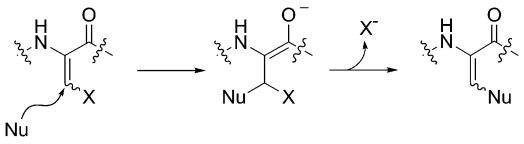
Chemical and Enzymatic Synthesis of Fluorinated-Dehydroalanine-Containing Peptides

Hao Zhou,[a] Dawn M. Z. Schmidt,[b] John A. Gerlt,*[b] and Wilfred A. van der Donk*[a]

Michael acceptors have long been recognized as reactive functionalities that may link a biologically active molecule to its cellular target. 1,2-Dehydro amino acids are potential Michael acceptors present in a large number of natural products, but their reactivity is modulated by the deactivating nature of the α-amino group engaged in an amide bond. We describe here the preparation of 3-fluoro-1,2-dehydroalanine moieties within peptides that significantly enhance the reactivity of the Michael acceptor. Two different routes were designed to access these compounds, one relying on chemical means to introduce the desired functionality and the second taking advantage of a peptide epimerase. In the chemical approach, the fluoro-Pummerer reaction of cysteine derivatives afforded 3-fluorocysteine residues that were oxidized to the corresponding sulfoxides, followed by thermolytic elimination to provide the desired 3-fluorodehydroalanines. The mechanism of the fluoro-Pummerer reaction was investigated and several possible pathways were ruled out. The enzymatic approach utilized the dipeptide epimerase YcjG from Escherichia coli. Difluorinated alanine was incorporated at the C terminus of a dipeptide by chemical means. The resulting peptide proved to be a substrate for YcjG, which catalyzed fluoride elimination to provide the 3-fluorodehydroalanine-containing peptide. Mechanistic investigations showed that fluoride elimination occurred faster than epimerization and at a rate close to that of epimerization of Ala-Ala.

Introduction

Michael acceptors have been popular functionalities for the design of enzyme inhibitors and active site affinity labels.[1–3] Dehydroalanines are potential Michael acceptors and are present in a large number of natural products, including the microcystins,[4–6] nodularin,[7–9] thiostrepton and other thiopeptides,[10–13] and the lantibiotics.[14–16] The electrophilicity of dehydroalanine (Dha) is significantly decreased compared to acrylamides as a result of the inherent enamine functionality present in dehydro amino acids. This effect may explain why natural products containing dehydroalanine residues often interact with their targets by noncovalent mechanisms despite the presence of the Michael acceptor. Microcystin does react with a cysteine residue on its target,[17–19] protein phosphatases Type 1 and 2A,[20–22] but kinetic studies have shown that the covalent linkage is formed subsequent to the initial inactivation.[23] Increasing the electrophilicity of dehydroalanine residues in natural products as well as in designed inhibitors may lead to the development of powerful tools for mechanistic biochemical studies or for use in cell biology and signal transduction. Introduction of an electron-withdrawing group on the terminal vinyl carbon atom would provide the desired increased reactivity. Moreover, if this group consisted of a good leaving group, the Michael addition could be rendered irreversible through an addition-elimination pathway (Scheme 1). Fluorine-substituted dehydroalanines would be particularly attractive for this purpose because of the small steric requirements of the fluorine substituent. We report here synthetic methodologies for the synthesis of 3-fluorodehydroalanine containing peptides by either chemical or enzymatic means.[24]



Scheme 1. *An electron-withdrawing good leaving group (X) increases the reactivity of dehydroalanine with regard to Michael addition and renders the addition – elimination irreversible.*

Results and Discussion

Chemical routes to fluorinated dehydroalanine

We envisioned that fluorine-substituted dehydroalanines could be accessed via synthons **1** or **3** (Scheme 2). In the case of **1**, thermal elimination would afford the desired product, whereas enolate generation with **3** was anticipated to induce fluoride

[a] *Prof. W. A. van der Donk, H. Zhou*
Department of Chemistry
University of Illinois at Urbana-Champaign
600 South Mathews Avenue
Urbana, IL 61801 (USA)
Fax: (+1) 217-244-5360
E-mail: vddonk@uiuc.edu

[b] *Prof. J. A. Gerlt, D. M. Z. Schmidt*
Department of Biochemistry
University of Illinois at Urbana-Champaign
600 South Mathews Avenue
Urbana, IL 61801 (USA)

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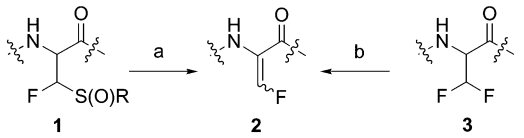
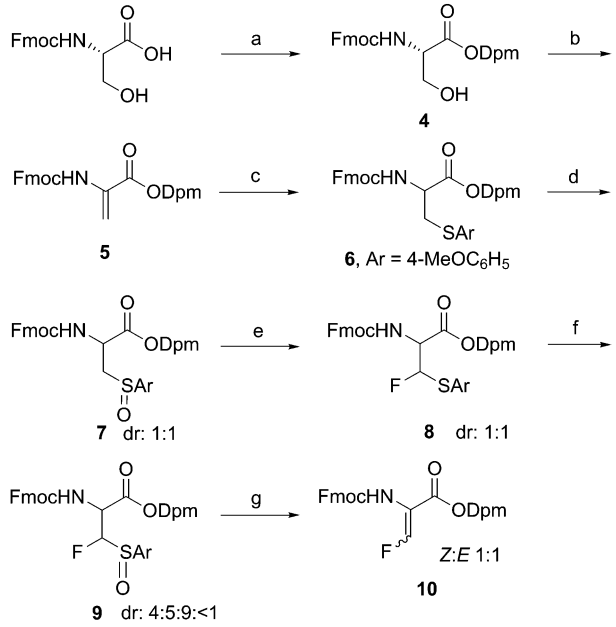


Scheme 2. *Synthons for fluorine-substituted dehydroalanine. a) Δ; b) base.*

elimination. This latter route is discussed in the section on enzymatic routes to the target structures.

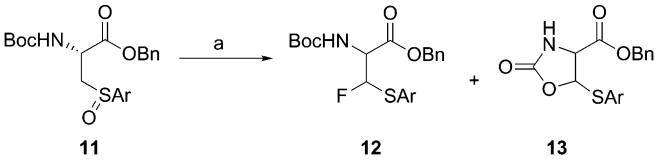
To access the required α-fluorosulfoxide analogues of cysteine, 9-fluorenylmethoxycarbonyl (Fmoc)-serine was transformed into the diphenylmethyl ester **4**, activated with methylsulfonyl chloride, and treated with base to produce dehydroalanine **5** (Scheme 3). Michael addition of 4-methoxybenzenethiol



Scheme 3. *Synthesis of fluorinated dehydroalanine derivatives using a fluoro-Pummerer reaction. a) $Ph_2C{=}NNH_2$, I_2, $PhI(OAc)_2$, 90%; b) MsCl, Et_3N, 82%; c) 4-MeOPhSH, Et_3N, THF, 70%; d)*m*CPBA, −40 °C, 95%; e) DAST, cat. $SbCl_3$, 70%; f)* m*CPBA, −20 °C to −5 °C, 64%; g) Δ, 74%. dr, diastereomeric ratio; Dpm (diphenylmethyl); DAST, diethylaminosulfur trifluoride;* m*CPBA,* meta*-chloroperbenzoic acid; THF, tetrahydrofuran; Ph, phenyl; Ac, acetyl.*

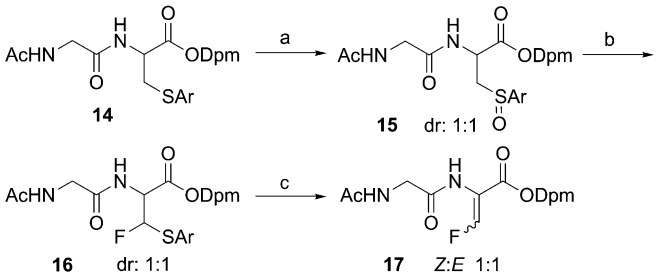
to **5** provided cysteine derivative **6**, which was oxidized with *m*CPBA to the corresponding sulfoxide **7** to give a 1:1 mixture of diastereomers. The mixture was converted into the fluorinated cysteine derivative **8** by a $SbCl_3$-catalyzed[25] fluoro-Pummerer rearrangement according to procedures previously reported by McCarthy[26] and Robins.[27] To probe whether the stereochemistry at the sulfur atom in sulfoxide **7** controls the diastereoselectivity of the fluoro-Pummerer reaction, the two diastereomers of **7** were separated by silica gel chromatography and treated in separate reactions with DAST/$SbCl_3$ in CH_2Cl_2. The product was obtained with identical diastereomer ratios for both transformations, which indicates that the configuration at the sulfur atom of the sulfoxide does not have an effect on the stereochemistry of the reaction. Fluorinated cysteine derivative **8** was oxidized with *m*CPBA to yield four diastereomers of the fluorinated sulfoxide **9**.[28] Subsequent thermolytic elimination in benzene at 80 °C afforded two 3-fluorodehydroalanine diastereomers in 74% yield. These two diastereomers were separated by silica gel chromatography, and the stereochemistry was assigned as described in our preliminary work.[29]

When the synthetic route in Scheme 3 was followed with *tert*-butyloxycarbonyl (Boc) as the N-protecting group, the reaction of sulfoxide **11** with DAST produced **13** as a major by-product (30%, Scheme 4), which was not obtained in appreciable amounts with **7**. Compound **13** is presumably formed by cyclization of the carbamate carbonyl oxygen atom onto a thiocarbenium intermediate (see below).



