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6-N-(N-Methylantranylamido)-4-Oxo-Hexanoic Acid: A New Fluorescent Protecting Group Applicable To A New Dna Sequencing Method

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**6-N-(N-METHYLANTHRANYLAMIDO)-4-OXO-HEXANOIC ACID : A
NEW FLUORESCENT PROTECTING GROUP APPLICABLE TO A NEW
DNA SEQUENCING METHOD**

Isabelle Rasolonjatovo* and Simon R. Sarfati

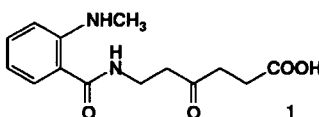
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Paris, France

Abstract : 6-Amino-4-oxo-hexanoic acid with a fluorescent probe attached to the amino function, derivative of the levulinic acid has been developed for protection of hydroxyl groups. It is introduced by reaction of its symmetrical anhydride and rapidly removed under mild conditions using a hydrazine-pyridinium acetate buffer at near neutral pH and room temperature. It can be used within the scope of a new DNA sequencing method and as a sensitive detectable protecting group.

Nowadays, research programs such as the human genome project and the growing field of genetic analysis have brought into light the need of rapid and easy sequencing techniques. We have recently described a new DNA sequencing method without gel electrophoresis¹. It consists in elongating enzymatically by 3'-modified nucleotides the 3'-OH end of a primer, annealed to a single-strand DNA template. Each nucleotide is characterized by the dye attached to the 3'-position *via* the same spacer. The presence of this label would induce base specific DNA termination. A convenient treatment could remove rapidly the tag thus enabling the identification of the incorporated nucleotide and then further elongation would be possible.

The spacer mimics the well known protecting group, the 4-oxo-pentanoic acid (levulinic acid), used in the oligonucleotide synthesis². It can be rapidly removed using a hydrazine-pyridinium acetate based buffer at room temperature and at neutral pH. Recently, we attempted without success to prepare a fluorescent spacer arm removable in the same neutral conditions³. These difficulties led us to adopt a different approach : we have designed a new spacer with a dye attached to the amino function.

The tag used in this study is the fluorescent levulinate analog **1**.



We have obtained the thymidine acylated with this analog at the 3'-position and synthesized the corresponding triphosphate **6** (FIG. 1). The latter will be used to screen a variety of DNA polymerases, in the aim to find enzymes deficient in editing activity described previously⁴.

Acylation of the 5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidine⁵ with **1**, in the presence of DCC and a catalytic amount of DMAP in dichloromethane at room temperature, led to compound **2** giving a 82 % yield. Removal of the DMTr group by treatment with 2% benzenesulfonic acid gave compound **3**. The 5'-OH group of the latter was phosphorylated following the phosphoramidite method.

O-phosphitylation was performed using the reactive reagent N,N'-diisopropyl dibenzyl phosphoramidite⁶ in the presence of 1H-tetrazole as the condensing agent, followed by oxidation, without isolation, of the phosphite diester intermediate with m-chloroperbenzoic acid (MCPBA) to give the corresponding phosphate triester **4** in 52% yield. This compound was debenzylated giving a 80% yield by catalytic hydrogenolysis using palladium on charcoal under hydrogen at atmospheric pressure in a mixture of CH₂Cl₂ / EtOH (1/4 : v/v) in the presence of tributylamine (2eq).

The method of preparation of nucleoside triphosphates which has been most employed⁷ involving condensation of an activated nucleoside, e.g., a nucleoside phosphoromorpholidate⁸ with inorganic pyrophosphate⁹ is not applicable for our purpose. This is due to a requirement for high temperature and basic conditions, under which esters are highly saponified. Thus, we turned to the preparation of compound **6**¹⁰ via the phosphoroimidazolate¹¹, prepared under mild conditions from compound **5** and 1,1'-carbonyldiimidazole (CDI).

Selectively removable protecting groups play an important role in multi-step synthesis of complex natural compounds. Recently, a 4-4'-dimethoxytrityl derivative of the levulinic acid has been developed and used successfully for the solid phase synthesis of oligodeoxynucleotides¹². It is easily introduced and removed as the levulinyl group and is highly detectable at low concentration without complex instrumentation. The new protecting group, 6-N-(N-methylantranilamido)-4-oxo-hexanoic acid **1** (FIG. 2), illustrates the same features.

