RADIOLABELED BIOTIN AMIDES FROM TRIAZENYL PRECURSORS: SYNTHESIS, BINDING, AND *IN-VIVO* PROPERTIES

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SUMMARY

The synthesis of *N*-(4-[$^{127/125/123}$I]iodobenzyl)biotin amides **4a - 4c** performed by the direct decomposition of *N*-[4-(3′,3′-dimethyltriazenyl)benzyl]biotin amide with sodium iodide in the presence of CF_3COOH is described. Iodinated in this way biotin formed a stable complex with avidin (K_d = 2.84 ± 0.45 x 10^{-15} M, n = 3.9 ± 0.6) which dissociated in the presence of excess native biotin with a rate constant of 0.034 ± 0.006 hr^{-1}. Blood clearance studies and the lack of thyroid uptake indicated that this compound was not deiodinated *in vivo* and behaved in circulation much like native biotin. This aryltriazene precursor method is suitable for labeling with short-lived radiohalides. It can be used to produce no-carrier-added derivatives of biotin for use in biologic studies and assays involving avidin or streptavidin.

Key Words: $^{123/125}$I-radioiodination; Triazene; Biotin; Iodobenzylbiotin Amides; Biodistribution

INTRODUCTION

The avidin-biotin complex (1) continues to serve as a versatile tool in the analysis of numerous biochemical interactions (2-5). Its strong, noncovalent binding has been recently used to specifically target biotin to avidin-antibody conjugates already localized on tumors (6-9). To fully explore the radioimmunotherapeutic and diagnostic potentials of this promising mode of site-selective delivery of radionuclides,

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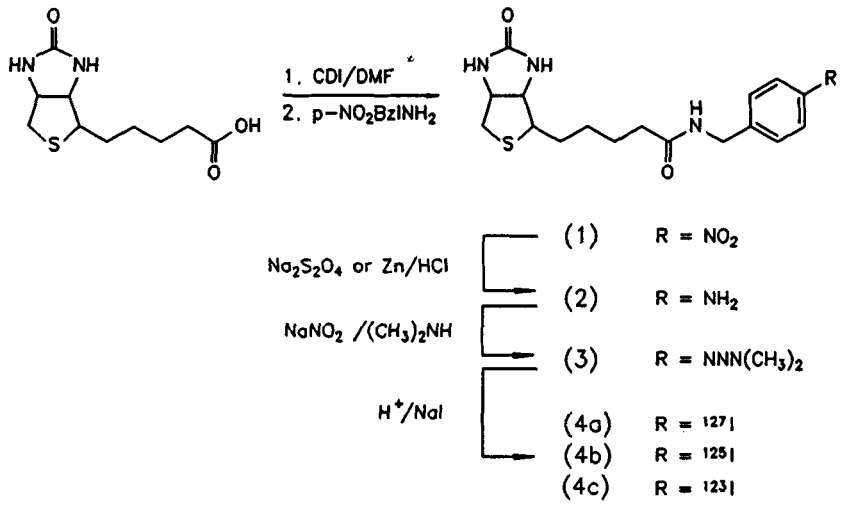
it is imperative to have a radiolabeled biotin with the highest possible specific activity. In addition, the biotin moiety must be biochemically intact. The synthesis of such a biotin carrier labeled with radioiodide is difficult because of the sensitivity of this molecule to oxidation (10,11) and because its preparation must be sufficiently fast to accommodate short-lived radiohalides.

We describe herein the synthesis of *N*-(4-[$^{127/125/123}$I]iodobenzyl)biotin amides **4a** - **4c** via the triazene of *N*-(4-aminobenzyl)biotin amide **2** by a method that fulfills these requirements. This approach involves trapping a diazonium ion with a secondary amine (12) followed by the decomposition of the thus formed triazene **3** to the corresponding radioiodinated biotin amide. Once prepared, compound **3** can be stored, and the final iodination step can be performed when required. Binding properties of the radiolabeled amide of biotin to avidin were studied under equilibrium dialysis conditions. The dissociation constant of this complex with avidin was also measured to determine the potential of **4b** to compete for binding sites on avidin *in vivo* where physiologic levels of biotin reach 3-6 ng/ml (13). Pharmacokinetic studies were conducted to determine the *in-vivo* behavior of this compound.

