Design and Synthesis of Visible Isotope-Coded Affinity Tags for the Absolute Quantification of Specific Proteins in Complex Mixtures

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Identification of proteins in complex mixtures by mass spectrometry is most useful when quantitative data is also obtained. We recently introduced isotope-coded affinity tags (ICAT reagents) for the relative quantification of proteins present in two or more biological samples. In this report, we describe a new generation of ICAT reagents that contain the following additional features: (1) a visible tag that allows the electrophoretic position of tagged peptides during separation to be easily monitored; (2) a photocleavable linker that allows most of the tag to be removed prior to mass spectrometric analysis; (3) an isotope tag that contains carbon-13 and nitrogen-15 atoms instead of deuterium to ensure precise comigration of light and heavy tagged peptides by reverse-phase HPLC. These reagents contain an iodoacetyl group that selectively reacts with peptide cysteine residues. Peptide modification chemistry is also reported that allows tagging of peptides that are devoid of cysteine. The synthesis of these visible isotope-coded affinity tags (VICAT reagents), and their reaction with peptides are described in this report. VICAT reagents containing a carbon-14 visible probe or an NBD fluorophore are described. These reagents are most useful for the determination of the absolute quantity of specific target proteins in complex protein mixtures such as serum or cell lysates.

INTRODUCTION

Mass spectrometry has emerged as one of the most useful techniques for the detection of proteins and protein-derived peptides. Since mass spectrometry often depends on internal standards for quantification, there have been recent efforts to develop novel chemical reagents that, when combined with mass spectrometry, provide protein abundance information on a quantitative level (*1*−*3*). Most of these methods involve the use of an internal standard, that is a compound identical to the analyte whose level is desired but is distinguished by different molecular mass due to heavy isotopic substitution. Along these lines, we have developed a reagent called the isotope-coded affinity tag (ICAT) and first applied this reagent to detect changes in the relative levels of specific proteins in yeast in response to changes in growth conditions (*4*). This is an example of a survey-type method in which abundance information is obtained on as many proteins as possible in a complex protein mixture (*5*, *6*). ICAT reagents are used to selectively tag the SH groups of peptide cysteine residues, with the introduction of a light and heavy isotope tag and a biotin residue for selective enrichment of tag peptides. Another cysteine peptide enrichment and isotope tagging scheme has been developed by Johnson and co-workers (*7*).

There is also a clear need for the development of reagents that allow a specific set of known proteins to be detected in complex mixtures and their absolute abundance determined. Detection of specific proteins in a complex mixture is usually carried out by one or more immunological methods including immunoblotting (western blotting) and ELISA. Although these methods are routinely used and provide valuable information, they have a number of limitations as discussed previously (*8*). For example, it often requires ∼1 year of work at a substantial cost to develop a highly specific antibody required for an ELISA-based detection of low levels of a specific protein in a complex biological fluid such as serum. Thus, it is not practical for a single laboratory to develop an immunological assay of a set of say 10−20 proteins, for example to evaluate the levels of proteins discovered by other techniques such as nucleic acid microarrays.

When the sequence of the peptide derived from the protein of interest is known, the mass spectrometer can be used to selectively monitor the particular parent ion mass of the target peptide of interest. Additional selective monitoring is provided by tandem mass spectrometry, in which a specific fragment ion or ions derived from the peptide parent ion is detected (specific or multiple reaction monitoring). Typically this type of experiment is done by separation of the peptide mixture on a reverse-phase, microbore HPLC column, which is interfaced directly with an electrospray ionization mass spectrometer. However, HPLC separation combined with specific reaction monitoring is probably not generally sufficient to detect specific peptides in complex peptide mixtures such as a trypsin digest of a eukaryotic cell lysate. This is because such tryptic digests may contain hundreds of thousands of distinct peptides, and the peptide of interest may be present in relatively small amounts compared to highly abundant peptides. For example, sequential separation is employed in the AQUA method (*9*), which makes use of protein electrophoresis (Laemmli gel) prior to

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combined HPLC/multiple reaction monitoring via tandem mass spectrometry for the absolute quantification of specific proteins in cell lysates.

In the present study, we describe the design and synthesis of modified ICAT reagents that contain a visible probe that allows tagged peptides to be detected by methods independent of mass spectrometry. These visible isotope-coded affinity tag reagents (VICAT reagents) are designed to be used in a analytical scheme in which peptides tagged with VICAT reagents are first resolved by isoelectric focusing (IEF) on a commercial gel strip containing an immobilized pH gradient. The presence of the visible probe allows the precise position of the desired tagged peptide to be immediately determined following one-dimensional separation by IEF. The desired region of the IEF gel strip is then excised, and eluted peptides are analyzed by combined reverse-phase, microbore HPLC/electrospray ionization tandem mass spectrometry. The visible probe also allows the absolute amount of the target peptide to be quantified at all steps in the process from postpeptide tagging to quantification in the mass spectrometer. This is an important feature since losses of analyte are inevitable in any multistep process.

In the present paper, we report the first phase of our development of VICAT reagents, the full experimental details for their synthesis and a demonstration of their reactivity with peptides and subsequent transformation prior to chromatographic separation and quantification by mass spectrometry. We also describe a peptide modification sequence, which allows tagging with ICAT and VICAT reagents of peptides that lack cysteine residues. In a subsequent, more biologically oriented study (*8*), we demonstrate the use of VICAT reagents for the absolute quantification of specific target proteins in eukaryotic cell lysates.

EXPERIMENTAL PROCEDURES

General Procedures. The structures of the VICAT reagents are shown in Figure 1, and their syntheses is outlined in Figures 2 and 4. Tetrahydrofuran (THF) was distilled from sodium/benzophenone under Ar, and methylene chloride (CH_2Cl_2) and triethylamine (TEA) were distilled from CaH_2 under Ar. Anhydrous dimethylformamide (DMF), pyridine, [^{13}C,^{15}N]-potassium cyanide, and 1,2-[$^{13}C_2$]-dibromoethane were purchased from Aldrich. Unless otherwise noted, all other compounds are reagent grade and were used as received. Unless otherwise noted, all nonaqueous reactions were carried out under Ar with oven-dried glassware. Electrospray ionization mass spectrometry (ESI-MS) was carried out on a Bruker Daltonics Esquire ion trap mass spectrometer, and MALDI data was obtained on a Applied Biosystems 4700 Proteomics Analyzer. Silica gel flash chromatography was performed using 230−400 mesh silica gel 60 (Merck, Darmstadt, Germany). Thin-layer chromatography was performed on aluminum-backed silica 60 plates with F254 indicator (Merck). Unless otherwise noted, reverse-phase HPLC separations were performed using a Vydac (Hisperia, CA) semipreparative column (up to 5 mg total loading, 10 μm packing, 10 mm × 250 mm, catalog no 218TP1010) at 4 mL/min, or a preparative column (up to 300 mg total loading, 10 μm packing, 20 mm × 250 mm, catalog no 218TP1022), or an a large preparative column (up to 1 g total loading, 10−15 μm packing, 5 × 25 cm, catalog no 218TP101550).

Synthesis of Sulfhydryl-Specific VICAT Reagents ($VICAT_{SH}$). *Biotin Tetrafluorophenyl Ester (**1**).* (+)-Biotin (4.5 g, 18.4 mmol) was dissolved in warm anhydrous DMF (90 mL). After the solution was cooled to room temperature, TEA (4.7 mL, 33.8 mmol) was added, followed by dropwise addition of tetrafluorophenyl trifluoroacetate (7.3 g, 27.4 mmol, (*10*)). After being stirred for 30 min at room temperature, the solvent was removed under reduced pressure. The residue was triturated with dry diethyl ether, and the product was filtered. The solid was dried under vacuum to afford 6.2 g (86%) of a white solid. ^{1}H NMR (300 MHz, d_6-DMSO) δ 7.9 (m, 1H); 6.4 (d, 2H); 4.35 (t, 1H); 4.15 (t, 1H); 3.2 (m, 1H); 2.75−2.9 (m, 3H), 2.6 (d, 1H); 1.4−1.8 (m, 6H).