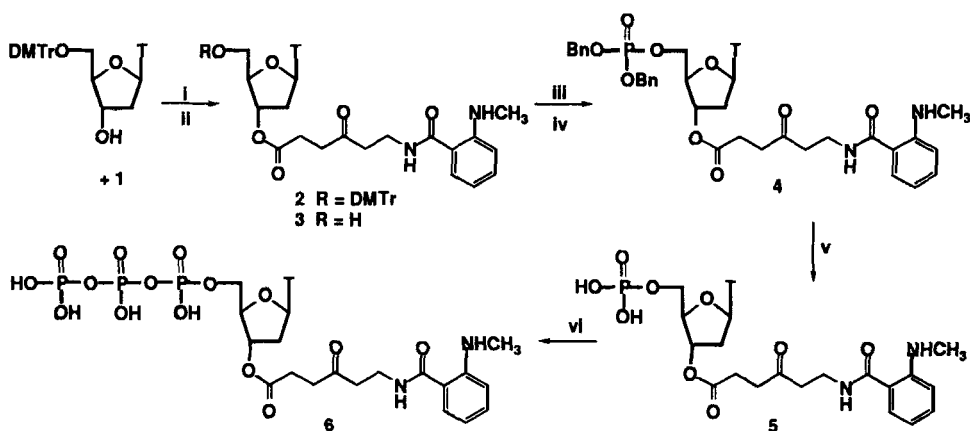


FIG. 1 i : DCC, DMAP, CH_2Cl_2 , r.t. ; ii : PhSO_3H 2%, CH_2Cl_2 -MeOH ; iii : $(\text{BnO})_2\text{PN}(\text{Pr}-i)_2$, 1H-tetrazole / CH_2Cl_2 , r.t. / N_2 ; iv : MCPBA / CH_2Cl_2 , -40 to 0°C , 45 min, N_2 ; v : H_2 -Pd / C, EtOH ; vi : CDI, $[\text{Bu}_2\text{NH}]^+$, $[\text{P}_2\text{O}_7]^{4-}$, DMF, MeCN

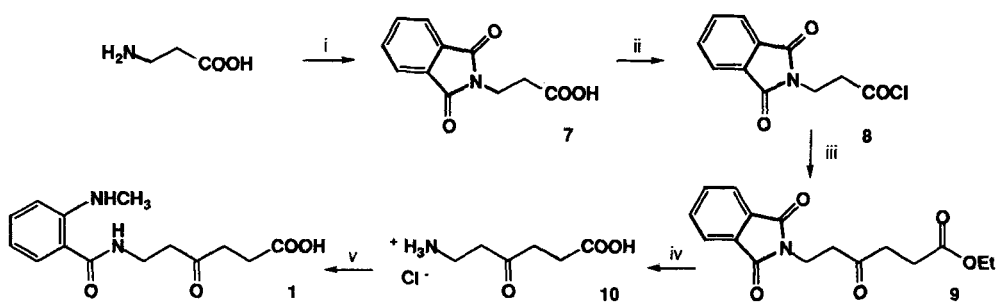


FIG. 2 i : phthalic acid ; ii : SOCl_2 ; iii : $\text{Zn}[(\text{CH}_2)_2\text{COOEt}]_2$, $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$; iv : HCl 6N ; v : N-methyl isatoic anhydride, pyridine

S. Wang and I. A. Scott have described an efficient four-step synthesis from glycine of the biologically important analog of the levulinic acid, the 5-amino-levulinic acid¹³. We have synthesized the 6-amino-4-oxo-hexanoic acid **5** using the same pathway starting from the β -alanine.

β -Alanine was reacted with phthalic acid to give the protected amino acid **7** giving a 96% yield. Treatment of compound **7** with thionyl chloride at room temperature afforded the acid chloride **8** in 100% yield. The key step is the formation of ω -phthalimido-4-oxo-hexanoic ethyl ester **9**, which was obtained in quantitative yield from palladium-catalysed coupling reaction of acid chloride **8** with the zinc homoenolate prepared according to a

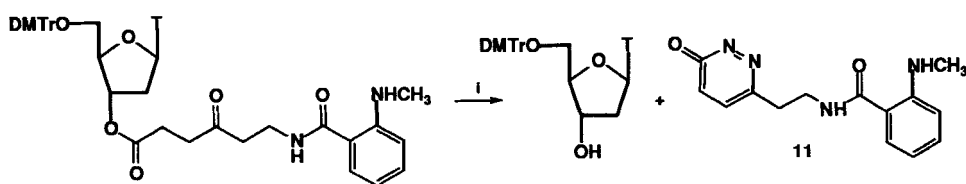


FIG. 3 i: N_2H_4 , H_2O , pyridine, acetic acid

published procedure¹⁴. Then, the phthalyl and ethyl groups were removed by hydrolysis of **9** with 6N HCl, at 110°C for 24 hrs to give the 6-amino-4-oxo-hexanoic acid hydrochloride **10** in 98% yield. The 6-amino function of compound **5** was labelled by N-methylisatoic anhydride in pyridine at room temperature, in the dark for 18hrs, to give the fluorescent compound **11**¹⁵ in 82% yield.

