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Facile Synthesis of Tetrafluorotyrosine and Its Application in pH Triggered Membrane Lysis

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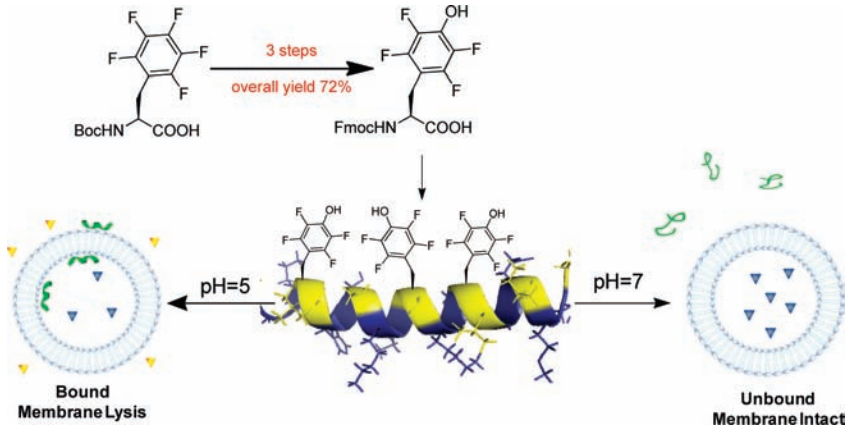
ABSTRACT



"Smart" peptides that lyse membranes in response to biological stimuli are of considerable interest. Herein, we report a facile synthesis of L-tetrafluorotyrosine (L-f$_4$Y) and its utilization as a pH sensing element. The synthetic route affords gram quantities of L-f$_4$Y within two days. Incorporation of this unnatural amino acid into magainin 2 gives a peptide that turns on its membrane-lytic activity upon mild acidification.

As the frontline of host-defense mechanisms across the biological world,[1] membrane-lytic peptides have attracted a great deal of attention, particularly in the search for novel antibiotics that are less prone to bacterial resistance.[2] Furthermore, inducible membrane lysis by a biological trigger holds great promise for a number of applications in biotechnology. For example, membrane-lytic peptides that respond to low pH have been utilized in liposomal drug delivery systems,[3] where acidification of endosomes triggers membrane lysis to release the encapsulated drug molecules. More recently, the pH low insertion peptide (pHLIP) was successfully applied to the imaging of acidic cancerous tissues.[4]

The design of pH-sensitive membrane-active peptides has been largely relying on the protonation of Glu or Asp side chains, which display pK_a values of 4.3 and 3.9 respectively. Residues with higher pK_a's are desirable considering the pH values of (patho)physiological relevance are generally above 5.5.[4a] We envisioned that pK_a values of a tyrosine side chain

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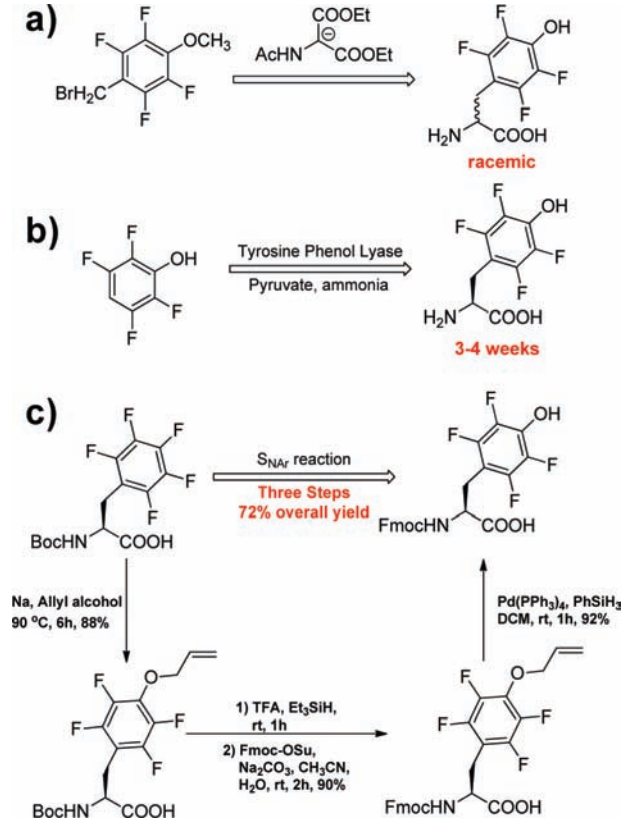
could be easily tuned to provide novel pH sensing elements in the design of membrane-lytic peptides.

