A Novel Radioiodination Reagent for Protein Radiopharmaceuticals with L-Lysine as a Plasma-Stable Metabolizable Linkage To Liberate *m*-Iodohippuric Acid after Lysosomal Proteolysis

Kouji Wakisaka,[†] Yasushi Arano,*[,†] Takashi Uezono,[†] Hiromichi Akizawa,[†] Masahiro Ono,[†] Keiichi Kawai,[‖] Yoshiro Ohomomo,[§] Morio Nakayama,[‡] and Hideo Saji[†]

Department of Patho-Functional Bioanalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606-01, Japan, Central Research Laboratories, Miyazaki Medical College, 5200 Kihara, Kiyotake-cho, Miyazaki-gun, Miyazaki 889-16, Japan, Osaka University of Pharmaceutical Sciences, 4-20-1, Nasahara, Takatsuki 569-11, Japan, and Faculty of Pharmaceutical Sciences, Kumamoto University, Oe-Honmachi, Kumamoto 863, Japan

Received September 10, 1996[⊗]

Radiochemical design of polypeptides using metabolizable linkages would be attractive to enhance target-selective localization of radioactivity for diagnostic and therapeutic nuclear medicine. However, while use of ester bonds as the linkage allows selective release of the designed radiometabolite from covalently conjugated polypeptides after lysosomal proteolysis in nontarget tissues, low plasma stability of ester bonds causes a decrease in radioactivity levels of the target. In pursuit of new metabolizable linkages that provide stable attachment of radiolabels with polypeptide in plasma while facilitating rapid and selective release of designed radiometabolites of rapid urinary excretion in lysosomes, a new radioiodination reagent with L-lysine as the metabolizable linkage to liberate *m*-iodohippuric acid (L-HML) was designed and synthesized. Stabilities of the metabolizable linkage in serum and cleavabilities of the linkage in lysosomal proteolysis in hepatic cells were investigated after conjugation of [^{131}I]-L-HML with galactosyl-neoglycoalbumin (NGA). For comparison, a radioiodination reagent with an ester bond to release *m*-iodohippuric acid (MIH) was conjugated with NGA under similar conditions. When incubated in human serum, [^{131}I]-L-HML-NGA liberated less than 3% of the initial radioactivity after 24 h, whereas [^{125}I]MIH-NGA released more than 60% of its radioactivity during the same interval. In biodistribution studies, [^{131}I]-L-HML-NGA demonstrated radioactivity elimination from murine liver at a rate and excretion route similar to [^{125}I]MIH-NGA. Analyses of murine urine after injection of [^{131}I]-L-HML-NGA indicated a single radioactivity peak at fractions identical to those of *m*-iodohippuric acid. Biodistribution studies of radioiodinated NGAs with D-lysine or cadaverine as the linkages demonstrated a delayed elimination rate from murine liver with significantly higher radioactivity being excreted in the feces at 24 h postinjection. Thus, L-HML is the first reagent that allows stable attachment of radiolabel with polypeptide in serum while facilitating selective release of a radiometabolite with rapid urinary excretion from covalently conjugated polypeptides after lysosomal proteolysis at a rate similar to that of ester bonds. Thus, L-HML is potentially useful for the radioiodination of polypeptides for diagnostic and therapeutic purposes.

Introduction

With the advent of protein and genetic engineering, monoclonal antibodies (mAbs) with less immunogenicity (e.g., humanized antibodies) or more favorable pharmacokinetic characteristics (e.g., single-chain Fv fragments) have become available for nuclear medicine.[1,2] Low molecular weight oligopeptides, such as octreotide and chemotactic peptides, have also attracted attention as new vehicles to deliver radioactivity to target tissues *in vivo*.[3,4] Such advances in carrier molecules would potentially solve various problems associated with the clinical use of radiolabeled murine mAbs. Thus, further development of the radiochemistry of polypeptides to reduce radioactivity localization in nontarget tissues would improve the diagnostic and therapeutic applications of the radiopharmaceuticals.

Prior studies of radiolabeled mAbs indicated that the vast majority of mAbs, especially those against solid tumors, do not or only slowly localize within tumor tissues. This is supported by the successful tumor visualizations by the pretargeting strategy, where radiolabeled compounds are administered many hours after administration of nonradiolabeled mAbs.[5−8] On the other hand, numerous studies demonstrated that mAbs are metabolized in nontarget tissues, such as the liver, when administered.[9−11] Furthermore, recent metabolic studies manifested that *in vivo* behaviors of radiometabolites generated after lysosomal proteolysis in nontarget tissues play a critical role in the radioactivity levels of radiolabeled polypeptides in nontarget tissues such as the liver.[12−16] These findings support the rationale behind the radiochemical design of polypeptides using metabolizable linkages, where a radiolabeled compound with rapid urinary excretion is attached to polypeptides *via* linkages cleavable in lysosomal proteolysis.

Based on the rapid urinary excretion and high stability against deiodination of *m*-iodohippuric acid, we have

* Address all correspondence to Yasushi Arano, Ph.D. Tel: 81-75-753-4566. Fax: 81-75-753-4568. E-mail: arano@pharm.kyoto-u.ac.jp.
[†] Kyoto University.
[‡] Kumamoto University.
[§] Osaka University of Pharmaceutical Sciences.
[‖] Miyazaki Medical College.
[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1997.

S0022-2623(96)00639-5 CCC: $14.00

developed a reagent for radioiodination of polypeptides with an ester bond to release a *m*-iodohippuric acid as the sole radiometabolite after lysosomal proteolysis, maleimidoethyl 3-iodohippurate (MIH).[17] Release of *m*-iodohippurate should facilitate rapid elimination of radioactivity from nontarget tissues, since lysosomes are common sites of proteolysis and the low pH environment of these organelles is well known.[18] In biodistribution studies, [^{131}I]MIH-conjugated mAb exhibited high target-to-nontarget ratios of radioactivity even at an early postinjection time.[19] However, [^{131}I]MIH-mAb also caused a decrease in the radioactivity levels in the target, presumably due to cleavage of the ester bond in serum and even at the target cell surface. In addition, when conjugated to Fab, MIH rapidly liberated high levels of *m*-iodohippuric acid in serum both *in vitro* and *in vivo*. These findings suggested that further development of metabolizable linkages that allow stable attachment of radiolabels with polypeptides in serum while facilitating rapid and selective release of radiometabolites of urinary excretion from the polypeptides after lysosomal proteolysis in nontarget tissues would provide target-selective radioactivity localization using polypeptides as the vehicles.

In recent metabolic studies of ^{111}In-labeled galactosylneoglycoalbumin (NGA) and mAbs, lysine adducts of ^{111}In chelates were predominantly obtained as the final radiometabolites after lysosomal proteolysis in hepatic cells within short postinjection times when ^{111}In chelates were attached to the proteins using ϵ amine groups of the lysine residues.[12−16,20] These findings indicated that proteolysis of polypeptides occurs at high rates in lysosomes or that the peptide bonds derived from L-lysine are rapidly cleaved in the organelles. They also suggested that L-lysine may constitute a plasma-stable metabolizable linkage that allows stable attachment of radiolabels with polypeptides in plasma while providing a selective release of the designed carboxylate compound from covalently conjugated polypeptides after lysosomal proteolysis.

To examine this hypothesis, we selected *m*-iodohippurate as the radiometabolite, and a new radioiodination reagent with L-lysine as the metabolizable linkage was designed and synthesized as outlined in Scheme 1. The novel reagent, 3′-(tri-*n*-butylstannyl)hippuryl N^{ϵ}-maleoyl-L-lysine (L-SHML, compound **8a**), possesses a maleimide group derived from the ϵ amine residue of L-lysine as a polypeptide conjugation site and a tri-*n*-butylstannyl group for high-yield and site-specific radioiodination.[17] The two functional groups are linked with a peptide bond derived from the α amine residue of L-lysine and the carboxylic acid of the hippuric acid derivative.

