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

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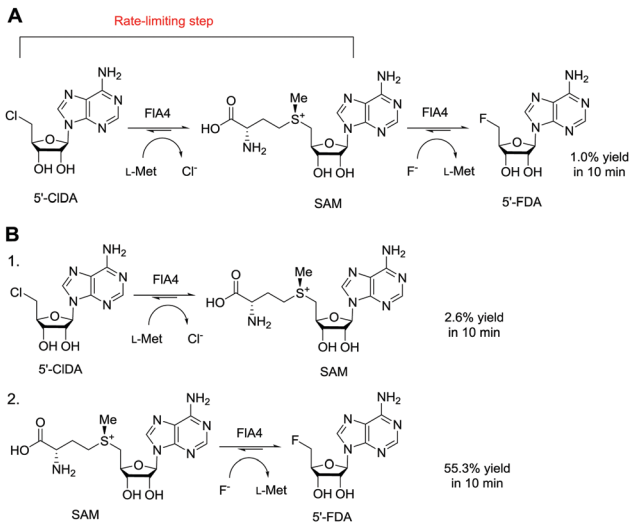
Enzymatic *trans*-halogenation enables radiolabeling under mild and aqueous conditions, but rapid reactions are desired. We developed a coupled chlorinase–fluorinase system for rapid *trans*-halogenation. Notably, the chlorinase shares a substrate tolerance with the fluorinase, enabling these two enzymes to cooperatively produce 5′-fluorodeoxy-2-ethynyladenosine (5′-FDEA) in up to 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 ions 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 an aqueous phase. The aqueous method of fluorination is especially desirable for positron emission tomography (PET) application. Aqueous [^{18}F]-fluoride ions generated in a 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 in two steps: (1) *in situ* SAM synthesis from 5′-ClDA and L-Met/L-selenomethionine (L-SeMet) and (2) 5′-FDA generation from SAM and fluoride ions.[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]



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, and 50 μM FlA4 at 47 °C. (B) Two separate reactions incubated at 47 °C: (1) SAM synthesis with 0.2 mM 5′-ClDA, 0.1 mM L-Met and 50 μM FlA4; and (2) fluorination reaction with 0.2 mM SAM, 80 mM NaF and 50 μM FlA4. Yield = [product] detected/[product] expected at full conversion.

The fluorinase was demonstrated to tolerate the acetylene functionality at the C-2 position of the adenine ring, leading to the 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.

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

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five reported ones (Scheme 1A). Comparing the two separate reaction steps, we found that the SAM synthesis is the rate-limiting step (Scheme 1B and Table S1, ESI†). 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 at 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, ESI†). 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 tangerina* NRRL B-24463 isolated from soil. Multiple sequence alignments showed that ClA1, ClA2 and SalL do not have the 22-residue loop region, which can be found in all five known fluorinases (FlA 92-113) (Fig. S1, ESI†).

Unlike SalL discovered from a high-chloride marine source,[8] the two new chlorinases were unveiled from soil bacteria. To find whether 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 and Fig. S2, ESI†). 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 (Fig. S3, ESI†). Though 14 GBLs with a 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 and Fig. S4 and S5, ESI†). The k_{cat}/K_M value 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

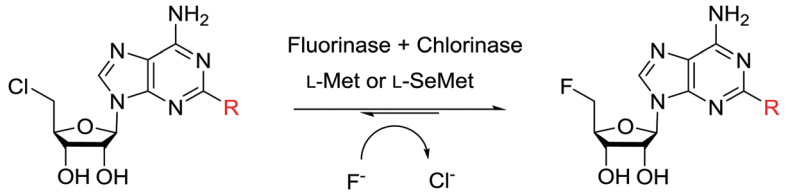
[a] Assays contain 200 mM NaCl and various concentrations of SAM. K_M refers to SAM K_M. [b] Assays contain 20 mM L-Met and various concentrations of 5′-ClDA. K_M refers to 5′-ClDA K_M.

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 and Fig. S6A, ESI†). The *trans*-halogenation reactions with the chlorinases coupled to FlA4 were compared with the reactions without the chlorinases. In the presence of L-Met, the *trans*-halogenation reaction with 50 μM FlA4 alone produced 5′-FDA in only 3.2% yield. Addition of 30 μM chlorinase improved the 5′-FDA yield by up to 25.6 fold. However, addition of 30 μM FlA4 just increased the yield by 1.5 fold. Although a higher concentration of the fluorinase is often believed to increase the product yields, a 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 the enzymatic *trans*-halogenation of 5′-ClDA to 5′-FDA.

To probe whether 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 and Fig. S6B, ESI†). The reactions were run under the same conditions for the *trans*-halogenation of 5′-ClDA to 5′-FDA. In the presence of L-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 in only 4.7% yield in 1 h. 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. A comparison of the conversion of 5′-ClDEA and L-SeMet to the SAM derivative by the three chlorinases showed that ClA2 exhibited the highest consumption rate of 5′-ClDEA to produce the SAM derivative (Fig. S7, ESI†). 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.

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



R: H 5′-ClDA
R: C≡CH 5′-ClDEA
R: H 5′-FDA
R: C≡CH 5′-FDEA

	Conversion of 5′-ClDA to 5′-FDA		Conversion of 5′-ClDEA to 5′-FDEA	
	5′-FDA yield (%)		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 mixture contains 0.2 mM 5′-ClDA/5′-ClDEA, 80 mM NaF and 0.1 mM L-Met/L-SeMet. The reaction mixtures were incubated at 37 °C for 1 h.

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 the exploitation of the coupled chlorinase–fluorinase system for the radiolabeling of cancer relevant peptides using either 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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