Brief Articles

Synthesis, Radiosynthesis, and Biological Evaluation of New Proteasome Inhibitors in a Tumor Targeting Approach

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Proteasome inhibition is a new strategy in cancer therapy. We synthesized three new peptide aldehyde inhibitors linked to the benzamide derivative structure to use their cytotoxic activity against malignant melanoma cells. Of these, **10** displayed the highest cytotoxicity (0.18 ± 0.16 μM). A radiosynthesis of the iodine aldehyde was performed. Its drug biodistribution showed that some selectivity of the benzamide group toward malignant melanoma tissue was conserved.

Introduction

The 26S proteasome is a multicatalytic protein complex that forms the central enzyme in intracellular protein degradation. It consists of a 20S proteolytic component capped with a regulatory 19S complex at each end, the role of which is to unfold the protein−substrates and stimulate proteolytic activity. This complex exhibits at least five endopeptidase activities: post glutamyl peptide hydrolyzing (PGPH[a]) activity, trypsin-like (T-L) activity, chymotrypsin-like (ChT-L) activity, and two minor peptidase activities that cleave peptide bonds after branched-chain amino acid preferring (BrAAP) or small neutral amino acid preferring (SNAAP) activities.[1] These proteolytic activities lend proteasomes a recycler function for damaged or misfolded proteins in the cell and a critical role in events in the regulation of the cell cycle.[2] Proteasomes thus offer a new target for anticancer drugs.[3] One proteasome inhibitor, the dipeptidylboronic acid bortezomib (Velcade, Figure 1),[4] is commercialized for its activity against several hematologic malignancies (particularly multiple myeloma) and solid tumors. Many other synthetic inhibitors of the proteasome have been developed.[3] These synthetic inhibitors possess a peptide structure necessary for specific recognition by the proteasome site and an inhibitory function that interacts with the N-terminal threonine (Thr 1) of one β-subunit.[5] According to Kisselev et al.,[3] peptide aldehyde inhibitors are an essential family for studies in cell cultures and tissues. The peptide aldehyde inhibitor MG132 (Figure 1) has been studied mainly for its significant cytotoxic activity.[3] The peptide aldehyde inhibitor family offers the advantage of dissociating from the proteasome, conferring rapid reversibility of action. However, the ubiquitous properties of the proteasome limit the use of the proteasome inhibitors in therapy. One way to overcome this limitation is to carry these drugs selectively to the target tumoral tissue. A new class of radiopharmaceuticals targeting melanic tissue has been developed by us: the *N*-(2-diethylaminoethyl)benzamide derived class, e.g., BZA (Figure 1). Two members have been successfully used in humans in a phase II clinical trial as radiopharmaceuticals in the diagnosis of disseminated melanoma.[6] We synthesized proteasome inhibitors vectorized with this benzamide group to target melanic tumoral tissue. Preliminary results showed that the tripeptide aldehyde tested (BZA-CO-LLL-H, Figure 1) presented the highest cytotoxicity toward human ocular melanoma IPC227F cells of the first peptide aldehyde inhibitors of the proteasome linked to the *N*-(2-diethylaminoethyl)benzamide structure (BZA-CO, Figure 1).[7] To confirm the targeting of tumoral melanic tissue, we then synthesized the iodine leucinal derivative.

Here, we report the synthesis, radiosynthesis, and pharmacological characterization of the iodine peptide aldehyde linked to the benzamide derivative structure (Figure 1). The effects on cytotoxicity of introducing an iodine atom and substituents on the aromatic ring into the peptide aldehyde structure are also discussed. We also report on the pharmacokinetic study of ^{125}I in C57Bl/6 J mice bearing the murine B16 melanoma to determine whether the selectivity of the benzamide group toward malignant melanoma tissue was preserved.