In the present study, NGA was used as a model polypeptide to estimate the fate of radiometabolites derived from L-HML after lysosomal proteolysis in hepatic parenchymal cells[12−17] where nonspecific localization of radioactivity of radiolabeled mAbs is observed.[21,22] A recent metabolic study indicated the formations of identical radiometabolites *in vivo* when NGA and mAb were radiolabeled with the same reagent.[23] [^{131}I]-L-HML-NGA was prepared as outlined in Scheme 1, and the stabilities of the linkage in human serum and the biodistribution of the radioactivity in mice were compared with [^{125}I]MIH-NGA. To further

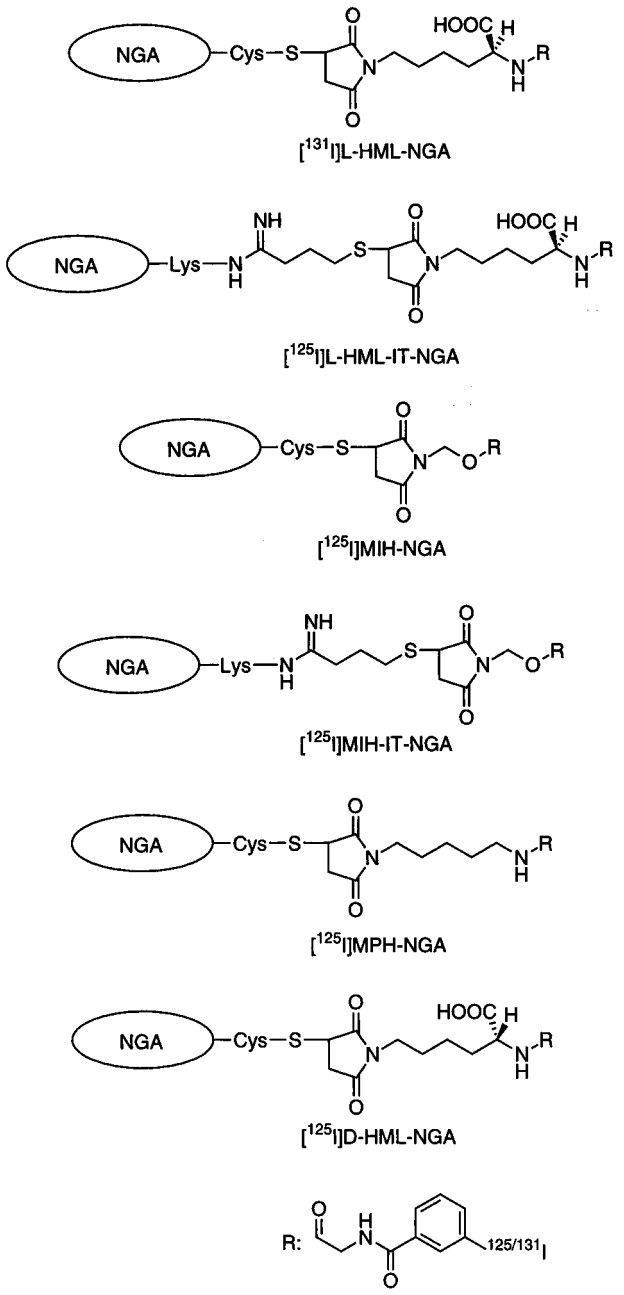


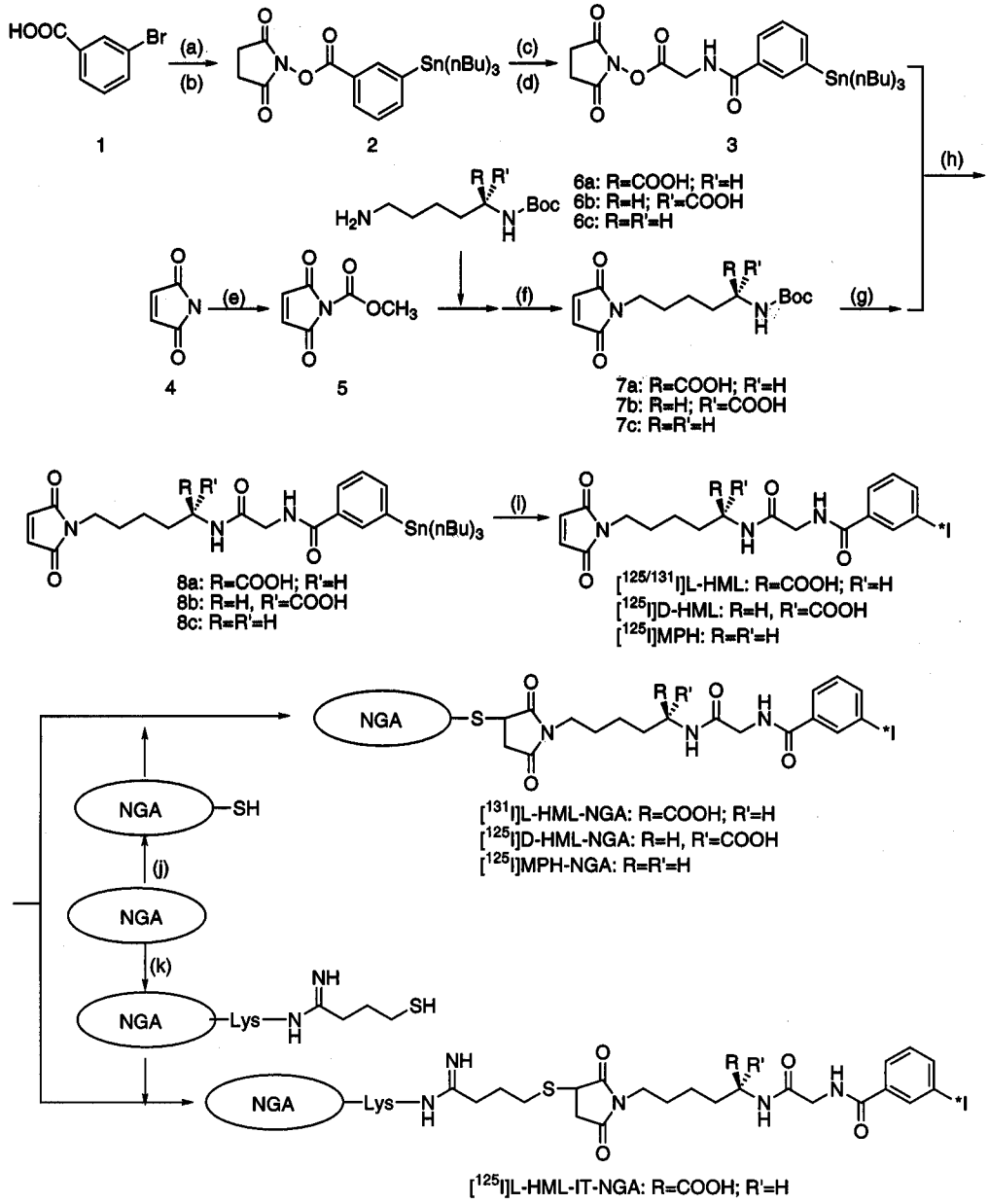
Figure 1. Chemical structures of radioiodinated NGAs used in this study. While L-HML possesses L-lysine as a metabolizable linkage, MIH, MPH, and D-HML have an ester bond, cadaverine, and D-lysine as the linkage, respectively. L-HML and MIH were conjugated with NGA by exposing its thiol group by DTT reduction (L-HML-NGA, MIH-NGA) or by modifying lysine residues with 2-iminothiolane (L-HML-IT-NGA and MIH-IT-NGA).

assess the role played by the peptide bond of L-HML, radioiodinated NGAs with D-lysine or cadaverine as linkages were also prepared, as illustrated in Figure 1, and similar studies were performed.

Chemistry

Syntheses of the Stannyl Precursor of L-HML and Its Derivatives. The stannyl precursor of L-HML (L-SHML, **8a**) was synthesized according to the procedure outlined in Scheme 1. The organotin intermediate, compound **2** (ATE), was prepared according to the procedure of Zalutsky et al.,[24] as described previously.[17] The active ester precursor of the hippurate derivative

Scheme 1[a]



[a] Regents: (a) *n*-BuLi, $(n\text{-Bu})_3$SnCl; (b) NHS, DCC; (c) glycine, $NaHCO_3$; (d) DSC, pyridine; (e) *N*-methylmorphine, methylchloroformate; (f) $NaHCO_3$; (g) HCl; (h) $(i\text{-Pr})_2$NEt; (i) *N*-chlorosuccinimide, Na[$^{125/131}$I]I; (j) dithiothreitol; (k) 2-iminothiolane.

(**3**) was synthesized by reaction of ATE with glycine in a 50% aqueous solution of acetonitrile followed by esterification of the resulting hippuric acid derivative with *N*,*N*′-disuccinimidyl carbonate in the presence of pyridine. N^{ϵ}-Maleoyl-L-lysine was prepared by reaction of *N*-(methoxycarbonyl)maleimide (**5**) with N^{α}-Boc-L-lysine (**6a**) in a saturated aqueous $NaHCO_3$ solution followed by deprotection of the Boc group of compound **7a** in the presence of dry HCl in ethyl acetate. Then, the active ester of compound **3** was condensed with the amine group of the maleimide derivative to produce the 3′-(tri-*n*-butylstannyl)hippuryl N^{ϵ}-maleoyl-L-lysine, compound **8a**.

