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One-pot three-step continuous enzymatic synthesis of 5-fluoro-5-deoxy-D-ribose

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ABSTRACT

The one-pot three-step continuous enzymatic synthesis of 5-fluoro-5-deoxy-D-ribose (5-FDR) from ATP and L-methionine using *S*-adenosyl-L-methionine synthase (MetK), fluorinase and methylthioadenosine nucleosidase (MtnN) in the presence of fluoride ion, was described. Especially, for the purpose of the sustainable development of fluorine chemistry, the reuse of fluoride ion generated from BF_4 ionic liquids and/or the biodegradation of BTF in the one-pot synthetic process to 5-FDR, was described.

1. Introduction

The challenge in fluorine chemistry to develop the recycle of fluorine resource is one of the most important factors for the purpose of the sustainable development [1–3]. Recently, we have reported that it is possible to generate fluoride ion from the biodegradation of fluorobenzene or benzotrifluoride and/or the degradation of BF_4 ionic liquids, and that 5′-FDA was produced from *S*-adenosyl-L-methionine (SAM) and the generated fluoride ion using fluorinase [4–6]. To date, the fluorination process with fluorinase (E.C.2.5.1.63) [7–18] and the transformation by nucleoside hydrolase from *Trypanosoma vivax* (*Tr*NH) [19] or Phytase (EC 3.1.3.1) [20,21], have been having an important impact on the development of fluorine science. While the enzymatic processes in fluorine science have been recognized to the useful synthetic technology [22–24], their synthetic value using the one-pot continuous enzymatic process still appears to be under development. Further, it is not known the practical one-pot enzymatic process to 5-FDR from ATP and L-methionine on the basis of the circulatory of the fluorine resource. Consequently, approaches for enzymatic technology with innovative mode of action are urgently required in fluorine chemistry.

In our continuous research for the sustainable development in fluorine chemistry, we would like to describe the one-pot three-step continuous enzymatic synthesis of 5-fluoro-5-deoxy-D-ribose (5-FDR) from ATP and L-methionine using *S*-adenosyl-L-methionine synthase (MetK), fluorinase and methylthioadenosine nucleosidase (MtnN) in the presence of fluoride ion. Especially, in attempting to develop the recycle of fluorine resource, the fluoride ion generated from BF_4 ionic liquids and/or the biodegradation of benzotrifluoride (BTF) was reused to establish the one-pot three-step continuous enzymatic practical process, producing 5-FDR via the production of SAM and 5′-FDA.

2. Results and discussion

2.1. One-pot three-step continuous enzymatic process for the synthesis of 5-FDR

For the development one-pot enzymatic process to 5-FDR from ATP and L-methionine, we have examined the one-pot three-step continuous enzymatic process using the SAM synthase (MetK) [25,26], fluorinase and methylthioadenosine nucleosidase (MtnN) in the presence of fluoride ion as shown in Scheme 1. Especially, to transform from 5′-FDA to 5-FDR in our route, we have used MtnN which is known as an enzyme for the hydrolysis of 5′-methylthioadenosine (5′-MTA), giving 5-methylthio-D-ribose and adenine [27]. At first, MetK and MtnN were amplified by PCR with primers shown in Table 1. [28,29].

To proceed the one-pot practical synthetic process which is constructed by the three-step continuous enzymatic reactions, we have examined the effect of concentration of fluoride ion on the SAM-supply from ATP and L-methionine with the SAM synthase (MetK). The results as shown in Fig. 1 (used; L-methionine: 2.5 mM, ATP: 2.5 mM, MetK: 50 μg/50 μl) suggest that the high concentration of fluoride ion decreases the SAM production. We have also found that it is possible the circulatory of L-methionine as shown in Scheme 1 on the basis of the results (L-methionine: 0.032 mM used; 5′-FDA: 0.039, 0.054, 0.035 mM produced) in Table 2. Therefore, we have examined the effect of concentration of L-methionine

