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(54) Title: CATHEPSIN B-TARGETING PROBES

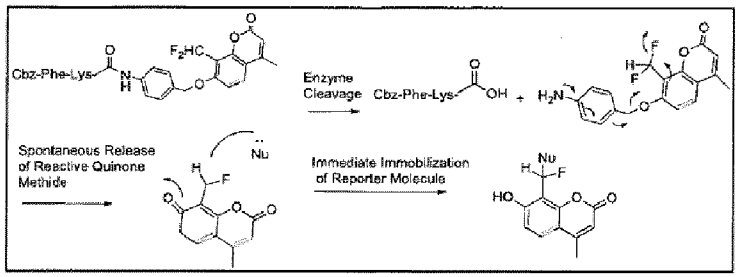

FIGURE 5

(57) Abstract: Compounds that are cathepsin B substrates and that are useful as imaging probes, for example in positron emission tomography (PET) or fluorescence imaging are described.

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CATHEPSIN B-TARGETING PROBES

Field

The present invention relates to compounds that are cathepsin B substrates. The compounds find particular use as imaging probes, for example in positron emission tomography (PET).

Background

The spread of cancer from a primary tumor to a secondary location, termed metastasis, is the cause of death in 90% of cancer patients.[1] A complex system of proteases called the proteolytic network degrades the extracellular matrix (ECM) components producing a permissive region for cancer cells to invade.[2, 3] Identifying legitimate therapeutic and diagnostic targets is challenging due to the number of different protease families operating in the tumor/tumor environment, the variety of tumor associated cell types which express and secrete proteases, and the numerous modes of enzyme regulation.

Cathepsin B (CTB, EC 3.4.22.1) is produced by a variety of tumor-associated cells and is a prominent member of the tumor promoting proteolytic network.[3-5] Mounting evidence suggests that CTB may be a potential diagnostic and prognostic biomarker for various cancers.[6-10] Interestingly, CTB appears to affect cancer progression dependent upon its localization with intracellular CTB involved in amplification of apoptosis.[11] In contrast, aggressive cancers have high extracellular CTB activity at the invading edge of a tumor and in the local ECM secreted by a variety of cell types.[12] Recent reports suggest CTB, together with the closely related enzyme cathepsin L (CTL, EC 3.4.22.15), may protect against anticancer chemotherapies such as Taxol, meaning CTB and/or CTL activity may be a marker to predict response to chemotherapy.[13] Accurate and sensitive tools capable of assessing CTB's activity in cell culture, animal models of aggressive cancer or cancer patients are needed to validate this protease as a high priority cancer marker or therapeutic target.

Discovering probes with sufficient membrane permeability, appropriate cellular localization, and rapid activation kinetics is a substantial task. Even more challenging is designing probes that are specific to a single protease due to the

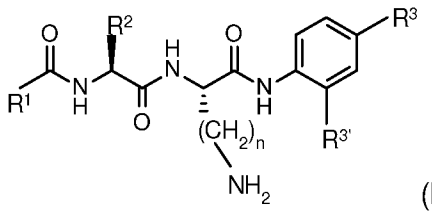
substrate overlap often observed between related and unrelated proteases. Blum et al. have developed elegant mechanism-based optical probes that covalently label CTB's active site but unfortunately lack specificity since these probes were efficiently recognized by CTL.[14] The commercially available probe $(Z\text{-}Arg\text{-}Arg)_2$-cresyl violet is an apparently CTB-specific substrate used for live cell studies[15] but fluorogenic intermediates of the hydrolysis reaction make it inappropriate for kinetic studies.[16]

Efforts here focused on CTB-specific fluorescent probes suitable for kinetic studies and live cell imaging; ideally relatively simple, cell-permeable compounds, particularly peptide-based derivatives.

Summary

One object of the invention is to provide peptide-based agents that can be cleaved by cathepsin B. Preferred compounds yield a fluorophore and/or a PET imaging agent upon cleavage by cathepsin B become that then becomes immobilized in a cellular milieu. Certain of the compounds are fluorescent while others can be labeled subsequent to immobilization.

A compound of the invention has formula (**I**):



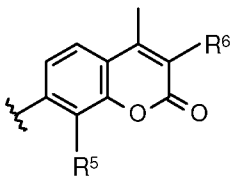
(**I**)

wherein:

R^1 is $PhCH_2O$-, CH_3 or $PhCH_2$-;

R^2 is $PhCH_2$-, $(CH_3)_2CH$- or $H_2N(CH_2)_m$- for $m \geq 4$;

R^3 and $R^{3'}$ are each H or $-CH_2OR^4$, and at least one of R^3 and $R^{3'}$ is $-CH_2OR^4$,wherein for each of R^3 and $R^{3'}$, R^4 is independently selected from the group consisting of:

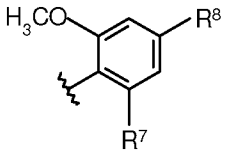


wherein:

R^5 is H, -C(O)H, -CHX_2, wherein each X is, independently of the other X, selected from the group consisting of:

H, F, Cl, Br, I, -NO_2, toluenesulfonate, methanesulfonate, trifluoromethansulfonate, perfluorobutanesulfonate, ethanesulfonate, benzenesulfonate, parachlorobenzenesulfonate, nitrobenzenesulfonate or methoxybenzenesulfonate, and both X are not simultaneously H,

R^6 is H, -C≡CH, N_3,

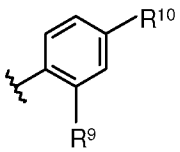


wherein:

R^7 is H, -C≡CH, N_3, -C(O)H, or -CHX_2, wherein each X is, independently of the other X, selected from the group consisting of:

H, F, Cl, Br, I, -NO_2, toluenesulfonate, methanesulfonate, trifluoromethansulfonate, perfluorobutanesulfonate, ethanesulfonate, benzenesulfonate, parachlorobenzenesulfonate, nitrobenzenesulfonate or methoxybenzenesulfonate, and both X are not simultaneously H, and

R^8 is H, -C≡CH, -C(O)H, N_3, -CHF_2, or –CH_2F,



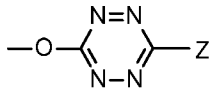
wherein each of R^9 and R^{10} is, independently of the other, H, N_3, -C(O)H, -CHX_2, wherein each X is, independently of the other X, selected from the group consisting of:

H, F, Cl, Br, I, -NO_2, toluenesulfonate, methanesulfonate, trifluoromethansulfonate, perfluorobutanesulfonate, ethanesulfonate, benzenesulfonate, parachlorobenzenesulfonate,

nitrobenzenesulfonate or methoxybenzenesulfonate, and both X are not simultaneously H,

$-CH_2Y$, wherein Y is selected from the group consisting of:

-OH, $-OC(O)NHAr^1$, wherein Ar^1 is $-C_6H_5$ in which 1, 2 or 3 hydrogen atoms is optionally and independently substituted by $-NO_2$, and



, where Z is selected from the group consisting of H, Cl, and Br.

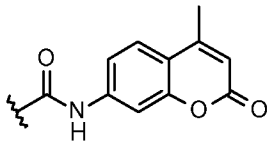
a fluorescent dye,

a radiolabelled substituent,

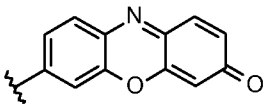
or $-C\equiv CR^{11}$ wherein R^{11} is H or Ar^2, in which Ar^2 is $-C_6H_5$ in which 1, 2 or 3 hydrogen atoms is optionally and independently substituted by F,

and

R^9 and R^{10} are not simultaneously H,



, and

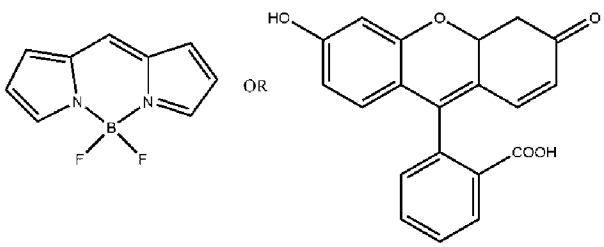


; and

$n \geq 4$,

including salts, hydrates and solvates thereof.

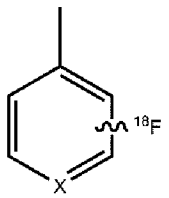
In certain embodiments, the compound includes fluorescent dye i.e., a fluorescent dye molecule is incorporated into the compound to form a part thereof, as exemplified herein. It is understood that the dye is covalently linked to the remainder of the compound. Such dyes include



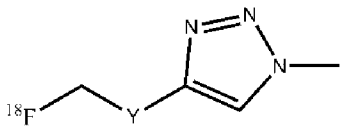
wherein one or more hydrogens bound to a carbon atom of the dye is optionally substituted with a methyl group. The structure on the left represents the borondipyrromethene (BODIPY) core. A BODIPY portion of the compound can be covalently linked at the carbon atom of the dye located at the para-position with respect to the boron atom.

In embodiments, the radiolabelled substituent comprises an ^{18}F atom. The radiolabelled substituent can be a C1-C20 alkyl group or C5-C8 aromatic group in which the aromatic ring is made up of carbon atoms or carbon and nitrogen atoms. The ring is optionally substituted with a C1-C20 alkyl or alkoxy group that is optionally substituted with said ^{18}F atom(s), and/or the ring bears one or more ^{18}F.

The radiolabelled substituent can be selected from the group consisting of:



Where X can be CH, N,



Where Y can be $(CH_2)m$ OR $O(CH_2)m$ with m = 1, 2, 3, 4, 5, 6

In embodiments, R^3 is the same as $R^{3'}$, or they are different.

The value of m and n are each typically 4, 5, 6, 7, 8, 9 or 10. The values of m and n are independent of each other.

In embodiments, the invention is a composition for use as an imaging probe, the composition including at least one compound having formula (**I**) as described above.

In embodiments, the invention is a pharmaceutical composition for use in PET and/or fluorescence imaging, the composition including at least one compound having formula (**I**) as described above. The composition can be one in which the compound(s) is dispersed or dissolved in a liquid medium suitable for injection.

The invention includes a method of PET and/or fluorescence imaging. The method includes administering to a patient in need thereof an effective amount of a composition(s) as described above, and scanning the subject with at PET and/or fluorescence imaging device. Such embodiments can be limited to a diagnostic method.

The invention includes method for studying the localization of PET probes within a tissue of a subject comprising: administering to the subject an effective amount of a compound(s) as described above, subjecting a tissue of the subject to irradiation of an electromagnetic radiation, wherein the electromagnetic radiation is absorbed by the compound, detecting fluorescence of the compound within the tissue, wherein the fluorescence of the compound within the tissue is indicative of the presence of a PET probe within the tissue.

Various embodiments of the invention are discussed throughout this specification. Any embodiment discussed with respect to one aspect of the invention applies to other aspects of the invention as well and vice versa. The embodiments in the detailed examples are understood to be embodiments of the invention that are applicable to all aspects of the invention.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

The term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive.

As used in this application, the words "comprising", "having", "including" or "containing" and any of their forms, are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

It should be noted here, that when discussing radical portions of a molecule, such as "-R", "R", etc., the connecting bond may or may not be included in various contexts for the sake of convenience, and the skilled person understands this.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various

changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Some embodiments of the invention are described, by way of example, with reference to the accompanying drawings and figures. The particulars shown are by way of example and purposes of illustrative discussion of embodiments of the invention. The description taken with drawings and figures makes apparent to the skilled person how embodiments of the invention can be practiced.

Figure 1 illustrate the general strategy for obtaining probes of the invention;

Figure 2(b) is a schematic representation of a probe employing PABA extend into the CTB S2' recognition site, and **Figure 2(c)** shows two fluorogenic peptides of the invention;

Figure 3 shows fluorescent microscope images taken of HeLa cells treated for 2 hours with **a)** 40 μM of **(3)** and **b)** 40 μM of **(4)**. Cells were treated overnight with CA-074Me and E64d followed by incubation with each probe for 2 hours. White bars represent 50 μm;

Figure 4 is a bar graph showing results obtained with HeLa cell lysates incubated with Z-Arg-Arg-AMC, compounds **3** and **4** using untreated cell lysates and cell lysates prepared from cells treated with CA-074Me or E64D. Bars represent the relative activity (%) compared to Z-Arg-Arg-AMC incubated with untreated lysates adjusted to total protein concentration minus background RFU with error bars depicting standard deviation from 4 independent trials;

Figure 5 illustrates a mechanism of cleavage of a particular agent by CTB followed by immobilization of a compound produced by said cleavage;

Figure 6 illustrates a general structure of an agent showing the CTB-targeting moiety and immobilizing compound, R^4, connected to the targeting moiety via a linker;

Figure 7 shows results obtained in a representative fluorescence microscopy experiment using the agent shown in **Figure 5**, to demonstrate the retention of the immobilized reporter molecule once activated by cathepsin B;

Figure 8 shows HPLC chromatogram of labelled bovine serum albumin (BSA) using Z-FK-PAB-CHF_2 HMC, compound **27**. One nanomolar of human Cathepsin B

was activated with 1 nM human thioredoxin. Fifty microliters of activated Cathepsin B was then added to 100 µl of 1.5 mM probe and incubated for 1 hour at 37°C in 30 mM acetate buffer at pH 5.5. Labelled BSA was derivatized with 3.0 mM $NaCNBH_3$ for 1 hour at 37°C. Fifty to twenty five microliter aliquots were injected in the HPLC, and resolved on a size-exclusion column (95% H_2O, 5% MeOH). Experiments show trapping of the QM reporter molecule by BSA once probe is activated by Cathepsin B: (**a**) BSA alone, (**b**) BSA and **27**, and (**c**) 1.0 mM **27**;

Figure 9 shows results obtained in a representative fluorescence microscopy experiment using (**a**) 1 µM compound **32**, and (**b**) 1 µM compound **33**;

Figure 10 shows images of Her2 positive breast cancer cells treated with (**a**) compound **32**, (**b**) APMA and compound **32**, (**c**) control, 4',6-diamidino-2-phenylindole (DAPI), (**d**) control, APMA and DAPI, (**e**) compound **33**, (**f**) APMA and compound **33**, (**g**) control, DAPI, and (**h**) control, APMA and DAPI. Images were taken using cells fixed with 4% paraformaldehyde;

Figure 11 shows fluorescence microscope images of breast cancer cells treated with **(a)** DAPI that stains the nucleus blue (first column), **(b)** compound **36** that is a green after activation by Cathepsin B, and **(c)** lysotracker red DND99® that stains the lysosomes. Compound **36** is activated in the lysosomes since the green fluorescence is clearly overlapping with the red lysotracker dye. In a control experiment breast cancer cells were treated with DAPI and lysotracker dye to demonstrate no green autofluorescence can be detected in cells.

Figure 12 shows results obtained under conditions described from **Figure 11**, with images taken at different time points and 20 X resolution;

Figure 13 demonstrates that the probe was specific to Cathepsin B, cells were pretreated with CA-074Me overnight prior to compound **32**.

Figure 14(a) shows an HPLC after compound **38** (100 µM) was incubated in assay buffer (30 mM acetate-NaOH, pH 5.5, 3.0 mM EDTA, 1.0 mM DTT, 10% DMSO) at 37 °C for 90 minutes. A 20 µL aliquot was injected into an HPLC fitted with a C8 column. The largest peak in the chromatogram at ~ 5 minute retention time is the intact probe clearly demonstrating chemical stability of the probe candidate. **(b)** In a second experiment, all conditions were identical to the first except 0.3 nM of activated Cathepsin B was included in the assay buffer. After 90 minutes, a 20 µL

aliquot was injected into the HPLC that showed a 40% reduction in the amount of compound **38** and new reaction product peaks formed.

Detailed Description

In one particular aspect, imaging probes having a peptide portion that provides high affinity and specificity to CTB, a self-destructive linker that spontaneously releases a reporter upon its enzymatic deprotection and a latent fluorophore which becomes highly fluorescent once enzymatically freed from the intact probe. This is illustrated generally in **Figure 1**. The prodrug linker *p*-aminobenzyl alcohol (PABA), originally reported by Katzenellenbogan,[17] can be coupled to peptides through its amino group, allowing conjugation of aniline-based fluorophores to the alcohol via a carbamate linkage. [18, 19]

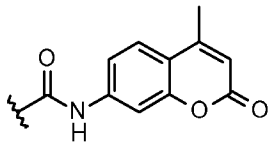
The evaluation of probe candidates typically determines the signal amplification after enzymatic activation as well as crude specificity studies by comparing the relative rates of probe turnover by competing proteases. Although these characteristics are critical in evaluating probe performance *in vitro*, such studies are largely qualitative making it difficult to compare lead probes to known substrates and existing imaging agents. In contrast, the specificity constant k_{cat}/K_M can be used quantitatively to rank structurally related probe candidates. In addition, the efficiency of lead compounds is evaluated by direct comparison to known substrates of the target protease and specificity assessed by comparing turnover rates by related proteases. Kinetic parameters may better predict probe performance in cellulo and possibly in vivo using benchmark kinetic values established by existing enzyme imaging agents used in humans.[20, 21]

Benchmark kinetic values of K_M, k_{cat} and k_{cat}/K_M for a series of two-component fluorogenic peptides using recombinant CTB and the closely related CTL were established. Previous studies showed that carboxybenzyl (Z)-protected fluorogenic dipeptides like Z-Phe-Arg-AMC are efficient substrates of CTB and CTL.[22] It is known that CTB and CTL prefer an aromatic group at P3 (corresponding to pocket S3 in **Figure 2(b**), a large hydrophobic amino acid at P2 (S2 in **Figure 2(b**) and a positively charged substituent at the P1 position (S1 in **Figure 2(b)**).[23] The compound Z-Phe-Lys-AMC **(1)** was thus prepared and characterized as substrate for

each of CTB and CTL. Although the synthesis of **1** was recently described[24] no kinetic values for this compound as a substrate for CTB or CTL are known to have been reported.

