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Establishment of Molecular Design Strategy To Obtain Activatable Fluorescent Probes for Carboxypeptidases

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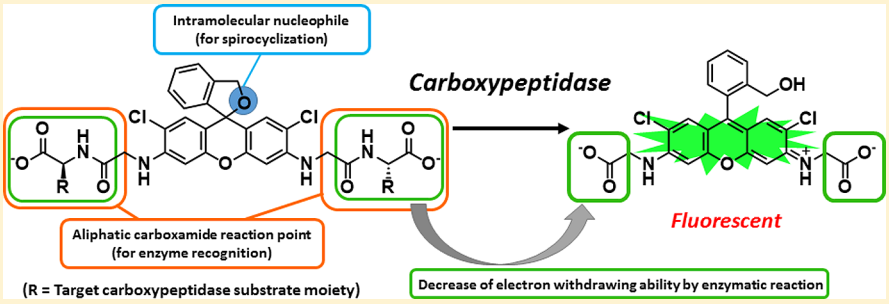
Supporting Information



ABSTRACT: Carboxypeptidases (CPs) are a family of hydrolases that cleave one or more amino acids from the C-terminal of peptides or proteins. However, methodology to monitor the activities of CPs is poorly developed. Here, we present the first versatile design strategy to obtain activatable fluorescent probes for CPs by utilizing intramolecular spirocyclization of rhodamine to translate the "aliphatic carboxamide to aliphatic carboxylate" structural conversion catalyzed by CPs into dynamic fluorescence activation. Based on this novel strategy, we developed probes for carboxypeptidases A and B. One of these probes was able to detect pancreatic juice leakage in mice *ex vivo*, suggesting that its suitability for intraoperative diagnosis of pancreatic fistula. This design strategy should be broadly applicable to CPs, as well as other previously untargetable enzymes, enabling development of fluorescent probes to study various pathological and biological processes.

INTRODUCTION

Carboxypeptidases (CPs) are a family of proteases that cleave one or more amino acids from the C-terminal of peptides or proteins,[1] and they have key roles in both physiological processes and various diseases. For example, carboxypeptidase A (CPA) and carboxypeptidase B (CPB) produced in the pancreas are responsible for protein degradation,[2] and angiotensin converting enzyme (ACE) controls blood pressure by regulating peptide hormone activity.[3,4] Expression levels of CPs are varied in various diseases: CPA and CPB in pancreatitis and pancreatic cancer,[5,6] prostate-specific membrane antigen (PSMA) in prostate cancer,[7,8] carboxypeptidase N (CPN) in breast cancer,[9] carboxypeptidase M (CPM) in several cancers,[10] and cathepsin A in galactosialidosis.[11] However, currently available methods for monitoring CP activities are HPLC analysis,[12] absorption spectrometry,[13,14] or coupled assay systems.[15,16] Their use is almost limited to *in vitro* study, and it is difficult to perform real-time imaging of CP activities.

Fluorescence imaging is a powerful methodology to monitor enzymatic activities in living cells and animals, being simple, safe, and sensitive, as well as offering high spatiotemporal resolution and signal activatability.[17] Various fluorogenic enzyme substrates (activatable fluorescent probes for enzymes) have been devised for real-time monitoring of enzyme activities of interest[18] and have been utilized not only in biological research but also for medical diagnosis or intraoperative visualization of diseases[19] including cancer,[20,21] Parkinson's

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disease,[22] or pancreatic fistula.[23] Thus, it is considered that activatable fluorescent probes for CPs will offer novel ways of studying enzymatic functions and disease diagnosis.

Despite their potential utility, only a few activatable fluorescent probes for CPs have been reported so far.[24,25] This is mainly from the difficulty in designing the suitable substrate analogue of CPs. Since CPs strictly recognize C-terminal amino acid, major strategies of designing activatable fluorescent probes for hydrolases such as incorporation of substrate moiety on an aromatic hydroxy or amino group of the scaffold fluorophore,[26−31] or sandwiching substrate moiety between a FRET donor−acceptor pair,[32,33] are not generally applicable to the enzyme. To our knowledge, only the currently available fluorogenic substrate is one utilizing the amino acid on substrate moiety as a FRET donor or static quencher,[24,25] but this strategy is only applicable to limited numbers of CPs that can accommodate those special amino acids for fluorescent readout.

Here, we propose a versatile and straightforward molecular design strategy for activatable fluorescent probes for CPs that translates chemical structural conversion by CPs ("aliphatic carboxamide to aliphatic carboxylate") directly into dynamic fluorescence activation. This was attained by utilization of intramolecular spirocyclization of rhodamines; we discovered that this molecular mechanism is highly suitable for converting the reaction of aliphatic carboxamide/carboxylate to robust fluorescence activation. Based on the versatility of the design, we succeeded in development of fluorescent probes for clinically important CPs, carboxypeptidase A (CPA: recognizing C-terminal bulky amino acid such as phenylalanine) and carboxypeptidase B (CPB: recognizing C-terminal basic amino acid such as arginine) only by changing the conjugated amino acid. Further, we showed that one of these probes is available for *ex vivo* detection of pancreatic fluid leakage, a clinically significant manifestation of pancreatic fistula.

RESULTS AND DISCUSSION

Molecular Design for Activatable Fluorescent Probes for CPs. First, we overviewed the known substrates of CPs to design the core structure of the probe that can be recognized by enzyme. It is known that hippuryl amino acid derivatives are widely used as standard substrates for CPs (Figure 1a), so we considered that it is desirable to conjugate a substrate amino acid to aliphatic carboxylate to form aliphatic carboxamide for versatile design of the probe. Therefore, the major chemical challenge in designing activatable fluorescent probes for CPs is how to translate the CP-catalyzed "aliphatic carboxamide to aliphatic carboxylate" structural conversion into dynamic fluorescence activation at physiological pH.