Results and Discussion

Synthesis of Peptide Inhibitors. The method studied to synthesize the vector structure precursors **5** and **6** used common intermediate **3** (Scheme 1). This intermediate, used to introduce an iodine atom into the structure, was obtained by the Wallach reaction. Using other methods for introducing an iodine atom ($R = NH_2$, NO_2, Br, $SnBu_3$), we obtained compounds with two position isomeric structures when we introduced an *N*,*N*-diethylethylenediamine chain. With the Wallach reaction, we developed a selective synthesis of precursors **5** and **6** using the steric hindrance of the triazene group. First, triazene **2** was prepared by diazotization of dimethyl aminoterephthalate **1** using sodium nitrite in hydrochloric acid solution. This was followed

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[a] Abbreviations: PGPH, post glutamyl peptide hydrolyzing; T-L, trypsin-like; ChT-L, chymotrypsin-like; BrAAP, branched-chain amino acid preferring; SNAAP, small neutral amino acid preferring; Cbz, carbobenzyloxy; DCC, dicyclocarbodiimide; HOBt, hydroxybenzotriazole; t_R, retention time; DMEM, Dulbecco's modified Eagle's medium.

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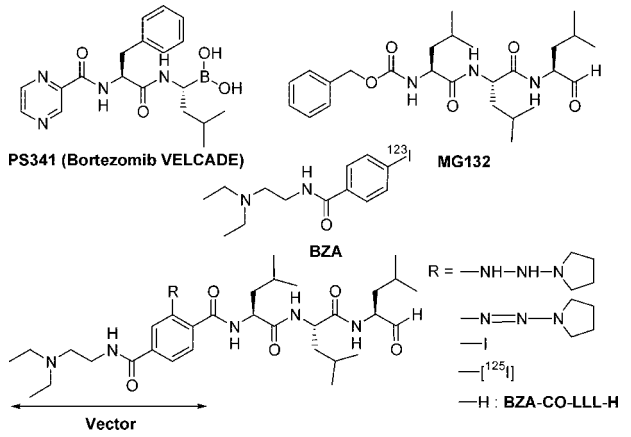
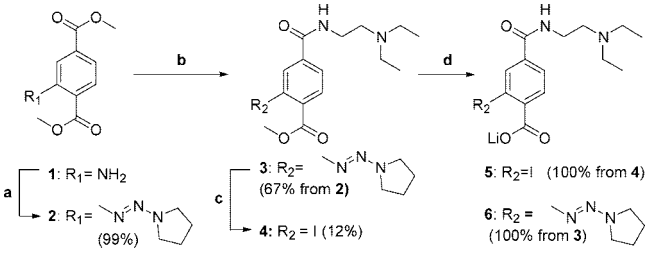


Figure 1. Pharmacomodulated proteasome inhibitors: coupling between BZA and proteasome inhibitors.

Scheme 1. Synthesis of Vector [a]



[a] Reagents: (a) (i) $NaNO_2$, H_2O/HCl, (ii) pyrrolidine, 1 N KOH; (b) $NH_2CH_2CH_2NEt_2$, $Al(CH_3)_3$, CH_2Cl_2, reflux; (c) KI, CH_3CN, CF_3COOH; (d) LiOH, THF/H_2O, room temp.

by immediate trapping with an excess of pyrrolidine in basic solution (99% from **1**, method A in Experimental Section). The reaction of 2-diethylaminoethylamine with **2** in the presence of trimethylaluminum gave the monosubstituted derivative **3** (67% from **2**), easily separated by crystallization in ethyl acetate (68%, method B in Experimental Section). Decomposition of triazene **3** with potassium iodine in trifluoroacetic acid gave the iodine compound with a yield of 12% (method C in Experimental Section).[8] Esters **3** and **4** were converted into their lithium salts **5** and **6** by saponification at room temperature with lithium hydroxide (method **D** in Experimental Section) in quantitative yield.

