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Fluorescent isotope-coded affinity tag (FCAT) I: Design and synthesis

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ABSTRACT

A novel class of isotope-coded affinity tag is proposed possessing a fluorescent feature, referred to as fluorescent isotope-coded affinity tag (FCAT), to provide a new tool for quantitative proteomics. The label is designed to bind cysteine containing proteins or peptides. The FCAT reagent comprises four functional elements: a specific chemical reactivity group toward sulfhydryl groups; a linker that can incorporate the stable isotopes; a hydroxymethylbenzoic residue (base labile group) to cleave off a large part of the label before MS analysis; and a fluorescent tag for absolute quantification. The fluorescent part of the tag is also planned to be utilized to isolate the FCAT-labeled peptides via antibody based pull-down method. In this paper, we report on the solid phase organic synthesis of the light isotope containing FCAT molecule. The new labeling reagent showed good reactivity with model cysteine containing peptides. The fluorophore group was also effectively cleaved off from the labeled products to accommodate easier MS based analysis.

1. Introduction

Recent efforts in proteomics are focused towards global studies that require quantitative and comparative analysis of a large number of proteins. Application of stable-isotope labeling in conjunction with mass spectrometry for the analysis of proteins and peptides has proven successful to comparatively detect gene expression differences at the protein level. Various emerging techniques have been introduced recently to fulfill this quest [1], such as stable-isotope labeling of amino acids in cell cultures (SILAC) [2–5], isotope-coded affinity tags (ICAT) [6–16], element-coded affinity tags (ECAT) [17,18], metabolic labeling (^{15}N or ^{13}C) [19,20], isobaric tags for relative and absolute quantitation by iTRAQ [21–25] and mass-coded abundance tagging (MCAT) [26].

In 1999, Gygi et al. introduced the first isotope-coded affinity tag (ICAT) reagent [6], which enabled relative abundance measurements in protein expression. Since then, ICAT and similar reagents have been widely applied in quantitative proteomics [7–16]. The ICAT method was based on a reagent that comprised three functional elements: a specific chemical reactive group towards sulfhydryl motifs (cysteine specificity), a linker to incorporate the stable isotopes (i.e. generating the heavy and light forms of the tag), and a biotin group to isolate the ICAT-labeled peptides. The strategy to investigate differential protein expression using stable-isotope-coded affinity tag based methods encompasses four major steps: (1) reduced pools of proteins from two different samples (e.g., cell lines, different cell states, etc.) are reacted with the light and heavy chain reagents, respectively; (2) the samples are then combined and subject to proteolysis (e.g., with trypsin), generating peptide fragments; (3) the labeled peptides are isolated by means of streptavidin coated beads through the biotin tag; (4) and finally the peptides are removed from the beads and separated by micro- or nanoLC followed by mass spectrometry. The advantage of the approach was that the heavy and light isotopically coded reagent labeled peptides assumed to be chemically identical, while their 8 Da mass difference was readily identified by mass spectrometry. The ratios of the heavy and light tag labeled peptides supposed to be representative of the original protein content ratio in the two samples, thus the relative expression level was readily determined. The corresponding proteins of interest were identified by MS/MS and computer based data search against relevant databases.

The stable-isotope-coded affinity tag method featured several advantages. It was compatible with the variable amounts of proteins harvested from body fluids, cells, or tissues. The alkylation reaction with the tag was highly specific and not sensitive to the presence of salts, detergents, and stabilizers (e.g., SDS, urea, guanidine hydrochloride). The complexity of the resulting peptide mixture was significantly reduced after the digestion step as only cysteine containing peptides were isolated. The approach enabled almost any type of biochemical, immunoaffinity, or physical fractionation that made possible the analysis of lower abundance proteins [27]. However, in spite of the above listed advantages, the ICAT approach also had some problems, such as during MS analysis the tethered biotin part of the reagent was fragmented from the peptides complicating spectral interpretation [28]. Also, with the increasing number of deuterium atoms utilized to

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increase the mass difference between the heavy and light reagent labeled peptides, some difference was found in their chromatographic behavior. In addition, when deuterium effect occurred, the concentration ratio of the isoforms varied continuously across the elution profile of the two components making quantification difficult [29].

All the above mentioned possible limitations with the use of ICAT reagents drove the field towards the development of modified versions of isotopically coded labeling reagents. Some of the problems with the original ICAT reagent have been solved since its inception [30,31]. However, to address absolute quantification (not only relative), Fluorescent isotope-coded affinity tag (FCAT) reagents were designed and synthesized. Fig. 1 exhibits our FCAT designs comprising the following four functional elements: (1) a fluorescein group, that is one of the most frequently used fluorophore labeling dyes in qualitative and quantitative analysis of biomolecules by HPLC and other separation methods. The popularity of fluorescein is mainly due to its biocompatibility, low price and readily available instrumentation, i.e., fluorescence detector [32]. In addition to the option of immunoprecipitation by anti-fluorescein antibody, in our approach it is more important that fluorescein also offers the possibility of quantification by fluorescence detection. It is important to note here that fluorescein labeled peptides/proteins can also be selectively enriched by means of IMAC stationary phase [33]; (2) a hydroxymethylbenzoic residue (base labile group) to cleave off the large fluorophore part of the reagent before MS analysis; (3) a linker to incorporate stable isotopes like ^{13}C, ^{15}N, but importantly, not deuterium and (4) a specific reactive group towards sulfhydryl residues, such as cysteines. In this paper, we delineate the FCAT design and provide the experimental details of the synthesis of the light chain isoforms of the three proposed FCAT structures, along with the complete characterization of the new reagents by MALDI-TOF MS, LC–MS and NMR. We also assessed the reactivity of the FCAT tag with model cysteine containing peptides.

2. Materials and methods

2.1. General

2-Chlorotrityl resin, TentaGel MB RAM resin, ethylenediamine, Fmoc-Leu-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Lys(ivDde)-OH, *N*,*N*-diisopropylethylamine (DIPEA), 1-hydroxybenzotriazole (HOBt), *N*,*N*′-diisopropylcarbodiimide (DIPCDI), 4-hydroxymethylbenzoic acid (HMBA), 1-(2-mesitylenesulfonyl)-3-nitro-1H-1,2,4 triazole (MSNT), 1-methylimidazole (MeIm), 5-carboxyfluorescein, triisopropylsilane (TIS), 2,5-dihydroxybenzoic acid (DHB), sinapinic acid (SA), 3-maleimidopropionic acid, iodoacetic acid, trityl chloride, hydrazine, sodium hydroxide (NaOH), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris base), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), acetic acid, acetic anhydride, piperidine, pyridine, ninhydrin, phenol, KCN and Kieselgel 60 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol, diethyl ether, *N*,*N*-dimethylformamide (DMF), absolute ethanol, dichlorometane (DCM), chloroform ($CHCl_3$), acetonitrile (ACN), isopropyl alcohol (IPA), trifluoroacetic acid (TFA) and hydrochloric acid (HCl) were obtained from Merck (Darmstadt, Germany). Peptide 1: Cys-Ala-Ser-Ile-Gln-Lys-NH_2 (CASIQK-NH_2), Peptide 2: Asn-Cys-Gln-Phe-Glu-Lys-OH (NCQFEK-OH) and Peptide 3 (control): Ser-Ala-Thr-Pro-Ala-Ser-Ala-Pro-Tyr-Pro-Leu-Ala-Gly-Gly-Gly-Ser-NH_2 (SATPASAPYPLAGGGS-NH_2) were acquired from FIDIC (Bogotá, Colombia). All reagents were used without further purification. PhyTip Polymer Reversed Phase pipette tips were purchased from PhyNexus (San Jose, CA, USA).

