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A coupled chlorinase-fluorinase system with high efficiency of trans-halogenation and a shared substrate tolerance†

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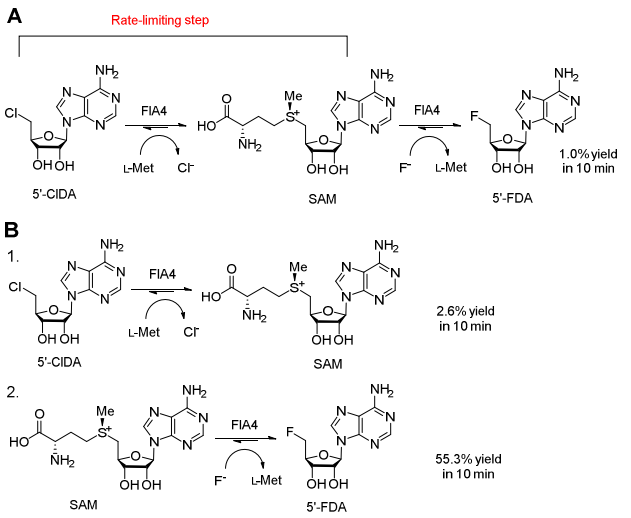
Enzymatic trans-halogenation enables radiolabeling under mild and aqueous conditions, but rapid reactions are desired. We develop a coupled chlorinase-fluorinase system for rapid trans-halogenation. Notably, the chlorinase shares a substrate tolerance with the fluorinase, enabling these two enzymes to cooperatively produce 5'-fluorodeoxy-2-ethynyladenosine (5'-FDEA) at upto 91.6% yield in 1 h.

S-adenosyl-L-methionine (SAM)-dependent nucleophilic halogenating enzymes are a newly discovered family of halogenases, which convert SAM and fluoride/chloride ion to 5'-fluoro-5'-deoxyadenosine (5'-FDA)/5'-chloro-5'-deoxyadenosine (5'-ClDA) and L-methionine (L-Met)[1] (**Scheme 1A**). To date, only five fluorinases[2-7] and one chlorinase[8] within this unique halogenase family have been discovered and characterized.

Fluorinases enable selective C-F bond formation under mild conditions in aqueous phase. The aqueous method of fluorination is especially desirable for positron emission tomography (PET) application. The aqueous [^{18}F]-fluoride ion generated in the cyclotron from [^{18}O]-water can be utilized directly by the fluorinase, and [^{18}F]-labeling of soluble biomolecules can be readily achieved in a buffer solution near physiological pH.[9]

Fluorinase-mediated trans-halogenation has emerged as a useful strategy for PET probe synthesis.[10-12] Fluorinases can catalyze the trans-halogenation of 5'-ClDA to 5'-FDA via two steps: 1) *in situ* SAM synthesis from 5'-ClDA and L-Met/L-seleno-methionine (L-SeMet); and 2) 5'-FDA generation from SAM and fluoride ion.[13] Thus, cheap and stable 5'-ClDA can be converted to [^{18}F]-5'-FDA,[14] a potential PET probe that can also be further converted to a wide range of potentially useful probes such as [^{18}F]-fluoroacetate,[15] [^{18}F]-fluororibose[16], and [^{18}F]-fluoronucleosides.[17] The fluorinase was demonstrated to tolerate the acetylene functionality at the C-2 position of the adenine ring, leading to conversion of 5'-chlorodeoxy-2-ethynyladenosine (5'-ClDEA) to 5'-fluorodeoxy-2-ethynyladenosine (5'-FDEA).[10] The acetylene moiety on FDEA enabled a "click" reaction to an azide-bearing arginylglycylaspartic acid (RGD) peptide. RGD-based PET tracers with high affinity and specificity for integrin $\alpha_v\beta_3$ have been used in clinical trials for tumor detection and staging.[18] Fluorinase-mediated trans-halogenation can even be employed for direct radiolabeling of RGD tethered to the C-2 position of the adenine ring.[10, 12] Due to the short half-life of F-18 ($t_{1/2}$ = 109.7 min), it is important to develop rapid enzymatic protocols.