*Biotin 2′-Aminoethylamide (**2**).* To a round-bottom flask containing ethylenediamine (8.5 g, 141.7 mmol) at 0 °C was transferred via cannula a solution of **1** (5 g, 12.8 mmol) in dry DMF (100 mL), also at 0 °C. After the mixture was stirred for 2 h at 0 °C, ethyl ether (100 mL) was added, and the solid was filtered and washed with ether. The white solid was left under vacuum overnight, and 3.15 g (86%) of the product was obtained. ^{1}H NMR (300 MHz, d_6-DMSO) δ 7.75 (bs, 1H); 6.4 (d, 2H); 4.35 (t, 1H); 4.15 (t,1H); 3.1 (m, 1H); 3.0 (q, 2H); 2.85 (dd, 1H); 2.5 (m, 3H); 2.0 (t, 2H), 1.2−1.7 (m, 6H). ESI-MS $(M + H^+)^+$: 287.0, $(2M + H^+)^+$: 573.0.

*Biotin 2′-(2″-Methylcarbonyl-1″-nitro-4″-phenylamino)-ethylamide (**3**).* To a round-bottom flask containing a solution of compound **2** (2.4 g, 8.4 mmol) in dry DMF (72 mL) and diisopropylethylamine (DIPEA) (2.9 mL, 16.7 mmol) at 0 °C was slowly added a solution of 5-bromomethyl-2-nitroacetophenone (*11*, *12*) (2.4 g, 9.3 mmol) in dry DMF (24 mL). After stirring for 3 h at 0 °C, the pH was adjusted to ∼5 with concentrated HCl (moist pH paper), and the solvent was removed in vacuo. The product was purified on a silica column packed with 5:1 CH_2Cl_2:MeOH. The column was developed with the same solvent, and the product was obtained in 64% yield (2.5 g). ^{1}H NMR (300 MHz, d_4-MeOH) δ 8.0 (d, 1H, J = 8.3 Hz); 7.75 (m, 2H); 4.45 (dd, 1H); 4.25 (dd,1H); 4.1 (s, 2H); 3.4 (t, 2H); 3.2 (m, 1H); 2.95 (m, 3H); 2.85 (d, 1H); 2.55 (s, 3H); 2.1 (t, 2H), 1.25−1.8 (m, 6H). ESI-MS $(M + H^+)^+$: 464.2, $(M + Na^+)^+$: 486.1.

*Compound **4**.* To a round-bottom flask containing a solution of compound **3** (2.5 g, 5.4 mmol) in MeOH (100 mL) and TEA (750 μL, 5.4 mmol) was added di-*tert*-butyl dicarbonate (1.3 g, 6.0 mmol). The mixture was stirred at 50 °C for 2 h and then cooled to room temperature. Sodium borohydride (650 mg,16.2 mmol) was added, and the reaction was stirred for an additional 1 h. Solvent was removed under reduced pressure and the residue resolubilized in CH_2Cl_2 (80 mL). The resulting solution was washed with water (40 mL) and brine (40 mL) and dried over anhydrous Na_2SO_4. Solvent was removed under reduced pressure to afford the product in 78% overall yield (2.4 g). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.8 (d, 1H, J = 8.4 Hz); 7.75 (bs, 1H); 7.2 (m, 1H); 6.9 (d, 1H); 5.9 (d, 1H); 5.35 (m, 1H); 4.4 (m, 3H); 4.25 (m,1H); 4.1 (d, 1H); 3.0−3.4 (m, 5H); 2.8 (dd, 1H); 2.65 (d, 1H); 2.1 (m, 2H); 1.8 (bs, 1H), 1.20−1.75 (m, 18H). ESI-MS $(M + Na^+)^+$: 588.5.

*N-(2-Aminoethyl)carbamate of **4** (**5**).* To a round-bottom flask containing a solution of compound **4** (224 mg, 0.4 mmol) in dry CH_2Cl_2 (4 mL) was added a solution of carbonyl diimidazole (CDI) (97.2 mg, 0.6 mmol) in dry CH_2Cl_2 (10 mL). After the mixture was stirred 2 h at room temperature in the presence of 4 Å molecular sieves, ethylenediamine (53.4 μL, 0.8 mmol) was added. After 1 h, the solvent was removed in a Speed-Vac (Savant Instruments). The residue was purified by HPLC: preparative C_{18} column, λ = 240 nm, 6 mL/min, solvent A = H_2O with 0.08% trifluoroacetic acid (TFA), solvent B =

CH_3CN with 0.08% TFA, gradient: 0′−20′: 0−20% B; 20′−70′: 20−70% B. The TFA salt of the product (244 mg, 79.7%) eluted between 38.7 and 44.3% B. ^{1}H NMR (300 MHz, d_4-MeOH) δ 7.9 (d, 1H); 7.5 (m, 1H); 7.35 (dd, 1H); 6.2 (m, 1H); 4.6 (bd, 2H); 4.45 (dd, 1H); 4.3 (m,1H); 3.25−3.5 (m, 6H); 3.2 (m, 1H); 3.0 (m, 2H); 2.8 (dt, 1H); 2.65 (dd, 1H); 2.2 (m, 2H); 1.25−1.8 (m, 18H). ESI-MS $(M + H^+)^+$: 652.4, $(M + Na^+)^+$: 674.3.

*Compound **6***. To a vial containing compound **5** (244 mg, 0.32 mmol) in dry THF (8 mL) in the presence of 4 Å molecular sieves was added DIPEA (112 μL, 0.64 mmol), followed by a solution of iodoacetic anhydride (170 mg, 0.48 mmol) in dry THF (2 mL). After the mixture was stirred 1.5 h at room temperature, the pH was adjusted to ∼5 with TFA (moist pH paper). The solvent was removed under reduced pressure and the residue purified by HPLC. Preparative C_{18} column, $\lambda = 240$ nm, 6 mL/min, gradient: 0′−20′: 0−20% B; 20′−80′: 20−70% B. The product (160 mg, 61%) eluted between 50.5 and 52% B. ^{1}H NMR (300 MHz, d_4-MeOH) δ 7.95 (d, 1H); 7.55 (m, 1H); 7.35 (dd, 1H); 6.2 (m, 1H); 4.55 (bs, 2H); 4.45 (dd, 1H); 4.25 (dd,1H); 3.65 (bs, 2H); 3.1−3.45 (m, 9H); 2.9 (dd, 1H); 2.65 (dd, 1H); 2.2 (m, 2H); 1.25−1.8 (m, 18H). ESI-MS $(M + Na^+)^+$: 842.2.

*Deprotection of **6** (**7**)*. To a vial containing a solution of compound **6** (160 mg, 0.2 mmol) in $CHCl_3$ (0.5 mL) was added TFA (200 μL). The reaction mixture was stirred for 1.5 h at room temperature. Solvent was removed in a Speed-Vac. MeOH was added, and the resulting solution was concentrated in a Speed-Vac. This procedure was repeated three times to remove excess TFA. The TFA salt of the product was obtained in 98.5% yield (160 mg). ^{1}H NMR (300 MHz, d_4-MeOH) δ 8.0 (d, 1H); 7.8 (bs, 1H); 7.6 (dd, 1H); 6.1 (m, 1H); 4.5 (m, 1H); 4.4 (bd, 2H); 4.3 (dd, 1H); 4.25 (dd,1H); 3.7 (bs, 2H); 3.55 (m, 2H); 3.0−3.45 (m, 7H); 2.9 (dd, 1H); 2.65 (dd, 1H); 2.2 (t, 2H); 1.25−1.8 (m, 9H). ESI-MS $(M + H^+)^+$: 720.