Using similar procedures, 3′-(tri-*n*-butylstannyl)hippuryl N^{ϵ}-maleoyl-D-lysine (D-SHML, **8b**) and *N*-(5-maleimidopentyl) 3′-(tri-*n*-butylstannyl)hippuric acid amide (SMPH, **8c**) were synthesized by reaction of compound **3** with N^{ϵ}-Maleoyl D-lysine and *N*-[5-(*tert*-butyloxycarbonyl)amino)pentyl]maleimide, respectively. N^{ϵ}-Maleoyl-D-lysine and *N*-[5-[(*tert*-butyloxycarbonyl)-amino]pentyl]maleimide were synthesized by reaction of *N*-(methoxycarbonyl)maleimide with N^{α}-Boc-D-lysine and *N*-Boc-cadaverine, respectively, in a saturated solution of aqueous $NaHCO_3$, according to the procedure of Keller et al.,[25] followed by deprotection of the Boc group with HCl in dry ethyl acetate or trifluoroacetic acid (TFA).

Radioiodination and Conjugation Reactions. [^{131}I]-L-HML was prepared by the organotin−halide exchange reaction in 1% AcOH/MeOH in the presence of NCS as an oxidant. Radiochemical yields of [^{131}I]-L-HML exceeded 86% when determined by TLC. Prior to conjugation with NGA, [^{131}I]-L-HML was treated with sodium bisulfite to reduce any unreacted electrophilic iodine species to iodide and to quench any excess NCS. This treatment prevented the iodine species from attaching to tyrosine residues of the protein. Furthermore, neutralization of excess NCS with sodium bisulfite prevented the protein from being exposed to the oxidant. Radioiodination of L-SHML, D-SHML, SMPH, and the

Table 1. Percent Radioactivity in NGA Fractions following Incubation of Radioiodinated NGAs in Human Serum[a]

reagents	% protein-bound radioactivity			
	1 h	3 h	6 h	24 h
[^{131}I]-L-HML-NGA	99.78 (0.53)	99.07 (0.36)	98.39 (0.32)	97.23 (0.54)
[^{125}I]-L-HML-IT-NGA	99.88 (0.19)	99.40 (0.27)	99.50 (0.27)	97.85 (0.35)
[^{125}I]MPH-NGA	100.07 (0.13)	99.12 (0.35)	98.56 (0.46)	97.26 (0.18)
[^{125}I]MIH-NGA	95.40 (0.37)	88.24 (0.64)	76.63 (1.25)	40.42 (0.50)
[^{125}I]MIH-IT-NGA	93.13 (1.06)	83.31 (0.60)	69.47 (0.33)	33.53 (0.59)

[a] Mean (SD) of four experiments for each point.

stannyl precursor of MIH with Na[^{125}I]I was performed by similar procedures. [^{125}I]-L-HML, [^{125}I]-D-HML, [^{125}I]-MPH, and [^{125}I]MIH were prepared at radiochemical yields of 86.2%, 62.9%, 80.4%, and 88.7%, respectively, as determined by TLC.

[^{131}I]-L-HML was conjugated with NGA by maleimide−thiol chemistry using thiolated NGA by exposing thiol groups with dithiothreitol (DTT) treatment,[26] as shown in Scheme 1. [^{125}I]-L-HML was also conjugated with thiolated NGA by 2-iminothiolane (2-IT) modification. DTT reduction exposed 0.9 thiol group/molecule of NGA, while 2-IT modification introduced 2.1 thiol groups/molecule of NGA as determined with 2,2′-dithiopyridine.[27] The conjugation reactions of radioiodinated HML with thiolated NGAs were then performed at a maleimide to thiol molar ratio of 1:1 to produce [^{131}I]-L-HML-NGA and [^{125}I]-L-HML-NGA with radiochemical yields of 61.6% and 58.1%, respectively, when determined by TLC. Similar results were obtained by CAE analyses. After purification by the centrifuged column procedure,[28] [^{131}I]-L-HML-NGA and [^{125}I]-L-HML-IT-NGA were obtained with respective radiochemical purities and specific activities of 95.3%, 3.19 MBq/mg and 97.9%, 2.85 MBq/mg.

[^{125}I]-D-HML-NGA, [^{125}I]MPH-NGA, [^{125}I]MIH-NGA, and [^{125}I]MIH-IT-NGA were also prepared by maleimide−thiol chemistry at a maleimide to thiol molar ratio of 1:1 after treating NGA with DTT or 2-IT as described above. Radiochemical purities and specific activities were 93.2%, 1.92 MBq/mg; 97.9%, 1.97 MBq/mg; 91.9%, 3.78 MBq/mg; and 93.1%, 2.73 MBq/mg for [^{125}I]-D-HML-NGA, [^{125}I]MPH-NGA, [^{125}I]MIH-NGA, and [^{125}I]-MIH-IT-NGA, respectively.

Biological Studies

Stability Assessment of the Metabolizable Linkages in Radioiodinated NGAs in Human Serum. Table 1 shows percentages of protein-bound radioactivity of [^{131}I]-L-HML-NGA, [^{125}I]-L-HML-IT-NGA, [^{125}I]-MPH-NGA, [^{125}I]MIH-NGA, and [^{125}I]MIH-IT-NGA after incubation in freshly prepared human serum at 37 °C. Both [^{125}I]MIH-NGA and [^{125}I]MIH-IT-NGA liberated radioactivity with incubation, and the latter liberated significantly more radioactivity than the former over the same incubation periods. The radioactivity released from the two conjugates had an R_f value similar to that of *m*-iodohippuric acid on TLC.

On the other hand, [^{131}I]-L-HML-NGA, [^{125}I]-L-HML-IT-NGA, and [^{125}I]MPH-NGA liberated less than 3% of the initial radioactivity after 24 h incubation. No significant differences were observed in radioactivity release of [^{131}I]-L-HML-NGA, [^{125}I]-L-HML-NGA, and [^{125}I]MPH-NGA.

Table 2. Biodistribution of Radioactivity after Concomitant Administration of [^{131}I]-L-HML-NGA and [^{125}I]MIH-NGA in Mice[a]

organ/tissue	biodistribution at time after injection				
	5 min	15 min	30 min	1 h	3 h
	[^{131}I]-L-HML-NGA				
liver	94.15 (3.51)	54.33 (2.56)	15.77 (1.14)	4.65 (0.47)	1.90 (0.19)
kidney	0.84 (0.12)	4.16 (0.73)	2.19 (0.47)	0.51 (0.22)	0.16 (0.08)
intestine	0.78 (0.09)	3.57 (0.18)	5.41 (1.05)	6.15 (0.78)	4.54 (0.46)
stomach	0.39 (0.01)	0.47 (0.10)	0.46 (0.06)	0.45 (0.08)	0.51 (0.20)
blood[b]	1.22 (0.06)	2.93 (0.42)	1.48 (0.22)	0.58 (0.08)	0.43 (0.02)
neck	0.07 (0.02)	0.25 (0.03)	0.12 (0.04)	0.05 (0.01)	0.03 (0.01)
	[^{125}I]MIH-NGA				
liver	93.60 (2.97)	58.69 (1.59)	18.09 (1.66)	4.42 (0.25)	1.12* (0.13)
kidney	1.17 (0.09)	2.86 (0.66)	2.36 (0.51)	0.73 (0.32)	0.14 (0.06)
intestine	0.65 (0.05)	2.95 (0.35)	9.02 (0.37)	5.49 (0.53)	1.15 (0.15)
stomach	0.28 (0.07)	0.50 (0.09)	0.60 (0.14)	0.60 (0.15)	0.62 (0.23)
blood[b]	0.87 (0.04)	1.84 (0.35)	1.58 (0.43)	0.41 (0.12)	0.17 (0.03)
neck	0.07 (0.01)	0.19 (0.02)	0.13 (0.03)	0.05 (0.01)	0.03 (0.01)

[a] Expressed as percent injected dose per organ. Mean (SD) of five animals for each point. [b] Expressed as percent injected dose per gram of tissue. *Significance determined by Student's paired *t*-test ($p < 0.0005$).