Fluorination has become an attractive strategy in biomolecular engineering because of the minimal steric perturbation introduced by replacing hydrogen with fluorine atoms.[5] On the other hand, the high electronegativity of fluorine may dramatically alter the electronic properties of a target molecule.[6] For example, fluorination of a tyrosine side chain preferentially stabilizes the phenolate, thus affording tyrosine isosteres with increased acidity. By varying the number of fluorine substitutions, tyrosine analogues can be created to display a wide range of pK_a values (~10 for the native tyrosine and 5.6 for the tetrafluorinated analogue f_4Y).[7] These fluorinated tyrosine analogues have been utilized as probes to elucidate the mechanisms of a number of enzymes, including kinases and ribonucleotide reductases.[7,8] Herein we show that f_4Y serves as an effective pH sensor and activates the membrane-lytic peptide magainin 2 (mag2) under mildly acidic conditions.

It came as a surprise to us that there was no chemical synthesis of f_4Y that is stereospecific. The only chemical synthesis of f_4Y was reported by Kang and co-workers that couples 4-methoxy-tetrafluorobenzyl bromide to diethylacetamidomalonate to give the racemic mixture of the *O*-methyl protected f_4Y (Scheme 1a).[9] A biosynthetic route for preparing fluorotyrosine analogues in the enantiomerically pure form has been developed by the Cole group and the Stubbe group.[7,8] This approach capitalizes on the enzymatic activity of tyrosine phenol lyase that converts fluorinated phenols to the corresponding L-fluorotyrosines in the presence of pyruvate and ammonia (Scheme 1b). While this strategy works well with mono-, di-, and trifluorine substituted phenols, the tetrafluorinated analogue appears to be a poor substrate for the enzyme. Consequently, the preparation of L-f_4Y takes multiple weeks and requires high concentrations of the enzyme.

We report a short and efficient synthesis of L-f_4Y with the commercially available L-pentafluorophenylalanine (dubbed L-f_5F for brevity) as the starting material (Scheme 1c). The key transformation of our synthesis is the regioselective nucleophilic addition−elimination (S_{NAr}) reaction of the pentafluorobenzyl moiety.[10] Due to the balance between the favorable inductive effect and the unfavorable resonance effect, fluorination on the *meta* position of the reaction center best accelerates the S_{NAr} reaction (by 106-fold relative to a

Scheme 1. Synthesis of Fmoc-L-f_4Y-OH



hydrogen) followed by the *ortho* position (57-fold). With resonance overwhelming the inductive effect, a fluorine substituent on the *para* position actually slows down the reaction by 2-fold. Given these considerations, the fluorine on Cζ of the f_5F side chain should be most activated (by two *meta* and *ortho* fluorines) toward S_{NAr} reactions. The Boc protected L-f_5F was chosen as the starting material. Initial attempts using hydroxide as the nucleophile failed to give any product in organic or aqueous media. The reaction proceeded nicely to completion with methoxide as the nucleophile. Sodium as the counterion afforded a slightly faster reaction than potassium presumably because sodium is a stronger Lewis acid (Table 1). Difficulty was encountered with the removal of the *O*-methyl protecting group: BBr_3 resulted in a messy mixture; even the milder alternative

Table 1. Summary of the S_{NAr} Reaction Conditions To Make f_4Y

entry	nucleophile (solvent)	Yield[b] (%)
1	KOH (*t*BuOH); KOH (DMSO); NaOH (H_2O)	0
2	KO*t*Bu (MeOH) → KOMe (MeOH)[a], 48 h	>90
3	NaOMe(MeOH), 36 h	>90
4	Na (allyl alcohol) → NaOallyl (allyl alcohol)[a]	88

[a] The nucleophile shown on the right-hand side of the arrow was generated *in situ*. [b] 0% yield means no product was identified by crude ^{19}F NMR.