To overcome this challenge, we focused on intramolecular spirocyclization as a mechanism of fluorescence activation. Spirocyclization of rhodamine or rhodol derivatives is a powerful technique for designing fluorescent probes, as the spirocyclized derivative is colorless and nonfluorescent due to separation of the π-conjugation system of the original fluorophore. Recently we have developed several fluorescent probes based on this mechanism by precisely controlling the pK_{cycl} values of the probes.[29,34−36] (pK_{cycl} is the pH at which the extent of spirocyclization is sufficient to reduce the absorbance of the compound to one-half of the maximum absorbance calculated from the pH titration curve.) Here, we focused on our previous finding that the electron-donating or -withdrawing ability of the alkyl substituent on the nitrogen atom of the

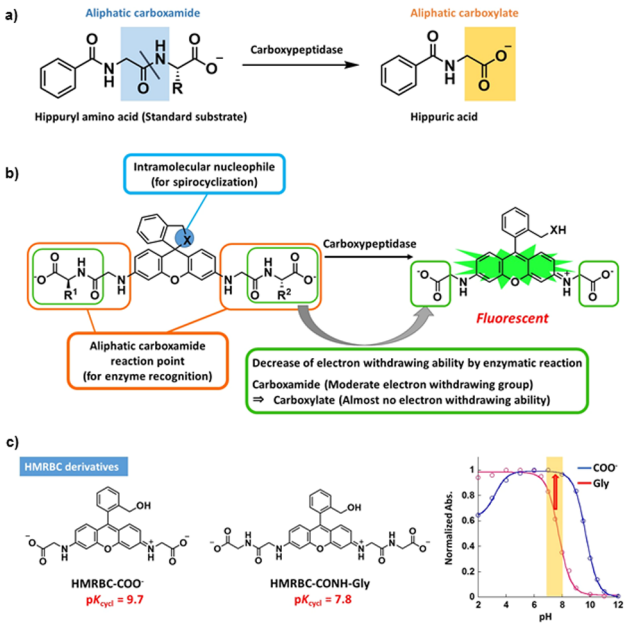

Figure 1. Molecular design strategy for activatable fluorescence probes for CPs. (a) Chemical structures and reaction scheme of hippuryl amino acid derivative with CP. (b) Molecular design of activatable fluorescent probe for CPs. (c) Chemical structures, pK_{cycl} values, and pH dependency of absorbance of HMRBC-COO$^-$ or HMRBC-CONH-Gly in 0.1 M sodium phosphate buffer at various pH's. In the titration curve, normalized absorbance at the wavelength in parentheses was plotted against pH. HMRBC-COO$^-$ (523 nm), HMRBC-CONH-Gly (513 nm).

xanthene moiety affected the pK_{cycl} value[35,36] (Figure S1), and we set out to utilize the decrease in electron-withdrawing ability caused by reaction with CPs (aliphatic carboxamide to aliphatic carboxylate) to induce a shift of the pK_{cycl} value that would provide a large fluorescence activation. Thus, we incorporated a substrate amino acid into aliphatic carboxylate arranged on the nitrogen atom of hydroxymethylrhodamine (Figure 1b).

To confirm the validity of this design strategy, we first prepared a hydroxymethyl rhodamine with two carboxymethyl groups on the nitrogen atoms, HMRBC-COO$^-$, and its glycine analogue HMRBC-CONH-Gly as model substrates of CPs. We found that the pK_{cycl} value of HMRBC-CONH-Gly was much lower than that of HMRBC-COO$^-$ (9.7 for HMRBC-COO$^-$ and 7.8 for HMRBC-CONH-Gly) (Figure 1c), indicating that it would be feasible to convert the structural change into a shift in pK_{cycl} value. However, based on the percentage of the open form (99% for HMRBC-COO$^-$ and 69% for HMRBC-CONH-Gly at pH 7.4), the activation ratio in the physiological pH range was still limited to 1.5-fold, due to the relatively high pK_{cycl} values of the probes, and therefore, we decided to modify the structure to lower the pK_{cycl} value so that we could obtain greater dynamic fluorescence activation in the physiological pH range.

Adjustment of pK_{cycl} Values To Develop Probes Capable of Sensing CPA and CPB Activities in the Physiological pH Range. To shift the pK_{cycl} value to the acidic side, we adopted two strategies. We previously showed that the pK_{cycl} value is correlated to the nucleophilicity of the intramolecular nucleophile and the LUMO energy level of the xanthene moiety.[29,34−37] In other words, raising the nucleophilicity of the intramolecular nucleophile or lowering the LUMO energy level of the xanthene moiety should be effective

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for shifting pK_{cycl} to the acidic side. Therefore, we prepared two candidate scaffolds, AMRBC-COO$^-$ (aminomethyl rhodamine with two carboxymethyl groups) and diClHMRBC-COO$^-$ (dichloro hydroxymethyl rhodamine with two carboxymethyl groups) (Figure 2a,b), by replacing the intramolecular