Scheme 1. Fluorinase-mediated trans-halogenation and its rate-limiting step. A) Trans-halogenation by FlA4: 0.2 mM 5'-ClDA, 80 mM NaF, 0.1 mM L-Met, 50 µM FlA4 at 47°C. B) Two separate reactions incubated at 47°C: 1) SAM synthesis with 0.2 mM 5'-ClDA,

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0.1 mM L-Met and 50 μM FlA4; 2) Fluorination reaction with 0.2 mM SAM, 80 mM NaF and 50 μM FlA4. Yield = [product] detected / [product] expected at full conversion.

Our results showed that fluorinase-mediated trans-halogenation is slow even for the fluorinase from *Streptomyces xinghaiensis* (FlA4), the most efficient fluorinase[7] among the five reported ones (**Scheme 1A**). Comparing the two separate reaction steps, we found that the SAM synthesis is the rate-limiting step (**Scheme 1B; Table S1**). Hence, fast *in situ* SAM synthesis will be the key to improving the overall trans-halogenation efficiency. The first SAM-dependent chlorinase SalL was reported to prefer the conversion of 5'-ClDA to SAM *in vitro*.[8] Here, our newly discovered SAM-dependent chlorinases, ClA1 and ClA2, showed that they are significantly more efficient in SAM synthesis from 5'-ClDA than the fluorinase FlA4. Based on this, we developed a coupled chlorinase-fluorinase system for efficient trans-halogenation of 5'-ClDA to 5'-FDA. The chlorinase was for the first time found to exhibit substrate tolerance on the C-2 position of the substrate and to work together with the fluorinase, enabling improved trans-halogenation of 5'-ClDEA to 5'-FDEA.

ClA1 (WP_078486934) was identified via BLAST (Basic Local Alignment Search Tool) search in the NCBI (National Center for Biotechnology Information) server. It shares 59.6% amino acid identity with SalL. Coding sequences (CDSs) for the ClA1 protein are present in the genomes of *Streptomyces ahygroscopicus* subsp. *wuyiensis* CK-15[19] and four strains of *Streptomyces albulus*,[20-23] all isolated from soil (**Table S2**). ClA2 (P077_11362) was identified via BLAST search of a collection of actinomycete genome sequences[24, 25] (PRJNA238534). It shares 52.7% amino acid identity with SalL. CDS for ClA2 is present in the genome of *Umezawaea tangerine* NRRL B-24463 isolated from soil. Multiple sequence alignment showed that ClA1, ClA2 and SalL do not have the 22-residue loop region, which can be found in all the five known fluorinases (FlA 92-113) (**Figure S1**).

Unlike SalL discovered from high-chloride marine source,[8] the two new chlorinases were unveiled from soil bacteria. To find if terrestrial bacteria could evolve more efficient chlorinases than SalL, kinetic studies of SalL, ClA1 and ClA2 were carried out for the chlorination reaction with varying concentrations of Cl^- (**Table S3; Figure S2**). The relative catalytic efficiencies (k_{cat}/K_M) of ClA1 and ClA2 are quite similar to that of SalL. The affinity of ClA1 for Cl^- is lower than that of SalL. These two new chlorinases may rely on subsequent steps in the biosynthetic pathway to pull the reaction forward.

Prediction of secondary metabolite clusters by antiSMASH (antibiotics & Secondary Metabolite Analysis Shell)[26] showed that *clA1* is within the putative biosynthetic gene cluster for γ-butyrolactone (GBL) biosynthesis. GBLs are small signaling molecules (also known as autoregulators) that trigger antibiotic biosynthesis and morphological development in *Streptomyces* species at nanomolar concentrations.[27] The gene encoding ClA1 is close to the genes coding for putative NAD(P)-dependent oxidoreductase, γ-butyrolactone biosynthesis enzyme and γ-butyrolactone receptor protein (**Figure S3**). Though 14 GBLs with shared GBL core and varied fatty acid side chains are known,[28] no halogenated GBL has ever been discovered.