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$NaSC_2H_5$[11] caused cleavage of the Boc protecting group, which made the purification difficult. Finally, we turned to sodium allyloxide, which reacted readily with Boc-f_5F to give the *O*-allyl protected L-f_4Y. After converting the Boc group to Fmoc, the *O*-allyl protecting group was removed by using $Pd(PPh_3)_4$ in the presence of $PhSiH_3$.[12] We confirmed the absence of epimerization by conjugating Fmoc-L-f_4Y-OH to a chiral amine, the product of which elutes as a single peak during HPLC analysis (Figure S1, Supporting Information (SI)). The lack of epimerization under such basic conditions is presumably because the negatively charged carboxylate prohibits the deprotonation of the α carbon. The synthetic route has an overall yield of 72% and can easily afford gram quantities of L-f_4Y for peptide synthesis.

A well characterized membrane-lytic peptide magainin 2 (mag2)[13] was used as a model system to test the potential of f_4Y in pH sensing. Mag2 is a 23-residue peptide that folds into an amphiphilic helix upon membrane binding. Three mag2 variants were synthesized on the Rink-MBHA amide resin using standard Fmoc/HBTU chemistry (Table 2, peptides **1**−**3**). A dansyl group was installed at the N-termini of all peptides to facilitate binding affinity measurement and concentration calibration. Because fluorination renders the f_4Y side chain much less nucleophilic than that of Tyr, the unprotected f_4Y is compatible with the standard peptide synthesis and cleavage conditions.[8a] However, the unprotected f_4Y did react with dansyl chloride to give the side chain dansylated peptide. To circumvent this problem, we used the protected f_4Y (Fmoc-f_4Y(OAllyl)-OH) in peptide synthesis. The allyl protecting group was removed on resin after the dansylation step by using $Pd(PPh_3)_4$ in the presence of $PhSiH_3$. Peptide **2** is the corresponding Tyr control for the designed pH-responsive peptide **1**. For comparison, we also synthesized peptide **3** incorporating three Glu residues, which have been widely used as pH sensing elements in previous reports.[3]

Table 2. Sequences of the Peptides Used in This Study[a]

WT mag2	GIGKFLHSAKKFGKAFVGEIMNS
1	dansyl-GIGK(**f_4Y**)LHSAKK(**f_4Y**)GKA(**f_4Y**)VGEIMNS
2	dansyl-GIGK**Y**LHSAKK**Y**GKA**Y**VGEIMNS
3	dansyl-GIGK**E**LHSAKK**E**GKA**E**VGEIMNS

[a] The wild type mag2 sequence (WT) was included for comparison; residues of interest are shown in bold:**f_4Y** (negative at pH7.0, neutral at pH5.0); **Y** (neutral in both); **E** (negative in both).

The membrane-lytic activities of peptides **1**−**3** were tested by using a calcein leakage assay.[14] Large unilamellar vesicles (LUVs, 70 nm in their hydrodynamic radii) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were prepared to encapsulate calcein at 40 mM. Significant self-quenching is expected at such a high concentration. The membrane lysis caused by the mag2 derivatives can be easily detected by the fluorescence increase due to calcein release and dilution.

The membrane-lytic activities of the mag2 mutants are shown in Figure 1a. Under neutral considitons (pH 7.0), no membrane lysis was observed upon mixing the peptides **1** or **3** with liposomes. The tyrosine variant **2** afforded a small degree (~18%, Table S1, SI) of membrane damage. Acidification to pH 5.0 by HCl addition caused little change for the control peptides **2** and **3**. In sharp contrast, the f_4Y mutant **1** displayed a dramatic increase in calcein fluorescence, showing that the f_4Y side chains indeed serve as effective pH sensors to turn on the membrane-lytic acitivity of mag2. The concentration dependence of the membrane-lytic potency yields an EC_{50} value of 3.7 μM at pH 5.0 (Figure S2, SI). Under neutral conditions (pH 7.0), no membrane damage was observed even at 10 μM peptide concentration. The membrane-lytic activity of peptide **1** was further evaluated