General synthetic pathways to obtain peptide structures are presented in Scheme 2. The final leucine derivative **12** was prepared by three different routes. Whenever possible, commercially available protected amino acids were used. In the first step, leucine-derivative-structured peptides **7** were prepared using standard peptide synthesis procedures, with DCC and HOBt as catalytic coupling agents[9] and a deprotection method. By route A, the Weinreb amide **7** was coupled to **6** to give **9** in 34% yield (method E in Experimental Section).The Weinreb amide **9** was reduced with $LiAlH_4$ to give aldehyde **10** (method **F** in Experimental Section).[10] The reaction must be quenched before secondary compound **11** is formed. Aldehyde **10** could be separated from **11** by flash chromatography (14% yield for **10**). The final compound **12** was obtained by the Wallach reaction from **10** (5% from **10**; not effective from **11**). For the two other routes, **8** was also obtained by the Wallach reaction from **9** in 77% yield (route B) or could be obtained by coupling between **5** and the Weinreb amide **7** in 13% yield (route **C**). The Weinreb amide **8** was then reduced by $LiAlH_4$ to give aldehyde **12** in 48% yield. In summary, the global synthesis yield of **12** was 0.2% through **6** (route A) and 0.5% through **5** (route C) or 8.3% (route B). The pure peptide aldehyde **12** was isolated as a white solid by flash chromatography and was tested as its hydrochloride.

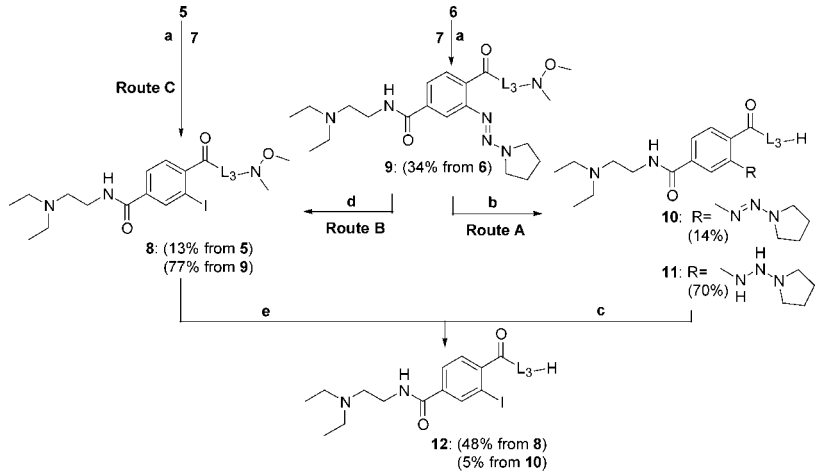
Synthesis of Labeled Compound [^{125}I]12. Our first approach to the carrier-free radioiodinated peptide aldehyde was by the Wallach triazene reaction. This method involves using a triazenyl precursor that undergoes nucleophilic radioiodine substitution when decomposed by acid in the presence of the radioiodine ion. We used the condition optimized with nonradiolabeled **8** to obtain iodine-125 labeled **8** (S4 in Supporting Information, route A). The highest yield was obtained when the reaction was carried out with acetonitrile in the presence of trifluoroacetic acid and left at room temperature for 4 h. On the basis of this information on the reaction conditions, radioiodination was undertaken using Na^{125}I. The radiochemical yield of 72% was detected by AMBIS after TLC and HPLC (t_R = 32.4 min for **9** vs 27.2 min for [^{125}I]**8**). The radiochemical purity was greater than 99.5%. The reduction of the Weinreb amide was performed with $LiAlH_4$ at −10 °C for 1 min and quenched with water. The lithium salts were eliminated by the method of Mihailovich. The reaction yield was only 7%, and the separation of [^{125}I]**8** and [^{125}I]**12** was very difficult, the difference between the two retention times being only 1 min.

Route B was also preferred to develop a radiosynthesis of **12** with a high iodine-125 specific activity. Because of the low yield (5%) for the introduction of an iodine atom into peptide aldehyde structure **10** with trifluoroacetic acid, conditions were optimized to obtain radioiodine compound **12**. The highest yield for the incorporation of ^{125}I (68%) was obtained with 30% methanesulfonic acid (v/v) in acetonitrile for 3 h. The yield fell to 5% when we used conditions with 5% methanesulfonic acid (v/v). The hydrochloride salt was formed by addition of ether/HCl solution. The mixture could be purified by semipreparative HPLC (see Experimental Section) because the difference between the two retention times was 5 min (t_R = 32.5 min for **10** vs 26 min for [^{125}I]**12**). After purification the radiochemical yield for [^{125}I]**12** by route B was 41% with a radiochemical purity of ≥99.3% as determined by HPLC analysis.