^{14}C-VICAT$_{SH}$(−28). To an Eppendorf tube containing a solution of compound **7** (7.34 mg; 8.8 μmol) in MeOH (272 μL) was added 1% [^{14}C]formaldehyde in water (v/v, 51 μL, 17.6 μmol, 1 mCi, specific activity 56 Ci/mol, Perkin-Elmer) followed by 1.2 M methanolic $NaCNBH_3$ (15 μL, 17.6 μmol) and 1.2 M methanolic DIPEA (22 μL, 26.4 μmol). The capped Eppendorf tube was placed in a shaker at 168 rpm for 6 h. The pH was adjusted to ∼5 (moist pH paper) with a 1.2 M aqueous solution of TFA (20 μL). The mixture was diluted with 30% CH_3CN in water and injected on to the HPLC column. Preparative C_{18} column, $\lambda = 240$ nm, 6 mL/min, gradient: 0′−20′: 0−20% B; 20′−80′: 20−70% B. The product (4.6 mg, 0.19 mCi) eluted between 34.4 and 36.7% B.

*N-(4-Aminobutyl)carbamate of **4** (**8**)*. To a round-bottom flask containing a solution of compound **4** (2.0 g, 3.54 mmol) in dry CH_2Cl_2 (20 mL) was added a solution of carbonyl diimidazole (860 mg, 5.31 mmol) in dry CH_2Cl_2 (20 mL). After the mixture was stirred 2 h at room temperature in the presence of 4 Å molecular sieves, 1,4-diaminobutane (putrecine) (623 mg, 7.08 mmol) was added. After an additional 2 h, the solvent was removed in a Speed-Vac. The residue was solubilized in water and the pH adjusted to ∼5 with TFA. The solution was injected onto the HPLC column. Large preparative C_{18} column, $\lambda = 240$ nm, 50 mL/min, gradient: 0′−10′: 20% B; 10′−60′: 70% B. The product as the TFA salt (2.15 g, 77%) eluted between 37 and 41.7% B. ^{1}H NMR (300 MHz, d_4-MeOH) δ 7.9 (d, 1H); 7.5 (m, 1H); 7.35 (dd, 1H); 6.2 (m, 1H); 4.6 (bd, 2H); 4.45 (dd, 1H); 4.3 (m,1H); 3.25−3.5 (m, 4H); 3.2 (m, 1H); 3.1 (m, 2H); 2.95 (m, 3H); 2.8 (dd, 1H); 2.2 (m, 2H); 1.25−1.8 (m, 22H). ESI-MS $(M + H^+)^+$: 680.5.

*N-(4-Iodoacetamidobutyl)carbamate of **4** (**9**)*. To a vial containing compound **8** (1.0 g, 1.26 mmol) in dry THF (15 mL) in the presence of 4 Å molecular sieves was added DIPEA (440 μL, 2.52 mmol), followed by a solution of iodoacetic anhydride (670 mg, 1.89 mmol) in dry THF (5 mL). After the mixture was stirred 1.5 h at room temperature, the pH was adjusted to ∼5 with TFA (moist pH paper). The solvents were removed under reduced pressure, and the residue was purified by HPLC. Large preparative C_{18} column, $\lambda = 240$ nm, 50 mL/min, gradient: 0′−10′: 20% B; 10′−80′: 70% B. The product (657 mg, 61%) eluted at 46.6% B. ^{1}H NMR (300 MHz, d_4-MeOH) δ 8.0 (d, 1H, $J = 8.3$ Hz); 7.6 (m, 1H); 7.4 (d, 1H, $J = 8.3$ Hz); 6.15 (m, 1H); 4.55 (d, 2H); 4.5 (dd, 1H); 4.3 (dd,1H); 3.65 (bs, 2H); 3.1−3.45 (m, 9H); 2.95 (dd, 1H); 2.75 (dd, 1H); 2.2 (t, 2H); 1.25−1.8 (m, 22H). ESI-MS $(M + H^+)^+$: 848.0; $(M + Na^+)^+$: 870.5.

*Deprotection of **9** (**10**)*. To a vial containing a solution of compound **9** (603 mg, 0.71 mmol) in $CHCl_3$ (3 mL) was added TFA (1.2 mL). The reaction mixture was stirred for 2 h at room temperature. Solvent was removed using in a Speed-Vac. MeOH was added, and the resulting solution was concentrated in the Speed-Vac. This procedure was repeated three times to remove excess TFA. The TFA salt of the product was obtained in 100% yield (611 mg). ^{1}H NMR (300 MHz, d_4-MeOH) δ 8.0 (d, 1H); 7.8 (bs, 1H); 7.6 (dd, 1H); 6.1 (m, 1H); 4.5 (m, 1H); 4.4 (bd, 2H); 4.3 (dd, 1H); 4.25 (dd,1H); 3.65 (bs, 2H); 3.55 (m, 2H); 3.1−3.35 (m, 7H); 2.95 (dd, 1H); 2.65 (dd, 1H); 2.25 (t, 2H); 1.4−1.8 (m, 13H). ESI-MS $(M + H^+)^+$: 748.4

^{14}C-VICAT$_{SH}$. To an Eppendorf tube containing a solution of compound **10** (7.54 mg; 8.8 μmol) in MeOH (272 μL), was added 1% [^{14}C]formaldehyde in water (v/v, 59 μL, 17.6 μmol, 1 mCi, 49.5 Ci/mole) followed by 1.2 M methanolic $NaCNBH_3$ (15 μL, 17.6 μmol) and 1.2 M methanolic DIPEA (22 μL, 26.4 μmol). The capped Eppendorf tube was placed in a shaker at 168 rpm for 6 h. The pH was adjusted to ∼5 (moist pH paper) with a 1.2 M aqueous solution of TFA (20 μL). The reaction was diluted with 30% CH_3CN:water and injected on to the HPLC column. Preparative C_{18} column, $\lambda = 240$ nm, 6 mL/min, gradient: 0′−10′: 0−20% B; 10′−70′: 20−70% B. The product (3.5 mg, 0.162mCi) eluted between 36.8 and 39.3% B.

VICAT$_{SH}$. The nonradioactive reaction was carried out as for ^{14}C-VICAT$_{SH}$ using compound **10** (1.09 g, 1.27 mmol), 37% formaldehyde in water (207 μL, 2.54 mmol), $NaCNBH_3$ (168 mg, 2.54 mmol), MeOH (50 mL) and DIPEA (662 μL, 3.81 mmol). The compound was purifed on the large preparative C18 column, $\lambda = 240$ nm, 50 mL/min, gradient: 0′−10′: 0−20% B; 10′−60′: 20−70% B. The product (900 mg, 81.8%) eluted at 32% B. ^{1}H NMR (300 MHz, d_4-MeOH) δ 8.0 (d, 1H); 7.8 (s, 1H); 7.6 (dd, 1H); 6.1 (m, 1H); 4.5 (dd, 1H); 4.25 (dd,1H); 3.65 (bs, 2H); 3.6 (m, 2H); 3.1−3.45 (m, 9H); 2.95 (m, 4H); 2.65 (dd, 1H); 2.25 (t, 2H); 1.25−1.8 (m, 13H). ESI-MS $(M + H^+)^+$: 762.3.

*[$^{15}N_2$,$^{13}C_4$]butanedinitrile (**11**)*. Potassium [^{13}C, ^{15}N]-cyanide (176.3 mg, 2.63 mmol) and [1,2-$^{13}C_2$]-dibromoethane (0.5 g, 2.63 mmol) were refluxed for 5 h in 70% ethanol (2 mL). The reaction mixture was cooled to room temperature and passed through a silica plug with benzene. The solvent was removed under reduced pressure to afford the product in 80% yield (90 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.0 (m, 2H); 2.55 (m, 2H).

*[$^{14}N_2$,$^{13}C_4$]-1,4-Butanediamine dihydrochloride (**12**)*. Compound **11** (137.1 mg, 1.59 mmol) and a 1 M solution of

borane in dry THF (16 mL, 15.9 mmol) were refluxed for 24 h. After the reaction was cooled to room temperature, anhydrous ethanol (18 mL) was added. The reaction was stirred for 24 h, and HCl gas was bubbled through the solution for 10 min, or until saturation. The di-chloride salt was filtered, washed with anhydrous ethanol, and dried under vacuum to afford 146.5 mg (55.2%) of a white solid. ^{1}H NMR (300 MHz, D_2O) δ 3.5 (m, 2H); 2.75 (m, 2H); 2.15 (m, 2H); 1.75 (m, 2H).