The conversion of 5'-ClDA to SAM is more efficient than the conversion of SAM to 5'-ClDA for both ClA1 and ClA2 (**Table 1; Figure S4 and S5**). The k_{cat}/K_M also showed that the two chlorinases are significantly more efficient in SAM formation from 5'-ClDA than the fluorinase FlA4 (**Table 1**). Thus, the rate-limiting step of fluorinase-mediated trans-halogenation can be accelerated by the chlorinases. This led us to couple the chlorinases, the robust SAM synthesis enzymes, to the fluorinase for improved overall trans-halogenation.

Table 1. Comparative kinetic data of ClA1, ClA2 and FlA4.

Enzyme	K_M (μM)	k_{cat} (min^{-1})	[k_{cat}/K_M] (mM^{-1} min^{-1})
Conversion of SAM to 5'-ClDA: [SAM][a]			
ClA1	15.69	4.68 ± 0.27	298.09
ClA2	2.48	0.67 ± 0.02	270.14
Conversion of 5'-ClDA to SAM: [5'-ClDA][b]			
ClA1	13.98	12.49 ± 0.99	893.42
ClA2	9.45	14.91 ± 0.92	1577.44
FlA4	29.87	0.69 ± 0.01	22.96

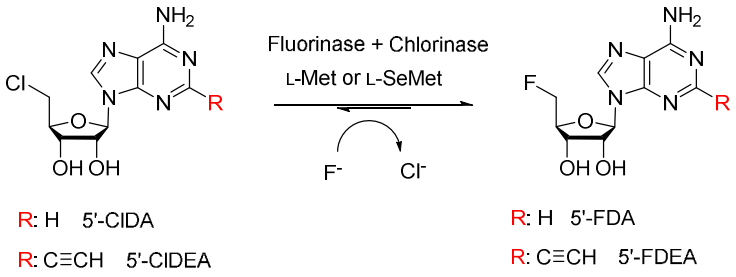
[a] Assays contain 200 mM NaCl and various concentrations of SAM. K_M refers to SAM K_M. [b] Assays contain 20 mM L-Met and various concentrations of 5'-ClDA. K_M refers to 5'-ClDA K_M.

The chlorinases were coupled to FlA4 for one-pot conversion of 5'-ClDA to 5'-FDA in the presence of L-Met or L-SeMet at 37°C for 1 h (**Table 2; Figure S6A**). The trans-halogenation reactions with chlorinase coupled to FlA4 were compared with the reactions without chlorinase. In the presence of L-Met, the trans-halogenation reaction with 50 μM FlA4 alone produced 5'-FDA at only 3.2% yield. Addition of 30 μM chlorinase improved 5'-FDA yield by up to 25.6 fold. However, addition of 30 μM FlA4 just increased the yield by 1.5 fold. Although higher concentration of the fluorinase is often believed to increase product yields, the cocktail of chlorinase and fluorinase achieved significantly higher yields than the fluorinase alone with the same total amount of enzyme. The trans-halogenation reactions were further improved in the presence of L-SeMet, leading to >90% 5'-FDA yields obtained by the coupling of FlA4 and the chlorinase. Thus, the chlorinase-fluorinase system is able to accelerate enzymatic trans-halogenation of 5'-ClDA to 5'-FDA.

To probe if the chlorinase can tolerate the C-2 position of the adenine ring of the substrate 5'-ClDA, we coupled the chlorinase to the fluorinase for the trans-halogenation of 5'-ClDEA to 5'-FDEA (**Table 2; Figure S6B**). The reactions were run under same

conditions for the trans-halogenation of 5'-ClDA to 5'-FDA. In the presence of L-

Table 2. Comparison of 5'-FDA/5'-FDEA yields in the presence of L-Met/L-SeMet.