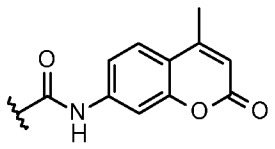
Issues of substrate specificity are common with two-component fluorogenic peptides such as Z-Phe-Arg-AMC due to efficient recognition by serine proteases.[25] However, Z-Arg-Arg-AMC is typically used for assaying CTB activity in cell lysates. The recognition of doubly cationic substrates like Z-Arg-Arg-AMC by CTB has been attributed to a glutamic acid residue at the top of the S2 binding pocket.[26] CTL lacks the equivalent Glu residue and prefers hydrophobic amino acids at the P2 position of substrates, explaining the selectivity of Z-Arg-Arg-AMC. Z-Lys-Lys-AMC **(2)** was prepared and evaluated as a substrate of CTB.

PABA derived prodrugs have been seen to be efficiently processed by CTB to release a cytotoxic drug.[27] This suggests that PABA can be tolerated at S1'. In addition, three-component peptides may offer higher specificity towards CTB because they occupy each of the core substrate binding positions S3 to S2' (**Figure 2(b)**.[28] To evaluate the efficiency of CTB probes bearing the PABA spacer, Z-Phe-Lys-PABA-AMC (**3**) and Z-Lys-Lys-PABA-AMC (**4**) compound I in which) (**Figure 2(c)**) were prepared. It is noted here that **3** is compound (**I**) in which R^1 = $PhCH_2O$-, R^2 = $PhCH_2$-, R^3 = -CH_2OR^4 in which R^4 is:



,

$R^{3'}$ = H, and n = 4: and compound **4** is compound (**I**) in which R^1 = $PhCH_2O$-, R^2 = $H_2N(CH_2)_m$-, R^3 = -CH_2OR^4 in which R^4 is:



,

$R^{3'}$ = H, m = 4, and n = 4.

As shown in **Table 1**, Z-Phe-Arg-AMC and Z-Arg-Arg-AMC were both excellent substrates of CTB. In contrast, CTL efficiently hydrolyzed Z-Phe-Arg-AMC but had 1% of CTB's relative activity towards Z-Arg-Arg-AMC. Peptide **1** was rapidly

hydrolyzed by both enzymes. In agreement with the data obtained for Z-Arg-Arg-AMC, compound **2** was an efficient substrate for CTB but a poor substrate for CTL suggesting that probes reliant on Z-Lys-Lys- may be selectively hydrolyzed by CTB. It was then determined how the PABA spacer affects recognition and turnover by CTB and CTL. The introduction of PABA into **3** and **4** resulted in a lower apparent K_M value for CTB when compared to their corresponding two component peptides. This indicates that PABA results in higher affinity towards CTB and may improve probe performance in cellulo. The lower apparent K_M value for **3** was partially offset by a lower k_{cat}, but an impressive specificity constant was maintained with $k_{cat}/K_M = 61$ mM^{-1} s^{-1} for CTB. Encouragingly, **4** sustained rapid turnover resulting in the highest specificity constant of all novel CTB substrates with $k_{cat}/K_M = 231$ $mM^{-1}s^{-1}$. For comparison purposes, ^{18}F-FHBG with a $k_{cat}/k_M = 43$ $mM^{-1}s^{-1}$ has been successfully used to image herpes simplex virus thymidine kinase activity in humans. CTL recognition of **3** suffered dramatically as no reliable kinetic data were obtained by increasing the CTL concentration to 10 nM, and **4** was also a very poor substrate of CTL as expected from the data obtained from Z-Arg-Arg-AMC and **2**.

TABLE 1

Kinetic parameters obtained for fluorogenic peptides as substrates of CTB and CTL

	Cathepsin B			Cathepsin L		
Probe	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)
Z-Phe-Arg-AMC	0.09 ± 0.01	36 ± 2	400 ± 70	$1.52 \pm 0.2 \times 10^{-3}$	0.38 ± 0.01	256 ± 36
Z-Arg-Arg-AMC	0.34 ± 0.06	56 ± 3	165 ± 37	0.024 ± 0.005	0.22 ± 0.01	9.2 ± 2.3
(**1**)	0.19 ± 0.03	26 ± 2	137 ± 13	$0.59 \pm 0.12 \times 10^{-3}$	0.39 ± 0.01	661 ± 149
(**2**)	0.25 ± 0.01	37 ± 1	148 ± 5	0.092 ± 0.001	0.14 ± 0.06	1.5 ± 0.6
(**3**)	0.082 ± 0.003	0.50 ± 0.04	61 ± 7	n.d.	n.d.	n.d.
(**4**)	0.16 ± 0.04	37 ± 2	231 ± 70	0.043 ± 0.01	0.11± 0.01	2.5 ± 0.8

All values were determined by an average of three trials ± standard deviation. Where indicated by n.d. no reliable kinetic data could be obtained using concentration of enzyme as high as 10 nM.

The activation of **3** and **4** could be visualized using fluorescent microscopy inside living HeLa cervical cancer cells[29] (see **Figure 3**). In agreement with enzyme kinetic data, **4** was the superior agent as strong fluorescence intensity was visible within 30 minutes of incubation. Similar results were observed when a Her2-positive breast cancer cell line derived from MD-MBA-231 called H2N[30] was treated with **3** and **4** (results not shown). Cell lines tested appeared to tolerate **4** based on the lack of observed changes in cell morphology, but HeLa and H2N cells incubated with **3** underwent rapid morphological changes reminiscent of cell death.

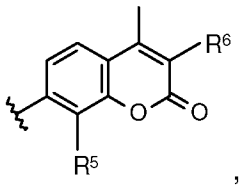
To evaluate the specificity of probe candidates towards CTB, the cell permeable, irreversible CTB inhibitor CA-074Me was used to treat both cell lines. As shown in **Figure 3**, cells inhibited with either CA-074Me or E64d were unable to efficiently activate **4** clearly demonstrating high CTB specificity of this probe in live cancer cells. In contrast, CA-074Me did not fully abolish intracellular fluorescence indicating that **3** was hydrolyzed inside the CTB-inhibited cells. However, the broad spectrum cysteine cathepsin inhibitor E64d prevented the onset of intracellular fluorescence suggesting that **3** is efficiently recognized by another cysteine

cathepsin such as Cathepsins F or S since they are E64 sensitive and known to efficiently hydrolyze Z-Phe-Arg-AMC.[31]

To determine if the enzyme kinetic values accurately predicted probe performance in a complex mixture, cell lysates were prepared from HeLa cells and incubated in the presence of Z-Arg-Arg-AMC, **3** and **4** (**Figure 4**). As predicted by the kinetic assays, probe **4** was the most efficient substrate. The relative hydrolysis of probe **3** was found to be similar to Z-Arg-Arg-AMC. To ensure that CA-074Me and E64d fully abolished the intracellular CTB activity and determine if the inhibited cells could turnover each probe, lysates were prepared from cells treated overnight with each inhibitor. CTB activity towards either Z-Arg-Arg-AMC or **4** was not observed in lysates from inhibited cells, demonstrating both inhibitors efficiently inactivated CTB and that probe **4** was highly specific towards this enzyme. In contrast, cell lysates treated with CA-074Me maintained about 35% of the original enzymatic activity towards **3** indicating hydrolysis by e.g., a competing proteases. In agreement with the live cell assays, lysates from HeLa cells treated with E64d or the cysteine protease inhibitor sodium chloroacetate were unable to hydrolyze **3** also indicating that the competing proteases are likely cysteine cathepsin enzymes.

Compound **3** was thus established to be an efficient and cell permeable substrate of CTB, not well tolerated by living cells, and activated by other cellular proteases. Compound **4** was found to be cell permeable, highly efficient probe capable of selectively detecting CTB activity in cell lysates and living cells.

The feasibility of use of a reactive quinone methide (QM) reporter molecule released after cleavage by CTB has also been established. This approach is illustrated in **Figure 5**. Compound **27** was prepared, **27** being a compound of formula (**I**) in which R^1 = $PhCH_2O$-, R^2 = $PhCH_2$-, n=4, $R^{3'}$ = H, and R^3 = -CH_2OR^4 in which R^4 is:



,

and R^5 is $–CHF_2$ and R^6 is H. Here, the agent provides a substrate moiety having high affinity to the target protease, CTB, a self-destructive linker, and QM reporter

molecule that becomes chemically activated and immobilized only after its release. Use of the self-destructive linker enables the attachment of reporter molecules to the probe via chemically and metabolically stable carbamates and ethers. Linkers can extend out of the enzyme's active site thus enabling the attachment of bulky reporter molecules maintaining low K_m values, and release of the QM outside of the enzyme active site will preserve the catalytic activity of the target enzyme resulting in amplification of signal.

In this example, the immobilized compound, containing a coumarin moiety, is fluorescent, and was found to be retained in HeLa cells. The difluoro compound can be e.g., conveniently ^{18}F-labeled making it useful as a PET imaging agent. The stable and prolonged retention of the reporter molecule that results from immobilization by nucleophilic attack by e.g., nearby proteins, permits sensitive molecular imaging studies of protease activity in living models of disease.

The substrate moiety is a dipeptide covalently linked at its carboxy-terminus to a self-immolative linker which in turn is covalently linked to a molecule to be released. The linker can be based on PABA, or it can be the *m*-substituted equivalent, or it can be both *p*- and *m*-substituted i.e., include two immobilizing molecules. The dipeptide includes either a large hydrophobic (e.g., phenylalanine) or positively charged amino acid derivative (denoted AAx) followed by (S)-lysine (Cbz-AAx-Lys) or other positively charged amino acid having side chain $–(CH_2)_nNH_2$ in which n is 4 (lysine) or greater. Preferably, n is 4, 5, 6, 7, 8, 9 or 10. In all of these cases, the conjugate acid of the side-chain amino group has a pK_a that is at least 5, so is positively charged in biological systems in which the agent is to be used i.e., in an environment having pH >5.

Figure 6 illustrates a general structure of an agent of the invention, as discussed above. The linker portion illustrated is *p*-substituted, but it can be *o*-substituted or the benzene ring can be tri-substituted having $–(CH_2)OR^4$ substituents at *o*- and *p*-positions with respect to the amino group.

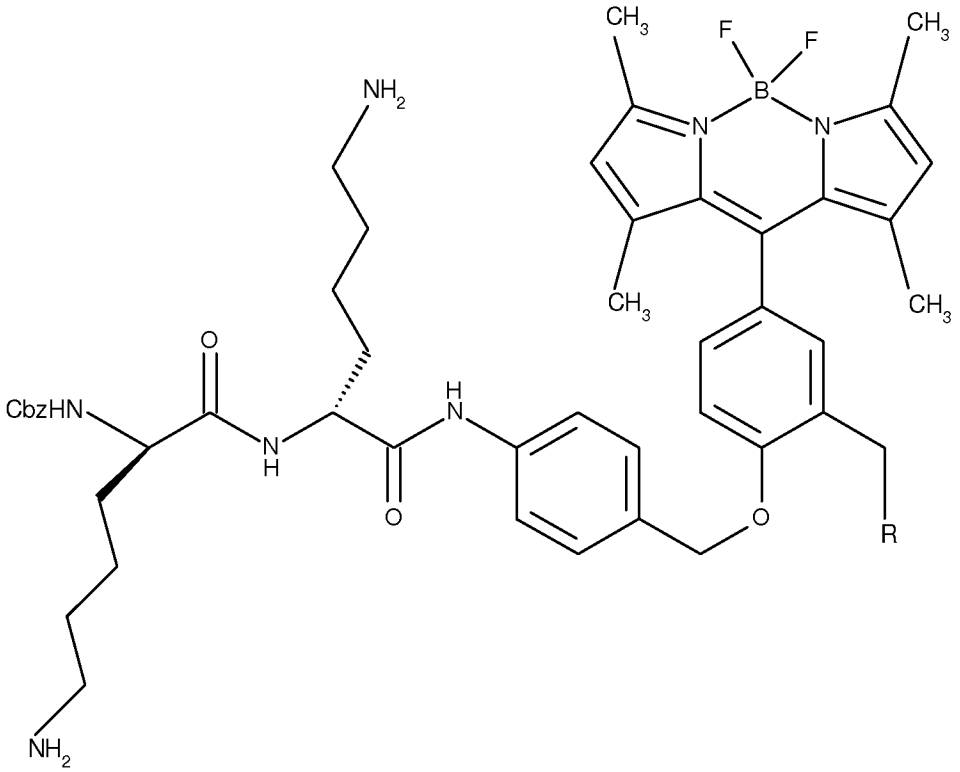
Experiments using HeLa cancer cells demonstrated that probe **27** is sufficiently cell permeable and efficiently activated by cathepsin B. Results are illustrated in **Figure 7**. It was thus demonstrated that the fluorescence was not washed out of cells while the control probe bearing 7-hydroxy-4-methyl coumarin

and incapable of chemical immobilization was readily wash out of the cells. In the experiments, the control probe was identical to the test compound but lacked the -CF_2 group (i.e. 7-hydroxy-4-methyl coumarin). Cathespin B processing released a fluorophore incapable of chemically immobilizing inside the cells and was therefore readily washed out. Further evidence of the feasibility of this approach is given by the results of **Figure 8** which show trapping of the QM reporter derived from molecule **27** by BSA once the probe is activated by Cathepsin B.

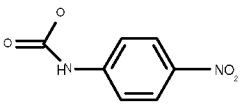
Compounds **32** and **33**, probes from which resorufin is generated upon protease cleavage were prepared. Experiments using HeLa cancer cells demonstrated that probes **32** and **33** are sufficiently cell permeable and efficiently activated by cathepsin B, and that compound **33** in particular has high specificity towards CTB in HeLa cells. See **Figure 9**. APMA is known to increase expression of Cathepsin B through protease truncation of the Her2 receptor. Dramatic increases in probe activation was observed in APMA-treated cells for compounds **32** and **33**. See **Figure 10**.

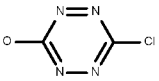
The invention thus includes embodiments that are probes which rely on a Cbz-AAx-Lys substrate (targeting) moiety in combination with the PABA self-immolative linker and a latent or activatable phenolic reporter that spontaneously produces a chemically reactive electrophile. Amino acid derivative x (AAx) can thus be an amino acid derivative having either an aromatic or amino containing groups.

Four probes cathepsin B probes **34**-**37** the incorporate the fluorophore BODIPY were produced:



34: R = F

35: R = 

36: R = 

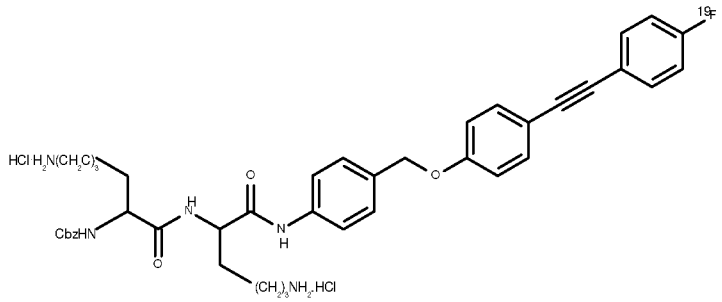
37: R = OH

Compounds **34**, **35** and **36**, once cleaved by Cathepsin B (**Figure 5**), the PABA decomposes to release BODIPY which then decomposes into a reactive quinone methide through spontaneous elimination of "HR". In the case of compound **37**, the quinone methide does not form.

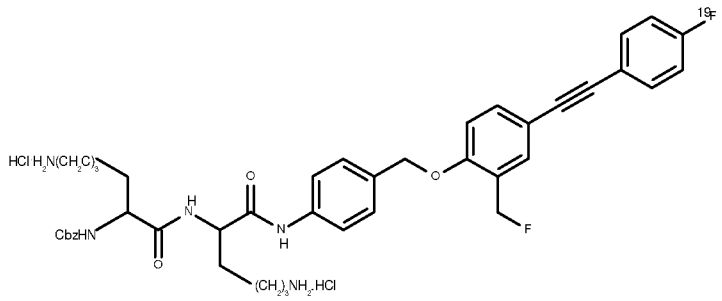
Probe **34** utilizes a fluorine as the leaving group to produce a reactive quinone methide. While compounds **35** and **36** utilize p-nitroaniline and chlorotetrazine, respectively, as a simultaneous quencher group (that turns BODIPY fluorescence off until it departs) and the leaving group to form the quinone methide. Compound **37** is

a control probe that is unable to form a reactive quinone methide. Of these compounds, **36** was found to be the most suitable for cell imaging

The invention includes specific ^{19}F-labelled probes **38** and **39**, radiolabelled so as to be useful as PET imaging agents:



38



39

In the case of compound **38**, lacking the $-CH_2F$ group, a reactive electrophile is not generated upon cleavage of the compound by Cathepsin B. The probe is thus a non-immobilizing agent. Cleavage of compound **39** by Cathepsin B produces a PABA intermediate in analogy to that shown in **Figure 5**, which decomposes to a methide electrophile that can undergo nucleophilic attack by e.g., nearby proteins and become immobilized.

