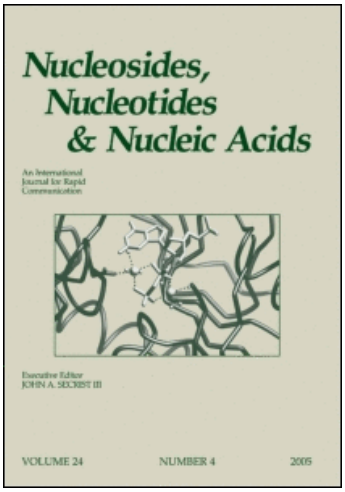

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Jonas Eriksson[a]; Baback Gharizadeh[b]; Nader Nourizad[a]; Pål Nyrén[a]
[a] Department of Biotechnology, SCFAB (Stockholm Centre for Physics, Astronomy, and Biotechnology), KTH (Royal Institute of Technology), Stockholm, Sweden [b] Stanford Genome Technology Center, Stanford University, Palo Alto, California, USA

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7-Deaza-2′-Deoxyadenosine-5′-Triphosphate as an Alternative Nucleotide for the Pyrosequencing Technology

Jonas Eriksson,[1,†] Baback Gharizadeh,[2,†] Nader Nourizad,[1] and Pål Nyrén[1,*]

[1]Department of Biotechnology, SCFAB (Stockholm Centre for Physics, Astronomy, and Biotechnology), KTH (Royal Institute of Technology), Stockholm, Sweden
[2]Stanford Genome Technology Center, Stanford University, Palo Alto, California, USA

ABSTRACT

A new adenosine nucleotide analog suitable for the Pyrosequencing method is presented. The new analog, 7-deaza-2′-deoxyadenosine-5′-triphosphate (c^7dATP), has virtually the same low substrate specificity for luciferase as the currently used analog, 2′-deoxyadenosine-5′-O-(1-thiotriphosphate) (dATPαS). The inhibitory effect dATPαS displays on the nucleotide degrading activity of apyrase was reduced significantly by substituting the c^7dATP for the dATPαS. Both analogs show high stability after long time storage at + 8°C. Furthermore, with the new nucleotide a read length of up to 100 bases was obtained for several templates from fungi, bacteria and viruses.

Key Words: DNA sequencing; Pyrosequencing technology; 7-deaza-2′-deoxyadenosine-5′-triphosphate; 2′-deoxyadenosine-5′-O-(1-thiotriphosphate); Luciferase.

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†Equal contribution to this work.

*Correspondence: Pål Nyrén, Department of Biotechnology, SCFAB (Stockholm Centre for Physics, Astronomy, and Biotechnology), KTH (Royal Institute of Technology), Stockholm SE-106 91, Sweden; Fax: +46-8-55378468; E-mail: paaln@biotech.kth.se.

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INTRODUCTION

The Pyrosequencing technology is based on an enzymatic system to monitor DNA synthesis in real time.[1,2] A detailed description of the Pyrosequencing technology can be found at htttp://www.pyrosequencing.com. In short, the sequencing reaction starts by the addition of one of the deoxynucleoside triphosphates, dNTPs. DNA polymerase catalyzes an incorporation admitted by the DNA base-pairing rule. The PP_i generated after each successful incorporation event is converted to ATP by ATP sulfurylase. The produced ATP is sensed by the luciferase that produces light. ATP and dNTPs are degraded by apyrase prior to the next dNTP addition. The dNTPs are added iteratively and the sequence result is presented in the form of a pyrogram (PSQ^{TM}96 System, Pyrosequencing AB, Uppsala, Sweden).

The natural dATP is not suitable for the Pyrosequencing chemistry because it functions as a substrate for luciferase.[3] Therefore, a modified dATP analog must be considered. Four criteria are important for such an analog: 1) the analog must be a good substrate for the DNA polymerase, 2) the analog should not function as a substrate for the luciferase, 3) the apyrase should be able to degrade the nucleotide, and 4) the apyrase should not be product inhibited.