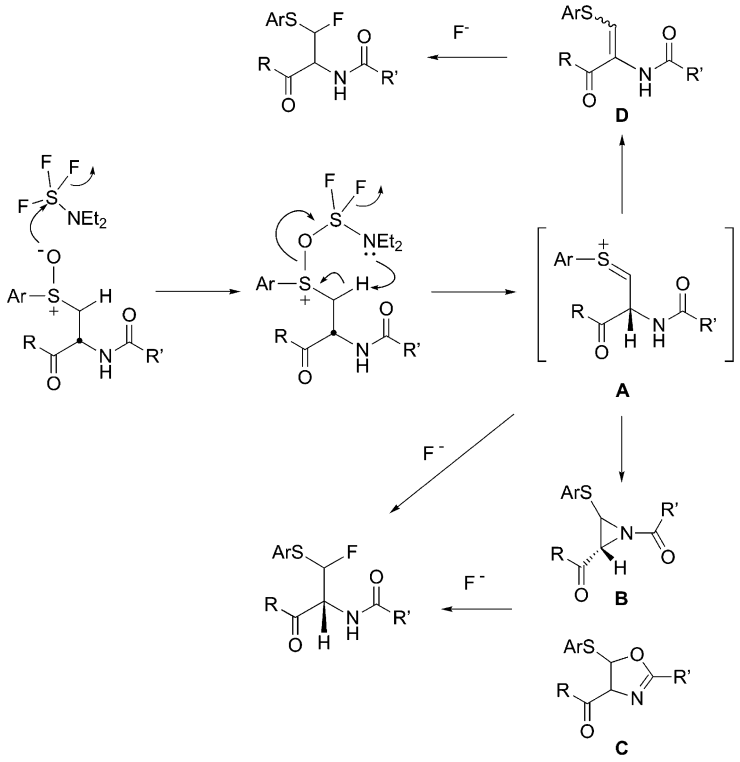
Scheme 4. *The synthetic route to fluorinated dehydroalanine derivatives was followed with a* t*Boc protection group, and the reaction of **11** with DAST was found to produce **13**.*

The methodology was applied next to the synthesis of a 3-fluorodehydroalanine-containing dipeptide as shown in Scheme 5. Dipeptide **14** was synthesized from acetyl glycine and **6** in 76% yield by solution phase techniques.[30] Oxidation of the sulfide to the sulfoxide, and treatment with DAST gave the desired product **16** in 65% yield. Two isomers of the target **17**, obtained after oxidation and thermal elimination at 80 °C, were separated by silica gel chromatography.



Scheme 5. *Synthesis of a fluorinated dehydroalanine containing dipeptide. a) $NaIO_4$, 93%; b) DAST, $SbCl_3$, 65%; c) 2 steps, 57%, i)* m*CPBA, ii) 80 °C, C_6H_6.*

A number of different mechanisms can be written for the fluoro-Pummerer reaction of cysteine derivatives (Scheme 6). Activation of the sulfoxide with DAST and subsequent elimination is believed to generate the thiocarbenium ion **A**.[25] The structure of the intermediate **A** is consistent with the observation that the diastereomeric ratio of the fluorinated product **8** is independent of the configuration at the sulfur atom in sulfoxide **7**. Intermediate **A** can be directly converted into the product by fluoride attack. Alternatively, intramolecular cyclization could generate aziridine **B** or oxazoline **C**. The latter possibility would

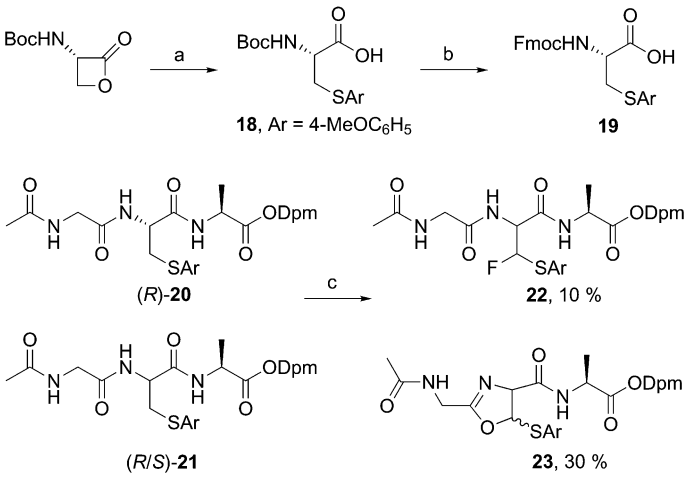


Scheme 6. Possible mechanisms for the fluoro-Pummerer reaction with cysteine derivatives. R, R′ = variable groups.

explain the formation of **13** (Scheme 4) when R′ is a *tert*-butyloxy group that can generate a relatively stable *tert*-butyl cation.[31] Both **B** and **C** may be converted into the observed product by ring opening through fluoride attack at the β-carbon atom. All three mechanisms maintain the stereochemical integrity at the α-carbon of the starting amino acid. On the other hand, deprotonation of intermediate **A** could produce 1,2-didehydrocysteine derivative **D**, which could undergo Michael addition by a fluoride ion to generate the product. If the starting sulfoxide were nonracemic at the α-carbon atom, this pathway would lead to loss of stereochemical purity.

In order to determine whether the stereochemical integrity at the α-carbon atom is maintained during the reaction, two tripeptides were prepared, one that contained (*R*)-*S*-methoxyphenyl cysteine and one that contained racemic *S*-methoxyphenyl cysteine (Scheme 7). Enantiomerically pure Boc-*S*-(4-methoxyphenyl)cysteine **18** was synthesized by ring opening of the serine β-lactone prepared by the procedure of Vederas.[32] The Boc protecting group was replaced with Fmoc to provide **19**. Two tripeptides **20** and **21** were prepared by standard Fmoc solution phase peptide synthesis by using either **19** or **6**, respectively. These two tripeptides were oxidized and reacted with DAST to give two and four diastereomers of **22**, respectively, as determined by analysis of the ^{19}F NMR spectra of the crude products (Figure 1). The presence of two diastereomeric products generated in the reaction of (*R*)-**20** strongly suggests that the stereochemical purity at the α-carbon atom of the cysteine derivative was retained, and is therefore evidence against the intermediacy of **D**. Although we cannot rule out the intermediacy of **B** and/or **C**, we favor direct attack of a fluoride anion on the thiocarbenium **A** as the mechanism for the fluoro-Pummerer reaction of cysteine derivatives.[33]

In the reactions of these tripeptides, an additional product was formed that was not observed with the fluoro-Pummerer reaction of either the protected amino acids or the dipeptides. This product appears to arise from a nonoxidative fluoro-Pummerer reaction, as has been observed in reactions of sulfoxides containing amides or carbamates with trifluoroacetic anhydride.[34, 35] While the exact structure of this byproduct could not be unambiguously established, the following observations suggest it is either sulfenamide **23** or **24** (Scheme 8). The ^{1}H NMR spectrum of the byproduct shows only one amide proton, which corresponds to the N-terminal acetamide as determined in a COSY experiment. The latter experiment also indicates the presence of three coupled protons at 5.55 (dd, $J = 10.1$, 8.6 Hz), 4.55 (dd, $J = 10.5$, 8.6 Hz), and 4.30 (t, $J = 8.5$ Hz) ppm that were assigned to the C-α and C-β protons of the central amino acid residue.[36] These observations indicate that the β-proton was not removed from the cysteine residue during the transformation, in contrast to the situation in the regular fluoro-Pummerer reaction. The signal of the amide proton of the C-terminal alanine residue was absent in the COSY spectrum, and the molecular weight of the by-product (as determined by high resolution FAB-mass spectrometry) agrees with both structures **23** and **24**. The most intense peak in the low-resolution FAB-MS spectrum was a fragment ion corresponding to loss of the *p*-methoxythiophenoxyl (*p*-MeOC$_6$H$_4$S) moiety. Such a fragment was not observed with any of the compounds containing this group on the β-carbon atom of the central amino acid residue. The products of nonoxidative Pummerer reactions have been proposed to arise



