

# Enzyme Catalyzed Synthesis of $\alpha,\beta$ -fluoromethylene-ATP and Optimization of Reaction Conditions

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**Abstract**— $\alpha,\beta$ -fluoromethylene-ATP is advantageously obtained via phosphorylation of corresponding ADP analogue using catalytic NTP (N=A or U), PEP, nucleoside diphosphate kinase (NDPK), and pyruvate kinase (PK). In order to find the optimized reaction conditions of synthesis of  $\alpha,\beta$ -CHF-ATP via the catalysis of ATP or UTP, the effect of the ratio of PK/NDPK and the dosage of catalyst (ATP, UTP) on the reaction yield and reaction rate were investigated. The yield and reaction rate are discussed in relation to the proportion of PK/NDPK and the amount and types of NTP. via the catalysis of ATP, When the ratio of PK/NDPK is 3, the ratio of ATP /  $\alpha,\beta$ -CHF-ADP is 5%, the reaction conditions are optimized. But, via the catalysis of UTP, When the ratio of PK/NDPK is 3, the reaction rate is small, so, further optimization of reaction conditions is necessary; Via the catalysis of UTP, when the ratio of PK/NDPK is 3, the ratio of UTP /  $\alpha,\beta$ -CHF-ADP is 30%, the reaction conditions are optimized.

**Keywords**— $\alpha,\beta$ -fluoromethylene-ATP; Catalysis; NTP; PK;

*Optimized;*

## I. INTRODUCTION

Methylenebisphosphonates are pyrophosphate analogues in which a carbon atom replaces the bridging oxygen atom between the two phosphate groups. Various substitutions on the bridging carbon with different side chains produce novel compounds. They are currently the major class of drugs used for the treatment of osteoporosis and other diseases characterized by excessive bone resorption. DNA polymerase (pol)  $\beta$  is a member of the X-family of DNA polymerases and plays an important role in the base excision repair that cleanses the genome of simple base lesions [1-6]. It has been the subject of extensive studies examining its key roles in repair and cancer [7-15]. Nucleoside-5'-triphosphate analogues in which the  $\alpha,\beta$ -bridging oxygen has been replaced with a

CXY group are useful chemical probes to investigate DNA polymerase catalytic and base-selection mechanisms [16-20].

Some  $\alpha,\beta$ -CXY-NTP analogue have been synthesized and studied as probes. However, the synthesis method mainly is chemical synthesis which is more complex and more slowly. we reported here  $\alpha,\beta$ -fluoromethylene-ATP is advantageously obtained via phosphorylation of corresponding ADP analogue using catalytic NTP (N=A or U), PEP, nucleoside diphosphate kinase (NDPK), and pyruvate kinase (PK) (Scheme 1). The yield and reaction rate are discussed in relation to the proportion of PK/NDPK and the amount and types of NTP and the respective optimized reaction conditions are found via the catalysis of ATP and UTP [21-25].

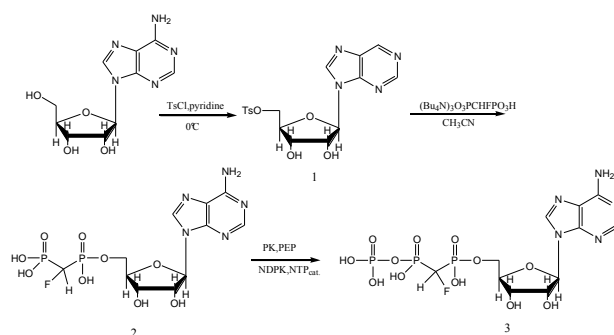


Figure 1. Synthesis of  $\alpha,\beta$ -CHF-ATP analogues

## II. EXPERIMENTAL

### A. Materials and methods

All reagents were purchased from commercial sources. Ion exchange HP-LC experiments were carried out using a Varian ProStar 210 (pump/Inject-tor) and Shimadzu SPD-10A VP (UV-visible detector, set to approx. full abs. Scale (1 volt output for the major UV peak,  $\epsilon_{\text{max}}$  wavelength ~274 nm for pyrimidine or ~266 for purine nucleotides) on Varian 10 mm x 100 mm PureGEL SAX or 21.4 mm x 250 mm SP15/25 NUCLEOGEL SAX (Macherey-Nagel) ion exchange columns. Analytical ion exchange HPLC on the SAX column was done using a 0-->50% linear gradient, A = water, B = 0.5 M LiCl, 30 min, 4 mL/min. Semi-preparative ion exchange HPLC on the SAX column was done using a 0 --> 100% linear gradient, A = water, B = 0.5 M TEAB/10% CH<sub>3</sub>CN, pH = 8, 20 min, 9 mL/min. NMR spectra were obtained using a Varian Mercury 400 NMR spectrometer. Chemical shifts ( $\delta$ ) are reported in parts per million (ppm) relative to internal residual CHCl<sub>3</sub> in CDCl<sub>3</sub> ( $\delta$  7.24, 1H), internal residual H<sub>2</sub>O in D<sub>2</sub>O (pH ~8,  $\delta$  4.8, 1H), external 85 % H<sub>3</sub>PO<sub>4</sub> ( $\delta$  0.00, 31P) or external CFC13 ( $\delta$  0.00, 19F).

