Maleimidoethyl 3-(Tri-*n*-butylstannyl)hippurate: A Useful Radioiodination Reagent for Protein Radiopharmaceuticals To Enhance Target Selective Radioactivity Localization

Yasushi Arano,† Kouji Wakisaka,† Yoshiro Ohmomo,§ Takashi Uezono,† Takahiro Mukai,† Hiroshi Motonari,† Hiromitsu Shiono,† Harumi Sakahara,‡ Junji Konishi,‡ Chiaki Tanaka,§ and Akira Yokoyama*,†

Faculty of Pharmaceutical Sciences and Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, and Osaka University of Pharmaceutical Sciences, Matsubara, 580, Japan

Received February 22, 1994®

In pursuit of radiolabeled monoclonal antibodies (mAbs) with rapid urinary excretion of radioactivity from nontarget tissues, radioiodinated mAbs releasing a *m*-iodohippuric acid from the mAbs in nontarget tissues were designed. A novel reagent, maleimidoethyl 3-(tri-*n*-butylstannyl)hippurate (MIH), was synthesized by reacting *N*-(hydroxyethyl)maleimide with *N*-Boc-glycine before coupling with *N*-succinimidyl 3-(tri-*n*-butylstannyl)benzoate (ATE). MIH possessed a maleimide group for mAb conjugation and a butylstannyl moiety for high-yield and site-specific radioiodination, and the two functional groups were linked via an ester bond to release *m*-iodohippuric acid. To investigate the fate of radiolabels after lysosomal proteolysis, hepatic parenchymal cells were used as a model nontarget tissue and ^{131}I-labeled MIH was conjugated with galactosyl-neoglycoalbumin (NGA). Further conjugation of [^{131}I]MIH with a mAb against osteogenic sarcoma (OST7) after reduction of its disulfide bonds was followed up. In murine biodistribution studies, [^{131}I]MIH-NGA exhibited rapid accumulation in the liver followed by radioactivity elimination from the liver at a rate that was identical to and faster than those of ^{131}I-labeled NGA via direct iodination ([^{131}I]NGA) and [^{131}I]ATE-labeled NGA, respectively. While [^{131}I]NGA indicated high radioactivity levels in the murine neck, stomach, and blood, such increases in the radioactivity count were not detectable by the administration of either [^{131}I]MIH-NGA or [^{131}I]ATE-NGA. At 6 h postinjection of [^{131}I]MIH-NGA, 80% of the injected radioactivity was recovered in the urine. Analyses of urine samples indicated that *m*-iodohippuric acid was the sole radiolabeled metabolite. In biodistribution studies using [^{131}I]-MIH-OST7 and [^{131}I]ATE-OST7, while both ^{131}I-labeled OST7s registered almost identical radioactivity levels in the blood up to 6 h postinjection, the former demonstrated a lower radioactivity level than [^{131}I]ATE-OST7 in nontarget tissues throughout the experiment. Such chemical and biological characteristics of MIH would enable high target/nontarget ratios in diagnostic and therapeutic nuclear medicine using mAbs and other polypeptides.

Introduction

An enhancement of radioactivity accumulation in the target and the target/nontarget radioactivity ratio enables reliable diagnosis and effective therapy using protein radiopharmaceuticals such as radiolabeled monoclonal antiboides (mAbs) in nuclear medicine. Previous appraoches to enhancing the target localization of radioactivity have included modifications of *in vivo* behaviors of mAbs by enzymatic fragmentation,[1] chemical modifications,[2–4] and *in vivo* stabilization of radiolabels attached to mAbs.[5–9] Recent studies have further suggested that radiolabeled metabolites generated in the body system play an important role in radioactivity localization after the injection of radiolabeled mAbs.[10–16]

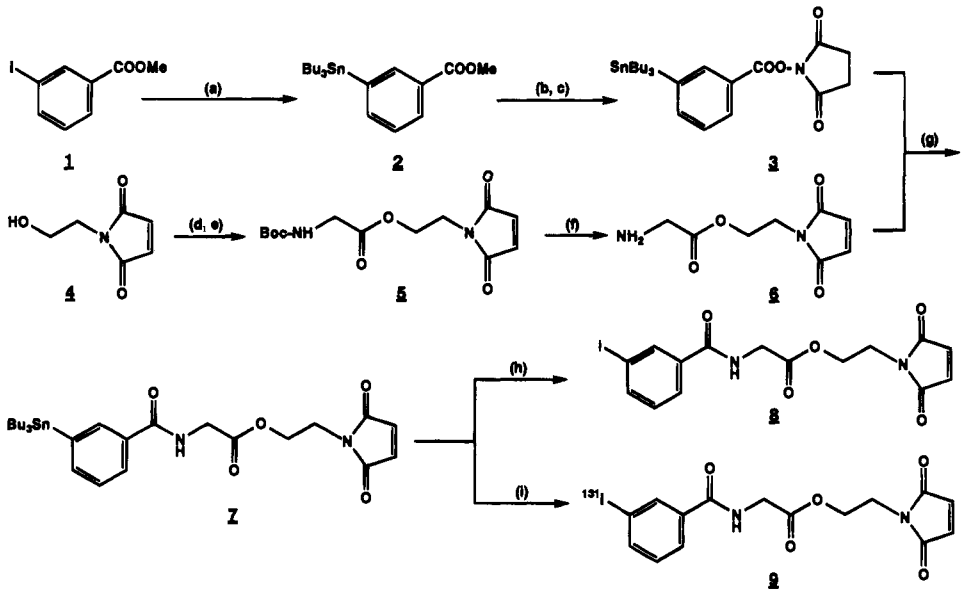
It was thought that radiochemical approaches to promoting rapid urinary excretion of radioactivity accumulated in the nontarget tissues would produce a higher target/nontarget radioactivity ratio. Such approaches would therefore enhance the diagnostic accuracy and therapeutic efficiency while maintaining a safe standard even when higher radioactivity doses were injected in patients to achieve higher radioactivity accumulation in target tissues. Moreover, since these radiochemical approaches are complementary to other approaches in modifying the *in vivo* behaviors of mAbs, a combination of the two different approaches would provide desirable radioactiivty localization of radiolabeled mAbs for diagnostic and therapeutic purposes.

In previous studies, we have developed a bifunctional cross-linker containing an ester bond for mAb conjugation with a gallium-67 (^{67}Ga) chelate of succinyldeferoxamine, a radiometal chelate indicating *in vivo* behaviors similar to those of radioiodinated hippuric acid.[17,18] This ^{67}Ga-labeled mAb registers lower levels of radioactivity in nontarget tissues without any decrease in radioactivity in the target.[19] This result prompted us to further pursue radiolabeled mAbs using other radionuclides of higher clinical applicability. Because of their extensive use in diagnosis and therapy, radioiodines were selected as the radionucldies of choice in this study. Moreover, use of radioiodines would provide an insight in understanding the structure/distribution relationship of this radiochemical approach with regard to other radionuclides. A novel radioiodination reagent releasing

* All correspondence should be directed to this author: Professor of the Department of Radiopharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan. TEL: 81-75-753-4556. FAX: 81-75-753-4568.
† Faculty of Pharmaceutical Sciences, Kyoto University.
‡ Faculty of Medicine, Kyoto University.
§ Osaka University of Pharmaceutical Sciences.
® Abstract published in *Advance ACS Abstracts,* July 1, 1994.

0022-2623/94/1837-2609$04.50/0

Scheme 1[a]



[a] Reagents: (a) $(Bu_3Sn)_2$, $Pd(Ph_3P)_4$; (b) NaOH; (c) *N,N'*-disuccinimidyl carbonate, pyridine; (d) $(CF_3SO_2)_2O$, pyridine; (e) *N*-Boc-glycine, *i*-Pr_2NEt; (f) CF_3COOH; (g) Et_3N; (h) I_2; (i) Na[^{131}I]I, *N*-chlorosuccinimide.

a radioiodinated metabolite of high *in vivo* stability against deiodination with prominent urinary excretion characteristics was thus developed.

Recent studies have indicated that the introduction of a radioiodine to the *meta* position of benzoic acid renders high resistance against *in vivo* deiodination.[5,20,21] In addition, hippuric acid, a conjugatoin product of benzoic acid and glycine in the liver, undergoes rapid urinary excretion from the liver.[22] Considering the high esterase activitylevels in many tissues,[23] we designed a radioiodination reagent that would release *m*-iodohippuric acid according to the procedure in Scheme 1. The novel reagent maleimidoethyl 3-(tri-*n*-butylstannyl)hippurate (MIH) possesses a maleimide group for antibody conjugation and a tri-*n*-butylstannyl group for high-yield and site-specific radioiodination. The two functional groups of this reagent are linked via an ester bond to release the *m*-iodohippuric acid upon cleavage.