*Scheme 7. Fluoro-Pummerer reaction on tripeptides with an S-phenylcysteine sulfoxide as the central residue. A peptide tentatively assigned as structure **23** was obtained as the major product. a) NaH, ArSH; b) i) TFA, ii) Fmoc-Osu, Et$_3$N, 85%; c) i) NaIO$_4$, ii) DAST, SbCl$_3$. TFA, trifluoroacetic acid*

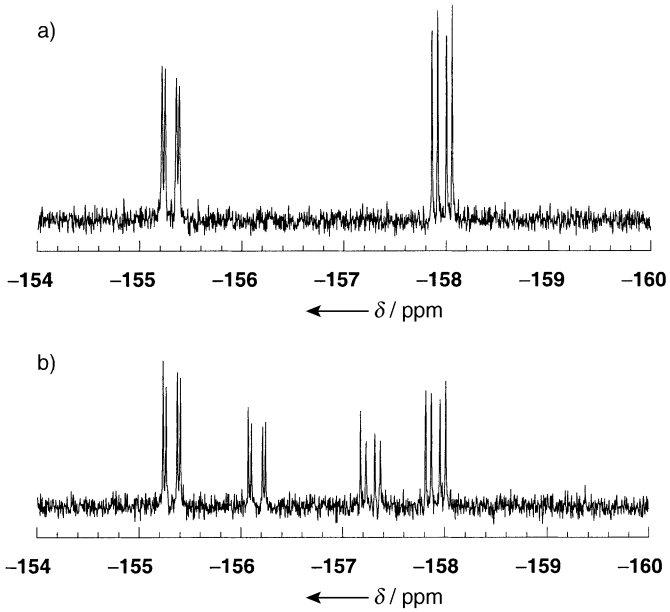
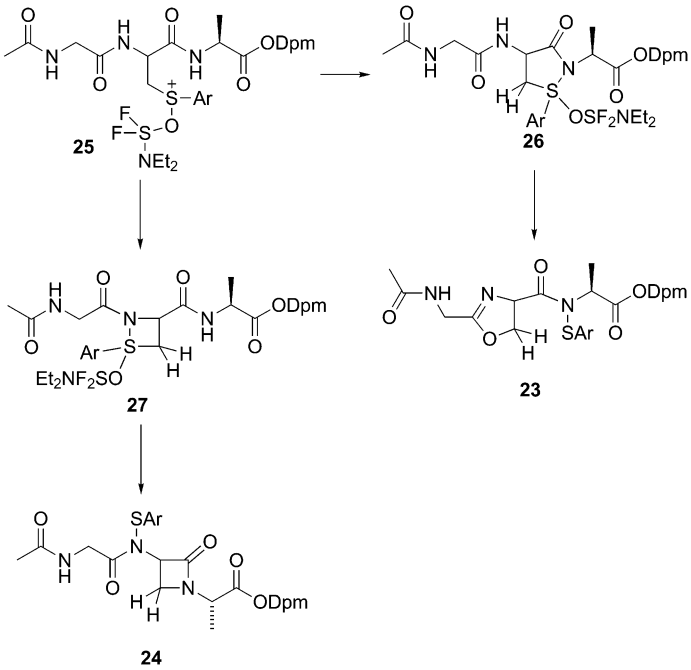


Figure 1. *^{19}F NMR spectra of **22** produced by a) the reaction of (R)-**20** with $NaIO_4$ followed by DAST treatment, and b) the reaction of DAST with the sulfoxides derived from (R/S)-**21** (Scheme 7).*



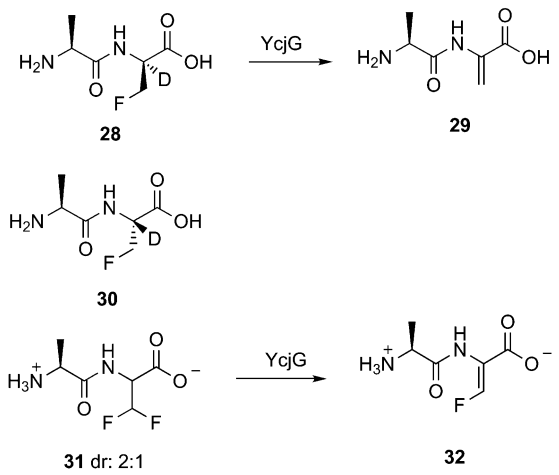
Scheme 8. *Two possible pathways leading to products **23** and **24** in an interrupted nonoxidative fluoro-Pummerer reaction.*

from an interrupted sequence in which an intramolecular nucleophile, typically the nitrogen atom of an amide or carbamate group,[34, 35] intercepts the activated sulfoxide, **25** in the case discussed here (Scheme 8). Two possible amide nitrogen atoms could participate in this process to give sulfurane **26** or **27**. Intramolecular cyclization involving a second peptide amide would then provide sulfenamide **23** or **24**, respectively. Since the major difference between dipeptide **14** and tripeptide **20**/**21** involves the additional C-terminal alanine residue, it is likely that **23** is the structure of the byproduct. Regardless of its identity, our studies show that the preparation of fluorinated dehydroalanines by using a fluoro-Pummerer reaction is successful for amino acids or dipeptides, but suffers from reduced yields with tripeptides because of formation of this side product.

Enzymatic routes to fluorinated dehydroalanines

In parallel with the efforts described above, we sought to convert difluoroalanine-containing peptides **3** (Scheme 2) into fluorinated dehydroalanines. Because of the strongly basic conditions required for the elimination of hydrogen fluoride from difluoroalanine, which could lead to racemization of other residues in the peptide, we focused our attention on enzymatic transformations. The basic premise underlying this strategy relied on the formation of enolate intermediates in enzymes that catalyze either epimerizations at the α-carbon atom of amino acids in peptides or dehydrations of serine or threonine residues. Upon enolate formation, it was anticipated that fluoride elimination would provide the desired structures. An added dimension to this methodology involves its potential application to activate a latent reactive functionality present in difluoroalanine that is unmasked into a reactive fluorinated dehydroalanine within the active site of the enzyme. Hence, if the enzyme does not contain any nucleophilic residues in its active site, this strategy would utilize the enzyme in question as a catalyst for the preparation of fluorinated dehydroalanines. On the other hand, if the protein does contain reactive active site residues, the enzyme would function as an unsuspecting target for mechanism-based inhibition (Scheme 1). Several enzymes catalyzing epimerization or dehydrations of peptides have been reported, such as the epimerase involved in the production of the venom of the funnel web spider *Agelenopsis aperta*,[37–39] the dehydratases involved in lantibiotic production,[14] and the L-Ala-D/L-Glu epimerase YcjG from *E. coli* whose substrate promiscuity has been recently demonstrated.[40] The latter property was particularly attractive as it would allow access to a variety of fluorinated-dehydroalanine-containing peptides.

YcjG catalyzes the epimerization of the C-terminal amino acids of various L-Ala-D/L-Xxx dipeptides, which include the murein peptide L-Ala-D-Glu, the preferred and presumably physiological substrate.[40] The protein is a member of the 'mechanistically diverse' enolase superfamily,[41, 42] as evidenced by the conservation of the ligands to a catalytically essential magnesium ion that stabilizes the enolate intermediate. Furthermore, Lys151 and Lys247, the two residues involved in a two-base mechanism of epimerization, are conserved in YcjG. In an initial proof of concept study, the broad spectrum antibiotic fludalanine,[43] the D-enantiomer of fluoroalanine deuterium labeled at the α-carbon atom, was incorporated into dipeptide **28** by using solution-phase peptide chemistry (Scheme 9). Incubation with YcjG resulted in the production of dipeptide **29**, which contains a dehydroalanine as determined by ^{1}H NMR spectroscopy. Monitoring of the reaction with a fluoride-selective electrode showed a time-dependent release of one equivalent fluoride anion (Figure 2). From the initial rate, a rate constant of



Scheme 9. *Incubation of the dipeptide epimerase YcjG with fluorinated dipeptides **28** and **31** produced dehydroalanine- and fluorodehydroalanine-containing peptides **29** and **32**, respectively.*