*N-([$^{14}N_2$,$^{13}C_4$]aminobutyl)carbamate of **4** (**13**).* A solution of compound **12** (30 mg, 0.18 mmol), and TEA (368 μL, 2.62 mmol) in DMSO (1 mL) was heated at 80 °C for 2 h. In another round-bottom flask containing a solution of compound **4** (25.4 mg, 0.045 mmol) in dry CH_2Cl_2 (1 mL) was added a solution of carbonyl diimidazole (10.9 mg, 0.067 mmol) in dry CH_2Cl_2 (0.5 mL). The reaction was stirred at room temperature for 2 h, and transferred to the DMSO solution that was being heated. The resulting reaction was kept at the same temperature for 1.5 h. The pH was adjusted to ~5 with TFA (moist pH paper), and the CH_2Cl_2 was evaporated under reduced pressure. The residue was diluted with water and injected on to the HPLC column. Preparative C_{18} column, λ = 240 nm, 6 mL/min, gradient: 0′−10′: 0−20% B; 10′−70′: 20−70% B. The product as the TFA salt (26.8 mg74.7%) eluted between 43.8% B. ^{1}H NMR (300 MHz, d_4-MeOH) δ 7.9 (d, 1H); 7.5 (m, 1H); 7.35 (dd, 1H); 6.2 (m, 1H); 4.6 (bd, 2H); 4.45 (dd, 1H); 4.3 (m,1H); 3.1−3.45 (m, 7H); 2.9 (m, 3H); 2.75 (dd, 1H); 2.2 (m, 2H); 1.25−1.8 (m, 22H). ESI-MS $(M + H^+)^+$: 686.3.

*Compound **14**.* The same procedure as described for compound **9** was used. The following amounts were used: Compound **13** (37.5 mg, 0.047 mmol); iodoacetic anhydride (25 mg, 0.07 mmol), DIPEA (16.3 μL, 0.094 mmol), and dry THF (0.6 mL). The product was purified by HPLC. Preparative C_{18} column, λ = 240 nm, 6 mL/min, gradient: 0′−10′: 0−20% B; 10′−70′: 20−70% B. The product (compound **16**) (24.5 mg, 61.3%) eluted between 50.8 and 53% B. ESI-MS $(M + H^+)^+$: 854.5; $(M + Na^+)^+$: 876.5.

*Compound **15**.* The same procedure as described for compound **10** was used. The following amounts were used: Compound **14** (24.5 mg, 0.029 mmol); TFA (160 μL), and $CHCl_3$ (240 μL). The TFA salt of the product was obtained in 94.8% yield (23.6 mg). ^{1}H NMR (300 MHz, d_4-MeOH) δ 8.0 (d, 1H); 7.8 (bs, 1H); 7.6 (dd, 1H); 6.1 (m, 1H); 4.5 (m, 1H); 4.4 (bd, 2H); 4.3 (dd, 1H); 3.65 (bs, 2H); 3.55 (m, 2H); 3.15−3.35 (m, 7H); 2.95 (dd, 2H); 2.70 (m, 1H); 2.65 (dd, 1H); 2.25 (t, 2H); 1.4−1.8 (m, 13H). ESI-MS $(M + H^+)^+$: 754.4.

VICAT$_{SH}$(+6). The same procedure as described for VICAT$_{SH}$ was used. The following amounts were used: Compound **15** (23.6 mg, 0.027 mmol); 1% aqueous solution of formaldehyde (83 μL; 0.054 mmol); 1.2 M methanolic $NaCNBH_3$ (23 μL; 0.054 mmol); 1.2 M methanolic DIPEA (34 μL; 0.081 mmol); and MeOH (800 μL). The reaction was diluted with 30% CH_3CN in water, and injected on to the HPLC column. Preparative C_{18} column, λ = 240 nm, 6 mL/min, gradient: 0′−10′: 0−20% B; 10′−70′: 20−70% B. The product (14.8 mg, 70.8%) eluted between 36.2 and 39% B. ^{1}H NMR (300 MHz, d_4-MeOH) δ 8.0 (d, 1H); 7.8 (s, 1H); 7.6 (dd, 1H); 6.15 (m, 1H); 4.45 (dd, 1H); 4.30 (dd,1H); 3.65 (bs, 2H); 3.6 (m, 2H); 3.1−3.5 (m, 7H); 2.9−3.0 (m, 6H); 2.6 (dd, 1H); 2.2 (t, 2H); 1.2−1.8 (m, 13H). ESI-MS $(M + H^+)^+$: 768.4; $(M + Na^+)^+$: 790.3.

^{14}C-VICAT$_{SH}$(+6). This compound was made using a variation of the procedure used to make ^{14}C-VICAT$_{SH}$. To a vial with a Teflon septum-lined screw cap containing a solution of compound **15** (17.3 mg; 20 μmol) in MeOH (765 μL) was added 1 M aqueous sodium acetate, pH 6.7 (139 μL). To a tube containing 60 μL of 0.33 M formaldehyde in water (19.8 μmol, prepared by diluting reagent grade 37% (w/v) formaldehyde with water), was added 49 μL of an aqueous solution of [^{14}C]formaldehyde in water (25 mCi/mL, 1.23 mCi, 21.9 μmol, 56 mCi/mmol, Perkin-Elmer). This mixture was transferred to the reaction vial. To the vial was added 1.2 M methanolic $NaCNBH_3$ (105 μL, 40 μmol). After the mixture was stirred for 30 min at room temperature, most of the solvent was removed with a stream of N_2. The residue was diluted with 100 μL of water containing 0.08% TFA, and the solution was injected onto the HPLC column. Preparative C18 column, λ = 240 nm, 6 mL/min, gradient: 0′−10′: 0−20% B; 10′−70′: 20−70% B. The TFA salt of the product (12.4 mg, 70.4% yield) eluted between 36.5 and 40% B. The compound was characterized by its HPLC retention, which matched that of the nonradiolabeled VICAT$_{SH}$(+6).

The specific radioactivity of ^{14}C-VICAT$_{SH}$(+6) was determined by accurately weighing several milligrams of the HPLC-purified material (after three rounds of evaporation to dryness in a Speed-Vac followed by dissolution in 0.4 mL of MeOH and drying in vacuo to constant weight), dissolving the compound in 1 mL of CH_3CN, and submitting a 5 μL aliquot to scintillation counting. The counter was calibrated using a vial of ^{14}C standard. Final specific activity is 22.0 mCi/mmol.

*Compound **16**.* To a vial containing a solution of compound **10** (5 mg; 5.81 mmol) in MeOH (179 μL) was added 1 M sodium acetate pH 6.7 (76 μL), followed by a 1.2 M methanolic solution of *tert*-butyl *N*-(2-oxyethyl)-carbamate (19.3 μL, 23.2 mmol, Aldrich) and a 1.2 M methanolic solution of $NaCNBH_4$ (19.3 μL, 23.2 mmol). The reaction was stirred at room temperature for 1 h. The solvent was partially removed, a mixture of 20% acetonitrile in water was added, and the reaction mixture was injected onto the HPLC column. Preparative C18 column, λ = 240 nm, 6 mL/min, gradient: 0′−20′: 0−20% B; 20′−80′: 20−100% B. The TFA salt of product **16** (5.8 mg, 92.6% yield) eluted between 53.8 and 58% B. ^{1}H NMR (300 MHz, d_1-$CHCl_3$/d_4-MeOH) δ 7.9 (d, 1H); 7.85 (m, 1H); 7.55 (m, 1H); 6.1 (m, 1H); 4.45 (m, 3H); 4.2 (m, 1H); 3.6 (s, 2H); 2.9−3.4 (m, 13H); 2.8 (dd, 1H); 2.6 (d, 1H); 2.2 (t, 2H); 1.25−1.65 (m, 22H). ESI-MS $(M + H^+)^+$: 891.0; $(M + Na^+)^+$: 913.5.