### B. Synthesis

#### 1) Synthesis of adenosine-5'-tosylate[3], 1

A quantity (534 mg, 2.0mmol) of adenosine is weighed out in a dried round bottom flask and dried by co-evaporation with pyridine. The dried nucleoside is then dissolved in 20 mL of anhydrous pyridine and cooled to 0°C. Then, 494 mg (2.0 mmol) of p-toluenesulfonyl chloride is added to the stirring nucleoside solution. The reaction is monitored by <sup>1</sup>H NMR. When the reaction has completed (17 h), the mixture is warmed to room temperature after which it is two-thirds concentrated in vacuo and added to 15 ml of ice-cold H<sub>2</sub>O. 60 ml of dried, chilled ethyl acetate is added and the organic layer is extracted and washed with bicarbonate solution (chilled). The organic layer is dried over MgSO<sub>4</sub>. The solvent is removed under reduced pressure and the product is dried and obtained as a colorless foam (337 mg -40%).

#### 2) Synthesis of tris(tetrabutylammonium) salt of (fluoromethylene) bis(phosphonic acid)

The (fluoromethylene)bis(phosphonic acid) [1-3] is dissolved in H<sub>2</sub>O. The aqueous solution is titrated to pH 7.6 with 40% tetrabutylammonium hydroxide. The solvent is removed under reduced pressure and dried leaving a colorless foam[1-3].

#### 3) Synthesis of adenosine- 5'-(fluoromethylene) bis (phosphonate), 2

A quantity (168.4mg, 0.4 mmol) of dried adenosine-5'-tosylate is added to a dried round bottom flask and cooled to 0°C. The dried tosylate is then dissolved in 1.5 mL of dried and distilled acetonitrile. In a separate flask, 514.2 mg (0.6 mmol) of the dried tris(tetrabutylammonium) salt of CHFBP is dissolved in 1.5 mL dried and distilled acetone-trile. The CHFBP solution is added to the tosylate solution dropwise. The reaction is monitored by

ion-exchange HPLC. After completion (48 h), the reaction mixture is diluted with 0.5M TEAB buffer (pH ~ 8) and purified via two-stage preparative ion-exchange HPLC on SAX followed by repurification on the C-18 column.  $\alpha,\beta$ -CHF-ADP( 2) is obtained as a triethylammonium salt. (71.4 mg - 40% yield). 1H: 8.46(s), 8.37 (s), 8.13(s),6.01 (d), 4.43(s),4.65(d),4.64 (d), 4.11 (m), 3.65(t),2.89 (s), 2.73(s),1.84 (s); 31P: 7.0 (m), 14.0 (m); 19F: -116.3 (d of d).

#### 4) Synthesis of $\alpha,\beta$ -fluoromethylene-adenosine triphosphate ( $\alpha,\beta$ -CHF- ATP), 3

##### a) Method A: using catalytic ATP synthesis

36 mg (0.08 mmol) of  $\alpha,\beta$ -CHF -ADP 2 are added to a round bottom flask and 24.5 mg of KCl and 21 mg of MgCl<sub>2</sub> as well as 25 mg of phosphoenol pyruvic acid (PEP) are added and prepared in 1mL of 50 mM HEPES buffer. In a separate eppendorf tube, 1 unit of pyruvate kinase (PK) are prepared and dissolved in 200  $\mu$ L HEPES buffer. In a separate eppendorf, 1 unit of nucleoside diphosphate kinase (NDPK) is prepared in 200  $\mu$ L HEPES buffer, as well. All of the solutions are kept at 0 °C prior to incubation. The PK solution is added to the dNDP solution. A catalytic amount of ATP is added to the NDPK solution prior addition to the reaction mixture. The reaction is incubated at 37 °C. The reaction is monitored by ion-exchange HPLC. However, before analysis the enzymes are removed from the product mixtures by passing them through an Amicon YM-10 micronfilter. After approximately 48 h, the enzymes are removed via filtration and the reaction mixture is purified via two-stage preparative ion exchange HPLC on SAX and then on C-18.  $\alpha,\beta$ -CHF-ATP 3 is obtained as the triethylammonium salt (15 mg). 1H: 8.46(s), 8.13(s),6.04 (d), 4.69(s),4.48(d),4.24 (d), 4.19 (m), 3.19(t),2.89 (s), 1.64 (s); 31P:1 4.29 (t), 1.23 (m), -6.20 (d); 19F: -119.6 (d of d).

