

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

Patricia Bottari,<sup>†</sup> Ruedi Aebersold,<sup>§</sup> Frantisek Turecek,<sup>\*,†</sup> and Michael H. Gelb<sup>\*,†,||</sup>

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 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 and 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

\* Corresponding authors. M.H.G.: phone (206) 543-7142; fax (206) 685-8665; e-mail gelb@chem.washington.edu. F.T.: phone (206) 685-2041; fax (206) 685-8665; e-mail turecek@chem.washington.edu.

<sup>†</sup> Department of Chemistry.

<sup>||</sup> Department of Biochemistry.

<sup>§</sup> Institute for Systems Biology.

## B

Bottari et al.

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

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

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

## 123 EXPERIMENTAL PROCEDURES

124 **General Procedures.** The structures of the VICAT  
125 reagents are shown in Figure 1, and their syntheses is  
126 outlined in Figures 2 and 4. Tetrahydrofuran (THF) was  
127 distilled from sodium/benzophenone under Ar, and methylene  
128 chloride ( $\text{CH}_2\text{Cl}_2$ ) and triethylamine (TEA) were  
129 distilled from  $\text{CaH}_2$  under Ar. Anhydrous dimethylformamide  
130 (DMF), pyridine, [ $^{13}\text{C}$ ,  $^{15}\text{N}$ ]-potassium cyanide, and  
131 1,2- [ $^{13}\text{C}_2$ ]-dibromoethane were purchased from Aldrich.  
132 Unless otherwise noted, all other compounds are reagent  
133 grade and were used as received. Unless otherwise noted,  
134 all nonaqueous reactions were carried out under Ar with  
135 oven-dried glassware. Electrospray ionization mass spectrometry  
136 (ESI-MS) was carried out on a Bruker Daltonics Esquire  
137 ion trap mass spectrometer, and MALDI data was  
138 obtained on a Applied Biosystems 4700 Proteomics  
139 Analyzer. Silica gel flash chromatography was performed  
140 using 230–400 mesh silica gel 60 (Merck, Darmstadt,  
141 Germany). Thin-layer chromatography was performed on  
142 aluminum-backed silica 60 plates with F254 indicator  
143 (Merck). Unless otherwise noted, reverse-phase HPLC  
144 separations were performed using a Vydac (Hisperia, CA)  
145 semipreparative column (up to 5 mg total loading, 10  $\mu\text{m}$   
146 packing, 10 mm  $\times$  250 mm, catalog no 218TP1010) at 4  
147 mL/min, or a preparative column (up to 300 mg total  
148 loading, 10  $\mu\text{m}$  packing, 20 mm  $\times$  250 mm, catalog no  
149 218TP1022), or an a large preparative column (up to 1 g  
150 total loading, 10–15  $\mu\text{m}$  packing, 5  $\times$  25 cm, catalog no  
151 218TP101550).

152 **Synthesis of Sulfhydryl-Specific VICAT Reagents**  
153 **(VICAT<sub>SH</sub>).** *Biotin Tetrafluorophenyl Ester (1).* (+)-Biotin  
154 (4.5 g, 18.4 mmol) was dissolved in warm anhydrous

DMF (90 mL). After the solution was cooled to room 155  
temperature, TEA (4.7 mL, 33.8 mmol) was added, 156  
followed by dropwise addition of tetrafluorophenyl tri- 157  
fluoroacetate (7.3 g, 27.4 mmol, (10)). After being stirred 158  
for 30 min at room temperature, the solvent was removed 159  
under reduced pressure. The residue was triturated with 160  
dry diethyl ether, and the product was filtered. The solid 161  
was dried under vacuum to afford 6.2 g (86%) of a white 162  
solid.  $^1\text{H}$  NMR (300 MHz,  $d_6$ -DMSO)  $\delta$  7.9 (m, 1H); 6.4 163  
(d, 2H); 4.35 (t, 1H); 4.15 (t, 1H); 3.2 (m, 1H); 2.75–2.9 164  
(m, 3H), 2.6 (d, 1H); 1.4–1.8 (m, 6H). 165

*Biotin 2'-Aminoethylamide (2).* To a round-bottom flask 166  
containing ethylenediamine (8.5 g, 141.7 mmol) at 0  $^\circ\text{C}$  167  
was transferred via cannula a solution of **1** (5 g, 12.8 168  
mmol) in dry DMF (100 mL), also at 0  $^\circ\text{C}$ . After the 169  
mixture was stirred for 2 h at 0  $^\circ\text{C}$ , ethyl ether (100 mL) 170  
was added, and the solid was filtered and washed with 171  
ether. The white solid was left under vacuum overnight, 172  
and 3.15 g (86%) of the product was obtained.  $^1\text{H}$  NMR 173  
(300 MHz,  $d_6$ -DMSO)  $\delta$  7.75 (bs, 1H); 6.4 (d, 2H); 4.35 (t, 174  
1H); 4.15 (t, 1H); 3.1 (m, 1H); 3.0 (q, 2H); 2.85 (dd, 1H); 175  
2.5 (m, 3H); 2.0 (t, 2H), 1.2–1.7 (m, 6H). ESI-MS ( $\text{M} +$  176  
 $\text{H}^+$ ): 287.0, ( $2\text{M} + \text{H}^+$ ): 573.0. 177

*Biotin 2'-(2'-Methylcarbonyl-1''-nitro-4''-phenylamino)-* 178  
*ethylamide (3).* To a round-bottom flask containing a 179  
solution of compound **2** (2.4 g, 8.4 mmol) in dry DMF (72 180  
mL) and diisopropylethylamine (DIPEA) (2.9 mL, 16.7 181  
mmol) at 0  $^\circ\text{C}$  was slowly added a solution of 5-bromo- 182  
methyl-2-nitroacetophenone (**11**, **12**) (2.4 g, 9.3 mmol) in 183  
dry DMF (24 mL). After stirring for 3 h at 0  $^\circ\text{C}$ , the pH 184  
was adjusted to  $\sim 5$  with concentrated HCl (moist pH 185  
paper), and the solvent was removed in vacuo. The 186  
product was purified on a silica column packed with 5:1 187  
 $\text{CH}_2\text{Cl}_2$ :MeOH. The column was developed with the same 188  
solvent, and the product was obtained in 64% yield (2.5 189  
g).  $^1\text{H}$  NMR (300 MHz,  $d_4$ -MeOH)  $\delta$  8.0 (d, 1H,  $J = 8.3$  190  
Hz); 7.75 (m, 2H); 4.45 (dd, 1H); 4.25 (dd, 1H); 4.1 (s, 2H); 191  
3.4 (t, 2H); 3.2 (m, 1H); 2.95 (m, 3H); 2.85 (d, 1H); 2.55 192  
(s, 3H); 2.1 (t, 2H), 1.25–1.8 (m, 6H). ESI-MS ( $\text{M} +$  193  
 $\text{H}^+$ ): 464.2, ( $\text{M} + \text{Na}^+$ ): 486.1. 194