*Compound **17**.* To a vial containing a solution of **16** (5 mg, 5.07 mmol) in $CHCl_3$ (30 μL) was added TFA (12 μL). The reaction mixture was stirred for 1.5 h at room temperature. Solvent was removed using a Speed-Vac. MeOH was added, and the resulting solution was concentrated in the Speed-Vac. This procedure was repeated three more times to remove excess TFA. The TFA salt of the product (compound **17**) was obtained in 95.6% yield (4.3 mg) as an oil. ^{1}H NMR (300 MHz, acetone-d_6) δ 7.9 (d, 1H); 7.7 (s,1H); 7.5 (d, 1H); 6.1 (m, 1H); 4.6 (m, 1H); 4.4 (m, 1H); 3.9 (m, 2H); 3.7 (s, 2H); 3.4−2.9 (m, 12H); 2.7 (m, 3H); 2.2 (t, 2H); 1.4−1.8 (13H, m). ESI-MS $(M + H^+)^+$: 791.5; (M + Na+)+: 813.5.

NBD-VICAT$_{SH}$. To a vial containing **17** (4.2 mg, 4.64 mmol) was added a solution of NBD-F (2.86 mg, 15.6 mmol, 70% pure, Dojindo Molecular Technologies) in DMF (220 μL), followed by DIPEA (3.15 μL, 18.1 mmol). After being stirred for 2 h at room temperature, the solvent was partially removed in a Speed-Vac. The residue was solubilized in 20% acetonitrile in water and injected onto the HPLC. Preparative C18 column, λ = 500 nm, 6 mL/min, gradient: 0′−20′: 0−20% B; 20′−

80′: 20−100% B. The orange solid product (3.5 mg, 81.4%) eluted between 61.3 and 69.7% B. ^{1}H NMR (300 MHz, CD_3CN) δ 8.43 (d, 1H); 7.65 (d, 1H); 7.6 (bs, 1H); 7.4 (d, 1H); 6.0 (m, 2H); 4.4 (m, 1H); 4.25 (m, 1H); 3.75 (d, 2H); 3.6 (s, 2H); 2.9−3.2 (m, 12H); 2.65 (m, 3H); 2.22 (t, 2H); 1.35−1.8 (m, 13H). ESI-MS $(M + H^+)^+$: 954.5, $(M + Na^+)^+$: 976.4.

NBD-VICAT$_{SH}$(−28). This compound was prepared from **7** as described for the preparation of NBD-VICAT$_{SH}$ starting from **10**. HPLC Preparative C18 column, $\lambda = 500$ nm, 6 mL/min, gradient: 0′−20′: 0−20% B; 20′−80′: 20−100% B. The product as an organge solid (4.6 mg, 43.8%) eluted at 51% B. ^{1}H NMR (500 MHz, CD_3CN) δ 8.43 (d, 1H); 7.7 (bs, 1H); 7.6 (bs, 1H); 7.4 (bs, 1H); 6.16 (bs, 1H); 6.05 (bs, 1H); 4.4 (m, 1H); 4.25 (m, 1H); 3.8 (m, 2H); 3.65 (s, 2H); 2.9−3.2 (m, 12H); 2.7 (m, 3H); 2.22 (t, 2H); 1.35−1.9 (m, 9H). ESI-MS $(M + H^+)^+$: 926.6, $(M + Na^+)^+$: 948.7.

Peptide Modification Reactions. *Conversion of Peptide Amines into Thioacetamido Groups*. The reaction sequence is shown in Figure 5. To a 1.5 mL Eppendorf tube was added 280 μL of DMF:0.1 M Hepes, pH 8.0 (1:1) (pH adjusted before DMF added), followed by 20 μL of 1 mM angiotensin I peptide in water (DRVYIHPFHL, Sigma) and then 100 μL of *N*-succinimidyl S-acetylthioacetate (SATA, Pierce, 40 mM in dry DMF, freshly prepared). After incubation for 2 h at room temperature, 40 μL of deacetylation solution (0.5 M NH_2OH, 25 mM EDTA, 50 mM sodium phosphate, pH 7.5, store at 4 °C) was added, and the mixture was incubated for 2 h at room temperature. Then 150 μL of tris(2-carboxylethyl)-phosphine (TCEP, Aldrich, 0.2 M in water, store at −20 °C) was added followed by a 1 h incubation at 37 °C. The pH was dropped to 3−4 by adding 50 μL of 2% TFA, and the mixture was loaded onto a C18 reverse-phase, solid-phase extraction cartridge (500 mg resin, Varian) previously washed with 80% CH_3CN/0.1% TFA and then equilibrated with 0.1% TFA. The cartridge was washed with 0.1% TFA (2 × 1 mL) and then with 5% CH_3CN/0.1% TFA (4 × 1 mL), and peptide was eluted into a new tube with 1 mL of 80% CH_3CN/0.1% TFA. The solution was concentrated to dryness (Speed-Vac) in an Eppendorf tube.

Conjugation of Thioacetylated Peptide with VICAT$_{SH}$. All steps involving VICAT$_{SH}$ reagents were carried out in normal room light (fluorescent overhead lights) and away from windows exposed to bright sunlight. The residue from the previous step was resuspended in 50 μL of 20% CH_3CN. Buffer (4.8 μL of 0.5 M sodium phosphate, 0.5 M sodium borate, pH 8.4) was added, followed by 5.2 μL of VICAT$_{SH}$ stock (22.9 mM in CH_3CN, stored at −20 °C). The tube was wrapped with foil to exclude light and left for 12 h at room temperature. The reaction was examined by HPLC by adding 100 μL of 2% TFA and injecting the solution onto the column (Vydac 218TP52) previously equilibrated with 0.08% TFA (solvent A). The column was developed as follows: 0−15 min, 0% B (CH_3CN/0.08% TFA); 15−20 min, 0−10% B; 20−60 min, 10% B, 60−110 min, 10−80% B. The flow rate was 0.2 mL/min, and the absorbance at 217 nm was monitored. Peak fractions were examined by ESI-MS and MALDI.

Photocleavage of VICAT$_{SH}$-Tagged Peptides. The desired peptide conjugate HPLC fraction (see above) was concentrated to dryness (Speed-Vac) in an Eppendorf tube and the residue dissolved in 100 μL of 20% CH_3CN/0.1% TFA. Neat 2-mercaptoethanol (1 μL) was added, and the tube was shaken in an Eppendorf vibrating rack mixer at room temperature with irradiation for 20 min at room temperature from a UV lamp (Black-Ray long wave ultraviolet lamp model 100AP, VWR) with the bulb face held 10 cm from the tube. The intensity of the lamp was routinely checked with a UV light meter (Mannix UV340, Professional Equipment, Inc.). The reaction mixture was examined by HPLC (same column as above) using a solvent program of 0−60 min, 0−70% B. Fractions were examined by ESI-MS and MALDI.

The photocleavage of bead-bound peptide conjugate was also carried out. A 50 μL aliquot of beads (streptavidin-Agarose, 1:1 suspension in buffer as supplied by the manufacturer, Sigma) was pelleted in an Eppendorf tube (1 min full speed spin in a microfuge), and the pellet was washed twice with 1 mL portions of phosphate-buffered saline and resuspended in 1 mL of the same buffer. The peptide−VICAT$_{SH}$ conjugation reaction mixture (see above) was added to the bead suspension. The tube was placed on a rocking platform for 1 h at room temperature. The beads were pelleted and washed twice with 1 mL portions of water and resuspended in 100 μL of 20% CH_3CN/0.1% TFA. 2-Mercaptoethanol (1 μL) was added, and the tube was irradiated with UV light as described above. A 2 μl aliquot was analyzed by MALDI.