2.2. Analytical methods

Reverse-phase HPLC (RP-HPLC) of the FCAT reagents and labeling reaction mixtures were performed on a ZORBAX Eclipse XDB-C18 column (Agilent, 250 × 4.6 mm, 5 μm) using a Transgenomic liquid chromatograph (Omaha, Nebraska, USA) with UV–

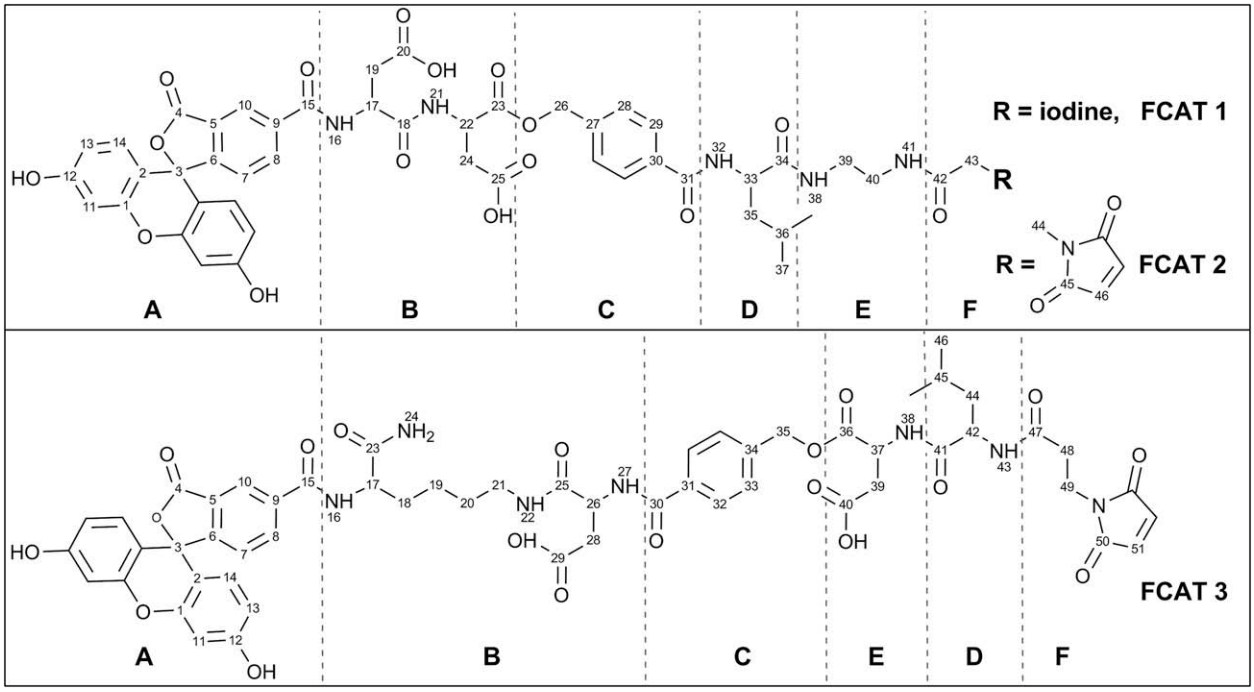


Fig. 1. Structures of the fluorescent isotope-coded affinity tag (FCAT) reagents. (A) Fluorescent affinity tag; (B) Spacer; (C) Base labile group; (D) Isotope-codeable linker; (E) Spacer; (F) Thiol-specific reactive group. Numbers shown for NMR data assignment.

vis detector (214 nm). For the analysis of the FCAT reagents, a linear gradient was applied from 5% to 95% acetonitrile (containing 0.05% TFA) in water (0.05% TFA) over 20 min at a flow rate of 0.8 mL/min at 35 °C. Labeling reaction mixtures were analyzed using a linear gradient from 30% to 70% acetonitrile (containing 0.05% TFA) in water (0.05% TFA) over 20 min at a flow rate of 0.8 mL/min at 35 °C. LC–MS analyses were performed in a chip-LC–MS system (Agilent 1100 with a chip-cube and ion trap MS XCT Ultra, Waldbronn, Germany) using Agilent HPLC-Chip: G4240-62001 ZORBAX 300SB-C18 5 μm (separation column: 43 mm × 75 μm; enrichment column: 4 mm, 40 nL). Solvent A was 0.1% formic acid containing water, and solvent B was acetonitrile (containing 0.1% formic acid and 5% water); a linear gradient was applied from 0% to 70% eluent B over 10 min at a flow rate of 0.5 μL/min. Thin layer chromatography (TLC) was carried out on Merck Kieselgel 60 F254 pre-coated glass plates with chloroform/methanol/acetic acid/water (70/25/2.5/2.5 vol) developing solvent, and visualized by UV illumination. Flash chromatography was performed on a 1.0 × 25 cm glass column packed with Kieselgel 60 (5 g, particle size 0.035–0.070 mm) using chloroform/methanol/acetic acid/water (87/10/2.5/0.5) eluent. The solid phase extraction columns (SUPELCO LC-18 with 0.5 g resin) were activated prior to use with 30 mL acetonitrile (containing 0.1% TFA) and equilibrated with 30 mL water (containing 0.1% TFA). MALDI-TOF MS analysis were performed on an Ultraflex III TOF–TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflectron mode, using MTP384 polished steel target (Bruker Daltonics), 2,5-dihydroxybenzoic acid or sinapinic acid as matrix; Laser: 500 shots and 25–30% power. NMR spectra were measured at 25 °C by a Varian NOVA 500 MHz instrument (Palo Alto, CA) equipped with a triple resonance probe head with z-gradient. Samples were dissolved in DMSO-*d*6 and TMS was used as internal standard. The labeled peptides were also analyzed by a Thermo Fisher LTQ (San Jose, CA) mass spectrometer, equipped with a nanoflow electrospray ionization source (Proxeon, Odense, Denmark). The samples were introduced directly into the ionization source. The electrospray conditions were as follows: spray potential: Ues = 2.02 kV; spray current: Ies = 0.67 μA; capillary temperature: 250 °C; capillary voltage: 36.93 V. Scanning was performed from 100 to 1000 *m/z*. The Excalibur software (Thermo Fisher) was used for data acquisition.

2.3. Synthesis of FCAT 1 and FCAT 2

2.3.1. Synthesis of compound 1

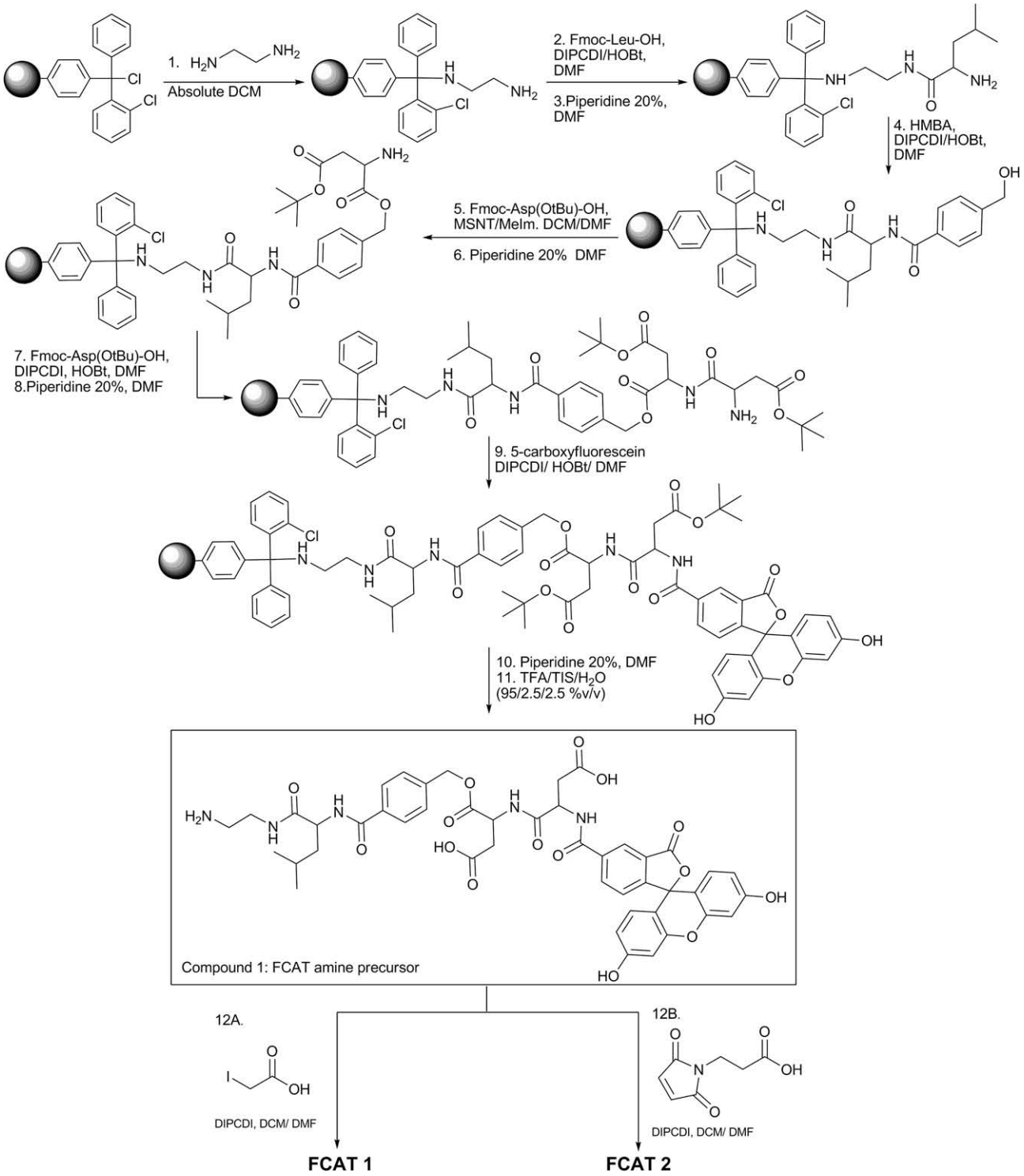
FCAT amine precursor (Compound 1) synthesis was preformed by solid phase organic synthesis as follows (Scheme 1) Step 1: Resin loading [34,35]: 2-chlorotrity resin (0.3 g, substitution ~1.5 mmol) was treated with ethylenediamine (90 μL, 1.35 mmol) dissolved in absolute dichloromethane (DCM, 2.0 mL). After shaking for 6 h at room temperature, the resin was capped twice with DCM/Methanol/DIPEA (75/20/5, v/v) for 20 min, and washed with DCM and ethyl ether. Finally, it was dried in vacuum. Step 2–3: Leucine residue incorporation: The resin from Step 1 was swelled in *N*,*N*-dimethylformamide (DMF) and treated with Fmoc-Leu-OH (0.54 mmol) activated with DIPCDI/HOBt (0.53/0.54 mmol) in DMF (3 mL) overnight. The incorporation of this amino acid was confirmed by ninhydrin test [36]. Then the resin was capped by a mixture of acetic anhydride (0.9 mmol) and DIPEA (1.8 mmol) in absolute DCM (2 mL). After this step the Fmoc group was removed using 25% piperidine in DMF (2 × 10 min) and the resin was washed with DMF (5 × 1 min), IPA (5 × 1 min) and finally with DCM again (5 × 1 min). Resin loading was determined by the quantitative Fmoc test [37] as 0.63 mmol/g. Step 4: Incorporation of base labile group [38]: the resin was treated with 2,5 molar excess of 4-hydroxymethylbenzoic acid (HMBA, 0.47 mmol) activated with DIPCDI/HOBt (0.46/0.47 mmol) and shaken for 2 h until detected negative by ninhydrin test. Step 5–6: Incorporation of the first aspartic residue [39]: MeIm (0.36 mmol) was added to a solution of Fmoc-Asp(OtBu)-OH (0.47 mmol) in absolute DCM/DMF (9/1 v/v, 2 mL) under N_2. The resulting solution was added to a MSNT (0.47 mmol) containing flask. After 1 min, the activated amino acid was added to the previously absolute DCM swelled resin and flushed with N_2. The reaction was left overnight with gentle agitation. The resin was then filtered and washed with DMF and DCM. Finally, as described above the Fmoc group was removed and the resin was washed. Step 7–8: Incorporation of the second aspartic residue: the swollen resin was treated with Fmoc-Asp(OtBu)-OH (0.47 mmol) activated with DIPCDI/HOBt (0.46/0.47 mmol) in DMF (3 mL) for 3 h. The incorporation of this amino acid was confirmed by ninhydrin test, and the Fmoc group was removed as described above. Step 9–10: Incorporation of the fluorophore [32]: The resin was treated with 5-carboxyfluorescein (0.38 mmol) activated with DIPCDI/HOBt (0.37/0.38 mmol) in DMF. When proven ninhydrin test negative (3 h), the reaction was stopped and the resin was washed with 25% piperidine in DMF (5 × 5 min), DMF (5 × 1 min), IPA (5 × 1 min), DCM (5 × 1 min) and diethyl ether (5 × 1 min), dried under vacuum and stored in a desiccator. Step 11: Cleavage [34,35]: the dried resin was treated with 95% TFA, 2.5% TIS and 2.5% H2O mixture (8 mL) for 3 h, then filtered and washed with TFA (0.5 mL). The solution was dried in a rotary vacuum evaporator. The solid product was washed with diethyl ether and dried. 145 mg of crude Compound 1 (FCAT amine precursor, yield 85.7%) were obtained and an aliquot was analyzed by TLC (R_f = 0.25) and MALDI-TOF MS (*m/z* = 896.28 $[M + H]^+$; calc $[M + H]^+$: 896.30). Compound 1 was then used for the synthesis of both FCAT 1 and 2.