Biological Studies. The studies of benzamide compounds had been conducted on mice carrying B16 melanoma. We therefore tested the cytotoxicity of our compounds on this same line. We also tested them on a human melanic cell line, IPC227F. Human ocular melanoma IPC227F and murine melanoma B16 cell line were maintained as monolayers in 75 cm^2 culture flasks in DMEM medium supplemented with 10% fetal calf serum. A population of 5×10^3 cells were seeded in 96-well plates and incubated for 24 h at 37 °C in a humidified atmosphere under 5% CO_2. The cells were then exposed for 48 h to increasing concentrations of peptide aldehydes **10**, **11**, **12**, or MG132 and BZA-CO-LLL-H as controls. The effect of these compounds on cell outgrowth was evaluated by assay with Hoechst dye 33342. As in previous optimization, we used BZA-CO-LLL-H[7] as control.

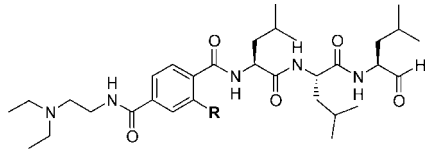
First, we demonstrate that the introduction of an iodine atom in the aromatic ring of the vector structure preserved the cytotoxic activity of these compounds. As shown in Table 1, the cytotoxicity of **12** was maintained ($IC_{50} = 1.79 \pm 0.42\ \mu M$) in B16 cells but decreased 5.6-fold in IPC227F cells ($IC_{50} = 3.58 \pm 1.9\ \mu M$) compared with the BZA-CO-LLL-H control ($IC_{50} = 1.3 \pm 0.1\ \mu M$ in B16 cells vs $0.64 \pm 0.07\ \mu M$ in IPC227F cells). We also investigated the cytotoxicity of intermediates **10** and **11**. The cytotoxicity of **10** was increased 3.5-fold in IPC227F cells ($IC_{50} = 0.18 \pm 0.16\ \mu M$) and 65-fold in B16 cells ($IC_{50} = 0.02 \pm 0.01 \mu M$) compared with the BZA-

Scheme 2. General Methods for the Preparation of Iodine Leucinal Derivatives[a]



[a] Reagents. **7**: TFA-Leu-Leu-Leu-NOMeMe. (a) DCC, HOBt, TEA, CH_2Cl_2/DMF, room temp; (b) $AlLiH_4$, THF, −80 °C; (c) NaI, CH_3CN, CH_3SO_3H; (d) NaI, CH_3CN, CF_3COOH; (e) $AlLiH_4$, THF, −80 °C.

Table 1. Concentration Inhibiting 50% of Cell Growth (IC_{50}) in B16 and IPC227F Cells[a]



Compound	R	IC_{50} (μM)	
		B16	IPC227F
MG132	-	0.042 ± 0.009	0.077 ± 0.009
BZA-CO-LLL-H	- H	1.3 ± 0.1	0.64 ± 0.07
10	—N=N—N⟨ ⟩	0.02 ± 0.01	0.18 ± 0.16
11	—NH—NH—N⟨ ⟩	0.53 ± 0.06	1.85 ± 0.63
12	- I	1.79 ± 0.42	3.58 ± 1.9

[a] Cbz = $PhCH_2OCO-$. BZA = $Et_2NH(CH_2)_2NHCOC_6H_4-$. IC_{50} values are determined from the survival curves. Each value is the mean ± SD from three independent experiments, each of which was performed in triplicate. All the compounds were tested as hydrochloride salts.

