The Synthesis of (2*S*)-4,4-Difluoroglutamyl γ-Peptides Based on Garner's Aldehyde and Fluoro-Reformatsky Chemistry

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Received 14 September 2002

Abstract: The development of optically active fluorinated synthetic building blocks of general utility is a current goal of organofluorine chemists. The serine-derived Garner aldehyde was converted to a general 4,4-difluoroamino acid building block via fluoro-Reformatsky reaction with ethyl bromodifluoroacetate. The utility of this building block was demonstrated by the synthesis of derivatives of (2*S*)-4,4-difluoroglutamine, (2*S*)-4,4-difluoroglutamic acid, and its incorporation into a fluorophore-containing isopeptide **2** designed as a mechanistic probe of γ-glutamyl hydrolase. Compound **2** proved to be a substrate for γ-glutamyl hydrolase and was hydrolyzed at a rate significantly slower than the corresponding non-fluorinated analog.

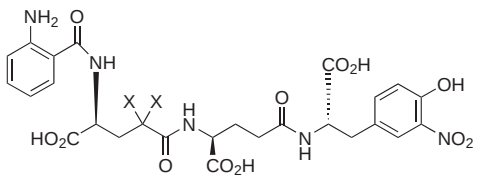
Key words: fluoroamino acids, fluoropeptides, stereoselective synthesis, γ-glutamyl hydrolase, fluorescence enzyme assay

Introduction

Fluorinated amino acids are valuable tools for bioorganic and medicinal chemists. Due to the properties of the fluorine atom, substitution of fluorine for hydrogen within a natural amino acid structure can impart dramatic effects on the chemical reactivity of the resulting product. This substitution is sterically conservative, and fluoroamino acids often possess unique and interesting reactivities within a biological system. Due to this fact, fluoroamino acids and larger molecules containing them enjoy widespread popularity and use in applications such as biochemical probes, alternate enzyme substrates, and enzyme inhibitors.[2,3] The power and utility of fluoroamino acids is illustrated by this laboratory's longstanding history of employing fluorinated glutamic acids to study the enzymatic synthesis and hydrolysis of folate and antifolate poly-γ-glutamate conjugates.[4–8]

A consequence of the popularity of fluoroamino acids is the existence of a large body of literature describing methods for the synthesis of these compounds.[9,10] As methods for the synthesis of these fluorinated compounds continue to evolve, more advanced secondary goals such as control of product stereochemistry[11–14] have gained importance. Another such desirable goal is the development of general fluorinated building blocks. Successful building blocks must not only be useful for the synthesis of multiple fluorinated products, but they must also allow easy incorporation of these products into larger and more complex molecules. In biochemical experiments involving fluoroglutamic acids, the most informative data are obtained when using stereochemically pure synthetic substrates. In addition, while some experiments involve single free fluoroglutamates, many more studies require their incorporation into much larger γ-linked polyglutamate molecules.[4] Thus, this situation represents an ideal application for a fluorinated building block.

Incorporation of fluoroglutamates into larger compounds is now a priority given the recent development of a peptide substrate **1**, which is internally quenched by fluorescence resonance energy transfer (FRET). Enzyme-catalyzed hydrolysis of this so-called FRET peptide results in release of the quenched fluorophore, *N*-(*o*-amino)benzoyl-L-glutamic acid (Abz-Glu).[15] This allows for a convenient continuous assay of γ-glutamyl hydrolase (E.C. 3.4.19.9, GH), an important enzyme involved in the metabolism of folate and antifolate poly-γ-glutamate conjugates.[16] In order to pursue a detailed kinetics examination of the effect of fluorination on the GH-catalyzed hydrolysis reaction, synthesis of the corresponding fluoropeptide, **2**, containing (L)-4,4-difluoroglutamic acid (4,4-F_2Glu) as a part of the poly-γ-glutamate chain, was desired (Figure 1).



X=H (**1**), X=F (**2**)

Figure 1 The structure of the peptide substrate **1** and its fluorinated analogue **2**.

Positioning fluorines adjacent to the amide bond undergoing enzymatic hydrolysis in **1** is expected to alter its chemical and electronic properties relative to the non-fluorinated compound and thus have an effect on the hydrolysis reaction. In the past, synthesis of compounds such as **2** first required the isolation of the free fluoroglutamates followed by re-protection and various peptide coupling reactions.[4] This approach involves numerous synthetic steps and it is sometimes necessary to use differ-

Synthesis 2002, No. 17, Print: 02 12 2002.
Art Id.1437-210X,E;2002,0,17,2616,2626,ftx,en;C05102SS.pdf.

ISSN 0039-7881

ent protection schemes based on the position of the fluoroglutamate in the desired synthetic peptide sequence. Thus, the synthesis of a universal fluorinated building block which can be used to synthesize **2** and other compounds incorporating fluoroamino acids into peptides and isopeptides at any position is desired.

The serine-derived oxazolidine known as Garner's aldehyde **3** has become one of the most popular building blocks in asymmetric synthesis due to its ease of preparation and flexibility for elaboration of the aldehyde carbonyl.[17] It has been used in a vast number of applications, including the synthesis of difluorinated amino acid derivatives.[18,19] In the context of the current work, it was proposed that reactions of the aldehyde carbonyl could be used to create fluoroamino acid side-chain structures, and then the oxazolidine ring could be employed as a differentially protected *N*-terminal nitrogen and α-carboxyl group through strategic deprotection and oxidation (Figure 2). This process could be used to synthesize both free amino acids **5** or larger molecules such as 4,4-F_2Glu-containing peptides (e.g., **2**) via intermediates having the general structure of **6**. For these reasons, Garner's aldehyde appeared to be an attractive starting material. This report demonstrates the utility of an enantiomerically pure serine aldehyde-derived 4,4-difluoroamino acid building block for the synthesis of a variety of fluorinated compounds. Of most significance is the 4,4-F_2Glu-containing peptide substrate **2**, for use in studying the effect of fluorination on the GH-catalyzed hydrolysis reaction.

Preparation of the N-Cbz Garner Aldehyde

The original form of the Garner aldehyde contains a *tert*-butyl carbamate (Boc) as the protecting group on nitrogen, and the majority of the work reported in the literature using this type of serine aldehyde also utilizes Boc protection.[17] Due to problems with selective acid-catalyzed oxazolidine hydrolysis encountered in preliminary experiments as well as the protecting group strategy designed for the synthesis of **2**, the known but less widely utilized Garner aldehyde derivative **3** containing a Cbz group was used in this work. Preparation of **3** is shown in Scheme 1. This route to **3** takes advantage of a number of improvements, which have been reported in the literature since Garner's original synthesis as well as addressing factors which are unique to the Cbz derivative. The starting material, D-serine, was esterified and then protected using benzyl chloroformate to yield the intermediate **8**. After Lewis acid-catalyzed condensation with 2,2-dimethoxypropane (DMP) to obtain the cyclic product **9**, the methyl ester was carefully reduced using $NaBH_4$ to obtain the primary alcohol **10**.[20] Finally, this alcohol was oxidized using catalytic 2,2,6,6-tetramethylpiperidine-*N*-oxide (TEMPO) and NaOCl as the stoichiometric oxidant.[21,22] This oxidation method was found to be superior to the more commonly used Swern oxidation for obtaining **3** due to the speed of the reaction, ease of carrying it out, the fact that it does not require dry conditions, and because no unpleasant byproducts such as dimethyl sulfide are produced.

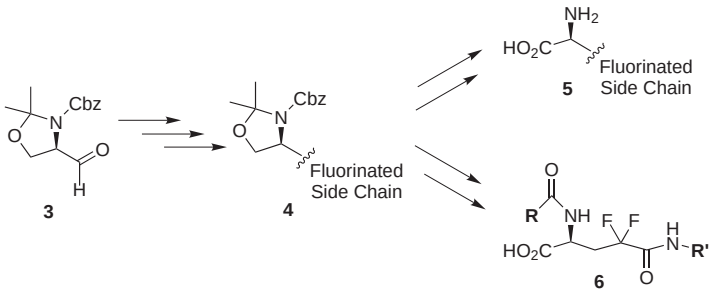
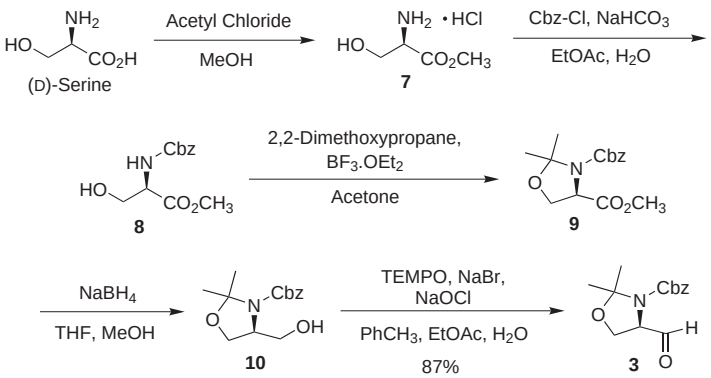


Figure 2 Proposed use of the Garner aldehyde as a fluoroamino acid building block.