Methods

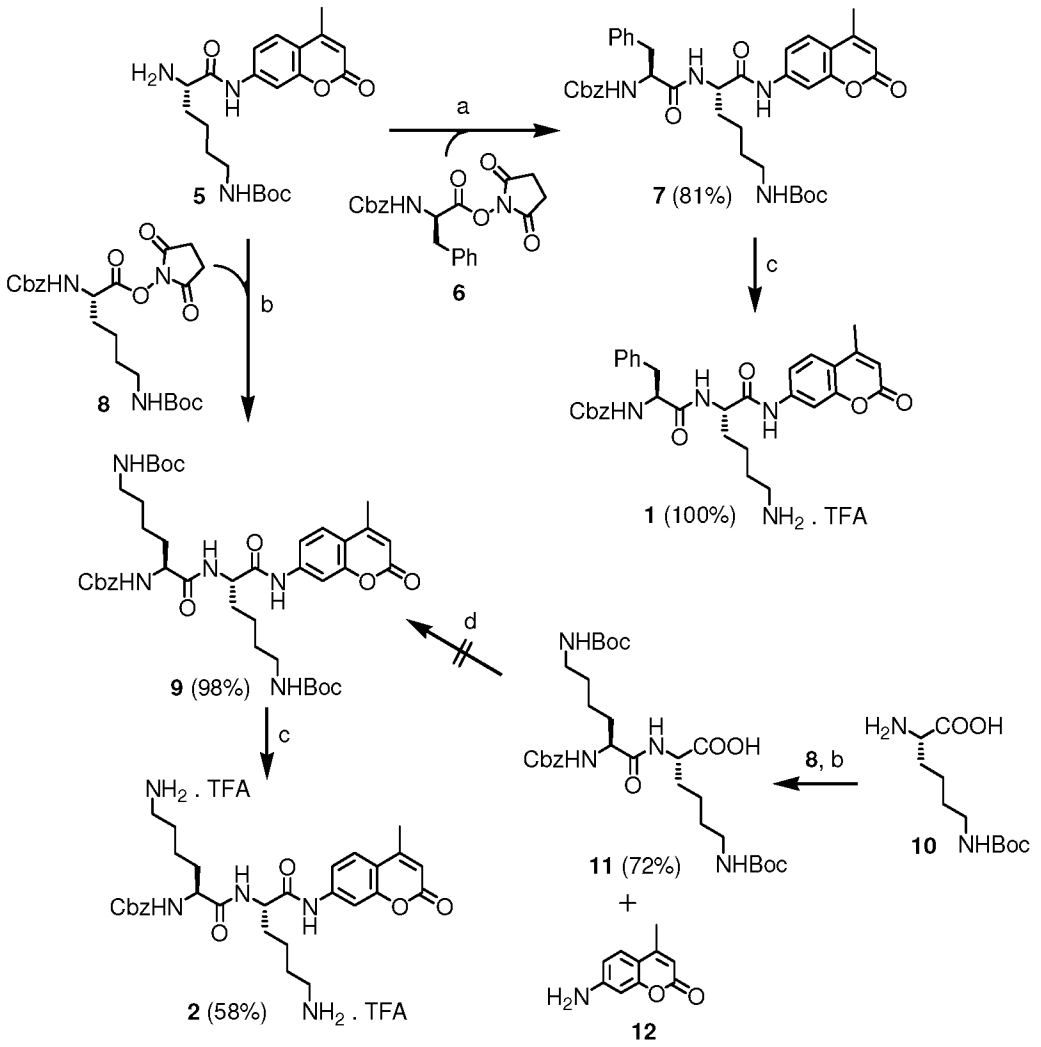
The synthesis of peptides **1 – 4,** compound characterization, the conditions for the enzyme assays and the protocol to assay the HeLa cell lysates can be found in the Supporting Information. For the fluorescence microscopy experiments, ~100,000 cells were seeded in a six well plate cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics-antimycotics incubated at 37 °C in a 5% CO_2 atmosphere. The cells were washed once with PBS and probes **3** and **4** (40 μM dissolved in Phenol Red free DMEM) were added and

incubated for 2 hours at 37 °C. Prior to florescence imaging the cells washed twice with PBS and the media replaced with Phenol Red free DMEM. Fluorescence was visualized and digitally captured using an inverted fluorescent microscope (Zeiss Axiovert 200, filter set with excitation maxima 365 nm and emission wavelength > 420 nm), digital camera attachment (QImaging QICAM Q21310), and Northern Eclipse software. For the inhibition experiments, cells were treated with CA074Me (50 μM) and E64D (50 μM) dissolved in serum free DMEM for 12 hours prior to probe addition.

Experimental

Reaction of H-Lys-*N*-ε-Boc-AMC (**5**)[32-34] with activated amino acid Cbz-Phe-OSu (**6**) in aqueous THF in presence of sodium bicarbonate[35] afforded the dipeptide Cbz-Phe-Lys-*N*-ε-Boc-AMC (**7**). Subsequent deprotection of the Boc group from **7** proceeded smoothly in a 50% TFA/CH_2Cl_2 solution at ice-bath temperature that upon removal of the solvents *in vacuo* produced compound Cbz-Phe-Lys-AMC (**1**). Similarly, Cbz-Lys-Lys-AMC (**2**) was easily prepared at gram scale levels using similar chemistry employed for the synthesis of **1** as shown in **Scheme 1**. Initially, a coupling reaction of Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-OH (**11**) which was available by the reaction of activated amino acid Cbz-Lys-*N*-ε-Boc-OSu (**8**) and H-Lys-*N*-ε-Boc-OH (**10**)[36] with 7-amino-4-methylcoumarin (AMC) (**12**) in presence of $POCl_3$ in pyridine at -15 °C following a similar literature procedure[33] did not work. Instead, coupling of **5** with **8** in DMF in presence of triethyl amine[36] proceeded smoothly to give Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε- Boc-AMC (**9**). Deprotection of Boc groups from **9** was performed using the same procedure as used for **7** to get compound **2** in pure form (**Scheme 1**).

SCHEME 1

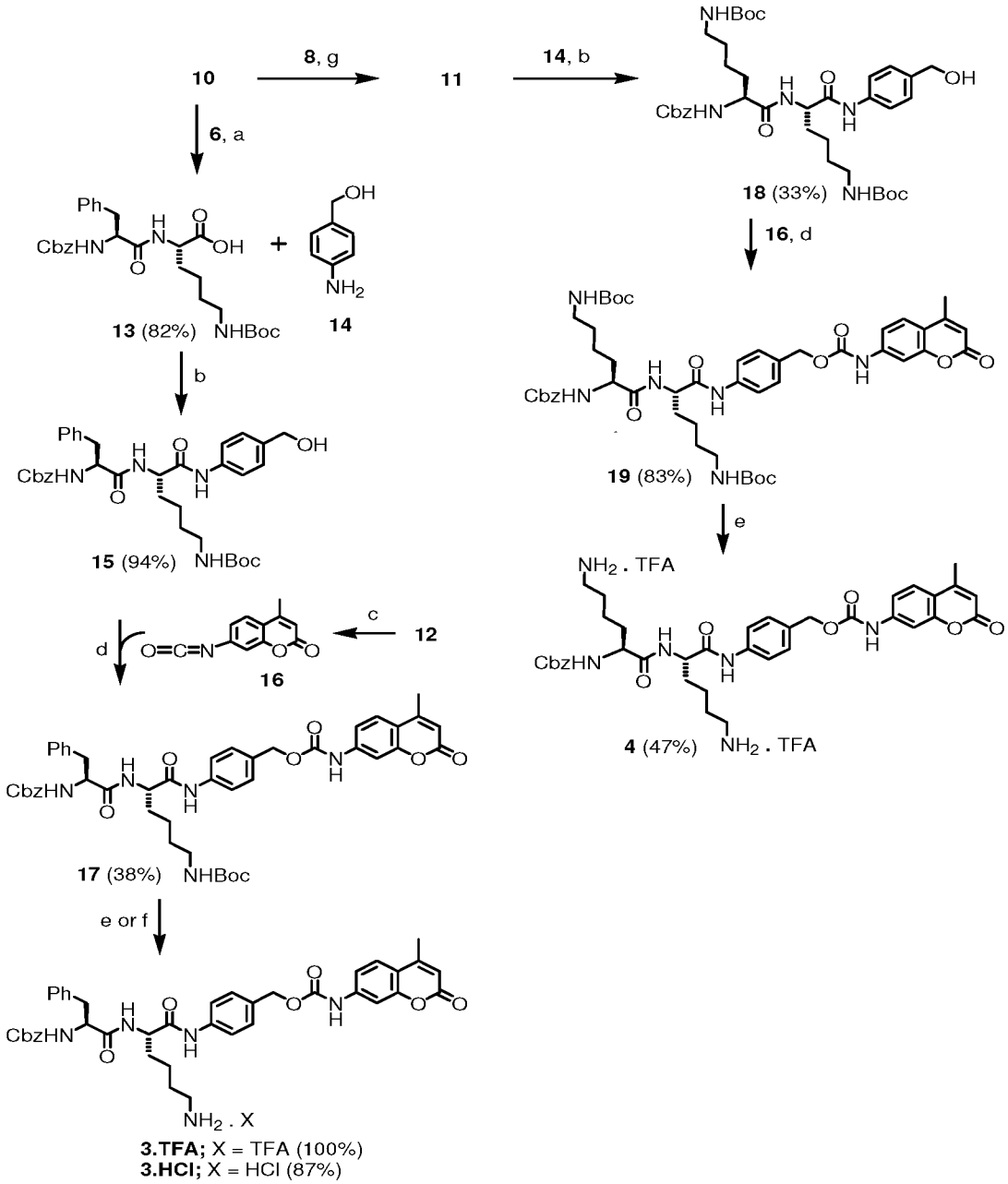


Reagents and conditions: (a) $NaHCO_3$, THF-H_2O, rt, 16 h; (b) Et_3N, DMF, 0 °C to rt, 16 h; (c) TFA-CH_2Cl_2 (1:1, v/v), ice-bath, 15 min.; (d) $POCl_3$, pyridine, -15 °C, 1 h.

The synthesis of the tripeptide was started with the reaction of **10** with **6** to get Cbz-Phe-Lys-*N*-ε-Boc-OH (**13**). Coupling of the linker *p*-aminobenzyl alcohol (PABA) (**14**) with **13** was carried out in presence of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)[37] to give tripeptide Cbz-Phe-Lys-*N*-ε-Boc-PAB-OH (**15**). Several efforts for the preparation of AMC carbamate with **15** by activating it with *p*-nitrophenyl chloroformate (PNP),[35] 1,1'-carbonyldiimidazole (CDI),[38] phosgene[39] were unsuccessful probably due to the low nucleophilicity of AMC. Efficient incorporation of AMC to yield carbamate Cbz-Phe-Lys-*N*-ε-Boc-PABC-AMC (**17**)

could only be accomplished following the reaction of **15** with 7-isocyanato-4-methylchromen-2-one (**16**) which was generated by refluxing of AMC **12** with phosgene.[40] On the other hand, Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-PAB-OH (**18**) was made by the reaction of **11** with PABA (**14**) using the same procedure as used for the synthesis of **15**. Subsequent elaboration of **18** to carbamate Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-PABC-AMC (**19**) was made following the same strategy as used for the synthesis of **17**. Finally, deprotection of Boc groups from both carbamates **17** and **19** with TFA/CH_2Cl_2 furnished TFA salts of probes Cbz-Phe-Lys-PABC-AMC (**3**) and Cbz-Lys-Lys-PABC-AMC (**4**). In addition, deprotection of Boc from **17** with methanolic-HCl proceeded well to give HCl salt of probe **3** (**Scheme 2**).

SCHEME 2

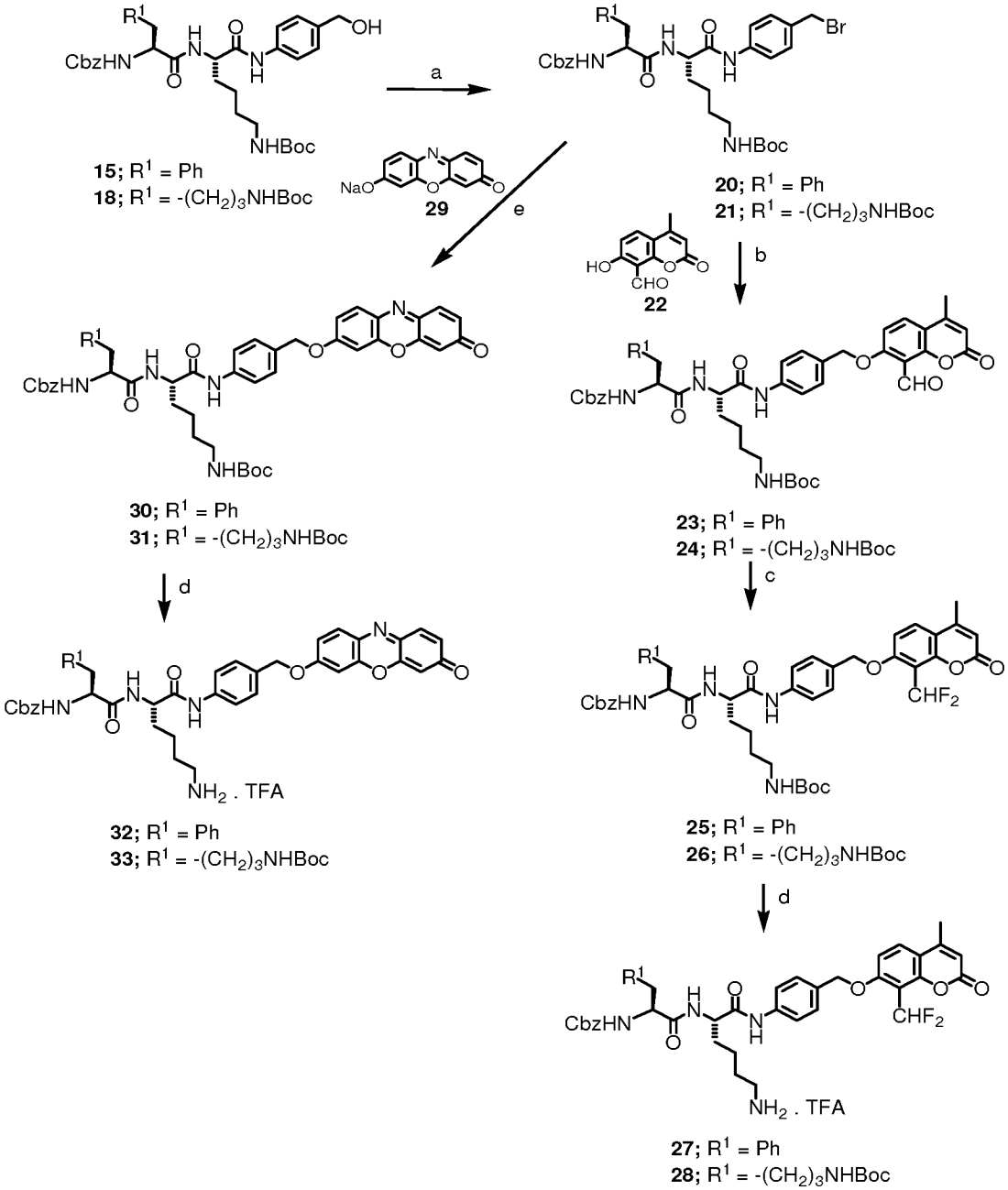


Reagents and conditions: (a) $NaHCO_3$, THF-H_2O, rt, 16 h; (b) THF, EEDQ, rt, 16 h; (c) 15% phosgene in toluene, 120°C, 16 h; (d) THF, 80 °C, 2 h; (e) TFA-CH_2Cl_2 (1:1, v/v), ice-bath, 15 min; (f) Methanolic-HCl (0.5M), rt, 16 h; (g) Et_3N, DMF, 0 °C to rt, 16 h.

Tripeptide bromides Cbz-Phe-Lys-*N*-ε-Boc-PAB-Br (**20**) and Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-PAB-Br (**21**) were efficiently made from the corresponding alcohols

15 and **18**, respectively by treating with KBr and $SOCl_2$ in presence of benzotriazole in DMF. Subsequent coupling reaction of 8-formyl-7-hydroxy-4-methylcoumarin (HMC) (**22**) with the corresponding bromides **20** or **21** proceeded well to get the respective products Cbz-Phe-Lys-*N*-ε-Boc-PAB-O-HMC (**23**) or Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-PAB-O-HMC (**24**) in good yields. Difluorination of **23** and **24** with diethylaminosulfur trifluoride (DAST) afforded Cbz-Phe-Lys-*N*-ε-Boc-PAB-O-DFHMC (**25**) and Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-PAB-O-DFHMC (**26**), respectively. Finally, deprotection of Boc groups from both HMC difluorides **25** and **26** with TFA/CH_2Cl_2 furnished TFA salts of probes Cbz-Phe-Lys-PAB-O-DFHMC (**27**) and Cbz-Lys-Lys-PAB-O-DFHMC (**28**). On the other hand, coupling reactions of bromides **20** or **21** with sodium resorufin (**29**) in DMF yielded the corresponding ether Cbz-Phe-Lys-*N*-ε-Boc-PAB-O-res (**30**) or Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-PAB-O-res (**31**), respectively. Deprotection of **30** and **31** with 50% TFA resulted the final probes **32** and **33** in high yields (**Scheme 3**).