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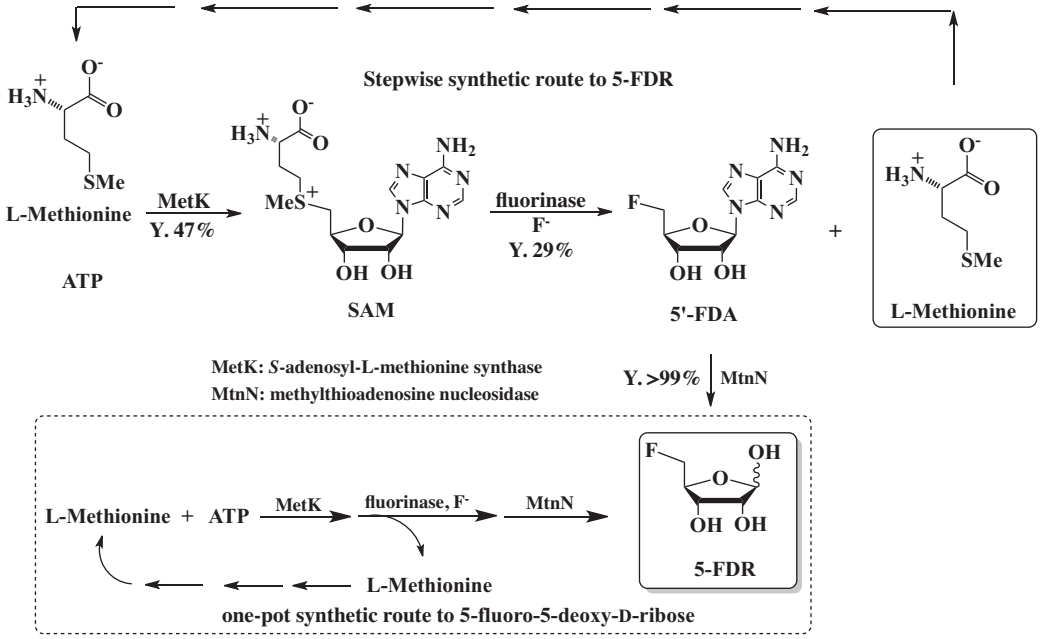

Scheme 1. One-pot three-step continuous enzymatic process of 5-FDR.

Table 1
MetK and MtnN were amplified by PCR with the following primers.

Primer name	Sequence (5′–3′)										Comment
metK-His f	ata	tta	cat	atg	gca	aaa	cac	ctt	ttt	ac	Including *Nde*I site
metK r	gcc	atg	gaa	ttc	gag	ctc	gtt	act	tca	ga	Including *Eco*R I site
mtnN f	agt	aaa	cat	atg	aaa	atc	ggc				Including *Nde*I site
mtnN	gac	agg	tcg	acc	agc	gcc	ctg	a			Including *Sal*I site
T7	taa	tac	gac	tca	cta	tag	gg				For sequence
RV	cag	gaa	aca	gct	atg	ac					For sequence
metK414	tat	cac	cta	tgc	aca	ccg	tc				For sequence

on the production of 5′-FDA using two-step continuous enzymatic process which was constructed with MetK and fluorinase. From the results as shown in Fig. 2 (used; KF: 2.5 mM, ATP: 2.5 mM, MetK: 50 μg/50 μl, fluorinase: 50 μg/50 μl), we have found that the high concentration of L-methionine decreases the 5′-FDA production.

In the next step, we have challenged the one-pot enzymatic process to obtain 5-FDR from ATP and L-methionine using MetK, fluorinase and MtnN in the presence of fluoride ion. On the basis of the results as shown in Figs. 1, 2 and Table 2, we have examined the following system using ATP (2.5 mM), L-methionine (0.25 mM), MetK enzyme (50 μg/50 μl), fluorinase (*flA* protein) (50 μg/50 μl), MtnN enzyme (50 μg/50 μl), KF (2.5 mM), $MgCl_2$ (5 mM) and KCl (10 mM) in Tris·HCl buffer solution. The one-pot three-step continuous enzymatic process was proceeded smoothly to produce 5-FDR, giving 22.6% conversion yield based on ATP (2.5 mM) used. The product (5-FDR) was analyzed by HPLC (column; Atlantis dC18Column, 4.6 × 150 mm, Waters). The conversion yield (22.6%) of 5-FDR from ATP was determined by ^{19}F NMR spectrum using 5′-FDA as an internal standard. In the one-pot three-step continuous enzymatic process, we have found that the conversion yield was increased up to 1.6 times more than that of stepwise enzymatic process as shown in Scheme 1.