2.3.2. FCAT 1 synthesis (Scheme 1, Step 12A)

Iodoacetic acid (0.1 mmol) was dissolved in absolute DCM (1 mL) and activated with DIPCDI (0.098 mmol) for 20 min. A solution of crude Compound 1 (30 mg) in DMF (1 mL) was added and the mixture was agitated for 2 h. Completion of the reaction was checked by TLC and ninhydrin test. When the reaction was finished, the solution was diluted with absolute ethanol (10 mL) and dried in a rotary vacuum evaporator. The solid product was dissolved in DCM/ethanol (5 mL, 5/3 v/v) and ether was added to precipitate the FCAT 1. It was followed by washing with ether and DCM then dried under vacuum. The yield was 32.1 mg of crude product, which was then purified by flash chromatography as described above (Analytical Methods). FCAT 1 containing fractions were collected and pooled followed by drying in a rotary vacuum evaporator. Then the FCAT 1 was dissolved in acetonitrile/water (3 mL, 2/8 v/v) and passed through a pre-activated solid phase extraction column (SUPELCO, 0.5 g). The SPE column was then washed with water −0.1% TFA mixture (20 mL), followed by water (20 mL), and acetonitrile/water mixture (20 mL, 2/8 v/v). The FCAT 1 was eluted with of acetonitrile/water (3 mL, 1/1 v/v). After drying, 15.4 mg of pure FCAT 1 (overall yield 43.4%) was obtained. The compound was analyzed by TLC (R_f = 0.43), RP-HPLC (t_R = 16.02 min), MALDI-TOF MS (*m/z* = 1064.40 $[M + H]^+$; 938.45 $[M + H-I]^+$; calculated $[M + H]^+$: 1064.20) and LC–ESI–MS (t_R = 8.6 min; *m/z* = 1064.8 $[M + H]^+$).

2.3.3. FCAT 2 synthesis (Scheme 1, Step 12B)

3-Maleimidopropionic acid (0.1 mmol) was dissolved in absolute DCM (1 mL) and activated with DIPCDI (0.098 mmol) for 20 min. The crude Compound 1 solution (30 mg, 1 mL of DMF) was added, and the mixture was agitated for 3 h. Completion of the reaction was checked by TLC and ninhydrin test. The reaction mixture was then dried and washed with ether. The residue

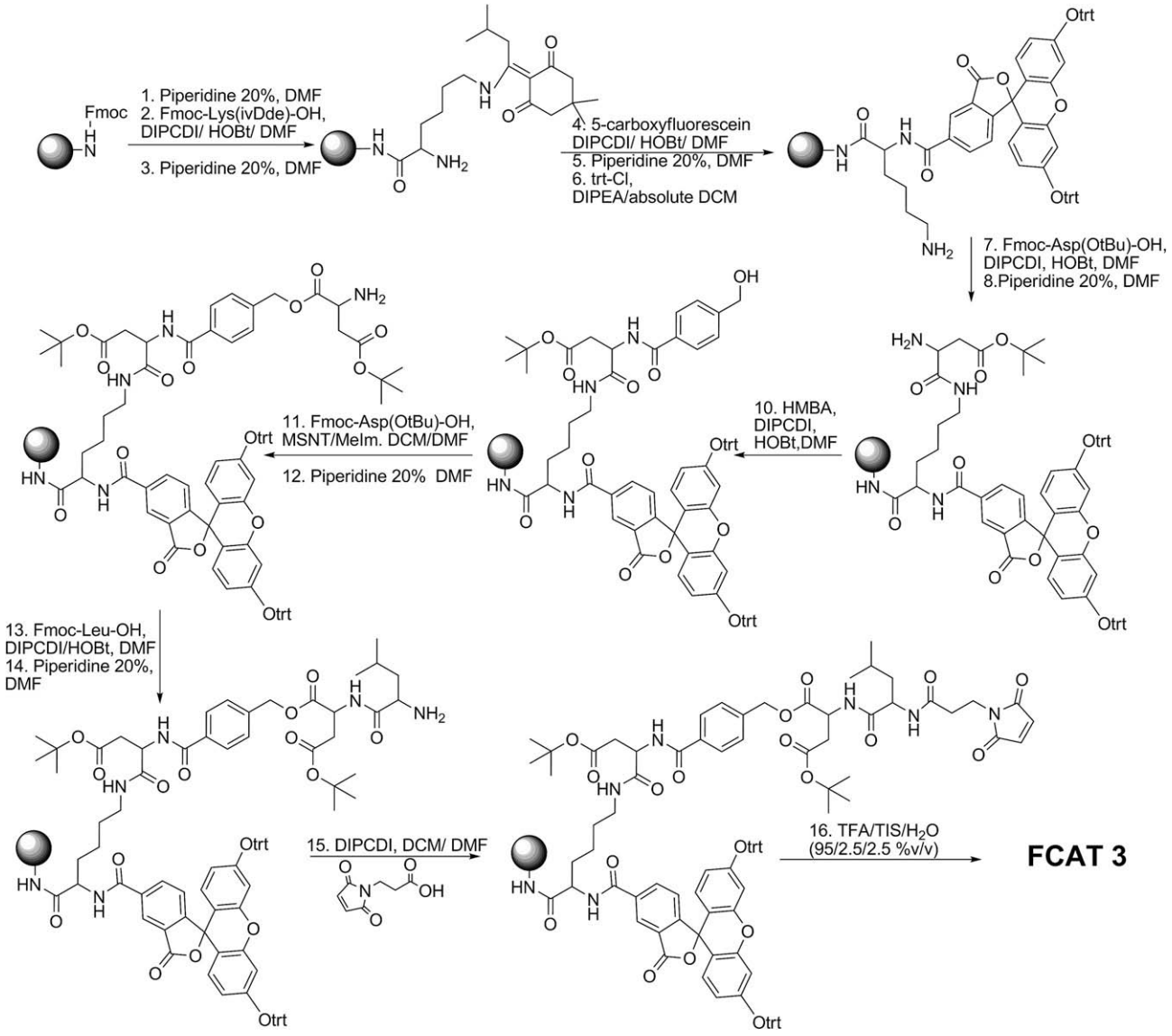


Scheme 1. Schematics of the synthesis of the FCAT 1 and 2 reagents.