One of the major advancements during the development of the Pyrosequencing technology came when the natural dATP was replaced with dATPαS.[4] The dATPαS matches some of the above stated criteria. The dATPαS works as a substrate for Klenow polymerase and is less than 0.05 % as effective as substrate for the luciferase. The introduction of dATPαS enabled the analysis of short sequences of DNA and the technology was found to be ideally suited for SNP-analysis. The applied dATPαS was a mixture of two isomers (Sp and Rp). However, a clear disadvantage by using the Sp/Rp mixture was the observation that the Rp-isomer was not used as a substrate by the Klenow polymerase. In addition, it was found that the apyrase was product inhibited. To obtain longer reads the dATPαS Sp/Rp mixture was replaced by a pure Sp-mixture[5] and thereby, the amount of S-nucleotide could be decreased. By utilizing the pure Sp-isomer the inhibition was significantly reduced and longer reads were obtained. However, the apyrase was to some degree still product inhibited. In this paper we have studied an alternative adenosine nucleotide analog. The implementation of this nucleotide in the Pyrosequencing technology is discussed.

MATERIAL AND METHODS

Chemicals and Solutions

Exonuclease-deficient Klenow DNA polymerase was from Pyrosequencing AB (Uppsala, Sweden). Deoxynucleoside triphosphates (dGTP, dCTP and dTTP) and T7 Sequenase v 2.0 were from Amersham Biosciences (Uppsala, Sweden). Pure 2′-deoxyadenosine-5′-O′-(1-thiotriphosphate) Sp-isomer (dATPαS) and adenosine 5′-phosphosulfate (APS) were from Biolog Life Science Institute (Bremen, Germany). 7-deaza-2′-deoxyadenosine-5′-triphosphate (c^7dATP) was from TriLink Biotechnologies (San Diego, USA). Polyvinylpyrrolidone (360,000 g/mol) (PVP) and apyrase were from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA), D-luciferin and purified firefly luciferase were from BioThema AB (Dalarö, Sweden).

Recombinant ATP sulfurylase and recombinant single-stranded DNA-binding protein (SSB) were produced according to earlier described procedures.[6,7]

Synthesis and Purification of Oligonucleotides

The following oligonucleotides were synthesized and HPLC purified by Interactiva (Ulm, Germany):

A EFFECT UP: 5′-(biotin)-GACGCTCAGTGGAACGAAAA-3′,
A EFFECT DOWN: 5′-GGTAACTGTCAGACCAAGTTTACTC-3′,
5A UP: 5′-(biotin)-CTCCCGTATCGTAGTTATCT-3′,
5A DOWN: 5′-CACGTTAAGGGATTTTGGTC-3′,
5A SEQ: 5′-TAAGGGATTTTGGTCATGAG-3′,
LS UP: 5′-(biotin)-GGGATCATGTAACTCGCCTTGA-3′,
LS DOWN: 5′-CGGGAGGGCTTACCATCTGG-3′,
LS SEQ: 5′-TCAGCAATAAACCAGCCAGCC-3′.

In Vitro Amplification

Amplifications for the 303 base-pair and 221 base-pair long fragments were performed in a total volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 1 unit of AmpiTaq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), 2 mM $MgCl_2$, 50 mM KCl, 0.1% (v/v) Tween 20, 0.2 mM dNTPs and 10 pmol of each primer. The PCR was carried out in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, Watertown, USA). A 303 base-pair long and a 221 base-pair long DNA fragment were amplified from the plasmid pUC19 (5 ng) (Biolabs, New England, USA) using primer pair LS UP/LS DOWN and 5A UP/5A DOWN, respectively. For the 303 base-pair long DNA fragment the thermocycler temperature program consisted of denaturation at 94°C for 30 s, annealing at 60°C for 45 s and extension at 72°C for 45 s during 32 cycles. The PCR was initiated with a 5 min activation step at 94°C and finished by a 7 min extension step at 72°C. For the 221 base-pair long DNA fragment the thermocycler temperature program consisted of denaturation at 94°C for 40 s, annealing at 55°C for 45 s and extension at 72°C for 45 s during 32 cycles. The PCR was initiated with a 2 min activation step at 94°C and finished by a 10 min extension step at 72°C.

A 144 base-pair long fragment was amplified from the plasmid Bluescript II KS-Phagem from Stratagene (Cedar Creek, Texas, USA) using primer pair A EFFECT UP/ A EFFECT DOWN. Amplification was performed in a total volume of 50 μl with the same composition as described above except that 25 mM Tris-HCl (pH 8.5), 10 ng Bluescript II KS-Phagem plasmid, 2 units of Taq polymerase and 5 mM KCl were used. The thermocycler temperature program consisted of denaturation at 94°C for 40 s, annealing at 50°C for 1 min and extension at 72°C for 1 min during 34 cycles. The PCR was initiated with a 5 min activation step at 94°C and finished by a 7 min extension step at 72°C.