2.2. One-pot enzymatic synthesis of 5-FDR exploiting the circulatory of fluorine resource

In the fluorine science, the recycle of fluorine resource is important factor for the purpose of the sustainable development. In our

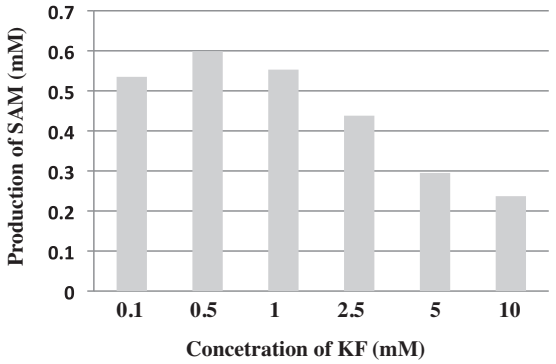

Fig. 1. Effect of KF concentration on production of SAM.

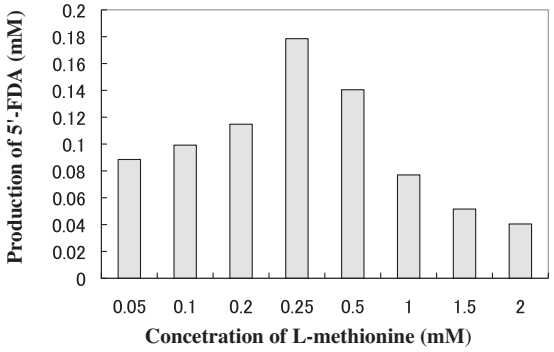

Fig. 2. Effect of L-Met concentration on production of 5′-FDA.

Table 2
Effect of KF and/or L-Met concentration on production of 5′-FDA.

L-Met conc. (mM)	KF conc. (mM)					
	0.1	0.316	1	3.16	10	31.6
0.032	0	0.014	0.039	0.054	0.035	0.025
0.1	0.018	0.041	0.071	0.086	0.052	0.030
0.316	0.032	0.052	0.069	0.084	0.056	0.034
1	0.031	0.010	0.024	0.036	0.055	0.029
3.16	0.028	0.003	0.007	0.014	0.026	0.025

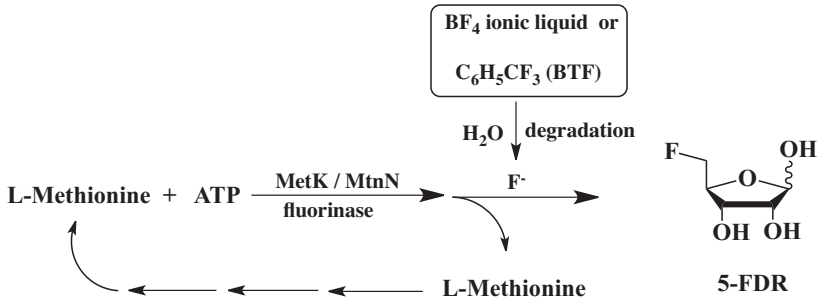

Table 3. Conversion yield

Ionic liquid or BTF	Conversion yield (%)[a]
[emim][BF_4]	10.2
[bmim][BF_4]	4.6
[hexylmim][BF_4]	3.1
BTF	12.7

a) Yield from ATP

Scheme 2. One-pot synthesis of 5-FDR exploiting BF_4 ionic liquid or BTF.