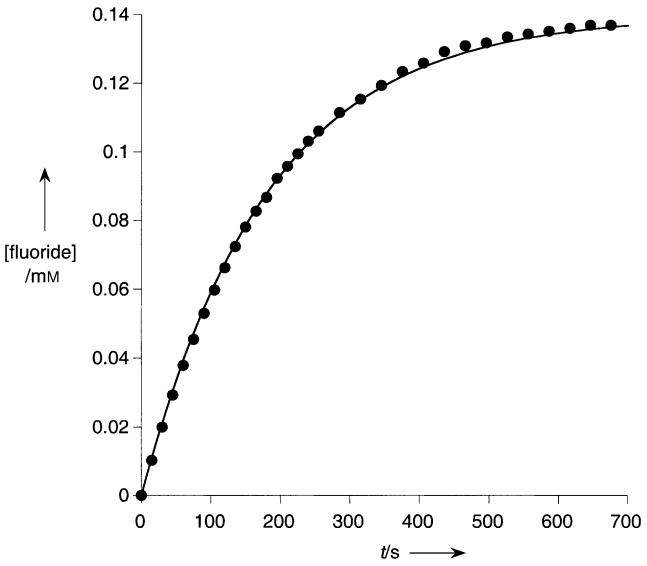


Figure 2. *Time dependence of the release of fluoride anion in the reaction of YcjG (2.8 μM) with dipeptide **28** (110 μM) measured with a fluoride-selective electrode. The instrument readings were converted into fluoride concentrations by using a standard curve generated under the same conditions with NaF. The solid line drawn through the experimental data points represents a least squares regression analysis using a single exponential equation for a first-order process. The initial rate determined from the first four data points was 0.23 s^{-1}.*

0.23 s^{-1} was estimated for the YcjG-catalyzed fluoride elimination compared to a k_{cat} value of 1.9 s^{-1} for epimerization of L-Ala-D-Met and 17 s^{-1} for L-Ala-D-Asp.[40] The slower rate observed with **28** is probably in part a reflection of an unsaturated concentration in the assay (150 μM), which is below or near the Michaelis constants K_M for L-Ala-D-Met (690 μM) and L-Ala-D-Asp (190 μM). Interestingly, when the reaction of YcjG with **28** was followed by ^{19}F NMR spectroscopy, no evidence was obtained for the formation of diastereomer **30**, which suggests that fluoride elimination from the enolate intermediate occurs faster than protonation to generate Ala-L-fluoroAla. Furthermore, deuterium washout into the solvent was not observed from Ala-D-fluoroAla (**28**); the splitting pattern of the starting dipeptide in the ^{19}F NMR spectrum remained unchanged over the course of the reaction. Since the active site bases in YcjG are lysine residues (i.e. polyprotic),[44a] this observation confirms that fluoride elimination is faster than return of a proton to the enolate intermediate. When **28** was incubated with mutants of the catalytic lysine residues of YcjG, K151R or K247R, fluoride release was seen only with the K247R mutant, which suggests that Lys151 is the *R*-specific base. YcjG K247R eliminated fluoride from **28** with a rate constant of 0.15 s^{-1}, less than twofold reduced from the rate for wild type YcjG. These results correlate with those seen for deuterium incorporation into L-Ala-L-Glu or L-Ala-D-Glu by YcjG K151R or YcjG K247R, in which Lys151 appears to be the *R*-specific base and Lys247 the *S*-specific base.[44b] Collectively, these observations validated the methodology and showed that YcjG is not inactivated by the dehydroalanine-containing product. This property is in contrast to the behavior of the epimerase of the spider toxin, which is inhibited by a dehydroalanine-containing analogue of its physiological substrate.[45] The ability of YcjG to produce dehydroalanine under mild conditions suggests that the protein, or in vitro evolved mutants, may have potential for dehydroalanine synthesis as an alternative to existing chemical methods.[46–48]

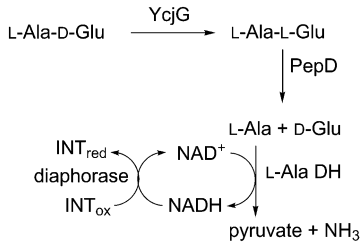
We next evaluated Ala-difluoroAla as a dipeptide precursor to Ala-fluoroDha. Racemic *N*-carboxybenzyl difluoroalanine was prepared by a previously reported route,[49] and subsequently converted into a 2:1 mixture of diastereomeric peptides **31**. Incubation of **31** with YcjG led to the production of the fluorinated dehydropeptide **32**. Only one isomer was detected by ^1H NMR spectroscopy, which was tentatively assigned as the *Z* isomer based on the chemical shift of the vinyl proton in comparison with that in the dipeptides described above. When the reaction was performed in D_2O, deuterium incorporation into the substrate was not observed; such incorporation would have been readily detected as a loss of the proton hyperfine interaction in the ^{19}F NMR spectrum. Hence, once again fluoride elimination from the enolate intermediate must be faster than deuteration of the enolate. Analysis of the reaction by using a fluoride-selective electrode indicated the production of 0.8 equiv. of fluoride per dipeptide at a similar rate (0.14 s^{-1}) as observed with **28**. Thus, YcjG is a useful catalyst to introduce fluorinated dehydroalanines into dipeptides.

Reactivity of fluorinated dehydroalanine

To evaluate the proposed mechanism of action of fluorine substituted dehydroalanines as presented in Scheme 1, both isomers of **10** were reacted with *p*-methoxybenzenethiol in the presence of diisopropylethylamine. Rapid conversion of *Z*-**10** into the corresponding *Z*-dehydrocysteine derivative was observed, which is consistent with an addition elimination mechanism. The *E* isomer of **10** produced predominantly *E*-*S*-aryldehydrocysteine, along with a small amount of the *Z* stereoisomer. The stereochemistry of the products was assigned based on the chemical shift of the vinylic proton in comparison with literature values for similar dehydrocysteine derivatives.[50] These results show that installation of a fluorinated dehydro-

alanine functionality at the site of Dha residues in natural products would result in a powerful covalent labeling agent.

The YcjG-catalyzed conversion of **31** into **32** reached complete conversion, which suggests that the enzyme does not react with the fluoroDha product. It was therefore unexpected that after incubation with **31**, YcjG showed no activity in a spectrophotometric assay developed previously.[40] This coupled assay utilizes L-Ala-D-Glu as substrate for YcjG, and relies on the broad specificity dipeptidase PepD to hydrolize the epimerized L-Ala-L-Glu to the individual amino acids (Scheme 10).



Scheme 10. *Enzymatic coupled assay for the determination of YcjG activity. DH, dehydrogenase.*

The deamination of L-Ala by L-Ala dehydrogenase (ADH) is driven to completion by using diaphorase from *Clostridium kuyveri* with the dye *p*-iodonitrotetrazolium violet (INT) as electron acceptor generating a convenient spectrophotometric readout. After completion of the reaction of YcjG with **31**, an aliquot was added to the assay mixture resulting in the complete absence of activity compared with a control sample (Figure 3, squares and closed circles). At first glance, these results suggested that YcjG had been inactivated in the reaction with **31**, presumably by the product **32**. To probe whether the inactivation was irreversible, the reaction mixture of YcjG and **31** was assayed again after 50 h. Curiously, after an initial lag phase, activity was recovered (Figure 3, ◇), and the duration of the lag phase decreased when aliquots of the mixture were assayed periodically over a period of days. The short lag phase evident in the control reaction is attributable to the high K_M value of PepD for its dipeptide substrates, reported to be in the range of 2–5 mM.[51]

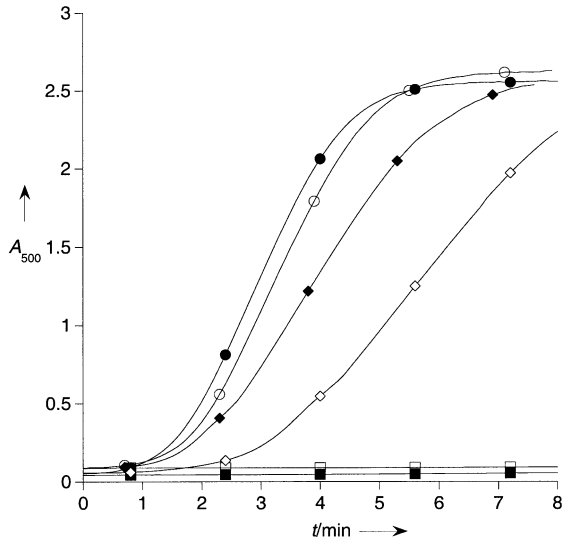


Figure 3. *Activity assays using the coupled assay shown in Scheme 10. The assays contained Tris (50 mM, pH 8.5), INT (1.5 mM), NAD$^+$ (1.5 mM), diaphorase (2 U), ADH (10 U), $CoCl_2$ (67 μM), PepD (200 μg), and L-Ala-D-Glu (350 μM). YcjG (10 μg) was added to the assay mixture after incubation of the mixture with **31** for 20 h (□), 28 h (■), 50 h (◇), 68 h (◆), and 140 h (○). In the absence of **31**, essentially no change in the spectrophotometric readout was observed over time, and a typical result is shown after a 20-h incubation (●). Spectra were collected every 6 s.*

The inhibition profile is not consistent with inactivation of YcjG but instead suggests that a compound produced in the reaction of YcjG with **31** inhibits another component of the assay mixture. Two other requirements must be met to explain the unusual behavior: 1) the inhibitor must be unstable and decompose to account for the shorter lag phase observed over time, and 2) one of the ingredients of the assay must act upon the inhibitor to explain the recovery of the activity only in the assay mixture. Whereas we were unable to determine the exact identity of the inhibiting species, as described in the Supporting Information, our data are consistent with PepD acting upon the fluorinated dehydropeptide **32** to produce a transient species that inhibits ADH or diaphorase.