Scheme 1 Synthesis of the *N*-Cbz Garner aldehyde.

Preparation of a 4,4-Difluoroamino Acid Building Block

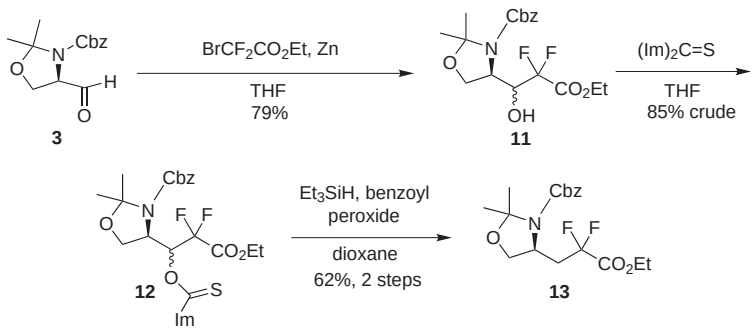
Synthesis of the key 4,4-difluoroamino acid building block **13** is shown in Scheme 2. It uses the general strategy of nucleophilic attack by a reactive difluorinated compound at the aldehyde carbonyl followed by deoxygenation of the epimeric secondary alcohol formed in the addition product. In this case, the addition of ethyl bromodifluoroacetate to the aldehyde carbonyl of **3** via a Reformatsky-type reaction[23] followed by deoxygenation leads to **13**, which contains the proper side-chain structure of 4,4-difluoroglutamic acid. Compound **13** is the desired 4,4-difluoroamino acid building block (**4**, Figure 2) because it can be used to synthesize (2*S*)-4,4-difluoroglutamate-containing products **6** or processed further to obtain other structurally related non-glutamate fluoroamino acids and derivatives **5**.

The addition of ethyl bromodifluoroacetate to aldehyde **3** in the presence of freshly activated zinc powder proceeded in good yield to give the product **11** as a mixture of diastereomeric alcohols. One of the two diastereomers was clearly formed in excess to the other, but the stereochemical identity and individual yields of the two isomers of **11** were not determined as the newly formed chiral alcohol was immediately removed in the subsequent step. The most popular method for the mild and selective deoxygenation of alcohols is the radical-based Barton–McCombie reaction.[24–26] Since its introduction, a variety of related procedures have been published describing alternatives to the sometimes-problematic tin hydrides utilized in the original Barton–McCombie method.[27–34] After evaluating these various deoxygenation reactions, it was determined that treatment of **11** with 1,1′-thiocarbonyldiimidazole in THF to obtain **12**, followed by deoxygenation with the triethylsilane/benzoyl peroxide system[29] (in dioxane for increased solubility of the starting material and product) was preferred. This provided the most reliable deoxygenation reaction and highest yields of the desired optically active 4,4-difluoroamino acid building block **13**.

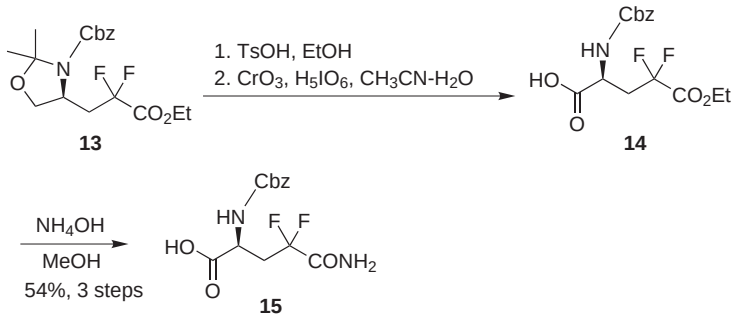
Synthesis of *N*-Cbz-(2S)-4,4-Difluoroglutamine

4,4-Difluoroglutamine is a compound of interest to biochemists who study glutamine-dependent amidotransferase enzymes.[35–37] The interest in 4,4-difluoroglutamine lies in examining the effect of fluorination on the chemistry of the glutamyl γ-carboxamido moiety in these enzymatic reactions. Prior to beginning this work, the preparation of optically active (2*S*)-4,4-difluoroglutamine had not been reported in the literature, and the preparation of the racemate had only appeared a single time in a publication from this laboratory.[38] Since this previous synthesis took advantage of a regiospecific aminolysis of a 4,4-difluoroglutamate diester, it seemed that the difluorinated building block **13** would be well suited for conversion to a single enantiomer of (2*S*)-4,4-difluoroglutamine. As this work was close to completion, a short report of the synthesis of (2*S*)-4,4-difluoroglutamine appeared in the literature.[39] The reported synthesis utilizes the *N*-Boc protected Garner aldehyde and a very similar strategy as that which is described in this work. A second report[40] also examined this type of strategy for the synthesis of (2*S*)-4,4-difluoroglutamine, but in that case the difluoroglutamine was not ultimately prepared.

The synthetic pathway developed here to obtain *N*-Cbz-(2*S*)-4,4-difluoroglutamine from **13** is shown in Scheme 3. The first step is hydrolysis of the oxazolidine ring to give the primary alcohol. As mentioned earlier, the Cbz derivative of the Garner aldehyde was used to help improve the selectivity of the hydrolysis reaction. In the case of **13**, undesired removal of the Cbz group was unlikely, but competing hydrolysis and/or transesterification at the activated α,α-difluoro ethyl ester required consideration. After examining the hydrolysis reaction using protic acids (TsOH, AcOH, HCl) in various solvents as well as Lewis acid-catalyzed hydrolysis (BF_3·2AcOH), it was determined that the best way to achieve selective hydrolysis and ring-opening of **13** was by using *p*-toluenesulfonic acid (TsOH) in refluxing 95% EtOH so any transesterification which took place still led to **14**. After hydrolysis, the alcohol product was immediately isolated and subjected to oxidation with periodic acid and catalytic



Scheme 2 Synthesis of a 4,4-difluoroamino acid building block.



Scheme 3 Synthesis of *N*-Cbz-(2*S*)-4,4-difluoroglutamine.

chromium trioxide[41] to give **14**. This minimizes any possibility of intramolecular attack of the intermediate primary alcohol on the activated α,α-difluoro ethyl ester to give an undesired lactone.

At this point, compound **14** can be converted to (2*S*)-4,4-difluoroglutamic acid by hydrolysis of the ethyl ester and removal of the Cbz group. This provides another synthetic route to this compound in addition to those already published.[13,40,42] Similarly, aminolysis of ester **14** gave the desired primary amide, *N*-Cbz-(2*S*)-4,4-difluoroglutamine **15**. Compound **15** can be used in peptide synthesis or deprotected to obtain the free amino acid, (2*S*)-4,4-difluoroglutamine, by hydrogenolysis according to the procedure developed previously in this laboratory for the deprotection of racemic **15**.[38] Unfortunately, the yield of this deprotection is significantly lower than that reported recently for the final deprotection of *N*-Boc-(2*S*)-4,4-difluoroglutamine.[39] In contrast, the oxazolidine hydrolysis, primary alcohol oxidation, and aminolysis of the more acid-stabile Cbz-protected **13** in this work appears to be more efficient and less problematic than the same sequence with the *N*-Boc derivative.[39a] These data, taken together, should ultimately lead to a more efficient synthesis of (2*S*)-4,4-difluoroglutamine.[39b]

Synthesis of a (2*S*)-4,4-Difluoroglutamate-Containing Peptide Substrate 2 for the Study of γ-Glutamyl Hydrolase

As described earlier, the (2*S*)-4,4-difluoroglutamate-containing peptide **2** is an important synthetic target for use in the study of GH. The building block **13** is equivalent to a differentially protected (2*S*)-4,4-difluoroglutamic acid molecule that can be utilized in a general method for synthesizing compounds such as **2**. Therefore, a goal of this research was to further demonstrate the general utility of **13** by using it in the synthesis of a (2*S*)-4,4-difluoroglutamate-containing Glu-γ-Glu analog and incorporating that precursor into the desired FRET peptide, **2**, a potential GH substrate.

The strategy for the synthesis of **2** is shown in Figure 3. This target can be divided into four basic segments (**A**–**D**), which were obtained independently and then assembled into the final product in a convergent manner. Segment **A**, *N*-Boc-*ortho*-aminobenzoic acid is commercially available. Segment **B** is compound **13**, already prepared from the *N*-Cbz Garner aldehyde **3** (Scheme 2). Segment **C** is L-glutamic acid-α-*tert*-butyl-ester-γ-(2-trimethylsilyl)ethyl ester which can be prepared via known glutamate protecting group chemistry[15] or a commercially available protected glutamic acid. Finally, segment **D**, L-3-nitrotyrosine-*tert*-butyl ester, has been prepared previously in this laboratory.[15]

The synthesis of the important protected (2*S*)-4,4-difluoroglutamate-containing Glu-γ-Glu analog **22** from segments **B** and **C** is shown in Scheme 4. Commercially available **16** was protected at the γ-carboxyl as a (2-trimethylsilyl)ethyl ester so that particular protecting group would have a different reactivity profile from the *tert*-butyl esters used throughout the rest of the molecule.[15] Removal of the Cbz group from **17** by hydrogenolysis gave the free amine **18** (Segment **C**, Figure 3). The preparation of **18** was carried out just prior to using this compound in order to avoid any possible cyclization and undesired pyroglutamate formation. To convert compound **13** to the corresponding carboxylate for coupling with **18**, the ethyl ester was hydrolyzed with NaOH and the sodium salt **19** was isolated.