##### b) Method B: using catalytic UTP synthesis

36 mg (0.08 mmol) of  $\alpha,\beta$ -CHF -ADP 2 are added to a round bottom flask and 24.5 mg of KCl and 21 mg of MgCl<sub>2</sub> as well as 25 mg of phosphoenol pyruvic acid (PEP) are added and prepared in 1mL of 50 mM HEPES buffer. In a separate eppendorf tube, 1 unit of pyruvate kinase (PK) are prepared and dissolved in 200  $\mu$ L HEPES buffer. In a separate eppendorf, 1 unit of nucleoside diphosphate kinase (NDPK) is prepared in 200  $\mu$ L HEPES buffer, as well. All of the solutions are kept at 0 °C prior to incubation. The PK solution is added to the dNDP solution. A catalytic amount of UTP is added to the NDPK solution prior addition to the reaction mixture. The reaction is incubated at 37 °C. The reaction is monitored by ion-exchange HPLC. However, before analysis the enzymes are removed from the product mixtures by passing them through an Amicon YM-10 micronfilter. After approximately 48 h, the enzymes are removed via filtration and the reaction mixture is purified via two-stage preparative ion exchange HPLC on SAX and then on C-18.  $\alpha,\beta$ -CHF-ATP 3 is obtained as the triethylammonium salt (15 mg). 1H: 8.46(s), 8.13(s),6.04 (d), 4.69(s),4.48(d),4.24 (d), 4.19 (m), 3.19(t),2.89 (s), 1.64 (s); 31P:1 4.29 (t), 1.23 (m), -6.20 (d); 19F: -119.6 (d of d).

### III. RESULTS AND DISCUSSION

In order to find the optimized reaction conditions of synthesis of  $\alpha,\beta$ -CHF -ATP via the catalysis of ATP or UTP, the effect of the ratio of PK/NDPK and the dosage of catalyst (ATP, UTP) on the reaction yield and reaction rate were investigated.

#### A. (一). The effect of the ratio of PK/NDPK on the reaction yield and reaction rate via the catalysis of ATP

The effect of the ratio of PK/NDPK on the reaction yield and reaction rate is studied, experimental data (see table 1).

TABLE I. THE EFFECT OF THE RATIO OF PK/NDPK ON THE REACTION YIELD AND REACTION RATE (THE REACTION TIME /30 MINUTES)

Exp .No	$\alpha,\beta$ -CHF-ADP/ $\mu$ mol	PEP/ $\mu$ mol	ATP/ $\mu$ mol	NDPK/ $\mu$ unit	PK/ $\mu$ unit	Yield /%	V/ $\mu$ mol/min /unitNDPK
1	0.5	1.25	0.025	0.04	0.04	36.6	0.15
2	0.5	1.25	0.025	0.04	0.08	43.5	0.18
3	0.5	1.25	0.025	0.04	0.12	44.5	0.19
4	0.5	1.25	0.025	0.04	0.24	46.9	0.20

#### B. (二). The effect of the ratio of PK/NDPK on the reaction yield and reaction rate via the catalysis of UTP

The effect of the ratio of PK/NDPK on the reaction yield and reaction rate is studied, experimental data (see table 2).

TABLE II. THE EFFECT OF THE RATIO OF PK/NDPK ON THE REACTION YIELD AND REACTION RATE (THE REACTION TIME /30 MINUTES)

Exp .No	$\alpha,\beta$ -CHF-ADP/ $\mu$ mol	PEP/ $\mu$ mol	ATP/ $\mu$ mol	NDPK/ $\mu$ unit	PK/ $\mu$ unit	Yield /%	V/ $\mu$ mol/min /unitNDPK
1	0.5	1.25	0.025	0.04	0.04	36.6	0.15
2	0.5	1.25	0.025	0.04	0.08	43.5	0.18
3	0.5	1.25	0.025	0.04	0.12	44.5	0.19
4	0.5	1.25	0.025	0.04	0.24	46.9	0.20