Since lysosome is a common organelle that digests protein and peptides,[12,24] pursuring the fate of radiolabel after lysosomal proteolysis of radioiodinated protein derived from MIH would allow a detailed evaluation of the present radiochemical design.[10,12] Furthermore, considering that parenchymal cells have been reported for the common site where mAbs in the murine liver are located,[16,25] MIH was conjugated with a galactosylneoglycoalbumin (NGA), which is known to be incorporated by hepatic parenchymal cells via receptor-mediated endocytosis immediately after injection.[26] The biodistribution of radioactivity after injection of [^{131}I]-MIH-labeled NGA in mice was compared with that of ^{131}I-labeled NGA via either direct radioiodination using the chloramine T method or indirect radioiodination with *N*-succinimidyl 3-(tri-*n*-butylstannyl)benzoate (ATE),[5] since both these methods are extensively estimated as an antibody radioiodination method in nuclear medicine. Chemical structures of the three radioiodinated NGAs, prepared by the respective method, are illustrated in Figure 1. Radioactivity excreted from the body was analyzed to estimate the fate of radiolabeled

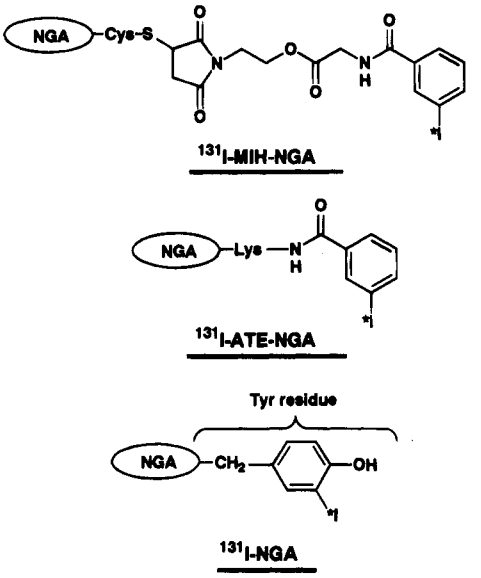


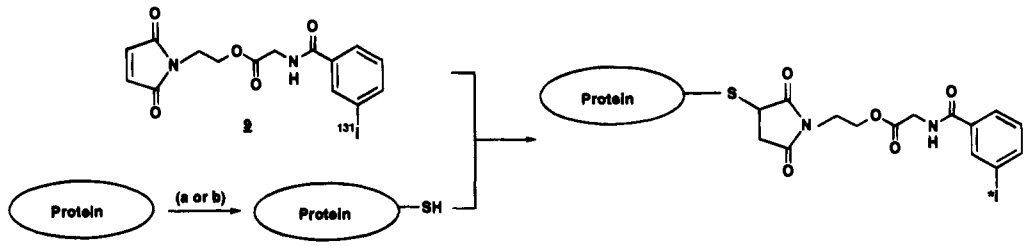
Figure 1. Chemical structures of radioiodinated NGAs used in this study. [^{131}I]MIH and [^{131}I]ATE were attached to cysteine and lysine residues of NGA, respectively. [^{131}I]NGA was prepared by incorporating ^{131}I to tyrosine residues of NGA.

metabolites. The mAb against osteogenic sarcoma (OST7) was also radioiodinated with MIH and ATE as reagents, and the radioactivity distribution was compared in normal mice. The above findings summarized that MIH potentially excreted the radiolabeled metabolite from hepatic parenchymal cells in urine as *m*-iodohippuric acid within a short postinjection time with negligible *in vivo* deiodination.

Chemistry

Synthesis and Iodination of MIH. A novel radioiodination reagent, MIH, containing an ester bond to facilitate the release of *m*-iodohippuric acid was synthesized according to the procedure outlined in Scheme 1. The synthetic steps to prepare the organotin intermediate **3** involved the use of methyl *m*-iodobenzoate (**1**) in a metal–halogen exchange reaction using hexabu-

Scheme 2[a]



[a] Reagents: (a) dithiothreitol for NGA; (b) 2-mercaptoethanol for OST7.

Table 1. Biodistribution of Radioactivity after iv Injection of [^{131}I]MIH-NGA, [^{131}I]ATE-NGA, and [^{131}I]NGA in Mice[a]

	time after injection					
	5 min	30 min	1 h	3 h	6 h	24 h
			[^{131}I]MIH-NGA			
liver	92.56 (2.94)	12.28 (2.73)	3.98 (0.65)	1.56 (0.21)	0.58 (0.03)	0.27 (0.03)
intestines	0.28 (0.04)	9.99 (1.88)	6.11 (0.81)	2.54 (0.16)	0.84 (0.32)	0.05 (0.02)
kidneys	0.65 (0.07)	5.34 (0.88)	2.79 (0.65)	0.14 (0.04)	0.06 (0.02)	0.02 (0.01)
spleen	0.21 (0.09)	0.12 (0.03)	0.10 (0.07)	0.07 (0.02)	0.07 (0.03)	0.04 (0.04)
stomach	0.18 (0.09)	0.59 (0.15)	0.56 (0.14)	0.52 (0.08)	0.45 (0.14)	0.05 (0.04)
neck	0.07 (0.02)	0.11 (0.02)	0.06 (0.01)	0.02 (0.01)	0.02 (0.02)	0.01 (0.01)
blood[b]	0.45 (0.05)	1.01 (0.20)	0.24 (0.06)	0.09 (0.04)	0.03 (0.01)	0.02 (0.01)
urine					78.35 (8.26)	90.60 (1.50)
feces					2.30 (1.55)	3.52 (1.34)
			[^{131}I]ATE-NGA			
liver	92.86 (4.66)	21.18 (0.72)	6.96 (0.82)	4.18 (0.49)	2.82 (0.48)	0.18 (0.03)
intestines	0.41 (0.04)	9.45 (0.70)	10.73 (1.51)	6.42 (0.63)	1.83 (0.57)	0.07 (0.03)
kidneys	0.80 (0.10)	6.42 (1.58)	1.71 (0.60)	0.70 (0.23)	0.31 (0.02)	0.02 (0.01)
spleen	0.02 (0.01)	0.07 (0.02)	0.09 (0.14)	0.01 (0.01)	0.02 (0.02)	0.02 (0.02)
stomach	0.16 (0.04)	0.65 (0.17)	0.43 (0.05)	0.36 (0.10)	0.32 (0.06)	0.04 (0.02)
neck	0.16 (0.01)	0.14 (0.03)	0.03 (0.03)	0.02 (0.01)	0.01 (0.01)	0.01 (0.01)
blood[b]	0.95 (0.14)	1.16 (0.19)	0.28 (0.08)	0.11 (0.04)	0.04 (0.02)	0.01 (0.01)
urine					63.20 (5.43)	83.90 (8.89)
feces					1.53 (0.49)	3.26 (1.60)
			[^{131}I]NGA			
liver	92.49 (5.77)	18.72 (1.41)	3.68 (0.44)	1.44 (0.20)	1.13 (0.33)	0.37 (0.22)
intestines	0.16 (0.02)	8.28 (0.71)	6.73 (1.30)	3.43 (0.07)	3.06 (1.66)	0.21 (0.10)
kidneys	0.01 (0.01)	2.07 (0.63)	1.73 (0.47)	0.83 (0.24)	0.57 (0.27)	0.04 (0.02)
spleen	0.06 (0.01)	0.30 (0.08)	0.23 (0.05)	0.10 (0.03)	0.06 (0.04)	0.02 (0.01)
stomach	0.25 (0.15)	15.65 (2.41)	25.55 (2.74)	17.50 (2.70)	13.22 (2.90)	0.11 (0.09)
neck	0.04 (0.02)	0.70 (0.07)	0.34 (0.05)	0.25 (0.03)	0.21 (0.04)	0.01 (0.01)
blood[b]	0.15 (0.04)	5.35 (0.50)	5.14 (0.92)	1.40 (0.44)	1.59 (0.72)	0.12 (0.07)
urine					26.84 (4.34)	79.66 (6.52)
feces					0.31 (0.25)	3.22 (0.52)

[a] Expressed as percent injected dose/organ. Mean (SD) for five animals for each point. [b] Expressed as percent injected dose/g.

tylditin. After base-catalyzed hydrolysis of the benzoate **2**, the carboxylic acid was converted to succinimide ester **3** in the presence of *N,N'*-disuccinimidyl carbonate. *N*-Boc-glycine was esterified by reacting with the triflate of **4** followed by deprotection of the Boc group in trifluoroacetic acid to yield **6**. Finally, the active ester of **3** was condensed with the amine group of **6** to produce maleimidoethyl 3-(tri-*n*-butylstannyl)hippurate (**7**) in relatively high yields. Iodination of compound **7** was accomplished by an organotin–halide exchange reaction in the inert solvent at room temperature with 91% yield to generate **8**.

Radioiodination and Conjugation Reactions. Radioiodination of **7** in methanol using *N*-chlorosuccinimide (NCS) as an oxidant produced radioiodinated MIH in 87–91% radiochemical yields when determined by TLC when compound **8** was used as a reference. Prior to conjugation of [^{131}I]MIH with either NGA or mAb (OST7), sodium metabisulfite was added to MIH medium to reduce any unreacted electrophilic iodine species to iodide and to quench any excess NCS. As such, this treatment prevented the iodine species from attaching to tyrosine moieties of the protein molecule. Furthermore, neutralization of excess NCS with sodium metabisulfide prevented the protein from exposure to the oxidant. Radioiodination of ATE according to previous procedures[5] yielded 82–90%.