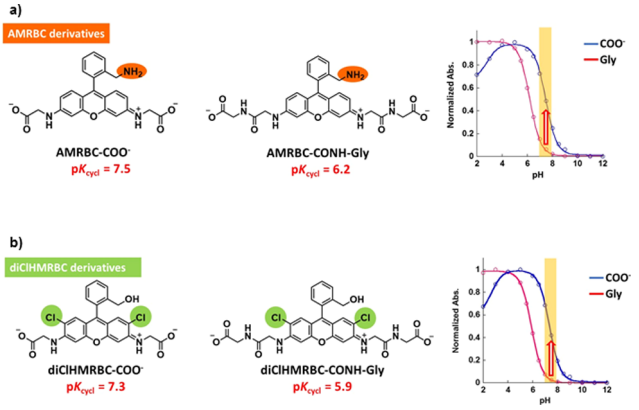

Figure 2. Adjustment of pK_{cycl} values to the physiological pH range. Chemical structures, pK_{cycl} values, and pH dependency of absorbance of (a) AMRBC derivatives or (b) diClHMRBC derivatives in 0.1 M sodium phosphate buffer at various pH's. In the titration curve, normalized absorbance at wavelength in parentheses was plotted against pH. AMRBC-COO$^-$ (528 nm), AMRBC-CONH-Gly (520 nm), diClHMRBC-COO$^-$ (529 nm), or diClHRBC-CONH-Gly (521 nm).

nucleophile with an aminomethyl group, which is considered to be a stronger nucleophile, or by introducing two chlorines into the xanthene core, which would lower the LUMO level of the xanthene moiety, respectively (Figure S2). We also prepared glycine analogues of these scaffolds, AMRBC-CONH-Gly and diClHMRBC-CONH-Gly, as model substrates. As expected, the pK_{cycl} values of both derivatives were lowered compared to those of HMRBCs: 7.5 for AMRBC-COO$^-$, 6.2 for AMRBC-CONH-Gly, 7.3 for diClHMRBC-COO$^-$, 5.9 for diClHMRBC-CONH-Gly. Thus, dynamic fluorescence activation could be achieved with these scaffolds.

Based on the newly developed scaffolds, we next set out to develop fluorescent probes for carboxypeptidase A (CPA) and carboxypeptidase B (CPB). As a substrate amino acid, we chose phenylalanine for CPA and arginine for CPB because CPA prefers bulky amino acids while CPB prefers basic ones,[6] and we prepared four derivatives, AMRBC-CONH-Phe, diClHMRBC-CONH-Phe, AMRBC-CONH-Arg, and diClHMRBC-CONH-Arg. The pK_{cycl} values of AMRBC-CONH-Phe and AMRBC-CONH-Arg were 6.0 and 5.8, and those of diClHMRBC-CONH-Phe and diClHMRBC-CONH-Arg were 5.6 and 5.0, respectively (Figure 3a, b). The photophysical properties of these compounds are summarized in the Supporting Information (Figures S3, S4 and Table S1). The percentage of the open form at the physiological pH of 7.4 was calculated to be 54% for AMRBC-COO$^-$, 6.4% for AMRBC-CONH-Gly, 4.8% for AMRBC-CONH-Phe, 3.6% for AMRBC-CONH-Arg, 45% for diClHMRBC-COO$^-$, 3.1% for diClHMRBC-CONH-Gly, 1.8% for diClHMRBC-CONH-Gly, and 0.79% for diClHMRBC-CONH-Arg. Although we do not have a clear explanation for the effects of the different kinds of amino acids on pK_{cycl} values, the results were favorable from the viewpoint of suppressing the background signal and obtaining enhanced fluorescence activation. Thus, though the pK_{cycl}