was then dried under vacuum, and 29.0 mg of crude FCAT 2 product was obtained. Finally, 18.7 mg of pure FCAT 2 (overall yield 54.2%) was obtained after purification by flash chromatography and SPE (as described for FCAT 1). The pure compound was analyzed by TLC (R_f = 0.43), RP-HPLC (t_R = 15.86 min), MALDI-TOF MS (m/z = 1047.33 $[M + H]^+$; calculated $[M + H]^+$: 1047.32) and LC–ESI–MS (tR = 8.5 min; m/z = 1047.9 $[M + H]^+$). The pure FCAT 1 and 2 reagents were stored in a desiccator and protected from light.

2.4. Synthesis of FCAT 3

Synthesis of FCAT 3 was preformed by solid phase organic synthesis as illustrated in Scheme 2. Step 1–2: Incorporation of lysine residue: TentaGel MB RAM resin (substitution 0.35 mmol/g, 140–170 μm) was chosen as solid support. To liberate its amino group, resin (200 mg, 0.07 mmol) was treated with 25% piperidine in DMF (2 × 10 min) and washed with DMF (5 × 1 min), IPA (5 × 1 min) and finally by DCM again (5 × 1 min). This was followed by treat-



Scheme 2. Schematics of the synthesis of the FCAT 3 reagent.

ment with Fmoc-Lys(ivDde)-OH (0.21 mmol) activated with DIPCDI/HOBt (0.20/0.21 mmol) in DMF (3 mL). The ninhydrin test was negative after 3 h, thus the reaction was stopped. Step 3–4: Incorporation of the fluorophore: The Fmoc group was removed from the lysine (as described before) and the resin was treated with 5-carboxyfluorescein (0.14 mmol) activated with DIPCDI/HOBt (0.14/0.14 mmol) in DMF. Reaction was agitated overnight or until testing negative with ninhydrin. Step 5–6: Protection of the hydroxyl groups on the fluorescein: the resin was treated with 25% piperidine in DMF, washed by DMF and absolute DCM, then dried in vacuum. The phenolic hydroxyl groups were then blocked by trityl chloride/DIPEA (0.7/1.4 mmol) dissolved in absolute DCM. After 2 h, the red color of the resin changed to yellow, indicating the end of the reaction. Step 7: Release of the lysine ε-amino group: The resin was treated with 2% hydrazine in DMF (2 mL, 4×3 min) followed by washing with DMF (7×1 min) and DCM (5×1 min). Step 8–9: Incorporation of the aspartic residue: The resin was treated with Fmoc-Asp(OtBu)-OH (0.21 mmol, activated with DIPCDI/HOBt; 0.20/0.21 mmol) in DMF (3 mL) for 3 h. Incorporation of this amino acid was confirmed by ninhydrin test. The Fmoc group was removed as described above. Step 10: Incorporation of the base labile group: The resin was treated with 4-hydroxymethylbenzoic acid (0.21 mmol, activated with DIPCDI/HOBt; 0.20/0.21 mmol) until detected negative by ninhydrin test (3 h). Step 11–12: Incorporation of the aspartic residue: MeIm (0.15 mmol) was added to a solution of Fmoc-Asp(OtBu)-OH (0.21 mmol) in absolute DCM/DMF (9/1 v/v, 2 mL) under N2. The resulting solution was added to a MSNT (0.21 mmol) containing flask. After 1 min, the activated amino acid was added to the previously absolute DCM swelled resin and flushed with N_2. The reaction was left overnight with gentle agitation. The resin was then filtered and washed with DMF and DCM. Then the Fmoc group was removed by 25% piperidine treatment and the resin was washed with DMF. Step 13–14: Incorporation of the leucine residue: The resin from Step 12 (Scheme 2) was swelled in DMF and treated with Fmoc-Leucine-OH (0.21 mmol activated with DIPCDI/HOBt; 0.20/0.21 mmol) in DMF (2 mL). Incorporation of this amino acid was confirmed by ninhydrin test after 4 h. Then the Fmoc group was removed. Step 15: Incorporation of the maleimide residue. The resin was treated by 3-maleimidopropionic acid (0.14 mmol, activated with DIPCDI; 0.13 mmol) in DCM (2 mL). This reaction was agitated overnight and checked by ninhydrin

test. Step 16: Cleavage of the final product: The dried resin was treated with 95% TFA, 2.5% TIS and 2.5% H2O mixture (4 mL) for 3 h, then filtered and washed with TFA (0.5 mL). The collected and pooled solution was dried in a rotary vacuum evaporator and the solid product was washed with DCM and diethyl ether followed by drying. Finally, 35.0 mg of crude FCAT 3 product (yield 44.3%) was obtained. The crude product was purified by flash chromatography and SPE, resulting in 12.2 mg of pure FCAT 3 (overall yield 15.4%). The pure compound was analyzed by TLC ($R_f = 0.36$), RP-HPLC ($t_R = 15.11$ min), MALDI-TOF MS ($m/z = 1132.23$ $[M + H]^+$; calculated $[M + H]^+$: 1132.37) and LC–ESI–MS ($t_R = 8.5$ min; $m/z = 1133.1$ $[M + H]^+$). The pure reagent was stored in a desiccator and protected from light.

2.5. Evaluation of FCAT reactivity

2.5.1. Peptide labeling with FCAT reagents

A terminal and a penultimate cysteine containing peptide were selected, respectively, to check the reactivity of the synthesized FCAT reagents: Peptide 1: CASIQK-NH_2 and Peptide 2: NCQFEK-OH. Stock peptide solutions (10 mM) were prepared in water. Each peptide (30 nmol) was reduced by adding tris(2-carboxyethyl)phosphine hydrochloride (6.7 mM TCEP, 9 μL) and incubating for 30 min at 25 °C. TCEP solution was prepared in 50 mM Tris–HCl buffer (pH 8.5) containing 0.1% SDS (w/v). The final pH was 7.4.

Labeling reaction with FCAT 1 (reacting group: iodoacetyl). Reduced peptides (10 nmol) were treated with FCAT 1 (40 nmol) in Tris–Hcl buffer pH 8.5 (4 mM final concentration) and the reaction mixture was incubated for 4 h at 37 °C, protected from light. The reaction mixture was kept overnight at 4 °C. Then 0.1 M DTT (10 μL) was added and incubated for 30 min at room temperature. Finally the solutions were diluted 10 times with 10% acetonitrile in water and desalted using PhyTip Polymer Reversed Phase pipette tips using 50% acetonitrile elution buffer.

Labeling reaction with FCAT 2 and 3 (reactive group: maleimide). 10 nmol of each reduced peptide was labeled with 40 nmol of FCAT 2 or 3 in 50 mM Tris–HCl buffer (pH 7.0) containing 0.1% SDS (w/v) for 4 h at 37 °C, protected from light. The reaction mixture was kept overnight at 4 °C, stopped by the addition of 0.1 M DTT and incubated for 30 min at room temperature. Finally, each solution was diluted 10 times with 10% acetonitrile in water and desalted by PhyTip Polymer Reversed Phase pipette tips using 50% acetonitrile elution buffer. The labeling efficiency was monitored by RP-HPLC, MALDI-TOF MS and LC–MS.

2.5.2. Cleavage of the fluorophore group

The FCAT reagent labeled model peptides were treated with sodium hydroxide (final concentration 0.15 M) for 15 min at room temperature to cleave off the fluorophore part of the molecule. After desalting by PhyTip Polymer Reversed Phase pipette tips (PhyNexus), the efficiency of the cleavage step was checked by MALDI-TOF MS.