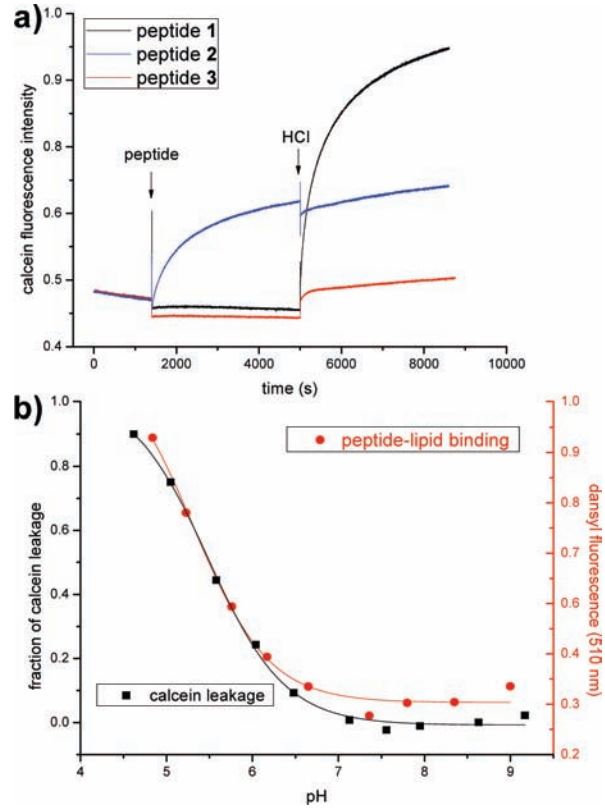

Figure 1. (a) kinetics of peptide induced cargo (calcein) leakage. The graph shows observed change in calcein fluorescence at 520 nm (λ_{ex} = 485 nm) upon addition of peptide (3 μM) into LUVs of POPC (500 μM lipids, pH 7.0), followed by the addition of 6 N HCl (12 μL) to reach pH 5.0. (b) pH profiles of peptide **1** induced calcein leakage (black squares) and the fraction of membrane-bound peptides (red dots); 3 μM peptide **1** and 500 μM lipids (POPC) were used in these experiments. Peptide-liposome binding was monitored by dansyl fluorescence at 510 nm with 340 nm excitation. The data were fitted into a sigmoidal function to determine the pK_a values: 5.5 ± 0.1 based on the leakage data and 5.3 ± 0.2 based on the binding curve.

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under varied pH conditions (Figure 1b, black squares). The pH dependence profile shows a clear sigmoidal curve with the middle point of transition at pH 5.5, which agrees nicely with the pK_a values of f_4Y.

To gain a further mechanistic understanding of the acid triggered membrane lysis, we evaluated the fraction of peptide **1** bound to the liposomes in buffers of varied pH values (Figure 1b, red dots). The peptide-lipid binding was conveniently monitored by recording the dansyl fluorescence, which is sensitive to the local environment. Peptide **1** shows little binding to vesicles under neutral conditions, and acidification elicits their partition onto liposomes. Interestingly, the binding curve overlaps with that of the membranelytic potency, indicating that the low pH induced peptide-vesicle binding accounts for the pH-dependent activity of peptide **1** toward membranes.

In summary, we have developed a short and effective synthesis that allows one to prepare grams of L-f_4Y within two days. This protocol compares favorably to the existing biosynthetic method that takes weeks to months. Our synthesis should be easily extendable to the preparation of D-f_4Y simply by using the D version of f_5F as the starting material. We further demonstrate that f_4Y functions as effective pH sensors in the design of 'smart" membranelytic peptides. The mag2 mutant harboring f_4Y residues responds quickly to the pH drop from 7.0 to 5.0. No pH activation was observed for the corresponding mutant that incorporates Glu resdiues as pH sensors. This is largely because the pK_a value of f_4Y falls into this physiologically relevant region, while that of Glu does not. In addition, the hydrophobic nature of the neutral f_4Y allows their incorporation onto the membrane-binding face of a peptide, whereas all previously reported pH sensing elements reside on the hydrophilic face of an amphipathic structure. Given that cancer tissues are often acidic, experiments are currently underway to investigate the potential of the pH-triggered membrane toxicity in killing cancer cells. Furthermore, we expect the ease in synthesis and unique physical property of f_4Y will make it suitable for a wide array of applications in protein biochemistry and engineering.

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Supporting Information Available: Experimental procedures and characterization of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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