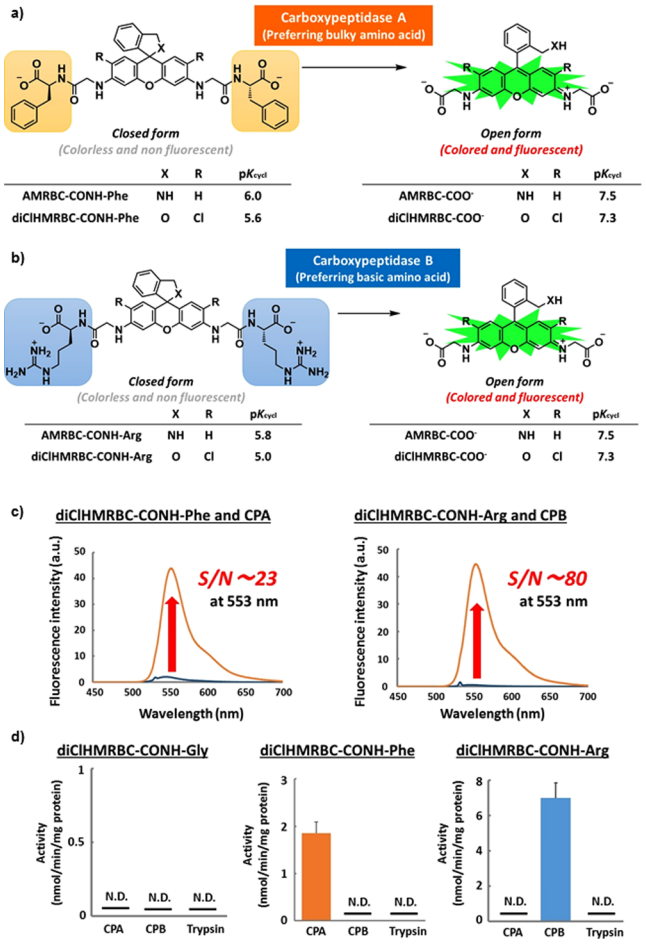

Figure 3. Enzyme reactions of the probes with CPA or CPB. (a, b) Reaction scheme of the probes with CPA or CPB and pK_{cycl} values of the derivatives. (a) AMRBC-CONH-Phe or diClHMRBC-CONH-Phe was recognized as a substrate by CPA and converted into AMRBC-COO$^-$ or diClHMRBC-COO$^-$, respectively. (b) AMRBC-CONH-Arg or diClHMRBC-CONH-Arg was recognized as a substrate by CPB and converted into AMRBC-COO$^-$ or diClHMRBC-COO$^-$, respectively. (c) Fluorescence spectra of 1 μM diClHMRBC-CONH-Phe or diClHMRBC-CONH-Arg upon enzymatic reaction and signal-to-noise (S/N) ratio. Reaction was performed in 0.80 mL of phosphate-buffered saline (pH 7.4) containing 0.01% CHAPS and 0.1% DMSO as a cosolvent using 3 μg proCPA activated by trypsin before addition or CPB at 25 °C. (Left) diClHMRBC-CONH-Phe was incubated with CPA for 15 h. (Right) diClHMRBC-CONH-Arg was incubated with CPB for 2 h. Blue and orange lines indicate the fluorescence spectra before and after enzymatic reaction, respectively. (d) diClHRBC-CONH-Gly, diClHMRBC-CONH-Phe, or diClHMRBC-CONH-Arg was reacted with CPA, CPB or trypsin. A 1 μM probe in phosphate-buffered saline (pH 7.4) containing 0.01% CHAPS and 0.1% DMSO as a cosolvent was reacted with 200 ng of enzyme at 25 °C (n = 3). The total assay volume was 20 μL, and the fluorescence increase was measured with a plate reader. Excitation/emission wavelengths were 485 nm/535 nm. Error bars represent SD.

values of the probes depended upon the kind of amino acid, the activation ratio (calculated from the percentages of the open form of the respective probe and COO$^-$ derivative) appeared to be sufficient for obtaining practically useful fluorescent probes: 8.4−15-fold in AMRBC and 15−58-fold in diClHMRBC, despite the low activation ratio of 1.5 fold in HMRBC.

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We then evaluated the reactivity of AMRBC-CONH-Phe or diClHMRBC-CONH-Phe with CPA and AMRBC-CONH-Arg or diClHMRBC-CONH-Arg with CPB. As expected, all of the probes were recognized as substrates, and a fluorescence increase was observed. The fluorescence activation was 18-fold for AMRBC-CONH-Phe or 23-fold for diClHMRBC-CONH-Phe with CPA, and 23-fold for AMRBC-CONH-Arg or 80-fold for diClHMRBC-CONH-Arg with CPB (Figure 3c and Figure S5). As we had expected, diClHMRBC derivatives showed higher activation ratios because of their smaller pK_{cycl} values. We also confirmed that the probes were cleaved to AMRBC-COO^- or diClHMRBC-COO^- by UPLC analysis (Figure S6), and the kinetic parameters were similar (Table S2). For further study, we focused on diClHMRBC derivatives because of their higher activation ratio, and first, we confirmed the substrate specificity. Indeed, these derivatives selectively reacted with their target enzyme among CPA, CPB and trypsin (Figure 3d and Figure S7; trypsin was examined because it was used for activating proCPA to CPA). Moreover, we examined the reactivity of the probes with other five proteases: cathepsin K, dipeptidylpeptidase-IV, γ-glutamyltranspeptidase, leucine aminopeptidase, and chymotrypsin. The probes did not react with any of these proteases, as expected, supporting their high selectivity (Figure S8). These results confirmed the suitability of the developed scaffolds to obtain activatable fluorescent probes for various CPs simply by changing the conjugated amino acid.

Fluorescence Detection of Pancreatic Fistula. Next, we examined whether CPA or CPB activity in human pancreatic juice can be detected with diClHMRBC-CONH-Phe or diClHMRBC-CONH-Arg, aiming at development of diagnosis tools for pancreatic fistula. Pancreatic fistula is one of the most serious complications after digestive surgery and can cause death.[38,39] However, it is difficult to prevent because of the inability to visualize leakage of pancreatic juice during surgery. Therefore, a fluorescent probe that could detect enzymatic activity in pancreatic juice would become a useful tool for detecting pancreatic fistula intraoperatively. For this purpose, we have so far developed gPhe-HMRG,[23] an activatable fluorescent probe for chymotrypsin, and we succeeded in detecting chymotrypsin activity in human pancreatic juice. But, although gPhe-HMRG can be used for imaging pancreatic fistula, it turned out that chymotrypsin activity varies from patient to patient. Thus, to achieve more accurate diagnosis of pancreatic fistula, it would be better to evaluate multiple enzyme activities in pancreatic juice.

So, we first set out to examine whether diClHMRBC-CONH-Phe and diClHMRBC-CONH-Arg could detect CP activities in eight samples of pancreatic juice from different patients. Although the pH of pancreatic juice is around 8.0, almost all of diClHMRBC-CONH-Phe or diClHMRBC-CONH-Arg exists as the closed form at this pH (ratio of open form <1%) and the ratio of the open form of diClHMRBC-COO^- is 18%. This should be sufficient for detection of CPA or CPB activity in pancreatic juice. In living systems, CPA and CPB exist as proenzymes in pancreatic juice, as is the case with chymotrypsin, and they are activated by trypsin after pancreatic juice is secreted into duodenum (Figure S9). Thus, we performed reaction of the probes with pancreatic juice in the presence of trypsin as an activator of proCPA or proCPB in this study. Marked fluorescence increases were observed with both probes and were inhibited by the respective CP inhibitors, (*S*)-benzylsuccinic acid and MGTA (Figure 4a).