3. Results and discussion

3.1. Design of the FCAT reagents

Considering the basic structural elements of the ICAT reagent, we designed an isotope labeled regent with a fluorescent part to support absolute quantification. The design of the FCAT 1, FCAT 2 and FCAT 3 molecules are shown in Fig. 1. The molecular structures contain some similar motifs. Section A in Fig. 1 depicts the fluorescein residue that supports quantification by fluorescent LC detection and also serves as affinity tag to selectively isolate the FCAT-labeled peptides either by anti-fluorescein antibody or by IMAC resins. For FCAT 1 and FCAT 2 aspartic acid and ethylenediamine, for FCAT 3 lysine and aspartic acid were used as spacers, respectively (Sections B and E). Section C shows the 4-hydroxymethylbenzoic acid (HMBA) group that was included into the structures to provide a cleavage site for the fluorophore group of the reagent by a simple base treatment before mass spectrometry. Leucine was chosen as stable-isotope carrying part (Section D), based on its availability with stable ^{13}C and ^{15}N labeled forms. Section F shows the reactive group of iodoacetyl for FCAT 1, or maleimide for FCAT 2 and FCAT 3. Both react with the sulfhydryl groups of cysteine containing peptides or proteins.

The FCAT reagent is planned to be used for absolute and relative quantification in proteomics by means of the following protocol: (1) the protein samples of interest are reduced and digested in order to generate free thiol group containing peptide mixture; (2) then the corresponding samples are labeled either with the light or the heavy FCAT reagent; (3) the two FCAT-labeled samples are then combined and the labeled peptides are partitioned by either affinity chromatography (using anti-fluorescein antibody or IMAC solid support); (4) the resulting sample with significantly decreased complexity is then analyzed by high resolution HPLC with fluorescent detection (absolute quantification); (5) finally, the pH of the HPLC column eluate is increased in order to cleave off the fluorescent motif that allows easier analysis of the resulting peptide pairs by mass spectrometry (relative abundance determination).

After the design of FCAT reagents, we demonstrated the following points: (1) possibility to synthesize this class of molecules; (2) reactivity with cysteine containing peptides; (3) option to cleavage off the fluorophore part from the labeled peptide and (4) possibility of peptide sequence determination. To test the above, we synthesized the light version of the FCAT reagents and used them to label model peptides.

3.2. Synthesis of the light FCAT reagents

After relevant retrosynthetic analysis, we considered solid phase organic synthesis (SPOS) as the best option to attain the FCAT molecules. FCAT 1 and FCAT 2 contain the same motifs in theirs structures, they only differ in their reactive groups, thus they can be produced from the same amine precursor (compound 1); complete schematics of the FCAT 1 and 2 syntheses are delineated in Scheme 1. The solid phase support of 2-chlorotrityl resin was chosen, because it provided a terminal amino group after cleavage. It was first loaded with ethylenediamine, and then coupled with one Fmoc-Leu-OH, one 4-hydroxymethylbenzoic acid, two Fmoc-Asp(OtBu)-OH residues as spacers, followed by one 5-carboxyfluorescein residue. The solid phase synthesis resin was then treated with a mixture of trifluoroacetic acid and relevant scavengers (water and TIS) to yield Compound 1. This amine precursor was checked by TLC and analyzed by MALDI-TOF MS. To obtain the target FCAT 1 and 2 structures, the crude Compound 1 solution was treated with iodoacetic acid or maleimidopropionic acid, respectively (Scheme 1, Step 12A and B).

FCAT 3 was also synthesized by solid phase organic synthesis as depicted in Scheme 2. TentaGel MB RAM resin was chosen for this synthesis process. The amine group was first deprotected by piperidine treatment followed by the incorporation of the Fmoc-Lys(ivDde)-OH. This residue allowed carrying out orthogonal synthesis. The α-amino group of the lysine was first deprotected then coupled to a 5-carboxyfluorescein residue. This step was followed by protection of the fluorescein hydroxyl groups using tritylation. After that, the lysine ε-amino group was deprotected by hydrazine treatment followed by coupling of one Fmoc-Asp(OtBu)-OH, one 4-hydroxymethylbenzoic acid, one Fmoc-Asp(OtBu)-OH, one Fmoc-Leu-OH and one maleimidopropionic residues. Finally,

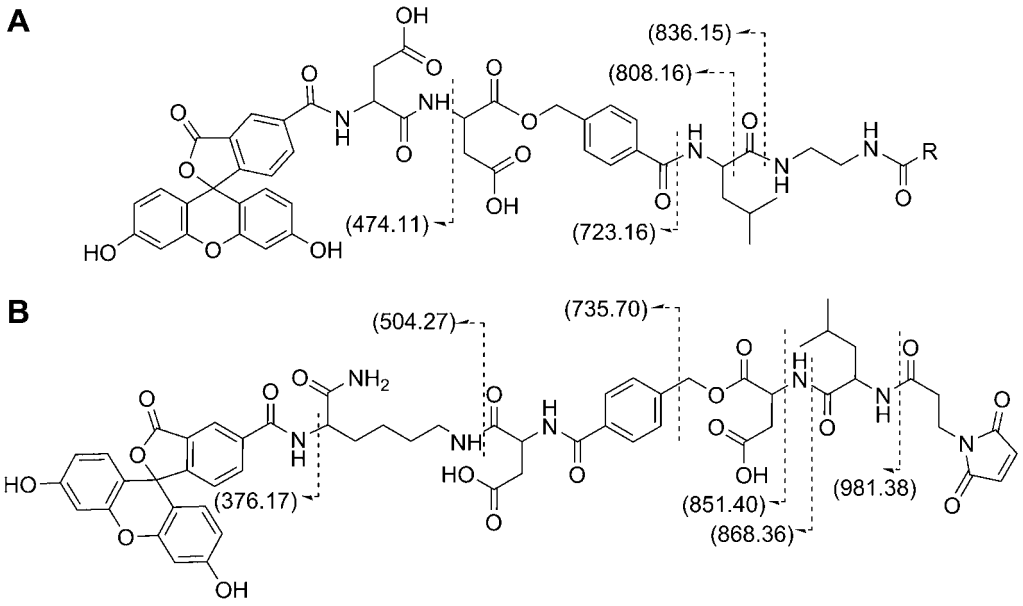


Fig. 2. MS/MS analysis of the FCAT reagents. Fragmentation pattern, (A) FCAT 1 and 2; (B) FCAT 3. The actual measured *m/z* values of the signals are shown in parenthesis.

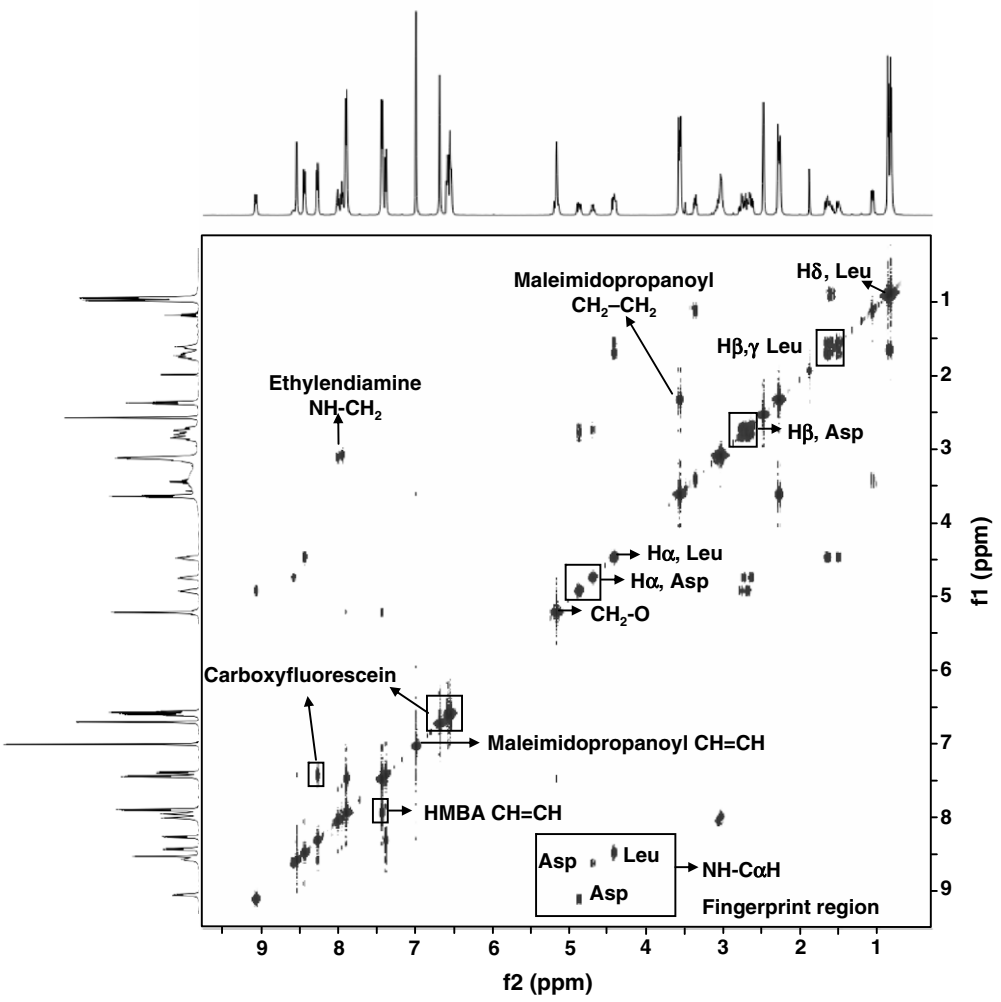


Fig. 3. ^{1}H-^{1}H COSY spectra of FCAT 2 (δ in ppm, 0 = TMS, DMSO-*d*6).

deprotection of all lateral chains of the FCAT 3 and its release form the resin was performed by addition of a mixture of trifluoroacetic acid and the scavengers of water and TIS.