*Compound 4.* To a round-bottom flask containing a 195  
solution of compound **3** (2.5 g, 5.4 mmol) in MeOH (100 196  
mL) and TEA (750  $\mu\text{L}$ , 5.4 mmol) was added di-*tert*-butyl 197  
dicarbonate (1.3 g, 6.0 mmol). The mixture was stirred 198  
at 50  $^\circ\text{C}$  for 2 h and then cooled to room temperature. 199  
Sodium borohydride (650 mg, 16.2 mmol) was added, and 200  
the reaction was stirred for an additional 1 h. Solvent 201  
was removed under reduced pressure and the residue 202  
resolubilized in  $\text{CH}_2\text{Cl}_2$  (80 mL). The resulting solution 203  
was washed with water (40 mL) and brine (40 mL) and 204  
dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Solvent was removed 205  
under reduced pressure to afford the product in 78% 206  
overall yield (2.4 g).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.8 (d, 207  
1H,  $J = 8.4$  Hz); 7.75 (bs, 1H); 7.2 (m, 1H); 6.9 (d, 1H); 208  
5.9 (d, 1H); 5.35 (m, 1H); 4.4 (m, 3H); 4.25 (m, 1H); 4.1 209  
(d, 1H); 3.0–3.4 (m, 5H); 2.8 (dd, 1H); 2.65 (d, 1H); 2.1 210  
(m, 2H); 1.8 (bs, 1H), 1.20–1.75 (m, 18H). ESI-MS ( $\text{M} +$  211  
 $\text{Na}^+$ ): 588.5. 212

*N-(2-Aminoethyl)carbamate of 4 (5).* To a round-bottom 213  
flask containing a solution of compound **4** (224 mg, 0.4 214  
mmol) in dry  $\text{CH}_2\text{Cl}_2$  (4 mL) was added a solution of 215  
carbonyl diimidazole (CDI) (97.2 mg, 0.6 mmol) in dry 216  
 $\text{CH}_2\text{Cl}_2$  (10 mL). After the mixture was stirred 2 h at 217  
room temperature in the presence of 4  $\text{Å}$  molecular sieves, 218  
ethylenediamine (53.4  $\mu\text{L}$ , 0.8 mmol) was added. After 1 219  
h, the solvent was removed in a Speed-Vac (Savant 220  
Instruments). The residue was purified by HPLC: pre- 221  
parative  $\text{C}_{18}$  column,  $\lambda = 240$  nm, 6 mL/min, solvent A = 222  
 $\text{H}_2\text{O}$  with 0.08% trifluoroacetic acid (TFA), solvent B = 223

224 CH<sub>3</sub>CN with 0.08% TFA, gradient: 0'–20': 0–20% B;  
225 20'–70': 20–70% B. The TFA salt of the product (244  
226 mg, 79.7%) eluted between 38.7 and 44.3% B. <sup>1</sup>H NMR  
227 (300 MHz, d<sub>4</sub>-MeOH) δ 7.9 (d, 1H); 7.5 (m, 1H); 7.35 (dd,  
228 1H); 6.2 (m, 1H); 4.6 (bd, 2H); 4.45 (dd, 1H); 4.3 (m, 1H);  
229 3.25–3.5 (m, 6H); 3.2 (m, 1H); 3.0 (m, 2H); 2.8 (dt, 1H);  
230 2.65 (dd, 1H); 2.2 (m, 2H); 1.25–1.8 (m, 18H). ESI-MS  
231 (M + H<sup>+</sup>)<sup>+</sup>: 652.4, (M + Na<sup>+</sup>)<sup>+</sup>: 674.3.

232 **Compound 6.** To a vial containing compound **5** (244  
233 mg, 0.32 mmol) in dry THF (8 mL) in the presence of 4  
234 Å molecular sieves was added DIPEA (112 μL, 0.64  
235 mmol), followed by a solution of iodoacetic anhydride (170  
236 mg, 0.48 mmol) in dry THF (2 mL). After the mixture  
237 was stirred 1.5 h at room temperature, the pH was  
238 adjusted to ~5 with TFA (moist pH paper). The solvent  
239 was removed under reduced pressure and the residue  
240 purified by HPLC. Preparative C<sub>18</sub> column, λ = 240 nm,  
241 6 mL/min, gradient: 0'–20': 0–20% B; 20'–80': 20–70%  
242 B. The product (160 mg, 61%) eluted between 50.5 and  
243 52% B. <sup>1</sup>H NMR (300 MHz, d<sub>4</sub>-MeOH) δ 7.95 (d, 1H); 7.55  
244 (m, 1H); 7.35 (dd, 1H); 6.2 (m, 1H); 4.55 (bs, 2H); 4.45  
245 (dd, 1H); 4.25 (dd, 1H); 3.65 (bs, 2H); 3.1–3.45 (m, 9H);  
246 2.9 (dd, 1H); 2.65 (dd, 1H); 2.2 (m, 2H); 1.25–1.8 (m,  
247 18H). ESI-MS (M + Na<sup>+</sup>)<sup>+</sup>: 842.2.

248 **Deprotection of 6 (7).** To a vial containing a solution of  
249 compound **6** (160 mg, 0.2 mmol) in CHCl<sub>3</sub> (0.5 mL) was  
250 added TFA (200 μL). The reaction mixture was stirred  
251 for 1.5 h at room temperature. Solvent was removed in  
252 a Speed-Vac. MeOH was added, and the resulting solution  
253 was concentrated in a Speed-Vac. This procedure  
254 was repeated three times to remove excess TFA. The TFA  
255 salt of the product was obtained in 98.5% yield (160 mg).  
256 <sup>1</sup>H NMR (300 MHz, d<sub>4</sub>-MeOH) δ 8.0 (d, 1H); 7.8 (bs, 1H);  
257 7.6 (dd, 1H); 6.1 (m, 1H); 4.5 (m, 1H); 4.4 (bd, 2H); 4.3  
258 (dd, 1H); 4.25 (dd, 1H); 3.7 (bs, 2H); 3.55 (m, 2H); 3.0–  
259 3.45 (m, 7H); 2.9 (dd, 1H); 2.65 (dd, 1H); 2.2 (t, 2H); 1.25–  
260 1.8 (m, 9H). ESI-MS (M + H<sup>+</sup>)<sup>+</sup>: 720.