CHEMISTRY

Scheme 1 shows the structures of the compounds in the synthetic route from biotin to the *N*-(4-[$^{127/125/123}$I]iodobenzyl)biotin amides **4a** - **4c**. The synthesis involved the initial activation of biotin with *N,N*-carbonyldiimidazole (CDI) to generate biotinylimidazolide (14) followed by a facile acylation of *p*-nitrobenzylamine to produce *N*-(4-nitrobenzyl)biotin amide **1** in a high yield (92%). This procedure gave a product of superior purity compared with that obtained by the direct coupling in the presence of dicyclohexylcarbodiimide (DCC) (15) or by the biotinyl chloride method (16). The material collected from the synthesis with DCC contained dicyclohexylurea, was difficult to separate, and the overall yield was low. The CDI coupling eliminated this

Scheme 1



problem since the only byproducts from the reaction were CO_2 and imidazole, both of which are easily removed. *p*-Nitrobenzylamide 1 was reduced with either 85% sodium hydrosulfite in aqueous ethanol or with zinc metal in dilute hydrochloric acid. Both reactions gave comparable yields (80%) of the amine 2. The first method may be more suitable in the reduction of nitro amides when these are substituted with groups sensitive to acid. The synthesis of the triazene of biotin benzylamide 3 involved the initial diazotization of the amine 2 with sodium nitrite followed by the treatment of the diazonium salt suspension with a large excess of dimethylamine at 0°C. The product was stable and could be purified by recrystallization or chromatography before iodination. A sample of 3 stored for three months at 4°C in a desiccator with the exclusion of light did not degrade. The use of dimethylamide had the advantage of a volatile dimethylamine released in the final iodination step. Therefore dimethyltriazenylamide 3 was used throughout this study. However the corresponding piperidino- and pyrrolidinotriazenyl derivatives of biotin 4 that were initially also synthesized gave even higher yields (85 and 87%, respectively, after purification).

The choice of solvent for the decomposition of triazene was very important. Guided by studies reported earlier on an aromatic fluorination via the aryltriazene decomposition (17), we examined the effect of the solvent polarity and acidity on the course of the triazene 3 iodination. The reaction in chloroform gave only traces of the iodination product (less than 2%) apparently due to the solvent-promoted fast homolytic degradation of the triazene. The use of tetrahydrofuran led to a complex product mixture which contained, in addition to the desired iodoamide 4a, many other species whose chromatographic separation was extremely difficult. Acidic solvents, such as glacial acetic acid, and to some extent acetonitrile, caused a notable decomposition of triazene before sodium iodide was added. When the reaction was conducted in acetonitrile at 0°C, the spontaneous decomposition of the triazene substrate was very slow and these conditions gave the best yields. The starting material was easily soluble in this solvent, and this allowed for a small total volume during radiolabeling, decreasing dilution effects on the efficiency of labeling.

The other major factor in the successful synthesis of [^{125}I/^{123}I]iodoanalogues 4b and 4c was the maintenance of anhydrous conditions during the process. When the reaction was carried out under aqueous conditions, the radioiodination yields were below 12%. Drying the commercial aqueous Na^{125}I/^{123}I, as described in the Experimental section, and the use of a molar excess of triazene optimized conditions for maximum radioiodine incorporation, increasing radiochemical yields to over 30%. The alternative method for obtaining dry Na^{125}I/^{123}I reported by Ku and Barrio (18) was also adequate, but extraction of the commercial aqueous iodide with 2-butanone and drying of the organic phase with $CaSO_4$ led to much greater losses of the radioactivity (up to 40%). It was especially evident when small volumes (less then 30 μl) of aqueous sodium iodide were used requiring the dilution of the aqueous layer to prevent formation of emulsions.

The separations of reaction products by flash column chromatography on silica gel and HPLC C_{18} columns were straightforward since the difference between

retention times of radiolabeled biotins and the unreacted triazene was more than 10 min. The unreacted iodide was retained on the top of a silica gel column and the iodine formed was eluted near the solvent front.

BINDING AND BIODISTRIBUTION STUDIES

Three methods for determining binding parameters of radiolabeled biotin **4b** were tested: the ultrafiltration procedure (19), the solid-phase assay in avidin-coated multiwell plastic plates, and the equilibrium dialysis method. The latter gave the most reproducible results. The measured K_d value was 2.84 ± 0.45 x 10^{-15} M and the measured number of binding sites was close to the expected value of 4 (n = 3.9 ± 0.6) although always with about 15% error. The binding of **4b** to dialysis bags was negligible (less than 0.01%) as determined from the total counts associated with bags and confirmed by the measured recovery of the total radioactivity in each experiment.