Conjugation of [^{131}I]MIH with either NGA or OST7 was achieved by reacting the thiolated proteins with the maleimide group of [^{131}I]MIH. After dithiothreitol (DTT) or 2-mercaptoethanol (2-ME) reduction of NGA or OST7, 1 or 6.7 thiol groups were exposed to each protein molecule, respectively (Scheme 2). The respective radiochemical yields of [^{131}I]MIH-labeled NGA and OST7 were 4.7% and 59.7% when determined by cellulose acetate electrophoresis. After purification through gel permeation column chromatography, [^{131}I]MIH-NGA and [^{131}I]MIH-OST7 with 93.2% and 95.6% radiochemical purities were obtained for subsequent studies, respectively. Size-exclusion HPLC of [^{131}I]MIH-labeled NGA and OST7 indicated a single radioactivity peak at the respective retention times of 16.8 and 15.2 min. Conjugation of [^{131}I]ATE with NGA and OST7 produced 39.4% and 45.2% yields, respectively. After gel permeation chromatography, both [^{131}I]ATE-labeled NGA (96.5% purity) and OST7 (94.6% purity) were used for subse-

Table 2. Statistical Analyses of Radioactivity after Injection of [^{131}I]MIH-NGA, [^{131}I]ATE-NGA, and [^{131}I]NGA in Mice[a]

	time after injection					
	5 min	30 min	1 h	3 h	6 h	24 h
			[^{131}I]MIH-NGA vs [^{131}I]ATE-NGA[c]			
liver	NS[b]	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
intestines	NS	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS
urine					$p < 0.05$	NS
			[^{131}I]MIH-NGA vs [^{131}I]NGA[d]			
liver	NS	NS	NS	NS	$p < 0.01$	NS
intestines	NS	NS	NS	$p < 0.01$	$p < 0.01$	NS
kidneys	$p < 0.001$	$p < 0.05$	$p < 0.05$	$p < 0.001$	$p < 0.01$	NS
stomach	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS
neck	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS
blood	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
urine					$p < 0.001$	NS

[a] Determined using unpaired t-test. [b] Not significant. [c] No significant differences were observed in kidneys, spleen, stomach, blood, neck, and feces. [d] No significant differences were observed in spleen and feces.

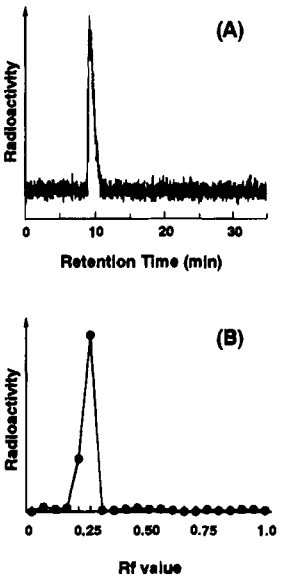


Figure 2. Analyses of radiolabeled metabolite excreted in the urine at 6 h postinjection of [^{131}I]MIH-NGA. Urine samples indicated a single radioactivity profile by both RP-HPLC (A) and TLC (B) analyses. These profiles correlated well with those of m-iodohippuric acid.

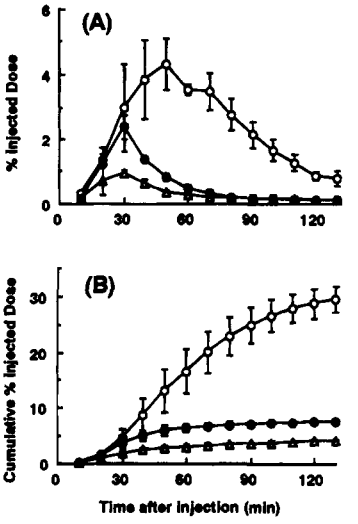


Figure 3. Radioactivity (expressed as % injected dose) excreted in the rat bile after iv injections of [^{131}I]MIH-NGA (●), [^{131}I]ATE-NGA (○), and [^{131}I]NGA (△). Radioactivity levels in bile at every 10 min after injection of each ^{131}I-labeled NGA (A) were recorded. Cumulative radioactivity in bile for 130 min after injection of each ^{131}I-labeled NGA (B) was also illustrated. At 50 min postinjection, [^{131}I]ATE-NGA excreted significantly higher ($p < 0.05$) radiolabels in bile than [^{131}I]-MIH-NGA and [^{131}I]NGA.

quent biodistribution studies. The [^{131}I]NGA (93.4% purity) was used for biodistribution studies after direct radioiodination of NGA with Na[^{131}I]I in the presence of chloramine T followed by purification through the gel permeation column chromatography.

Biological Evaluation

***In Vivo* Studies using NGA.** The radioactivity distributions after administration of [^{131}I]MIH-NGA, [^{131}I]ATE-NGA, and [^{131}I]NGA are shown in Table 1. At 5 min postinjection of each ^{131}I-labeled NGA, more than 92% of the injected radioactivity was accumulated in the liver. [^{131}I]MIH-NGA thereafter manifested almost similar to and significantly higher than [^{131}I]NGA and [^{131}I]ATE-NGA in radioactivity elimination rate from the liver, respectively (Tables 1 and 2). [^{131}I]MIH-NGA demonstrated the fastest rate of radioactivity excretion from the body with most of the radioactivity being excreted in the urine. While [^{131}I]NGA exhibited high levels of radioactivity in the neck, stomach, and blood from 0.5 to 6 h postinjection, such high radioactivity counts were not observed in similar tissues following injection of either [^{131}I]MIH-NGA or [^{131}I]ATE-NGA. Although most of the radioactivity was excreted in the urine at 24 h postinjection, high radioactivity levels were detected in the intestines from 0.5 to 1 h postinjection of the respective ^{131}I-labeled NGA.

TLC and reversed-phase HPLC (RP-HPLC) analyses of urine samples at 6 h postinjection of [^{131}I]MIH-NGA indicated a single radioactivity peak with a retention time (9.4 min; Figure 2A) and an R_f value (0.25; Figure 2B) similar to those of m-iodohippuric acid.

On investigating the radioactivity levels excreted in rat bile (expressed as a percentage of the injected dose) at 10-min intervals and the cumulative amount of radioactivity in bile for 130 min after iv injection of the three ^{131}I-labeled NGAs, [^{131}I]MIH-NGA demonstrated a hepatobiliary excretion rate faster than and similar to those of [^{131}I]ATE-NGA and [^{131}I]NGA, respectively (Figure 3A). The amount of radioactivity excreted in rat bile 130 min after [^{131}I]ATE-NGA injection registered the highest value (28.9% of the injected dose), whereas only 7.5% and 4.1% were recovered with the injection of [^{131}I]MIH-NGA and [^{131}I]NGA at a similar postinjection period, respectively (Figure 3B).

Table 3. Biodistribution of Radioactivity after Injection of [^{131}I]MIH-OST7 and [^{131}I]ATE-OST7 in Normal Mice[a]

	time after injection						
	10 min	1 h	3 h	6 h	24 h	48 h	72 h
blood							
MIH	33.64 (1.48)	29.30 (1.43)	25.11 (1.53)	20.31 (2.53)	9.64 (0.33)	5.78 (0.47)	4.22 (0.53)
ATE	38.38 (1.61)	31.31 (1.53)	26.13 (1.07)	23.13 (1.11)	14.39 (1.26)	12.64 (0.50)	11.50 (0.81)
significance[c]	NS[d]	NS	NS	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$
lung							
MIH	10.37 (1.13)	7.08 (1.15)	8.06 (0.92)	7.60 (0.76)	4.06 (0.78)	1.98 (0.35)	1.52 (0.14)
ATE	11.91 (0.66)	11.10 (1.56)	7.97 (1.14)	6.90 (0.48)	5.55 (0.49)	4.25 (1.14)	3.55 (0.68)
significance[c]	NS	NS	NS	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$
liver							
MIH	9.81 (1.29)	8.51 (0.71)	6.43 (0.69)	5.39 (0.73)	2.46 (0.33)	1.53 (0.08)	1.05 (0.09)
ATE	15.66 (1.21)	14.38 (1.74)	11.17 (1.40)	9.83 (0.62)	4.94 (0.48)	3.84 (0.76)	3.34 (0.11)
significance[c]	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
intestine							
MIH	0.80 (0.16)	1.96 (0.26)	1.93 (0.16)	1.27 (0.25)	0.67 (0.08)	0.40 (0.02)	0.28 (0.03)
ATE	0.85 (0.13)	2.03 (0.34)	2.88 (0.09)	2.43 (0.15)	1.06 (0.12)	0.91 (0.09)	0.83 (0.07)
significance[c]	NS	$p < 0.05$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
kidneys							
MIH	6.08 (0.51)	5.35 (0.81)	5.00 (0.62)	4.45 (0.73)	2.08 (0.41)	1.12 (0.02)	0.82 (0.09)
ATE	6.31 (0.39)	8.15 (1.05)	7.40 (1.12)	6.57 (0.53)	4.23 (0.40)	3.27 (0.59)	2.73 (0.49)
significance[c]	NS	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.001$	$p < 0.001$
spleen							
MIH	7.15 (1.52)	4.84 (0.83)	3.67 (0.67)	3.19 (0.39)	1.41 (0.24)	0.92 (0.12)	0.49 (0.12)
ATE	7.07 (1.55)	6.38 (0.61)	5.70 (0.66)	5.48 (0.84)	3.37 (0.53)	2.35 (0.21)	1.83 (0.29)
significance[c]	NS	$p < 0.05$	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.001$
stomach[b,e]							
MIH	0.54 (0.11)	0.92 (0.10)	1.04 (0.11)	0.84 (0.09)	0.28 (0.04)	0.20 (0.03)	0.14 (0.03)
ATE	0.44 (0.13)	0.81 (0.13)	1.31 (0.19)	1.30 (0.17)	0.49 (0.11)	0.42 (0.04)	0.40 (0.07)
neck[b,e]							
MIH	0.34 (0.15)	0.75 (0.18)	0.51 (0.02)	0.45 (0.14)	0.32 (0.02)	0.16 (0.04)	0.09 (0.02)
ATE	0.66 (0.02)	0.83 (0.22)	0.72 (0.18)	0.55 (0.03)	0.34 (0.14)	0.34 (0.01)	0.25 (0.17)
urine[b]							
MIH					23.39 (1.99)		
ATE					10.09 (0.84)		
significance[c]					$p < 0.001$		
feces[b,e]							
MIH					0.89 (0.22)		
ATE					0.55 (0.16)		

[a] Expressed as percent injected dose/g of tissue. Mean (SD) for five mice for each point. [b] Expressed as percent injected dose. [c] Determined using unpaired t-test. [d] Not significant. [e] No significant differences were observed throughout the postinjection time.