***In Vivo* Studies.** The biodistribution of radioactivity after concomitant administration of [^{131}I]-L-HML-NGA and [^{125}I]MIH-NGA in mice is shown in Table 2. At 5 min postinjection, more than 93% of the injected radioactivity was found in the liver for both radioiodinated NGAs. After this postinjection interval, [^{131}I]-L-HML-NGA showed elimination of radioactivity from the liver at a rate similar to that of [^{125}I]MIH-NGA. At 15 min postinjection, both radioiodinated NGAs exhibited a transient increase in the radioactivity levels in the blood and kidney. Low radioactivity levels in the neck and stomach were observed with the two NGAs throughout the experimental period. At 24 h postinjection, excretion of more than 85% of the injected radioactivity in the urine was observed for both NGAs (Table 6). When murine urine was analyzed at 6 h postinjection of [^{131}I]-L-HML-NGA by reversed-phase (RP) HPLC and TLC, more than 93% and 95% of the radioactivity in the urine showed a retention time and an R_f value identical to those of *m*-iodohippuric acid, respectively.

Tables 3 and 4 show biodistributions of radioactivity after concomitant administration of [^{131}I]-L-HML-NGA with [^{125}I]MPH-NGA or [^{125}I]-D-HML-NGA, respectively. After accumulation of more than 93% of the injected radioactivity in the liver, both [^{125}I]MPH-NGA and [^{125}I]-D-HML-NGA showed radioactivity elimination from the liver at rates significantly slower than that of [^{131}I]-L-HML-NGA. Though [^{125}I]MPH-NGA and [^{125}I]-D-HML-NGA also indicated a transient increase in the radioactivity levels in the blood and kidney at 15 min postinjection, the radioactivity levels in these tissues were significantly lower than those observed with [^{131}I]-

Table 3. Biodistribution of Radioactivity after Concomitant Administration of [^{131}I]-L-HML-NGA and [^{125}I]MPH-NGA in Mice[a]

	biodistribution at time after injection				
organ/tissue	5 min	15 min	30 min	1 h	3 h
	[^{131}I]-L-HML-NGA				
liver	95.63	50.14	15.34	4.29	1.84
	(1.83)	(3.51)	(1.29)	(0.38)	(0.23)
kidney	0.76	5.30	2.30	0.31	0.13
	(0.05)	(0.93)	(0.96)	(0.06)	(0.04)
intestine	0.66	4.66	4.59	5.75	5.43
	(0.06)	(0.16)	(0.37)	(0.25)	(0.31)
stomach	0.26	0.57	0.30	0.29	0.33
	(0.04)	(0.14)	(0.04)	(0.05)	(0.06)
blood[b]	1.22	3.14	1.28	0.54	0.31
	(0.08)	(0.17)	(0.23)	(0.03)	(0.05)
neck	0.05	0.23	0.09	0.05	0.03
	(0.02)	(0.04)	(0.02)	(0.01)	(0.01)
	[^{125}I]MPH-NGA				
liver	93.75	77.12*	34.19$^{£}$	10.12*	5.72$^{\#}$
	(1.63)	(1.83)	(2.68)	(0.92)	(1.35)
kidney	0.80	1.62¶	1.72	0.77	0.38
	(0.08)	(0.19)	(0.29)	(0.35)	(0.02)
intestine	0.71	7.17	33.84*	55.62*	59.36*
	(0.06)	(0.81)	(2.21)	(4.52)	(3.38)
stomach	0.24	0.46	0.54	0.61	1.48
	(0.05)	(0.09)	(0.18)	(0.06)	(0.17)
blood[b]	0.76	0.68$^{¢}$	0.64	0.32	0.23
	(0.08)	(0.04)	(0.08)	(0.03)	(0.05)
neck	0.06	0.06	0.07	0.04	0.04
	(0.02)	(0.01)	(0.03)	(0.01)	(0.01)

[a] Expressed as percent injected dose per organ. Mean (SD) of five animals for each point. [b] Expressed as percent injected dose per gram of tissue. Significance determined by Student's paired *t*-test ($^{\#}p < 0.05$, ¶$p < 0.005$, $^{£}p < 0.001$, $^{¢}p < 0.0005$, *$p < 0.0001$).

Table 4. Biodistribution of Radioactivity after Concomitant Administration of [^{131}I]-L-HML-NGA and [^{125}I]-D-HML-NGA in Mice[a]

	biodistribution at time after injection				
organ/tissue	5 min	15 min	30 min	1 h	3 h
	[^{131}I]-L-HML-NGA				
liver	93.30	53.79	16.81	7.60	2.96
	(2.41)	(4.04)	(0.59)	(0.90)	(0.15)
kidney	1.07	4.49	1.55	0.61	0.27
	(0.11)	(1.28)	(0.22)	(0.29)	(0.08)
intestine	1.77	4.88	7.76	9.83	8.60
	(0.18)	(0.35)	(0.85)	(0.64)	(0.46)
stomach	0.20	0.50	0.88	0.78	0.69
	(0.07)	(0.11)	(0.10)	(0.24)	(0.23)
blood[b]	1.90	3.14	1.63	0.82	0.50
	(0.12)	(0.35)	(0.31)	(0.15)	(0.11)
neck	0.13	0.28	0.17	0.12	0.05
	(0.02)	(0.05)	(0.05)	(0.03)	(0.01)
	[^{125}I]-D-HML-NGA				
liver	93.14	66.90$^{¢}$	27.49$^{¢}$	12.60¶	3.62
	(2.53)	(5.33)	(3.16)	(2.04)	(0.57)
kidney	1.26	2.97¶	1.73	0.72	0.34
	(0.11)	(0.76)	(0.19)	(0.09)	(0.07)
intestine	1.14	5.11	21.60*	34.28*	40.82*
	(0.10)	(0.64)	(2.27)	(2.30)	(2.14)
stomach	0.24	0.65	2.00	1.40	1.09
	(0.05)	(0.14)	(0.66)	(0.54)	(0.33)
blood[b]	1.15	1.78*	1.11	0.54	0.34
	(0.11)	(0.25)	(0.13)	(0.09)	(0.06)
neck	0.15	0.20	0.18	0.11	0.04
	(0.03)	(0.02)	(0.05)	(0.01)	(0.01)

[a] Expressed as percent injected dose per organ. Mean (SD) of five animals for each point. [b] Expressed as percent injected dose per gram of tissue. Significance determined by Student's paired *t*-test (¶$p < 0.005$, $^{¢}p < 0.0005$, *$p < 0.0001$).

L-HML-NGA. In addition, both [^{125}I]MPH-NGA and [^{125}I]-D-HML-NGA demonstrated significantly higher radioactivity levels in the intestine than [^{131}I]-L-HML-NGA 30 min after injection. This was reflected in the significantly higher radioactivity excreted in the feces at 24 h postinjection of both [^{125}I]MPH-NGA and [^{125}I]-D-HML-NGA (Table 6). Figure 2 illustrates TLC radioactivity profiles of the urine samples obtained at 6 h postinjection of [^{125}I]MPH-NGA (Figure 2A) and [^{125}I]-D-HML-NGA (Figure 2B) in mice, where 53.2% and 83.9% of the radioactivity in the urine were detected at an R_f value similar to that of *m*-iodohippuric acid, respectively. Similar findings were obtained by RP-HPLC analyses of the urine samples (data not shown).

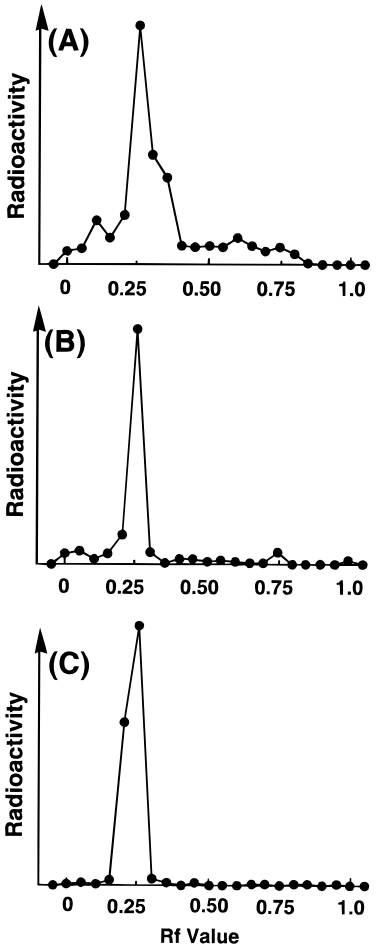


Figure 2. Radioactivity profiles of murine urine 6 h postinjection of [^{125}I]MPH-NGA (A), [^{125}I]-D-HML-NGA (B), and [^{125}I]-L-HML-IT-NGA (C) by TLC. While 53.2% and 83.9% of the radioactivity in the urine samples were detected at an R_f value similar to that of *m*-iodohippuric acid following administration of [^{125}I]MPH-NGA and [^{125}I]-D-HML-NGA, respectively, more than 94% of the radioactivity was detected at the *m*-iodohippuric acid fractions after injection of [^{125}I]-L-HML-NGA. Similar results were obtained by RP-HPLC analyses of the urine samples.