Dissociation of **4b** from its complex with avidin was measured under conditions similar to those used in equilibrium dialysis. A solution of **4b** (4.5 x 10^{-6} μM) was incubated with excess avidin and then exposed to 12 μM native biotin. The course of the reaction was assessed by measuring concentrations of radiolabeled biotin inside and outside of the dialysis bag. The kinetics of this process followed a typical pseudo-first-order dissociation curve. The data extrapolated to the initial value of 100% revealing that there was no anomalous dissociation as reported by others for some 1:1 biotin-avidin complexes (20). The rate of dissociation as measured in our studies was 0.034 hr^{-1}, about 3.5 times faster than the rate reported for biotinamidoethyl-3-(3-[^{125}I]iodo-4-hydroxyphenyl)propionamide at 20°C (20). Our studies were conducted at 37°C to approximate physiologic conditions.

Biodistribution studies were done following iv injection of either **4b** or **4b**-avidin. Clearance rates are summarized in Table I. There was no significant difference in the

in-vivo behavior of free 4b and 4b complexed with avidin. Elimination of radioactivity from blood followed what appeared to be triphasic first-order kinetics (Figure 1). The initial, fast stage was over after about 9 min and only during this time was there a measurable difference in concentrations of free biotin and its complex; for example, at 3 min % injected dose per gram blood (% ID/g) for 4b was 8.6 and for its complex, 21. For thyroid the fast period was not evaluated, but %

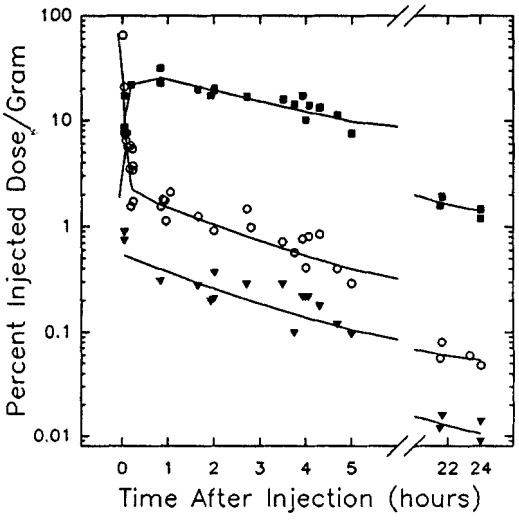


Figure 1. Clearance of ^{125}I from liver (■), blood (○) and thyroid (▾) in mice injected with 4b or 4b-avidin. Data for both agents are shown on same plot. For clarity, the same symbols were used for 4b and 4b-avidin in each curve.

Table I. Blood and Tissue Clearance Rates for 4b and Its Complex with Avidin[a]

Tissue	$k_o \pm \sigma_{ko}$ [min^{-1}]	$k_1 \pm \sigma_{k1}$ [min^{-1}]	$k_2 \pm \sigma_{k2}$ [min^{-1}]
blood[b]	$1.53 \pm 0.14 \times 10^{-1}$	$2.42 \pm 0.41 \times 10^{-3}$	$7.91 \pm 0.36 \times 10^{-4}$
thyroid		$6.44 \pm 1.03 \times 10^{-3}$	$2.02 \pm 0.12 \times 10^{-3}$
kidney		$6.42 \pm 0.56 \times 10^{-3}$	$1.41 \pm 0.11 \times 10^{-3}$
liver[c]		$4.28 \pm 0.50 \times 10^{-3}$	$1.47 \pm 0.33 \times 10^{-3}$
liver[d]			$2.06 \pm 0.12 \times 10^{-3}$

[a]There is no statistically significant difference between clearance rates of 4b and 4b-avidin. Data for both agents have been analyzed together in each tissue with the exception of the first phase in blood. The fast, first stage of blood clearance (k_0) for 4b does not appear to have a corresponding step for 4b-avidin.
[b] n = 5 at each time point in blood analysis; n = 2 in other tissues.
[c]biphasic analysis.
[d]monophasic analysis.

ID/g at 3 min was 7.72 and 7.34 for free biotin and the complex, respectively. The elimination of radioactivity through the kidneys paralleled blood clearance curves. The absorptive phase in liver lasted about 30 min reaching an uptake of over 32% ID/g. Once the distribution equilibrium was attained, the elimination of radioactivity followed first-order kinetics. However it is not clear whether it proceeded in a mono- or biphasic fashion. Analysis of the data in either mode revealed no statistically significant differences.