By the data of table 1 and table 2 data, the relation of the ratio of PK/NDPK and the yield of reaction can be found ( see Fig .2)

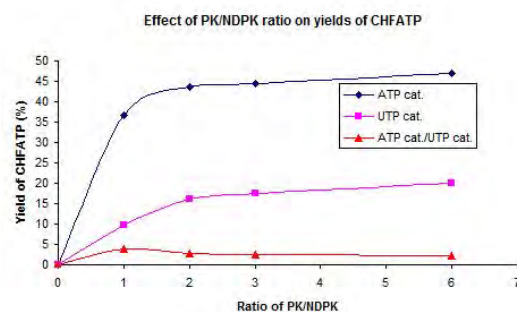


Figure 2. the relation of the ratio of PK/NDPK and the yield of reaction

According to Fig .2, via the catalysis of ATP, When the ratio of PK/NDPK is 3, the ratio of ATP /  $\alpha,\beta$ -CHF -ADP is 5%, the reaction conditions are optimized. But ,via the catalysis of UTP, When the ratio of PK/NDPK is 3, the reaction rate is small,so.,further optimization of reaction conditions is necessary. We study the relationship between the amount of UTP and reaction yield and reaction rate when the ratio of PK/NDPK is 3. The experimental data are shown in Table 3.

TABLE III. THE EFFECT OF THE AMOUNT OF UTP ON THE REACTION YIELD AND REACTION RATE (THE REACTION TIME /30 MINUTES)

Exp .No	$\alpha,\beta$ -CHF-ADP/ $\mu$ mol	PEP/ $\mu$ mol	UTP/ $\mu$ mol	NDPK/ $\mu$ unit	PK/ $\mu$ unit	Yield /%	V/ $\mu$ mol/min /unitNDPK
1	0.5	1.25	0.03	0.04	0.12	21.9	0.09
2	0.5	1.25	0.06	0.04	0.12	31.1	0.13
3	0.5	1.25	0.10	0.04	0.12	35.1	0.15
4	0.5	1.25	0.15	0.04	0.12	39.9	0.17

By the data of table 3 data, When the ratio of PK/NDPK is 3, the effect of the amount of UTP on the reaction yield and reaction rate can be found ( see Fig .3)

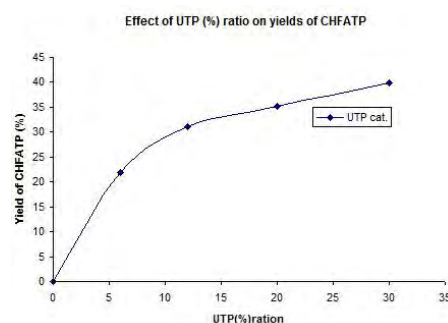


Figure 3. The effect of the amount of UTP on the reaction yield

According to Fig .3, via the catalysis of UTP, When the ratio of PK/NDPK is 3, the ratio of UTP /  $\alpha,\beta$ -CHF -ADP is 30% ,the reaction conditions are optimized.