Conclusion

In summary, we have developed new methodology to prepare fluorine-substituted dehydroalanines within dipeptides relying on either chemical or enzymatic installation of the olefin. The chemical strategy is currently limited to peptides in which the fluorinated dehydroalanine residue occupies the C-terminal position because of a side reaction in the fluoro-Pummerer reaction that prevents C-terminal extension. The enzymatic route provides dipeptides that can be protected at the N terminus to provide building blocks that can be used for C-terminal extension. Given the broad substrate specificity of YcjG at both the first and second position,[40] the enzymatic route is the preferred method for the synthesis of these targets. Directed evolution of YcjG may further increase its utility. Given the well-known success of fluorine containing pharmaceuticals,[52–55] including fluoropeptides,[56] the current approach expands the arsenal of fluorinated moieties that can be used in the preparation of bioactive molecules. Efforts to introduce these reactive Michael acceptors into natural products are underway and will be reported in due course.

Experimental section

All NMR spectra were recorded on Varian U400, U500, or UI500NB spectrometers. ^1H spectra were referenced to $CHCl_3$ at 7.26 ppm and ^{13}C spectra were referenced to $CDCl_3$ at 77.23 ppm. ^{19}F spectra were referenced externally to 1% $C_6F_6/CDCl_3$ at −162.0 ppm. All spectra were taken in $CDCl_3$ unless otherwise indicated. Mass spectrometry (MS) experiments were carried out by the Mass Spectrometry Laboratory at the University of Illinois at Urbana-Champaign (UIUC). Elemental analysis was performed by the Microanalysis Laboratory at UIUC. Infrared (IR) spectra were taken on a Mattson Galaxy Series FTIR

5000. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} plates. Compounds and solvents were obtained from Fisher, Aldrich, and Chem-Impex. Anhydrous $MgSO_4$ was used for drying organic solutions.

2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-acrylic acid benzhydryl ester (5): Compound **4**[57] (1.01 g, 2.06 mmol) was dissolved in anhydrous CH_2Cl_2 (25 mL). The solution was cooled to 0 °C. Methylsulfonyl chloride (MsCl; 0.26 g, 2.26 mmol) was added dropwise and the reaction mixture was stirred for 10 min. Then triethylamine (0.46 g, 4.52 mmol) was added and the reaction was stirred for an additional 24 h at 0 °C. The solution was diluted with CH_2Cl_2 (80 mL) and washed with saturated aqueous NH_4Cl, $NaHCO_3$, and brine. The organic layer was dried and concentrated. Purification by silica gel chromatography (hexane/ethyl acetate 5:1) provided the desired product as a white powder (0.80 g, 82%). R_f: 0.38; m.p.: 109–110 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.28$ (t, $J = 7.01$ Hz, 1H, $CHCH_2$), 4.48 (d, $J = 6.73$ Hz, 2H, CH_2), 6.07 (s, 1H, CH_2), 6.40 (br s, 1H, CH_2), 7.07 (s, 1H, $CH(Ph)_2$), 7.33–7.45 (m, 15H, Ph, NH), 7.62 (d, $J = 7.43$ Hz, 2H, Ph), 7.80 (d, $J = 7.36$ Hz, 2H, Ph) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta =$ 47.3 (CH/CH_3), 67.5 (C/CH_2), 79.3 (CH/CH_3), 106.9 (C/CH_2), 120.4 (CH/CH_3), 125.3 (CH/CH_3), 127.4 (CH/CH_3), 127.5 (CH/CH_3), 128.1 (CH/CH_3), 128.6 (CH/CH_3), 128.8 (CH/CH_3), 129.0 (CH/CH_3), 131.5 (C/CH_2), 139.7 (C/CH_2), 141.6 (C/CH_2), 143.9 (C/CH_2), 153.5 (C/CH_2), 163.4 (C/CH_2) ppm; IR: 3408, 3066, 3033, 1736, 1519, 1316, 1105, 981, 736 cm^{-1}; HRMS (FAB^+): calcd for $C_{31}H_{25}NO_4$: 475.1784; found: 475.1783; elemental anal.: calcd for $C_{31}H_{25}NO_4$: C, 78.30; H, 5.30; N, 2.95; found: C, 78.25; H, 5.28; N, 3.11.

2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-3-(4-methoxy-phenylsulfanyl)-propionic acid benzhydryl ester (6): Compound **5** (0.143 g, 0.3 mmol) was placed in a 10-mL round-bottomed flask. The flask was flushed with N_2 and anhydrous THF (4 mL) was added. Triethylamine (0.061 g, 0.6 mmol) was added, followed by 4-methoxybenzenethiol (0.042 g, 0.3 mmol). The solution was stirred for 18 h, diluted with Et_2O (80 mL), washed with saturated $NaHCO_3$ (50 mL) and 5% $KHSO_4$ (50 mL), dried, filtered, and concentrated. The mixture was purified by flash chromatography (hexane/ethyl acetate 5:1) to give the product as a white powder (two diastereomers, 0.129 g, 70%). R_f: 0.17; m.p.: 107–108 °C; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 3.31$ (B of ABX, $J_{ab} = 14.2$ Hz, $J_{bx} = 5.5$ Hz, 1H, CH_2), 3.46 (A of ABX, $J_{ab} = 14.2$ Hz, $J_{ax} = 4.5$ Hz, 1H, CH_2), 3.80 (s, 3H, OCH_3), 4.18 (t, $J = 7.19$ Hz, 1H, CH), 4.30–4.36 (m, 2H, CH_2), 4.76 (X of ABX, 1H, CH), 5.76 (d, $J =$ 8.29 Hz, 1H, NH), 6.78 (d, $J = 8.74$ Hz), 2H, Ph), 6.88 (s, 1H, $CH(Ph)_2$), 7.31–7.38 (m, 20H, Ph) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 38.9$ (C/CH_2), 47.3 (CH/CH_3), 54.5 (CH/CH_3), 55.5 (CH/CH_3), 67.5 (C/CH_2), 78.8 (CH/CH_3), 114.9 (CH/CH_3), 120.2 (CH/CH_3), 125.0 (C/CH_2), 125.4 (CH/CH_3), 127.3 (CH/CH_3), 127.4 (CH/CH_3), 128.0 (CH/CH_3), 128.4 (CH/CH_3), 128.8 (CH/CH_3), 128.9 (CH/CH_3), 134.6 (CH/CH_3), 139.6 (C/CH_2), 140.0 (C/CH_2), 141.5 (C/CH_2), 144.0 (C/CH_2), 155.8 (C/CH_2), 159.7 (C/CH_2), 169.9 (C/CH_2) ppm; IR: 3425, 1720, 1500, 1235, 728 cm^{-1}; HRMS (FAB^+): calcd for $C_{38}H_{33}NO_5S$: 615.2079; found: 615.2075; elemental anal.: calcd for $C_{38}H_{33}NO_5S$: C, 74.12; H, 5.40; N, 2.27; found: C, 73.91; H, 5.36; N, 2.38.