DISCUSSION

Several radioiodinated derivatives of biotin have been reported (20-24). The preparation of these derivatives always involves the initial radiolabeling of a small molecule followed by its conjugation to biotin. The resulting product is frequently a mixture containing monoiodo-, diiodo- and unlabeled precursors (22) which after coupling to biotin gives products of low specific activity with structures not strictly defined. Since the binding characteristics of biotin to avidin, particularly the dissociation kinetics, have been reported to vary depending on the number of iodine atoms attached to the prosthetic group (20,21), one needs to be careful in the interpretation of the biologic and assay data. The method reported here provides rapid access to high specific activity (no-carrier-added) radioiodobiotins. The synthesis involves the use of a triazenyl derivative of biotin which undergoes nucleophilic iodine substitution when decomposed by acids in the presence of the iodide ion. The use of *N*-triazenylarylbiotin amides gives considerable flexibility to the choice of molecules that can be coupled with biotin and then, using the same chemistry, converted into labeled derivatives. The literature reports that radiofluorination (17) as well as radioiodination (18,25,26) can be accomplished via the triazene method. Although the reported yields (20-50%) are modest in most cases, short reaction times and relatively fast purification methods compensate for these losses of radioactivity,

particularly when radioisotopes with short half-lives are used. In the biotin system, the fact that the substitution is nucleophilic rather than electrophilic has a distinct significance. The sulfur atom on the biotin ring is sensitive to a variety of oxidants (11). Typical electrophilic radioiodinations require strong oxidants such as Chloramine-T, *N*-chlorosuccinimide, or peracetic acid; moreover, the iodinating species thus produced also have oxidative properties. In fact, it is our experience that all of these reagents (including ICl) cause substantial oxidation of biotin to its sulfoxides and sulfones, and the recovery of radioiodinated biotin is less than 10% (Baranowska-Kortylewicz *et al.*, unpublished results).

There are several other advantages to the triazene method. It has been noted previously that since the iodination is in the final stage of the synthetic route, the use of short-lived isotopes is facilitated. The products are always no-carrier-added, single species. The method allows for the preparation of a variety of modified biotin amides. The interactions of biotin amide 4b with avidin confirm the preservation of the binding properties of the biotin ring during our synthesis. The dissociation constant is only about 10 times lower than that of native biotin. The dissociation of 4b from its complex with avidin in the presence of biotin at concentrations about 10,000-fold higher than physiologic levels is slow. Approximately 2% of 4b per hour is lost from the complex at 37°C giving further indication that the biotin molecule is intact. Similarly strong interactions should be observed *in vivo* with avidin-antibody conjugates localized on tumor cell surfaces. The lack of thyroid uptake shows that the radiolabeled compound 4b is quite stable and does not deiodinate. The clearance of radioactivity from this organ is parallel to the blood clearance and one day after injection falls below detectable levels.

The considerable specific accumulation of 4b in the liver is reminiscent of the behavior of native biotin (28). Similar observations were reported with ^{14}C- or ^{3}H-labeled biotin in chicks and rats (29,30). Approximately 16% of the injected biotin-$^{14}COOH$ was incorporated in the liver after 4 hours (28). This is not surprising in view

of the fact that hepatocytes appear to have an active transport system for biotin (31,32). However it was expected that since cells *in vitro* internalize ^{3}H-biotin-avidin by absorptive pinocytosis at a much faster rate than free biotin (33) (approximately 7-fold greater), there would be a detectable difference in the radioactive content of the liver in animals treated with **4b**-avidin and **4b** alone. Unexpectedly, the **4b**-avidin complex behaved remarkably like free biotin amide (**4b**). The replacement of radiotracer with physiologic biotin is unlikely when *in-vitro* dissociation kinetics are considered (see Results section). The transport of biotin into mammalian cells has been reported to be controlled by two processes: an active transport mediated by specialized protein carrier at concentrations between 5 nM and 50 μM, and a second nonsaturable component, linear above 75 μM, driven by diffusion (33,34). Since the concentrations of no-carrier-added preparations in mice at the time of injection of 5 μCi are 1 to 2 nM, and thus within the range of the active transport process, it seems justified to assume that the rate of uptake of **4b**-avidin and **4b** at nM concentrations via carrier-mediated processes is similar.

The triazene method for the radiolabeling of biotin reported here produces a high specific activity product with unimpaired biologic properties. The synthetic scheme is adaptable to labeling with fluorine, bromine and astatine.