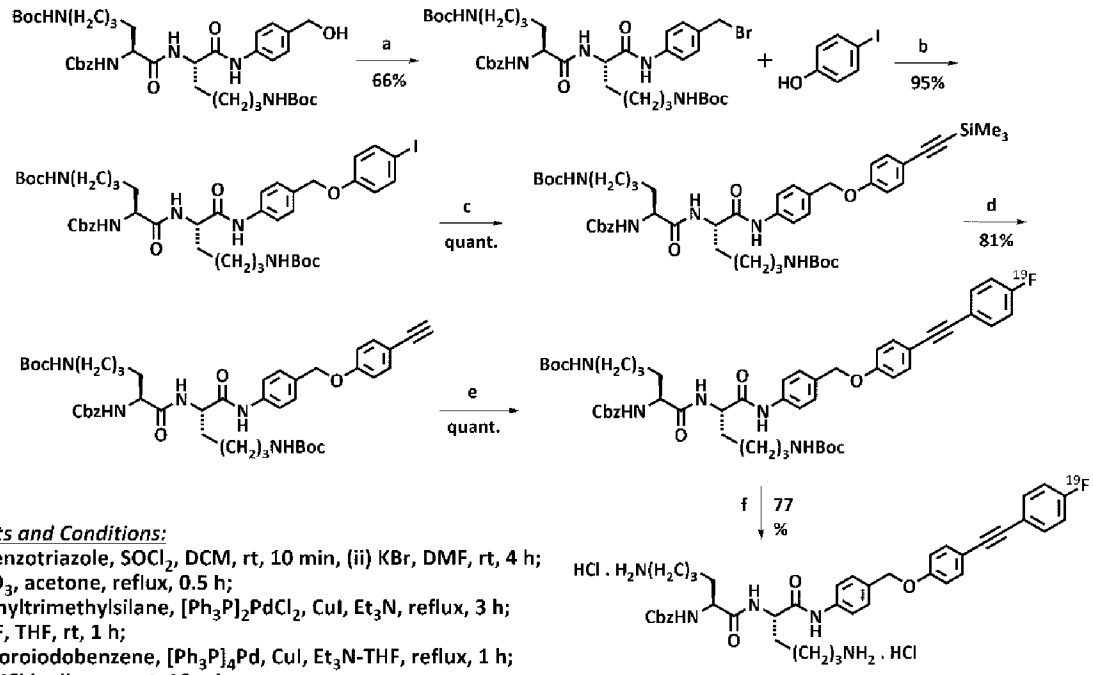
SCHEME 3



Reagents and conditions: (a) i) benzotriazole, $SOCl_2$, CH_2Cl_2, rt, 5 min; ii) KBr, DMF, rt, 4 h; (b) **22**, acetone, K_2CO_3, reflux, 0.5 h; (c) DAST, CH_2Cl_2, rt, 16 h; (d) TFA-CH_2Cl_2 (1:1, v/v), ice-bath, 15 min; (e) **29**, DMF, K_2CO_3, rt, 16 h.

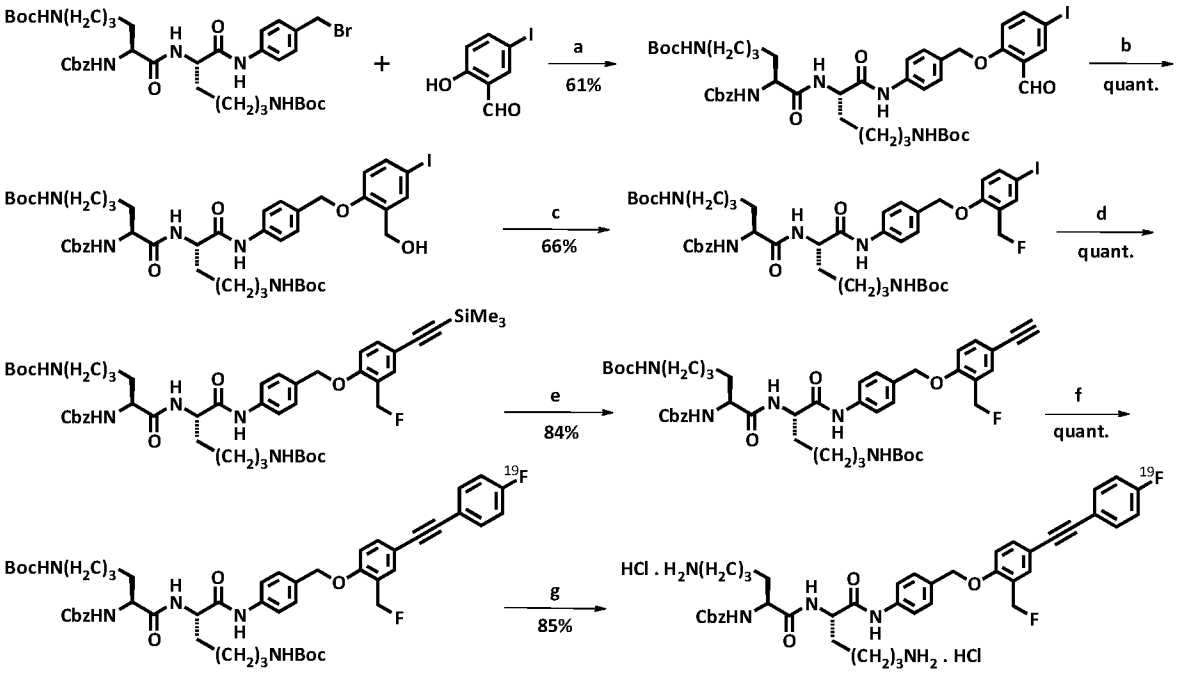
Synthetic schemes for compounds **38** and **39** are shown in **Schemes 4** and **5** respectively.

SCHEME 4



Reagents and Conditions:
(a) (i) benzotriazole, $SOCl_2$, DCM, rt, 10 min, (ii) KBr, DMF, rt, 4 h;
(b) K_2CO_3, acetone, reflux, 0.5 h;
(c) ethynyltrimethylsilane, $[Ph_3P]_2PdCl_2$, CuI, Et_3N, reflux, 3 h;
(d) TBAF, THF, rt, 1 h;
(e) 4-fluoroiodobenzene, $[Ph_3P]_4Pd$, CuI, Et_3N-THF, reflux, 1 h;
(f) 4 M HCl in dioxane, rt, 10 min.

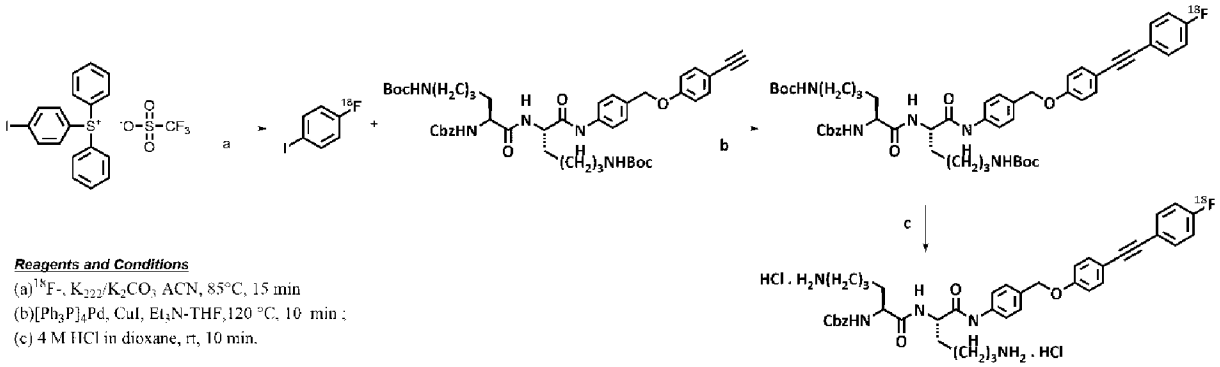
SCHEME 5



Reagents and Conditions:

(a) K_2CO_3, acetone, reflux, 0.5 h;
(b) $NaBH_4$, MeOH, rt, 2 h;
(c) (i) benzotriazole, $SOCl_2$, DCM, rt, 10 min, (ii) KF, DMF, rt, 1.5 h;
(d) ethynyltrimethylsilane, $[Ph_3P]_4Pd$, CuI, Et_3N-THF, reflux, 1.5 h;
(e) TBAF, THF, rt, 0.5 h;
(f) 4-fluoroiodobenzene, $[Ph_3P]_4Pd$, CuI, Et_3N-THF, reflux, 1 h;
(g) 4 M HCl in dioxane, 10 min.

SCHEME 6



Reagents and Conditions

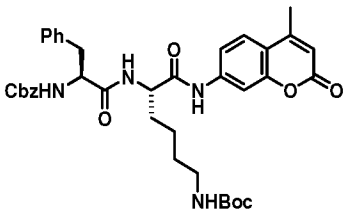
(a) ^{18}F-, K_{222}/K_2CO_3 ACN, 85°C, 15 min
(b) $[Ph_3P]_4Pd$, CuI, Et_3N-THF, 120 °C, 10 min ;
(c) 4 M HCl in dioxane, rt, 10 min.

General Remarks

^1H and ^{13}C NMR spectra were acquired in Lakehead University Instrumentation Laboratory (LUIL) on a Varian Unity Inova 500 MHz spectrometer in DMSO-d_6 with TMS as the internal standard, where *J* (coupling constant) values were estimated in hertz (Hz). Microanalyses were measured for C, H, N in LUIL using Elementar Vario

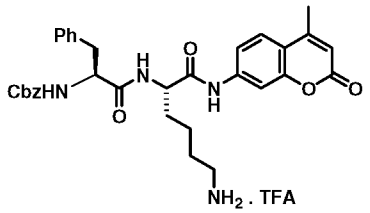
EL and were within ±0.4% of theoretical values. Thin layer chromatography (TLC) and silica gel column chromatography were performed using TLC Silica Gel 60 F_{254} (EMD) and SiliaFlash®P60 (SiliCycle), respectively. Compounds **5**,[32-34] **6**,[43] **10**,[41] **11**[36] and **13**[42] were prepared according to literature procedures. Compound **8** was prepared using the same procedure as described for **6** and known in literature.[44] All other reagents and solvents were purchased from Sigma-Aldrich, Acros Organics, Alfa Aesar, ChemTech, and Fisher, and were used without further purification.

Synthesis and Characterization of Compounds



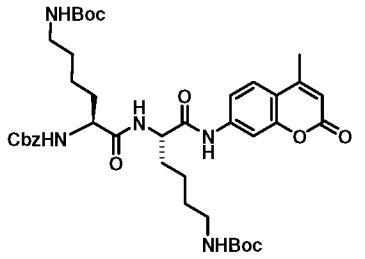
7

Cbz-Phe-Lys-*N*-ε-Boc-AMC (7): To a solution of **5** (212 mg, 0.52 mmol) in THF – H_20 (3.5:1.5 v/v, 5 mL) and $NaHCO_3$ (44 mg, 0.52 mmol) was added **6** (206 mg, 0.52 mmol) in THF (3.5 mL) and stirred at room temperature for 16 h. The reaction mixture was then concentrated *in vacuo* and extracted with ethyl acetate (15 mL x 3). The combined organic phase was washed with brine (25 mL) and dried (Na_2SO_4). After filtration, the solvent was removed from filtrate and the crude residue was purified by silica gel column chromatography using dichloromethane – methanol (9:1 v/v) as eluent to obtain the pure title compound **7**. White solid, 288 mg (81%); ^{1}H NMR (DMSO-d_6) δ 1.28-1.45 (m, 4H), 1.35 (s, 9H), 1.60-1.70 (m, 1H), 1.70-1.81 (m, 1H), 2.41 (s, 3H), 2.75 (dd, J = 10.9, 13.7 Hz, 1H), 2.83-2.95 (m, 2H), 3.05 (dd, J = 3.6, 15.3 Hz, 1H), 4.31-4.39 (m, 1H), 4.39-4.46 (m, 1H), 4.96 (s, 2H), 6.28 (d, J = 1.3 Hz, 1H), 6.79 (t, J = 5.6 Hz, 1H), 7.16-7.36 (m, 10H), 7.47-7.54 (m, 2H), 7.74 (d, J = 8.8 Hz, 1H), 7.79 (d, J = 1.9 Hz, 1H), 8.33 (d, J = 7.6 Hz, 1H), 10.52 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.5, 23.3, 28.7, 29.8, 32.2, 37.9, 54.2, 56.5, 65.7, 77.8, 106.2, 112.8, 115.6, 115.7, 126.4, 126.7, 127.3, 127.9, 128.2, 128.5, 128.8, 129.7, 137.5, 138.5, 142.7, 153.5, 154.1, 156.0, 156.4, 160.5, 171.9, 172.3. Anal. Calcd for $C_{38}H_{44}N_4O_8$: C, 66.65; H, 6.48; N, 8.18. Found: C, 66.40; H, 6.74; N, 8.10.



1

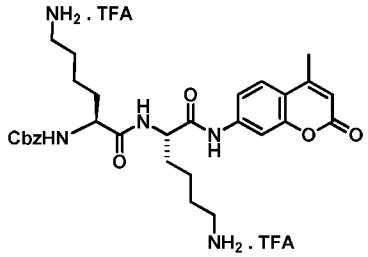
Cbz-Phe-Lys-AMC . TFA (1): To a solution of trifluoroacetic acid – DCM (1:1 v/v, 2 mL) at ice-bath temperature was added **7** (200 mg, 0.29 mmol) and stirred for 15 min. After reaction, diethyl ether was added to the reaction mixture to precipitate out the solids. Then the solids were centrifuged out and successively washed with diethyl ether (5 mL x 2) and ethyl acetate (5 mL x 2), and dried under high vacuum in dark to get the pure title compound **1**. White solid, 204mg (100%); ^{1}H NMR (DMSO-d_6) δ 1.23-1.80 (m, 6H), 2.33 (s, 3H), 2.61-2.78 (m, 3H), 2.94-3.04 (m, 1H), 4.23-4.32 (m, 1H), 4.32-4.43 (m, 1H), 4.89 (s, 2H), 6.21 (d, *J* = 1.2 Hz, 1H), 7.08-7.31 (m, 10H), 7.45 (dd, *J* = 2.1, 8.6 Hz, 1H), 7.48 (d, *J* = 8.6 Hz, 1H), 7.66 (d, *J* = 8.6 Hz, 1H), 7.70-7.86 (m, 4H), 8.38 (d, *J* = 7.6 Hz, 1H), 10.54 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.5, 22.9, 27.2, 31.8, 37.8, 45.0, 54.1, 56.5, 65.7, 106.2, 112.8, 115.6, 115.8, 117.7 (*J* = 300 Hz), 126.4, 126.7, 127.9, 128.2, 128.5, 128.8, 129.7, 137.5, 138.5, 142.6, 153.6, 154.1, 156.4, 158.9 (*J* = 31 Hz), 160.5, 171.8, 172.3. Anal. Calcd for $C_{35}H_{37}F_3N_4O_8$: C, 60.17; H, 5.34; N, 8.02. Found: C, 59.82; H, 5.52; N, 7.95.



9

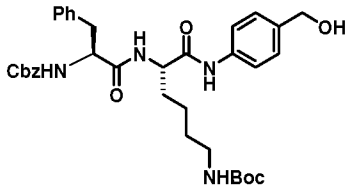
Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-AMC (9): To a solution of **8** (592 mg, 1.24 mmol) in dry DMF (5 mL) was added **5** (500 mg, 1.24 mmol) and triethylamine (173 µL, 1.24 mmol) at 0 °C and stirred for 30 min and then at room temperature for 16 h. The reaction mixture was then evaporated *in vacuo* and the residue was dissolved in ethyl acetate (20 mL) and successively washed with 1 M citric acid (10 mL x 2), brine (15 mL) and dried (Na_2SO_4). After filtration, the solvent was removed from filtrate and the crude residue was purified by silica gel column chromatography using ethyl

acetate – hexanes (4:1 v/v) as eluent to obtain the pure title compound **9**. White solid, 935 mg (98%); ^{1}H NMR (DMSO-d_6) δ 1.29-1.44 (m, 6H), 1.37 (s, 18H), 1.45-1.79 (m, 6H), 2.40 (s, 3H), 2.79-2.95 (m, 4H), 3.96-4.08 (m, 1H), 4.33-4.42 (m, 1H), 5.04 (s, 2H), 5.53- 5.61 (m, 1H), 6.28 (s, 1H), 6.77 (t, *J* = 5.0 Hz, 2H), 7.26-7.45 (m, 5H), 7.49 (dd, *J* = 2.0, 8.5 Hz, 1H), 7.72 (d, *J* = 8.5 Hz, 1H), 7.78 (d, *J* = 2.0 Hz, 1H), 8.14 (d, *J* = 7.6 Hz, 1H), 10.45 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.4, 23.2, 23.3, 24.9, 25.8, 28.7, 28.7, 29.7, 29.7, 32.1, 33.8, 54.0, 55.1, 65.9, 77.8, 77.8, 106.1, 112.8, 115.5, 115.7, 126.4, 128.2, 128.2, 128.8, 137.5, 142.6, 153.5, 154.1, 156.0, 156.5, 157.1, 160.5, 172.0, 172.7. Anal. Calcd for $C_{40}H_{55}N_5O_{10}$: C, 62.73; H, 7.24; N, 9.14. Found: C, 63.12; H, 7.10; N, 8.92.