RP-HPLC analyses of bile samples after [^{131}I]MIH-NGA iv injection revealed a major and a minor peak at respective retention times of 9–10 and 4–5 min (Figure 4A). The former, which represented over 75% of the radioactivity excreted in the bile, registered a retention time similar to that of m-iodohippuric acid. The latter peak represented only 25% of the recovered bile radioactivity. Similar results were observed with TLC analyses of the bile (Figure 4B). The major peak indicated an R_f value similar to that m-iodohippuric acid.

Plasma Stability of [^{131}I]MIH-OST7. At 1 h postincubation of both [^{131}I]MIH-OST7 and [^{131}I]ATE-OST7 in 50% murine plasma at 37 °C, no significant differences in radioactivity release were observed between the two ^{131}I-labeled OST7s. However, while radioactivity in the protein fraction remained unchanged for [^{131}I]-ATE-OST7, gradual release of radioactivity from the conjugate was observed with [^{131}I]MIH-OST7 thereafter. At 6 and 24 h postincubation, [^{131}I]MIH-OST7 released 9% and 18% of the initial radioactivity (Figure 5). TLC and paper chromatography analyses indicated that the radioactivity released from [^{131}I]MIH-OST7 showed R_f values similar to those of m-iodohippuric acid.

***In Vivo* Studies using mAb (OST7).** When injected into mice, both [^{131}I]MIH-OST7 and [^{131}I]ATE-OST7 had similar levels of radioactivity in the blood up to 6 h postinjection (Table 3). The former had a radioactivity clearance rate from blood significantly higher than that of [^{131}I]ATE-OST7 after this postinjection interval. At the respective postinjection intervals, [^{131}I]MIH-OST7 also manifested significantly lower radioactivity levels in all tested tissues. Although most of the radioactivity was excreted in urine after injecting the two ^{131}I-labeled

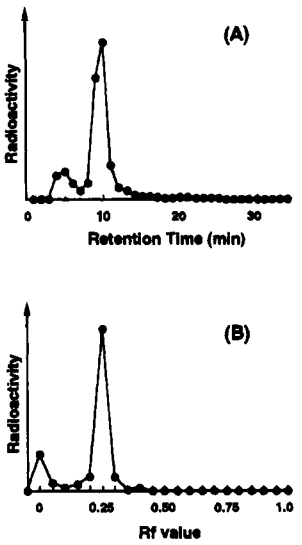


Figure 4. Analyses of radioactivity excreted in rat bile after [^{131}I]MIH-NGA injection. Bile samples contained a major (>75%) and a minor (<25%) radioactivity species by RP-HPLC (A) and TLC (B) analyses. The major radioactivity species had a retention time and an R_f value similar to those of m-iodohippuric acid.

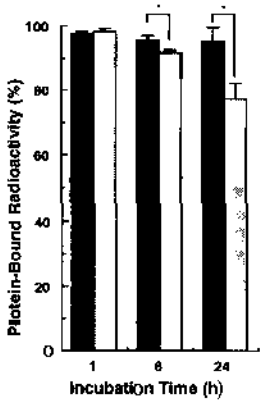


Figure 5. Stability of ([131]I]MJH-OST7 (light column) and ([131]I]-ATE-OST7 (dark column) in 50% murine plasma. While no significant differences in the radioactivity release were observed between the two at 1 h postincubation, ([131]I]MIH-OST7 released significantly ($p^* < 0.05$) higher levels of radiolabel at 6 and 24 h postincubation.

OST7s, higher levels were registered by the use of [[131]I]-MIH-OST7.

Results and Discussion

Numerous raidoiodination reagnets for mAbs have been developed to stabilize the radioiodine atom against *in vivo* deiodination.[5,6,20,21,27,28] In this study, we designed a radioiodination reagent not only possessing high resistance against *in vivo* deiodination but also releasing a radioiodinated metabolite with high urinary excretion after lysosomal proteolysis in nontarget tissues. Among the various monoiodinated hippuric acid analogs, specificity in the renal organic anion transport system increases in the order of *ortho* > *para* > *meta* substitution.[29] However, the introduction of an iodine atom at the *meta* position of benzoate renders a higher *in vivo* stability than *para* substitution.[20,21] Since the stability against *in vivo* deiodination of a radioiodination reagent for mAbs was thought to be more important than the slightly higher specificty in the renal organic anion transport system, MIH was designed to release *m*-iodohippuric acid to serve as a potential radiolabeled metabolite after cleavage of the ester bond.

In preparing MIH, compound **6** was the key intermediate product. In our initial attempt to synthesize maleimidoethyl 3-iodohippurate (**8**), direct esterification of *m*-iodohippuric acid with (2-hydroxyethyl)maleimide (**4**) in the presence of dicyclohexylcarbodiimide generated poor yields of product **8**. Such a synthetic route was thus unsuitable for the preparation of compound **7**. Instead, Boc-glycine was esterified with the hydroxy group of (2-hydroxyethyl)maleimide (**4**) after activating the hydroxy group of compound **4** by trifluoromethanesulfonic anhydride. Following deprotection of the Boc group of **5**, the resulting amine of **6** was conjugated with the active ester of **3** (ATE) to generate MIH **7**. TLC analyses of radioiodinated MIH using compound **8** as a reference confirmed that the organotin–halide exchange reaction produced a high yield with site-specific radioiodination.

Conjugation of [[131]I]MIH with OST7 yielded 60% of the radioiodinated OST7 when crude radioiodinated MIH was used. Since the reduction of OST7 generated 6.7 thiol groups/molecule of OST7, the molar ratio of the reaction between the maleimide and thiol groups was appropriated to 1:1. This radiochemical yield was higher than that of [[131]I]ATE-OST7 (45%) by our hands, although literature cited higher values for the latter conjugation reaction.[5,20] These results indicated that the use of a maleimide group as the protien conjugation site was useful not only for the site-specific modification of mAbs but also in attaining high conjugation yield. Our findings reinforced previously documented similar conjugation yields between a radioiodinated maleimide derivative and thiol groups of mAb.[27] However, on radioiodination of NGA with [[131]I]MIH, a yield of only 4.7% for [[131]I]MIH-NGA was achieved. Since only one thiol group was liberated per NGA molecule under the present DTT reduction conditions,[30] the maleimide/thiol molar ratio in this reaction was 10:1. The low yield of this reaction was, therefore, due to the presence of nonradiolabeled maleimide compoudns, which interfered with the conjugation of radioiodinated MIH to a small number of thiol groups in the NGA molecule.

Since parenchymal cells serve as a common site where antibodies in the murine liver are located,[16,25] the *in vivo* fate of radiolabels after lysosomal proteolysis in hepatic parenchymal cells was investigated to evluate the ability of MIH to excrete radioactivity from nontarget tissues to urine. Since the radiochemical purity of each [131]I-labeled NGA was over 92%, radioactivity counts after administration of the three radioiodinated NGAs would reflect the fate of the radiolables after parenchymal incorporation and lysosomal proteolysis in the liver.[10,12] Following rapid radioactivity elimination from the liver, [[131]I]NGA demonstrated a high radioactivity accumulation in the stomach and blood (Table 1). When injected in rats, [125]I-labeled asialo-fetuin is rapidly digested to [[125]I]iodotyrosine in hepatic lysosomes before being deiodinated within the cytoplasm of hepatocytes. As such, only free iodide was found in the plasma after administering the radiolabeled protein.[31] Rapid radioactivity elimination from the liver and high radioactivity levels in the neck, stomach, and blood after [[131]I]NGA injection could well be explained by the rapid digestion of [[131]I]NGA to [[131]I]iodotyrosine ensued with deiodination of the metabolite by hepatocytes. Significant *in vivo* redistribution of the radiolabeled metabolite derived from [[131]I]NGA was well reflected by the delayed radioactivity excretion from the body system at **6** h postinjection. Both [[131]I]ATE-NGA and [[131]I]MIH-NGA displayed rapid and almost quantitative accumulation in the liver, whereas [[131]I]MIH-NGA demonstrated radioactivity elimination from the liver at a rate higher than and similar to those of [[131]I]ATE-NGA and [[131]I]-NGA after iv administration, respectively. (Tables 1 and 2). Furthermore, the radioactivity counts of [[131]I]MIH-NGA in the neck and stomach were comparable to and much lower than those of [[131]I]ATE-NGA and [[131]I]NGA, respectively. Of the three [131]I-labeled NGAs, [[131]I]MIH-NGA had the fastest excretion rate of radioactivity to urine. From our findings, [[131]I]MIH-NGA generated radiolabeled metabolites with high urinary excretion characteristics following lysosomal proteolysis and their resistance against *in vivo* deiodination was remarkably high. Urine samples at **6** h postinjection of [[131]I]MIH-NGA portrayed a single radioactivity peak on both RP-HPLC and TLC analyses. In addition, the radiolabeled metabolite possessed a retention time and an R_f value similar to those of *m*-iodohipuuric acid (Figure 2). This

strongly suggests that the release of *m*-[^{131}I]iodohippuric acid occurs promptly after lysosomal proteolysis in hepatic parenchymal cells, followed by rapid elimination of the radioiodinated metabolite from liver to urine.