Preparation of Single-Stranded DNA Template

Streptavidin-coated super paramagnetic beads (100 μg) (Dynabeads™ M280-Streptavidin, Dynal A.S., Oslo, Norway) were washed one time by 100 μl washing

buffer (10 mM Tris-HCl (pH 7.5), 2 M NaCl, 1 mM EDTA, 0.1% Tween 20). The biotinylated PCR product (25-50 µl) was added to the washed beads and the solution was incubated for 30 min at +43°C. Unbound DNA was removed by washing three times with 100 µl of washing buffer. NaOH (10 µl 0.15 M) was added to the beads and the solution was incubated for 3 min. Subsequently, ssDNA was obtained by removing the supernatant. After a washing step, the immobilized ssDNA was dissolved in 10 µl 0.1 M Tris-acetate (pH 7.75). A specific sequencing primer (A EFFECT DOWN, 5A SEQ or LS SEQ) was hybridized to each of the three templates, as described earlier.[4]

Pyrosequencing Technology

Analyses were performed at 28°C in a volume of 50 µl on an automated bench-top PSQ™96 System (Pyrosequencing AB, Uppsala, Sweden). Five to ten microliters of the immobilized and primed ssDNA template were added to the Pyrosequencing reaction mixture (final volume 50 µl), containing 5-8 U Klenow DNA polymerase, 50 mU apyrase, 0.5–1 µg purified luciferase, 2.5 µg SSB, 28 mU ATP sulfurylase, 0.1 M Tris-acetate (pH 7.75), 0.5 mM EDTA, 5 mM magnesium acetate, 0.1% BSA, 2 mM DTT, 5 µM APS, 0.4 mg/ml PVP and 100 µg/ml D-luciferin. The sequencing procedure was carried out by stepwise elongation of the primer-strand upon sequential addition of the different dNTPs with simultaneous degradation of nucleotides by apyrase.

Luciferase Assay

The standard assay volume was 0.2 ml and contained 40 ng purified luciferase, 0.1 M Tris-acetate (pH 7.75), 0.5 mM EDTA, 5 mM magnesium acetate, 0.1% bovine serum albumin, 2 mM DTT, 0.4 mg/ml PVP and 100 µg/ml D-luciferin. The assay was started by the addition either of ATP, dATP, dATPαS or c^7dATP. The generated light was detected by a LKB 1251 luminometer (BioOrbit, Turku, Finland) connected to a LKB 2210 recorder.

Apyrase Inhibition Study

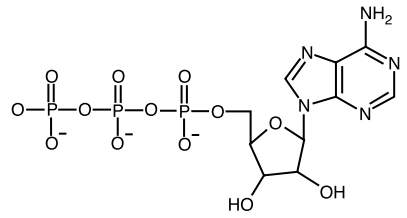
The effect of dATPαS and c^7dATP on the rate of apyrase-catalyzed ATP degradation was studied by a bioluminometric assay as described earlier.[5] The luminescence was analyzed with the PSQ™96 System.

Table 1. Substrate specificity of firefly luciferase.

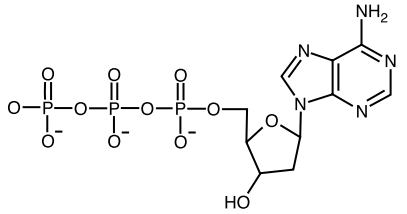
Nucleotide	Light emission relative to ATP (%)
ATP	100.0
dATP	2.5
c^7dATP	0.006
dATPαS	0.005

Stability Test

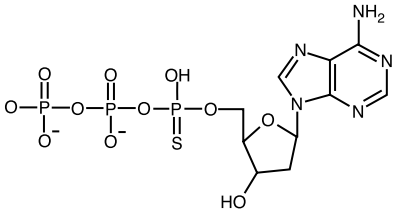
All nucleotides were treated with inorganic pyrophosphatase (EC 3.6.1.1) to remove any PP_i-contamination. Two millimolar solutions of dATPαS and c^7dATP were prepared in 100 mM Tris-acetate (pH 7.75). The samples were held at +8°C and aliquots were taken at 0 days, 10 days, 1 month and 3 months for analysis. The analysis was performed by sequencing of a region of a PCR-generated 221 base-pair long DNA-fragment containing a homopolymeric region with 5T. The quality of the sequence