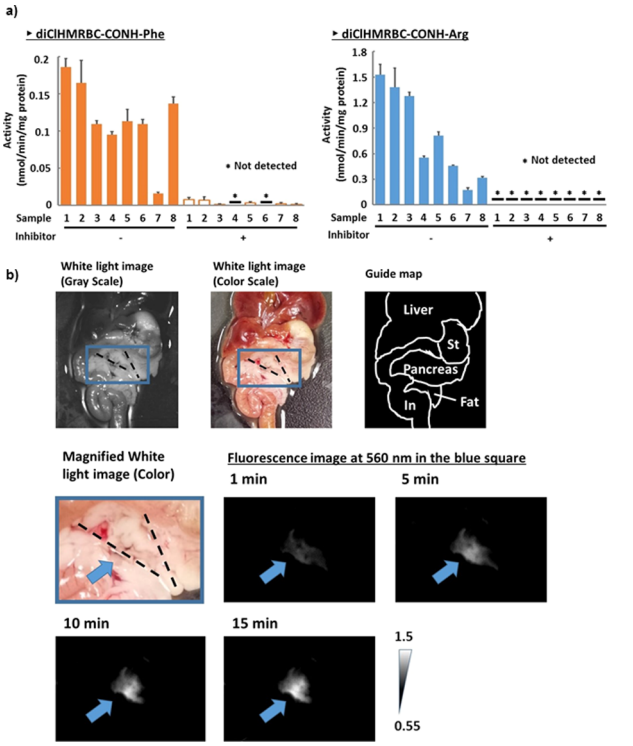

Figure 4. Detection of CPA or CPB activities in pancreatic juice. (a) Fluorescence increase rates of 1 μM probe and 1.25 μg pancreatic juice from 8 different patients with or without 100 μM inhibitor, (*S*)-benzylsuccinic acid for CPA or MGTA for CPB, in phosphate-buffered saline (pH 7.4) containing 0.01% CHAPS and 0.1% DMSO as a cosolvent in the presence of 1.5 μg of trypsin as an activator of proCPA or proCPB in pancreatic juice. Error bars represent SD (n = 4). (b) Detection of pancreatic juice leakage from cut mouse pancreas *ex vivo*. Mouse organs were resected, and the pancreas was cut along the black dotted lines. 50 μM diClHMRBC-CONH-Arg in phosphate-buffered saline (pH 7.4) containing 1% DMSO as a cosolvent in the presence of 100 μg/mL trypsin as an activator of proCPB in pancreatic juice was applied onto cut mouse pancreas. Fluorescence increase in the blue square was acquired with a Maestro imaging system. Blue arrows indicate the area where fluorescence increased. Excitation/emission wavelengths were 523 nm/560 nm. Abbreviations in the guide map: St, stomach; In, intestine.

This result shows that we could detect CPA or CPB activities in pancreatic juice with the developed probes. On the other hand, no fluorescence increase was observed in the case of abdominal dropsy, which did not contain pancreatic juice (Figure S10). Compared to gPhe-HMRG, diClHMRBC-CONH-Arg showed a faster reaction rate (Figure S11). Interestingly, there was almost no correlation between chymotrypsin activity and CPA or CPB activity, in spite of the strong correlation between CPA and CPB activities (Figure S12). These results support the idea that our probes for CPs, in combination with gPhe-HMRG, would enable more accurate diagnosis of pancreatic fistula by detecting multiple enzyme activities in pancreatic juice.

Finally, in order to examine if diClHMRBC-CONH-Arg works *ex vivo*, we tried to detect pancreatic juice leakage from mouse pancreas. Indeed, when diClHMRBC-CONH-Arg was applied to cut mouse pancreas, we could visualize pancreatic juice leakage in terms of a marked fluorescence increase (Figure 4b). On the other hand, no significant fluorescence increase was seen when the probe was applied before cutting the pancreas (Figure S13). Further, the fluorescence increase was

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inhibited in the presence of CPB inhibitor even after cutting the pancreas (Figures S14 and S15). We also examined the reactivity of the probe toward mouse tissue lysate and serum, and we found that it is highly selective for pancreas lysate in the presence of trypsin (Figure S16). These data indicate that diClHMRBC-CONH-Arg is a promising candidate as an intraoperative diagnostic tool for pancreatic fistula.

CONCLUSIONS

We have designed a scaffold that can translate the CP-catalyzed "aliphatic carboxamide to aliphatic carboxylate" structural conversion into dynamic fluorescence activation based on intramolecular spirocyclization of rhodamine. The spirocyclic equilibrium of the new scaffolds, diClHMRBC-COO^- or AMRBC-COO^-, was precisely adjusted by optimizing the nucleophilicity of the intramolecular nucleophile and/or the electrophilicity of the fluorophore. We demonstrated the validity of this design strategy by developing activatable fluorescent probes for CPA or CPB simply by changing the substrate amino acid on the scaffold. In addition to these results, our preliminary work has confirmed that this design strategy is also applicable to angiotensin converting enzyme, which has dipeptidyl carboxypeptidase activity (Figure S17). Further, we succeeded in detecting CPA and CPB activities in pancreatic juice with diClHMRBC-CONH-Phe and diClHMRBC-CONH-Arg, respectively, and we visualized pancreatic juice leakage in an *ex vivo* mouse model with diClHMRBC-CONH-Arg. To our knowledge, this is the first report of a practical design strategy for activatable fluorescent probes for CPs; such probes have a variety of potential applications, for example as medical diagnostic tools and as research tools for studying the biological roles of CPs, as well as screening and evaluation of CP inhibitors. It is particularly noteworthy that the pK_{cycl} value of the scaffold is basically regulated by the electron-withdrawing ability of the aliphatic functional group and is highly tunable by modification of the structure. This means that the scaffold should also be applicable for other enzymes that induce chemical conversions leading to reduced electron-withdrawing ability, and we believe that this platform could also be available to develop activatable fluorescent probes for various other enzymes that have not so far been targetable.