Although more than 79% of the injected radioactivity was excreted in the urine at 24 h postinjection of the three ^{131}I-labeled NGAs, all the ^{131}I-labeled NGAs indicated a higher radioactivity localization in intestines than in feces (Table 1). This implied that while most of the radiolabeled metabolites derived from each ^{131}I-labeled NGA were excreted in the urine directly from the liver to the blood stream, some of the radiolabeled metabolites were excreted via hepatobiliary excretion and subsequently reabsorbed from the gut lumen to the blood. To further understand the excretion pathways of radiolabeled metabolites from the liver, each ^{131}I-labeled NGA was injected in rats and radioactivity excreted in the bile was assessed. The elimination rate of [^{131}I]MIH-NGA was similar to and higher than those of [^{131}I]NGA and [^{131}I]ATE-NGA, respectively (Figure 3). The amount of radioactivity eliminated from the liver via hepatobililary excretion was highest for [^{131}I]ATE-NGA followed by [^{131}I]MIH-NGA and [^{131}I]NGA (Figure 3). Analyses of the bile after [^{131}I]MIH-NGA injection indicated that the major radiolabeled metabolite (75% in the bile) had a chromatographic retention time and an R_f value similar to those of *m*-iodohippuric acid (Figure 4). These results suggested that [^{131}I]MIH-NGA released most of the radioactivity (>90%) in a form of *m*-[^{131}I]iodohippuric acid after lysosomal proteolysis in hepatic parenchymal cells. The major excretion pathway of this metabolite was from the liver to the blood stream followed by urinary excretion via the kidney. Although 7% of injected radioactivity was excreted in the bile from the liver, most of the radioactivity (75% excreted in bile) was reabsorbed from the gut lumen to the blood and eventually excreted in the urine. Analyses of the bile indicated that only 1−2% of the injected radioactivity of [^{131}I]MIH-NGA was metabolized to unidentified compounds. These metabolites might be responsible for the radioactivity excreted in feces.

Recent studies on the metabolism of [^{125}I]ATE-conjugated F(ab')$_2$ have indicated that more than 90% of the radioactivity in urine had a RP-HPLC retention time corresponding to an iodobenzoic acid−lysine conjugate.[32] A similar result has also been reported when the major urinary metabolites of *p*-iodobenzoate conjugates of mAbs were investigated.[33] Therefore, lower elimination rate of radiolabels from the liver and higher radioactivity levels in the intestine after [^{131}I]ATE-NGA injection reflected the biological characteristics of radiolabeled metabolites derived from MIH and ATE in the liver.

In biodistribution of mice with mAb, [^{131}I]MIH-OST7 demonstrated lower radioactivity levels in nontarget tissues such as the liver, kidney, and spleen with higher radioactivity levels in the urine when compared with [^{131}I]ATE-OST7 (Table 3). This radioactivity localization reflects the rapid release of *m*-[^{131}I]iodohippuric acid from [^{131}I]MIH-OST7 in nontarget tissues and subsequent elimination via urinary excretoin, as characterized by using NGA. [^{131}I]MIH-OST7 exhibited a radioactivity level in blood up to 6 h postinjection similar to that of [^{131}I]ATE-OST7. However, the former indicated a higher rate of radioactivity elimination from blood after this postinjection time. This could be attributable to gradual cleavage of the ester bond in [^{131}I]MIH-OST7, as indicated by *in vitro* plasma incubation (Figure 5). Since our previous studies have indicated that the ester bond between mAb and a ^{67}Ga chelate of succinyldeferoxamine would be stabilized to a higher degree in target tissues than in blood because of the formation of antigen−antibody complexes,[18,19] the radioactivity distribution of [^{131}I]MIH-labeled mAb might be favorable for target selective radioactivity localization.

In conclusion, this newly designed raidoiodination reagent, MIH, rendered high yields and site-specific radioiodination of the *meta* position of the hippuric acid moiety, releasing the radioiodinated hippuric acid upon cleavage of the ester bond in nontarget tissues. The radioiodinated hippuric acid displayed high stability against *in vivo* deiodination and rapid urinary excretion. Thus, although further estimation using the nude mice model may be required, these chemical and biological characteristics would enable MIH to serve as a useful radioiodination reagent for protein radiopharmaceuticals in diagnostic and therapeutic nuclear medicine.

Experimental Section

Melting points were determined on a Yanagimoto micro-melting point apparatus (Kyoto, Japan) and are uncorrected. Infrared (IR) spectra were recorded with a JASCO IR-700 spectrometer (Tokyo, Japan). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer (Tokyo, Japan), and the chemical shifts are reported in ppm downfield from an internal tetramethylsilane standard. High-resolution mass spectra (HRMS) were obtained with the Hitachi M-80 model. Size-exclusion HPLC and RP-HPLC were performed with Cosmosil 5Diol-120 (7.5 × 600 mm) and Cosmosil 5C18-AR (4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan) columns, respectively. Size-exclusion HPLC and RP-HPLC were eluted at a flow rate of 1 mL/min with 0.1 M phosphate buffer (pH 6.8) and a mixture of 0.1% aqueous phosphoric acid and acetonitrile (7:3), respectively. Phosphate buffer (PB) and phosphate-buffered saline (PBS) were prepared as necessary with reagent grade disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride (Nalcalai Tesque), and reagent grade water (milli-Q: Millipore Ltd., Tokyo, Japan). To facilitate urine and feces collection at 6 and 24 h postinjection of radiolabeled proteins, mice were housed in metabolic cages (metabolica, MM type; Sugiyama-Gen Iriki Co. Ltd., Tokyo, Japan).

Synthesis of Neoglycoalbumin (NGA). Cyanomethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside, synthesized according to the procedure of Lee et al.,[34] was conjugated with human serum albumin (HSA, A-3782; Sigma Co., St. Louis, MO) according to the procedure of Stowell et al.[35] When determined with the phenol−sulfuric acid reaction,[36] 44 galactoses were attached to each HSA molecule.

Monoclonal Antibody. The monoclonal antibody against osteogenic sarcoma (OST7, IgG_1) was produced by standard hybridoma technology.[37]

Synthesis of Methyl 3-(Tri-*n*-butylstannyl)benzoate (2). Methyl-3-iodobenzoate (1) (1.31 g, 5.0 mmol) and hexa-*n*-butylditin (3.48 g, 6.0 mmol) were dissolved in dry toluene (20 mL), and a catalytic amount of tetrakis(triphenylphoshine)-palladium (60 mg, 0.05 mmol) was added. The mixture was refluxed with stirring for 15 h under argon atmosphere. After cooling, the reaction mixture was filtered through Celite. The filtrate was concentrated *in vacuo*. The oily residue was then purified by column chromatography on silica gel eluted with chloroform−hexane (1:1) to yield **2** (1.90g, 89%) as a colorless oil. IR ($CHCl_3$): 2956, 2926, 1715, 1460, 1437, 1279, 1118 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 0.85−1.59 (27H, m, $SnBu_3$), 3.92 (3H, s, CH_3), 7.39 (1H, t, J = 7.5 Hz, aromatic), 7.65 (1H, dt,

J = 7.5, 1.5 Hz, aromatic), 7.96 (1H, dt, J = 7.5, 1.5 Hz, aromatic), 8.13 (1H, t, J = 1.5 Hz, aromatic), CI-HRMS calcd for $C_{20}H_{35}O_2Sn$ (MH^+): m/z 427.1659. Found: 427.1661.

Synthesis of *N*-Succinimidyl 3-(Tri-*n*-butylstannyl)-benzoate (3). Methyl 3-(tri-*n*-butylstannyl)benzoate (**2**) (2.13 g, 5.0 mmol) was dissolved in NaOH (1 M, 10 mL) and methanol (40 mL), and the mixture was refluxed with stirring for 1 h. Methanol was removed *in vacuo*. The resulting solution was acidified with 1 M HCl before extraction with chloroform (10 mL × 3). Combined organic layers were washed with water (10 mL × 3) and dried over sodium sulfate. The solvent was removed *in vacuo* to yield crude 3-(tri-*n*-butylstannyl)benzoic acid (2.05 g).