Compounds **18** and **19** were then coupled using DCC to form the peptide bond of the (2*S*)-4,4-F_2Glu-γ-Glu precursor, **20**. The best method for the selective hydrolysis of the oxazolidine ring in **20** was determined by once again testing the methods outlined earlier for the hydrolysis of **13**. In this case, however, harsh conditions such as refluxing alcohols were avoided and a focus was placed on milder methods. Ultimately, the use of TsOH in wet acetonitrile at room temperature was successful for obtaining the desired alcohol, **21**, which was once again oxidized using periodic acid and catalytic chromium trioxide[41] to give the intermediate carboxylic acid. The acid was converted to its *tert*-butyl ester by alkylation with *N*,*N*′-dicyclohexyl-*O*-*tert*-butylisourea[43] to give the important differentially protected (2*S*)-4,4-F_2Glu-γ-Glu building block **22**.

Final assembly of the FRET peptide **2** is shown in Scheme 5. The Cbz group of **22** was removed by hydrogenolysis and the amine was immediately used for DCC-

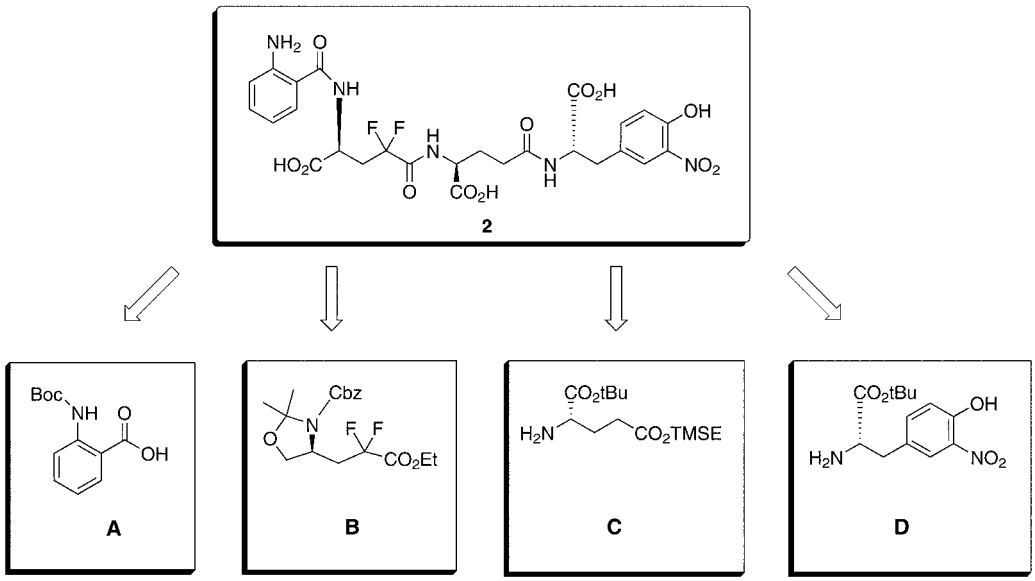
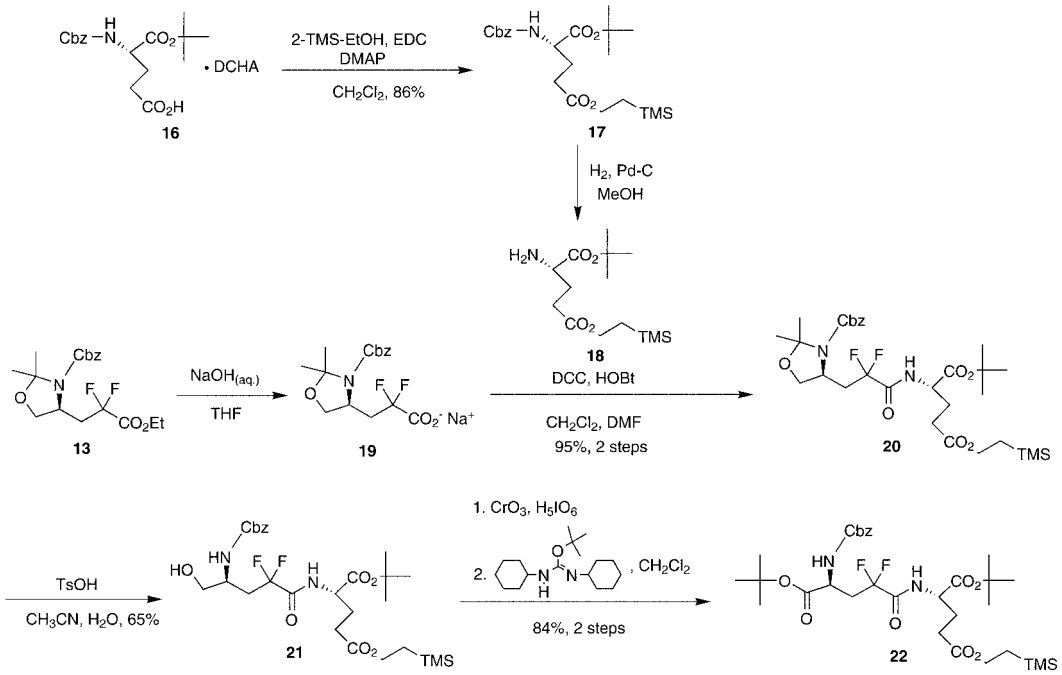


Figure 3 Strategy for the synthesis of the GH FRET substrate **2**.

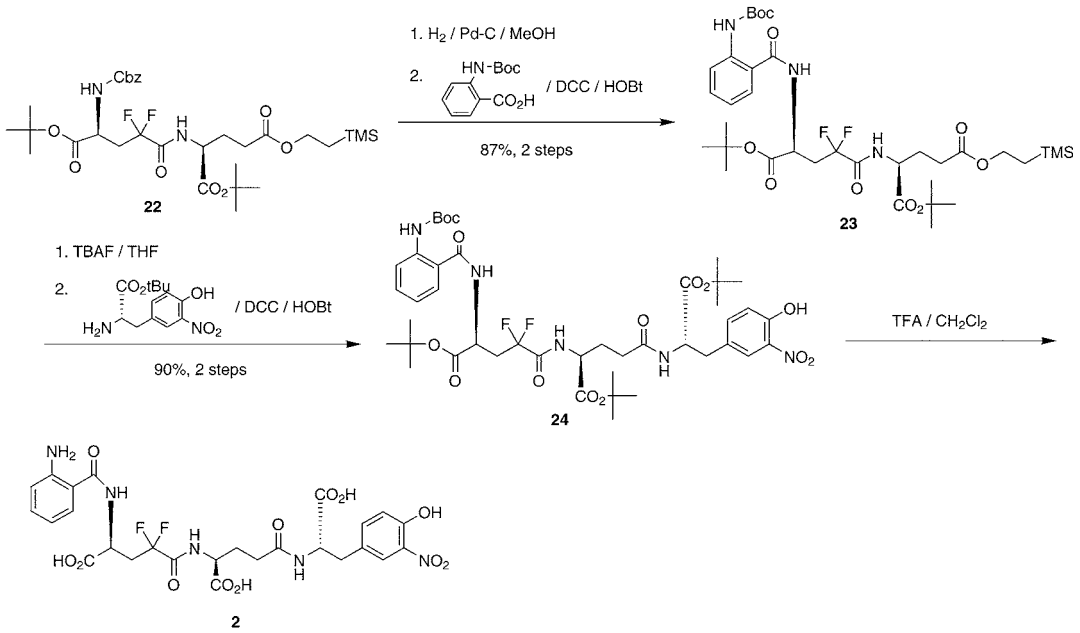
mediated coupling with *N*-Boc-*ortho*-aminobenzoic acid to give **23**. To prepare for the final DCC coupling, the TMSE ester of **23** was removed using TBAF. The intermediate carboxylic acid was purified by silica gel column chromatography and then coupled to (L)-3-nitrotyrosine-*tert*-butyl ester again using DCC to give the fully protected FRET substrate **24**. Finally, the protecting groups of **24** were removed in one step using anhydrous TFA in CH_2Cl_2 to yield the final desired product **2**. After removal of the excess TFA, the final product **2** was purified by DEAE ion exchange chromatography. Compound **2** exhibits two closely eluting peaks of almost equal area (1.5:1) by HPLC, and LC-MS analysis indicates that both peaks contain only the expected product mass. Based on the behavior of this compound as a GH substrate (See Biochemistry Section), we conclude that the two peaks may be due to the presence of conformational isomers of **2** in solution.

Biochemistry

Compound **2** was incubated extensively with GH until complete hydrolysis was demonstrated by LC-MS. The analysis of the hydrolysis mixture showed complete con-



Scheme 4 Synthesis of the protected Glu-γ-Glu analog **22**.