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# REFERENCES

- [1] Krylov, I. S.; Kashemirov, B. A.; Hilfinger, J.; McKenna, C. E. Evolution of an Amino Acid Based Prodrug Approach: Stay Tuned. *Mol. Pharmaceutics* .2013, 10, 445–458.
- [2] Sun, S. T.; McKenna, C. E., Farnesyl pyrophosphate synthase modulators: a patent review (2006-2010). *Expert Opin Ther Pat* 2011, 21 (9), 1433-1451..
- [3] McKenna, C. E.; Kashemirov, B. A.; Eriksson, U.; Amidon, G. L.; Kish, P. E.; Mitchell, S.; Kim, J.-S.; Hilfinger, J. M. Cidofovir peptide conjugates as prodrugs. *J. Organomet. Chem.* 2005, 690, 2673–2678.
- [4] De Clercq, E.; Neyts, J. Antiviral agents acting as DNA or RNA chain terminators. *Handb. Exp. Pharmacol.* 2009, 189, 53-84.
- [5] Hol, . Phosphonomethoxyalkyl analogs of nucleotides. *urr. Pharm. Des.* 2003, 9, 2567–2592.
- [6] Keriann, O.; WU Yue.; Valeria, M. Z.; Boris, A. K.; David, D. S.; William, A. B.; Samuel, H. W.; Charles, E. M.; Myron, F. G. Effect of  $\beta,\gamma$ -CHF- and  $\beta,\gamma$ -CHCl-dGTP Halogen Atom Stereochemistry on the Transition State of DN Polymerase  $\beta$ . *Biochemistry* .2012, 51, 8491–8501.
- [7] Iwanaga, A., Ouchida, M., Miyazaki, K., Hori, K., Mukai, T. Functional mutation of DN polymerase  $\beta$  found in human gastric cancer: Inability of the base excision repair in vitro. *Mutat. Res.* 1999, 435, 121–128.
- [8] Horton, J. K.; Wilson, S. H. Hypersensitivity phenotypes associated with genetic and synthetic inhibitor-induced base excision repair deficiency. *DNA Repair*. 2007, 6, 530-543.
- [9] Madhusudan, S.; Middleton, M. R. The emerging role of DNA repair proteins as predictive, prognostic and therapeutic targets in cancer. *Cancer Treat. Rev.* 2005, 31, 603-617.
- [10] Sucato, C. A.; Upton, T. G.; Kashemirov, B. A.; Osuna, J.; Oertell, K., Beard; W. A., Wilson, S. H.; Florian, J.; Warshel, A.; McKenna, C. E.; Goodman, M. F. DNA polymerase  $\beta$  fidelity: Halomethylene- modified leaving groups in pre-steady-state kinetic analysis reveal differences at the chemical transition state. *Biochemistry* .2008, 47, 870-879.
- [11] van Beek, E.; Pieterman, E.; Cohen, L.; Lowik, C.; Papapoulos, S., Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. *Biochem Biophys Res Commun* 1999, 264 (1), 108-11.
- [12] Kavanagh, K. L.; Guo, K.; Dunford, J. E.; Wu, X.; Knapp, S.; Ebetino, F. H.; Rogers, M. J.; Russell, R. G.; Oppermann, U., The molecular mechanism of nitrogen-containing bisphosphonates as antiosteoporosis drugs. *Proc Natl Acad Sci U S A* 2006, 103 (20), 7829-34.
- [13] Ogura, K.; Koyama, T., Enzymatic Aspects of Isoprenoid Chain Elongation. *Chem Rev* 1998, 98 (4), 1263-1276.
- [14] Liang, P. H.; Ko, T. P.; Wang, A. H., Structure, mechanism and function of prenyltransferases. *Eur J Biochem* 2002, 269 (14), 3339-54.
- [15] Szkopinska, A.; Plochocka, D., Farnesyl diphosphate synthase; regulation of product specificity. *Acta Biochim Pol* 2005, 52 (1), 45-55.
- [16] Sacchettini, J. C.; Poulter, C. D., Biochemistry - Creating isoprenoid diversity. *Science* 1997, 277 (5333), 1788-1789.
- [17] Holstein, S. A.; Hohl, R. J., Isoprenoids: Remarkable diversity of form and function. *Lipids* 2004, 39 (4), 293-309.
- [18] Lynen, F.; Agranoff, B. W.; Egger, H.; Henning, U.; Moslein, E. M., Gamma-Gamma-Dimethyl-Allyl-Pyrophosphate and Geranyl-Pyrophosphate, Biological Preliminary Stages of Squalene .6. Biosynthesis of Terpenes. *Angew Chem Int Edit* 1959, 71 (21), 657-663.
- [19] Liang, P. H., Reaction kinetics, catalytic mechanisms, conformational changes, and inhibitor design for prenyltransferases. *Biochemistry (Mosc)* 2009, 48 (28), 6562-70.
- [20] Mo, H.; Elson, C. E., Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. *Exp Biol Med (Maywood)* 2004, 229 (7), 567-85.
- [21] Moreno, S. N.; Li, Z. H., Anti-infectives targeting the isoprenoid pathway of *Toxoplasma gondii*. *Expert Opin Ther Targets* 2008, 12 (3), 253-63.
- [22] Wiemer, A. J.; Hohl, R. J.; Wiemer, D. F., The intermediate enzymes of isoprenoid metabolism as anticancer targets. *Anticancer Agents Med Chem* 2009, 9 (5), 526-42.
- [23] Wiemer, A. J.; Hsiao, C. H.; Wiemer, D. F., Isoprenoid metabolism as a therapeutic target in gram-negative pathogens. *Curr Top Med Chem* 2010, 10 (18), 1858-71.
- [24] Coxon, F. P.; Helfrich, M. H.; Van't Hof, R.; Sebt, S.; Ralston, S. H.; Hamilton, A.; Rogers, M. J., Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. *J Bone Miner Res* 2000, 15 (8), 1467-76.
- [25] Dunford, J. E.; Thompson, K.; Coxon, F. P.; Luckman, S. P.; Hahn, F. M.; Poulter, C. D.; Ebetino, F. H.; Rogers, M. J., Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo.