /1H, t/, 5.55 /1H, m/, 4.40 /3H, m/, 4.10 /2H, m/, 2.90 /1H, m/, 2.14, 2.11, 2.08 /9H, 3s/, 1.27-1.14 /18H, 6s/.

Reaction of VIa with pyridine. VIa /122 mg, 0.2 mmol/ was dissolved in dry pyridine /1 ml/ and kept in dark at room temperature. After 48 hrs tlc analysis /system C/ revealed almost quantitative conversion into fluorescent product R_f 0.05. Mixture was treated with water /5 ml/ and concentrated in order to remove pyridine and to precipitate traces of VIa. Aqueous solution was lyophilized to yield IIa /X=$ArSO_3^-$/ as a powder 118 mg, 80% yield. λ_{max}/H_2O/ 272 nm /ε 10100/, 299 nm /7200/ sh, λ_{min}244 nm /5000/; δ_H /D_2O/ 8.97 /2H,m/, 8.09 /1H, s/, 7.85 /2H, m/, 7.33 /2H, m/, 6.18 /2H, s/, 5.51 /1H, d, J=4.5 Hz/, 5.01 /1H, m/, 4.79 /2H, m/, 3.10 /2H, m/, 1.83 /1H, m/, 1.13, 1.06, 1.03 /9H, 3s/, 0.12 /18H, d/.

Pyridine assisted arenesulphonations of Ia /Table 2, entries 2-5/. 2',3',5"-Tri-O-acetylinosine Ia /48/ /0.39 g, 1 mmol/ was dissolved in pyridine /7 ml/ and treated with 5 mmol of one of the following condensing agents TPSCl, TPST, TPSNT, TPSTe in dark. All reactions were terminated after 17 hrs. Entry 2. Mixture was evaporated to dryness, residue treated with water /50 ml/ and obtained suspension centrifuged. Supernatant containing fluorescent product R_f 0.37 /system D/ was treated with Dowex 1 /HCO_3^-/ up to pH 6.5 under conditions given for isolation of IIa /X=Cl/, concentrated, passed through Dowex 1 /Cl^-/ /3 ml/ and lyophilized to yield pure IIa /X=Cl/, 98 mg /20%/. Residue obtained after initial treatment with water, containing unhydrolyzed TPSCl, unreacted Ia, R_f 0.10, and small amounts of VIa, R_f 0.55, and VIIa R_f 0.45 /all in system C/ was dissolved in chloroform /25 ml/, dried over Na_2SO_4, concentrated, and fractionated on silica gel in chloroform containing methanol /0-3%, 10%/. Appropriate fractions were collected and evaporated to give: VIa as a glass /33 mg, 5%/, VIIa as solid /53 mg, 8%/ and Ia as a glass /236 mg, 60%/. Entry 3. Tlc analysis revealed the presence of unreacted Ia only. Entry 4. Tlc analysis showed total consumption of Ia, the presence of fluorescent IIa /X=$ArSO_3^-$/, R_f 0.05 as a main product and small amounts of IIIa R_f 0.36, VIIa R_f 0.45 and VIa R_f 0.55 /system C/. Mixture was evaporated, the residue dissolved in chloroform applied on the silica gel column and eluted with chloroform containing methanol /0-5%, 20%/. Appropriate fractions gave: IIa /X=$ArSO_3^-$/ as a solid /370 mg, 50%/, IIIa /95 mg, 20%/, VIa /26 mg, 4%/ and VIIa /59 mg, 9%/ /all as a glass/. Entry 5. Tlc analysis revealed total consumption of Ia and the presence of numerous products. IIa /X=$ArSO_3^-$/ as the major one was isolated from semi-preparative tlc plate /silica gel, system B/; its amount /45%/ was estimated by UV analysis.