Scheme 5 Final assembly of the GH FRET substrate **2**.

sumption of the starting material (T_R 34 min) with the concomitant appearance of a peak (T_R 17 min) with a mass of 356.1, corresponding to $[M + H]^+$ of a Glu-γ-Tyr(NO_2) product. A mass of 303.1, corresponding to $[M + H]^+$ for the presumed second product, Abz-F_2Glu, was observed as a minor mass in a peak at $T_R = 4$ minutes. Since this compound was not available as an intermediate in the synthesis of **2** for use as an authentic HPLC standard, we were unable to confirm the formation of Abz-F_2Glu by LC-MS. No LC peaks or $[M + H]^+$ ions corresponding to the hydrolysis products were observed in control reactions without GH. These data demonstrate that **2** is a substrate for GH and appears to be hydrolyzed at the internal γ-glutamyl bond, as expected from results with similar substrates.[15,44] Having demonstrated that compound **2** is a substrate for GH, it remained to determine if the placement of fluorines adjacent to the amide bond undergoing enzymatic hydrolysis had an effect on the rate of the reaction relative to the non-fluorinated peptide substrate **1**.

The progress of the hydrolysis reaction was monitored by observing the increase in fluorescence of the sample as **2** was hydrolyzed and the intramolecular fluorescence quenching was relieved (See Experimental Section). A direct comparison of the initial rates (fluorescence counts per s) between 0.5, 2.5, and 10 μM **1** and **2** under identical conditions is shown in Figure 4. Although **2** was hydrolyzed, the rate was significantly slower than for the corresponding non-fluorinated substrate **1**. This is consistent with preliminary results obtained by an HPLC assay method demonstrating that methotrexate-based γ-glutamyl peptides containing (2*RS*,4*RS*)-4,4-F_2-Glu-γ-Glu and (2*S*,4*S*)-4-F-Glu-γ-Glu are slow substrates for GH when compared to the corresponding Glu-γ-Glu peptides.[45,46] If the rate difference observed here is due to an alteration of the rate of breakdown of a reaction intermediate rather than a difference in binding affinity, **2** will provide a particularly interesting mechanistic probe of the GH reaction. Further investigation of the basis for the slow hydrolysis of **2** is currently underway.

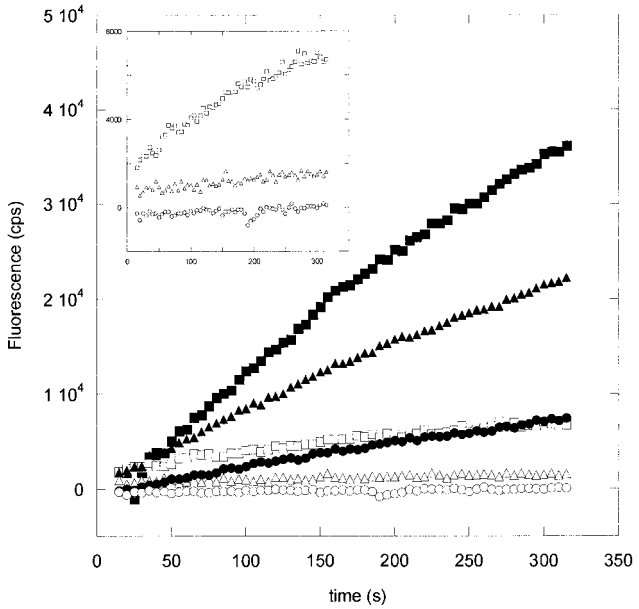


Figure 4 A comparison of initial rates between 0.5 (●), 2.5 (▲) and 10 μM (■) fluorogenic peptide **1** and 0.5 (○), 2.5 (△), and 10 μM (□) fluoropeptide **2**. Each point shown for the 6 runs is an average of duplicate exeriments. Inset: Expanded view of data for fluoropeptide **2**.

Conclusions

The development of optically active fluorinated synthetic building blocks of general utility is a current goal of organofluorine chemists. The serine-derived Garner aldehyde was successfully converted to the general 4,4-difluoroamino acid building block **13**, through a fluoro-Reformatsky reaction with ethyl bromodifluoroacetate. The utility of this building block for synthesizing difluoroamino acids was demonstrated by the synthesis of *N*-Cbz-(2*S*)-4,4-difluoroglutamine (**15**), via an intermediate **14**, which could also be converted to (2*S*)-4,4-difluoroglutamic acid. The ability of **13** to be conveniently incorporated into larger structures was demonstrated with the synthesis of a (2*S*)-4,4-difluoroglutamate-containing isopeptide **2**, designed as a potential mechanistic probe of γ-glutamyl hydrolase. The initial biochemical evaluation of **2** demonstrates that it is a substrate for γ-glutamyl hydrolase and is hydrolyzed at a rate significantly slower than the corresponding non-fluorinated FRET peptide **1**. Given the utility of the Garner aldehyde and building block **13**, this chemistry and the various intermediates involved should continue to be of interest to organofluorine chemists and biochemists.

TLC was performed with standard Sigma-Aldrich brand silica gel 60 F-254 plates. Column chromatography was performed with silica gel 60 (230–400 mesh). Melting points were obtained on a Thomas–Hoover Mel-Temp apparatus and are uncorrected. ^{1}H NMR, ^{13}C and ^{19}F NMR spectra were recorded on Bruker Avance DRX300 and DRX500 spectrometers using the X-WinNMR software. Chemical shifts are reported in parts per million (ppm) upfield or downfield from tetramethylsilane (internal standard for ^{1}H and ^{13}C) or trifluoroacetic acid (external standard for ^{19}F). The NMR spectra of some intermediates containing the oxazolidine structure derived from **3** exhibited line broadening and doubling of some signals due to slow or restricted rotation within the molecule. NMR spectra of these compounds were obtained at elevated temperatures when it aided in simplifying their appearance. IR spectra were recorded on a Nicolet 5-DX spectrometer. Mass spectra were obtained with a VG 70-250-S mass spectrometer made by Micromass (UK) and an Opus data system. HPLC chromatograms were obtained with Waters 510 and 6000A model pumps with a 996 diode array detector using Millenium version 3.20 software and a Vydac C_{18} column. A gradient elution of 15% to 20% solvent A (0.1% AcOH, 0.02% TFA in MeCN) in solvent B (0.1% AcOH, 0.02% TFA in H_2O) over 25 min followed by an increase to 95% solvent A in 15 min was used unless otherwise specified. LC-MS data were collected from ThermoFinnigan Surveyor HPLC components with a Vydac C_{18} column and a Finnigan LCQ.

Compound **1** was prepared as previously described.[15] (*S*)-4-Hydroxymethyl-2,2-dimethyloxazolidine-3-carboxylic acid benzyl ester **10** was synthesized from D-serine according to the procedure described by Delle Monache et al.[20] for the preparation of its enantiomer. *N*-Cbz-(L)-glutamic acid-α-*tert*-butyl ester dicyclohexylammonium salt **16** was purchased from Chem Impex International, Inc., Wood Dale, IL, USA. *N*,*N*′-Dicyclohexyl-*O*-*tert*-butylisourea[43] was prepared by stirring DCC in *tert*-butyl alcohol (1.5 equiv) at 35 °C with CuCl (catalytic). The reaction was complete (approximately 24 h) when the IR spectrum indicated the loss of the carbodiimide (2100 cm^{-1}) and the appearance of the isourea (1660 cm^{-1}). The mixture was diluted with $CHCl_3$ and all volatile components were evaporated in vacuo to give the desired isourea product as a yellow oil which was used without further purification. THF was freshly distilled from sodium/benzophenone. CH_2Cl_2 was freshly distilled from CaH_2. 1,4-Dioxane was distilled from $LiAlH_4$. Zinc powder was washed with 1 N HCl for 1 min followed by washings with distilled water, EtOH, Et_2O, and drying completely under high vacuum. *N*-Boc-*o*-Aminobenzoic acid (Boc-Abz-OH) was obtained from Advanced ChemTech, Louisville, KY, USA. All other reagents and starting materials were obtained from Sigma–Aldrich or Fisher–Acros and used without further purification. Air- and moisture-sensitive reactions were run in flame- or oven-dried (T >100 °C overnight) glassware under dry nitrogen or argon.

(4*R*)-4-Formyl-2,2-dimethyloxazolidine-3-carboxylic Acid Benzyl Ester (3)

(*S*)-4-Hydroxymethyl-2,2-dimethyloxazolidine-3-carboxylic acid benzyl ester **10**[20] (1.85 g, 7.0 mmol) and NaBr (720 mg, 7.0 mmol, 1 equiv) were dissolved in a vigorously stirred solvent mixture of toluene–EtOAc–H_2O (6:6:1) and then cooled to 0 °C in an ice-water bath before the addition of solid TEMPO (22 mg, 0.14 mmol, 0.02 equiv). An aqueous solution of NaOCl (573 mg, 7.7 mmol, 1.1 equiv, 0.35 M) and $NaHCO_3$ (1.7 g, 20.3 mmol, 2.9 equiv) was continuously added dropwise over 1 h. The reaction mixture was stirred an additional 15 min after complete addition of the oxidant solution and then H_2O was added to the flask. The layers were separated and the aqueous layer was extracted with Et_2O. The combined organic layers were washed with solutions of KI (4 mg/mL) in 10% aq $KHSO_4$, 10% aq $Na_2S_2O_3$, and then brine, and dried (Na_2SO_4). The solvents were removed in vacuo and the resulting crude product was purified by silica gel column chromatography (hexanes–EtOAc, 1:1) to give the desired aldehyde **3**, as a colorless oil (1.60 g, 87%); R_f 0.56 (hexanes–EtOAc, 2:1).