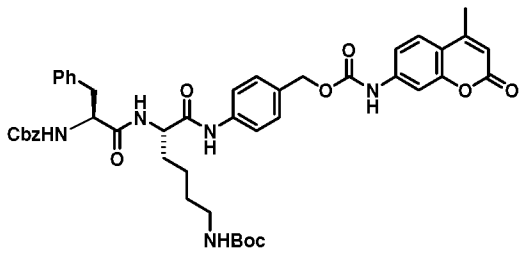
2

Cbz-Lys-Lys-AMC . 2TFA (2): To a solution of trifluoroacetic acid – DCM (1:1 v/v, 1 mL) at ice-bath temperature was added **9** (100 mg, 0.13 mmol) and stirred for 15 min. After reaction, diethyl ether was added to the reaction mixture to precipitate out the solids. Then the solids were centrifuged out and successively washed with diethyl ether (5 mL x 2) and ethyl acetate (5 mL x 2), and dried under high vacuum in dark to get the pure title compound **2**. White solid, 60 mg (58%). ^{1}H NMR (DMSO-d_6) δ 1.28-1.83 (m, 12H), 2.41 (s, 3H), 2.69-2.84 (m, 4H), 3.98-4.11 (m, 1H), 4.34-4.47 (m, 1H), 5.04 (s, 2H), 6.28 (d, *J* = 1.2 Hz, 1H), 7.27-7.56 (m, 7H), 7.67-7.94 (m, 8H), 8.23-8.34 (m, 1H), 10.59 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.5, 22.9, 22.9, 27.1, 27.2, 31.7, 31.7, 39.1, 45.0, 53.9, 54.8, 65.9, 106.2, 114.1, 115.6, 115.7, 117.7 (*J* = 300 Hz), 126.5, 128.2, 128.3, 128.8, 137.5, 142.6, 153.6, 154.1, 156.5, 158.7 (*J* = 31 Hz), 160.5, 171.9, 172.7. Anal. Calcd for $C_{34}H_{41}F_6N_5O_{10}.H_2O$: C, 50.31; H, 5.34; N, 8.63. Found: C, 50.58; H, 5.35; N, 8.79



15

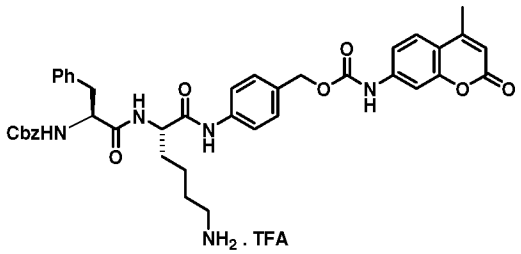
Cbz-Phe-Lys-*N*-ε-Boc-PAB-OH (15): To a solution of **13** (0.83 g, 1.57 mmol) and PABA (**14**) (232 mg, 1.89 mmol) in THF (15 mL) was added EEDQ (467 mg, 1.89 mmol) and stirred at room temperature under argon for 16 h. After reaction, solvent was removed under reduced pressure and the residue was taken into diethyl ether (25 mL) to form a suspension. The solid was filtered out and washed with diethyl ether (25 mL x 2) and dried under vacuum to get the pure title compound **15**. Pale yellow solid, 0.93 g (94%). ^{1}H NMR (DMSO-d_6) δ 1.20-1.50 (m, 4H), 1.36 (s, 9H), 1.50-1.60 (m, 1H), 1.60-1.80 (m, 1H), 2.74 (dd, *J* = 11, 13.5 Hz, 1H), 2.80-2.95 (m, 2H), 3.03 (dd, *J* = 5.5, 13.5 Hz, 1H), 4.30-4.37 (m, 1H), 4.37-4.50 (m, 1H), 4.44 (d, *J* = 18 Hz, 2H), 4.96 (s, 2H), 5.12 (t, *J* = 5.5 Hz, 1H), 6.79 (t, *J* = 5.5 Hz, 1H), 7.15-7.35 (m, 12H), 7.51 (d, *J* = 8.5 Hz, 1H), 7.56 (d, *J* = 8.0 Hz, 2H), 8.21 (d, *J* = 8.0 Hz, 1H), 10.01 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 23.2, 28.7, 29.7, 32.5, 37.9, 40.5, 53.9, 56.5, 63.1, 65.7, 77.8, 119.5, 126.7, 127.4, 127.9, 128.1, 128.5, 128.8, 128.7, 137.5, 137.9, 138.0, 138.5, 156.0, 156.3, 170.8, 172.0. Anal. Calcd for $C_{35}H_{44}N_4O_7$: C, 66.44; H, 7.01; N, 8.85. Found: C, 66.32; H, 7.23; N, 8.84.



17

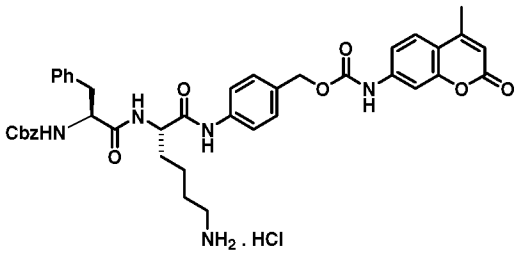
Cbz-Phe-Lys-*N*-ε-Boc-PABC-AMC (17): AMC (**12**) (0.25 g, 1.43 mmol) was taken in a 25 mL flask and a solution of 15% phosgene in toluene 10 mL was added to it. The reaction mixture was refluxed at 120 °C for 16 h under argon. After reaction, the mixture was cooled to room temperature and argon was bubbled into the solution for 10 minutes to remove excess phosgene gas. Then the solvents were removed under reduced pressure to dryness to get 7-isocyanato-4-methylchromen-2-one (**16**) as a

white powdered solid (*ca.* 0.29 g) which was used immediately without further purification. A solution of isocyanate **16** (*ca.* 0.29 g, 1.43 mmol) and **15** (0.6 g, 0.95 mmol) in dry THF (5 mL) was stirred at 80 °C under argon for 2 h. After reaction, solvent was removed under reduced pressure to get the crude product **17** which contained some unreacted isocyanate **16** and alcohol derivative **15**. To remove the isocyanate **16**, the crude product was dissolved in dichloromethane (25 mL) and washed successively with 10% aqueous HCl solution (15 mL x 3), water (15 mL), saturated aqueous $NaHCO_3$ solution (15 mL), water (15 mL) and brine (15 mL), and dried ($MgSO_4$). After filtration, the solvents were removed from the filtrate and the solid was taken in ethyl acetate (20 mL) which dissolved unreacted alcohol derivative **15** only and the product was filtered out from the slurry. Repeating the procedure two more times afforded analytically pure title compound **17** as a white solid, 0.3 g (38%). The combined filtrates were evaporated under reduced pressure to recover the alcohol derivative **15** as a pale yellow solid, 0.25 g (42%). Compound **17**: ^{1}H NMR (DMSO-d_6) δ 1.20-1.50 (m, 4H), 1.35 (s, 9H), 1.60-1.70 (m, 1H), 1.70-1.80 (m, 1H), 2.51 (s, 3H), 2.74 (dd, J = 9.5, 13.5 Hz, 1H), 2.90 (d, J = 6 Hz, 2H), 3.05 (d, J = 13.5 Hz, 1H), 4.34 (d, J = 6.5 Hz, 1H), 4.41 (d, J = 6.5 Hz, 1H), 4.96 (s, 2H), 5.14 (s, 2H), 6.24 (s, 1H), 6.78 (s, 1H), 7.15-7.35 (m, 10H), 7.40 (d, J = 8.5 Hz, 2H), 7.51 (d, J = 8.5 Hz, 1H), 7.56 (s, 1H), 7.64 (d, J = 8.5 Hz, 3H), 7.69 (d, J = 8.5 Hz, 1H), 8.23 (d, J = 7.5 Hz, 1H), 10.14 (s, 1H), 10.27 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.4, 23.2, 28.7, 29.8, 32.4, 37.9, 45.0, 54.0, 54.0, 56.5, 65.7, 66.5, 77.8, 104.9, 104.9, 112.4, 114.7, 114.8, 119.6, 126.5, 126.7, 127.9, 128.1, 128.5, 128.7, 129.6, 129.7, 131.4, 137.5, 138.5, 139.4, 143.2, 153.6, 153.7, 154.3, 156.0, 156.3, 156.3, 160.5, 171.1, 172.0. MS $(M + Na)^+$ 856. Anal. Calcd for $C_{46}H_{51}N_5O_{10}.1/2H_2O$: C, 65.54; H, 6.22; N, 8.30. Found: C, 65.19; H, 6.27; N, 8.14.



3.TFA

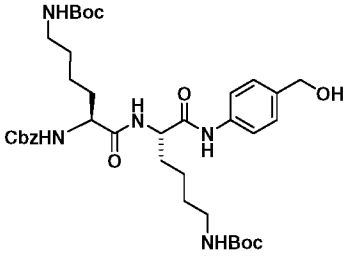
Cbz-Phe-Lys-PABC-AMC . TFA (3.TFA): To a solution of trifluoroacetic acid – DCM (1:1 v/v, 2 mL) at ice-bath temperature was added **17** (200 mg, 0.24 mmol) and stirred for 15 min. After reaction, diethyl ether was added to the reaction mixture to precipitate out the solids. The solids were centrifuged out and successively washed with diethyl ether (5 mL x 2) and ethyl acetate (5 mL x 2), and dried under high vacuum in dark to get the pure title compound **3.TFA**. White solid, 204 mg (100%). ^{1}H NMR (DMSO-d_6) δ 1.30-1.84 (m, 6H), 2.39 (s, 3H), 2.73-2.83 (m, 3H), 3.05 (d, *J* = 9.9 Hz, 1H), 4.30-4.37 (m, 1H), 4.40-4.47 (m, 1H), 4.95 (s, 2H), 5.15 (s, 2H), 6.25 (s, 1H), 7.17-7.33 (m, 10H), 7.41 (d, *J* = 8.5 Hz, 3H), 7.53 (d, *J* = 8.9 Hz, 1H), 7.57 (bs, 1H), 7.66 (d, *J* = 8.9 Hz, 2H), 7.70 (d, *J* = 8.0 Hz, 1H), 7.78 (bs, 3H), 8.34 (d, *J* = 8.0 Hz, 1H), 10.21 (s, 1H), 10.29 (s, 1H). ^{13}C NMR (DMSO-d_6) 18.0, 22.4, 26.8, 31.6, 37.4, 38.7, 53.4, 56.1, 65.3, 66.0, 104.5, 111.9, 114.3, 114.4, 119.2, 126.0, 126.3, 127.5, 127.7, 128.1, 128.3, 129.2, 129.3, 131.1, 137.0, 138.1, 138.9, 142.8, 153.2, 153.3, 153.9, 155.9, 160.1, 170.6, 171.8. Anal. Calcd for $C_{43}H_{44}F_3N_5O_{10}.1/2H_2O$: C, 60.27; H, 5.29; N, 8.17. Found: C, 60.08; H, 5.20; N, 7.97.



3.HCl

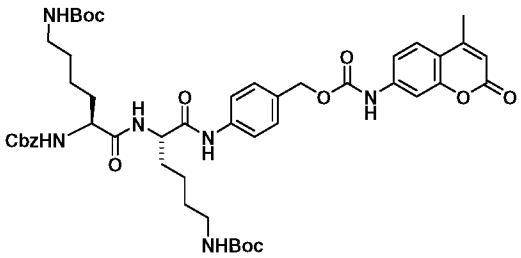
Cbz-Phe-Lys-PABC-AMC . HCl (3.HCl): A solution of **17** (100 mg, 0.12 mmol) in methanolic-HCl (0.5M, 5 mL) was stirred at room temperature for 16 h. After reaction, solvents were removed *in vacuo* to dryness and the residue was taken in diethyl ether and stirred for 10 min. The suspension was centrifuged, the supernatant was discarded, and the process was repeated twice to get the pure title compound **3.HCl**. White solid, 80 mg (87%). ^{1}H NMR (DMSO-d_6) δ 1.31-1.52 (m, 2H), 1.52-1.86 (m, 4H), 2.38 (s, 3H), 2.65-2.84 (m, 3H), 2.99-3.12 (m, 1H), 4.28-4.40 (m, 1H), 4.40-4.51 (m, 1H), 4.95 (s, 2H), 5.14 (s, 2H), 6.23 (s, 1H), 7.16-7.36 (m, 10H), 7.37-7.46 (m, 3H), 7.51-7.60 (m, 2H), 7.66-7.73 (m, 3H), 8.03 (bs, 3H), 8.43 (d, *J* = 8.3 Hz, 1H), 10.31 (s, 1H), 10.37 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.5, 22.8, 27.0, 31.9, 37.8, 38.8, 53.9, 56.6, 65.7, 66.5, 105.0, 112.4, 114.7, 114.8, 119.6, 126.5, 126.7, 127.9,

128.1, 128.5, 128.8, 129.6, 129.7, 131.4, 138.6, 139.5, 143.2, 153.7, 154.3, 156.4, 160.5, 171.1, 172.2. Anal. Calcd for $C_{41}H_{44}ClN_5O_8.2H_2O$: C, 61.07; H, 6.00; N, 8.69. Found: C, 60.85; H, 5.79; N, 8.69.



18

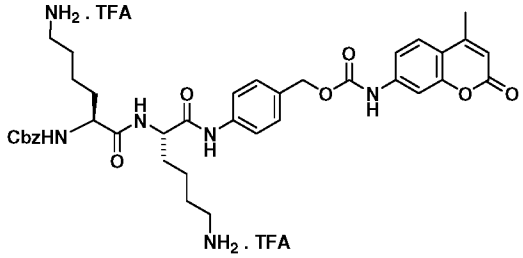
Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-PAB-OH (18): To a solution of **11** (3.71 g, 6.10 mmol) and PABA (**14**) (0.9 g, 7.32 mmol) in THF (50 mL) was added EEDQ (1.81 g, 7.32 mmol) and stirred at room temperature under argon for 16 h. After reaction, solvent was removed under reduced pressure and the residue was taken into diethyl ether (50 mL) to form a suspension. The solid was filtered out and washed with diethyl ether (50 mL x 2) and dried under vacuum to get the pure title compound **18**. White solid, 1.42 g (33%). ^{1}H NMR (DMSO-d_6) δ 1.14-1.75 (m, 12H), 1.36 (s, 9H), 1.37 (s, 9H), 2.88 (bs, 4H), 3.95-4.05 (m, 1H), 4.31-4.40 (m, 1H), 4.44 (d, *J* = 5.6 Hz, 2H), 5.04 (s, 2H), 5.12 (t, *J* = 5.6 Hz, 1H), 6.77 (t, *J* = 5.6 Hz, 2H), 7.24 (d, *J* = 8.0 Hz, 2H), 7.28-7.40 (m, 4H), 7.43 (d, *J* = 8.0 Hz, 1H), 7.55 (d, *J* = 8.0 Hz, 2H), 8.05 (d, *J* = 8.0 Hz, 1H), 9.96 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 23.2, 23.3, 28.7, 29.7, 29.7, 32.1, 32.4, 53.8, 55.2, 63.1, 65.9, 77.8, 119.4, 127.3, 128.2, 128.2, 128.8, 137.5, 137.9, 138.0, 156.0, 156.0, 156.5, 170.9, 172.5. Anal. Calcd for $C_{37}H_{55}N_5O_9.1/2H_2O$: C, 61.48; H, 7.81; N, 9.69. Found: C, 61.50; H, 7.84; N, 9.79.



19

Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-PABC-AMC (19): A solution of isocyanate **16** (*ca.* 2.29 g, 11.43 mmol) and **18** (0.9 g, 1.26 mmol) in dry THF (80 mL) was stirred at 80 °C under argon for 2 h. After reaction, solvent was removed under reduced pressure

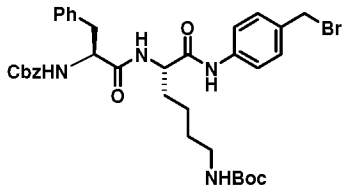
and the residue was successively suspended in diethyl ether (100 mL), stirred for 10 min and filtered, and then repeated with ethyl acetate (50 mL x 2), stirred 10 min and filtered to get the pure title compound **19**. Pale yellow solid, 0.96 g (83%). ^{1}H NMR (DMSO-d_6) δ 1.21-1.45 (m, 8H), 1.34 (s, 9H), 1.37 (s, 9H), 1.45-1.77 (m, 4H), 2.39 (s, 3H), 2.81-2.94 (m, 4H), 3.98-4.04 (m, 1H), 4.34-4.41 (m, 1H), 5.01 (s, 2H), 5.16 (s, 2H), 6.23 (d, *J* = 1.2 Hz, 1H), 6.70-6.83 (m, 2H), 7.28-7.44 (m, 9H), 7.56 (d, *J* = 1.9 Hz, 1H), 7.63 (d, *J* = 8.4 Hz, 2H), 7.69 (d, *J* = 8.8 Hz, 1H), 8.04 (d, *J* = 7.8 Hz, 1H), 10.07 (s, 1H), 10.27 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 14.5, 18.5, 21.2, 23.2, 23.3, 28.7, 28.7, 29.7, 29.7, 32.1, 32.3, 53.8, 55.2, 60.2, 65.9, 66.5, 77.8, 104.9, 112.4, 114.7, 114.8, 119.6, 126.4, 128.2, 128.2, 128.8, 129.6, 131.4, 137.5, 139.4, 143.2, 153.6, 153.7, 154.3, 156.0, 156.0, 156.5, 160.5, 171.2, 172.6. Anal. Calcd for $C_{48}H_{62}N_6O_{12}$.1/2H_2O: C, 62.39; H, 6.87; N, 9.09. Found: C, 62.57; H, 6.96; N, 8.91.



4

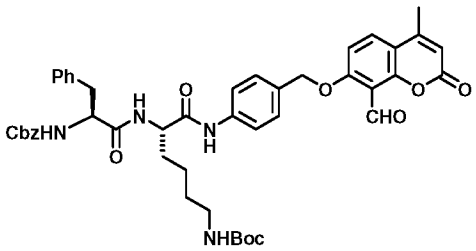
Cbz-Lys-Lys-PABC-AMC . 2TFA (4): To a solution of trifluoroacetic acid – DCM (1:1 v/v, 2 mL) at ice-bath temperature was added **19** (200 mg, 0.22 mmol) and stirred for 15 min. After reaction, diethyl ether was added to the reaction mixture to precipitate out the solids and the suspension was centrifuged, discarding the supernatant. The solids obtained were dissolved in water (2 mL) washed with ethyl acetate (5 mL x 2), and the aqueous phase was freeze dried in dark to get the pure title compound **4**. White solid, 48 mg (47%). ^{1}H NMR (DMSO-d_6) δ 1.23-1.80 (m, 12H), 2.40 (s, 3H), 2.70-2.82 (m, 4H), 4.00-4.08 (m, 1H), 4.36-4.44 (m, 1H), 5.03 (s, 2H), 5.15 (s, 2H), 6.25 (d, *J* = 1.2 Hz, 1H), 7.28-7.48 (m, 9H), 7.57 (d, *J* = 2 Hz, 1H), 7.64 (d, *J* = 8.5 Hz, 2H), 7.67-7.81 (m, 7H), 8.17 (d, *J* = 7.9 Hz, 1H), 10.15 (s, 1H), 10.28 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.5, 22.9, 22.9, 27.1, 27.2, 31.7, 32.0, 39.1, 45.0, 53.7, 54.9, 65.9, 66.4, 104.9, 112.4, 114.7, 114.8, 117.7 (*J* = 300 Hz), 119.6, 126.5, 128.2, 128.3, 128.8, 129.6, 131.5, 137.5, 139.3, 143.2, 153.7, 153.7, 154.3,

156.5, 158.7 (J = 31 Hz), 160.5, 171.1, 172.5. Anal. Calcd for $C_{42}H_{48}F_6N_6O_{12}.1/2H_2O$: C, 53.00; H, 5.19; N, 8.83. Found: C, 52.62; H, 5.34; N, 8.84.