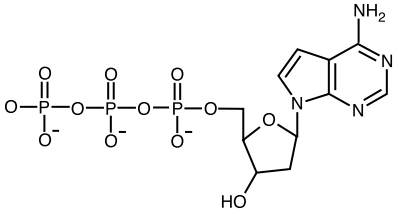
adenosine-5'-triphosphate (ATP)



2'-deoxyadenosine-5'-triphosphate (dATP)



2'-deoxyadenosine-5'-O-(1-thiotriphosphate) (dATPαS)



7'-deaza-2'-deoxyadenosine-5'-triphosphate (c^7dATP)

Figure 1. Structures of the adenine nucleotides studied.

results obtained by the stored nucleotides was compared with data obtained with newly prepared nucleotides.

RESULTS

The Substrate Specificity of Firefly Luciferase

The generation of light by firefly luciferase is a two-step process. In the first reaction, luciferase reacts with D-luciferin and ATP in the presence of Mg^{2+} to form a luciferyl-adenylate complex and release of PP_i. In the second reaction, the luciferyl-adenylate complex undergoes oxidative decarboxylation with subsequent release of CO_2, oxyluciferin, AMP and light (λ_{max} = 562 nm). Most nucleotides other than ATP are virtually inactive in the bioluminescence reaction with the exception for e.g., dATP[3] and APS.[8] The light emission by luciferase in the presence either of ATP, dATP, dATPαS or c^7dATP is shown in Table 1. Both dATPαS and c^7dATP showed more than 400-fold lower activity compared to dATP and about 16 000-fold lower activity than ATP (the natural substrate for luciferase) (Fig. 1).

Effect of dATPαS and c^7dATP on Apyrase Activity

In Fig. 2, the effect of dATPαS and c^7dATP on the ATP degradation efficiency of apyrase is shown. The experiments show the degradation of ATP by apyrase in the presence of dATPαS (Fig. 2a) and c^7dATP (Fig. 2b). The observed initial light increase

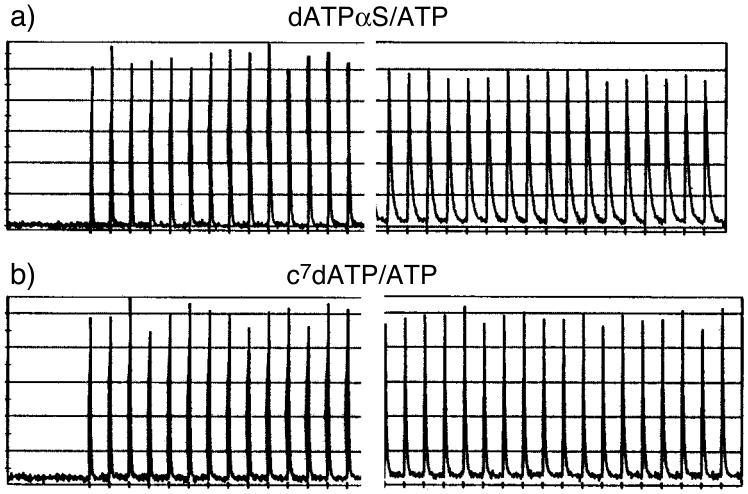


Figure 2. The effect of different adenosine nucleotides on the apyrase activity. The reaction was started by addition of 0.2 μl of a solution containing (a) 0.4 pmol ATP and 320 pmol dATPαS or (b) 0.4 pmol ATP and 320 pmol c^7dATP. The addition was repeated 100 times with 1-min intervals. Only the first 14 and the last 17 additions are shown. The reaction was detected by the luciferase system. See the text and the Material and Methods for details.