2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-3-(4-methoxy-benzenesulfinyl)-propionic acid benzhydryl ester (7): Compound **6** (0.391 g, 0.635 mmol) was dissolved in CH_2Cl_2 (25 mL) and the solution was cooled to −40 °C. A solution of *m*CPBA (Aldrich tech. grade, 57% peracid content determined by titration,[58] 0.160 g, 0.528 mmol) in CH_2Cl_2 (3 mL) was added dropwise. The solution was stirred at −40 °C and additional *m*CPBA (0.040 g, 0.132 mmol) was added to complete the reaction, as determined by TLC. The mixture was diluted with CH_2Cl_2 (100 mL), washed with 10% aqueous $Na_2S_2O_3$ (30 mL), water (30 mL), and brine (30 mL). The organic layer was dried, filtered, and concentrated. Purification by silica gel (hexane/ethyl acetate 1:1) gave the product as a white powder (0.381 g, 95%). R_f: 0.22; two diastereomers: m.p.: 69–71 °C. ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 3.25–3.33$ (m, 2H, CH_2), 3.36–3.43 (m, 2H, CH_2), 3.81 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 4.20 (t, $J = 7.02$ Hz, 2H, CH), 4.29–4.40 (m, 4H, CH_2), 4.73 (q, $J = 6.04$ Hz, 1H, CH), 4.85–4.89 (m, 1H, CH), 5.98 (br s, 1H, NH), 6.40 (t, $J = 3.7$ Hz, 1H, NH), 6.92 (s, 1H, $CH(Ph)_2$), 6.97–7.00 (m, 5H, Ph, $CH(Ph)_2$), 7.27–7.78 (m, 40H, Ph) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 50.7$ (CH/CH_3), 51.6 (CH/CH_3), 55.7 (CH/CH_3), 55.8 (CH/CH_3), 67.7 (C/CH_2), 67.8 (C/CH_2), 79.1 (CH/CH_3), 79.3 (CH/CH_3), 115.2 (CH/CH_3), 125.5 (CH/CH_3), 126.6 (CH/CH_3), 127.3 (CH/CH_3), 128.0 (CH/CH_3), 128.5 (CH/CH_3), 139.4 (C/CH_2), 141.5 (C/CH_2), 143.9 (C/CH_2), 156.0 (C/CH_2), 162.6 (C/CH_2), 169.4 (C/CH_2) ppm. IR: 3270, 3063, 1723, 1594, 1496, 1450, 1304, 1260, 1173, 1087, 1030, 830, 759, 740, 700 cm^{-1}; HRMS (FAB^+): calcd for $C_{38}H_{33}NO_6S$: 631.2029; found: $[M^+ + H]$ 632.2106; elemental anal.: calcd for $C_{38}H_{33}NO_6S$: C, 72.25; H, 5.27; N, 2.22; found: C, 71.89; H, 5.28; N, 2.26.

2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-3-fluoro-3-(4-methoxy-phenylsulfanyl)-propionic acid benzhydryl ester (8): Compound **7** (0.238 g, 0.377 mmol) was dissolved in anhydrous CH_2Cl_2 (2 mL) in a 5-mL round-bottomed flask under N_2. DAST (0.091 g, 0.565 mmol) was added, followed by a solution of antimony trichloride ($SbCl_3$; 0.009 g, 0.04 mmol) in CH_2Cl_2 (0.3 mL). The solution was stirred at RT for 4 h until TLC showed complete consumption of starting material. An ice-cold $NaHCO_3$ solution was added and the mixture was stirred for 5–10 min. The mixture was diluted with CH_2Cl_2 (80 mL), and the organic layer was washed with brine (30 mL), dried, and concentrated. Purification over silica gel (hexane/ethyl acetate 5:1) gave the product as two diastereomers (0.167 g, 70%). R_f: 0.18; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.80$ (s, 3H, OCH_3), 4.26 (t, $J =$ 7.4 Hz, 1H, CH), 4.40–4.48 (m, 2H, CH_2), 5.06–5.17 (m, 1H, CH), 5.76 (t, $J = 9.7$ Hz, 1H, NH), 6.05 (dd, $J = 53.0$ Hz, 3.3 Hz, CHF), 6.19 (dd, $J =$ 54.0 Hz, 3.5 Hz, CHF), 6.86 (d, $J = 8.5$ Hz, 2H, Ph), 6.99 (s, 1H, $CH(Ph)_2$), 7.32–7.79 (m, 20H, Ph) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) $\delta = 47.2$ (CH/CH_3), 55.6 (CH/CH_3), 58.3 (CH/CH_3), 58.5 (CH/CH_3), 67.8 (C/CH_2), 68.0 (C/CH_2), 79.6 (CH/CH_3), 79.7 (CH/CH_3), 102.8 (d, $J = 224$ Hz, CH/CH_3), 114.9 (CH/CH_3), 115.1 (CH/CH_3), 120.2 (CH/CH_3), 122.1(CH/CH_3), 125.4 (CH/CH_3), 127.4 (CH/CH_3), 127.6 (CH/CH_3), 128.6 (CH/CH_3), 128.9 (CH/CH_3), 135.8 (CH/CH_3), 135.9 (CH/CH_3), 139.2 (C/CH_2), 141.6 (C/CH_2), 143.9 (C/CH_2), 156.4 (C/CH_2), 160.8 (C/CH_2), 167.2 (C/CH_2) ppm; ^{19}F NMR (376 MHz, $CDCl_3$): (two diastereomers) $\delta = -156.0$ (dd, $J =$ 56.2 Hz, 15.3 Hz, CHF), −157.4 (dd, $J = 56.5$ Hz, 26.4 Hz, CHF) ppm; IR: 3318, 3065, 3033, 1727, 1592, 1494, 1450, 1249, 1178, 1030, 740, 699 cm^{-1}; HRMS (FAB^+): calcd for $C_{38}H_{32}O_5NSF$ 633.1985; found: 633.1984.

2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-3-fluoro-acrylic acid benzhydryl ester (10): Compound **8** (0.126 g, 0.199 mmol) was dissolved in CH_2Cl_2 (5 mL) and the solution was cooled to −20 °C. A solution of *m*CPBA (57% peracid content, see the method used for **7**) (0.066 g, 0.219 mmol) in CH_2Cl_2 (2 mL) was added, and the reaction mixture was stirred at −20 to −5 °C for 1.5 h. The solution was diluted with CH_2Cl_2 (80 mL), and washed with 10% aqueous $Na_2S_2O_3$, water (30 mL), and brine (30 mL). The organic layer was dried and concentrated to give product **9** as four diastereomers. Compound **9** was placed in a 10-mL flask without purification and anhydrous benzene (5 mL) was added. The solution was refluxed overnight. The solvent was removed under reduced pressure. Purification over silica gel (hexane/ethyl acetate 5:1) gave product **10** as two diastereomers that were separable (0.046 g, 47%). (*E* isomer): R_f: 0.24; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 4.22$ (t, $J = 6.84$ Hz, 1H, CH), 4.42 (d, $J = 6.91$ Hz, 2H, CH_2), 6.56 (s, 1H, NH), 7.02 (s, 1H, $CH(Ph)_2$), 7.30–7.43 (m, 14H, Ph), 7.56 (d, $J = 7.30$ Hz, 2H, Ph), 7.71 (d, $J = 7.63$ Hz, 2H, Ph), 8.18 (d, $J = 77.3$ Hz, 1H, CHF) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 47.1$ (CH/

CH_3), 67.8 (C/CH_2), 79.5 (CH/CH_3), 116.3 (d, $^2J_{CF}=17.86$ Hz, C/CH_2), 120.3 (C/CH_2), 125.2 (CH/CH_3), 127.2 (CH/CH_3), 127.4 (CH/CH_3), 128.1 (CH/CH_3), 128.4 (CH/CH_3), 128.9 (CH/CH_3), 139.7 (C/CH_2), 141.6 (C/CH_2), 143.7 (C/CH_2), 152.1 (d, $^1J_{CF}=276.17$ Hz, CH/CH_3), 153.5 (C/CH_2), 162.4 (C/CH_2) ppm; ^{19}F NMR (376 MHz, $CDCl_3$): $\delta=-130.9$ (d, $J=$ 78.1 Hz, CHF) ppm; IR: 3403, 3065, 3023, 1709, 1519, 1495, 1450, 1384, 1365, 1277, 1216, 1143, 1045, 759, 740, 700 cm^{-1}. (*Z* isomer): R_f: 0.19; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=4.20$ (t, $J=7.10$ Hz, 1H, CH), 4.41 (d, $J=6.91$ Hz, 2H, CH_2), 6.08 (s, 1H, NH), 6.98 (s, 1H, $CH(Ph)_2$), 7.28–7.42 (m, 14H, Ph), 7.57 (d, $J=7.51$ Hz, 2H, Ph), 7.66 (d, $J=74.63$ Hz, 1H, CHF), 7.77 (d, $J=7.59$ Hz, 2H, Ph) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=47.1$ (CH/CH_3), 68.2 (C/CH_2), 78.8 (CH/CH_3), 114.9 (d, $^2J_{CF}=5.52$ Hz, C/CH_2), 120.3 (C/CH_2), 125.3 (CH/CH_3), 127.3 (CH/CH_3), 128.0 (CH/CH_3), 128.5 (CH/CH_3), 128.6 (CH/CH_3), 128.9 (CH/CH_3), 139.4 (C/CH_2), 141.5 (C/CH_2), 143.8 (C/CH_2), 152.8 (d, $^1J_{CF}=283$ Hz, CH/CH_3), 153.4 (C/CH_2), 163.1 (d, $^1J_{CF}=11.6$ Hz, C/CH_2) ppm; ^{19}F NMR (376 MHz, $CDCl_3$): $\delta=-117.2$ (d, $J=72.6$ Hz, CHF) ppm; IR: 3318, 3065, 3033, 1728, 1674, 1496, 1674, 1496, 1450, 1267, 1230, 1181, 1047, 910, 758, 740 cm^{-1}; HRMS (FAB^+): calcd for $C_{31}H_{24}NO_4F$: 493.1689; found: [M^++H] 494.1768.