The removal of the tag has been tested. As expected, the ester bond in **3** can be removed quantitatively in less than 5 minutes at room temperature using a hydrazine-pyridinium acetate buffer at near neutral pH to furnish the 5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidine and compound **11**¹⁶ (FIG 3).

The 3'-modified-TTP **6** will be tested for incorporation activity by a variety of DNA polymerases. The subsequent removal of the tag and reinitiation of the DNA synthesis will be examined to verify the feasibility of this DNA sequencing method.

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- 10 ^1H NMR (CDCl_3) δ_{H} : 1.91 (s, 3H, $\text{CH}_3\text{-T}$); 2.26 (m, 2H, H-2'+H-2''), 2.67(m, 2H, OCOCH_2), 2.80 (s, 3H, NHCH_3); 2.96 (m, 4H, $2\times\text{COCH}_2$); 3.60 (m, 2H, NHCH_2), 4.18 (m, 2H, H-5'+H-5''); 4.32 (m, 1H, H-4'); 5.40 (m, 1H, H-3'); 6.08 (t, 1H, $\text{J}_{1',2'}=\text{J}_{1,2''}$ 8.14 Hz, H-1'); 6.68 (t, 1H, H-Ant); 6.74 (d, 1H, H-ant); 7.32 (t, 1H, H-Ant); 7.34 (d, 1H, H-Ant); 7.62 (s, 1H, H-6); ^{13}C NMR (H_2O) δ_{C} : 12.44 ($\text{CH}_3\text{-T}$); 29.09 ($\text{OCOCH}_2\text{CH}_2$); 30.42 (NCH_3); 35.02 ($\text{CH}_2\text{C=O}$); 36.98 ($\text{CH}_2\text{C=O}$); 37.98 (C-2'); 41.95 (NHCH_2); 66.61 (d, $\text{J}_{\text{C,P}} = 4.9$ Hz, C-5'); 76.95 (C-3'); 83.51 (d, $\text{J}_{\text{C,P}} = 10.4$ Hz, C-4'); 85.46 (C-1'); 112.59 (C-5); 112.79-117.21-118.19-129.04-133.57-172.21 C-aromatics); 137.89 (C-5); 152.16 ((C-2); 167.25 (C-4); 175.01 (NHCO); 181.99 (OCO); 213.25 (C=O); ^{31}P NMR (D_2O) δ_{P} -22.52 (1P, t, $\text{J}_{\text{P}\gamma}$, $\text{P}\alpha=\text{J}_{\text{P}\gamma}$, $\text{P}\beta$ 16.68 Hz, $\text{P}\gamma$), -11.22 (1P, d, $\text{J}_{\text{P}\alpha}$, $\text{P}\gamma$ 16.01 Hz, $\text{P}\alpha$), -10.11 (1P, d, $\text{J}_{\text{P}\beta}$, $\text{P}\gamma$ 16.01 Hz, $\text{P}\beta$).
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- 15- ^1H NMR (CDCl_3) δ_{H} : 2.55 (m, 2H, CH_2COOH); 2.61 (m, 2H, $\text{CH}_2\text{CH}_2\text{COOH}$); 2.69 (m, 2H, NHCH_2CH_2); 3.55 (q, 2H, NHCH_2); 6.45 (t, 1H, H-Ant); 6.53 (d, 1H, H-Ant); 6.63 (bt, 1H, NHCH_2); 7.16 (m, 2H, H-Ant); 7.96 (bs, 2H, $\text{NHCH}_3 + \text{COOH}$); ^{13}C NMR (CDCl_3) δ_{C} : 208.8 (CO); 177.10 (COOH); 169.75 (CONH); 150.29-132.95-; 127.49-114.81-114.73-111.19 (C aromatics); 42.18 (NHCH_2); 29.75 (CH_3); 37.13 (NHCH_2CH_2); 34.37 [C(O)CH_2]; 27.81 (CH_2COOH).
- 16- ^1H NMR (CDCl_3) δ_{H} : 2.40 (m, 4H, $\text{NHCOCH}_2 + \text{NHCOCH}_2\text{CH}_2$); 2.49 (t, 2H, $\text{CONHCH}_2\text{CH}_2$); 2.75 (s, 3H, CH_3); 3.61 (q, 2H, CONHCH_2); 6.43 (t, 1H, H-Ant); 6.53 (d, 1H, H-Ant); 6.61 (bt, 1H, NHCH_2); 6.59 (m, 3H, $\text{H-Ant} + \text{NHCH}_3$); 8.8 (s, 1H, N=NH); ^{13}C NMR (CDCl_3) δ_{C} : 170.05 (CONH); 167.55 (CONH); 154.07 (C=N); 150.58-135.45-132.94-115.21-114.60-111.13 (C aromatics); 36.26 (NHCH_2); 35.65 (NHCH_2CH_2); 29.77 (CH_3); 25.95 (COCH_2CH_2); 26.15 (COCH_2).