261 <sup>14</sup>C-VICAT<sub>SH</sub>(-28). To an Eppendorf tube containing  
262 a solution of compound **7** (7.34 mg; 8.8 μmol) in MeOH  
263 (272 μL) was added 1% [<sup>14</sup>C]formaldehyde in water (v/v,  
264 51 μL, 17.6 μmol, 1 mCi, specific activity 56 Ci/mol,  
265 Perkin-Elmer) followed by 1.2 M methanolic NaCNBH<sub>3</sub>  
266 (15 μL, 17.6 μmol) and 1.2 M methanolic DIPEA (22 μL,  
267 26.4 μmol). The capped Eppendorf tube was placed in a  
268 shaker at 168 rpm for 6 h. The pH was adjusted to ~5  
269 (moist pH paper) with a 1.2 M aqueous solution of TFA  
270 (20 μL). The mixture was diluted with 30% CH<sub>3</sub>CN in  
271 water and injected on to the HPLC column. Preparative  
272 C<sub>18</sub> column, λ = 240 nm, 6 mL/min, gradient: 0'–20':  
273 0–20% B; 20'–80': 20–70% B. The product (4.6 mg, 0.19  
274 mCi) eluted between 34.4 and 36.7% B.

275 **N-(4-Aminobutyl)carbamate of 4 (8).** To a round-bottom  
276 flask containing a solution of compound **4** (2.0 g, 3.54  
277 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (20 mL) was added a solution of  
278 carbonyl diimidazole (860 mg, 5.31 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub>  
279 (20 mL). After the mixture was stirred 2 h at room  
280 temperature in the presence of 4 Å molecular sieves, 1,4-  
281 diaminobutane (putrescine) (623 mg, 7.08 mmol) was  
282 added. After an additional 2 h, the solvent was removed  
283 in a Speed-Vac. The residue was solubilized in water and  
284 the pH adjusted to ~5 with TFA. The solution was  
285 injected onto the HPLC column. Large preparative C<sub>18</sub>  
286 column, λ = 240 nm, 50 mL/min, gradient: 0'–10': 20%  
287 B; 10'–60': 70% B. The product as the TFA salt (2.15 g,  
288 77%) eluted between 37 and 41.7% B. <sup>1</sup>H NMR (300 MHz,  
289 d<sub>4</sub>-MeOH) δ 7.9 (d, 1H); 7.5 (m, 1H); 7.35 (dd, 1H); 6.2  
290 (m, 1H); 4.6 (bd, 2H); 4.45 (dd, 1H); 4.3 (m, 1H); 3.25–  
291 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<sup>+</sup>)<sup>+</sup>: 680.5.

292 **N-(4-Iodoacetamidobutyl)carbamate of 4 (9).** To a vial  
293 containing compound **8** (1.0 g, 1.26 mmol) in dry THF  
294 (15 mL) in the presence of 4 Å molecular sieves was  
295 added DIPEA (440 μL, 2.52 mmol), followed by a solution  
296 of iodoacetic anhydride (670 mg, 1.89 mmol) in dry THF  
297 (5 mL). After the mixture was stirred 1.5 h at room  
298 temperature, the pH was adjusted to ~5 with TFA (moist  
299 pH paper). The solvents were removed under reduced  
300 pressure, and the residue was purified by HPLC. Large  
301 preparative C<sub>18</sub> column, λ = 240 nm, 50 mL/min, gradi-  
302 ent: 0'–10': 20% B; 10'–80': 70% B. The product (657  
303 mg, 61%) eluted at 46.6% B. <sup>1</sup>H NMR (300 MHz, d<sub>4</sub>-  
304 MeOH) δ 8.0 (d, 1H, J = 8.3 Hz); 7.6 (m, 1H); 7.4 (d, 1H,  
305 J = 8.3 Hz); 6.15 (m, 1H); 4.55 (d, 2H); 4.5 (dd, 1H); 4.3  
306 (dd, 1H); 3.65 (bs, 2H); 3.1–3.45 (m, 9H); 2.95 (dd, 1H);  
307 2.75 (dd, 1H); 2.2 (t, 2H); 1.25–1.8 (m, 22H). ESI-MS (M  
308 + H<sup>+</sup>)<sup>+</sup>: 848.0; (M + Na<sup>+</sup>)<sup>+</sup>: 870.5.

309 **Deprotection of 9 (10).** To a vial containing a solution  
310 of compound **9** (603 mg, 0.71 mmol) in CHCl<sub>3</sub> (3 mL) was  
311 added TFA (1.2 mL). The reaction mixture was stirred  
312 for 2 h at room temperature. Solvent was removed using  
313 in a Speed-Vac. MeOH was added, and the resulting  
314 solution was concentrated in the Speed-Vac. This proce-  
315 dure was repeated three times to remove excess TFA. The  
316 TFA salt of the product was obtained in 100% yield (611  
317 mg). <sup>1</sup>H NMR (300 MHz, d<sub>4</sub>-MeOH) δ 8.0 (d, 1H); 7.8 (bs,  
318 1H); 7.6 (dd, 1H); 6.1 (m, 1H); 4.5 (m, 1H); 4.4 (bd, 2H);  
319 4.3 (dd, 1H); 4.25 (dd, 1H); 3.65 (bs, 2H); 3.55 (m, 2H);  
320 3.1–3.35 (m, 7H); 2.95 (dd, 1H); 2.65 (dd, 1H); 2.25 (t,  
321 2H); 1.4–1.8 (m, 13H). ESI-MS (M + H<sup>+</sup>)<sup>+</sup>: 748.4

322 <sup>14</sup>C-VICAT<sub>SH</sub>. To an Eppendorf tube containing a  
323 solution of compound **10** (7.54 mg; 8.8 μmol) in MeOH  
324 (272 μL), was added 1% [<sup>14</sup>C]formaldehyde in water (v/v,  
325 59 μL, 17.6 μmol, 1 mCi, 49.5 Ci/mole) followed by 1.2 M  
326 methanolic NaCNBH<sub>3</sub> (15 μL, 17.6 μmol) and 1.2 M  
327 methanolic DIPEA (22 μL, 26.4 μmol). The capped  
328 Eppendorf tube was placed in a shaker at 168 rpm for 6  
329 h. The pH was adjusted to ~5 (moist pH paper) with a  
330 1.2 M aqueous solution of TFA (20 μL). The reaction was  
331 diluted with 30% CH<sub>3</sub>CN:water and injected on to the  
332 HPLC column. Preparative C<sub>18</sub> column, λ = 240 nm, 6  
333 mL/min, gradient: 0'–10': 0–20% B; 10'–70': 20–70%  
334 B. The product (3.5 mg, 0.162 mCi) eluted between 36.8  
335 and 39.3% B.