	Conversion of 5'-ClDA to 5'-FDA 5'-FDA Yield (%)		Conversion of 5'-ClDEA to 5'-FDEA 5'-FDEA Yield (%)	
Reaction[a]	L-Met	L-SeMet	L-Met	L-SeMet
50 μM FlA4	3.20 ± 0.07	11.00 ± 0.06	0.87 ± 0.05	4.68 ± 0.06
80 μM FlA4	4.69 ± 0.10	16.23 ± 0.08	1.54 ± 0.03	7.79 ± 0.08
50 μM FlA4 + 30 μM SalL	69.89 ± 0.08	90.18 ± 0.40	6.47 ± 0.38	58.31 ± 0.08
50 μM FlA4 + 30 μM ClA1	81.77 ± 0.26	98.04 ± 0.27	5.76 ± 0.16	59.70 ± 0.18
50 μM FlA4 + 30 μM ClA2	73.47 ± 0.32	96.63 ± 0.18	12.37 ± 0.29	91.60 ± 0.13

[a] Each reaction contains 0.2 mM 5'-ClDA/5'-ClDEA, 80 mM NaF and 0.1 mM L-Met/L-SeMet. Reactions were incubated at 37°C for 1 h.

Met, the trans-halogenation efficiencies decreased dramatically compared to the conversion of 5'-ClDA to 5'-FDA. L-SeMet[10, 11, 13] was used instead of L-Met to improve the trans-halogenation efficiency on 5'-ClDEA. In the presence of L-SeMet, the reaction with 50 μM FlA4 alone produced 5'-FDEA at only 4.7% yield in 1h. Addition of 30 μM FlA4 only increased the yield by 1.7 fold. Impressively, addition of 30 μM ClA2 led to the highest 5'-FDEA yield (91.6%), with a 19.6-fold improvement. Comparison of the conversion of 5'-ClDEA and L-SeMet to the SAM derivative by the three chlorinases showed that ClA2 exhibited highest consumption rate of 5'-ClDEA to produce the SAM derivative (**Figure S7**). Thus, the chlorinase is able to tolerate the linear acetylene moiety at the C-2 position of the adenine ring and work together with the fluorinase for highly efficient trans-halogenation of 5'-ClDEA to 5'-FDEA.

In conclusion, we discovered two new SAM-dependent chlorinases from soil bacteria and developed a coupled chlorinase-fluorinase system for highly improved trans-halogenation reactions. The chlorinase was for the first time demonstrated to tolerate the modification at the C-2 position of the adenine ring and act cooperatively with the fluorinase to accelerate the trans-halogenation of 5'-ClDEA to 5'-FDEA. The acetylene group will enable the linkage with an azide tethered peptide via a "click" reaction ("two step" strategy).[11] The coupled chlorinase-fluorinase system offers the prospect of developing rapid radiolabeling protocols under mild and aqueous conditions. Future work will be focused on exploitation of the coupled chlorinase-fluorinase system for radiolabeling of cancer relevant peptides either by a "two step" strategy[11] or a "last step" protocol[10, 12] if the chlorinase can further tolerate a tethered peptide at the C-2 position of the adenine ring.

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Conflicts of interest

There are no conflicts to declare.

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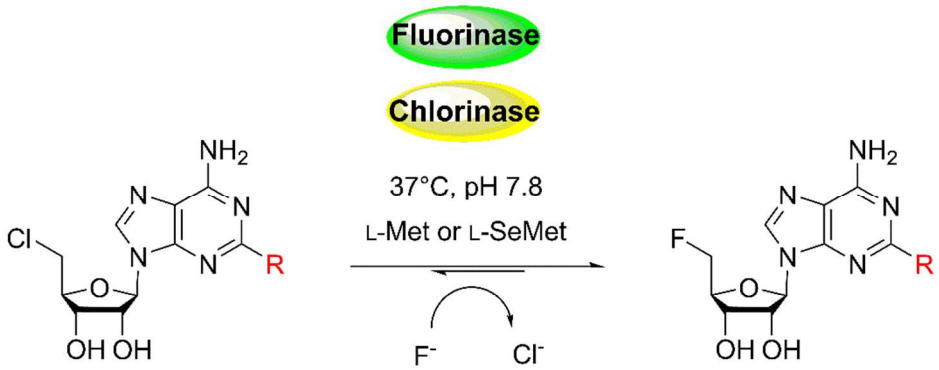

R: H 5'-ClDA

R: C≡CH 5'-ClDEA

R: H 5'-FDA

R: C≡CH 5'-FDEA

SAM-dependent chlorinases exhibited tolerance to the modification at the C-2 position of the adenosine substrate and acted cooperatively with the fluorinase for rapid trans-halogenation.