Table 5 shows biodistributions of radioactivity after concomitant administration of [^{131}I]-L-HML-NGA and [^{125}I]-L-HML-IT-NGA in mice. No significant differences were observed in the radioactivity levels in any tissues tested, such as the liver, kidney, intestine, and blood. Excretion of similar amounts of the injected radioactivity in the urine at 24 h postinjection was observed for both L-HML-conjugated NGAs (Table 6). TLC analysis of the radioactivity in the urine at 6 h postinjection of [^{125}I]-L-HML-IT-NGA indicated a single radioactivity peak (over 94%) at an R_f value identical to that of *m*-iodohippuric acid, as shown in Figure 2C. Similar

Table 5. Biodistribution of Radioactivity after Concomitant Administration of [^{131}I]-L-HML-NGA and [^{125}I]-L-HML-IT-NGA in Mice[a]

	biodistribution at time after injection				
organ/tissue	5 min	15 min	30 min	1 h	3 h
	[^{131}I]-L-HML-NGA				
liver	93.88	54.25	17.18	5.70	3.01
	(2.70)	(4.45)	(1.34)	(0.27)	(0.44)
kidney	0.28	4.91	4.92	0.96	0.21
	(0.03)	(0.67)	(1.30)	(0.33)	(0.05)
intestine	0.35	2.30	4.98	5.54	5.14
	(0.02)	(0.26)	(0.98)	(0.57)	(0.41)
stomach	0.17	0.46	0.30	0.35	0.30
	(0.03)	(0.03)	(0.04)	(0.07)	(0.17)
blood[b]	1.13	3.19	1.72	0.80	0.48
	(0.10)	(0.10)	(0.31)	(0.18)	(0.05)
neck	0.07	0.25	0.21	0.07	0.03
	(0.02)	(0.04)	(0.03)	(0.01)	(0.02)
	[^{125}I]-L-HML-IT-NGA				
liver	93.67	55.86	18.14	5.92	3.00
	(2.09)	(4.41)	(0.91)	(0.20)	(0.38)
kidney	0.33	4.38	4.64	1.04	0.22
	(0.04)	(0.64)	(1.18)	(0.32)	(0.06)
intestine	0.43	2.26	4.79	5.17	4.96
	(0.09)	(0.22)	(0.76)	(0.51)	(0.44)
stomach	0.24	0.50	0.36	0.42	0.35
	(0.05)	(0.04)	(0.05)	(0.06)	(0.15)
blood[b]	1.17	2.76	1.70	0.73	0.41
	(0.08)	(0.14)	(0.14)	(0.02)	(0.04)
neck	0.11	0.25	0.19	0.07	0.03
	(0.02)	(0.04)	(0.04)	(0.01)	(0.01)

[a] Expressed as percent injected dose per organ. Mean (SD) of five animals for each point. [b] Expressed as percent injected dose per gram of tissue.

Table 6. Cumulative Radioactivity Excreted in the Urine and Feces at 24 h Postinjection of [^{131}I]-L-HML-NGA and ^{125}I-Radioiodinated NGAs[a]

	urine	feces
[^{125}I]MIH-NGA	85.34 (8.86)	4.10 (1.88)
[^{131}I]-L-HML-NGA	87.32 (3.28)	5.87 (1.65)
significance[b]	NS[c]	$p < 0.01$
[^{125}I]MPH-NGA	25.46 (3.12)	58.83 (5.82)
[^{131}I]-L-HML-NGA	84.45 (4.62)	3.28 (0.61)
significance[b]	$p < 0.0001$	$p < 0.0001$
[^{125}I]-D-HML-NGA	39.06 (1.92)	40.97 (2.11)
[^{131}I]-L-HML-NGA	80.78 (2.15)	4.40 (0.82)
significance[b]	$p < 0.0001$	$p < 0.0001$
[^{125}I]-L-HML-IT-NGA	78.26 (1.48)	4.03 (0.76)
[^{131}I]-L-HML-NGA	80.07 (6.23)	2.83 (0.77)
significance[b]	NS	NS

[a] Expressed as % injected dose. Mean (SD) of five animals for each experiment following concomitant administration. [b] Determined by Student's paired *t*-test. [c] Not significant.

results were obtained by RP-HPLC analysis of the urine samples (data not shown).

Discussion

The new radioiodination reagent using L-lysine as the metabolizable linkage, L-HML, and its organotin precursor, L-SHML, were prepared as outlined in Scheme 1. To apply L-lysine as metabolizable linkages, L-lysine should be conjugated to polypeptides *via* the ϵ amine residue without providing an additional linkage that can potentially be cleaved in lysosomes. Formation of inter- and intramolecular cross-linking should also be prevented during the conjugation reactions with polypeptides for further applications. Since previous studies have indicated high-yield and site-specific conjugation reactions between maleimide groups and thiolated polypeptides without inducing unfavorable side reactions[17,19,29−31] and high stability of the resulting thioether bond in lysosomal proteolysis,[14] the maleimide group was selected as the polypeptide binding site and the ϵ amine residue of L-lysine was converted to a maleimide group. In addition, similar conjugation reactions of L-HML and MIH with thiolated polypeptides would render the estimation of L-HML more reliable since MIH generates *m*-iodohippuric acid from covalently conjugated polypeptides following lysosomal proteolysis.[17,19] Other reference reagents, D-HML and MPH, were also designed to possess maleimide groups as the polypeptide conjugation sites for accurate estimations.

Since the stabilities of metabolizable ester bonds *in vivo* can be well estimated by *in vitro* incubation studies using freshly prepared serum,[17,19,32] stabilities of the metabolizable linkage of L-HML were evaluated by incubating [^{131}I]-L-HML-conjugated polypeptides in freshly prepared human serum. To estimate the ability of L-HML in liberating *m*-iodohippuric acid from covalently conjugated polypeptides after lysosomal proteolysis in hepatocytes, NGA was selected as a model polypeptide.[12,14−16] The NGA molecule used holds 44 galactose molecules/molecule of HSA, and previous studies indicated that administrations of 9 μg of the protein/mouse allowed specific localization in hepatic cells immediately after administration.[12,17]

In the preparation of L-HML-conjugated NGA by maleimide−thiol chemistry, two representative procedures are known for thiolation of NGA. In addition, our previous studies of gallium-67 (^{67}Ga)-labeled mAbs with an ester bond showed that esterase-mediated hydrolysis of the ester bond is affected by the thiolation chemistry of mAbs, presumably due to steric interference of the bulky protein molecule blocking access of esterase to the ester bond.[30] A similar phenomenon was observed in MIH-conjugated IgG and Fab, where the ester bond of MIH-IgG showed a much higher stability than that of MIH-Fab.[19] Since enzymatic hydrolyses are also involved in the cleavage of the peptide bond of L-HML, steric interference of the NGA molecule against the enzymes was considered when estimating both serum stability and lysosomal cleavability of the metabolizable linkage of L-HML. Therefore, L-HML was conjugated to NGA by DTT reduction or 2-IT modification (Scheme 1).

The serum incubation studies demonstrated that the peptide bond in L-HML possesses stabilities significantly higher than and comparable to the ester bond in MIH and the amide bond in MPH, respectively. Similar stabilities of [^{131}I]-L-HML-NGA and [^{125}I]-L-HML-IT-NGA also indicated that steric interference of NGA molecules would not be involved in the high serum stability of the peptide bond of L-HML.

In biodistribution studies, similar radioactivity distribution was observed between [^{131}I]-L-HML-NGA and [^{125}I]MIH-NGA, suggesting the selective release of *m*-iodohippuric acid from [^{131}I]-L-HML-NGA after lysosomal proteolysis in hepatic cells at a rate similar to that of [^{125}I]MIH-NGA (Tables 2 and 6). This was further supported by the urine analyses and similar radioactivity distributions between [^{131}I]-L-HML-NGA and [^{125}I]-L-HML-IT-NGA in mice (Tables 5 and 6 and Figure 2C). The gathered *in vitro* and *in vivo* findings suggested that L-HML allows stable attachment of *m*-iodohippuric

acid with polypeptide in serum while providing selective release of the radiolabeled compound from covalently conjugated polypeptide after lysosomal proteolysis at a rate similar to that of the ester bond of MIH. Such characteristics of L-HML would facilitate clearance of radioactivity from nontarget tissues to urine without impairing radioactivity accumulation in the target delivered by polypeptides.