2-(2-Acetylamino-acetylamino)-3-(4-methoxy-benzenesulfinyl)-propionic acid benzhydryl ester (15): The same procedure was followed as given for compound **7** (93%, two diastereomers). M.p.: 54–56 °C; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=2.01$ (s, 3H, CH_3), 2.03 (s, 3H, CH_3), 3.16–3.23 (m, 2H, CH_2), 3.31–3.39 (m, 2H, CH_2), 3.84 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.88–4.03 (m, 4H, CH_2), 4.90 (q, $J=6.03$ Hz, 1H, CH), 5.01–5.06 (m, 1H, CH), 6.28–6.31 (m, 2H, NH), 6.86 (s, 1H, $CH(Ph)_2$), 6.94–6.70 (m, 5H, Ph, $CH(Ph)_2$), 7.28–7.77 (m, 26H, Ph, NH) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=23.1$ (CH/CH_3), 43.2 (C/CH_2), 49.4 (CH/CH_3), 55.8 (CH/CH_3), 58.0 (C/CH_2), 79.1 (CH/CH_3), 115.2 (CH/CH_3), 126.2 (CH/CH_3), 126.5 (CH/CH_3), 127.2 (CH/CH_3), 127.5 (CH/CH_3), 128.4 (CH/CH_3), 128.7 (CH/CH_3), 133.7 (C/CH_2), 139.3 (C/CH_2), 139.5 (C/CH_2), 162.5 (C/CH_2), 169.0 (C/CH_2), 169.8 (C/CH_2), 171.0 (C/CH_2) ppm; IR: 3294.9, 3054.8, 1746.8, 1668.0, 1496.7, 1265.5, 738.1, 703.4 cm^{-1}; HRMS (FAB^+): calcd for $C_{27}H_{28}N_2O_6S$: 508.1746; found: 508.1747.

2-(2-Acetylamino-acetylamino)-3-fluoro-3-(4-methoxy-phenylsulfanyl)-propionic acid benzhydryl ester (16): A similar procedure was followed as given for compound **8** (yield 62%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=2.00$ (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 3.99 (dd, $J=5.1$ Hz, 1.2 Hz, 1H, CH_2), 4.04 (d, $J=5.1$ Hz, 1H, CH_2), 5.24–5.33 (m, 1H, CH), 5.94 (dd, $J=52.8$ Hz, 3.5 Hz, 0.5H, CHF), 6.09 (dd, $J=53.7$ Hz, 2.6 Hz, 0.5H, CHF), 6.17 (1H, NH), 6.80 (1H, NH), 6.84 (d, $J=6.9$ Hz, 2H, Ph), 6.97 (s, 1H, $CH(Ph)_2$), 7.27–7.44 (m, 12H, Ph) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=23.1$ (CH/CH_3), 43.4 (C/CH_2), 55.6 (CH/CH_3), 56.3 (CH/CH_3), 79.7 (CH/CH_3), 102.2 ($^1J_{C\text{-}F}=229$ Hz, CH/CH_3), 115.1 (CH/CH_3), 128.6 (CH/CH_3), 128.8 (CH/CH_3), 135.8 (CH/CH_3), 139.2 (C/CH_2), 160.8 (C/CH_2), 166.7 (C/CH_2), 166.9 (C/CH_2), 169.2 (C/CH_2), 169.6 (C/CH_2), 170.7 (C/CH_2) ppm; ^{19}F NMR (376 MHz, $CDCl_3$): (two diastereomers) $\delta=-155.6$ (dd, $J=54.4$ Hz, 14.2 Hz, CHF), -157.4 (dd, $J=$ 54.7 Hz, 25.6 Hz, CHF) ppm; IR: 3416.6, 3054.9, 1750.1, 1671.2, 1495.7, 1265.6, 737.0, 704.4 cm^{-1}; HRMS (FAB^+): calcd for $C_{27}H_{27}N_2O_5SF$: 510.1703; found: [M^++H] 511.1703.

2-(2-Acetylamino-acetylamino)-3-fluoro-acrylic acid benzhydryl ester (17): The same procedure was followed as given for compound **10** (yield 57%). (*E* isomer): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=2.03$ (s, 3H, CH_3), 3.99 (d, $J=5.47$ Hz, 2H, CH_2), 6.20 (br s, 1H, NH), 7.00 (s, 1H, $CH(Ph)_2$), 7.30–7.41 (m, 10H, Ph), 7.80 (s, 1H, NH), 8.50 (d, $J=78.4$ Hz, 1H, CHF) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=23.1$ (CH/CH_3), 44.1 (C/CH_2), 79.6 (CH/CH_3), 116.3 (d, $^2J_{CF}=18.7$ Hz, C/CH_2), 127.2 (CH/CH_3), 128.4 (CH/CH_3), 128.9 (CH/CH_3), 140.2 (d, $^1J_{CF}=275$ Hz, C/CH_2), 140.5 (C/CH_2), 162.2 (C/CH_2); 167.3 (C/CH_2), 171.1 (C/CH_2) ppm; ^{19}F NMR (376 MHz, $CDCl_3$): $\delta=-127.0$ (d, $J=78.4$ Hz, CHF) ppm; IR: 3283, 1659, 1527, 1374, 1269, 1225, 1183, 1144, 699 cm^{-1}. (*Z* isomer): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=2.04$ (s, 3H, CH_3), 4.05 (d, $J=5.28$ Hz, 2H, CH_2), 6.43 (br s, 1H, NH), 6.93 (s, 1H, $CH(Ph)_2$), 7.30–7.37 (m, 10H, Ph), 7.60 (d, $J=74.50$ Hz, 1H, CHF), 7.79 (s, 1H, NH) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=23.1$ (CH/CH_3), 43.9 (C/CH_2), 78.8 (CH/CH_3), 119.2 (d, $^2J_{CF}=5.63$ Hz, C/CH_2), 127.4 (CH/CH_3), 128.6 (CH/CH_3), 128.9 (CH/CH_3), 139.4 (C/CH_2), 157.0 (d, $^1J_{CF}=330$ Hz, CH/CH_3), 162.8 (d, $^3J_{CF}=17.4$ Hz, C/CH_2), 167.4 (C/CH_2), 171.2 (C/CH_2) ppm; ^{19}F NMR (376 MHz, $CDCl_3$): $\delta=-113.9$ (d, $J=76.09$ Hz, CHF); IR: 3282, 1732, 1664, 1654, 1522, 1497, 1266, 1098, 699 cm^{-1}; HRMS (FAB^+): calcd for $C_{20}H_{19}N_2O_4F$: 370.1329; found: [M^++H] 371.1406.