CO-LLL-H control. Contrary to **10**, the reduction of the N−N bond in the triazene group was correlated with a 3-fold decrease in the cytotoxicity of BZA-CO-LLL-H in B16 and IPC227F cells. This increase in toxicity of **10** may be explained by the alkylating effect of the triazene group, found in dacarbazine, added to the action of the proteasome inhibitor. The inhibition of the proteasome associated with its alkylating action may possibly lead to another type of compound. Generally, the cytotoxicity of these compounds was higher on the B16 murine cells than on human IPC227F cells.

Second, to verify the conservation of BZA affinity for melanic tissue, we chose the radioiodine compound [^{125}I]**12**. The pharmacokinetic study of [^{125}I]**12** was carried out in C57Bl/6J mice bearing murine B16 melanoma. Postinjection points at 15 min, 30 min, 1 h, 3 h, and 6 h were chosen to determine the distribution of the compound in various organs and tissues (drug

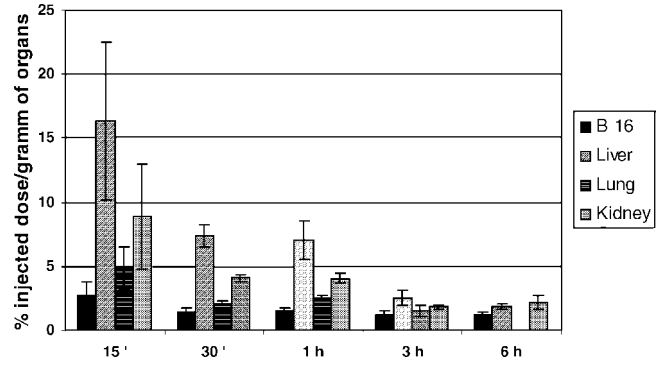


Figure 2. Drug biodistribution of [^{125}I]**12**.

biodistribution method developed in Supporting Information). In the tumor, the level of radioactivity was only 2.7% of the injected dose per unit mass of organs, whereas it was 9.5%/ID in the mice treated with BZA.[6] Radioactivity was distributed in many tissues, especially in the metabolizing and eliminating organs, i.e., the lung, kidney, and liver (16.3%, 4.9%, and 8.9%, respectively, as shown in Figure 2). However, the lungs were also a frequent tissue site for melanoma metastasis, and the high concentration of the compound [^{125}I]**12** in these organs could be explained by the presence of σ receptors in tissues such as liver, kidney, and lung.[11] Compound [^{125}I]**12** seems to be more fully metabolized than BZA (5.95% in liver for BZA vs 16.3% for [^{125}I]**12**). No radioactivity was measured in the brain; [^{125}I]**12** did not cross the blood−brain barrier. The elimination of [^{125}I]**12** was mainly fecal, 87% of the radioactivity injected being found in the feces (2/3) and in urine (1/3) in 72 h. The ratio between B16 cells and blood was greater than or equal to 1 (see S1 in Supporting Information). Some selectivity of the benzamide group toward malignant melanoma tissue was also conserved; the affinity might well be increased by synthesizing this compound with another vector structure derived from BZA.

Conclusion

We report a selective synthesis and radiosynthesis of new leucine iodine peptides linked to the benzamide structure. We investigated different methods to obtain an iodine aldehyde (**12**). The different iodine compounds were obtained by the Wallach triazene reaction and the aldehyde by Weinreb amide reduction. The radiosynthesis of [^{125}I]**12** gave the highest radiochemical

yield by route B (41% with a radiochemical purity of ≥99.3% as determined by HPLC analysis). Cytotoxic assay showed that **10** had the highest cytotoxicity (0.18 ± 0.16 μM) probably because of the presence of the triazene group. The drug biodistribution of [^{125}I]**12** was also studied in mice bearing a subcutaneous implantation of malignant melanoma cells. These experiments showed an accumulation of 2.7% DI/g of this compound in B16 tumors at 15 min, with 1.2% of this aldehyde remaining in the tumor at 3 and 6 h after administration, thus giving a tumor/blood ratio greater than or equal to 1. Also, some selectivity of the benzamide group toward malignant melanoma tissue was conserved. The affinity of the compounds might be increased by using another vector structure derived from BZA and might also offer a better selectivity for melanoma cells.[6] A derivative of **10** bearing a triazene group will be tested in in vitro and in vivo experiments on tumor-bearing animals with radiolabeled compounds to determine whether this new compound has a greater selectivity for malignant melanoma and metastases.