EXPERIMENTAL SECTION

Materials and Instruments. Reagents and solvents were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Sigma-Aldrich, Dojindo, Kanto Chemical Co., Watanabe Chemical Industries, Merck Millipore, and R&D Systems, and were used without further purification. NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR, a JEOL JNM-LA400 instrument at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR or a JEOL JNM-ECZ400S instrument at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. All chemical shifts (δ) are reported in ppm relative to internal standard tetramethylsilane (δ = 0.0 ppm), or relative to the signals of residual solvent $CDCl_3$ (7.26 ppm for ^{1}H, 77.16 ppm for ^{13}C), CD_3OD (3.31 ppm for ^{1}H, 49.00 ppm for ^{13}C), acetone-*d6* (2.04 ppm for ^{1}H) or DMSO-*d6* (2.50 ppm for ^{1}H, 39.52 ppm for ^{13}C), and coupling constants are given in Hz. Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuToF (ESI). Preparative HPLC was performed on an Inertsil ODS-3 (10.0 × 250 mm) column (GL Sciences Inc.) using an HPLC system composed of a pump (PU-2080, JASCO) and a detector (MD-2015 or FP-2025, JASCO). Eluent A (H_2O containing 0.1% TFA), eluent B (80% acetonitrile and 20% H_2O containing 0.1% TFA), eluent E (H_2O containing 100 mM triethylammonium acetate) and eluent F (80% acetonitrile and 20% H_2O containg 100 mM triethylammonium acetate) were used for HPLC purification. UPLC analyses were performed on a Waters Acquity UPLC (H class)/QDa quadrupole MS analyzer or Acquity UPLC (H class)/Xevo TQD quadrupole MS/MS analyzer equipped with an Acquity UPLC BEH C18 column (Waters). Eluent C (H_2O containing 0.1% formic acid) and eluent D (80% acetonitrile and 20% H_2O containing 0.1% formic acid) were used for UPLC analyses. Gel-permeation chromatography (GPC) purification was performed on a recycle preparative HPLC LC-9110 NEXT (Japan Analytical Industry) equipped with a JAIGEL-2HR column (20 mm × 600 mm, Japan Analytical Industry).

UV–vis Absorption and fluorescence spectroscopy. UV–visible spectra were obtained on a Shimadzu UV-1800. Fluorescence spectroscopic studies were performed on a Hitachi F7000. The slit width was 5 nm for both excitation and emission. The photomultiplier voltage was 400 V. Relative fluorescence quantum yields were obtained by comparing the area under the emission spectra of the test samples with standard samples and were calculated according to the following equation

$$\Phi_x/\Phi_{st} = [A_{st}/A_x][n_x^2/n_{st}^2][D_x/D_{st}]$$

where st = standard; x = sample; A = absorbance at the excitation wavelength; n = refractive index; and D = area under the fluorescence spectra on an energy scale. Optical properties of probes (1 μM) were examined in 0.1 M sodium phosphate buffer containing 0.1% DMSO as a cosolvent. For enzyme reaction, proCPA activated by trypsin before addition or CPB (3 μg) was added to probes (1 μM) in phosphate-buffered saline (pH 7.4) containing 0.01% CHAPS and 0.1% DMSO as a cosolvent, and the solution was incubated at 25 °C.

Determination of pK_{cycl} Value. Absorption spectra of compounds were measured in 0.1 M sodium phosphate buffer at various pH values. For compounds with n acid–base equilibria (n = 1 or 2), pH profiles of absorbance (Abs) were fitted to the following formula to determine pK_a values

$$\text{Abs or FI} = \frac{c_0 + \sum_{k=1}^{n} c_k \cdot 10^{k\cdot \text{pH} - \sum_{l=1}^{k} pK_{al}}}{1 + \sum_{k=1}^{n} 10^{k\cdot \text{pH} - \sum_{l=1}^{k} pK_{al}}}$$

where $pK_{a1} < pK_{a2} < \cdots < pK_{an}$; c_n = constant.

Enzymes. Recombinant human procarboxypeptidase A (2856-ZN) was supplied by R&D Systems. Carboxypeptidase B from porcine pancreas (10103233001) was supplied by Roche. Trypsin (T4799), human recombinant procathepsin K (SRP6001), leucine aminopeptidase from porcine kidney (L5006), human recombinant dipeptidylpeptidase-IV (D3446) and chymotrypsin from bovine pancreas (C4129) were supplied by Sigma. γ-glutamyltranspeptidase from bovine kidney (46557003) was supplied by Oriental Yeast Co. Ltd. Activation of procarboxypeptidase A by trypsin was performed by diluting procarboxypeptidase A to 100 μg/mL with 1 μg/mL trypsin in phosphate-buffered saline (pH 7.4) followed by incubation at room temperature for 1 h. Activation of procathepsin K was performed by adjusting the pH to 4.0 by adding an equal volume of 100 mM NaOAc pH 3.9, 10 mM DTT, 5 mM EDTA followed by incubation at room temperature for 40 min.

Enzyme Assay. Unless otherwise mentioned, enzyme assay was performed in phosphate-buffered saline (pH 7.4) containing 0.01% CHAPS at 25 °C. Half area 384-well plates (Corning 3677) were used for the assay (20 μL reaction volume). Fluorescence was detected with a plate reader, EnVision 2103 Multilabel Reader (PerkinElmer), with appropriate filter settings. Activity was calculated according to the following formula:

$$\text{activity} = (\text{fluorescence increase rate})/(\text{fluorescence intensity of product in enzyme or pancreatic juice} - \text{fluorescence intensity of probe just after addition of enzyme or pancreatic juice})/(\text{protein concentration})$$

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Kinetic Assay. Enzyme reaction for kinetic study was performed in 20 μL of phosphate-buffered saline (pH 7.4) containing 0.01% CHAPS and 0.1% DMSO as a cosolvent using 200 ng proCPA activated by trypsin before addition or CPB at 37 °C. Clear 384-well plates (Nunc 262160) were used for the assay. Initial velocity, calculated from the change in absorbance at 530 nm, was plotted against substrate concentration. Absorbance was measured with a plate reader, SH-9000 (Corona Electric Co. Ltd.). The results were fitted to the Michaelis−Menten equation for calculating the apparent kinetic parameters

$$V = V_{max}[S]/(K_m+[S])$$

where V = initial velocity and [S] = substrate concentration.