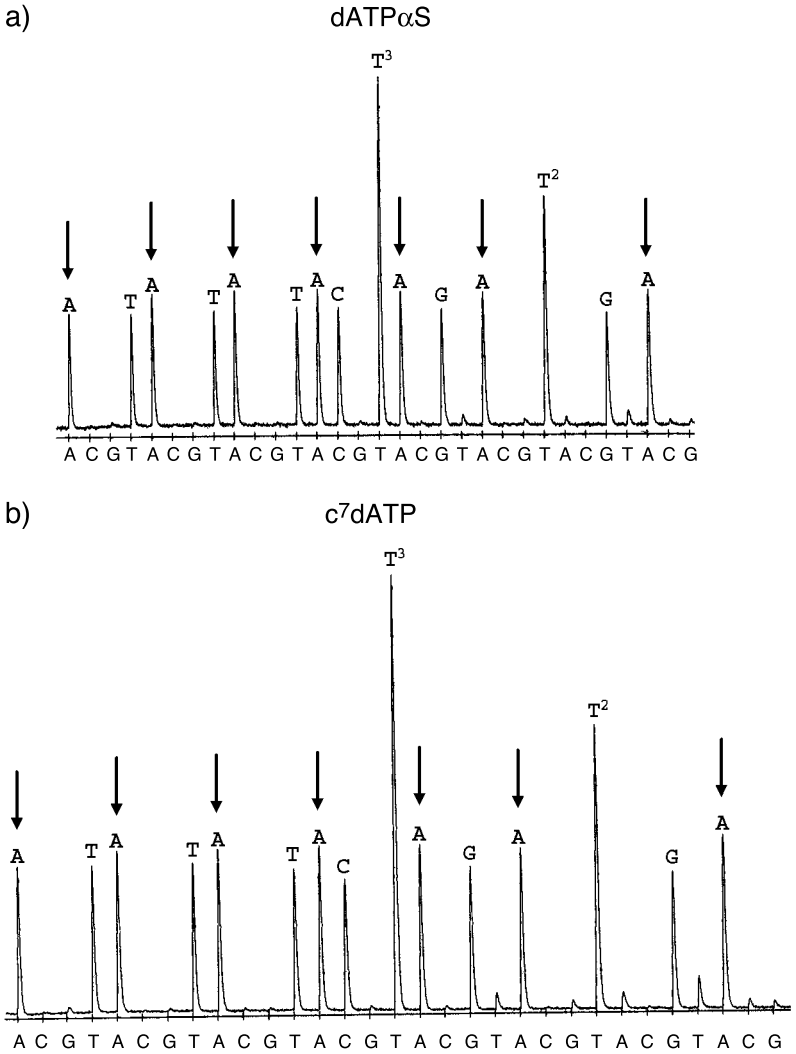


Figure 3. Pyrograms illustrating the A-effect. Sequencing was performed on a 131-base-long PCR-generated DNA fragment in the presence of (a) dATPαS or (b) c^7dATP. Note the higher signals (arrows) for the adenine nucleotides. The nucleotides were added with 65 s intervals and the order is indicated on the bottom of the traces. The manually read sequence is indicated above the traces. See the text and the Material and Methods for details.

after each addition is due to the reaction between ATP and luciferase and the following decrease is due to ATP degradation by apyrase. After every addition of the ATP/dATPαS nucleotide mixture, a continuous decrease in apyrase activity could be observed as an increased peak width (Fig. 2a). However, when c^7dATP was used at the same concentration as dATPαS the inhibition of the apyrase was significantly reduced