One flask conversion of 2',3',5'-tri-O-acetylinosine into 2',3',5'-tri-O-acetyladenosine. Phosphorylation mixture obtained as for preparation of IIa /X=Cl/ /scale 1 mmol, tlc revealed total consumption of Ia/ was concentrated, residue dissolved in dioxane /20 ml/ and dimethylamine bubbled through with

cooling /ext. temp.+10°C/ until mixture became orange. After 10 min. mixture was concentrated, coevaporated with pyridine, diluted with pyridine /ca 8 ml/ and treated with acetic anhydride /2 ml/ for 3 hrs. Reaction mixture was evaporated few times with ethanol and residue purified on short column chromatography in chloroform containing methanol /0-8%/. Appropriate fractions were collected and evaporated to glass /360 mg, 92%/ which after crystallization from hot ethanol gave crystals of 2',3',5'-tri-O-acetyladenosine, 298 mg /76%/ mp. 171-173°C /48/. Found: C 48.60, H 4.79, N 17.90, $C_{16}H_{19}N_5O_7$ requires: C 48.80, H 4.83, N 17.18%; δ_H /$CDCl_3$/ 8.28 /1H, s/, 8.11 /1H, s/, 7.30 /2H, bs/, 6.15 /1H, d, J=6.0 Hz/, 5.85 /1H, t/, 5.28 /1H, m/, 4.27 /3H, bs/, 2.03, 1.96, 1.94 /9H, 3s/; R_f 0.20 /C/.

Stability tests for IIa and IIb /X=Cl/.

a/ IIa and IIb were stable in HCl aq. in pH range 1.5-6.5 and 80% AcOH aq. for 24 hrs as tested by tlc /system D/ and by UV analysis.

b/ 10 mM solutions of IIa in sat. $NaHCO_3$ or 1M TEAB buffer pH 7.5 were kept over period 17 hrs. Tlc /system D/ and UV analysis revealed formation of Ia in trace quantities.

c/ To a solution of IIb /75 mg, 0.3 mmol/ in chloroform-methanol 4:1 v/v /5 ml/ silica gel H60 /1 g/ was added and suspension stirred overnight. After this time silica gel was filtered off, washed with chloroform-methanol 1:1v/v /5 ml/ and filtrates evaporated. Obtained residue was partitioned between water /2 ml/ and chloroform /3 x 2 ml/. Chloroform layer was dried /Na_2SO_4/ and evaporated to give crystalline solid mp. 147°C of 6-methoxy-9-methylpurine /25 mg, 50%/. Found: C 50.9, H 4.9, N 33.6, $C_7H_8N_4O$ requires: C 51.2, H 4.8, N 34.1%; λ_{max}/H_2O/ 253 nm /ε 11000/ λ_{min} 222 nm /2300/; δ_H /$CDCl_3$/ 8.56 /1H, s/, 7.89 /1H, s/, 4.19 /3H, s/, 3.89 /3H, s/.

d/ IIa /24 mg, 0.05 mmol/ was treated with triethylamine-pyridine 1:1 v/v /0.2 ml/ overnight. After this time tlc analysis /system A/ showed total transformation of the substrate to numerous coloured unidentified products.

e/ IIb /24 mg, 0.1 mmol/ was treated with triethylamine-pyridine-water 3:5:1 v/v /0.2 ml/ during 6 hrs. After this time tlc analysis /system B/ revealed transformation of IIb R_f 0.1 into numerous coloured products and 9-methyladenine R_f 0.45, identical with authentic sample.

f/ IIb /50 mg, 0.2 mmol/ was suspended in dioxane /0.3 ml/ and treated with 1M dioxane-water 1:1 v/v solution of N^1,N^1,N^3,N^3-tetramethylguanidine salt of 2-pyridinealdoxime /0.3 ml, 0.33 mmol/. Tlc analysis /system B/ revealed that fluorescent IIb disappeared after 30 min. /mixture turned deep yellow/; after 17 hrs total conversion into 9-methylhypoxanthine, R_f 0.30, and 9-methyladenine, R_f 0.45, in ratio ca 1:1 was found by semi-preparative tlc /system B/ followed by UV analysis.

g/ IIa /49 mg, 0.1 mmol/ treated with 0.5M dioxane-water 1:1 v/v solution of above oxime reagent /1 ml, 0.5 mmol/ disappeared immediately /system D/ and gradual formation of partially deacetylated adenosine was observed within 6 hrs. Mixture of partially deacetylated adenosines was isolated by semi-preparative tlc in system A and peracetylated to yield 2',3',5'-tri-O-acetyladenosine as crystalline solid /28 mg, 72% yield, analytical data as above/.

h/ Both IIa and IIb treated with conc. aqueous ammonia were converted quantitatively within 1 hr at room temp. into adenosine and 9-methyladenine respectively as determined by tlc /systems B,D/ and UV analysis.

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Abbreviations: see Table 1.

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