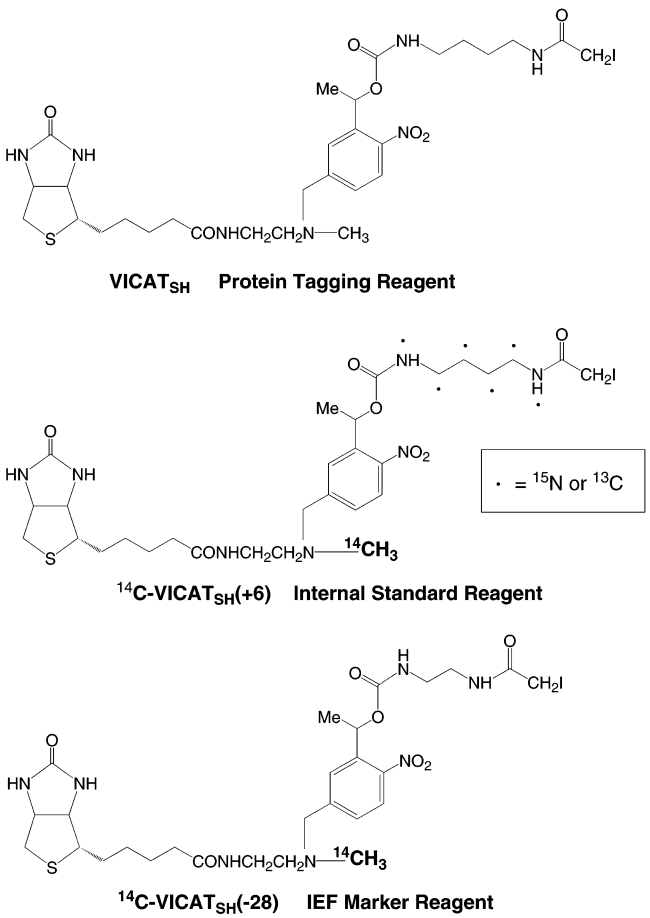

Figure 1. VICAT$_{SH}$ reagents.

RESULTS AND DISCUSSION

VICAT Reagents. The present paper describes the design and synthesis of VICAT reagents and their reaction with peptides. In a subsequent study we will illustrate the usefulness of these reagents to determine the absolute quantity of specific target proteins in complex protein mixtures (*8*). The set of three VICAT reagents is shown in Figure 1. These reagents contain an iodoacetyl group and thus react specifically with the

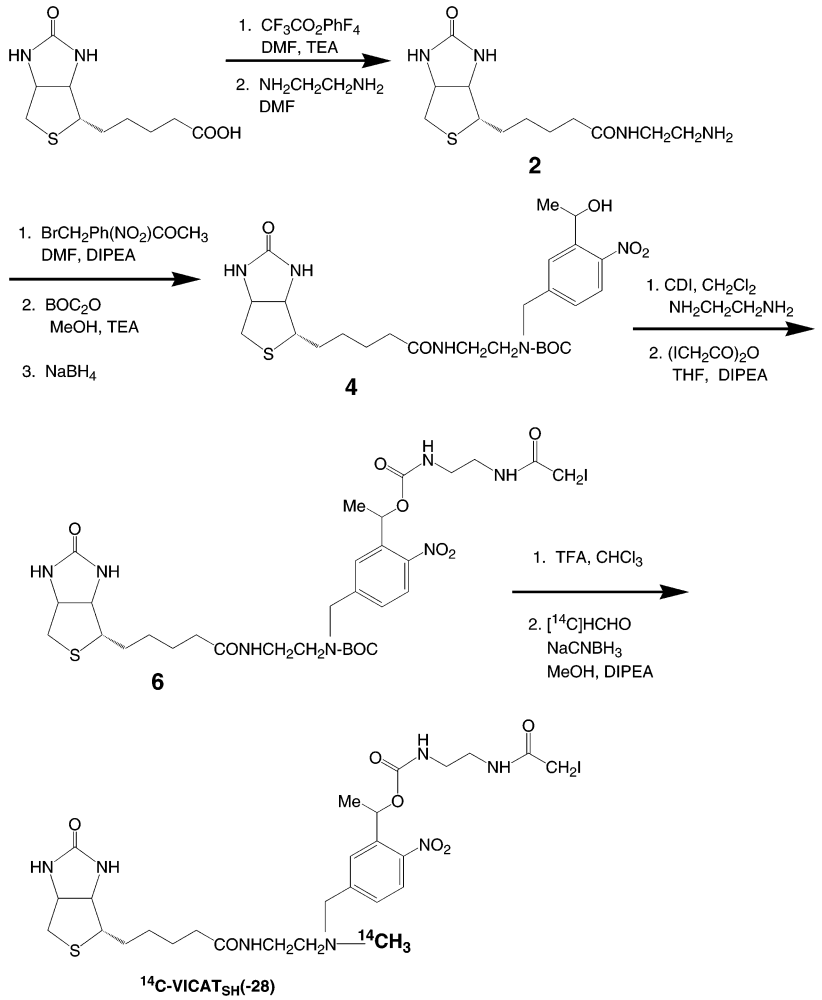

Figure 2. Synthesis of ^{14}C-VICAT$_{SH}$(−28).

SH group of cysteine-containing peptides. Thus we refer to these as VICAT$_{SH}$ reagents. We also show below that peptides without cysteine can be modified to contain an SH group which can be reacted with VICAT reagents. The VICAT reagents are used for (1) derivatization of SH-containing peptide fragments derived from all of the proteins in the sample including the target protein of interest, (2) preparation of an internal standard suitable for absolute quantification of the target protein of interest, and (3) preparation of a marker suitable for locating the tagged peptide derived from the target protein of interest following separation on an IEF gel strip. The VICAT-derivatized peptides are distinguished by the different masses of the tag introduced into the peptide as shown below.

The first reagent, VICAT$_{SH}$, is the "protein tagging" reagent as it is used to tag the SH groups present in peptides generated by proteolytic digestion of all of the proteins present in the biological sample to be analyzed. This reagent contains a 1,4-diaminobutane linker attached to the iodoacetyl group (Figure 1).

The second reagent, VICAT$_{SH}$(+6) is chemically identical to VICAT$_{SH}$ except for the presence of four ^{13}C and two ^{15}N labels in the diaminobutane linker (Figure 1). VICAT$_{SH}$(+6) is used as the "internal standard" reagent to tag the authentic synthetic peptide (prepared by solid-phase peptide synthesis) whose sequence is identical to the tryptic peptide derived from the target protein of interest. The mass difference of 6 Da between the sample-derived and internal standard peptides is sufficient for the distinction of both singly and doubly charged ions produced by electrospray ionization. A precise amount of the VICAT$_{SH}$(+6)−peptide conjugate is added to the sample and serves as an internal standard that is differentiated in the mass spectrometer from the protein sample-derived, tagged peptide. Integration of the ion chromatogram peaks, observed by mass spectrometry, from the sample-derived and internal standard conjugates in the mass spectrometry analysis provides the absolute abundance of the former and thus the absolute abundance of the specific protein in the complex protein mixture. We also prepared ^{14}C-VICAT$_{SH}$(+6), which can also be used to prepare the internal standard. This reagent is radiolabeled and of known specific activity and thus offers the ability to readily determine the absolute quantity of purified internal standard by scintillation counting.