Experimental Section

General procedures and synthesis of **6**, **9**−**11**, and [^{125}I]**8** are described in Supporting Information.

General Method A: (*E*)-Dimethyl 2-(Pyrrolidin-1-yldiazenyl)-terephthalate (2). To a solution of dimethyl aminoterephthalate (**1**) (1 equiv, 61.8 mmol, 12.90 g) in water (100 mL) was added a solution of 37% HCl to obtain a red suspension, and the mixture was vigorously stirred for 30 min at 0 °C under a nitrogen atmosphere. To the reaction mixture was added dropwise a solution of sodium nitrite (1.3 equiv, 81.2 mmol, 5.56 g) in 20 mL of water at 0 °C. The mixture was stirred for 45 min, and 8 mL of pyrrolidine (1.5 equiv, 92.7 mmol) and 12 mL of 1 N potassium hydroxide at 0 °C were then added. The mixture was stirred for 3 h at room temperature, made alkaline to pH 12 with NaOH, and extracted with CH_2Cl_2 (3 × 100 mL). The organic layer was dried over $MgSO_4$ and concentrated in part under vacuum to obtain 17.79 g of **2** (yield 99.0%): mp 83 ± 1 °C; TLC R_f = 0.8 (alumina, CH_2Cl_2). Anal ($C_{14}H_{17}N_3O_4$) C, H, N.

General Method B: (*E*)-Methyl 4-((2-(Diethylamino)ethyl)-carbamoyl)-2-(pyrrolidin-1-yldiazenyl)benzoate (3). To a solution of *N*,*N*-diethylethylenediamine (1 equiv, 1.72 mmol, 0.24 mL) in 20 mL of CH_2Cl_2 was added dropwise a solution of 2 M trimethylaluminum in hexane (50 mL, 100 mmol) at 0 °C under a nitrogen atmosphere. The mixture was stirred at 0–5 °C for 2 h and was added to a solution of ester **2** (3 equiv, 5.1 mmol, 1.51 g) in 60 mL of dichloromethane. The mixture was refluxed for 77 h and then quenched with water (40 mL). The white precipitate obtained was filtered and extracted with CH_2Cl_2 (4 × 100 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in part under vacuum. The pure **3** was obtained by recrystallization in ethyl acetate (yield, 68%): beige solid; mp 100 ± 1 °C; TLC R_f = 0.4 (alumina, CH_2Cl_2). Anal. ($C_{19}H_{29}N_5O_3$) C, H, N.

General Method C: Methyl 4-((2-(Diethylamino)ethyl)carbamoyl)-2-iodobenzoate (4). To a solution of **3** (1 equiv, 2.66 mmol, 1.00 g), potassium iodide (2 equiv, 5.33 mmol, 884 mg) in acetonitrile (CH_3CN, 1.5 mL) at −10 °C was added a solution of trifluoroacetic acid (2 equiv, 5.33 mmol, 3.9 mL). The mixture was stirred at room temperature under a nitrogen atmosphere. The crude mixture in 10 mL of water was extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layers were washed with brine (50 mL), dried over $MgSO_4$, and concentrated under vacuum. The crude product was purified by flash chromatography (aluminum oxide, CH_2Cl_2/gradient of MeOH from 0 to 3%) to give 126.6 mg of **4** (yield, 12.0%): yellow solid; mp >200 ± 1 °C; TLC R_f = 0.9 (alumina, CH_2Cl_2/MeOH, 95:5).