All three FCAT reagents were purified by flash chromatography using SilicaGel 60 with the overall yield of 43.4%, 54.2% and 15.4% for FCAT 1, FCAT 2 and FCAT 3, respectively. The resulting purified compounds were analyzed by TLC, RP-HPLC, MALDI-TOF MS and LC–MS techniques. All the synthesized products were obtained in good quality, based both on HPLC analysis with UV detection and MS spectra information (Fig. 1 in supporting information shows the LC–MS analysis of FCAT reagents). MS/MS analysis of the relevant peaks corresponded to the molecular masses of each FCAT were performed. Fig. 2 shows the fragmentation pattern of each FCAT molecules. Please note that FCAT 1 and 2 had the same fragmentation pattern. The purified FCAT 1, 2 and 3 products were also analyzed by ^{1}H NMR, and 2D NMR spectroscopy. ^{1}H-^{1}H COSY was used to establish ^{1}H -^{1}H connectivities. The two aspartic acids and the leucine residues are clearly identified in NH–CαH fingerprint region of the FCAT 2 ^{1}H-^{1}H COSY spectra as shown in Fig. 3. It is also possible to recognize the amino acid proton spin systems consisting of HN, Hα, Hβ, Hδ, and for the hydroxymethylbenzoic, maleimidopropanoyl, carboxyfluorescein and ethylendiamine residues. HSQC spectra provided ^{1}H -^{13}C connectivities allowing to determinate chemical shifts of some carbon atoms. Finally, sequential connections and fluorescein quaternary carbons were identified from the HMBC spectra. Tables 1 and 2 (in supporting information) summarize the complete chemical shift assignments for FCAT 1, 2 and 3.

Fractions collected during the flash chromatography purification step were analyzed by MALDI-TOF MS to find any possible side products. Apparently, FCAT 1 dimers with and without iodine were found (*m/z* 1999.87 and 1873.97, respectively), maybe due to self-alkylation, similar to as it was reported earlier in the case of ICAT reagents [40]. These side reactions might represent a stability issue with the FCAT 1 reagent. A product resulting from the loss of iodine was also found ($m/z = 938.45$ $[M + H - I]^{+}$). For FCAT 3, the main side product (22%) could be a hydrolysis product ($m/z = 753.25$), caused by an incomplete coupling reaction over the hydroxyl group of HMBA residue or an acid hydrolysis of the ester during the cleavage step.

3.3. FCAT reactivity

The applicability of the FCAT reagent was tested in two reactions. The first reaction was designed to demonstrate that the FCAT molecule can readily react with cysteine containing model peptides (both in terminal and internal locations). The second reaction attempted to prove that the fluorophore group can be easily cleaved off from labeled peptides.

3.3.1. FCAT reactivity with model peptide

Peptide 1 (CASIQK-NH_2), and Peptide 2 (NCQFEK-OH) were selected to check the reactivity of the synthesized FCAT reagents. Each peptide was reduced using TCEP at pH 7.4, followed by labeling with four molar excess of FCAT 1, 2 or 3 reagents. Labeling reactions with FCAT 1 were performed in Tris–HCl buffer (pH 8.5). The same buffer was used at pH 7.0 for the maleimide group containing FCAT 2 and 3. Completion of the FCAT 1, 2 and 3 reactions with cysteine containing peptides was analyzed by MALDI-TOF MS and RP-HPLC. In all instances, the labeling reaction efficiency was measured by HPLC and found to be >99%. Table 1 summarizes all peaks/signals present in the MALDI-TOF MS spectra for labeling reaction of FCAT 1, 2 and 3, with both cysteine containing peptides, and the control peptide (without cysteine). In addition to the characteristic signals for the labeled peptides, peaks for FCAT

Table 1
Reaction of FCAT 1, FCAT 2 and FCAT 3 tags with the model and control peptides

Peptide	Labeled products m/z $[M + H]^{+}$			Fluorophore cleavage products m/z $[M + H]^{+}$		
	FCAT 1 (1063.20)	FCAT 2 (1046.32)	FCAT 3 (1131.37)	FCAT 1	FCAT 2	FCAT 3
1: CASIQK (647.34)	1583.76 (1582.63)	1694.62 (1693.66)	1779.88 (1778.71)	995.56 (994.53)	1124.69 (1105.56)	1063.62 (1044.49)
	1090.36[a1]	1201.33[a1]	1286.53[a1]			
	2025.66[a2]	2247.33[a2]	1382.62[b1]			
	1186.44[b1]					
2: NCQFEK (767.33)	1703.78 (1702.61)	1814.90 (1813.65)	1899.81 (1898.70)	1115.59 (1114.51)	1244.73 (1225.54)	1183.67 (1164.48)
	1090.42[a1]	1201.56[a1]	1286.61[a1]			
	2025.73[a2]	2247.70[a2]	1382.68[b1]			
	1186.51[b1]	1297.64[b1]				
3: SATPASAPYPLAGGGS(1401.69)	1402.83 1090.32[a1]	1402.79 1201.37[a1]	–	–	–	–

Summary of MALDI-TOF MS analysis. The calculated monoisotopic masses are shown in parentheses.
[a1] Labeled DTT with one molecule of FCAT.
[a2] Labeled DTT with two molecules of FCAT.
[b1] Labeled TCEP with FCAT.