The third reagent, ^{14}C-VICAT$_{SH}$(−28), contains the radioactive label and functions as the "IEF marker" for peptide visualization on the IEF strip. This reagent is used to derivatize the same synthetic peptide used to prepare the internal standard. ^{14}C-VICAT$_{SH}$(−28) contains a shorter (two-carbon) diamine linker that is attached to the iodoacetyl group (Figure 1). The purpose of using a shorter linker is 2-fold. First, the tagged peptide formed after photocleavage (see below) is more polar than the tagged peptides derived from the other VICAT$_{SH}$ reagents and is expected to have a slightly shorter reverse-phase HPLC retention time than the internal standard and sample protein-derived tagged

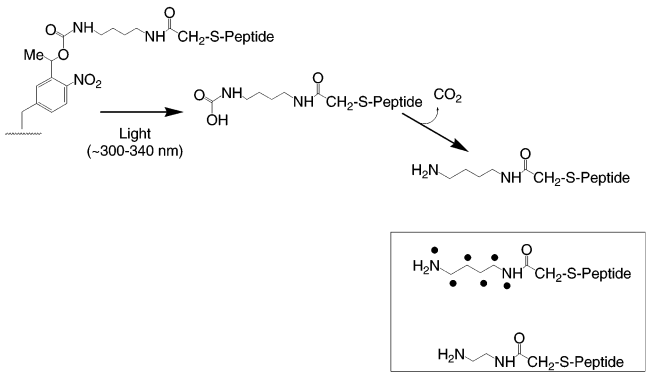

Figure 3. Photocleavage of VICAT$_{SH}$-tagged peptide. Shown is the photocleavage of the peptide tagged with VICAT$_{SH}$, which generates the carbamic acid, which spontaneously decarboxylates to give the peptide derived from the sample protein of interest bearing a tag with the diaminobutane linker. Shown in the box are the tagged peptides resulting from photocleavage of the internal standard and IEF marker, respectively. The black dots on the internal standard tag designate ^{15}N and ^{13}C labels.

peptides, both of which have a four-carbon linker in their tags. Second, the tag left on the IEF marker peptide is 28 Da lighter than the tag left on the sample protein-derived peptide and 34 Da ligher than the tag left on the internal standard and so is readily distinguished by mass spectrometry. Both factors are important for achieving a high dynamic range in the detection of peptides from low-level sample proteins in the presence of a substantially larger (10−1000-fold) amount of the visible marker applied to the IEF strip.

Following IEF, peptides are eluted from the appropriate region of the IEF gel strip using the IEF marker as a guide (*8*). The biotinyl moiety of these VICAT$_{SH}$ reagents is used for affinity capture of tagged peptides using streptavidin−agarose. Peptides are then released from the solid phase by photocleavage as shown in Figure 3. This has the advantage that the impurities in the sample after IEF, including soluble ampholytes, are not carried forward to the next step. Note that a small isotope tag remains on the peptides after photocleavage (Figure 3). This allows differentiation between the sample-derived and internal standard peptides in the mass spectrometer. The use of ^{15}N and ^{13}C present in VICAT$_{SH}$(+6) is advantageous over substitution with deuterium since the latter sometimes causes a slight change in the reverse-phase HPLC retention time of the isotope tagged peptide relative to that of the peptide tagged with the light isotope. The lack of precise coelution of heavy and light tagged peptides complicates the accurate integration of ion peaks observed in the mass spectrometer. Note that the peptide derived from the IEF marker bears a tag that is two methylene groups shorter than the tags left on the sample-derived and internal standard peptides (Figure 3). The IEF marker is typically used in much larger quantities than the internal standard because mass spectrometry is more sensitive than ^{14}C radiometric analysis. Since the IEF marker-derived peptide is 28 Da lighter than the sample-derived peptide, it is easily excluded during selective ion monitoring with the mass spectrometer. The use of a relatively large amount of IEF marker actually offers the important advantage of serving as a carrier to minimize nonspecific losses of trace amounts of sample-derived and internal standard peptides present throughout the analytical process. Such nonspecific losses can be severe when analyzing femtomole amounts of material.

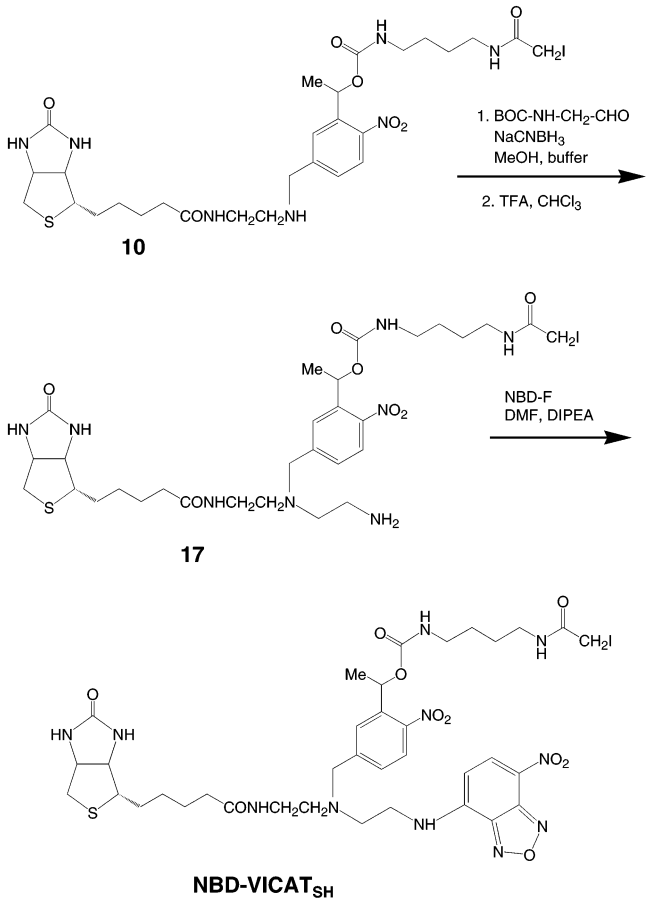

Figure 4. Synthesis of NBD-VICAT$_{SH}$.

Synthesis of VICAT$_{SH}$ Reagents. A highly efficient synthesis of VICAT$_{SH}$ reagents was developed as shown in Figure 2. This scheme allows the radiolabel of ^{14}C-VICAT$_{SH}$(−28) to be introduced in the last synthetic step using readily available ^{14}C-H_2CO. All of the steps proceed in acceptable yields. The heavy atom substituted 1,4-diaminobutane used to prepare VICAT$_{SH}$(+6) was readily prepared by a simple two-step scheme starting from the relatively inexpensive heavy isotopic materials [^{13}C,^{15}N]-potassium cyanide and 1,2-[$^{13}C_2$]-dibromoethane.

Using this scheme, we have been able to prepare gram quantities of VICAT$_{SH}$ reagent, sufficient for tens of thousands of VICAT$_{SH}$-based analyses. The other two reagents, ^{14}C-VICAT$_{SH}$(+6) and ^{14}C-VICAT$_{SH}$(−28), are required in relatively small amounts since each VICAT$_{SH}$-based analysis requires femtomole amounts of internal standard and nanomole amounts of IEF marker.

We have also designed a variant of VICAT$_{SH}$ reagents in which the visible probe is an NBD fluorophore rather than a radiolabel. As shown in Figure 4, intermediate **10** can be reductively aminated with commercially available BOC-NHCH$_2$CHO. Removal of the BOC group provides primary amine **17**, which can be reacted with a variety of different amine-reactive fluorophores including NBD-F as shown in Figure 4. Thus, both radioactive and fluorescent VICAT$_{SH}$ reagents can be prepared from a common synthetic intermediate. The availability of VICAT$_{SH}$ reagents bearing a fluorophore as the visible probe is advantageous in laboratories that are not approved for use of radioisotopes. The use of these fluorimetric reagents for the absolute quantification of specific proteins in complex protein mixtures is being currently developed in our laboratories. In cases were ^{14}C-VICAT$_{SH}$ is used, it may be noted that the carbon-14

Table 1. Thioacetylation of Peptide Amino Groups

peptide[a]	product	yield[b]
DRVYIHPFHL	(thioacetyl)DRVYIHPFHL	quantitative
pyroELYENKPRRPYIL	pyroELYENK(thioacetyl)PRRPYIL	quantitative
HDMNKVLDL	(thioacetyl)HDMNK(thioacetyl)VLDL	quantitative
ALEPPEPKKSRRCVLL	(thioacetyl)ALEPPEPK(thioacetyl)K(thioacetyl)SRRCVLL	quantitative

[a] pyroE is pyroglutamic acid. [b] Yields were determined by inspection of the HPLC trace (absorbance at 214 nm) and by MALDI mass spectrometry.