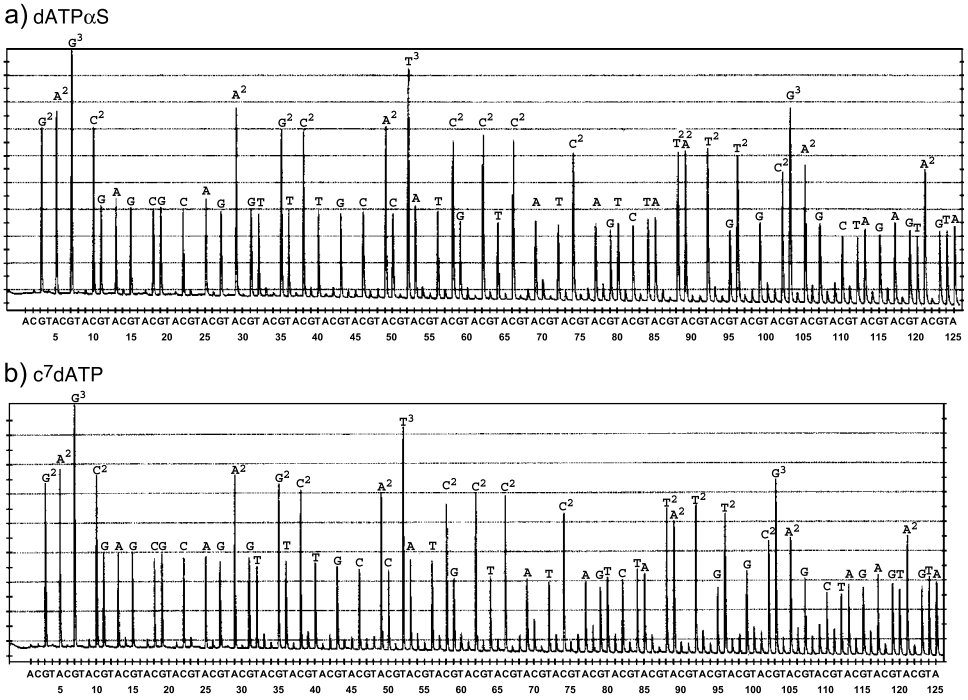


Figure 4. Pyrosequencing data obtained using (a) dATPαS and (b) c^7dATP. Sequencing was performed on a 303-base-long PCR-generated fragment in the presence of (a) dATPαS and (b) c^7dATP. Only the first 85 bases are shown. The read length limit was 140 bases for both nucleotides. The nucleotides were added with 65 s intervals and the order is indicated on the bottom of the traces. The manually read sequence is indicated above the traces. See the text and the Material and Methods for details.

(Fig 2b). The lower inhibition observed in the presence of c^7dATP was at the same level as earlier observed in the presence of dTTP.[5]

The Stability of c^7dATP and dATPαS

For evaluation of the storage stability of c^7dATP and dATPαS a 221 base-pair long fragment containing a 5T region was sequenced with nucleotides stored for different time intervals. The stability criterion was based upon successful incorporation of 5A to the 5T region. Both c^7dATP and dATPαS were stable for at least 3 months when stored in 2 mM solutions at +8°C. No reduction in sequence performance or increased PP_i levels could be detected within the time of 3 month (data not shown).

A-Effect

Since the introduction of dATPαS to the Pyrosequencing technology a slightly higher signal (5–15%) has been observed after the incorporation of A-nucleotides

compared to the other three natural nucleotides. This distinguished characteristic, as illustrated in Fig. 3, is refereed to as an A-effect. Higher concentrations of dATPαS and c^7dATP compared to the other nucleotides were needed for Klenow polymerase to successfully (100%) incorporate A-nucleotides in templates with homopolymeric T-regions (data not shown). The A-effect was concentration dependent and by using about 50% lower concentration of c^7dATP or dATPαS the A-effect disappeared (data not shown). However, at the lower nucleotide concentration the incorporation in poly-T regions were inefficient. By substituting Sequenase 2.0 (a modified T7 DNA polymerase) for Klenow polymerase this later problem could be circumvented (data not shown). It is worth noting that no increase in signal height was observed with higher concentrations of the other nucleotides (data not shown).

Pyrosequencing Technology

In Fig. 4b a part of the result from sequencing of a 303 base-pair long PCR generated template using the c^7dATP is shown. The sequence quality was equal to that obtained in the presence of the dATPαS (Fig. 4a) and the obtained read-lengths were also equal (140 bases for both dATPαS and c^7dATP). Several templates were successfully sequenced using c^7dATP. Various bacterial species could be typed and the samples generated a read length of over 130 bases (data not shown). Furthermore, the c^7dATP was used for genotyping clinically important Human Papilloma Viruses (HPV) (data not shown). All results and performances with c^7dATP equally matched sequence results achieved with dATPαS (data not shown).

DISCUSSION

Pyrosequencing is a non-electrophoretic DNA sequencing method based upon the sequencing-by-synthesis principle. To increase the signal-to-noise ratio in the sequencing reaction the natural dATP was replaced by a α-phosphate modified nucleotide (dATPαS).[4,5] In this paper we have studied the possibility to use a base-modified nucleotide (c^7dATP) in the Pyrosequencing technology.