recent report for the time course of the degradation of several types of BF_4 ionic liquids, we have revealed slowly degradation of BF_4 ionic liquids in the buffer solution [5]. Furthermore, we have examined the assembly of the fluorine resource circulatory system exploiting fluorinase using the above system which is constructed from ATP (2.5 mM), L-methionine (0.25 mM), MetK enzyme (50 μg/50 μl), fluorinase (*flA* protein) (50 μg/50 μl), MtnN enzyme (50 μg/50 μl), $MgCl_2$ (5 mM), KCl (10 mM) and BF_4 ionic liquid in Tris·HCl buffer solution. After incubating for 24 h at 37 °C, 5-FDR was analyzed by HPLC. From the results as shown in Table 3 and Scheme 2, we have found that one-pot three-step continuous enzymatic process is smoothly proceeded by using fluoride ion derived from the degradation of BF_4 ionic liquids. In the above reaction system, there is no fluorine resource except the degradation of BF_4 ionic liquid.

Further, as we have reported that it is possible to biodegradate benzotrifluoride (BTF) by microorganism [5], we have examined the resuse of the fluorine atom released from the biodegradation of BTF by microorganism to make clear the recycle of the fluorine resource. In the biodegradation, fluoride ion (1.51 mM) was detected by fluoride ion selective electrode. In the reaction system derived from ATP (2.5 mM), L-methionine (0.25 mM), MetK enzyme (50 μg/50 μl), fluorinase (*flA* protein) (50 μg/50 μl), MtnN enzyme (50 μg/50 μl), $MgCl_2$ (5 mM), KCl (10 mM) in Tris·HCl buffer solution, the above benzotrifluoride cultivation layer (fluoride ion: 1.51 mM) was added. The one-pot three-step continuous enzymatic process proceeded smoothly to produce 5-FDR in the conversion yield 12.7% based on ATP (2.5 mM) used.

Table 3
Conversion yield.

Ionic liquid or BTF	Conversion yield (%)[a]
[emim][BF4]	10.2
[bmim][BF_4]	4.6
[hexylmim][BF_4]	3.1
BTF	12.7

[a] Yield from ATP.

3. Conclusion

We have established the one-pot three-step continuous enzymatic synthesis of 5-FDR from ATP and L-methionine using *S*-adenosyl-L-methionine synthase (MetK), fluorinase and methylthioadenosine nucleosidase (MtnN) in the presence of fluoride ion. Furthermore, we have also mentioned the one-pot enzymatic synthetic process to 5-FDR using a fluoride ion generated from the degradation of fluorinated materials. This is an example of the circulatory of fluorine resource for the one-pot three-step continuous enzymatic synthesis of fluorinated intermediate.

4. Experimental

4.1. General

All commercially available reagents were used without further purification. The ^{19}F NMR (470 MHz) spectra were recorded in ppm downfield from internal standard C_6F_6 in DMSO-d_6 using a VXR 500 instrument.

4.2. Materials and methods

4.2.1. Bacterial strains and plasmids

(a) *E. coli* JM109 and BL21(DE3) pLysS were used for cloning and enzyme engineering, respectively. The fluorinase gene (*flA*) was amplified from the *Streptomyces cattleya* NBRC14057 genome using primers reported previously [8]. Only a single, silent mutation in *flA* was detected by CEQ8000 DNA analysis (Beackman Coulter). The gene was cloned in pET28b+ (Novagen) and consequently fused to the His-tag-containing peptide in the *N*-terminal of the enzyme, termed pETflA.

(b) MetK gene was amplified by polymerase chain reaction using PCR primers (metK-His f, metK r) from *E. coli* MG1655 genome (Table 1). Then, *metK* was inserted into pET28b(+) vector, bearing pET28b-metK His. Cloned metK was confirmed by sequence analysis (primers: T7, RV, metK414). *E. coli* BL21 (DE3) pET28b-metK His transformed with this plasmid was cultured for the

desired enzyme expression after IPTG induction. Harvested cells were suspended in 50 mM Tris·HCl buffer (pH 7.5) and then lysed by sonication to prepare the whole cell lysate fraction excluding the debris fraction with centrifugation. The metK protein was purified by Ni^{2+}-affinitiy chromatography (HisTrap HP: GE Healthcare) and the protein concentration was quantified according to the Bradford method.