336 **VICAT<sub>SH</sub>.** The nonradioactive reaction was carried out  
337 as for <sup>14</sup>C-VICAT<sub>SH</sub> using compound **10** (1.09 g, 1.27  
338 mmol), 37% formaldehyde in water (207 μL, 2.54 mmol),  
339 NaCNBH<sub>3</sub> (168 mg, 2.54 mmol), MeOH (50 mL) and  
340 DIPEA (662 μL, 3.81 mmol). The compound was purified  
341 on the large preparative C<sub>18</sub> column, λ = 240 nm, 50  
342 mL/min, gradient: 0'–10': 0–20% B; 10'–60': 20–70%  
343 B. The product (900 mg, 81.8%) eluted at 32% B. <sup>1</sup>H NMR  
344 (300 MHz, d<sub>4</sub>-MeOH) δ 8.0 (d, 1H); 7.8 (s, 1H); 7.6 (dd,  
345 1H); 6.1 (m, 1H); 4.5 (dd, 1H); 4.25 (dd, 1H); 3.65 (bs, 2H);  
346 3.6 (m, 2H); 3.1–3.45 (m, 9H); 2.95 (m, 4H); 2.65 (dd,  
347 1H); 2.25 (t, 2H); 1.25–1.8 (m, 13H). ESI-MS (M + H<sup>+</sup>)<sup>+</sup>:  
348 762.3.

349 [<sup>15</sup>N<sub>2</sub>, <sup>13</sup>C<sub>4</sub>]butanedinitrile (**11**). Potassium [<sup>13</sup>C, <sup>15</sup>N]-  
350 cyanide (176.3 mg, 2.63 mmol) and [1,2-<sup>13</sup>C<sub>2</sub>]-dibromo-  
351 ethane (0.5 g, 2.63 mmol) were refluxed for 5 h in 70%  
352 ethanol (2 mL). The reaction mixture was cooled to room  
353 temperature and passed through a silica plug with  
354 benzene. The solvent was removed under reduced pres-  
355 sure to afford the product in 80% yield (90 mg). <sup>1</sup>H NMR  
356 (300 MHz, CDCl<sub>3</sub>) δ 3.0 (m, 2H); 2.55 (m, 2H).

357 [<sup>14</sup>N<sub>2</sub>, <sup>13</sup>C<sub>4</sub>]-1,4-Butanediamine dihydrochloride (**12**). Com-  
358 pound **11** (137.1 mg, 1.59 mmol) and a 1 M solution of  
359 360

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. <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O) δ 3.5 (m, 2H); 2.75 (m, 2H); 2.15 (m, 2H); 1.75 (m, 2H).

*N*-([<sup>14</sup>N<sub>2</sub>, <sup>13</sup>C<sub>4</sub>]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<sub>2</sub>Cl<sub>2</sub> (1 mL) was added a solution of carbonyl diimidazole (10.9 mg, 0.067 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (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<sub>2</sub>Cl<sub>2</sub> was evaporated under reduced pressure. The residue was diluted with water and injected on to the HPLC column. Preparative C<sub>18</sub> 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 mg, 74.7%) eluted between 43.8% B. <sup>1</sup>H NMR (300 MHz, d<sub>4</sub>-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)<sup>+</sup>: 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<sub>18</sub> 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)<sup>+</sup>: 854.5; (M + Na)<sup>+</sup>: 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<sub>3</sub> (240 μL). The TFA salt of the product was obtained in 94.8% yield (23.6 mg). <sup>1</sup>H NMR (300 MHz, d<sub>4</sub>-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)<sup>+</sup>: 754.4.

*VICAT<sub>SH</sub>(+6)*. The same procedure as described for VICAT<sub>SH</sub> 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<sub>3</sub> (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<sub>3</sub>CN in water, and injected on to the HPLC column. Preparative C<sub>18</sub> 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. <sup>1</sup>H NMR (300 MHz, d<sub>4</sub>-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)<sup>+</sup>: 768.4; (M + Na)<sup>+</sup>: 790.3.

<sup>14</sup>C-VICAT<sub>SH</sub>(+6). This compound was made using a variation of the procedure used to make <sup>14</sup>C-VICAT<sub>SH</sub>. 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 [<sup>14</sup>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<sub>3</sub> (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<sub>2</sub>. The residue was diluted with 100 μL of water containing 0.08% TFA, and the solution was injected onto the HPLC column. Preparative C<sub>18</sub> 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 VICAT<sub>SH</sub>(+6).

The specific radioactivity of <sup>14</sup>C-VICAT<sub>SH</sub>(+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<sub>3</sub>CN, and submitting a 5 μL aliquot to scintillation counting. The counter was calibrated using a vial of <sup>14</sup>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-oxoethyl)-carbamate (19.3 μL, 23.2 mmol, Aldrich) and a 1.2 M methanolic solution of NaCNBH<sub>3</sub> (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 C<sub>18</sub> 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. <sup>1</sup>H NMR (300 MHz, d<sub>1</sub>-CHCl<sub>3</sub>/d<sub>4</sub>-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)<sup>+</sup>: 891.0; (M + Na)<sup>+</sup>: 913.5.

*Compound 17*. To a vial containing a solution of **16** (5 mg, 5.07 mmol) in CHCl<sub>3</sub> (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. <sup>1</sup>H NMR (300 MHz, acetone-*d*<sub>6</sub>) 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)<sup>+</sup>: 791.5; (M + Na)<sup>+</sup>: 813.5.

*NBD-VICAT<sub>SH</sub>*. 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 C<sub>18</sub> column, λ = 500 nm, 6 mL/min, gradient: 0'–20': 0–20% B; 20'–

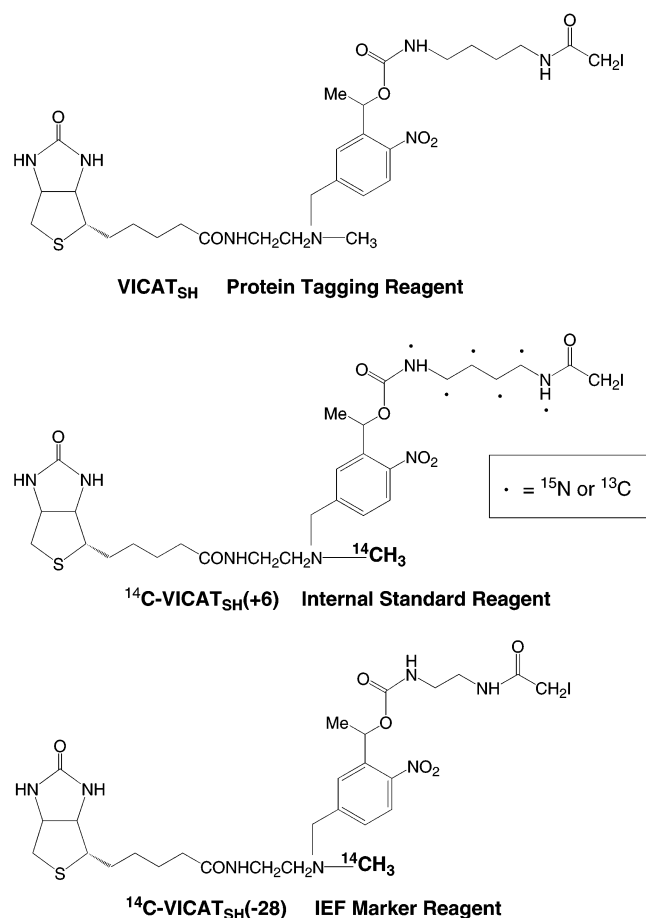
499 80': 20–100% B. The orange solid product (3.5 mg,  
500 81.4%) eluted between 61.3 and 69.7% B. <sup>1</sup>H NMR (300  
501 MHz, CD<sub>3</sub>CN) δ 8.43 (d, 1H); 7.65 (d, 1H); 7.6 (bs, 1H);  
502 7.4 (d, 1H); 6.0 (m, 2H); 4.4 (m, 1H); 4.25 (m, 1H); 3.75  
503 (d, 2H); 3.6 (s, 2H); 2.9–3.2 (m, 12H); 2.65 (m, 3H); 2.22  
504 (t, 2H); 1.35–1.8 (m, 13H). ESI-MS (M + H<sup>+</sup>)<sup>+</sup>: 954.5,  
505 (M + Na<sup>+</sup>)<sup>+</sup>: 976.4.