Collection of Clinical Samples. Collection of pancreatic juice or abdominal dropsy from patients was conducted with the approval of the Institutional Ethics Review Board of the University of Tokyo. This study is registered in the UMIN Clinical Trials Registry (registration number: UMIN000003654; http://www.umin.ac.jp/ctr/index.htm). Informed consent was obtained from all patients. After collection, the samples were stored at −80 °C.

***Ex Vivo* Imaging of Pancreatic Juice Leakage.** All procedures were approved by the Animal Care and Use Committee of the University of Tokyo. C57BL/6JJcl mice (female, 8-week-old) were purchased from CLEA Japan, Inc. Mice were euthanized with isoflurane, and tissues including liver, stomach, pancreas, intestine, and spleen were resected. Before or after cutting of the pancreas, 50 μM diClHMRBC-CONH-Arg in phosphate-buffered saline (pH 7.4) containing 1% DMSO as a cosolvent with 20 μg trypsin as an activator of proCPB (100 μL) was applied onto the pancreas. Fluorescence images were captured with a Maestro In-Vivo Imaging System (CRi, Inc.) equipped with an excitation filter set at 523 nm (503−548 nm) and a 560 nm emission long path filter.

Preparation of Mouse Tissue Lysate. Black mice (C57BL/6JJcl, male, 7−8 weeks old) were euthanized, and tissues were collected and placed in 1.5 mL plastic tubes. The tubes were freeze-dried in liquid nitrogen immediately after collection and kept at −80 °C. To prepare the lysate, freeze-dried tissues (100−200 mg) were placed in a glass homogenizer, and 2× volume of phosphate buffer (pH 7.4, containing $CaCl_2$ and $MgCl_2$) was added. The tissues were homogenized, and the homogenate was placed in 1.5 mL plastic tubes and centrifuged (3000 rpm × 10 min at 4 °C). The supernatant was collected as tissue lysate. The lysates were aliquoted and stored at −80 °C.

Computation Details.[40−45] Time-dependent density functional theory (TDDFT) calculations were performed at the CAM-B3LYP40−44 level as implemented in Gaussian 0945. The 6-31+G(d) basis set was used for all atoms. The number of imaginary frequencies was 0 for all structures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b11014.

Synthesis, experimental details, and photophysical properties of the compounds (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Skidgel, R. A.; Erdös, E. G. *Immunol. Rev.* **1998**, *161*, 129.
(2) Whitcomb, D. C.; Lowe, M. E. *Dig. Dis. Sci.* **2007**, *52*, 1.
(3) Skeggs, L. T.; Marsh, W. H.; Kahn, J. R.; Shumway, N. P. *J. Exp. Med.* **1954**, *99*, 275.
(4) Patchett, A.; Harris, E.; Tristram, E.; Wyvratt, M.; Wu, M.; Taub, D.; Peterson, E.; Ikeler, T.; Ten Broeke, J.; Payne, L. *Nature* **1980**, *288*, 280.
(5) Matsugi, S.; Hamada, T.; Shioi, N.; Tanaka, T.; Kumada, T.; Satomura, S. *Clin. Chim. Acta* **2007**, *378*, 147.
(6) Fernández, D.; Pallarès, I.; Vendrell, J.; Avilés, F. X. *Biochimie* **2010**, *92*, 1484.
(7) Chang, S. S.; Reuter, V. E.; Heston, W.; Bander, N. H.; Grauer, L. S.; Gaudin, P. B. *Cancer Res.* **1999**, *59*, 3192.
(8) Lapidus, R. G.; Tiffany, C. W.; Isaacs, J. T.; Slusher, B. S. *Prostate* **2000**, *45*, 350.
(9) Li, Y.; Li, Y.; Chen, T.; Kuklina, A. S.; Bernard, P.; Esteva, F. J.; Shen, H.; Ferrari, M.; Hu, Y. *Clin. Chem.* **2014**, *60*, 233.
(10) Denis, C. J.; Lambeir, A.-M. *Expert Opin. Ther. Targets* **2013**, *17*, 265.
(11) Galjart, N. J.; Morreau, H.; Willemsen, R.; Gillemans, N.; Bonten, E.; d'Azzo, A. *J. Biol. Chem.* **1991**, *266*, 14754.
(12) Deiteren, K.; Surpateanu, G.; Gilany, K.; Willemse, J. L.; Hendriks, D. F.; Augustyns, K.; Laroche, Y.; Scharpé, S.; Lambeir, A.-M. *Biochim. Biophys. Acta, Proteins Proteomics* **2007**, *1774*, 267.
(13) Peterson, L. M.; Holmquist, B.; Bethune, J. *Anal. Biochem.* **1982**, *125*, 420.
(14) Mock, W. L.; Liu, Y.; Stanford, D. J. *Anal. Biochem.* **1996**, *239*, 218.
(15) Willemse, J.; Leurs, J.; Verkerk, R.; Hendriks, D. *Anal. Biochem.* **2005**, *340*, 106.
(16) Chang, Y.-C.; Chao, P.-W.; Tung, C.-H. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3931.
(17) Ueno, T.; Nagano, T. *Nat. Methods* **2011**, *8*, 642.
(18) Komatsu, T.; Urano, Y. *Anal. Sci.* **2015**, *31*, 257.
(19) Garland, M.; Yim, J. J.; Bogyo, M. *Cell chemical biology* **2016**, *23*, 122.
(20) Ofori, L. O.; Withana, N. P.; Prestwood, T. R.; Verdoes, M.; Brady, J. J.; Winslow, M. M.; Sorger, J.; Bogyo, M. *ACS Chem. Biol.* **2015**, *10*, 1977.
(21) Onoyama, H.; Kamiya, M.; Kuriki, Y.; Komatsu, T.; Abe, H.; Tsuji, Y.; Yagi, K.; Yamagata, Y.; Aikou, S.; Nishida, M.; Mori, K.; Yamashita, H.; Fujishiro, M.; Nomura, S.; Shimizu, N.; Fukayama, M.; Koike, K.; Urano, Y.; Seto, Y. *Sci. Rep.* **2016**, *6*, 26399.
(22) Li, L.; Zhang, C.-W.; Chen, G. Y.; Zhu, B.; Chai, C.; Xu, Q.-H.; Tan, E.-K.; Zhu, Q.; Lim, K.-L.; Yao, S. Q. *Nat. Commun.* **2014**, *5*, 3276.
(23) Yamashita, S.; Sakabe, M.; Ishizawa, T.; Hasegawa, K.; Urano, Y.; Kokudo, N. *Br. J. Surg.* **2013**, *100*, 1220.
(24) Latt, S.; Auld, D.; Vallee, B. *Anal. Biochem.* **1972**, *50*, 56.
(25) Carmel, A.; Yaron, A. *Eur. J. Biochem.* **1978**, *87*, 265.