Ac-Gly-Cys(SAr)-Ala-ODpm (*R*-20): Fmoc-Ala-ODpm (0.157 g, 0.328 mmol) was dissolved in $CHCl_3$ (4 mL). 4-AMP (4-aminomethyl-piperidine; 0.45 mL) was added, and the reaction mixture was stirred at RT for 2 h 30 min. The mixture was diluted with $CHCl_3$ (80 mL) and washed with 10% aqueous phosphate buffer (pH 5.5, 3 × 10 mL), water (30 mL), and brine (30 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated in a 100-mL round-bottomed flask to a volume of approximately 2 mL. Fmoc-Cys(Ar)-OH (0.147 g, 0.328 mmol) in $CHCl_3$ (3 mL) was added directly to the concentrated solution obtained in the Fmoc deprotection, along with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; 0.171 g, 0.328 mmol) and *N*,*N*-diisopropylethylamine (DIEA; 0.11 mL, 0.656 mmol). After stirring for 2 h, the reaction mixture was diluted with $CHCl_3$ (80 mL) and washed with aq. 1 N HCl (30 mL), sat. aq. $NaHCO_3$ (30 mL), water (30 mL), and brine (30 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated to produce Fmoc-Cys(Ar)-Ala-ODpm (0.185 g, 0.270 mmol). The dipeptide obtained was dissolved in $CHCl_3$ (3 mL). 4-AMP was added, and the reaction mixture was stirred at ambient temperature for 2 h. The mixture was diluted with $CHCl_3$ (80 mL) and washed with 10% aqueous phosphate buffer (pH 5.5, 3 × 10 mL), water (30 mL), and brine (30 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated to a volume of approximately 3 mL. Ac-Gly-OH (0.032 g, 0.27 mmol), PyBop (0.141 g, 0.270 mmol), and DIEA (0.1 mL, 0.540 mmol) were added. After stirring for 1 h and 45 min, the reaction mixture was diluted with $CHCl_3$ (80 mL) and washed with aqueous 1 N HCl (30 mL), sat. aq. $NaHCO_3$ (30 mL), water (30 mL), and brine (30 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated. Purification over silica gel chromatography gave the desired product (0.085 g, 46%). $R_f=0.14$ (CH_2Cl_2:MeOH: 20:1); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=1.41$ (d, $J=6.9$ Hz, 3H, CH_3), 1.98 (s, 3H, CH_3), 3.14 (m, 2H, CH_2), 3.76 (s, 3H, OCH_3), 3.87 (d, $J=5.1$ Hz, 2H, CH_2), 4.48 (q, $J=6.6$ Hz, 1H, CH), 4.59 (q, $J=7.2$ Hz, 1H, CH), 6.26 (t, $J=5.2$ Hz, 1H, NH), 6.81 (d, $J=9.0$ Hz, 2H, Ph), 6.85 (d, $J=7.5$ Hz, 1H, NH), 6.88 (s, 1H, $CH(Ph)_2$), 7.32 (m, 12H, Ph) ppm; ^{13}C NMR (500 MHz, $CDCl_3$, APT): $\delta=18.2$ (CH/CH_3), 23.1 (CH/CH_3), 37.9 (C/CH_2), 43.4 (C/CH_2), 48.8 (CH/CH_3), 52.8 (CH/CH_3), 55.5 (CH/CH_3), 78.4 (CH/CH_3), 115.0 (CH/CH_3), 124.6 (C/CH_2), 127.2 (CH/CH_3), 127.3 (CH/CH_3), 128.4 (CH/CH_3), 128.8 (CH/CH_3), 128.9 (CH/CH_3), 133.9 (CH/CH_3), 137.4 (C/CH_2), 139.8 (C/CH_2), 169.1 (C/CH_2), 169.5 (C/CH_2), 171.0 (C/CH_2), 171.7 (C/CH_2) ppm; HRMS (FAB^+): calcd for $C_{30}H_{34}N_3O_6S$: [M+H] 564.2090; found: 564.2089.

Ac-Gly-Cys(SOAr)-Ala-ODpm: The same procedure was followed as given for compound **7** to give two diastereomers. ^{1}H NMR (500 MHz, $CDCl_3$): diastereomer A: $\delta=1.40$ (d, $J=7.1$ Hz, 3H, CH_3), 1.96 (s, 3H, CH_3), 3.08–3.34 (m, 2H, CH_2), 3.85 (s, 3H, CH_3), 3.86 (d, $J=5.2$ Hz, 2H, CH_2), 4.55 (q, $J=7.3$ Hz, 1H, CH), 4.77 (q, $J=6.7$ Hz, 1H, CH), 6.45 (br s, 1H, NH), 6.85 (s, 1H, C*H*$(Ph)_2$), 7.02 (d, $J=8.8$ Hz, 2H, Ph), 7.26–7.89 (m, 14H, Ph + 2NH) ppm; diastereomer B: $\delta=1.47$ (d, $J=7.3$ Hz, 3H, CH_3), 2.01 (s, 3H, CH_3), 3.08–3.34 (m, 2H, CH_2), 3.84 (s, 3H, CH_3), 3.86 (d, $J=5.2$ Hz, 2H, CH_2), 4.66 (q, $J=7.2$ Hz, 1H, CH), 5.00 (q, $J=5.8$ Hz, 1H, CH), 6.59 (br s, 1H, NH), 6.86 (s, 1H, C*H*$(Ph)_2$), 6.98 (d, $J=8.8$ Hz, 2H, Ph), 7.26–7.89 (m, 14H, Ph + 2NH) ppm; ^{13}C NMR (500 MHz,

$CDCl_3$, attached proton test, mixture of diastereomers): $\delta = 17.6$ (CH/CH_3), 23.1 (CH/CH_3), 43.6 (C/CH_2), 49.0 (CH/CH_3), 49.1 (CH/CH_3), 55.8 (CH/CH_3), 56.5 (C/CH_2), 78.2 (CH/CH_3), 115.2 (CH/CH_3), 126.5 (CH/CH_3), 127.2 (CH/CH_3), 127.3 (CH/CH_3), 128.3 (CH/CH_3), 128.8 (CH/CH_3), 132.7 (C/CH_2), 139.7 (C/CH_2), 140.0 (C/CH_2), 162.6 (C/CH_2), 169.0 (C/CH_2), 169.3 (C/CH_2), 171.5 (C/CH_2) ppm; HRMS (FAB^+): calcd for $C_{30}H_{34}N_3O_7S_1$: [M+H] 580.2117; found: 580.2117.

Ac-Gly-Cys(3-F-SAr)-Ala-ODpm (**22**): The same procedure was followed as given for compound **8**. ^{19}F NMR (376 MHz): $\delta = -157.5$ (dd, $J = 54.0$ Hz, 15.3 Hz), -158.4 ($J = 54.3$ Hz, 28.1 Hz) ppm; HRMS (FAB^+): calcd for $C_{30}H_{33}N_3O_6S_1F_1$: [M+H] 582.2074; found: 582.2090.

Enzymatic preparation of fluorinated dehydroalanines: YcjG and PepD were expressed and purified as described previously.[40] Dipeptides **28** and **31** were prepared by solution phase peptide chemistry from fludalanine and D/L-difluoroalanine as described in the Supporting Information. Fludalanine was a gift from Merck and difluoroalanine was prepared as described previously (see the Supporting Information).[49] The reaction of the enzyme with the dipeptides was monitored by ^1H and ^{19}F NMR spectroscopy in D_2O. The samples contained the peptides (1.1-mM final concentrations), YcjG (100 μg, 2.7×10^{-3} μM), $MgCl_2$ (10 mM), and phosphate buffer, (20 mM, pD 7). ^1H NMR of **29** (400 MHz): $\delta = 1.39$ (d, 3H, $J = 7.1$ Hz, CH_3), 3.98 (q, 1H, $J = 7.1$ Hz, CH), 5.58 (s, 1H, Dha), 5.78 (s, 1H, Dha) ppm; LRMS (ESI): calcd for $C_6H_{11}N_2O_3$: [M+H] 159.1; found: 159.2. ^1H NMR of **31** (400 MHz, D_2O): $\delta = 1.38$ (d, 3H, $J = 7.4$ Hz, CH_3), 3.84 – 3.91 (m, 1H, $CHCH_3$), 7.29 (d, 1H, $J = 77.5$ Hz, CHF) ppm; ^{19}F NMR (400 MHz, D_2O): $\delta = -125.3$ (d, $J = 75.6$ Hz, CHF) ppm; LRMS (ESI): calcd for $C_6H_{10}F_1N_2O_3$: [M+H] 177.1; found: 177.1.

Time-dependent release of fluoride: A solution containing H_2O (4.4 mL), L-Ala-D-Ala(F) (0.1 mL, 5.56 mM), aq. NaF (0.5 mL, 0.142 mM), $MgCl_2$ (1 M, 10 μL), and phosphate buffer (1 M, 20 μL, pH 7) was placed in a 10-mL plastic container. A fluoride-selective electrode connected to a pH/mV meter was inserted into the solution to measure the voltage. After YcjG (100 μg) had been added to the above solution, the readings were recorded every 15 s. A standard curve (mV versus log[fluoride]) was prepared by measuring mV values of various aq. NaF solutions of the following concentrations: 0.0173, 0.0956, 0.161, 0.179, 0.322, 0.533, 0.728, and 1.61 mM. The predicted final fluoride concentration after enzymatic conversion of L-Ala-D-Ala(F) (**28**) to **29** for a 1:1 stoichiometry was 0.11 mM, and the experimentally observed value was 0.13 mM.

Supporting information: Synthetic schemes for the preparation of dipeptides **14**, **28**, and **31**, and compounds **18**, **19**, and FmocAla-ODpm; figures showing the pH dependence of the assay of YcjG after incubation with **31**; experiments investigating the origin of the lag phase in the YcjG assay.

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