506 **NBD-VICAT<sub>SH</sub>(-28)**. This compound was prepared  
507 from **7** as described for the preparation of NBD-VICAT<sub>SH</sub>  
508 starting from **10**. HPLC Preparative C18 column, λ = 500  
509 nm, 6 mL/min, gradient: 0'–20': 0–20% B; 20'–80': 20–  
510 100% B. The product as an orange solid (4.6 mg, 43.8%)  
511 eluted at 51% B. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>CN) δ 8.43 (d,  
512 1H); 7.7 (bs, 1H); 7.6 (bs, 1H); 7.4 (bs, 1H); 6.16 (bs, 1H);  
513 6.05 (bs, 1H); 4.4 (m, 1H); 4.25 (m, 1H); 3.8 (m, 2H); 3.65  
514 (s, 2H); 2.9–3.2 (m, 12H); 2.7 (m, 3H); 2.22 (t, 2H); 1.35–  
515 1.9 (m, 9H). ESI-MS (M + H<sup>+</sup>)<sup>+</sup>: 926.6, (M + Na<sup>+</sup>)<sup>+</sup>:  
516 948.7.

517 **Peptide Modification Reactions. Conversion of Pep-**  
518 **ptide Amines into Thioacetamido Groups.** The reaction  
519 sequence is shown in Figure 5. To a 1.5 mL Eppendorf  
520 tube was added 280 μL of DMF:0.1 M Hepes, pH 8.0 (1:  
521 1) (pH adjusted before DMF added), followed by 20 μL of  
522 1 mM angiotensin I peptide in water (DRVYIHPFHL,  
523 Sigma) and then 100 μL of *N*-succinimidyl *S*-acetylthio-  
524 acetate (SATA, Pierce, 40 mM in dry DMF, freshly  
525 prepared). After incubation for 2 h at room temperature,  
526 40 μL of deacetylation solution (0.5 M NH<sub>2</sub>OH, 25 mM  
527 EDTA, 50 mM sodium phosphate, pH 7.5, store at 4 °C)  
528 was added, and the mixture was incubated for 2 h at  
529 room temperature. Then 150 μL of tris(2-carboxylethyl)-  
530 phosphine (TCEP, Aldrich, 0.2 M in water, store at –20  
531 °C) was added followed by a 1 h incubation at 37 °C. The  
532 pH was dropped to 3–4 by adding 50 μL of 2% TFA, and  
533 the mixture was loaded onto a C18 reverse-phase, solid-  
534 phase extraction cartridge (500 mg resin, Varian) previ-  
535 ously washed with 80% CH<sub>3</sub>CN/0.1% TFA and then  
536 equilibrated with 0.1% TFA. The cartridge was washed  
537 with 0.1% TFA (2 × 1 mL) and then with 5% CH<sub>3</sub>CN/  
538 0.1% TFA (4 × 1 mL), and peptide was eluted into a new  
539 tube with 1 mL of 80% CH<sub>3</sub>CN/0.1% TFA. The solution  
540 was concentrated to dryness (Speed-Vac) in an Eppendorf  
541 tube.

542 **Conjugation of Thioacetylated Peptide with VICAT<sub>SH</sub>**  
543 All steps involving VICAT<sub>SH</sub> reagents were carried out  
544 in normal room light (fluorescent overhead lights) and  
545 away from windows exposed to bright sunlight. The  
546 residue from the previous step was resuspended in 50  
547 μL of 20% CH<sub>3</sub>CN. Buffer (4.8 μL of 0.5 M sodium  
548 phosphate, 0.5 M sodium borate, pH 8.4) was added,  
549 followed by 5.2 μL of VICAT<sub>SH</sub> stock (22.9 mM in CH<sub>3</sub>CN,  
550 stored –20 °C). The tube was wrapped with foil to exclude  
551 light and left for 12 h at room temperature. The reaction  
552 was examined by HPLC by adding 100 μL of 2% TFA  
553 and injecting the solution onto the column (Vydac  
554 218TP52) previously equilibrated with 0.08% TFA (sol-  
555 vent A). The column was developed as follows: 0–15 min,  
556 0% B (CH<sub>3</sub>CN/0.08% TFA); 15–20 min, 0–10% B; 20–  
557 60 min, 10% B, 60–110 min, 10–80% B. The flow rate  
558 was 0.2 mL/min, and the absorbance at 217 nm was  
559 monitored. Peak fractions were examined by ESI-MS and  
560 MALDI.

561 **Photocleavage of VICAT<sub>SH</sub>-Tagged Peptides.** The de-  
562 sired peptide conjugate HPLC fraction (see above) was  
563 concentrated to dryness (Speed-Vac) in an Eppendorf  
564 tube and the residue dissolved in 100 μL of 20% CH<sub>3</sub>CN/  
565 0.1% TFA. Neat 2-mercaptoethanol (1 μL) was added, and  
566 the tube was shaken in an Eppendorf vibrating rack  
567 mixer at room temperature with irradiation for 20 min



**Figure 1.** VICAT<sub>SH</sub> reagents.

568 at room temperature from a UV lamp (Black-Ray long  
569 wave ultraviolet lamp model 100AP, VWR) with the bulb  
570 face held 10 cm from the tube. The intensity of the lamp  
571 was routinely checked with a UV light meter (Mannix  
572 UV340, Professional Equipment, Inc.). The reaction  
573 mixture was examined by HPLC (same column as above)  
574 using a solvent program of 0–60 min, 0–70% B. Fractions  
575 were examined by ESI-MS and MALDI.