To further understand the role played by the peptide bond derived from L-lysine of L-HML in releasing *m*-iodohippuric acid from covalently conjugated polypeptide, radioiodinated NGAs using D-lysine (D-HML) or cadaverine (MPH) were prepared (Figure 1). Although analyses of urine indicated the generation of *m*-iodohippuric acid from the two conjugates (Figure 2), high radioactivity levels in the intestine and feces indicated that radiometabolites other than *m*-iodohippuric acid were predominantly generated after lysosomal proteolysis of both [^{125}I]MPH-NGA and [^{125}I]-D-HML-NGA (Tables 3, 4, and 6). These findings suggested that use of the α amine residue of L-lysine would be essential for the rapid and selective release of *m*-iodohippuric from covalently conjugated polypeptides by lysosomal enzymes.

In conclusion, a novel radioiodination reagent for polypeptides using L-lysine as the metabolizable linkage was designed and synthesized. The peptide bond of L-HML remained stable in human serum but liberated *m*-iodohippuric acid selectively from the covalently conjugated NGA after lysosomal proteolysis in hepatic cells at a rate similar to that of the ester bond of MIH. Thus, L-HML compensated for the labile characteristics of the ester bond in serum while preserving the ability to liberate the designed radiometabolite, *m*-iodohippuric acid, after lysosomal proteolysis of covalently conjugated polypeptide. Such characteristics render L-HML a potentially useful radioiodination reagent for diagnostic and therapeutic applications using not only polypeptides of high molecular weight but also peptides of lower molecular weights. The present findings provide insight in designing radiolabeling reagents with metabolizable linkages using metallic radionuclides of more clinical importance. The present studies may also provide a good basis for further development of peptide-based metabolizable linkages for targeting radioactive compounds as well as drugs or toxins.

Experimental Section

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JNM-EX 270 (270 MHz) spectrometer (JOEL Ltd., Tokyo, Japan), and the chemical shifts are reported in ppm downfield from an internal tetramethylsilane standard. Fast atom bombardment mass spectra (FAB-MS) were obtained with a JMS-HX/HX110 A model instrument (JOEL Ltd.). Na[^{131}I]I and Na[^{125}I]I were supplied by Daiichi Radioisotopes Labs and Daiichi Kagaku (Tokyo, Japan), respectively, and diluted with phosphate buffer (PB; 0.1 M, pH 7.4) to 37 MBq (1 mCi)/2.7−27 μL for Na[^{131}I]I and 37 MBq (1 mCi)/13−24 μL for Na[^{125}I]I. RP-HPLC was performed with a Cosmosil 5C18-AR column (4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan) equilibrated and eluted at a flow rate of 1 mL/min with a mixture of 0.1% aqueous phosphoric acid and acetonitrile (7:3). TLC analyses were performed with Kieselgel 60 (Merck Art 5553). Radiochemical purities of radioiodinated NGAs were determined by cellulose acetate electrophoresis (CAE) and TLC analyses. CAE was run at an electrostatic field of 0.8 mA/cm for 45 min in veronal buffer (I = 0.05, pH 8.6), while TLC was developed with a mixture of methanol−water (4:1). To facilitate collection of urine and feces after injection of radioiodinated proteins, mice were housed in metabolic cages (metabolica, MM type; Sugiyama-Gen Iriki Co., Ltd., Tokyo, Japan). N^{α}-Boc-D-lysine and human serum albumin (HSA) were obtained from Watanabe Chemical Industries Ltd. (Hiroshima, Japan) and Sigma Co. (A-3782; St. Louis, MO), respectively. Maleimidoethyl 3′-(tri-*n*-butylstannyl)hippurate was synthesized as described previously.[17] Other reagents were of reagent grade and used as received.

Synthesis of Galactosyl-neoglycoalbumin (NGA). Cyanomethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside, prepared by the method of Lee et al.,[33] was conjugated with HSA according to the procedure of Stowell et al.[34] The number of galactose molecules attached per molecule of HSA was 44 as determined by the phenol−sulfuric acid reaction.[35]

Synthesis of *N*-Succinimidyl 3-(Tri-*n*-butylstannyl)-hippurate (3). To a solution of glycine (0.108 g, 1.44 mmol) in 7.25 mL of 0.1 N NaOH containing $NaHCO_3$ (0.122 g, 1.44 mmol) was added a solution of *N*-succinimidyl 3-(tri-*n*-butylstannyl)benzoate (compound **2**; 0.73 g, 1.44 mmol) prepared by the method of Zalutsky et al.[24] in 7.25 mL of acetonitrile. The mixture was refluxed with stirring for 3 h. After cooling to 0 °C, the solution was acidified to pH 3 with 1 N HCl before extraction with ethyl acetate (50 mL × 3). The organic layers were combined and dried over anhydrous calcium sulfate. After removing the solvent, the residue was dissolved in dry acetonitrile (15 mL). *N*,*N*′-Disuccinimidyl carbonate (DSC; 0.48 g, 1.87 mmol) and dry pyridine (0.15 mL) were added to the solution, and the mixture was refluxed with stirring for 2.5 h. After removal of volatile components *in vacuo*, the residue was chromatographed on silica gel using a mixture of ethyl acetate−hexane (1:1) as an eluent to produce compound **3** (0.49 g, 60%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 0.85−1.67 (27H, m, SnBu_3), 2.83 (4H, s, succinimide), 4.72 (2H, d, NHCH_2), 6.86 (1H, t, N*H*), 7.38 (1H, t, aromatic), 7.68 (1H, d, aromatic), 7.72 (1H, d, aromatic), 7.96 (1H, t, aromatic). FAB-MS calcd for $C_{25}H_{39}N_2O_5Sn$ (MH^+): *m*/*z* 586. Found: 586.

Synthesis of *N*-Succinimidyl 3-Iodohippurate. This compound was synthesized by the reaction of 3-iodobenzoyl chloride and glycine, as described previously.[17] Mp: 152−153 °C. ^{1}H-NMR ($CDCl_3$): δ 2.82 (4H, s, succinimide), 4.43 (2H, d, NHCH_2), 7.32 (1H, t, aromatic), 7.89 (1H, d, aromatic), 7.96 (1H, d, aromatic), 8.23 (1H, t, aromatic), 9.27 (1H, t, *NH*). FAB-MS calcd for $C_{13}H_{12}N_2O_5I$ (MH^+): *m*/*z* 403. Found: 403. Anal. ($C_{13}H_{11}N_2O_5I$) C, H, N.

Synthesis of N^{ϵ}-Maleoyl-α-(*tert*-butyloxycarbonyl)-L-lysine (7a). This compound was synthesized according to the procedure of Keller et al.[25] *N*-(Methoxycarbonyl)maleimide (compound **5**; 0.93 g, 6.0 mmol) was added to a solution of N^{α}-Boc-L-lysine (1.58 g, 6.0 mmol) in saturated aqueous $NaHCO_3$ (30 mL) at 0 °C. After vigorous stirring for 40 min at the same temperature, the mixture was stirred for an additional 50 min at room temperature. After cooling to 0 °C again, the solution was acidified to pH 3.0 with concentrated H_2SO_4 before extraction with ethyl acetate (50 mL × 3). The organic layers were combined and dried over anhydrous calcium sulfate. After removing the solvent *in vacuo*, the residue was chromatographed on silica gel using a mixture of chloroform−methanol (5:1) as an eluent to produce compound **7a** (1.71 g, 87%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 1.21−1.67 (6H, m, $(CH_2)_3$), 1.44 (9H, s, *Boc*), 3.52 (2H, t, NCH_2), 4.13 (1H, q, *CH*), 5.08 (1H, d, *NH*), 6.70 (2H, s, maleimide). FAB-MS calcd for $C_{15}H_{24}N_2O_6$ (MH^+): *m*/*z* 327. Found: 327. Anal. ($C_{15}H_{23}N_2O_6 \cdot {}^3/_5H_2O$) C, H, N.

Synthesis of N^{ϵ}-Maleoyl-α-(*tert*-butyloxycarbonyl)-D-lysine (7b). This compound was synthesized by reaction of N^{α}-Boc-D-lysine with compound **5** as described above in 51% yield. ^{1}H-NMR ($CDCl_3$): δ 1.22−1.67 (6H, m, $(CH_2)_3$), 1.45 (9H, s, *Boc*), 3.52 (2H, t, NCH_2), 4.13 (1H, q, *CH*), 5.12 (1H, d, *NH*), 6.70 (2H, s, maleimide). FAB-MS calcd for $C_{15}H_{24}N_2O_6$ (MH^+): *m*/*z* 327. Found: 327. Anal. ($C_{15}H_{23}N_2O_6 \cdot {}^1/_{10}CHCl_3$) C, H, N.