N,N'-Disuccinimidyl carbonate (1.54 g, 6.0 mmol) was added to a mixture of crude 3-(tri-*n*-butylstannyl)benzoic acid (2.05 g) and pyridine (0.47 g, 6.0 mmol) in dry acetonitrile (30 mL). The mixture was refluxed with stirring for 3 h. After removal of volatile components *in vacuo*, the residue was chromatographed on silica gel using ether–hexane (8:2) as an eluent to produce compound **3** (1.80 g, 71%) as a colorless oil. IR ($CHCl_3$): 2958, 2926, 1771, 1742, 1219, 1068, 1006 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 0.85–1.59 (27H, m, $SnBu_3$), 2.92 (4H, s, succinimide), 7.45 (1H, t, J = 7.5 Hz, aromatic), 7.76 (1H, dt, J = 7.5, 1.5 Hz, aromatic), 8.06 (1H, dt, J = 7.5, 1.5 Hz, aromatic), 8.20 (1H, t, J = 1.5 Hz, aromatic). CI-HRMS calcd for $C_{23}H_{36}NO_4Sn$ (MH^+): m/z 510.1666. Found: 510.1661.

Synthesis of 2-Maleimidoethoxy *N*-*t*-Boc-glycinate (5). A solution of trifluoromethanesulfonic anhydride (3.68 g, 13 mmol) in dry methylene chloride (4 mL) was added to a solution containing both *N*-(2-hydroxyethyl)maleimide (**4**) (1.41 g, 10 mmol) (17) and 2,6-dimethylpyridine (1.40 g, 13 mmol) in dry methylene chloride (16 mL) under cooling in an ice bath. After stirring at 0 °C for 1 h, a solution of *N*-Boc-glycine (2.28 g, 13 mmol) and *N,N*-diisopropylethylamine (1.68 g, 13 mmol) in dry methylene chloride (10 mL) was added to the reaction mixture and then the mixture stirred for 3 h at room temperature. The reaction mixture was washed with water (20 mL) followed by 1 M HCl (20 mL) and finally with water again (20 mL). The organic layer was dried over sodium sulfate and evaporated *in vacuo* to yield crude crystals. Recrystallization from chloroform–ether afforded **5** (1.29 g, 43%). Mp: 91–93 °C. IR (KBr): 3224, 3082, 1763, 1709, 1403, 1246, 1161 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 1.45 (9H, s, Boc), 3.81 (2H, t, J = 5.2 Hz, OCH_2CH_2N), 3.88 (2H, d, J = 5.6 Hz, $NHCH_2$), 4.31 (2H, t, J = 5.2 Hz, OCH_2CH_2N), 5.00 (1H, br, NH), 6.74 (2H, s, maleimide). CI-HRMS calcd for $C_{13}H_{19}N_2O_6$ (MH^+): m/z 299.1243. Found: 299.1250. Anal. Calcd for $C_{13}H_{16}N_2O_6$: C, 52.35; H, 6.08; N, 9.39. Found: C, 52.51; H, 6.05; N, 9.33.

Synthesis of 2-Maleimidoethoxy Glycinate (6). Trifluoroacetic acid (5 mL) was added to a solution of compound **5** (0.60 g, 2.0 mmol) in dry chloroform (5 mL) and stirred for 10 min at room temperature. Volatile components were removed *in vacuo* to yield deprotected amine **6** as trifluoroacetic acid salt. The salt was used for subsequent stages of the reaction without further purification.

Synthesis of Maleimidoethyl 3-(Tri-*n*-butylstannyl)-hippurate (7). A mixture of compound **3** (1.02 g, 2.0 mmol), compound **6** (0.40 g, 2.0 mmol as free base), and triethylamine (1.01 g, 10 mmol) in tetrahydrofuran (20 mL) was stirred at reflux for 5 h. After removal of the solvent, the residue was chromatographed on silica gel using chloroform–ether (1:1) as an eluent to produce **7** as a colorless oil (0.60 g, 51%). IR ($CHCl_3$): 2958, 2926, 1745, 1714, 1662, 1513, 1406 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 0.85–1.58 (27H, m, $SnBu_3$), 3.84 (2H, t, J = 5.1 Hz, OCH_2CH_2N), 4.23 (2H, d, J = 5.2 Hz, $NHCH_2$), 4.35 (2H, t, J = 5.1 Hz, OCH_2CH_2N), 6.65 (1H, t, J = 5.2 Hz, NH), 6.70 (2H, s, maleimide), 7.39 (1H, t, J = 7.5 Hz, aroamtic), 7.61 (1H, dt, J = 7.5, 1.5 Hz, aromatic), 7.71 (1H, dt, J = 7.5, 1.5 Hz, aromatic), 7.92 (1H, t, J = 1.5 Hz, aromatic). CI-HRMS calcd for $C_{27}H_{41}N_2O_5Sn$ (MH^+): m/z 593.2037. Found: 593.2043.

Synthesis of Maleimidoethyl 3-Iodohippurate (8). Iodine (0.1 M) in chloroform was added at ambient temperature to a solution of **7** (0.59 g, 1.0 mmol) in chloroform (10 mL) until the solution remained pink. The reaction mixture was washed with 10% aqueous sodium thiosulfate (10 mL) and water (10 mL) and dried over sodium sulfate prior to removal of the solvent *in vacuo*. The oil was chromatographed on silica gel using chloroform–ether (1:1) as an eluent to produce **8** as a pale yellow viscous oil (0.39 g, 91%). IR ($CHCl_3$): 1745, 1714, 1668, 1518, 1433, 1406, 1197 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 3.84 (2H, t, J = 5.1 Hz, OCH_2CH_2N), 4.22 (2H, d, J = 5.2 Hz, $NHCH_2$), 4.35 (2H, t, J = 5.1 Hz, OCH_2CH_2N), 6.67 (1H, t, J = 5.2 Hz, NH), 6.74 (2H, s, maleimide), 7.20 (1H, t, J = 7.9 Hz, aroamtic), 7.79 (1H, dt, J = 7.9, 1.6 Hz, aromatic), 7.86 (1H, dt, J = 7.9, 1.6 Hz, aromatic), 8.17 (1H, t, J = 1.6 Hz, aromatic). HRMS calcd for $C_{15}H_{13}IN_2O_5$ (M^+): m/z 427.9871. Found: 427.9880. Anal. Calcd for $C_{15}H_{13}IN_2O_5$: C, 42.08; H, 3.06; N, 6.54. Found: C, 42.01; H, 3.10; N, 6.33.

Synthesis of *m*-Iodohippuric Acid. This compound was synthesized according to the procedure of Jonson et al.[38] with some modifications. Thionyl chloride (60.0 mL, 823 mmol) was added slowly to *m*-iodobenzoic acid (20.0 g, 80.6 mmol), and the mixture was stirred at 60 °C until the acid was completely dissolved. After cooling to room temperature, the reaction mixture was distilled under reduced pressure to yield *m*-iodobenzoyl chloride (15.7 g, 73%) at 159–160 °C/23 mmHg. The *m*-iodobenzoyl chloride (5.3 g, 20 mmol) was added slowly to a cold solution of glycine (1.5 g, 20.0 mmol) in 3 M NaOH (15 mL) and benzene (15 mL). After stirring for 1 h at room temperature, the reaction mixture was treated with concentrated HCl to produce a white precipitate. Analytically pure *m*-iodohippuric acid was obtained after recrystallization of the precipitate in acetonitrile (2.7 g, 32%). Mp: 155–156 °C (lit.[38] mp 155–156 °C). Anal. Calcd for $C_9H_6NO_3I$: C, 35.43; H, 2.64; N, 4.59; O, 15.73. Found: C, 35.29; H, 2.74; N, 4.63; O, 15.74.

Radioiodination of MIH and Protein Labeling. MIH was radioiodinated in the presence of NCS.[6] MIH was dissolved in methanol containing 1% acetic acid (0.56 mg/mL), and 81.4 μL of this solution was mixed with 22.2 μL of NCS in methanol (0.5 mg/mL) in a sealed vial followed by the addition of Na[^{131}I]I (8.18 μL). The reaction was allowed to proceed at room temperature for 45 min before quenching with aqueous sodium bisulfide (11.1 μL, 0.72 mg/mL). The radiochemical purity of radioiodinated MIH was determined by TLC (Merck Art 5553) developed with a mixture of chloroform–ether (1:1). MeOH was removed by a flow of N_2 prior to subsequent conjugation reactions with the protein.

NGA was reduced with 5 molar excess of DTT to expose one thiol group.[30] Briefly, freshly prepared 375 μL of DTT (0.45 mg/mL) in 0.1 M PB (pH 6.8) containing 0.3 M NaCl was added to 375 μL of NGA (40 mg/mL) in the same buffer. After incubating the mixture for 30 min at room temperature, excess DTT was removed by the Diaflow system (8 MC Model; Amicon Grace, Tokyo, Japan) with 20 volumes of well-degassed 0.1 M PB containing 2 mM EDTA (pH 6.0). The protein concentration was adjusted to 5 mg/mL. Under these conditions, none of the 17 disulfide bonds of HSA was accessible to DTT reduction. A small portion of this mixture was sampled, and the number of thiol groups was estimated with 2,2'-dithiodipyridine.[39] This solution (100 μL) was then added to a reaction vial containing the dried residue of crude radioiodinated MIH prepared as described above. The reaction was allowed to proceed at room temperature for 2 h. The crude radiolabeled NGA was then purified by Sephadex G-25 column chromatography (0.8 × 16 cm) equilibrated and eluted with 0.1 M PB (pH 6.0).