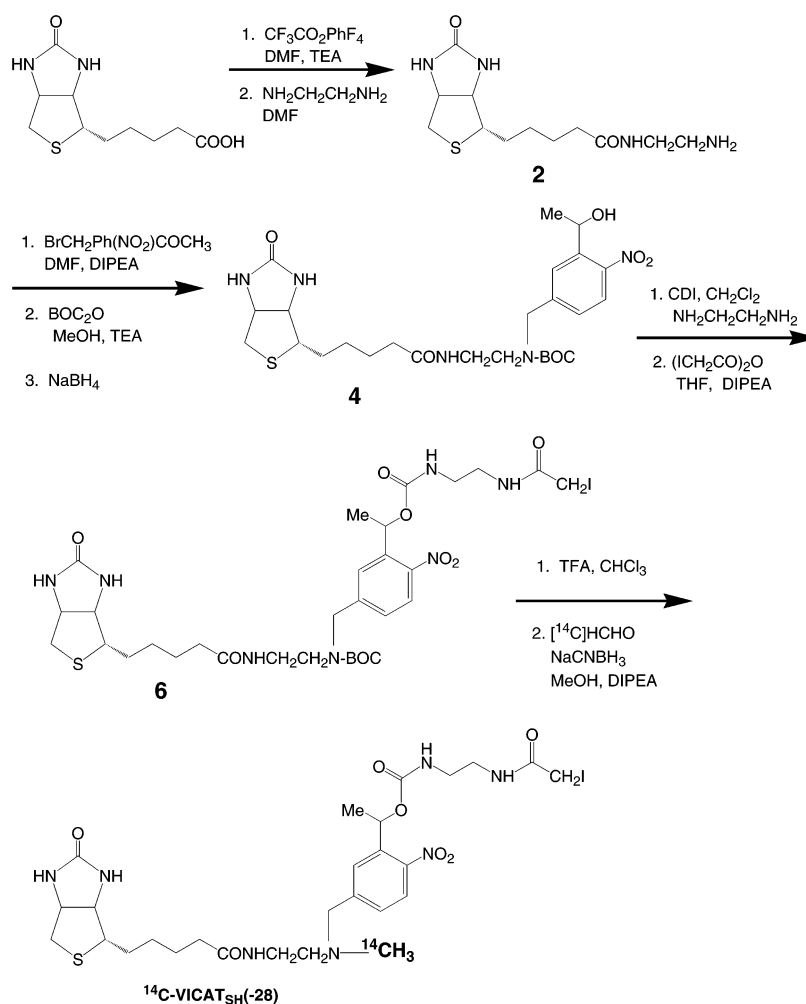
576 The photocleavage of bead-bound peptide conjugate  
577 was also carried out. A 50 μL aliquot of beads (strep-  
578 tavidin-Agarose, 1:1 suspension in buffer as supplied by  
579 the manufacturer, Sigma) was pelleted in an Eppendorf  
580 tube (1 min full speed spin in a microfuge), and the pellet  
581 was washed twice with 1 mL portions of phosphate-  
582 buffered saline and resuspended in 1 mL of the same  
583 buffer. The peptide–VICAT<sub>SH</sub> conjugation reaction mix-  
584 ture (see above) was added to the bead suspension. The  
585 tube was placed on a rocking platform for 1 h at room  
586 temperature. The beads were pelleted and washed twice  
587 with 1 mL portions of water and resuspended in 100 μL  
588 of 20% CH<sub>3</sub>CN/0.1% TFA. 2-Mercaptoethanol (1 μL) was  
589 added, and the tube was irradiated with UV light as  
590 described above. A 2 μL aliquot was analyzed by MALDI.

## RESULTS AND DISCUSSION

591  
592 **VICAT Reagents.** The present paper describes the  
593 design and synthesis of VICAT reagents and their  
594 reaction with peptides. In a subsequent study we will  
595 illustrate the usefulness of these reagents to determine  
596 the absolute quantity of specific target proteins in  
597 complex protein mixtures (8). The set of three VICAT  
598 reagents is shown in Figure 1. These reagents contain  
599 an iodoacetyl group and thus react specifically with the

F

Bottari et al.



**Figure 2.** Synthesis of  $^{14}\text{C}$ -VICAT<sub>SH</sub>(-28).

600 SH group of cysteines-containing peptides. Thus we refer  
 601 to these as VICAT<sub>SH</sub> reagents. We also show below that  
 602 peptides without cysteine can be modified to contain an  
 603 SH group which can be reacted with VICAT reagents.  
 604 The VICAT reagents are used for (1) derivatization of SH-  
 605 containing peptide fragments derived from all of the  
 606 proteins in the sample including the target protein of  
 607 interest, (2) preparation of an internal standard suitable  
 608 for absolute quantification of the target protein of inter-  
 609 est, and (3) preparation of a marker suitable for locating  
 610 the tagged peptide derived from the target protein of  
 611 interest following separation on an IEF gel strip. The  
 612 VICAT-derivatized peptides are distinguished by the  
 613 different masses of the tag introduced into the peptide  
 614 as shown below.

615 The first reagent, VICAT<sub>SH</sub>, is the “protein tagging”  
 616 reagent as it is used to tag the SH groups present in  
 617 peptides generated by proteolytic digestion of all of the  
 618 proteins present in the biological sample to be analyzed.  
 619 This reagent contains a 1,4-diaminobutane linker at-  
 620 tached to the iodoacetyl group (Figure 1).

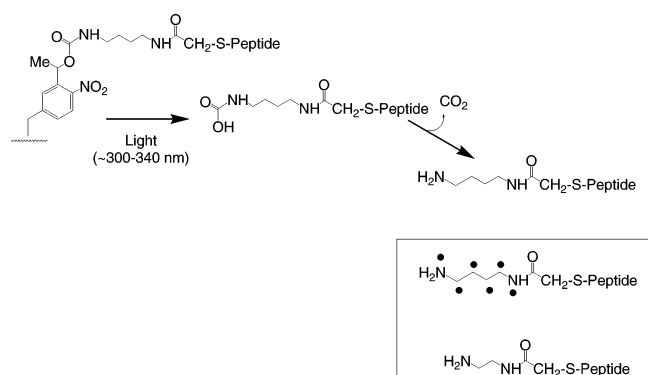
621 The second reagent, VICAT<sub>SH</sub>(+6) is chemically identi-  
 622 cal to VICAT<sub>SH</sub> except for the presence of four  $^{13}\text{C}$   
 623 and two  $^{15}\text{N}$  labels in the diaminobutane linker (Figure 1).  
 624 VICAT<sub>SH</sub>(+6) is used as the “internal standard” reagent  
 625 to tag the authentic synthetic peptide (prepared by solid-  
 626 phase peptide synthesis) whose sequence is identical to  
 627 the tryptic peptide derived from the target protein of  
 628 interest. The mass difference of 6 Da between the sam-  
 629 ple-derived and internal standard peptides is sufficient for

630 the distinction of both singly and doubly charged ions  
 631 produced by electrospray ionization. A precise amount  
 632 of the VICAT<sub>SH</sub>(+6)-peptide conjugate is added to the  
 633 sample and serves as an internal standard that is  
 634 differentiated in the mass spectrometer from the protein  
 635 sample-derived, tagged peptide. Integration of the ion  
 636 chromatogram peaks, observed by mass spectrometry,  
 637 from the sample-derived and internal standard conju-  
 638 gates in the mass spectrometry analysis provides the  
 639 absolute abundance of the former and thus the absolute  
 640 abundance of the specific protein in the complex protein  
 641 mixture. We also prepared  $^{14}\text{C}$ -VICAT<sub>SH</sub>(+6), which can  
 642 also be used to prepare the internal standard. This  
 643 reagent is radiolabeled and of known specific activity and  
 644 thus offers the ability to readily determine the absolute  
 645 quantity of purified internal standard by scintillation  
 646 counting.