20

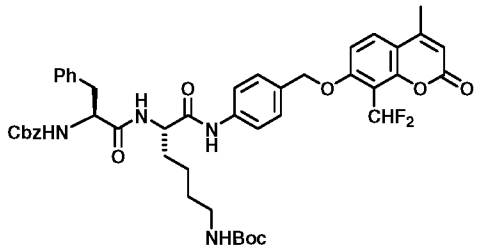
Representative Synthesis of Cbz-Phe-Lys-*N*-ε-Boc-PAB-Br (20): To a solution of **15** (3.16 g, 5 mmol) and benzotriazole (0.59 g, 5 mmol) in DCM (10 mL) was added thionyl chloride (0.4 mL, 5.50 mmol) and stirred at room temperature for 5 min. Then KBr (0.60 g, 5 mmol) followed by DMF (10 mL) were added to the reaction mixture and stirred for additional 4 hours. After reaction, water (25 mL) was added and extracted with ethyl acetate (50 mL x 2). The combined organic phase was successively washed with water (50 mL), brine (50 mL), and dried (Na_2SO_4). After filtration, the filtrate was evaporated *in vacuo,* and the solids were washed several times with ethyl acetate to get the pure compound as a white solid 2.71 g (78%). [M - Br]$^+$ 615.



23

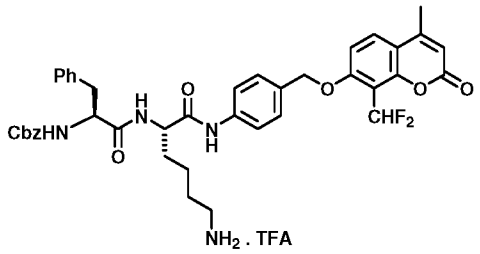
Representative Synthesis of Cbz-Phe-Lys-*N*-ε-Boc-PAB-O-HMC (23): To a solution of **20** (2.0 g, 2.88 mmol) and **22** (2.94 g, 14.40 mmol) in dry acetone (50 mL) was added anhydrous K_2CO_3 (2.0 g, 14.40 mmol) and refluxed for 0.5 h. After reaction, acetone was removed and water (25 mL) was added and extracted with ethyl acetate (50 mL x 2). The combined organic phase was successively washed with water (50 mL), brine (50 mL), and dried (Na_2SO_4). After filtration, the filtrate was evaporated *in vacuo,* and the crude product was purified by silica gel column chromatography using EtOAC : hexanes (4 :1 v/v) as eluents to get the pure title compound **23**. Pale yellow solid, 1.06 g (45%). ^{1}H NMR (DMSO-d_6) δ 1.18-1.40 (m,

13H), 1.50-1.60 (m, 1H), 1.60-1.72 (m, 1H), 2.36 (s, 3H), 2.69 (dd, *J* = 9.5, 13.5 Hz, 1H), 2.79-2.89 (m, 2H), 2.98 (d, *J* = 13.5 Hz, 1H), 4.24-4.31 (m, 1H), 4.31-4.39 (m, 1H), 4.90 (s, 2H), 5.28 (s, 2H), 6.25 (s, 1H), 6.70 (s, 1H), 7.09-7.31 (m, 11H), 7.38-7.47 (m, 3H), 7.59 (d, *J* = 8.5 Hz, 2H), 7.92 (d, *J* = 8.5 Hz, 1H), 8.15 (d, *J* = 8.5 Hz, 1H), 10.01 (s, 1H), 10.44 (s, 1H).



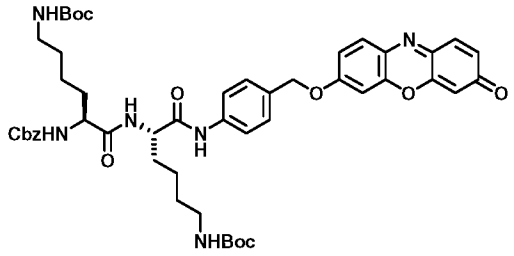
25

Representative Synthesis of Cbz-Phe-Lys-*N*-ε-Boc-PAB-O-DFHMC (25): To a solution of **23** (0.50 g, 0.61 mmol) in dry CH_2Cl_2 (8 mL) was added DAST (160 µL, 1.22 mmol) and stirred at room temperature for 16 h. The reaction mixture was quenched with ice-water and the organic phase was separated and washed with brine (5 mL), and dried ($MgSO_4$). After filtration, the filtrate was evaporated *in vacuo,* and the crude product was purified by silica gel column chromatography using EtOAC : hexanes (4 :1 v/v) as eluents to get the pure title compound **25**. Pale yellow solid, 142 mg (28%). $[M + Na]^+$ 863.



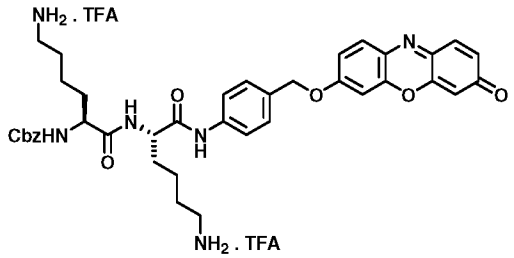
27

Representative Synthesis of Cbz-Phe-Lys-PAB-O-DFHMC . TFA (27): To a solution of trifluoroacetic acid – DCM (1:1 v/v, 0.5 mL) at ice-bath temperature was added **25** (100 mg, 0.12 mmol) and stirred for 10 min. After reaction, diethyl ether was added to the reaction mixture to precipitate out the solids. The solids were centrifuged out and washed with diethyl ether (2 mL x 2) and dried under high vacuum in dark to get the pure title compound **27**. White solid, 52 mg (51%).



31

Representative Synthesis of Cbz-Lys-*N*-ε-Boc-Lys-*N*-ε-Boc-PAB-O-res (31): To a solution of **21** (0.5 g, 0.64 mmol) and **29** (0.75 g, 3.20 mmol) in dry DMF (50 mL) was added anhydrous K_2CO_3 (0.44 g, 3.20 mmol) and stirred at room temperature for 16 h. After reaction, water (200 mL) was added and extracted with ethyl acetate (100 mL x 2). The combined organic phase was successively washed with water (100 mL x 2), brine (100 mL), and dried (Na_2SO_4). After filtration, the filtrate was evaporated *in vacuo,* and the crude product was purified by silica gel column chromatography using CH_2Cl_2 : MeOH (15 :1 v/v) as eluents to get the pure title compound **31**. Red solid, 0.35 g (60%).



33

Representative Synthesis of Cbz-Lys-Lys-PAB-O-res . 2TFA (33): To a solution of trifluoroacetic acid – DCM (1:1 v/v, 2 mL) at ice-bath temperature was added **31** (200 mg, 0.22 mmol) and stirred for 15 min. After reaction, diethyl ether was added to the reaction mixture to precipitate out the solids. The solids were centrifuged out, dissolved in water (3 mL) and washed with ethyl acetate (5 mL x 2). The aqueous part was then evaporated using freeze dryer in dark to get the pure title compound **33**. Red solid, 140 mg (68%).^{1}H NMR (DMSO-d_6) δ 1.14-1.71 (m, 12H), 2.54-2.77 (m, 4H), 3.88-4.02 (m, 1H), 4.25-4.37 (m, 1H), 4.95 (s, 2H), 5.15 (s, 2H), 6.20 (d, J = 1.9 Hz, 1H), 6.71 (dd, J = 1.9, 9.8 Hz, 1H), 7.04 (dd, J = 2.5, 8.8 Hz, 1H), 7.11 (d, J = 2.5 Hz, 1H), 7.21-7.33 (m, 5H), 7.36 (d, J = 8.2 Hz, 3H), 7.46 (d, J = 9.8 Hz, 1H),

7.57 (d, J = 8.2 Hz, 2H), 7.67 (bs, 6H), 7.71 (d, J = 8.8 Hz, 1H), 8.13 (bs, 1H), 10.10 (s, 1H). Anal. Calcd for $C_{43}H_{46}F_6N_6O_{11}.4/5H_2O$: C, C, 54.29; H, 5.04; N, 8.83. Found: C, 53.98; H, 4.88; N, 8.66.

Enzyme Kinetic Studies

To obtain the K_M, k_{cat} and k_{cat}/K_M parameters for each compound as a substrate of CTB or CTL, concentrations of fluorogenic peptide were varied between ~ 0.5 KM – 5 KM while maintaining a constant concentration of activated enzyme. The reaction mixture (150 µl final volume) consisted of 30 mM acetate-NaOH, pH 5.5, 3.0 mM EDTA, 2.0 mM DTT, 10% DMSO, and 0.33 to 3.3 nM human cathepsin B or L in 96 well black plates. The samples were pre-warmed at 37°C for 30 min, and the reaction was initiated upon addition of the enzyme. The activity was monitored spectrophotometrically for the release of 7-amino-4-methyl-coumarin (AMC; excitation 380 nm; emission 460 nm). The amount of AMC released from the reaction was determined by a standard curve in assay buffer. Incubation of the prodrug inspired probes in the assay buffer without enzyme demonstrated that the probes were stable for at least 6 hours with very little spontaneous hydrolysis of AMC as monitored by fluorescence or HPLC (data not shown). The assay to quantify active enzyme by titration with E64 was adapted from Barrett *et al.*[45] Briefly, human cathepsin enzyme (~10 nM) in assay buffer (30 mM acetate-NaOH, pH 5.5, 3.0 mM EDTA, 1.0 mM DTT) was inactivated with E64 (final concentrations ranging from 1.5 to 7.0 nM) for 20 min at 37 °C. Following the inactivation reaction, an aliquot of this mixture was added to a solution containing the substrate (30 µM z-RR-AMC or z-FK-AMC in 10% DMSO 90% assay buffer) at 37 °C. The residual enzymatic activity was monitored spectrophotometrically for the release of 7-amino-4-methyl-coumarin and plotted versus the concentration of E64 to graphically determine the amount of active enzyme.

Preparation of Cell Lysates from HeLa Cervical Cancer Cells

The cell lysates and assay were adapted from Giusti *et al.*[46]. Briefly, cells were grown to ~ 80% confluency and washed with Phenol Red free DMEM, treated with lyse buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) and centrifuged (12,000 rpm) in a 1.5 mL tube. For the CTB activity assays,

100 µL of the lysis solution was added to a black 96 well plate and diluted with 100 µL activation buffer (100 mM Acetate, pH 5.5, 5 mM DTT, 5 mM EDTA) followed by 15 minute incubation at 37 °C. A 100 µL aliquot of the activated lysate was then added to a 50 µL solution containing 300 µM probe, in 30% DMSO and 70% buffer (100 mM acetate buffer, 5 mM EDTA, pH 5.5). The enzymatic reaction proceeded at 37 °C for 1 hour after which 50 µL was withdrawn and added to 100 µL of sodium choloroacetate (200 mM) to stop the reaction. The fluorescence at 460 nm was measured and normalized to total protein concentration (Bradford Assay) and expressed as a percentage of AMC released from experiments using Z-Arg-Arg-AMC. As a control, spontaneous AMC hydrolysis was determined in assay buffer without lysate while hydrolysis by other protease classes was assessed by pre-treating lysates with sodium chloroacetate (200 mM in assay buffer). Levels of free AMC were similar in both control reactions and therefore used as a blank which was subtracted from the total RFU in each experiment.

Fluorescent Probes

MDA MB 231 breast cancer cells grown on cover slips were treated with PBS buffer (control) or compound **36** for 4h at 37°C. Results are shown in **Figures 6** to **8**. The compound turns green after Cathepsin B hydrolysis. Cells were then treated with DAPI (blue, to stain nucleus) and lysosome tracker (red) and incubated for another 30 minutes. Cells were then washed with PBS fixed with paraformaldehyde onto slides and fluorescence microscopy images were taken at 100X. The probe activation is clearly visualized in the lysosomes of cells.

Radiolabelled Probes

Turnover of compound **38,** a non-radioactive version of a potential Cathepsin B PET probe, in presence and absence of Cathepsin B was examined using HPLC. In order to visualize Cathepsin B activity *in vivo* using PET, a fluorinated probe candidate must be an efficient substrates of the enzyme. In the first experiment, compound **38** (100 µM) was incubated in assay buffer (30 mM acetate-NaOH, pH 5.5, 3.0 mM EDTA, 1.0 mM DTT, 10% DMSO) at 37 °C for 90 minutes. A 20 µL aliquot was injected into an HPLC fitted with a C8 column and detected at 254 nm. In the second experiment, Cathepsin B (0.3 nM) was included in the assay buffer.

As can be seen in **Figure 14**, the largest peak in the chromatogram at ~ 5 minute retention time corresponds to intact compound **38** clearly demonstrating high chemical stability of the probe candidate. In a second experiment, a 20 µL aliquot was injected into the HPLC showing a 40% reduction in the total amount of compound **32**. New reaction product peaks can be seen at longer retention times. This experiment clearly shows compound **38** is an efficient substrate of Cathepsin B. A series of HPLC experiments performed at varying concentrations of compound **32** allowed to estimate k_{cat}/K_M of 30,000 $mM^{-1}s^{-1}$.

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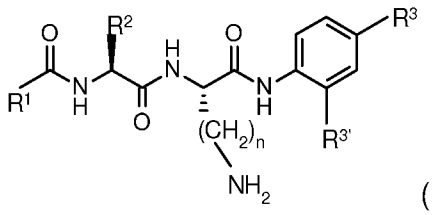
WHAT IS CLAIMED IS:

1. A compound of formula (**I**):



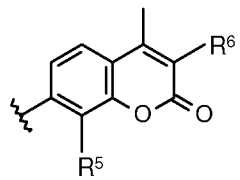
(**I**)

wherein:

R^1 is $PhCH_2O$-, CH_3 or $PhCH_2$-;

R^2 is $PhCH_2$-, $(CH_3)_2CH$- or $H_2N(CH_2)_m$- for $m \geq 4$;

R^3 and $R^{3'}$ are each H or $-CH_2OR^4$, and at least one of R^3 and $R^{3'}$ is $-CH_2OR^4$,wherein for each of R^3 and $R^{3'}$, R^4 is independently selected from the group consisting of:

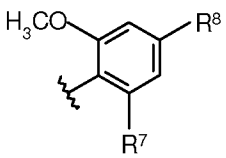


wherein:

R^5 is H, -C(O)H, $-CHX_2$, wherein each X is, independently of the other X, selected from the group consisting of:

H, F, Cl, Br, I, $-NO_2$, toluenesulfonate, methanesulfonate, trifluoromethansulfonate, perfluorobutanesulfonate, ethanesulfonate, benzenesulfonate, parachlorobenzenesulfonate, nitrobenzenesulfonate or methoxybenzenesulfonate, and both X are not simultaneously H,

R^6 is H, $-C\equiv CH$, N_3,

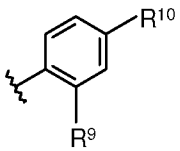


wherein:

R^7 is H, -C≡CH, N_3, -C(O)H, or -CHX_2, wherein each X is, independently of the other X, selected from the group consisting of:

H, F, Cl, Br, I, -NO_2, toluenesulfonate, methanesulfonate, trifluoromethansulfonate, perfluorobutanesulfonate, ethanesulfonate, benzenesulfonate, parachlorobenzenesulfonate, nitrobenzenesulfonate or methoxybenzenesulfonate, and both X are not simultaneously H, and

R^8 is H, -C≡CH, -C(O)H, N_3, -CHF_2, or –CH_2F,

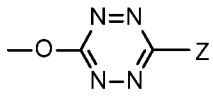


wherein each of R^9 and R^{10} is, independently of the other, H, N_3, -C(O)H, -CHX_2, wherein each X is, independently of the other X, selected from the group consisting of:

H, F, Cl, Br, I, -NO_2, toluenesulfonate, methanesulfonate, trifluoromethansulfonate, perfluorobutanesulfonate, ethanesulfonate, benzenesulfonate, parachlorobenzenesulfonate, nitrobenzenesulfonate or methoxybenzenesulfonate, and both X are not simultaneously H,

-CH_2Y, wherein Y is selected from the group consisting of:

-OH, -OC(O)NHAr^1, wherein Ar^1 is –C_6H_5 in which 1, 2 or 3 hydrogen atoms is optionally and independently substituted by –NO_2, and



, where Z is selected from the group consisting of H, Cl, and Br.

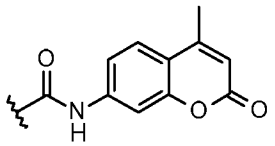
a fluorescent dye,

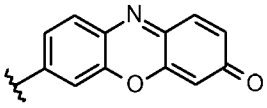
a radiolabelled substituent,

or -C≡CR^{11} wherein R^{11} is H or Ar^2, in which Ar^2 is –C_6H_5 in which 1, 2 or 3 hydrogen atoms is optionally and independently substituted by F,

and

R^9 and R^{10} are not simultaneously H,


, and

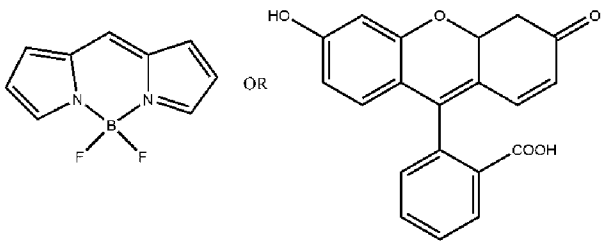

; and

n ≥ 4,

including salts, hydrates and solvates thereof.