^{1}H NMR (DMSO-d_6, 300 MHz, 80 °C): δ = 1.50 (s, 3 H), 1.56 (s, 3 H), 4.13 (d, 2 H, *J* = 5.1 Hz), 4.52 (dt, 1 H, *J* = 5.1, 1.4 Hz), 5.11 (s, 2 H), 7.35 (m, 5 H), 9.57 (s, 1 H).

^{13}C NMR (DMSO-d_6, 75.5 MHz, 80 °C): δ = 23.46, 25.24, 62.80, 64.08, 66.00, 94.00, 127.06, 127.42, 127.92, 136.03, 151.50, 198.60.

(4*R*)-4-[(1*RS*)-2-Ethoxycarbonyl-2,2-difluoro-1-hydroxyethyl]-2,2-dimethyloxazolidine-3-carboxylic Acid Benzyl Ester (11)

A stirred suspension of zinc powder (2.8 g, 43.2 mmol, 3.0 equiv) in THF was heated to reflux temperature in a flask equipped with a condenser and drying tube and a solution of the aldehyde **3** (3.8 g, 14.4 mmol) and ethyl bromodifluoroacetate (3.79 g, 18.7 mmol, 1.3 equiv) in THF was added dropwise over 15 min. After complete addition, the reaction mixture was stirred an additional 15 min before being cooled to r.t. and quenched with 1 N aq $KHSO_4$. The mixture was diluted with CH_2Cl_2 and then filtered through Celite. The filtrate was condensed in vacuo and the resulting residue was partitioned between EtOAc and H_2O. The EtOAc layer was washed with brine, dried (Na_2SO_4), and condensed in vacuo. The resulting crude product was purified by silica gel column chromatography (CH_2Cl_2–EtOAc, 16:1) and the desired product **11**, was obtained as a colorless oil consisting of a 3:1 mixture of diastereomers (4.4 g, 79%); R_f 0.58, 0.50 (CH_2Cl_2– EtOAc, 16:1). A sample of only the major (lower R_f) diastereomer was isolated and utilized for spectral characterization.

^{1}H NMR (DMSO-d_6, 500 MHz, 80 °C): δ = 1.28 (t, 3 H, *J* = 7.1 Hz), 1.47 (s, 3 H), 1.54 (s, 3 H), 3.97 (dd, 1 H, *J* = 8.7, 7.1 Hz), 4.15 (dd, 1 H, *J* = 8.8, 3.5 Hz), 4.20 (m, 1 H), 4.29 (q, 2 H, *J* = 7.1 Hz), 4.53 (m, 1 H), 5.14 (s, 2 H), 6.0 (br, 1 H), 7.33–7.89 (m, 5 H).

^{13}C NMR (DMSO-d_6, 125.75 MHz, 80 °C): δ = 13.64, 23.76 (br), 25.49, 55.99, 62.19, 62.34, 65.91, 68.30 (m), 93.08, 114.90 (t, $^1J_{C-F}$ = 256 Hz), 127.27, 127.43, 127.91, 136.08, 151.63, 162.40 (t, $^2J_{C-F}$ = 31.3 Hz).

^{19}F NMR (DMSO-d_6, 282.38 MHz, 60 °C): δ = –44.5 (dm, $^2J_{F\text{-}F}$ = 258 Hz), –35.8 (d, $^2J_{F\text{-}F}$ = 258 Hz).

CI-MS (NH_3): *m*/*z* (%) = 388.1 (MH^+, 90.8), 330.1 (100.0), 108.1 (23.7).

CI-HRMS (NH_3): *m*/*z* calcd for $C_{18}H_{24}F_2NO_6$ (MH^+) 388.1572, found 388.1556.

Anal. Calcd for $C_{18}H_{23}F_2NO_6$: C, 55.81; H, 5.98; N, 3.62. Found: C, 55.38; H, 5.99; N, 3.67.

(4*S*)-4-(2-Ethoxycarbonyl-2,2-difluoroethyl)-2,2,-dimethyloxazolidine-3-carboxylic Acid Benzyl Ester (13)

The diastereomeric alcohols **11** (500 mg, 1.29 mmol) and 1,1′-thiocarbonyldiimidazole (346 mg, 1.94 mmol, 1.5 equiv) were dissolved in THF and stirred at 30–40 °C for 48 h. The THF was removed in vacuo and the resulting residue was dissolved in a minimum of EtOAc and purified by silica gel column chromatography (hexanes–EtOAc, 1:1) to give the desired intermediate **12** as a colorless oil containing an inseparable mixture of diastereomers (548 mg, 85%). The intermediate **12** derived from only the lower R_f diastereomer of **11** was used for characterization; R_f 0.52 (hexanes–EtOAc, 1:1).

^{1}H NMR (DMSO-d_6, 300 MHz, 80 °C): δ = 1.18 (t, 3 H, *J* = 7.0 Hz), 1.23 (s, 3 H), 1.41 (s, 3 H), 4.18 (dd, 1 H, *J* = 9.9, 7.0 Hz), 4.27 (m, 2 H), 4.38 (d, 1 H, *J* = 9.9 Hz), 4.61 (d, 1 H, *J* = 6.5 Hz), 5.18 (s, 2 H), 6.69 (dd, 1 H, *J* = 15.2, 10.7 Hz), 7.12 (m, 1 H), 7.33–7.41 (m, 5 H), 7.77 (m, 1 H), 8.44 (m, 1 H).

^{19}F NMR (DMSO-d_6, 282.38 MHz, 80 °C): δ = –38.7 (d, $^2J_{F\text{-}F}$ = 266 Hz), –35.6 (dd, $^2J_{F\text{-}F}$ = 266 Hz, $^3J_{H\text{-}F}$ = 85 Hz).

FAB-MS (NBA with Na^+): *m*/*z* (%) = 498.1 (MH^+, 21.6), 91.0 (100.0).

FAB-HRMS (with Na^+): *m*/*z* calcd for $C_{22}H_{26}F_2N_3O_6S$ (MH^+) 498.1510, found 498.1521.

Compound **12** (431 mg, 0.87 mmol) and triethylsilane (7 mL) were added to a flask equipped with a condenser and drying tube and dioxane was added slowly until the solution was clear and homogeneous. The reaction mixture was stirred and heated at reflux temperature while portions of solid benzoyl peroxide (10 mg) were added at 15 min intervals until complete consumption of the starting material was observed by TLC. The reaction was cooled to r.t. and repeatedly diluted with EtOAc and concentrated in vacuo to remove the triethylsilane and obtain the crude product. This crude residue was purified by silica gel column chromatography (hexanes–EtOAc, 4:1) and the desired product **13**, was obtained as a colorless oil (200 mg, 62% yield from **11**); R_f 0.75 (hexanes–EtOAc, 1:1); $[\alpha]_D^{23}$ +19.0 (*c* = 0.95, MeOH).

^{1}H NMR (DMSO-d_6, 300 MHz, 75 °C): δ = 1.26 (t, 3 H, *J* = 7.1 Hz), 1.45 (s, 3 H), 1.52 (s, 3 H), 2.30–2.51 (m, 2 H), 3.82 (dd, 1 H, *J* = 9.2, 1.5 Hz), 4.04 (dd, 1 H, *J* = 9.2, 5.5 Hz), 4.20 (m, 1 H), 4.27 (q, 2 H, *J* = 7.1 Hz), 5.12 (s, 2 H), 7.37 (m, 5 H).

^{13}C NMR (DMSO-d_6, 75.47 MHz, 75 °C): δ = 14.11, 24.11, 27.24, 37.74 (m), 52.21, 63.58, 66.81, 67.67, 93.79, 115.74 (t, $^1J_{C\text{-}F}$ = 250 Hz), 128.16, 128.41, 128.90, 137.07, 152.08, 163.47 (t, $^2J_{C\text{-}F}$ = 32 Hz).

^{19}F NMR (DMSO-d_6, 282.38 MHz, 75 °C): δ = –26.4 (d, $^2J_{F\text{-}F}$ = 263 Hz), –28.6 (dt, $^2J_{F\text{-}F}$ = 262 Hz, $^3J_{H\text{-}F}$ = 19 Hz).

FAB-MS (NBA): *m*/*z* (%) = 372.2 (MH^+, 33.6), 328.2 (23.4), 91.0 (100.0).

FAB-HRMS (NBA): *m*/*z* calcd for $C_{18}H_{24}F_2NO_5$ (MH^+) 372.1622, found 372.1633.

Anal. Calcd for $C_{18}H_{23}F_2NO_5 \cdot 0.7H_2O$: C, 56.33; H, 6.40; N, 3.65. Found: C, 56.33; H, 6.00; N, 3.52.