In radiolabeling OST7 with [^{131}I]MIH, OST7 was treated with 2-ME. Briefly, OST7 (10 mg/mL), in well-degassed 0.1 M PBS (pH 7.0) containing 2 mM EDTA, was allowed to react with 1000 molar excess of 2-ME at room temperature for 30 min. Excess 2-ME was then removed in a manner similar to that of DTT removal. The number of thiol groups exposed by this treatment was determined with 2,2'-dithiodipyridine.[39] This solution (5 mg/mL, 250 μL) was added to the reaction vial containing crude [^{131}I]MIH. After agitating the reaction mixture gently for 2 h at room temperature, 27 μL of iodoacetamide (10 mg/mL) in 0.1 M PB (pH 6.0) was added. The reaction mixture was incubated for an additional 30 min to alkylate the nonreacted thiol groups.[19] The radiolabeled protein was subsequetly purified by Sephadex G-25 column

chromatography (0.8 × 16 cm) equilibrated and eluted with 0.1 M PB (pH 6.0). Radiochemical purities of [^{131}I]MIH-labeled NGA and OST7 were determined by cellulose acetate electrophoresis run at an electrostatic field of 0.8 mA/cm for 45 min in a veronal buffer (I = 0.05, pH 8.6).

Radioiodination of ATE and Protein Labeling. ATE was radioiodinated according to the procedure as described above except that 0.45 mg/mL ATE in 1% AcOH/MeOH was used in place of MIH. The radiochemical purity of [^{131}I]ATE was determined by TLC developed with a mixture of 30% ethyl acetate in hexane.[5] After evaporating the MeOH, the residue was directly used for subsequent conjugation with either NGA or OST7.

Radioiodination of NGA or OST7 with [^{131}I]ATE was performed according to the procedure of Zalutsky et al.[5] with slight modifications: 200 or 540 μL or NGA or OST7 (5 mg/mL) in 0.2 M borate buffer (pH 8.5) was added to a dried residue of crude [^{131}I]ATE prepared as described above. After gentle incubation at room temperature for 1 h, crude radioiodinated proteins were purified by Sephadex G-25 column chromatography (0.8 × 16 cm) equilibrated and eluted with 0.1 M PB (pH 7.4). Radiochemical purities of [^{131}I]ATE-NGA and OST7 were determined by cellulose acetate electrophoresis under the conditions described above.

Preparation of [^{131}I]NGA. Direct radioiodination of NGA was performed by the chloramine T method.[21] To 500 μL of NGA (4 mg/mL in 0.1 M PB, pH 7.4) was added 3 μL of Na[^{131}I]I diluted with the same buffer. Chloramine T (1 mg/mL, 10 μL), freshly prepared in the same buffer, was then added. After incubation of the mixture at room temperature for 10 min, the reaction was terminated by adding 6 μL of aqueous sodium bisulfite (0.72 mg/mL). In a manner similar to other radioiodinated proteins, [^{131}I]NGA was purified. The radiochemical purity of this protein was determined by cellulose acetate electrophoresis as described above.

Mice Biodistribution of Radioiodinated NGAs. Each radioiodinated NGA was diluted with 0.1 M PBS (pH 6.0) to adjust the protein concentration to 90 μg/mL. Biodistribution studies were conducted by injecting the respective radioiodinated NGA iv in 6-week-old ddY mice.[40] Groups of five mice each were administered with 9 μg of the respective protein prior to sacrificing the animals at 5 min and 0.5, 1, 3, 6, and 24 h postinjection by decapitation. Tissues of interest were removed and weighted, and the radioactivity was determined with a well counter (ARC 2000; Aloka, Tokyo, Japan).

At 6 h postinjection of [^{131}I]MIH-NGA, urine samples were collected and analyzed by TLC developed with a mixture of chloroform–methanol–water (15:8:1). Under these conditions, free iodine, m-iodohippuric acid, and m-iodobenzoic acid exhibited R_f values of 0.1, 0.25, and 0.4, respectively. The urine samples were further analyzed by RP-HPLC after filtration of the samples through a 10-kDa cutoff ultrafiltration membrane (Myrex; Millipore Ltd., Tokyo, Japan). In the RP-HPLC analyses, free iodine, m-iodohippuric acid, and m-iodobenzoic acid were eluted at retention times of 2.9, 9.4, and 30.8 min, respectively.

To estimate the amount of radioactivity excreted by hepatobiliary excretion following iv injection of the three [^{131}I]NGAs, the common bile duct of male Wistar rats (250–300 g) was cannulated. Each ^{131}I-labeled NGA (50 μg) was administered through the femoral vein. Bile samples were collected every 10 min for 130 min. The radioactivity excreted in bile after [^{131}I]MIH-NGA injection was analyzed by TLC developed with a mixture of chloroform–methanol–water (15:8:1) and RP-HPLC.

Plasma Stability of [^{131}I]MIH-OST7. [^{131}I]MIH-OST7 was diluted to 0.5 mg/mL with 0.1 M PBS (pH 6.0), and 20 μL of this solution was added to a mixture of 115 μL of freshly prepared murine plasma and 115 μL of 20 mM PBS (pH 7.4). After incubating for 1, 6, and 24 h at 37 °C, samples were taken from the reaction mixture. The percentage of radioactivity bound to antibody was determined by TLC developed with a mixture of chloroform–methanol–water (15:8:1) and paper chromatography (No. 50; Advantec Toyo, Tokyo, Japan) developed with a mixture of water and methanol (20:80). Radiolabeled proteins, free iodine, and m-iodohippuric acid exhibited respective R_f values of 0–0.05, 0.6–0.65, and 0.9–0.95 in this paper chromatography system. Percentage of the radioactivity released from [^{131}I]ATE-OST7 was also determined under similar conditions.

Mice Biodistribution of Radioiodinated OST7s. The radiolabeled OST7 was diluted to 200 μg/mL with 0.1 M PBS (pH 6.0). Biodistribution studies of radioactivity after [^{131}I]-MIH-OST7 and [^{131}I]ATE-OST7 injections in 6-week-old male ddY mice were monitored at 1, 3, 6, 24, 48, and 72 h postinjection. Groups of five mice, each receiving 20 μg of the respective radioiodinated OST7, were used for the experiments. Organs of interest were then removed and weighted, and the radioactivity was determined.

Statistical Analysis. Data were expressed as the mean ± standard deviation when appropriate. Each result was statistically analyzed using unpaired t-test.

Acknowledgment. The authors are grateful to Daiichi Radioisotope Labs (Tokyo, Japan) for their kind gift of Na[^{131}I]I.

References

(1) Yokota, T.; Milenic, D. E.; Whitlow, M.; Schlom, J. Rapid Tumor Penetration of a Single-Chain Fv and Comparison with Other Immunoglobulin Forms. *Cancer Res.* **1992**, *52*, 4302–3408.
(2) Khaw, B. A.; Kilbanov, A.; O'Donnell, S. M.; Saito, T.; Nossiff, N.; Slinkin, M. A.; Newell, J. B.; Strauss, H. W.; Torchilin, V. P. Gamma Imaging with Negatively Charge-Modified Monoclonal Antibody: Modification with Synthetic Polymers. *J. Nucl. Med.* **1991**, *32*, 1742–1751.
(3) Slinkin, M. A.; Curtet, C.; Faivrechauvet, A.; Saimaurel, C.; Gestin, J. F.; Torchilin, V. P.; Chatal, J. F. Biodistribution of Anti-CEA F(ab')$_2$ Fragments Conjugated with Chelating Polymers – Influence of Conjugate Electron Charge on Tumor Uptake and Blood Clearance. *Nucl. Med. Biol.* **1993**, *20*, 443–452.
(4) Wang, T. S. T.; Fawwaz, R. Z.; Anderson, P. O. Reduced Hepatic Accumulation of Radiolabeled Monoclonal Antibodies with Indium-111-Thioether-Poly-L-Lysine-DTPA-Monoclonal Antibody TP41.2 F(ab')$_2$. *J. Nucl. Med.* **1992**, *33*, 570–574.
(5) Zalutsky, M. R.; Narula, A. S. A method for the Radiohalogenation of Proteins Resulting in Decreased Thyroid Uptake of Radioiodine. *Appl. Radiat. Isot.* **1987**, *38*, 1051–1055.
(6) Wilbur, D. S.; Hadley, S. W.; Hylarides, M. D.; Abrams, P. G.; Morgan, A. C.; Reno, J. M.; Fritzberg, A. R. Development of a Stable Radioiodinating Reagent to Label Monoclonal Antibodies for Radiotherapy of Cancer. *J. Nucl. Med.* **1989**, *30*, 216–226.
(7) Kurth, M.; Pelegrin, A.; Rose, K.; Offord, R. E.; Pochon, S.; Mach, J.-P.; Buchegger, F. Site-Specific Conjugation of a Radioiodinated Phenethylamine Derivative to a Monoclonal Antibody Resulted in Increased Radioactivity Localization in Tumor. *J. Med. Chem.* **1993**, *36*, 1255–1261.
(8) Craig, A. S.; Helps, I. M.; Jankowski, K. J.; Parker, D.; Beeley, N. R. A.; Boyce, B. A.; Eaton, M. A. W.; Millican, A. T.; Millar, K.; Phipps, A.; Rhind, S. K.; Harrison, A.; Walker, C. Towards Tumour Imaging with Indium-111 Labelled Macrocycle-Antibody Conjugates. *J. Chem. Soc., Chem. Commun.* **1989**, 794–796.
(9) Blend, M. J.; Greager, J. A.; Atcher, R. W.; Brown, J. M.; Brechbiel, M. W.; Gansow, O. A.; Gupta, T. K. D. Improved Sarcoma Imaging and Reduced Hepatic Activity with Indium-111-SCN-Bz-DTPA Linked to MoAb 19–24. *J. Nucl. Med.* **1988**, *29*, 1810–1816.
(10) Arano, Y.; Mukai, T.; Uezono, T.; Wakisaka, K.; Motonari, H.; Akizawa, H.; Taoka, Y.; Yokoyama, A. A. Biological Method to Evaluate Chelating Agents to Label Antibodies with Metallic Radionuclides. *J. Nucl. Med.* **1994**, *35*, 890–898.
(11) Deshpande, S. V.; Subramanian, R.; McCall, M. J.; DeNardo, S. J.; DeNardo, G. L.; Meares, C. F. Metabolism of Indium Chelates Attached to Monoclonal Antibody: Minimal Transchelation of Indium from Benzyl-EDTA chelate In Vivo. *J. Nucl. Med.* **1990**, *31*, 218–224.
(12) Duncan, J. R.; Welch, M. J. Intracellular Metabolism of Indium-111-DTPA-Labeled Receptor Targeted Proteins. *J. Nucl. Med.* **1993**, *34*, 1728–1738.
(13) Himmelsbach, M.; Wahl, R. L. Studies on the Metabolic Fate of ^{111}In-labeled Antibodies. *Nucl. Med. Biol.* **1989**, *16*, 839–845.
(14) Motta-Hennessy, C.; Sharkey, R. M.; Goldenberg, D. M. Metabolism of Indium-111-Labeled Murine Monoclonal Antibody in Tumor and Normal Tissue ofthe Athymic Mouse. *J. Nucl. Med.* **1990**, *31*, 1510–1519.
(15) Paik, C. H.; Sood, V. K.; Le, N.; Cioloca, L.; Carrasquillo, J. A.; Reynolds, J. C.; Neumann, R. D.; Reba, R. C. Radiolabeled Products in Rat Liver and Serum After Administration of Antibody-Amide-DTPA-Indium-111. *Nucl. Med. Biol.* **1992**, *19*, 517–522.