2. A compound of claim 1, wherein the fluorescent dye is a substituent containing a borondipyrromethene (BODIPY) core.

3. A compound of claim 1, wherein said fluorescent dye is



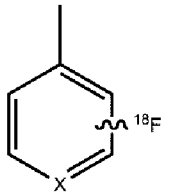
wherein one or more hydrogens bound to a carbon is optionally substituted with a methyl group.

4. A compound of claim 2, wherein the BODIPY of the compound is covalently linked at the carbon located at the para-position with respect to the boron atom.

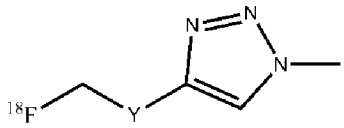
5. A compound of claim 1, wherein the radiolabelled substituent comprises an ^{18}F atom.

6. The compound of claim 5, wherein the radiolabelled substituent is a C1-C20 alkyl group or C5-C8 aromatic group consisting of carbon atoms or carbon and nitrogen atoms, and optionally substituted with a C1-C20 alkyl or alkoxy group substituted with said ^{18}F atom(s).

7. The compound of claim 6, wherein the radiolabelled substituent is selected from the group consisting of:.



Where X can be CH, N,



Where Y can be $(CH_2)m$ OR $O(CH_2)m$ with m = 1, 2, 3, 4, 5, 6

8. A compound as defined by any one of claims 1 to 7, wherein R^3 is the same as $R^{3'}$.

9. A compound as defined by any one of claims 1 to 7, wherein R^3 is the same as $R^{3'}$ are different from each other.

10. A compound as defined by any one of claims 1 to 9, wherein m = 4, 5, 6, 7, 8, 9 or 10.

11. A compound as defined by any one of claims 1 to 10, wherein n = 4, 5, 6, 7, 8, 9, or 10.

12. A composition for use as an imaging probe comprising at least one compound selected from the group consisting of compounds as defined in any of claims 1 to 11.

13. A composition as defined by claim 12, for use in PET imaging, said compound comprising ^{18}F as defined by any one of claims 1 and 5 to 11.

14. A composition as defined by claim 12, for use in fluorescence imaging, said compound comprising a fluorescent dye.

15. A composition as defined by claim 14, wherein said compound is as defined by any one of claims 2 to 4.

16. A pharmaceutical composition for use in PET and/or fluorescence imaging comprising a compound as defined in any one of claims 1 to 11, and a pharmaceutically acceptable carrier.

17. The pharmaceutical composition according to claim 16 wherein the compound is dispersed or dissolved in a liquid medium suitable for injection.

18. A method of PET and/or fluorescence imaging comprising administering to a patient in need thereof an effective amount of the composition as defined by any one of claims 12 to 18, and scanning the subject with a PET and/or fluorescence imaging device.

19. A method for studying the localization of PET probes within a tissue of a subject comprising: administering to the subject an effective amount of a compound as defined by claim 13, subjecting a tissue of the subject to irradiation of an electromagnetic radiation, wherein the electromagnetic radiation is absorbed by the compound, detecting fluorescence of the compound within the tissue, wherein the fluorescence of the compound within the tissue is indicative of the presence of a PET probe within the tissue.

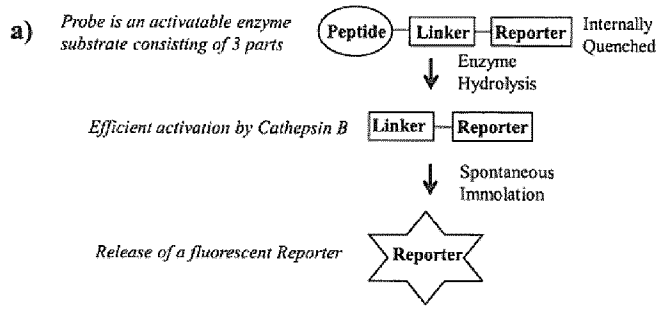
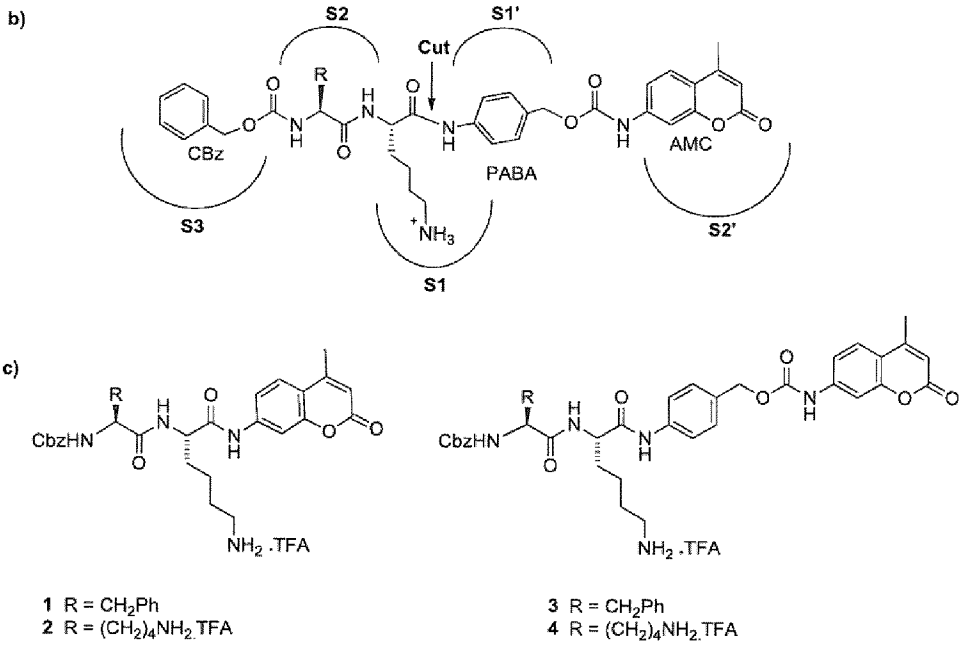


FIGURE 1



1 R = CH_2Ph
2 R = $(CH_2)_4NH_2.TFA$

3 R = CH_2Ph
4 R = $(CH_2)_4NH_2.TFA$

FIGURE 2

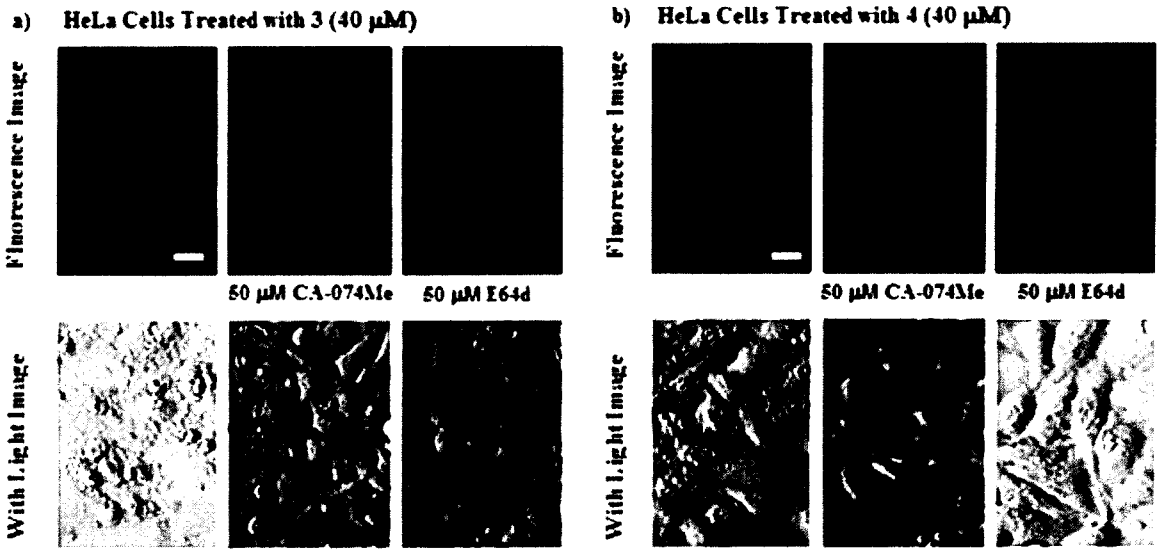


FIGURE 3

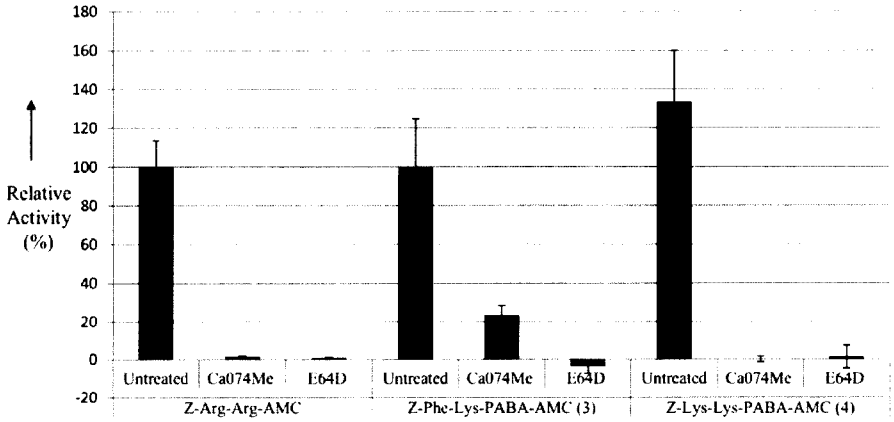


FIGURE 4

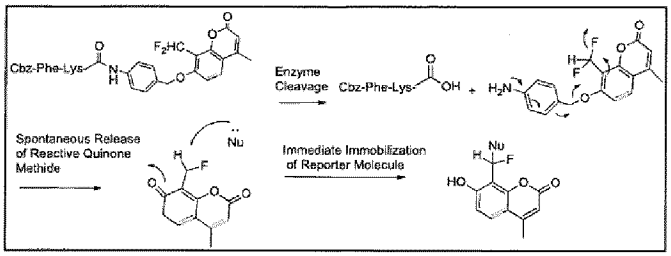


FIGURE 5

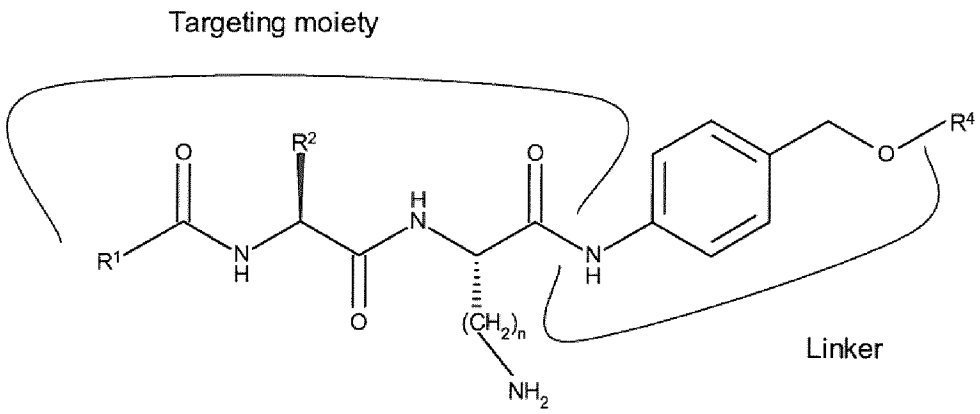


FIGURE 6

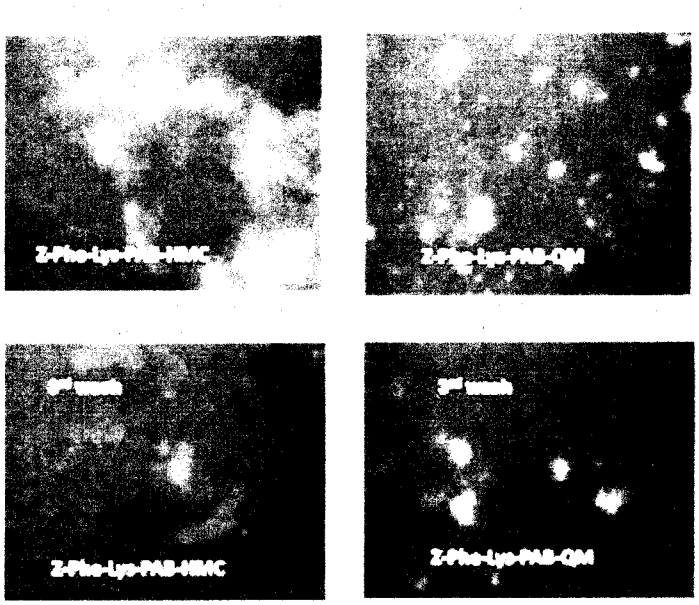
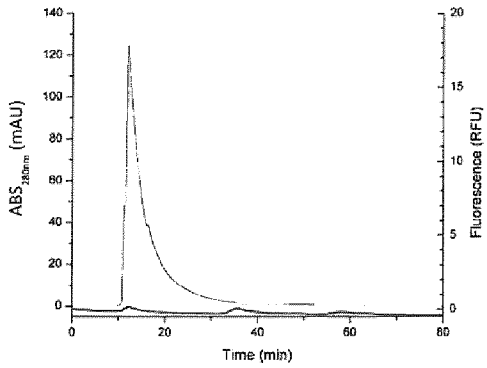
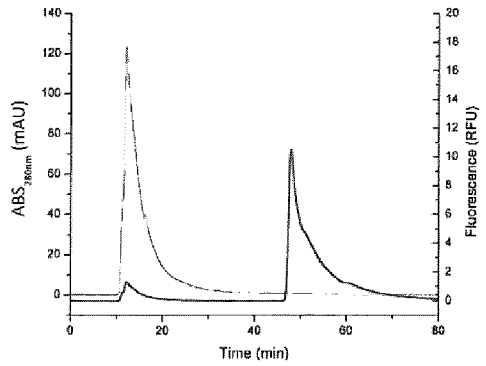


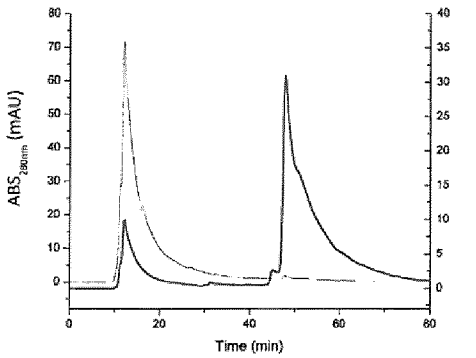
FIGURE 7


ABS280nm (mAU)
Fluorescence (RFU)
Time (min)

(a)

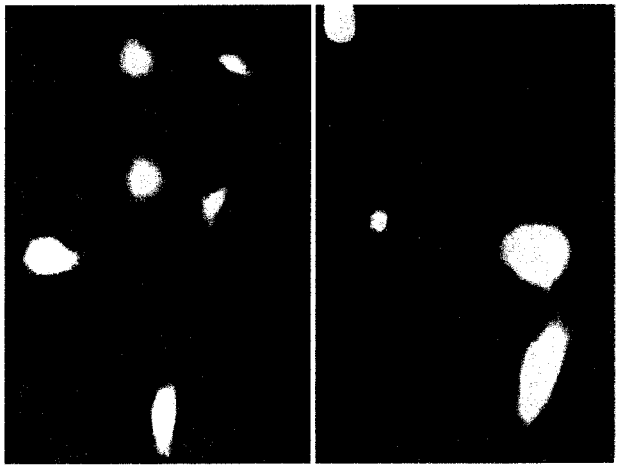

ABS280nm (mAU)
Fluorescence (RFU)
Time (min)

(b)


ABS280nm (mAU)
Time (min)

(c)

FIGURE 8



(a)

(b)

