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

Patricia Bottari,† Ruedi Aebersold,§ Frantisek Turecek,*,† and Michael H. Gelb*,†

Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195, and Institute for Systems Biology, Seattle, Washington 98103-8904. Received September 22, 2003; Revised Manuscript Received December 18, 2003

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 an 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
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 isoelution 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 in IEF gels. We 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 (CH2Cl2) and triethylamine (TEA) were distilled from CaH2 under Ar. Anhydrous dimethylformamide (DMF), pyridine, [13C,15N]-potassium cyanide, and (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. [1H NMR (300 MHz, d6-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′-Aminoethylamid(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. [1H NMR (300 MHz, d6-DMSO) δ 7.5 (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′-phenylaminomethylene)ethy lamid(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-bromo-1H-1,2,3-triazole (4.5 mL, 30.9 mmol) dissolved in DMF (90 mL). After the solution was cooled to room temperature in the presence of 4 Å molecular sieves, [1H NMR (300 MHz, d6-DMF) δ 7.75 (m, 2H); 4.45 (dd, 1H); 4.25 (dd, 1H); 4.1 (s, 2H); 3.4 (t, 2H); 3.4 (m, 2H); 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-tet-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 was resolubilized in CH2Cl2 (80 mL). The resulting solution was washed with water (40 mL) and brine (40 mL) and dried over anhydrous Na2SO4. Solvent was removed under reduced pressure to afford the product in 78% overall yield (2.4 g). [1H NMR (300 MHz, CDCl3) 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 CH2Cl2 (4 mL) was added a solution of carbonyl diimidazole (CDI) (97.2 mg, 0.6 mmol) in dry CH2Cl2 (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 C18 column, λ = 240 nm, 6 mL/min, solvent A = H2O with 0.08% trifluoroacetic acid (TFA), solvent B =
CH$_3$CN 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) $\delta$ 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 $\mu$L, 0.64 mmol), followed by a solution of iodocarboxylic 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 was 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 49.3% B. $^1$H NMR (300 MHz, $d_4$-MeOH) $\delta$ 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 (dd, 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 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) $\delta$ 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 (m, 2H); 1.25–1.8 (m, 18H). 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 $\mu$mol) in MeOH (272 $\mu$L) was added 1% 7$^{14}$Cformaldehyde in water (v/v, 51 $\mu$L, 17.6 $\mu$mol), 1 mL, specific activity 50 Ci/mol, Perkin-Elmer) followed by 1.2 mL methanolic NaCNBH$_3$ (15 $\mu$L, 7.6 $\mu$mol) and 1.2 mL methanolic DIPEA (22 $\mu$L, 24.4 $\mu$mol). 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 $\mu$L). The reaction was diluted with 30% CH$_3$CN:water and injected onto 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 8 (28). To a round-bottom flask containing a solution of compound 4 (2.0 g, 3.54 mmol) in dry CH$_2$Cl$_2$ (20 mL) was added a solution of carbonyl diimide (860 mg, 5.31 mmol) in dry CH$_2$Cl$_2$ (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) and DIPEA (662 $\mu$L, 3.81 mmol). The compound was purified on the large preparative C$_{18}$ column, $\lambda$ = 240 nm, 50 mL/min, gradient: 0–10': 0–20% B; 10–60': 20–70% B. The product (900 mg, 81.8% eut) eluted at 32% B. $^1$H NMR (300 MHz, $d_4$-MeOH) $\delta$ 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 [13C, 15N]-cyanide (176.3 mg, 2.63 mmol) and [1,2-$^{13}$C$_2$]-dibromomethylene (0.5 g, 2.63 mmol) were refluxed for 5 h in 7% 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$_3$) $\delta$ 3.0 (m, 2H); 2.55 (m, 2H).

$^{15}$N$_2$,$^{12}$C$_4$]-1,4-Butanediamine dihydrochloride (12). Compound 11 (137.1 mg, 1.59 mmol) and a 1 M solution of...
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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 μL, prepared by diluting reagent grade 37% (w/v) formaldehyde with water), was added 49 μL of an aqueous solution of [14C]formaldehyde in water (25 mCi/mL, 1.23 mCi, 21.9 μL, 56 mCi/mmole, Perkin-Elmer). This mixture was transferred to the reaction vial. To the vial was added 1.2 M methanolic NaCNBH₄ (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 N₂. The residue was dissolved 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 nonradio-labeled VICATSH(+)6).

The specific radioactivity of 14C-VICATSH(+)6) was determined by accurately weighing several milligrams of the HPLC-purified material (after three rounds of evaporation 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 CH₃CN, and submitting a 5 μL aliquot to scintillation counting. The counter was calibrated using a vial of 14C standard. Final specific activity is 22.0 mCi/mmol.