***N*-Cbz-(L)-4,4-Difluoroglutamine (15)**

Compound **13** (86 mg, 0.23 mmol) was dissolved in 95% EtOH and *p*-toluenesulfonic acid monohydrate (11 mg, 0.05 mmol, 0.25 equiv) was added. The solution was stirred and heated at reflux until TLC (hexanes–EtOAc, 1:1) indicated the complete loss of the starting material (R_f 0.75) and formation of the desired product alcohol (R_f 0.44) (5 h). The reaction was cooled to r.t. and the solvent was removed in vacuo. The resulting colorless oil was immediately dissolved in wet MeCN (1.4 mL, 0.75 vol% H_2O) and cooled to 0 °C in an ice-water bath. This solution was treated with a stock solution containing H_5IO_6 (132 mg, 0.58 mmol, 2.5 equiv) and CrO_3 (0.25 mg, 2.50 μmol, 1.1 mol%) in wet MeCN (1.4 mL, 0.75 vol% H_2O) added dropwise over 40 min. After complete addition, the reaction mixture was stirred for an additional 20 min. Any active oxidant remaining in the heterogeneous reaction mixture was quenched with the addition of *i*-PrOH (1 mL) and stirring for 10 min until a light green color was observed. All solvents were removed in vacuo to leave a crude solid residue. This residue was dissolved in half-sat. aq NaCl and this solution was extracted with CH_2Cl_2. The combined extracts were dried (Na_2SO_4) and condensed in vacuo to leave the crude carboxylic acid product **14** as a colorless oil. This oil was dissolved in MeOH (5 mL) and the solution was cooled to 0 °C in an ice-water bath before 30% aq NH_4OH (1.5 mL) was added. The reaction mixture was stirred for 1 h and then the flask was warmed to r.t. The solvents were removed in vacuo and the remaining residue was redissolved in MeOH and the solution was acidified (pH 2.0) with 1 N $KHSO_4$. Removal of the solvents again in vacuo left the crude product as a white solid residue which was purified by silica gel column chromatography eluting with a solution consisting of 62.5% EtOAc, 30% hexanes, and 7.5% AcOH. The product-containing fractions were combined and the solvent was removed in vacuo. Removal of the final traces of AcOH was facilitated by repeatedly dissolving the product in 1:5 EtOAc–cyclohexane and evaporating in vacuo. The desired product **15** was obtained as a white solid (40 mg, 54% yield in 3 steps from **13**); R_f 0.38 (EtOAc–hexanes–AcOH, 62.5:30.0:7.5); mp 146–149 °C; $[\alpha]_D^{23}$ –14.8 (*c* = 0.75, MeOH).

^{1}H NMR (CD_3OD, 300 MHz): δ = 2.50–2.76 (m, 2 H), 4.45 (dd, 1 H, *J* = 9.7, 3.3 Hz), 5.09 (s, 2 H), 7.33 (m, 1 H).

^{13}C NMR (CD_3OD, 75.5 MHz): δ = 36.50 (t, $^2J_{C\text{-}F}$ = 23.7 Hz), 50.22, 67.68, 119.74 (d, $^1J_{C\text{-}F}$ = 252 Hz), 128.72, 128.95, 129.44, 138.13, 158.32, 168.28 (t, $^2J_{C\text{-}F}$ = 29 Hz), 174.25.

^{19}F NMR (CD_3OD, 282.38MHz, 80 °C): δ = –31.31 (dt, $^2J_{F\text{-}F}$ = 257, $^3J_{H\text{-}F}$ = 16 Hz), –30.21 (dt, $^2J_{F\text{-}F}$ = 257, $^3J_{H\text{-}F}$ = 16 Hz).

DCI-MS (NH_3): *m*/*z* (%) = 334.0 ($[M + NH]^+$, 6.6), 169.1 (65.4), 139.0 (139.0), 106.0 (63.8), 88.0 (74.3).

DCI-HRMS (NH_3): *m*/*z* calcd for $C_{13}H_{18}F_2N_3O_5$ ($[M + NH_4]^+$) 334.1214, found 334.1218.

***N*-Cbz-(L)-Glutamic Acid-α-*tert*-butyl Ester γ-(2-Trimethylsilyl)ethyl Ester (17)**

N-Cbz-(L)-glutamic acid-α-*tert*-butyl ester dicyclohexylammonium salt (**16**; 800 mg, 1.54 mmol) and 2-(trimethylsilyl)ethanol (260 mg, 2.2 mmol, 1.4 equiv) were dissolved in CH_2Cl_2. DMAP (188 mg, 1.54 mmol, 1.0 equiv) and then 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 383 mg, 2.0 mmol, 1.3 equiv) were added to the solution and the mixture was stirred at r.t. for 36 h. The mixture was diluted with CH_2Cl_2 and the CH_2Cl_2 layer was washed with 0.5 N aq HCl, H_2O, and brine. The organic layer was dried (Na_2SO_4) and condensed in vacuo to give the crude product, which was purified by silica gel column chromatography (hexanes–EtOAc, 2:1). The desired product **17**, was obtained as a colorless oil (580 mg, 86%); R_f 0.45 (hexanes– EtOAc, 2:1).

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.03 (s, 9 H), 0.97 (m, 2 H), 1.46 (s, 9 H), 1.81–2.41 (m, 4 H), 4.16 (m, 2 H), 4.29 (m, 1 H), 5.10 (s, 2 H), 5.43 (d, 1 H), 7.34 (m, 5 H).

^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = –1.34, 17.42, 28.09, 28.11, 30.53, 54.00, 63.04, 67.07, 82.54, 128.25, 128.29, 128.67, 136.43, 156.07, 171.14, 173.06.

FAB-MS (NBA with Na^+) *m*/*z* (%) = 460.1 (MNa^+, 13.8), 354.0 (10.3), 310.1 (18.82), 91.0 (100.0).

FAB-HRMS (NBA with Na^+): *m*/*z* calcd for $C_{22}H_{35}NNaO_6Si$ (MNa^+) 460.2131, found 460.2120.

Anal. Calcd for $C_{22}H_{35}NO_6Si$: C, 60.38; H, 8.06; N, 3.20. Found: C, 60.17; H, 7.96; N, 3.02.

N-Cbz-Difluorooxazolidine-γ-(L)-glutamic Acid-α-*tert*-butyl Ester γ-(2-Trimethylsilyl)ethyl Ester (20)

Compound **13** (250 mg, 0.67 mmol) was dissolved in THF and cooled to 0 °C in an ice-water bath before 1 N NaOH (870 μL, 0.87 mmol, 1.3 equiv) was added. The solution was stirred at 0 °C for 1 h. The solvents were removed in vacuo and the solid residue was re-dissolved in H_2O, frozen in a –78 °C bath, and lyophilized to give the desired sodium salt **19** as a white hygroscopic solid which was used without further purification. The differentially protected glutamate **17** (550 mg, 1.26 mmol) was dissolved in MeOH–*i*-PrOH (4:1) and 10% Pd/C (100 mg) was added. The mixture was shaken in a Parr apparatus with H_2 (40 psi) for 90 min. The reaction was diluted with an equal volume of EtOAc and filtered through Celite to remove the catalyst. The solvents were removed in vacuo to give the desired amine **18** as a colorless oil, which was used without further purification. CH_2Cl_2 (5 mL) was added to the sodium salt **19** (0.67 mmol) and DMF was slowly added until the mixture was completely homogeneous. The amine **18** (303 mg, 1.0 mmol, 1.5 equiv), HOBt (135 mg, 1.0 mmol, 1.5 equiv), and finally DCC (206 mg, 1.0 mmol, 1.5 equiv) were added to the solution and it was allowed to stir at r.t. for 48 h. The reaction mixture was diluted with CH_2Cl_2 and filtered through Celite. The filtrate was concentrated in vacuo to give the crude product which was purified by two sequential silica gel columns: 1st (hexanes–EtOAc, 2:1), 2nd ($CHCl_3$–EtOAc, 95:5). The desired amide product **20** was obtained as a colorless oil (402 mg, 95% yield in two steps from **13**); R_f 0.32 ($CHCl_3$–EtOAc, 95:5); $[\alpha]_D^{23}$ +17.1 (*c* = 0.70, $CHCl_3$).

^{1}H NMR (DMSO-d_6, 500 MHz, 55 °C): δ = 0.02 (s, 9 H), 0.94 (m, 2 H), 1.40 (s, 9 H), 1.43 (s, 3 H), 1.50 (s, 3 H), 1.96 (m, 1 H), 2.07 (m, 1 H), 2.33 (m, 2 H), 2.36 (t, *J* = 7.8 Hz), 3.86 (dd, *J* = 9.2, 1.4 Hz), 4.00 (dd, *J* = 9.1, 5.5 Hz), 4.13 (m, 2 H), 4.21 (m, 2 H), 5.11 (s, 2 H), 7.30–7.37 (m, 5 H), 8.81 (d, 1 H, *J* = 7.3 Hz).