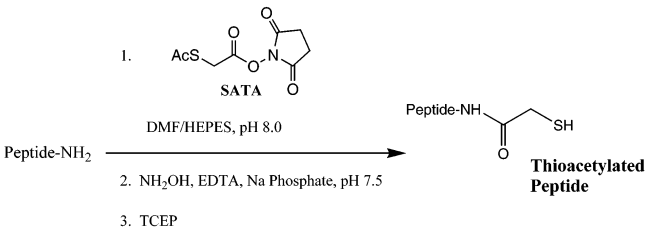

Figure 5. Thioacetylation of amino groups in peptides and reaction with $VICAT_{SH}$ reagents.

remains bound to the solid-phase streptavidin−agarose following photocleavage; thus, radioisotopes do not come in contact with the mass spectrometer.

Introduction of the SH Group into Peptides That Lack Cysteine. The use of ICAT and $VICAT_{SH}$ reagents to selectively tag cysteine-containing peptides has the advantage that complex mixtures of peptides, such as those derived from a cell lysate, are greatly simplified. However, in some cases it is desirable to tag peptides that lack cysteine, i.e., to quantify a protein that lacks cysteine or to quantify a protein segment that contains a post-translational modification and that also lacks cysteine. Thus, we desired a simple and quantitative method to introduce SH groups into peptides lacking cysteine. We chose the modification of amino groups since every tryptic peptide will contain an N-terminal amino group (except for the N-terminal peptide of N-terminally modified proteins), and those that have a lysine at the C-terminus will also have an ϵ-amino group. It is possible to design peptide-tagging reagents that contain a functional group such as an acylating agent that reacts with peptide amino groups. However, agents such as active esters that efficiently acylate amino groups also undergo hydrolysis in water, and thus an excess of tagging reagent is required, which is not desirable when using isotopically substituted tagging reagents. Thus, we desired a simple method for the conversion of peptide amino groups into sulfhydryl groups using inexpensive reagents and prior to tagging with $VICAT_{SH}$ reagents. This has the advantage that a large excess of acylating agent can be used to drive the amino group modification to completion and that the same ICAT and $VICAT_{SH}$ reagents can be used for peptides that contain or lack cysteine.

The previously described reagent *N*-succinimidyl-S-acetylthioacetate (SATA) is known to react with the amino group to give the amide which can be treated with hydroxylamine, resulting in the replacement of the amino group with a 2-sulfhydryl-acetamido group (Figure 5) (*13*). We examined this reaction sequence with a variety of peptides containing one or two amino groups (Table 1). A concentration of SATA of 10 mM was found to give quantitative acylation of peptide amino groups after 2 h at room temperature. Acylation studies at pH 6−9 revealed incomplete reaction at pH 6 (presumably due to protonation of the peptide amino groups) and at pH 9 (presumably due to hydrolysis of SATA). Addition of NH_2OH in slight excess over the amount of SATA led to incomplete deacylation; thus, a large excess (>100 mM) was optimal. It was found that after deacylation, a significant amount of the disulfide peptide-$NHCOCH_2$-$SSCH_2COOH$ was detected by MALDI and HPLC. Thus, the reaction mixture was treated with tricarboxylethylphosphine (TCEP), which led to quantitative reduction to the free thiol.

Using the optimized conditions, we obtained quantitative thioacetylation of amino groups in the peptides shown in Table 1. Thioacetylated peptides were examined by reverse phase HPLC, and single symmetrical product peaks were observed in all cases (not shown). Furthermore, MALDI mass spectrometry analysis of the HPLC product fraction showed only the desired product, with no trace of starting material or side products (not shown). The peptides in Table 1 have one to three amino groups, which were fully thioacetylated in all cases. Amino acids with nucleophile-containing side chains other than the amino group, Arg, Asp, Cys, Glu, His, Ser, and Tyr were not thioacetylated (any acylation of these nucleophiles during reaction with SATA would be reversed during treatment with NH_2OH).

Reaction of Peptides with $VICAT_{SH}$ Reagents. Thioacetylated angiotensin I peptide [(thioacetyl)DRVYIHPFHL] was desalted by solid-phase extraction on a C18 reverse-phase cartridge and then subjected to tagging with $VICAT_{SH}$. Conjugation of peptide SH group with $VICAT_{SH}$ reagent (~6 equiv based on peptide SH groups) was carried out in 20% CH_3CN/aqueous buffer to ensure good solubility of most peptides and tagging reagent and at pH 8.4, which is standard for reaction of peptide SH groups with iodoacetamido reagents. Reverse-phase HPLC analysis of the reaction mixture (not shown) showed the desired tagged peptide (confirmed by ESI-MS and MALDI), remaining $VICAT_{SH}$ reagent (confirmed by ESI-MS and MALDI), a trace amount of $VICAT_{SH}$-TCEP adduct (ESI-MS and MALDI gave $m/z = 884$), and a trace amount of $VICAT_{SH}$ cyclization product (ESI-MS and MALDI analyses show a m/z of 634, which is presumed to result from an intramolecular reaction of the tertiary amine of $VICAT_{SH}$ with the iodoacetyl group). No other HPLC peaks were observed, and spiking the reaction mixture with nontagged thioacetylated angiotensin I peptide confirmed that peptide tagging went to completion.

Thioacetylated angiotensin I tagged with $VICAT_{SH}$ was subjected to solution phase photocleavage or to affinity capture with streptavidin−agarose followed by on-resin photocleavage. HPLC analysis (not shown) of both reaction mixtures revealed only two peaks, one due to the desired photocleavage product, NH_2-$(CH_2)_4$−$NHCOCH_2SCH_2CONH$-peptide, and the other due to the photofragmented $VICAT_{SH}$ reagent containing the 2-nitrosoacetophenone moiety (products confirmed by ESI-MS and MALDI). Only these two products were seen when both photocleavage reaction mixtures were examined by MALDI prior to HPLC (not shown). This indicates that the $VICAT_{SH}$-tagged peptide undergoes quantitative photocleavage to the desired peptide containing the isotope tag (see above).

Application of VICAT$_{SH}$ Reagents and Future Prospects. There are numerous examples where the use of VICAT$_{SH}$ reagents will be useful. A major advantage of VICAT$_{SH}$ reagents is that they permit the absolute quantification of specific proteins in complex mixtures (*8*). In contrast, immunological methods require the lengthy and costly production of highly specific antibodies. Furthermore, immunoblot analyses (western blots) provide only qualitative data. Microarray nucleic analysis of changes in gene expression often need to be validated at the protein level. Furthermore, the search for markers in readily available biological fluids such as serum that have diagnostic value for human diseases will require a method for the absolute quantification of specific proteins. Microarray analyses often suggest tens if not hundreds of proteins that might be upregulated in human tumors for example. The validation of these candidate proteins using immunological methods is probably not practical, especially for a single laboratory effort.

The tagging of cysteine SH groups by VICAT$_{SH}$ reagents offers an important simplification of the peptide mixture in that many peptides will be removed following the purification of tagged peptides with streptavidin−agarose. Nevertheless, in some cases it will be beneficial to tag peptides that lack cysteines with VICAT reagents. A simple and efficient method to introduce SH groups into peptides lacking cysteine has been developed, and such peptides may be tagged with the same VICAT$_{SH}$ reagents used to modify cysteine-containing peptides. In a subsequent study (*8*), we will illustrate the usefulness of these reagents to determine the absolute quantity of specific target proteins in complex protein mixtures.

During the completion of the present study, the AQUA method was introduced for obtaining the absolute quantity of proteins in complex mixtures (*9*). The availability of two methods is beneficial, especially since it seems unlikely that a single method will provide useful data in every attempted experiment. It may be noted that with both the AQUA and VICAT$_{SH}$ methods, the amount of added internal standard should be within an order of magnitude of the amount of sample-derived peptide, given the issues of dynamic range in the mass spectrometry analysis. A detailed comparison of the AQUA and VICAT$_{SH}$ methods will be presented (*8*). VICAT$_{SH}$ reagents are available from the authors upon request.

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