**Compound 15.** 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-oxethyl)-carbamate (19.3 μL, 23.2 mmol, Aldrich) and a 1.2 M methanolic solution of NaCNBH₄ (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. The reaction mixture 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 reaction mixture 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 reaction mixture 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 reaction mixture 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 reaction mixture 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 reaction mixture 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 reaction mixture 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 reaction mixture 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 reaction mixture 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 reaction mixture 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 reaction mixture 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.
80°: 20–100% B. The orange solid product (3.5 mg, 81.4%) eluted between 61.3 and 69.7% B. ¹H NMR (300 MHz, CD₃CN) 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, λ = 500 nm, 6 mL/min, gradient: 0’–20’: 0–20% B; 20’–80’: 20–100% B. The product as an orange solid (4.6 mg, 43.8%) eluted at 51% B. ¹H NMR (500 MHz, CD₃CN) 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 Thiaoacetamido 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 (DRYH1020HFL, 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, the tube was shaken in an Eppendorf vibrating rack away from windows exposed to bright sunlight. The reaction was examined by HPLC by adding 100 µL of 0.5 M sodium phosphate, pH 7.5, store at 4 °C) was added followed by a 1 h incubation at 37 °C. The pH was dropped to 3 and then 100 µL of 100% DMF:0.1 M Hepes, pH 8.0 (1:1) (pH adjusted before DMF added) was added followed by 20 µL of 1 mM angiotensin I peptide in water (DRYH1020HFL, 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, the residue from the previous step was resuspended in 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% CH₃CN/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% CH₃CN/0.1% TFA (4 × 1 mL), and peptide was eluted into a new tube with 1 mL of 80% CH₃CN/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% CH₃CN. 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 CH₃CN, 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 (CH₃CN/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% CH₃CN/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 by adding 100 µL of 2% TFA into the vial, and the mixture was loaded onto a C18 reverse-phase, solid-phase extraction cartridge (500 mg resin, Varian) previously washed with 80% CH₃CN/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% CH₃CN/0.1% TFA (4 × 1 mL), and peptide was eluted into a new tube with 1 mL of 80% CH₃CN/0.1% TFA. The solution was concentrated to dryness (Speed-Vac) in an Eppendorf tube.

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 1. VICAT_Sh reagents.](image-url)
SH group of cysteine-containing peptides. Thus we refer to these as VICATSH 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, VICATSH, 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, VICATSH(+6) is chemically identical to VICATSH except for the presence of four 13C and two 15N labels in the diaminobutane linker (Figure 1). VICATSH(+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 VICATSH(+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 14C-VICATSH(+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, 14C-VICATSH(-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. 14C-VICATSH(-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 photolysis (see below) is more polar than the tagged peptides derived from the other VICATSH reagents and is expected to have a slightly shorter reverse-phase HPLC retention time than the internal standard and sample protein-derived tagged peptide.

Figure 2. Synthesis of 14C-VICATSH(-28).
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 lighter 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. 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 15N and 13C 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 14C 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.

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 14C-VICAT SH (−28) to be introduced in the last synthetic step using readily available 14C-H2CO. 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 [13C,15N]-potassium cyanide and 1,2-[13C2]-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, 14C-VICAT SH (±6) and 14C-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-NH-CH2-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 where 14C-VICAT SH is used, it may be noted that the carbon-14

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 15N and 13C labels.

Figure 4. Synthesis of NBD-VICAT SH.
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 VICATSH 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 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 protein), and those that have a lysine at the C-terminus for the N-terminal peptide will contain an N-terminal amino group (except 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 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 protein), 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 VICATSH 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 VICATSH 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-sulfonylhydroxamido 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 NH3OH 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-NHCOCH2-SSCH2-COHN revealed incomplete reaction at pH 6 (presumably due to deacylation; thus, a large excess (>100 mM) was optimal. It was found that after deacylation, a significant amount of the disulfide peptide-NHCOCH2-SSCH2-COHN was detected by MALDI and HPLC. Thus, the reaction mixture was treated with tricarboxyethylphosphine (TCEP), which led to quantitative reduction to the free thiol.

Using the optimized conditions, we obtained quantitative thioacylation of amino groups in the peptides shown in Table 1. Thioacylated 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 thioacylated 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 thioacylated (any acylation of these nucleophiles during reaction with SATA would be reversed during treatment with NH3OH).

**Reaction of Peptides with VICATSH Reagents.** Thioacylated angiotensin I peptide ([thioacyl]DRVYIHPFHL) was desalted by solid-phase extraction on a C18 reverse-phase cartridge and then subjected to tagging with VICATSH. Conjugation of peptide SH group with VICATSH reagent (~6 equiv based on peptide SH groups) was carried out in 20% CH3CN/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 VICATSH reagent (confirmed by ESI-MS and MALDI), a trace amount of VICATSH-TCEP adduct (ESI-MS and MALDI gave m/z = 884), and a trace amount of VICATSH 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 VICATSH with the iodoacetyl group). No other HPLC peaks were observed, and spiking the reaction mixture with nontagged thioacylated angiotensin I peptide confirmed that peptide tagging went to completion.

Thioacylated angiotensin I tagged with VICATSH 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, NH2-(CH2)4-NHCOCH2-SSCH2-COHN peptide, and the other due to the photofragmented VICATSH 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 VICATSH-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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LITERATURE CITED


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