^{13}C NMR (DMSO-d_6, 125.75 MHz, 60 °C): δ = –1.89, 16.53, 25.13, 26.42, 27.24, 29.90, 39.62, 51.99, 61.50, 65.81, 66.57, 80.85, 92.69, 116.18 (t, $^1J_{C\text{-}F}$ = 254 Hz), 127.16, 127.44, 127.97, 136.20, 151.08, 163.08 (t, $^2J_{C\text{-}F}$ = 29 Hz), 169.20, 171.63;

^{19}F NMR (DMSO-d_6, 470.56 MHz, 55 °C): δ = –28.04 (m).

FAB-MS (NBA with Na^+) *m*/*z* (%) = 651.2 (MNa^+, 39.8), 487.1 (49.5), 91.0 (100.);

FAB-HRMS (NBA with Na^+): *m*/*z* calcd for $C_{30}H_{46}F_2N_2NaO_8Si$ (MNa^+) 651.2889, found 651.3008.

N-Cbz-(l)-4-Aminobutyryl-3,3-difluoro-(2-hydroxymethyl)-(L)-glutamic Acid-α-*tert*-butyl Ester γ-(2-Trimethylsilyl)ethyl Ester (21)

Compound **20** (31 mg, 0.05 mmol) was dissolved in MeCN and then H_2O (40 μL) and $TsOH{\cdot}H_2O$ (3 mg, 0.015 mmol, 0.3 equiv) were added. The solution was stirred at r.t. for 19 h. The solvents were removed in vacuo. The crude product was purified by silica gel column chromatography (hexanes–EtOAc, 1:1) and the desired alcohol product **21**, was obtained as a colorless oil (19 mg, 65%); R_f 0.41 (hexanes–EtOAc, 1:1).

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.02 (s, 9 H), 0.97 (m, 2 H), 1.46 (s, 9 H), 1.94–2.52 (m, 6 H), 2.95 (br, 1 H), 3.68 (m, 2 H), 3.96 (m, 1 H), 4.17 (m, 2 H), 4.45 (m, 1 H), 5.07 (dd, 2 H, *J* = 18, 12 Hz), 5.49 (d, 1 H, *J* = 8.3 Hz), 7.27–7.35 (m, 5 H).

^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = –1.30, 17.47, 27.04, 28.14, 30.47, 35.26 (t, $^2J_{C\text{-}F}$ = 23 Hz), 48.28, 52.74, 63.45, 64.89, 67.09, 83.43, 117.33 (t, $^1J_{C\text{-}F}$ = 253 Hz), 128.26, 128.34, 128.74, 136.52, 156.42, 164.60 (t, $^2J_{C\text{-}F}$ = 29 Hz), 170.11, 173.26.

^{19}F NMR ($CDCl_3$, 282.38 MHz): δ = –27.1 (dt, $^2J_{F\text{-}F}$ = 261 Hz, $^3J_{H\text{-}F}$ = 16 Hz), –28.6 (dt, $^2J_{F\text{-}F}$ = 261 Hz, $^3J_{H\text{-}F}$ = 17 Hz).

N-Cbz-(2*S*)-4,4-Difluoroglutamic Acid-α-*tert*-butyl Ester-γ-glutamic Acid-α-*tert*-butyl-γ-(2-trimethylsilyl)ethyl Ester (22)

Compound **21** (48 mg, 0.08 mmol) was dissolved in wet MeCN (1.0 mL, 0.75 vol% H_2O) and cooled to 0 °C in an ice-water bath. A stock solution containing H_5IO_6 (46 mg, 0.20 mmol, 2.5 equiv) and CrO_3 (0.09 mg, 1.1 mol%) in wet MeCN (1.0 mL, 0.75 vol% H_2O) was slowly added dropwise over 40 min. After complete addition the mixture was stirred for an additional 1.5 h before being quenched with aq phosphate buffer [prepared by dissolving Na_2HPO_4 (600 mg) in H_2O (10 mL)]. The solution was extracted with EtOAc. The combined extracts were dried (Na_2SO_2), filtered, and evaporated in vacuo to leave the crude carboxylic acid intermediate. The carboxylic acid (48 mg, 0.08 mmol) was dissolved in CH_2Cl_2 and cooled to 0 °C in an ice-water bath. A solution of *N*,*N*′-dicyclohexyl-*O*-*tert*-butylisourea (56 mg, 0.2 mmol, 2.5 equiv) in CH_2Cl_2 was added and after 15 min the ice-water bath was removed and the mixture stirred for 24 h. An additional portion of *N*,*N*′-dicyclohexyl-*O*-*tert*-butylisourea[43] (56 mg, 0.2 mmol, 2.5 equiv) in CH_2Cl_2 was added and the mixture stirred for an additional 12 h. The reaction was diluted with CH_2Cl_2, filtered, and the filtrate was condensed to give the crude product as a light yellow oil. The crude product was purified by two sequential silica gel columns: 1st (hexanes–EtOAc, 2:1), 2nd ($CHCl_3$–EtOAc, 95:5) to give the desired product, **22**, as a colorless oil (47 mg, 89% yield in two steps from **21**); R_f 0.49 ($CHCl_3$–EtOAc, 95:5).

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.03 (s, 9 H), 0.97 (m, 2 H), 1.47 (s, 18 H), 2.02–2.74 (m, 6 H), 4.17 (m, 2 H), 4.46 (m, 2 H), 5.10 (dd, 2 H, *J* = 16, 12 Hz), 5.57 (d, 1 H, *J* = 8 Hz), 7.21 (d, 1 H, *J* = 8 Hz), 7.35 (m, 5 H).

^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = –1.28, 17.47, 27.16, 28.05, 28.16, 29.94, 35.76 (t, $^2J_{C\text{-}F}$ = 23 Hz), 49.94, 52.66, 63.36, 67.20, 83.14, 83.34, 116.95 (t, $^1J_{C\text{-}F}$ = 254 Hz), 128.28, 128.34, 128.71, 136.48, 155.86, 163.62 (t, $^2J_{C\text{-}F}$ = 29 Hz), 169.64, 169.98, 173.13;

^{19}F NMR ($CDCl_3$, 282.38 MHz): δ = –28.17 (t, *J* = 16 Hz).

N-Boc-Abz-4,4-Difluoroglutamic Acid-α-*tert*-butyl-γ-glutamic Acid-α-*tert*-butyl-γ-(2-trimethylsilyl)ethyl Ester (23)

Compound **22** (45 mg, 0.068 mmol) was dissolved in THF and a suspension of 10% Pd/C (12 mg, catalytic) in THF was added. The mixture was cooled to 0 °C in an ice-water bath and then vigorously stirred under an atmosphere of H_2 (1 atm) for 2 h. The reaction mixture was diluted with EtOAc, the catalyst was removed by filtration, and the filtrate was concentrated in vacuo to give the desired intermediate deprotected amine, which was immediately used without further purification. This amine was dissolved in anhyd DMF and then *N*-Boc-*ortho*-aminobenzoic acid (25 mg, 0.102 mmol, 1.5 equiv), HOBt (14 mg, 0.102 mmol, 1.5 equiv), and DCC (21 mg, 0.102 mmol, 1.5 equiv) were added in that order. The mixture stirred at r.t. for 4 d. The reaction mixture was diluted with EtOAc, filtered, and then the solvents were removed in vacuo to give the crude product. This crude residue was dissolved in a minimum of EtOAc and purified by silica gel column chromatography (hex-

anes–EtOAc, 2:1) to give the desired product **23**, as a colorless oil (44 mg, 87% yield in two steps from **22**); R_f 0.57 (hexanes–EtOAc, 2:1).

^{1}H NMR ($CDCl_3$, 500 MHz): δ = 0.02 (s, 9 H), 0.97 (m, 2 H), 1.41 (s, 9 H), 1.49 (s, 9 H), 1.50 (s, 9 H), 2.03 (m, 1 H), 2.19 (m, 1 H), 2.34 (m, 2 H), 2.79 (m, 2 H), 4.15 (m, 2 H), 4.40 (m, 1 H), 4.79 (m, 1 H), 7.00 (t, 1 H, *J* = 7.5 Hz), 7.14 (d, 1 H, *J* = 7.6 Hz), 7.29 (d, 1 H, *J* = 7.5 Hz), 7.43 (t, 1 H, *J* = 7.3 Hz), 7.51 (d, 1 H, *J* = 6.9 Hz), 8.36 (d, 1 H, *J* = 8.4 Hz), 10.07 (s, 1 H).

^{13}C NMR ($CDCl_3$, 125.75 MHz): δ = –1.34, 17.44, 27.11, 28.04, 28.52, 30.36, 35.37 (t, $^2J_{C\text{-}F}$ = 23.7 Hz), 48.66, 52.68, 63.35, 80.37, 83.31, 83.38, 116.87 (t, $^1J_{C\text{-}F}$ = 254 Hz), 119.10, 119.87, 121.65, 127.15, 132.96, 140.67, 153.18, 163.78 (t, $^2J_{C\text{-}F}$ = 28.6 Hz), 168.74, 169.41, 169.73, 173.06.