FIGURE 9

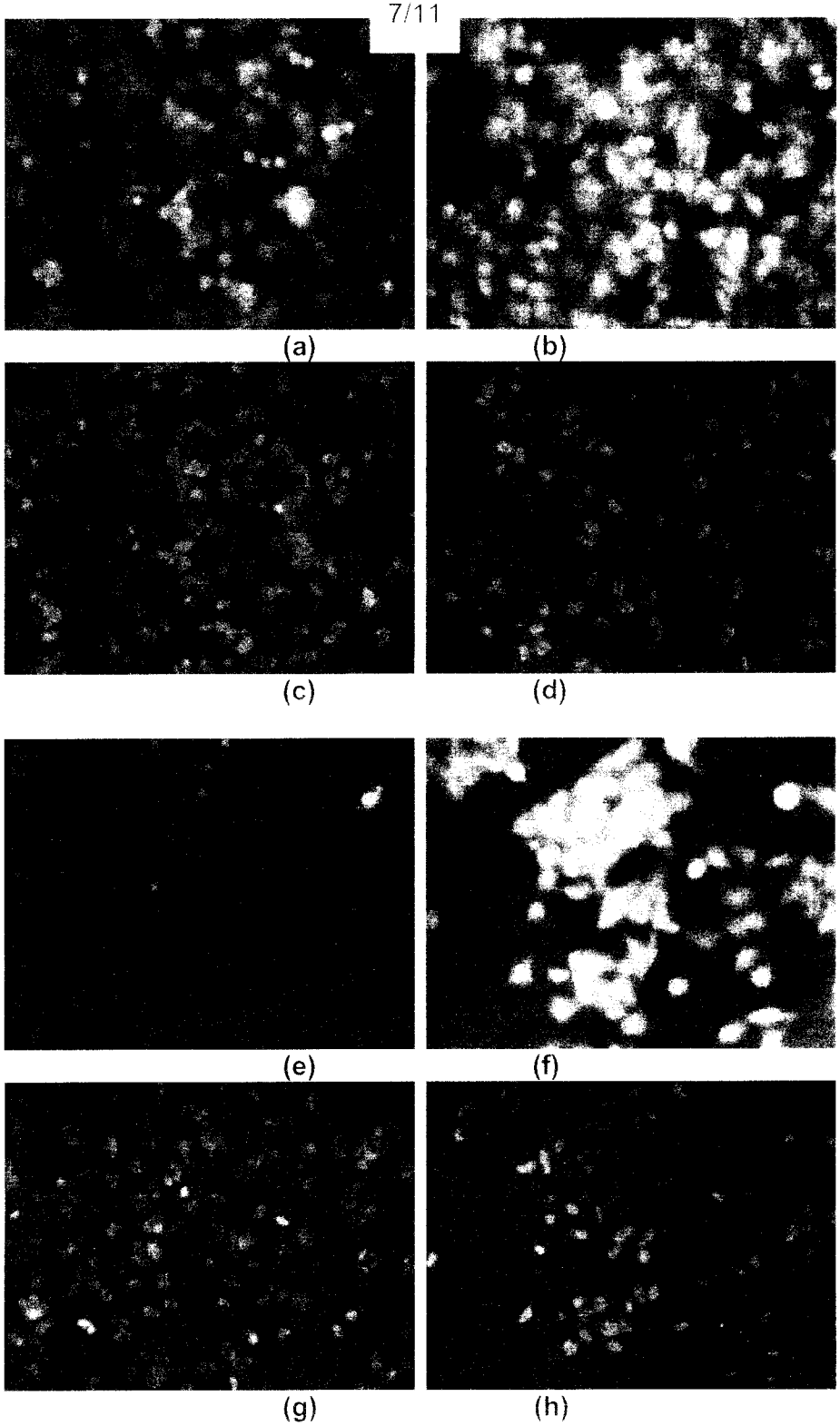


FIGURE 10

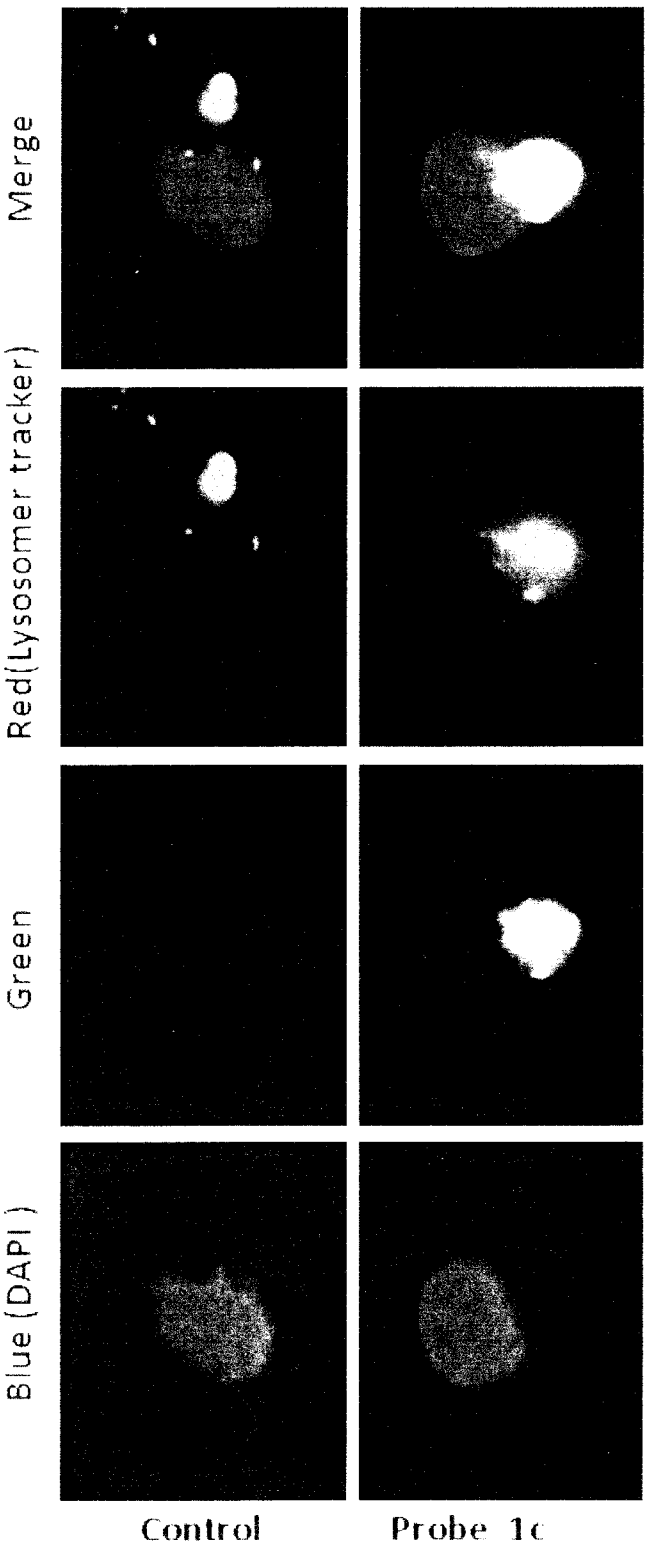

Merge
Red(Lysosomer tracker)
Green
Blue (DAPI)
Control
Probe 1c

FIGURE 11

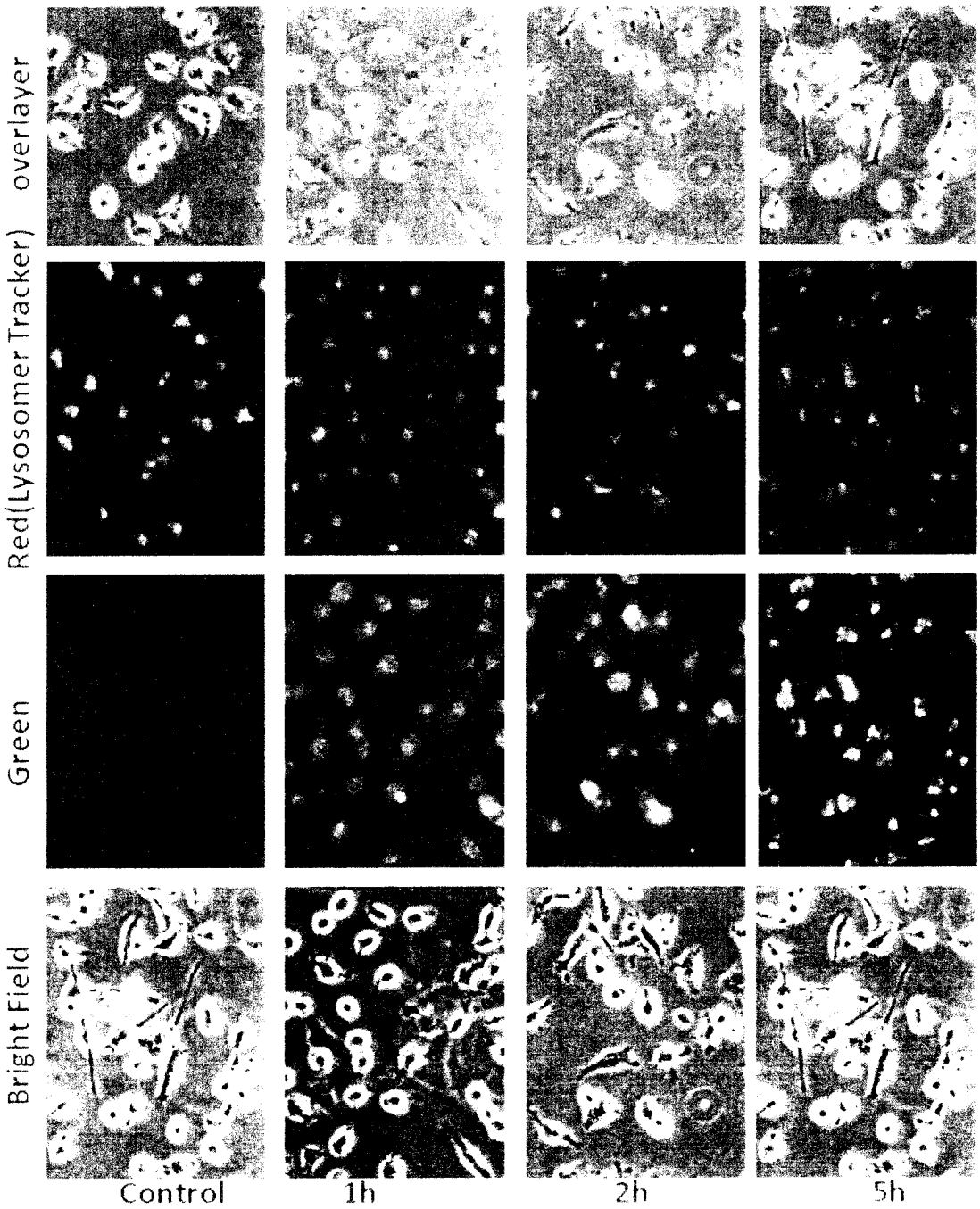

overlayer
Red(Lysosomer Tracker)
Green
Bright Field
Control
1h
2h
5h

FIGURE 12

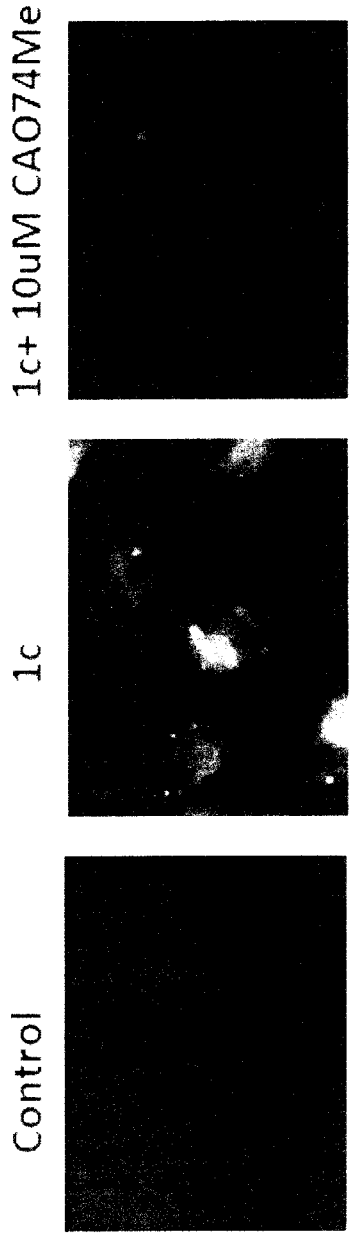
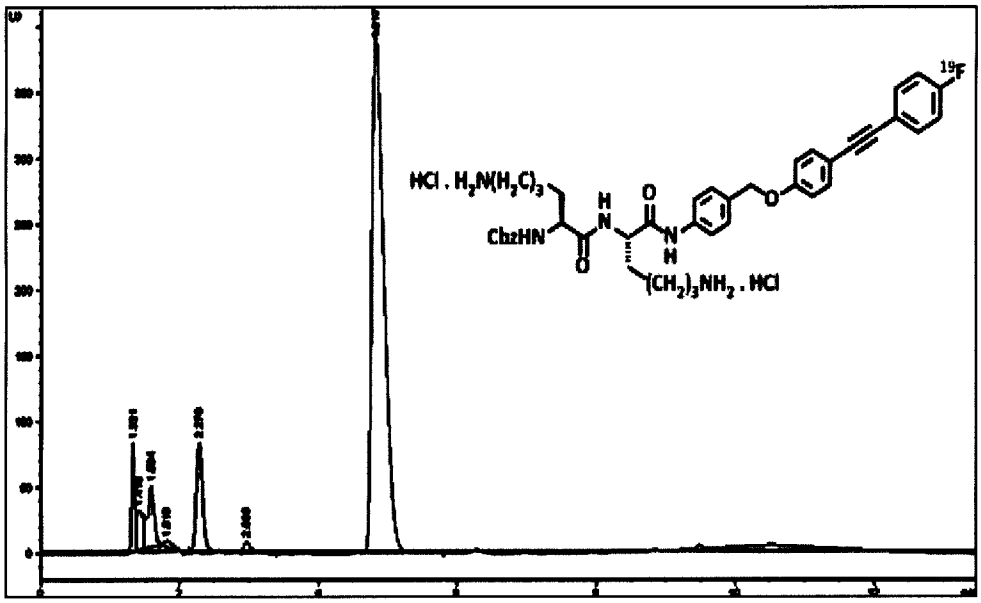
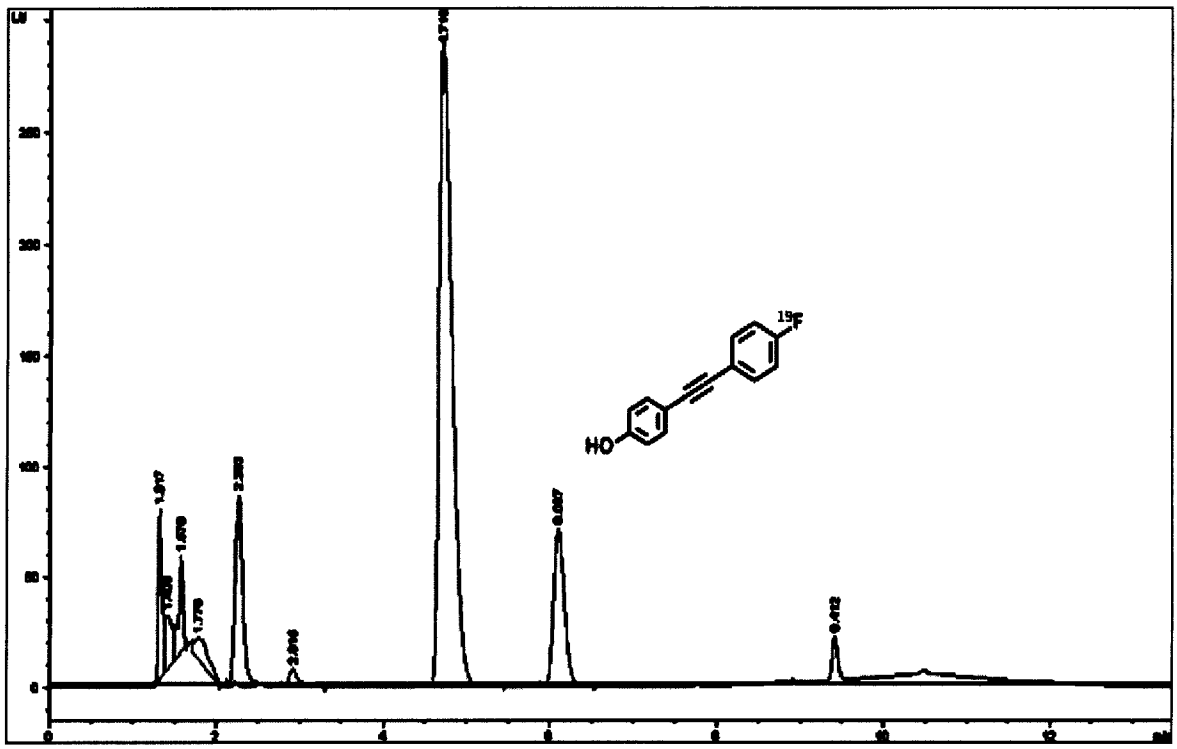


FIGURE 13



(a)



(b)

FIGURE 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2015/050136

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07K 5/06*** (2006.01) , ***A61K 49/00*** (2006.01) , ***A61K 51/08*** (2006.01) , ***C07K 5/065*** (2006.01) , ***C07K 5/068*** (2006.01) , ***C07C 237/22*** (2006.01), ***C07D 265/38*** (2006.01) , ***C07D 311/16*** (2006.01)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***C07K 5/06*** (2006.01) , ***A61K 49/00*** (2006.01) , ***A61K 51/08*** (2006.01) , ***C07K 5/065*** (2006.01) , ***C07K 5/068*** (2006.01) , ***C07C 237/22*** (2006.01), ***C07D 265/38*** (2006.01) , ***C07D 311/16*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Structure search in CAS Registry database and MARPAT database; Key word search in Questel-Orbit, Scopus, Canadian Patent Database, and BIOSIS databases

Key words: cathepsin B substrate, imaging, PET, probe, targeting, cathepsin B, inventor names

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RYU, JU HEE et al., "*Cathepsin B-sensitive nanoprobe for in vivo tumor diagnosis*". Journal of Materials Chemistry, 2011, Vol. 21, pp. 17631-17634, ISSN 0959-9428 (see whole document)	1-19
A	Baudy, A.R. et al., "*Non-invasive optical imaging of muscle pathology in mdx mice using cathepsin caged near-infrared imaging*", Molecular Imaging and Biology, 2011, Vol. 13, pp. 462-470, ISSN 1536-1632	1-19
P, X	CHOWDHURY, M.A. et al., "*Prodrug-inspired probes selective to cathepsin B over other cysteine cathepsins*". Journal of Medicinal Chemistry, 18 June 2014 (18-06-2014), Vol. 57, pp. 6092-6104, ISSN 0022-2623 (see whole document; Peptide 3 and Peptide 4)	1-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent but published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 26 May 2015 (26-05-2015)	Date of mailing of the international search report 18 June 2015 (18-06-2015)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Christiane Hansen (819) 934-5144