There are some limitations for how a nucleotide can be modified to still be able to function as a substrate in the Pyrosequencing reaction. Modifications can be performed in either the phosphate part, the ribose part or the base part of the adenosine nucleotide. Modifications in the phosphate part must take place on the α-phosphate. The remaining two phosphates must be unchanged due to their destiny to be converted to ATP and thereafter be able to react with luciferase. Analogs containing boron instead of oxygen on the α-phosphate have been described.[9] Our currently used analog (dATPαS) have a sulphur in this position. This substitution makes the dATPαS less then 0.05% as effective as substrate for the luciferase compared to dATP. Still, the requirements for the Pyrosequencing technology on the performances of incorporation and extension rates of dATPαS with Klenow polymerase were matched.

Introduction of methyl and ethyl labels at the 4′-position in the 2′-deoxyribose part do not affect the 1-base incorporation efficiency by Klenow polymerase,[10] but once incorporated further DNA synthesis is reduced 2000-fold.[11]

In the base part of the adenosine nucleotide, position 1 and 6 are important for the base pairing. Position 2 is important for the DNA structure, e.g. 2-aminoadenine substitution for adenine introduces changes in the minor groove of DNA and creates an additional hydrogen bond in the base pair with thymine.[12] The N3 position is proposed to have a important role for the fidelity of DNA polymerases.[13] Substitution by fluorescin-15[14] or 2-(4-imidazolyl)ethylamino in position 8 showed low incorporation efficiency by polymerase.[15] Analogs with bromo- and hydroxy-groups replacements in position 8 are not substrates for firefly luciferase, but have low incorporation efficiency and consequently could not be used by the Pyrosequencing technology (unpublished observations). The new analog c^7dATP tested in this work is modified in position 7. The c^7dATP was shown to have low substrate specificity for luciferase, comparable to dATPαS (Table 1). In addition, the c^7dATP can be incorporated by Klenow polymerase with good efficency.

Apyrase is a nucleotide hydrolyzing enzyme that converts NTP and dNTP to first NDP and dNDP and then to NMP and dNMP. Any modified dATP in the Pyrosequencing method must be able to be degraded by apyrase. We found that both dATPαS and c^7dATP were degraded by apyrase (Fig. 2). However, dATPαS had an inhibiting effect on the apyrase activity (Fig. 2a). By replacing the dATPαS by c^7dATP the inhibition was reduced (Fig. 2b). The most probable explanation for the observation is that there is a difference between the intermediate products dADPαS and c^7dADP. The dADPαS seems to be less efficiently degraded by the apyrase and might be an inhibitor for the apyrase. On the other hand, c^7dADP is efficiently degraded by the apyrase.

The A-effect [a 5–15% higher than expected peak-hight (Fig. 3a)] has been known for the dATPαS analog ever since it was introduced to the Pyrosequencing technology. The effect is related to the adenine nucleotide alone and gets more pronounced as the concentration increases. No similar effect has been observed with the guanine, thymine or cytosine nucleotides. The origin is still unknown. One hypothesis is that a side reaction by luciferase is involved where a di-nucleoside-tetra-phosphate (dNp_4dN) is formed.[16,17] The dATP is a better substrate for this reaction than dGTP, dCTP and dTTP. This side reaction might increase the turnover of the enzyme and thereby increase the light-yield by 5–15 % when adenine nucleotides are used. To test the hypothesis we used the c^7dATP and hoped that this nucleotide would be more equal to dGTP, dCTP and dTTP. However, we could not observe any difference between the new nucleotide c^7dATP (Fig. 3b) and the old nucleotide dATPαS (Fig 3a).

It is worth noting that no disadvantage with the new nucleotide compared to dATPαS was found. The lower inhibition found for c^7dATP did not had any pronounced effect on the read length obtained for up to 100 bases, but might be important for obtaining longer reads in the future. Clone-checking and bacterial typing are two obvious applications for which longer reads will be an advantage.

In conclusion, we introduce a new nucleotide, c^7dATP, with desirable features as a suitable alternative for dATPαS in the Pyrosequencing technology. In addition, apyrase can efficiently hydrolyse c^7dATP with no sign of product inhibition. In low concentration and at low temperature the stability is high for several months. The new nucleotide may open up new possibilities for real-time DNA extension assays and for obtaining longer read lengths with the Pyrosequencing technology.

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