^{19}F NMR ($CDCl_3$, 282.38 MHz): δ = –25.8 (dt, $^2J_{F\text{-}F}$ = 264 Hz, $^2J_{H\text{-}F}$ = 15.2 Hz), –28.4 (dt, $^2J_{F\text{-}F}$ = 264 Hz, $^2J_{H\text{-}F}$ = 16.9 Hz).

FAB-MS (3-NBA with Na^+): *m*/*z* (%) = 766.5 (MNa^+, 7.6), 329.1 (21.5), 307.1 (21.4), 176.1 (54.3), 154.1 (100.0), 136.1 (70.8).

FAB-HRMS (3-NBA with Na^+): *m*/*z* calcd for $C_{35}H_{55}F_2N_3NaO_{10}Si$ (MNa^+) 766.3522, found 766.3526.

***N*-Boc-Abz-4,4-Difluoroglutamic Acid-α-*tert*-butyl-γ-glutamic Acid-α-*tert*-butyl-γ-3-nitrotyrosine-*tert*-butyl Ester (24)**

Compound **23** (40 mg, 0.054 mmol) was dissolved in THF and cooled to 0 °C in an ice-water bath. A 1 M aq solution of TBAF (81 μL, 1.5 equiv) was added and the reaction mixture was stirred for 2 h. The reaction was quenched with the addition of sat. aq NH_4Cl and then all of the solvents were removed in vacuo. The resulting crude acid product was partially purified by silica gel column chromatography (2% AcOH in $CHCl_3$–EtOAc, 3:1) (R_f 0.38). All product-containing fractions were combined and concentrated in vacuo to give a residue corresponding to slightly greater than 100% yield. This carboxylic acid and L-3-NO_2-tyrosine-*tert*-butyl ester (28 mg, 0.10 mmol, 1.8 equiv) were dissolved in anhyd DMF and HOBt (11 mg, 0.081 mmol, 1.5 equiv) and then DCC (17 mg, 0.081 mmol, 1.5 equiv) were added. The yellow reaction mixture stirred at r.t. for 48 h. The DMF was removed in vacuo and the resulting yellow residue was dissolved in EtOAc, filtered, and then the filtrate was concentrated in vacuo to give the crude product. This crude material was purified by silica gel column chromatography (hexanes–EtOAc, 1:5) to give the desired product **24**, as a light yellow oil (44 mg, 90% yield in 2 steps from **23**); R_f 0.42 (hexanes–EtOAc, 1:5).

^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.42–1.50 (36 H), 2.20–3.10 (8 H), 4.2–4.5 (3 H), 6.50 (1 H), 6.99–7.86 (7 H), 8.33 (1 H), 10.00 (1 H), 10.48 (1 H).

FAB-MS (3-NBA with Na^+): *m*/*z* (%) = 930.3 (MNa^+, 31.4), 640.2 (23.6), 176.0 (22.6), 154.0 (36.7), 120.0 (100.0).

FAB-HRMS (3-NBA with Na^+): *m*/*z* calcd for $C_{43}H_{59}F_2N_5NaO_{14}$ (MNa^+) 930.3924, found 930.3942.

***N*-Abz-4,4-Difluoroglutamyl-γ-glutamyl-γ-3-nitrotyrosine (2)**

Compound **24** was dissolved in CH_2Cl_2 (1.5 mL) and cooled to 0 °C in an ice-water bath. An equal volume of anhyd TFA was added dropwise. The mixture was allowed to warm to r.t. and stirred for a total of 24 h. The volatile components of the mixture were removed in vacuo to leave a yellow-brown residue which was repeatedly triturated with CH_2Cl_2 and concentrated. The resulting yellow solid was kept under high vacuum for 24 h to give the crude solid product **2**.

^{1}H NMR (D_2O, 300 MHz): δ = 1.66 (m, 1 H), 1.86 (m, 1 H), 2.10 (m, 2 H), 2.69–3.15 (m, 4 H), 4.00 (m, 1 H), 4.70 (m, 1 H), 6.95–7.82 (m, 7 H).

FAB-MS (3-NBA w/Na^+): *m*/*z* (%) = 662 ($M + Na^+$, 1.6).

FAB-HRMS (DTT/DTE w/Na^+): *m*/*z* calcd for $C_{26}H_{27}F_2N_5NaO_{12}$, 662.1522 $[M + Na]^+$, found 662.1507.

A fraction of the crude material (3.2 mg) was purified by ion exchange chromatography using Whatman DE52 cellulose in a mini-column. DE52 (3.0 g) was prepared following the manufacturer's directions. The column was packed in a glass pipette to a height of 8 cm and equilibrated in 0.02 M $Et_3NH_2CO_3$, pH 8, conductivity 1.22 mS. A solution of crude **2** in 0.02 M $Et_3NH_2CO_3$ was applied to the column and eluted with a linear gradient from 0.02 M $Et_3NH_2CO_3$ (100 mL) to 1.0 M $Et_3NH_2CO_3$ (pH 8, conductivity 30.0 mS, 100 mL). Fractions containing **2** were identified by UV absorbance spectra and were selectively combined based on comparison of chromatograms (HPLC) obtained with a gradient elution of 15 to 20% solvent A (0.1% AcOH, 0.02% TFA in MeCN) in solvent B (0.1% AcOH, 0.02% TFA in H_2O) over 25 min followed by an increase to 95% solvent A over 15 additional min. The total column recovery was 90.3% based on UV absorbance. Selected fractions each containing greater than 90% of the desired product by HPLC were combined and lyophilized to provide 1.1 mg (34.4%) of the desired product **2**; HPLC: T_R = 22.9, 24.8 min.

^{1}H NMR: (D_2O, 300 MHz): δ = 1.19–1.24 [t, 21 H, $(CH_3CH_2)_3N$], 1.65–2.06 (m, 4 H), 2.68–3.04 (m, 4 H), 3.10–3.18 [q, 13 H, $(CH_3CH_2)_3N$], 3.88 (m, 1 H), 4.33–4.37 (m, 1 H), 4.51–4.54 (m, 1 H), 6.78–6.84 (m, 2 H), 7.02–7.05 (d, 1 H), 7.26 (m, 1 H), 7.36–7.45 (m, 2 H), 7.84–7.87 (d, 1 H).

The low integration observed for $(CH_3CH_2)_3N$ in the ^{1}H NMR spectrum suggests the lyophilized material contains ca. 2 $(CH_3CH_2)_3N$ per molecule.

^{19}F NMR: (D_2O, 282 MHz): δ = –25.8 (dt), –28.2 (dt), –29.4 (dt), –32.7 (dt).

LC-MS: T_R 34 min; $[M + H]^+$ *m*/*z* calcd 640.2, found 640.1.

Fluorogenic Assay

The assay was carried out as previously described[15] with the following modifications. Concentrations of stock solutions of **1** and **2** were based on UV absorbance at 285 nm (ε = 5080 $M^{-1}cm^{-1}$) in 0.1 M NaOH. Histidine-tagged recombinant rat GH, expressed in the same construct as described for human GH,[47] was used in this assay. An aliquot of GH (10 μL, 33 nM) in storage buffer (25 mM NaOAc, 50 mM BME, 1 mM octyl-β-glucopyranoside, 1 mM EDTA, and 0.1 M NaCl) was incubated at 37 °C for 30 min and then added to 90 μL of assay buffer containing the desired concentration of substrate, 50 mM BME, 50 mM NaOAc, pH 6.0, at 37 °C in a quartz microcuvette. Fluorescence was monitored using a Spex Fluoromax-2 with the excitation wavelength set at 325 nm and emission at 415 nm with the temperature maintained at 37 °C by a circulating water bath. Controls with storage buffer replacing GH were used to correct for the low intrinsic fluorescence of the substrate due to incomplete quenching at each concentration. The fluorescence of the controls did not increase significantly over the time of the assay.

LC-MS Identification of Fragments from GH Cleavage of 2

A solution of 100 μM (1 mL) **2** in assay buffer was hydrolyzed with GH (100 nM). The sample was monitored by fluorescence as described above and the enzyme-catalyzed hydrolysis of **2** was judged to be complete when the addition of more GH yielded no further increase in fluorescence. A control with only storage buffer added in place of GH was subjected to the same conditions. Samples were centrifuged at 8,000 rpm for 15 min, filtered through a 0.2 μm syringe filter, and lyophilized. The resulting residue was dissolved in double-distilled H_2O (200 μL) and injected onto a Vydac C_{18} column which was eluted with the gradient described in general procedures. UV absorbance was monitored at 278 nm and mass spectra of the two product peaks were obtained: T_R 4.0, *m*/*z* = 303.1 $(M + H)^+$ and T_R 18.5, *m*/*z* = 356.1 $(M + H)^+$.

Acknowledgments

This research was supported by a grant from the National Cancer Institute (CA28097). We thank Mr. Scott Harrison for technical assistance with synthetic chemistry and thank the American Chemical Society, Division of Fluorine Chemistry, for a Moissan Summer Undergraduate Research Fellowship in Fluorine Chemistry awarded to Mr. Harrison, Ms. Joyce Reese for assistance in the careful preparation of this manuscript, and Dr. Thomas J. Ryan for generously providing purified recombinant γ-glutamyl hydrolase.

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