647 The third reagent,  $^{14}\text{C}$ -VICAT<sub>SH</sub>(-28), contains the  
 648 radioactive label and functions as the “IEF marker” for  
 649 peptide visualization on the IEF strip. This reagent is  
 650 used to derivatize the same synthetic peptide used to  
 651 prepare the internal standard.  $^{14}\text{C}$ -VICAT<sub>SH</sub>(-28) con-  
 652 tains a shorter (two-carbon) diamine linker that is  
 653 attached to the iodoacetyl group (Figure 1). The purpose  
 654 of using a shorter linker is 2-fold. First, the tagged  
 655 peptide formed after photocleavage (see below) is more  
 656 polar than the tagged peptides derived from the other  
 657 VICAT<sub>SH</sub> reagents and is expected to have a slightly  
 658 shorter reverse-phase HPLC retention time than the  
 659 internal standard and sample protein-derived tagged

## Visible Isotope-Coded Affinity Tags

G



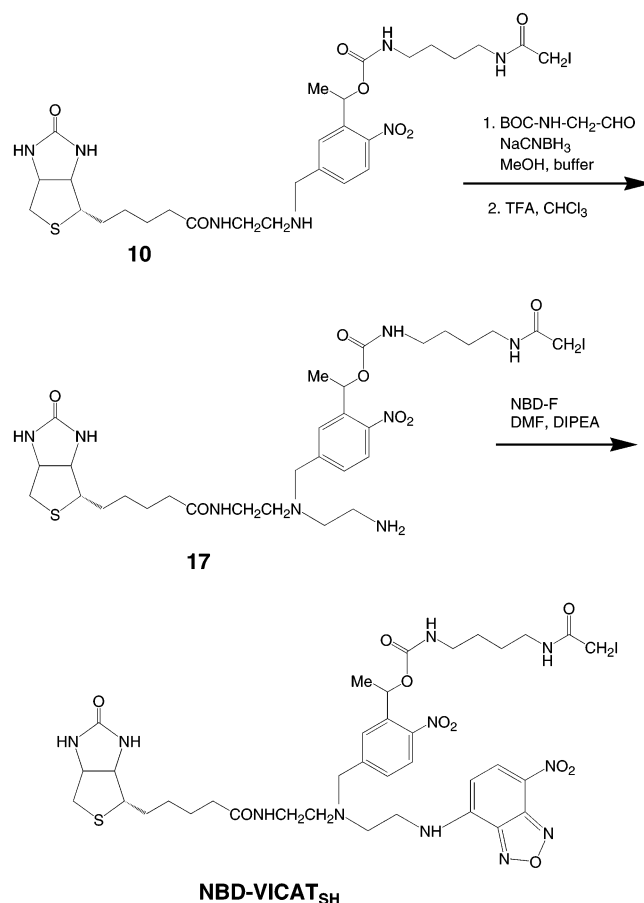
**Figure 3.** Photocleavage of VICAT<sub>SH</sub>-tagged peptide. Shown is the photocleavage of the peptide tagged with VICAT<sub>SH</sub>, which generates the carbamic acid, which spontaneously decarboxylates to give the peptide derived from the sample protein of interest bearing a tag with the diaminobutane 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 <sup>15</sup>N and <sup>13</sup>C labels.

660  
661  
662  
663  
664  
665  
666  
667  
668  
669

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.

670  
671  
672  
673  
674  
675  
676  
677  
678  
679  
680  
681  
682  
683  
684  
685  
686  
687  
688  
689  
690  
691  
692  
693  
694  
695  
696  
697  
698  
699  
700  
701  
702  
703  
704  
705

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<sub>SH</sub> 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 <sup>15</sup>N and <sup>13</sup>C present in VICAT<sub>SH</sub>(+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 <sup>14</sup>C radiometric analysis. Since the IEF marker-derived peptide is 28 AMU 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<sub>SH</sub>.

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

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

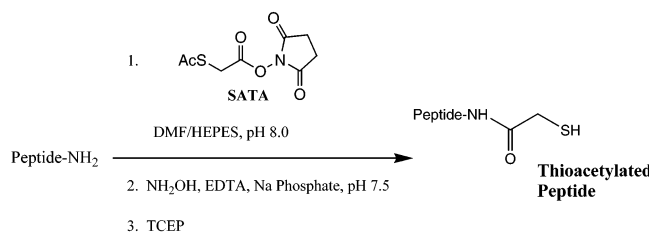
We have also designed a variant of VICAT<sub>SH</sub> reagents in which the visible probe is an NBD fluorophore rather than a radiolabel. As shown in Figure 4, intermediate **10** can be reductively aminated with commercially available BOC-NHCH<sub>2</sub>CHO. Removal of the BOC group provides primary 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<sub>SH</sub> reagents can be prepared from a common synthetic intermediate. The availability of VICAT<sub>SH</sub> 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 <sup>14</sup>C-VICAT<sub>SH</sub> is used, it may be noted that the carbon-14

706  
707  
708  
709  
710  
711  
712  
713  
714  
715  
716  
717  
718  
719  
720  
721  
722  
723  
724  
725  
726  
727  
728  
729  
730  
731  
732  
733  
734  
735  
736  
737  
738  
739

**Table 1. Thioacetylation of Peptide Amino Groups**

peptide <sup>a</sup>	product	yield <sup>b</sup>
DRVYIHPFHL	(thioacetyl)DRVYIHPFHL	quantitative
pyroELYENKPRRPYIL	pyroELYENK(thioacetyl)PRRPYIL	quantitative
HDMNKVLDL	(thioacetyl)HDMNK(thioacetyl)VLDL	quantitative
ALEPPEPKSRRCVLL	(thioacetyl)ALEPPEPK(thioacetyl)K(thioacetyl)SRRCVLL	quantitative

<sup>a</sup> pyroE is pyroglutamic acid. <sup>b</sup> 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<sub>SH</sub> 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<sub>SH</sub> 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<sub>SH</sub> 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<sub>SH</sub> 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<sub>2</sub>OH 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<sub>2</sub>-SSCH<sub>2</sub>COOH 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<sub>2</sub>OH).