Synthesis of *N*-[5-[(*tert*-Butyloxycarbonyl)amino]-pentyl]maleimide (7c). To a solution of cadaverine (1,5-diaminopentane; 4.45 g, 43.6 mmol) dissolved in dry chloroform (120 mL) was added dropwise a solution of $(Boc)_2O$ (4.75 g,

21.8 mmol) in dry chloroform (120 mL) at 0 °C. The mixture was stirred vigorously at the same temperature for 1 h. After filtration of the precipitate, the filtrate was evaporated *in vacuo*, and the residue was chromatographed on silica gel using a mixture of chloroform−methanol−28% aqueous ammonia solution (80:16:3) as an eluent to produce compound **6c** (3.00 g, 68%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$): δ 1.21−1.55 (6H, m, $(CH_2)_3$), 1.37 (2H, t, NH_2), 1.41 (9H, s, *Boc*), 2.69 (2H, t, NH_2CH_2), 3.11 (2H, q, $NHCH_2$), 4.57 (1H, br, *NH*). Anal. ($C_{15}H_{23}N_2O_6 \cdot {}^1/_{10}CHCl_3$) C, H, N.

Compound **7c** was then synthesized by reaction of compound **6c** with compound **5** as described above in 87% yield. Mp: 58−59 °C. ^{1}H-NMR ($CDCl_3$): δ 1.20−1.66 (6H, m, $(CH_2)_3$), 1.44 (9H, s, *Boc*), 3.10 (2H, q, $NHCH_2$), 3.52 (2H, t, NCH_2), 4.54 (1H, br, *NH*), 6.70 (2H, s, maleimide). FAB-MS calcd for $C_{14}H_{23}N_2O_4(MH^+)$: *m*/*z* 283. Found: 283. Anal. ($C_{14}H_{22}N_2O_4 \cdot {}^1/_{12}CHCl_3$) C, H, N.

Synthesis of 3′-(Tri-*n*-butylstannyl)hippuryl N^ϵ-Maleoyl-L-lysine (8a). Compound **7a** (0.56 g) was treated with 4 M HCl in dry ethyl acetate (5 mL) for 30 min at room temperature before addition of 15 mL of dry ether at 0 °C. The HCl salt of N^ϵ-maleoyl-L-lysine (0.3 g) precipitated as white crystals. A mixture of compound **3** (215 mg, 0.38 mmol), the HCl salt of N^ϵ-maleoyl-L-lysine (100 mg, 0.38 mmol), and *N*,*N*-diisopropylethylamine (69.5 μL, 0.40 mmol) in dry acetonitrile (4 mL) was stirred at room temperature for 1 h. After filtration, the filtrate was evaporated *in vacuo*, and the residue was chromatographed on silica gel using a mixture of chloroform−methanol−acetic acid (250:50:1) as an eluent to produce compound **8a** (198 mg, 78%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 0.85−1.94 (33H, m, $SnBu_3$, $(CH_2)_3$), 3.49 (2H, t, NCH_2), 4.24 (2H, d, $NHCH_2$), 4.29 (1H, q, *CH*), 6.86 (2H, s, maleimide), 7.38 (1H, t, aromatic), 7.60 (1H, d, aromatic), 7.72 (1H, d, aromatic), 7.84 (1H, t, aromatic). FAB-MS calcd for $C_{31}H_{46}N_3O_6Sn$ (M^-): *m*/*z* 676. Found: 676.

Synthesis of 3′-(Tri-*n*-butylstannyl)hippuryl N^ϵ-Maleoyl-D-lysine (8b). This compound was synthesized according to the procedure as described above except using N^α-Boc-D-lysine in place of N^α-Boc-L-lysine as the starting material in 70% yield. ^{1}H-NMR ($CDCl_3$): δ 0.85−1.94 (33H, m, $SnBu_3$, $(CH_2)_3$), 3.49 (2H, t, NCH_2), 4.27 (2H, d, $NHCH_2$), 4.64 (1H, q, *CH*), 6.64 (2H, s, maleimide), 7.36 (1H, t, aromatic), 7.60 (1H, d, aromatic), 7.72 (1H, d, aromatic), 7.91 (1H, t, aromatic). FAB-MS calcd for $C_{31}H_{46}N_3O_6Sn$ (M^-): *m*/*z* 676. Found: 676.

Synthesis of *N*-(5-Maleimidopentyl) 3′-(Tri-*n*-butylstannyl)hippuric Acid Amide (8c). To a solution of compound **7c** (120 mg, 42.5 mmol) in dry chloroform (1.5 mL) was added 1.5 mL of TFA, and the mixture was stirred for 30 min at room temperature. Volatile components were removed *in vacuo* to yield 5′-aminopentylmaleimide as the TFA salt. A mixture of compound **3** (80 mg, 0.20 mmol), 5′-aminopentylmaleimide TFA salt (59 mg, 0.20 mmol), and *N*,*N*-diisopropylethylamine (41.7 μL, 0.24 mmol) in dry tetrahydrofuran (TFA; 7 mL) was stirred at room temperature for 1.5 h. After filtration of the precipitate, the solvent was removed *in vacuo*, and the residue was chromatographed on silica gel using a mixture of ethyl acetate−hexane (4:1) as an eluent to produce compound **8c** (58 mg, 63%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 0.85−1.63 (33H, m, $SnBu_3$, $(CH_2)_3$), 3.27 (2H, q, $NHCH_2$), 3.51 (2H, t, NCH_2), 4.21 (2H, d, $NHCH_2$), 4.29 (1H, q, *CH*), 6.26 (1H, t, *NH*), 6.67 (2H, s, maleimide), 7.01 (1H, t, *NH*), 7.36 (1H, t, aromatic), 7.61 (1H, d, aromatic), 7.71 (1H, d, aromatic), 7.90 (1H, t, aromatic). FAB-MS calcd for $C_{30}H_{47}N_3O_4Sn$ (M^-): *m*/*z* 632. Found: 632.

Synthesis of 3′-Iodohippuryl N^ϵ-Maleoyl-L-lysine. A mixture of *N*-succinimidyl 3-iodohippurate (138 mg, 0.34 mmol), the HCl salt of N^ϵ-maleoyl-L-lysine (90 mg, 0.34 mmol), and *N*,*N*-diisopropylethylamine (62.0 μL, 0.36 mmol) in dry acetonitrile (4 mL) was stirred at room temperature for 24 h. After filtration, the filtrate was evaporated *in vacuo*, and the residue was chromatographed on silica gel using a mixture of chloroform−methanol−acetic acid (40:5:2) as an eluent to produce 3′-iodohippuryl N^ϵ-maleoyl-L-lysine (145 mg, 76%) as a colorless oil. ^{1}H-NMR (CD_3OD): δ 0.87−1.95 (6H, m, $(CH_2)_3$), 3.49 (2H, t, NCH_2), 4.08 (2H, d, $NHCH_2$), 4.37 (1H, q, *CH*), 6.77 (2H, s, maleimide), 7.25 (1H, t, aromatic), 7.87 (1H, d, aromatic), 7.91 (1H, d, aromatic), 8.24 (1H, t, aromatic). FAB-MS calcd for $C_{19}H_{21}N_3O_4I$ (MH^+): *m*/*z* 514. Found: 514. Anal. ($C_{19}H_{20}N_3O_6I \cdot {}^2/_5CHCl_3$) C, H, N.

Synthesis of *N*-(5-Maleimidopentyl) 3′-Iodohippuric Acid Amide. A mixture of *N*-succinimidyl 3-iodohippurate (82 mg, 0.15 mmol), 5′-aminopentylmaleimide TFA salt (43 mg, 0.15 mmol), and *N*,*N*-diisopropylethylamine (30.2 μL, 0.17 mmol) in dry THF (5 mL) was stirred at room temperature for 1.5 h. After filtration of the precipitate, the solvent was removed *in vacuo*, and the residue was chromatographed on silica gel using ethyl acetate as an eluent to produce *N*-(5-maleimidopentyl) 3′-iodohippuric acid amide (80 mg, 85%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 1.25−1.66 (6H, m, $(CH_2)_3$), 3.29 (2H, q, $NHCH_2$), 3.51 (2H, t, NCH_2), 4.19 (2H, d, $NHCH_2$), 6.45 (1H, t, *NH*), 6.69 (2H, s, maleimide), 7.18 (1H, t, aromatic), 7.32 (1H, t, NH), 7.79 (1H, d, aromatic), 7.84 (1H, d, aromatic), 8.19 (1H, t, aromatic). FAB-MS calcd for $C_{18}H_{20}N_3O_4I$ (MH^+): *m*/*z* 470. Found: 470. Anal. ($C_{18}H_{20}N_3O_4I$) C, H, N.