(16) Sands, H.; Jones, P. J. Methods for the Study of the Metabolism of Radiolabeled monoclonal Antibodies by Liver and Tumor. *J. Nucl. Med.* **1987**, *28*, 390–398.
(17) Arano, Y.; Matsushima, H.; Tagawa, M.; Koizumi, M.; Endo, K.; Konishi, J.; Yokoyama, A. A Novel Bifunctional Metabolizable Linker for the Conjugation of Antibodies with Radionuclides. *Bioconjugate Chem.* **1991**, *2*, 71–76.
(18) Arano, Y.; Matsushima, H.; Tagawa, M.; Inoue, T.; Koizumi, M.; Hosono, M.; Sakahara, H.; Endo, K.; Konishi, J.; Yokoyama, A. A New Design of Radioimmunoconjugate Releasing Hippurate-Like Radiometal Chelate for Tumor-Selective Radioactivity Delivery. *Nucl. Med. Biol.* **1994**, *21*, 63–69.
(19) Arano, Y.; Inoue, T.; Mukai, T.; Wakisaka, K.; Sakahara, H.; Konishi, J.; Yokoyama, A. Discriminated Release of a Hippurate-like Radiometal Chelate in Non-Target for Target Selective Radioactivity Localization Using pH-dependent Dissociation of Reduced Antibody. *J. Nucl. Med.* **1994**, *35*, 326–333.
(20) Garg, P. K.; Slade, S. K.; Harrison, C. L.; Zalutsky, M. R. Labeling Proteins Using Aryl Iodide Acylation Agents: Influence of *Meta* vs *Para* Substituition on *In Vivo* stability. *Nucl. Med. Biol.* **1989**, *16*, 669–673.
(21) Wilbur, D. S.; Hadley, S. W.; Grant, L. M.; Hylarides, M. D. Radioiodinated Iodobenzoyl Conjugates of a Monoclonal Antibody Fab Fragment. In Vivo Comparisons with Chloramine-T-Labeled Fab. *Bioconjugate Chem.* **1991**, *2*, 111–116.
(22) Goldstein, A.; Arnow, L.; Kalman, S. M. Chemical pathways of drug metabolism. *Principles of Drug Action*; John Wiley & Sons: New York, 1968; pp 242–267.
(23) Heymann, E. Hydrolysis of carboxylic ester and amides. *Metabolic Basis of Detoxification: Metabolism of Functional Groups*; Academic Press: New York, 1982; pp 229–241.
(24) Santoro, L.; Rebout, A.; Journet, A. M.; Colomb, M. G. Major Involvement of Cathepsin B in the Intracellular Proteolic Processing of Endogenous IgGs in U937 Cells. *Mol. Immunol.* **1993**, *30*, 1033–1039.
(25) Boyle, C. C.; Paine, A. J.; Mather, S. J. The Mechanism of Hepatic uptake of a Radiolabeled Monoclonal Antibody. *Int. J. Cancer* **1992**, *50*, 912–917.
(26) Ashwell, G.; Harford, J. Carbohydrate-Specific Receptors of the Liver. *Annu. Rev. Biochem.* **1982**, *51*, 531–554.
(27) Hylarides, M. D.; Wilbur, D. S.; Reed, M. W.; Hadley, S. W.; Schroeder, J. R.; Grant, L. M. Preparation and in Vivo Evaluation of an N-(p-[^{125}I]Iodophenethyl)maleimide-Antibody Conjugate. *Bioconjugate Chem.* **1991**, *2*, 435–440.
(28) Khawli, L. A.; Kassis, A. I. Synthesis of ^{125}I Labeled N-Succinimidyl p-Iodobenzoate for Use in Radiolabeling Antibodies. *Nucl. Med. Biol.* **1989**, *16*, 727–733.
(29) Essig, A.; Taggart, J. Y. Competitive inhibition of renal transport of p-aminohippurate by other monosubstituted hippurates. *Am. J. Physiol.* **1960**, *199*, 509–512.
(30) Sogami, M.; Nagoka, S.; Era, S.; Honda, M.; Noguchi, K. Resolution of human mercapt- and nonmercaptalbumin by high-performance liquid chromatography. *Int. J. Pept. Protein Res.* **1984**, *24*, 96–103.
(31) LaBadie, J. H.; Chapman, K. P.; Aronson, N. N., Jr. Glycoprotein Catabolism in Rat Liver. Lysosomal Digestion of Iodinated Asialo-fetuin. *Biochem. J.* **1975**, *152*, 271–279.
(32) Garg, P. K.; Garg, S.; Zhao, X. G.; Welsh, P. C.; Zalutsky, M. R. Catabolism of Label from MEL-14 F(ab')$_2$ Fragment Radiohalogenated Using N-Succinimidyl 3-Halobenzoates. *J. Labelled Compd. Radiopharm.* **1994**, *35*, 310–312.
(33) Axworthy, D. B.; Wilbur, D. S.; Schroff, R. W.; Hadley, S. W.; Hylarides, M. D.; Wahl, R. L.; Hanelin, L.; Fritzberg, A. R. Identification of the Major Urinary Metabolites of *para*-Iodobenzoate Conjugates of an Antibody Fragment in Humans. *J. Nucl. Med.* **1988**, *29*, 1325, Abstract.
(34) Lee, Y. C.; Stowell, C. P.; Krantz, M. J. 2-Imino-2-methoxyethyl 1-Thioglycosides: New Reagents for Attaching Sugars to Proteins. *Biochemistry* **1976**, *15*, 3956–3963.
(35) Stowell, C. P.; Lee, Y. C. Preparation of Some New Neoglycoproteins by Amidination of Bovine Serum Albumin Using 2-Imino-2-methoxyethyl 1-Thioglycosides. *Biochemistry* **1980**, *19*, 4899–4904.
(36) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **28**, 350–356, 1956.
(37) Hosoi, S.; Nakamura, T.; Higashi, S.; Yamamuro, T.; Toyama, S.; Shinimiya, K.; Mikawa, H. Detection of human osteosarcoma-associated antigen(s) by monoclonal antibodies. *Cancer Res.* **1982**, *42*, 654–659.
(38) Johnson, T. B.; Meade, H. A. Ortho-, Meta-, and Paraiodohippuric Acids. *Am. Chem. J.* **1906**, *36*, 294–301.
(39) Grassetti, D. R.; Murray, J. F. M., Jr. Determination of Sulfhydryl Groups with 2,2'- or 4,4'-Dithiodipyridine. *Arch. Biochem. Biophys.* **1967**, *119*, 41–49.
(40) Imai, S.; Morimoto, J.; Tsubura, T.; Esaki, K.; Michalides, R.; Holmes, R. S.; Deimling, O.; Hilgers, J. Genetic marker patterns and endogenous mammary tumor virus genes in inbred mouse strains in Japan. *Exp. Anim.* **1986**, *35*, 263–273.