**Reaction of Peptides with VICAT<sub>SH</sub> 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<sub>SH</sub>. Conjugation of peptide SH group with VICAT<sub>SH</sub> reagent (~6 equiv based on peptide SH groups) was carried out in 20% CH<sub>3</sub>CN/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<sub>SH</sub> reagent (confirmed by ESI-MS and MALDI), a trace amount of VICAT<sub>SH</sub>-TCEP adduct (ESI-MS and MALDI gave *m/z* = 884), and a trace amount of VICAT<sub>SH</sub> 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<sub>SH</sub> 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<sub>SH</sub> 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<sub>2</sub>-(CH<sub>2</sub>)<sub>4</sub>-NHCOCH<sub>2</sub>SCH<sub>2</sub>CONH-peptide, and the other due to the photofragmented VICAT<sub>SH</sub> 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<sub>SH</sub>-tagged peptide undergoes quantitative photocleavage to the desired peptide containing the isotope tag (see above).

847 **Application of VICAT<sub>SH</sub> Reagents and Future**  
 848 **Prospects.** There are numerous examples where the use  
 849 of VICAT<sub>SH</sub> reagents will be useful. A major advantage  
 850 of VICAT<sub>SH</sub> reagents is that they permit the absolute  
 851 quantification of specific proteins in complex mixtures  
 852 (8). In contrast, immunological methods require the  
 853 lengthy and costly production of highly specific antibod-  
 854 ies. Furthermore, immunoblot analyses (western blots)  
 855 provide only qualitative data. Microarray nucleic analysis  
 856 of changes in gene expression often need to be validated  
 857 at the protein level. Furthermore, the search for markers  
 858 in readily available biological fluids such as serum that  
 859 have diagnostic value for human diseases will require a  
 860 method for the absolute quantification of specific proteins.  
 861 Microarray analyses often suggest tens if not hundreds  
 862 of proteins that might be upregulated in human tumors  
 863 for example. The validation of these candidate proteins  
 864 using immunological methods is probably not practical,  
 865 especially for a single laboratory effort.

866 The tagging of cysteine SH groups by VICAT<sub>SH</sub> re-  
 867 agents offers an important simplification of the peptide  
 868 mixture in that many peptides will be removed following  
 869 the purification of tagged peptides with streptavidin-  
 870 agarose. Nevertheless, in some cases it will be beneficial  
 871 to tag peptides that lack cysteines with VICAT reagents.  
 872 A simple and efficient method to introduce SH groups  
 873 into peptides lacking cysteine has been developed, and  
 874 such peptides may be tagged with the same VICAT<sub>SH</sub>  
 875 reagents used to modify cysteine-containing peptides. In  
 876 a subsequent study (8), we will illustrate the usefulness  
 877 of these reagents to determine the absolute quantity of  
 878 specific target proteins in complex protein mixtures.

879 During the completion of the present study, the AQUA  
 880 method was introduced for obtaining the absolute quan-  
 881 tity of proteins in complex mixtures (9). The availability  
 882 of two methods is beneficial, especially since it seems  
 883 unlikely that a single method will provide useful data in  
 884 every attempted experiment. It may be noted that with  
 885 both the AQUA and VICAT<sub>SH</sub> methods, the amount of  
 886 added internal standard should be within an order of  
 887 magnitude of the amount of sample-derived peptide,  
 888 given the issues of dynamic range in the mass spectrom-  
 889 etry analysis. A detailed comparison of the AQUA and  
 890 VICAT<sub>SH</sub> methods will be presented (8). VICAT<sub>SH</sub> re-  
 891 agents are available from the authors upon request.

892 **ACKNOWLEDGMENT**

893 This work was supported by NIH grant DK67859  
 894 (M.H.G. and F.T.) and by the National Heart, Lung, and  
 895 Blood Institute, NIH, under contract no. NO1-HV-28179  
 896 (R.A.).

LITERATURE CITED

(1) Gygi, S. P., and Aebersold, R. (2000) Mass spectrometry and proteomics. *Curr. Opin. Chem. Biol.* 4, 489–494. 898  
 899  
 (2) Mo, W., and Karger, B. L. (2002) Analytical aspects of mass spectrometry and proteomics. *Curr. Opin. Chem. Biol.* 6, 666–675. 900  
 901  
 (3) Turecek, F. (2002) Mass spectrometry in coupling with affinity capture-release and isotope-coded affinity tags for quantitative protein analysis. *J. Mass Spectrom.* 37, 1–14. 903  
 904  
 (4) Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 17, 994–999. 906  
 907  
 (5) Aebersold, R., and Mann, M. (2003) Mass spectrometry-based proteomics. *Nature* 422, 198–207. 910  
 911  
 (6) Liu, H., Lin, D., and Yates, J. R. (2002) Multidimensional separations for protein/peptide analysis in the post-genomic era. *Biotechniques* 32, 898–902. 912  
 913  
 (7) Shen, M., Guo, L., Wallace, A., Fitzner, J., Eisenman, J., Jacobson, E., and Johnson, R. S. (2003) Isolation and Isotope Labeling of Cysteine- and Methionine-containing Tryptic Peptides: Application to the Study of Cell Surface Proteolysis. *Mol. Cell Proteomics* 2, 315–324. 915  
 916  
 (8) Lu, Y., Bottari, P., Turecek, F., Aebersold, R., and Gelb, M. H. (2003) Visible Isotope-Coded Affinity Tags for the Absolute Quantification of Specific Proteins in Complex Mixtures. Submitted for publication. 917  
 918  
 (9) Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W., and Gygi, S. P. (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U.S.A.* 100, 6940–6945. 920  
 921  
 (10) Gamper, H. B., Reed, M. W., Cox, T., Viroso, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Meyer, R. B. (1993) Facile preparation of nuclease resistant 3' modified oligodeoxynucleotides. *Nucleic Acids Res.* 21, 145–150. 922  
 923  
 (11) Doppler, T., Schmid, H., and Jurgen, H. (1979) On the Photochemistry of 2,1-Benzisoxazoles (Anthraniles) and on the Thermal and Photochemical Decomposition of 2-Azidoacylbenzenes in Strong Acidic Solution. *Helv. Chim. Acta* 62, 271. 924  
 925  
 (12) Senter, P. D., Tansey, M. J., Lambert, J. M., and Blattler, W. A. (1988) Novel photocleavable protein cross-linking reagents and their use in the preparation of antibody-toxin conjugates. *Photochem. Photobiol.* 42, 231–237. 926  
 927  
 (13) Duncan, R. J. S., and Weston, P. D. (1983) A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. *Anal. Biochem.* 132, 68–73. 928  
 929  
 930  
 931  
 932  
 933  
 934  
 935  
 936  
 937  
 938  
 939  
 940  
 941  
 942  
 943  
 944