(c) MtnN gene was amplified by polymerase chain reaction from PCR primers (mtnN f, mtnN r) (Table 2). Then, *mtnN* was inserted into pET28b(+) vector, bearing pET28b-mtnN. Cloned metK was confirmed by sequence analysis (primers: T7, RV). *E. coli* BL21(DE3)pET28b-mtnN transformed with this plasmid was cultured for the desired enzyme expression after IPTG induction. Harvested cells were suspended in 50 mM Tris·HCl buffer (pH 7.5) and then lysed by sonication to prepare the whole cell lysate fraction excluding the debris fraction with centrifugation. The metK protein was purified by Ni^{2+}-affinitiy chromatography (HisTrap HP: GE Healthcare) and the protein concentration was quantified according to the Bradford method.

4.3. General procedure

(a) The mixture of ATP (2.5 mM), L-methionine (0.25 mM), MetK enzyme (50 μg/50 μl), fluorinase (*flA* protein) (50 μg/50 μl), MtnN enzyme (50 μg/50 μl), KF (2.5 mM), $MgCl_2$ (5 mM), KCl (10 mM) in Tris·HCl buffer solution was incubated. After incubating for 24 h at 37 °C, the conversion yield (22.6%) of 5-FDR from ATP was determined by ^{19}F NMR spectrum using 5′-FDA as an internal standard in DMSO-d_6. 5′-FDA: ^{19}F NMR; −67.4 ppm, td, $J_{F\text{-}Hgem}$ = 46.9 Hz, $J_{F\text{-}Hvic}$ = 29.0 Hz; 5-FDR: ^{19}F NMR; (−64.2 ppm, td, $J_{F\text{-}Hgem}$ = 47.7 Hz, $J_{F\text{-}Hvic}$ = 24.0 Hz).

(b) The mixture of ATP (2.5 mM), L-methionine (0.25 mM), MetK enzyme (50 μg/50 μl), fluorinase (*flA* protein) (50 μg/50 μl), MtnN enzyme (50 μg/50 μl), $MgCl_2$ (5 mM), KCl (10 mM) and [emim][BF_4] ionic liquid (2.5 mM) in Tris·HCl buffer solution, was incubated. After incubating for 24 h at 37 °C, the conversion yield (10.2%) of 5-FDR from ATP was determined by ^{19}F NMR spectrum using 5′-FDA as an internal standard in DMSO-d_6.

4.4. Biodegradation of benzotrifluoride (BTF)

Into the test tube production medium (4 ml) derived from starch (0.5%), sucrose (0.5%), N.Z. Amine (0.25%), peptone (0.25%), yeast extract (0.2%), extract ehlich (0.1%), KH_2PO_4 (0.1%), $MgSO_4{\cdot}7H_2O$ (0.05%) and buffer solution consisted of Na_2HPO_4 and KH_2PO_4, 200 μl of culture was inoculated. Into the above test tube, benzotrifluoride (4–16 mM) was added, and then the degradation of benzotrifluoride was carried out at 28 °C for 2 weeks aerobically. Fluoride ion (1.51 mM) was detected by ISE combination fluoride (ION pH/mV/ORP).

4.5. Fluorination using the cultivation layer of the degradation of benzotrifluoride (BTF)

(a) Into the mixture of ATP (2.5 mM), L-methionine (0.25 mM), MetK enzyme (50 μg/50 μl), fluorinase (*flA* protein) (50 μg/50 μl), MtnN enzyme (50 μg/50 μl), $MgCl_2$ (5 mM), KCl (10 mM) in Tris·HCl buffer solution, and cultivation layer (concentration of fluoride ion: 1.51 mM) was added, and then the mixture was incubated. After incubating for 24 h at 37 °C, the conversion yield (12.7%) of 5-FDR from ATP was determined by ^{19}F NMR spectrum using 5′-FDA as an internal standard in DMSO-d_6.

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