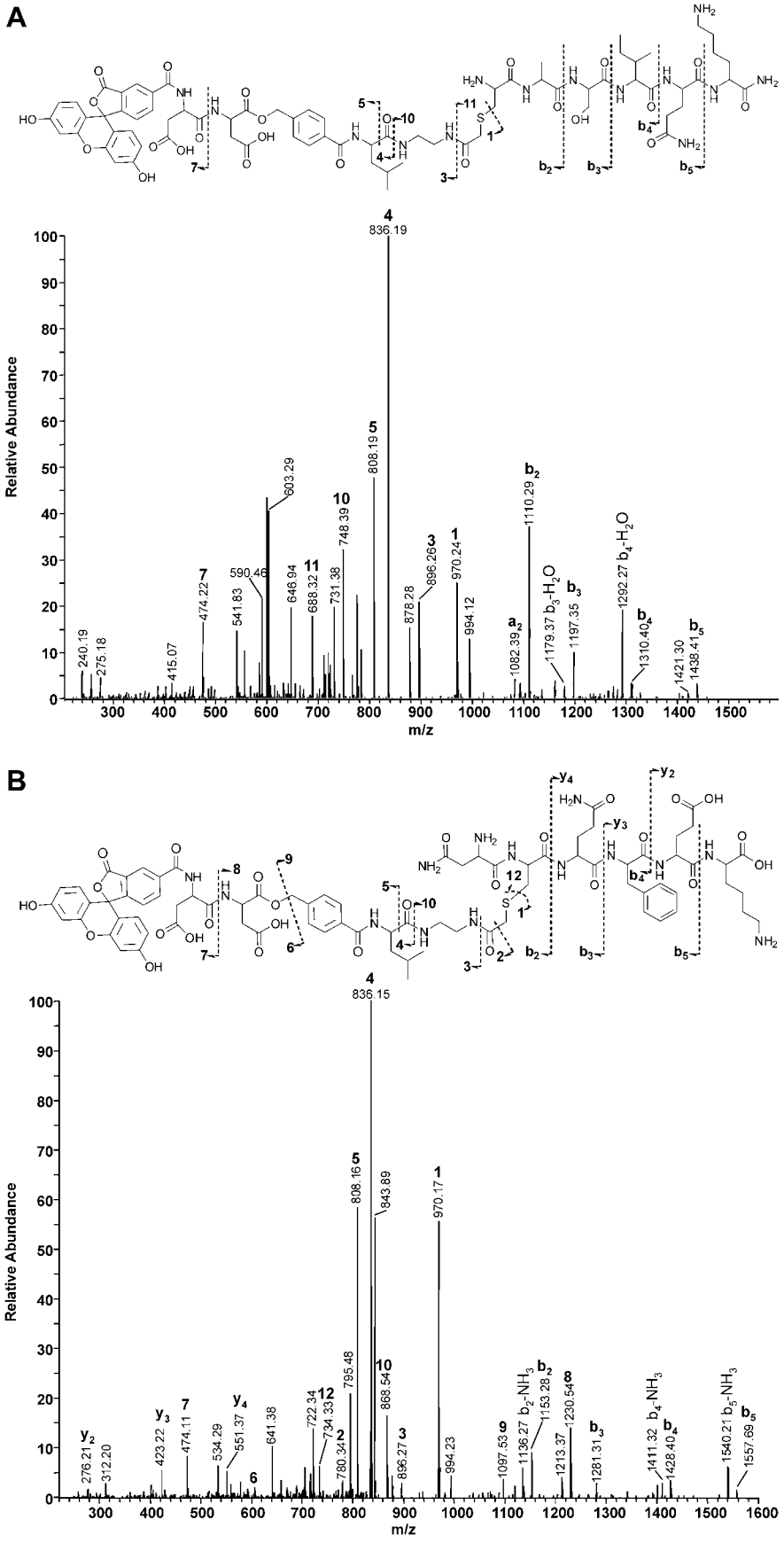


Fig. 4. MS/MS analyses of the labeling reaction products. (A) Peptide 1 labeled with FCAT 1 (*m/z* 792.37 ion was monitored). (B) Peptide 2 labeled with FCAT 1 (*m/z* 852.30 ion was monitored). Conditions are in Section 2.

conjugated reducing agents were also found (i.e., FCAT 1 with DTT *m/z* 1090.36 and TCEP 1186.44, respectively). In the RP-HPLC analysis of labeling reaction mixture with FCAT 1, besides the expected peaks, some additional peaks were detected, collected and then analyzed by MALDI-TOF MS. They corresponded to FCAT 1 decomposition products (dimer, *m/z* 1999.24; loss of iodine, *m/z* 938.14 and dehydrated, *m/z* 1046.14 forms). A control reaction was also performed with a peptide having no cysteine residue (Peptide 3: SATPASAPYPLAGGGS-NH_2). In this latter instance, we only observed peaks corresponding to labeled DTT, suggesting the high specificity of the FCAT reagent towards thiol groups.

MS/MS analysis (LTQ) of the labeled products proved the prospective sequencing option. Despite of the large fluorescent residue, signals corresponding to b and y ions characteristic from peptide fragmentation pattern can be observed. As an example Fig. 4 shows the fragmentation pattern of Peptide 1 (panel A) and Peptide 2 (panel B) labeled with FCAT 1. Peptide 1 labeled with FCAT 1 principally generated b ions. Peptide 2 labeled with FCAT 1 generated b and y ions. In both spectra peaks 4, 5 and 7 correspond to the fragmentation pattern of FCAT 1 (Fig. 2).

Additionally, a longer peptide (TCAYTNHTVLPEALER-OH) was labeled with FCAT 2 in order to address possible limitation issues in regard to peptides length that still can be sequenced by analyzing the MS/MS fragmentation pattern of the FCAT conjugates. MS/MS analysis of the labeled 16-mer allowed adequate determination of the peptide sequence (Fig. 5). The spectra feature the signals of all corresponding b (b_2–b_8) and y (y_3–y_{14}) ions, characteristic of the peptide. FCAT related peaks also identifiable in the pattern (1–7).

3.3.2. Cleavage of the fluorophore group

Table 1 shows the MALDI-TOF MS analysis of the cleavage reaction products of the FCAT-labeled peptides. For FCAT 1 labeled pep-

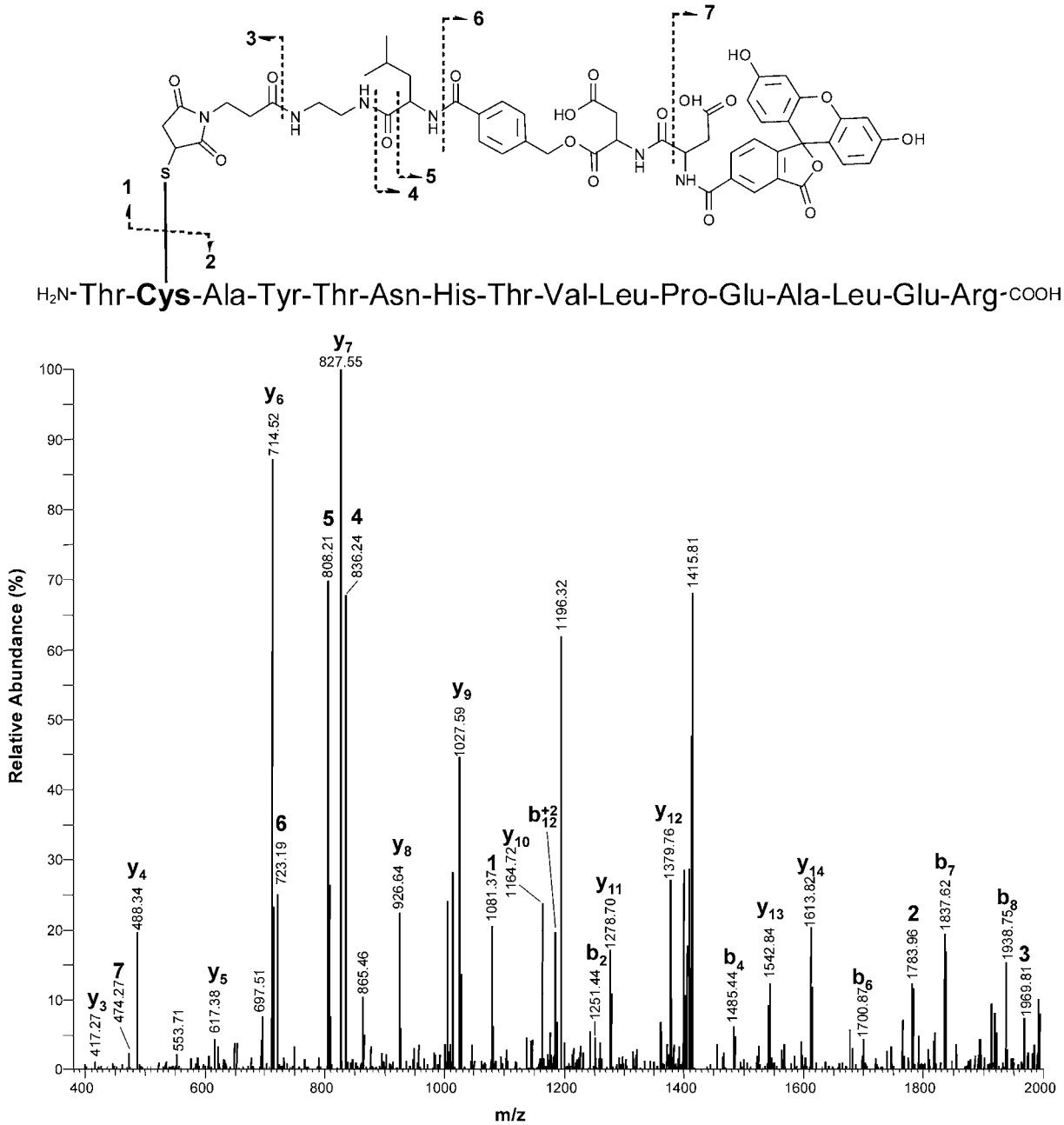


Fig. 5. MS/MS analysis of the FCAT 2 labeled 16-mer of TCAYTNHTVLPEALER (*m/z* 1492.97 ion was monitored). Conditions are in Section 2.