General Method D: Lithium 4-((2-(Diethylamino)ethyl)carbamoyl)-2-iodobenzoate (5). To a solution of lithium hydroxide monohydrate (0.13 g, 3.18 mmol, 1.5 equiv) in water (3.5 mL) was added dropwise a solution of **4** (2.12 mmol, 859.1 mg) in THF (3.5 mL) at 0 °C. The mixture was allowed to reach room temperature and then stirred for 1 h. The mixture was evaporated under reduced pressure and the crude product was washed with acetone and dried to give 842 mg of **5** (yield, 100%): white solid; mp >200 ± 1 °C, TLC R_f = 0.67 (RP18; H_2O/CH_3CN/TFA, 40:60:0.1%).

***N*^{4}-(2-(Diethylamino)ethyl)-2-iodo-*N*^{1}-(1-(1-(1-(methoxy(methyl)amino)-4-methyl-1-oxopentan-2-ylamino)-4-methyl-1-oxopentan-2-ylamino)-4-methyl-1-oxopentan-2-yl)terephthalamide (8).** The iodination of Weinreb amide **9** (1 equiv, 0.51 mmol, 380 mg) was achieved using method C.

The crude product was purified by flash chromatography (aluminum oxide, CH_2Cl_2/gradient of MeOH from 0 up to 2%) to give 290 mg of **11** (yield, 73%): beige solid; mp 120 ± 1 °C; TLC R_f = 0.4 (alumina, CH_2Cl_2/MeOH, 95:5). Anal. ($C_{34}H_{57}IN_6O_6$) C, H, N.

***N*^{4}-(2-(Diethylamino)ethyl)-2-iodo-*N*^{1}-(4-methyl-1-(4-methyl-1-(4-methyl-1-oxopentan-2-ylamino)-1-oxopentan-2-ylamino)-1-oxopentan-2-yl)terephthalamide (12).** A solution of lithium aluminum hydride in ether (1 M/THF, 1.1 equiv, 0.2 mmol) was added dropwise at −90 °C under a nitrogen atmosphere to a solution of **8** (1 equiv, 0.17 mmol, 130 mg) in anhydrous THF (5 mL). The mixture was stirred for 2 h and 30 min and then allowed to warm to 0 °C. The reaction was quenched by adding water (5 mL), and excess lithium aluminum hydride was eliminated by the Mihailovic method. The resulting crude mixture was extracted with CH_2Cl_2 (2 × 50 mL). The combined organic layers were dried over $MgSO_4$, concentrated under vacuum, and purified by flash chromatography (aluminum oxide, CH_2Cl_2/gradient of MeOH from 2% to 3%) to give 59.1 mg of **12** (yield, 48%): yellow solid; mp >200 °C; TLC R_f = 0.4 (alumina, CH_2Cl_2/MeOH, 95:5). Anal. ($C_{32}H_{52}IN_5O_5 \cdot HCl \cdot 1H_2O$) C, H, N.

***N*^{4}-(2-(Diethylamino)ethyl)-2-iodo-*N*^{1}-(4-methyl-1-(4-methyl-1-(4-methyl-1-oxopentan-2-ylamino)-1-oxopentan-2-ylamino)-1-oxopentan-2-yl)terephthalamide ([^{125}I]12).** To a solution of **10** (8 μmol, 5.31 mg) in acetonitrile (CH_3CN, 50 μL) at −10 °C was added 2.1 mCi of $Na^{125}I$ in 0.1 N NaOH, followed by a solution of methanesulfonic acid (37 μL). The mixture was stirred at room temperature and made alkaline with 400 μL of a saturated solution of $NaHCO_3$ to give [^{125}I]**12** (radiochemical yield, 68%). The crude product was purified with HPLC to give 860 μCi of [^{125}I]**12** (yield, 41%): TLC R_f = 0.4 (alumina, CH_2Cl_2/MeOH, 95:5); HPLC t_R = 32.5 min.

Supporting Information Available: Experimental details of the synthesis of the compounds described here, spectroscopic data, elemental analysis results, details of all biological methods, ratio of the injected dose of [^{125}I]**12** in B16 tumor and in blood, main fragmentations of $[M + H]^+$ ions from electrospray of aldehyde **12**, and the synthesis scheme for radiolabeled [^{125}I]**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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