Preparation of [^{131}I]-L-HML-NGA. The stannyl precursor of L-HML (L-SHML) was radioiodinated with Na[^{131}I]I in the presence of *N*-chlorosuccinimide (NCS) as previously described.[17] Briefly, L-SHML was dissolved in methanol containing 1% acetic acid (0.64 mg/mL), and 16.3 μL of this solution was mixed with 2 μL of Na[^{131}I]I. After addition of 4.44 μL of NCS in methanol (0.5 mg/mL), the reaction mixture was incubated at room temperature for 25 min. Aqueous sodium bisulfite (2.22 μL, 0.72 mg/mL) was then added to quench the reaction. The radiochemical yield of [^{131}I]-L-HML was determined by TLC developed with a mixture of chloroform−methanol−acetic acid (40:5:2). Methanol was removed by an N_2 flow prior to subsequent conjugation reaction with NGA.

NGA was pretreated with 5 molar excess of DTT to expose 1 thiol group/molecule of the protein. Under these conditions, none of the 17 disulfide bonds of HSA were accessible to DTT reduction.[26] Briefly, 50 μL of freshly prepared DTT (0.45 mg/mL) in well-degassed 0.1 M PB (pH 6.8) containing 0.3 M NaCl was added to 50 μL of NGA (40 mg/mL) in the same buffer. After incubation for 30 min at room temperature, a 100 μL solution of 0.1 M PB (pH 6.0) containing 2 mM ethylenediaminetetraacetic acid (EDTA) was added. Excess DTT was removed by the centrifuged column procedure[28] using a Sephadex G-50 column (Pharmacia Biotech, Tokyo, Japan) equilibrated with well-degassed 0.1 M PB (pH 6.0) containing 2 mM EDTA. A small portion of this mixture was sampled, and the number of thiol groups was estimated with 2,2′-dithiopyridine.[27] The filtrate (100 μL) was then added to the reaction vial containing crude [^{131}I]-L-HML. After gentle agitation of the reaction mixture for 1.5 h at room temperature, crude [^{131}I]-L-HML-NGA was purified by the centrifuged column procedure using a Sephadex G-50 column equilibrated with 0.1 M PB (pH 7.4).

Preparation of [^{125}I]-L-HML-IT-NGA. Radioiodination of L-SHML with Na[^{125}I]I was performed as described above using Na[^{125}I]I in place of Na[^{131}I]I. Before conjugation with [^{125}I]-L-HML, NGA was treated with a 30 molar excess of 2-IT, as previously reported.[30] Briefly, 177 μL of freshly prepared 2-IT (1.0 mg/mL) in well-degassed 0.16 M borate buffer (BB; pH 8.0) containing 2 mM EDTA was added to 120 μL of NGA (25 mg/mL) in the same buffer. After gentle stirring at room temperature for 1 h, excess 2-IT was removed by the centrifuged column procedure as described above. The number of thiol groups introduced by this treatment was determined with 2,2′-dithiopyridine. The filtrate solution (100 μL) was then added to the reaction vial containing 9.8 MBq (265 μCi) of crude [^{125}I]-L-HML. After gentle agitation of the reaction mixture for 1.5 h at room temperature, the radiolabeled NGA was purified in a manner similar to [^{131}I]-L-HML-NGA.

Preparation of [^{125}I]-D-HML-NGA. Radioiodination of compound **8c** with Na[^{125}I]I and subsequent conjugation with NGA were performed by the procedures similar to those used for [^{131}I]-L-HML-NGA.

Preparation of [^{125}I]MPH-NGA. MPH was radioiodinated by the procedures similar to those used for [^{125}I]-D-HML-NGA except that 0.60 mg/mL compound **8b** dissolved in methanol containing 1% acetic acid was used in place of D-SHML. Radiochemical purity of [^{125}I]MPH was determined by TLC

developed with ethyl acetate. After evaporation of the methanol, the residue was directly used for subsequent conjugation with NGA pretreated with DTT, as described.

Preparation of [^{125}I]MIH-NGA and [^{125}I]MIH-IT-NGA. MIH was radioiodinated by the procedures similar to those used for [^{131}I]-L-HML-conjugated NGAs except that 0.56 mg/mL of the stannyl precursor of MIH was used in place of L-SHML. Radiochemical purity of [^{125}I]MIH was determined by TLC developed with a mixture of chloroform−ether (1:1). After evaporation of methanol, the residue was directly used for subsequent conjugation with thiolated NGAs prepared by DTT reduction or 2-IT treatment.

Serum Stability of Radioiodinated NGAs. [^{131}I]-L-HML-NGA, [^{125}I]-L-HML-IT-NGA, [^{125}I]MPH-NGA, [^{125}I]MIH-NGA, and [^{125}I]MIH-IT-NGA were diluted to 0.5 mg/mL with 0.1 M PBS (pH 6.0), and 20 μL of each solution was added to 230 μL of freshly prepared human serum. After incubation for 1, 3, 6, or 24 h at 37 °C, samples were taken from the solutions, and the percentages of radioactivity bound to proteins were analyzed by paper chromatography (No. 50; Advantec Toyo Inc., Tokyo, Japan) developed with a mixture of methanol−water (4:1). Under these conditions, protein-bound radioactivity and other small metabolized radioactivities had R_f values of 0 and 0.6−0.9, respectively. Each value was calculated by dividing the protein-bound radioactivity at various intervals by the radiochemical purity of the freshly prepared respective radioiodinated protein. To determine the radioactivities released from the conjugates, samples were also analyzed by TLC developed with chloroform−methanol−water (15:8:1). Under these conditions, *m*-iodohippuric acid, free iodine, and *m*-iodobenzoic acid had R_f values of 0.25, 0.1, and 0.4, respectively.

Biodistribution of Radioiodinated NGAs in Mice. [^{131}I]-L-HML-NGA, [^{125}I]MIH-NGA, [^{125}I]MPH-NGA, [^{125}I]-D-HML-NGA, and [^{125}I]-L-HML-IT-NGA were diluted with 0.1 M PBS (pH 6.0) to adjust the protein concentration to 90 μg/mL. Aliquots of 2 mL of [^{131}I]-L-HML-NGA were mixed with the same volume of [^{125}I]MIH-NGA, [^{125}I]MPH-NGA, [^{125}I]-D-HML-NGA, or [^{125}I]-L-HML-IT-NGA prior to administration. Biodistribution of radioactivity was determined after intravenous administration of each mixture to 6-week-old ddY male mice.[36] Groups of five mice, each receiving 9 μg of radioiodinated NGA, were sacrificed at 5, 15, and 30 min, 1 and 3 h postinjection. Organs of interest were removed and weighed, and radioactivity was determined with a multichannel well counter (ARC 2000; Aloka, Tokyo, Japan). A window from 24 to 93 keV was used for counting ^{125}I and one from 280 to 440 keV for ^{131}I. Correlation factors to eliminate crossover of ^{131}I activity into ^{125}I were determined by counting the ^{131}I standard in both windows. The crossover of ^{125}I activity into the ^{131}I channel was negligible.

To determine the amount and the routes of the radioactivity excreted from the body, mice were housed in metabolic cages for 24 h after administration of the respective preparation. At 6 h postinjection, urine samples were collected and analyzed by TLC developed with a mixture of chloroform−methanol−water (15:8:1). The urine samples were also analyzed by RP-HPLC after filtration through a 10-kDa cutoff ultrafiltration membrane (Microcon 10; Amicon Grace, Tokyo, Japan). On RP-HPLC analyses, *m*-iodohippuric acid, free iodine, and *m*-iodobenzoic acid were eluted at retention times of 9.4, 2.9, and 30.8 min, respectively.

Statistical Analysis. Data are expressed as means (standard deviation) where appropriate. Results were statistically analyzed using unpaired *t*-test for *in vitro* studies and paired *t*-test for *in vivo* studies. Differences were considered significant when the *p* value was less than 0.05.

Acknowledgment. The authors are grateful to Dr. I. Yomoda of Daiichi Radioisotopes Labs, Tokyo, Japan, for providing Na[^{131}I]I. The authors also thank Dr. Naoshige Akimoto (Faculty of Pharmaceutical Sciences, Kyoto University) for taking FAB-MS spectra. This study was supported in part by a Grant-in-Aid for Developing Scientific Research (numbers 08557135, 07672419, and 09672279) from the Ministry of Education, Science and Culture of Japan.

Supporting Information Available: Radioactivity profiles of murine urine at 6 h postinjection of [^{131}I]-L-HML-NGA analyzed by RP-HPLC (A) and TLC (B); the major radioactivity (over 93.5% for A and over 95% for B) was detected at a retention time (A) and an R_f value (B) identical to those of *m*-iodohippuric acid (1 page). Ordering information can be found on any current masthead page.

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JM9606397