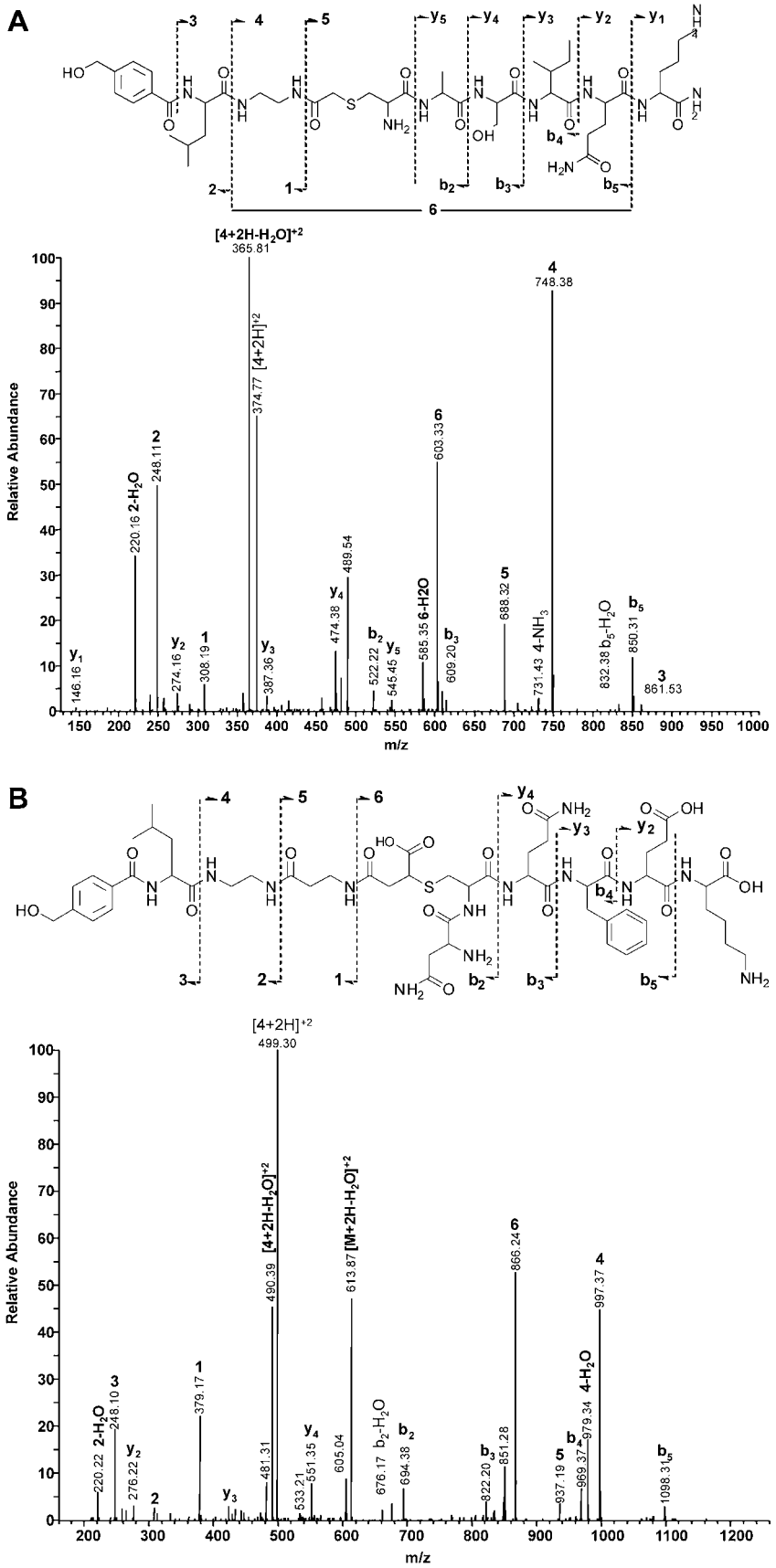


Fig. 6. MS/MS analyses of the fluorophore cleavage reaction products. (A) Peptide 1 labeled with FCAT 1 (*m/z* 498.11 ion was monitored). (B) Peptide 2 labeled with FCAT 2 (*m/z* 622.61 ion was monitored).

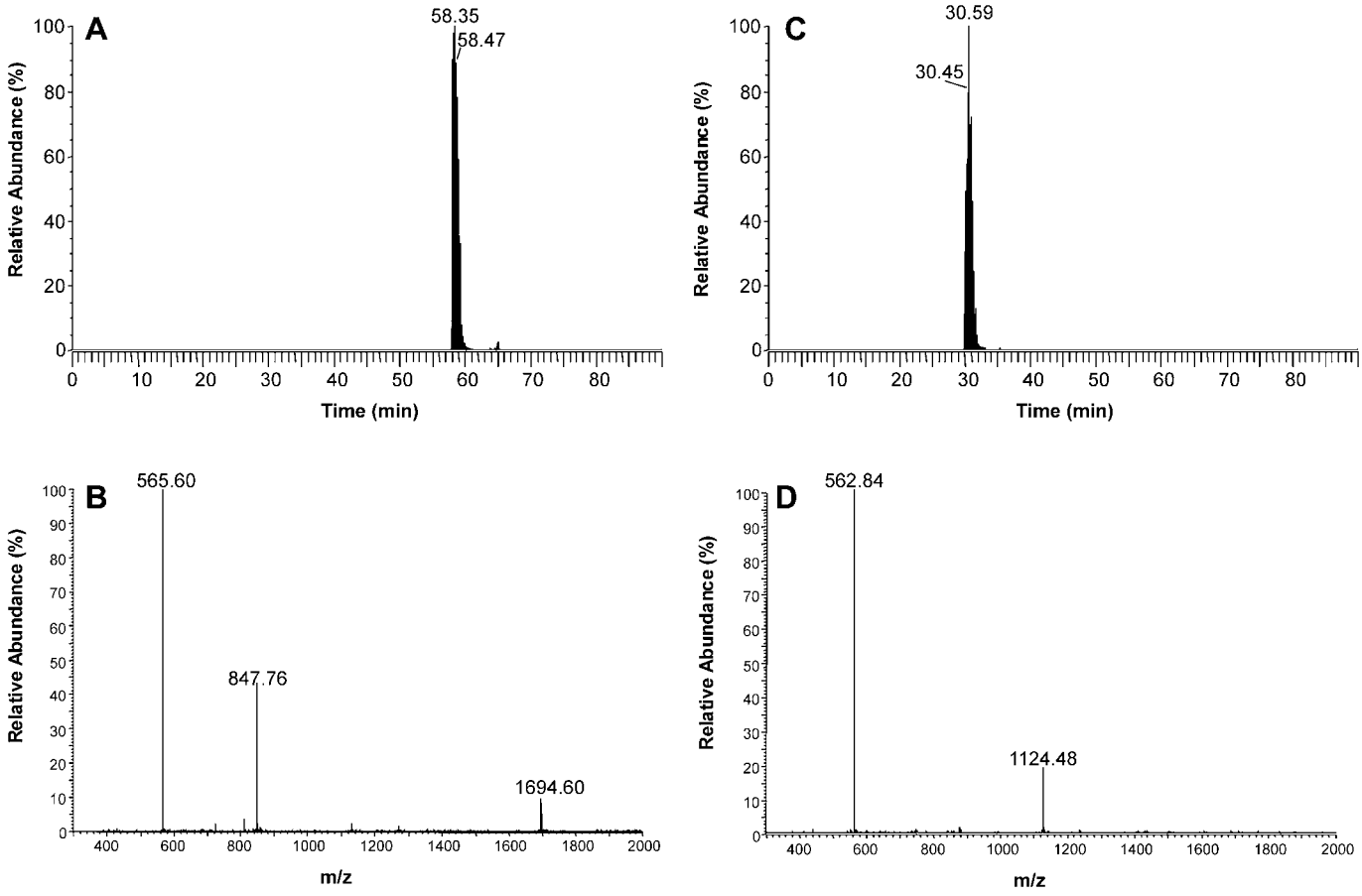


Fig. 7. LC/MS analysis of FCAT 2 labeled Peptide 1 before and after fluorophore motif removal. (A) Total ion current trace of FCAT 2 labeled Peptide 1; (B) MS spectra at 58.41 min retention time ($[M + H]^+ = 1694.60$; $[M + 2H]^{+2} = 847.76$; $[M + 3H]^{+3} = 565.60$). (C) Total ion current trace of fluorophore motif removal reaction product; (D) MS spectra at 30.54 min retention time ($[M + H]^+ = 1124.48$; $[M + 2H]^{+2} = 562.84$).

tides the predicted products were found as shown by the MS/MS analysis in Fig. 6A depicting the b and y ions. On the other hand, while the anticipated peaks were not found in the FCAT 2 and FCAT 3 labeled peptides (e.g., *m/z* 1105.56 and 1044.49 for Peptide 1), the cleavage products had unexpected peaks with 18 Da higher (e.g., 1124.69 and 1063.62 for Peptide 1). MS/MS analysis of the cleavage products suggested water addition, probably via the opening of the succinimide ring. Fig. 6B shows the MS/MS analysis of Peptide 2 labeled with FCAT 2. The efficiency of the fluorophore group removal was higher than 99%. The example in Fig. 7 depicts the LC/MS analysis traces of FCAT 2 labeled Peptide 1 before and after the fluorophore group cleavage.

4. Conclusions

A new class of isotope-coded affinity reagents was designed, dubbed as Fluorescent isotope-coded affinity tag (FCAT), to label cysteine containing peptides or proteins. The fluorophore part of the tag offered the option for absolute quantification by means of fluorescent detection. The molecules were synthesized by SPOS. Best synthesis yield was obtained for the FCAT 2 reagent. FCAT 2 also showed higher stability than FCAT 1, as this latter showed self-alkylation, dehydration and deiodination reactions. All molecules were well characterized by MALDI-TOF MS, NMR and LC–MS. Reactivity of the FCAT tags were tested on terminal and internal cysteine containing model peptides. Our results also suggested that FCAT labeling supports peptides MS/MS based peptide sequencing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2008.08.005.

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