DOI: 10.1021/jacs.7b11014
J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

(26) Widholm, J. M. *Stain Technol.* **1972**, *47*, 189.
(27) Li, L.; Ge, J.; Wu, H.; Xu, Q.-H.; Yao, S. Q. *J. Am. Chem. Soc.* **2012**, *134*, 12157.
(28) Urano, Y.; Kamiya, M.; Kanda, K.; Ueno, T.; Hirose, K.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 4888.
(29) Sakabe, M.; Asanuma, D.; Kamiya, M.; Iwatate, R. J.; Hanaoka, K.; Terai, T.; Nagano, T.; Urano, Y. *J. Am. Chem. Soc.* **2013**, *135*, 409.
(30) Xie, Y.; Ge, J.; Lei, H.; Peng, B.; Zhang, H.; Wang, D.; Pan, S.; Chen, G.; Chen, L.; Wang, Y. *J. Am. Chem. Soc.* **2016**, *138*, 15596.
(31) Kawaguchi, M.; Okabe, T.; Okudaira, S.; Hanaoka, K.; Fujikawa, Y.; Terai, T.; Komatsu, T.; Kojima, H.; Aoki, J.; Nagano, T. *J. Am. Chem. Soc.* **2011**, *133*, 12021.
(32) Mizukami, S.; Kikuchi, K.; Higuchi, T.; Urano, Y.; Mashima, T.; Tsuruo, T.; Nagano, T. *FEBS Lett.* **1999**, *453*, 356.
(33) Blum, G.; Mullins, S. R.; Keren, K.; Fonovic, M.; Jedeszko, C.; Rice, M. J.; Sloane, B. F.; Bogyo, M. *Nat. Chem. Biol.* **2005**, *1*, 203.
(34) Kenmoku, S.; Urano, Y.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2007**, *129*, 7313.
(35) Asanuma, D.; Sakabe, M.; Kamiya, M.; Yamamoto, K.; Hiratake, J.; Ogawa, M.; Kosaka, N.; Choyke, P. L.; Nagano, T.; Kobayashi, H.; Urano, Y. *Nat. Commun.* **2015**, *6*, 6463.
(36) Iwatate, R. J.; Kamiya, M.; Urano, Y. *Chem. - Eur. J.* **2016**, *22*, 1696.
(37) Uno, S.-n.; Kamiya, M.; Yoshihara, T.; Sugawara, K.; Okabe, K.; Tarhan, M. C.; Fujita, H.; Funatsu, T.; Okada, Y.; Tobita, S.; Urano, Y. *Nat. Chem.* **2014**, *6*, 681.
(38) Lee, J. H.; Hwang, D. W.; Lee, S. Y.; Hwang, J. W.; Song, D. K.; Gwon, D. I.; Shin, J. H.; Ko, G.-Y.; Park, K.-M.; Lee, Y.-J. *Planta Med.* **2012**, *78*, 309.
(39) Sakamoto, Y.; Shimada, K.; Esaki, M.; Kajiwara, T.; Sano, T.; Kosuge, T. *Journal of the American College of Surgeons* **2007**, *204*, 334.
(40) Lee, C.; Yang, W.; Parr, R. G. *Phys. Rev. B: Condens. Matter Mater. Phys.* **1988**, *37*, 785.
(41) Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 1372.
(42) Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 5648.
(43) Becke, A. D. *Phys. Rev. A: At., Mol., Opt. Phys.* **1988**, *38*, 3098.
(44) Yanai, T.; Tew, D. P.; Handy, N. C. *Chem. Phys. Lett.* **2004**, *393*, 51.
(45) Carsten Hille, M. B.; Bressel, L.; Munzke, D.; Primus, P.; Löhmannsröben, H.-G.; Dosche, C. *Anal. Bioanal. Chem.* **2008**, *391*, 1871.

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J. Am. Chem. Soc. XXXX, XXX, XXX−XXX