EXPERIMENTAL

Sodium [^{125}I]iodide (specific activity 2200 Ci/mmol) in 10^{-3} to 10^{-5} M NaOH solution was purchased from DuPont (Billerica, MA) and no-carrier-added sodium [^{123}I]iodide was supplied by Atomic Energy Canada Limited (Vancouver, Canada). All chemicals were reagent grade. High performance liquid chromatography (HPLC) was done using reverse phase C_{18} μ-Bondapack (Waters, Milford, MA) and Maxsil 5 silica gel, 250 x 4.6 mm, analytical columns, or Maxsil 5, 250 x 10 mm, a normal phase

semipreparative column (Phenomenex, Rancho Palos Verdes, CA). The radioactive compounds were detected with a NaI(Tl) 3-in crystal well detector. The radioactivity of collected fractions and mixtures was estimated in an ion chamber dose calibrator (Nuclear Associates) and precisely measured in a Packard Autogamma 500 counter. Thin layer chromatography (TLC) was done on silica gel plates (Merck silica gel 60, F-254) which were also used for autoradiography on Kodak XTL-2 film. Melting points (mp) were determined on a hot stage and are uncorrected. Elemental analyses were performed by Galbraith Laboratories (Knoxville, TN). The identity of the radiolabeled compounds was verified by comparing their retention times (R_t) on HPLC and retention fronts (R_f) on TLC with the [^{127}I]iodine-substituted analogues. Radiochemical purities were higher than 99%.

***N*-(4-Nitrobenzyl)biotin Amide (1).** D(+)-Biotin (1 g, 4.10 mmol) was dissolved in 20 ml of anhydrous *N,N*-dimethylformamide (DMF) under nitrogen at 80°C, and *N,N'*-carbonyldiimidazole (0.67 g, 4.13 mmol) was added. When evolution of CO_2 ceased, the mixture was cooled and stirring was continued for 2 hr at 25°C. During this time the intermediate came out of the solution as a flocculent white precipitate. An excess of p-nitrobenzylamine, prepared from its hydrochloride (1.5 g, 7.95 mmol), was added and the reaction continued at 4°C overnight. The crude product obtained after the evaporation of DMF was washed with 50 ml of CH_2Cl_2 and purified on a silica gel column (CH_2Cl_2/CH_3OH, 20:3, v/v) to give 1.42 g (92%) of 1. Recrystallization from methanol produced white needles with mp 240-241°C. HPLC analysis gave a single peak with R_t = 16 min on a silica gel column with eluant A EtOAc, eluant B CH_3OH; flow rate 1 ml min^{-1}; initial composition 7% B with a 1% min^{-1} linear gradient of B. ^{1}HNMR (DMSO-d_6) δ 0.96-1.30 (6H, m, C*H*$_2$C*H*$_2$C*H*$_2$), 1.86 (2H, m, C*H*$_2$CO), 2.43 (2H, m, C*H*$_2$S), 3.12 (1H, d/d, C*H*S), 3.52 (2H, d, PhC*H*$_2$), 4.15 (2H, m, 2 x N*H*), 7.12 (2H, d, Ph), 7.70 (2H, d, Ph), 8.15 (1H, t, PhCH$_2$N*H*). Anal. Calcd. for $C_{17}H_{20}N_4SO_4$: %C 53.97; %H 5.82; %N 14.82. Found: %C 54.07; %H 5.96; %N 14.93.

***N*-(4-Aminobenzyl)biotin Amide (2).**

a. Reduction of 1 with Sodium Hydrosulfite. To a stirred solution of 1 (1 g, 2.6 mmol) in 120 ml of hot (60°C) ethanol, sodium hydrosulfite (85%) (2 g, 9.8 mmol) dissolved in 8 ml of water was added. The mixture was refluxed for 1 hr, filtered hot, and evaporated down to about 50 ml. Water (100 ml) was added and the pH adjusted to 8.5 with concentrated ammonia. The collected precipitate was applied to a silica gel column which was eluted with $CHCl_3/CH_3OH$ (20:3, v/v) giving 0.76 g (81%) of 2 as a yellow solid.

b. Reduction of 1 with Zinc Metal. Compound 1 (1 g) was suspended in 60 ml of 0.2 N HCl, and 6 g of zinc metal was added. After 2 hr the mixture was filtered and the clear solution neutralized with 6 N ammonia to pH 8. The precipitate was collected following purification on a silica gel column as described above and gave 0.72 g (78%) of 2 as a light yellow solid. Crystallization from EtOAc with CH_3OH produced white crystals with mp 210-213°C. HPLC analysis of the purified material on a silica gel column gave a single peak with R_t = 22 min; eluant A EtOAc, eluant B CH_3OH; flow rate 1 ml min^{-1}; initial composition 7% B with a 1% min^{-1} linear gradient of B. ^{1}HNMR (DMSO-d_6) δ 1.10 - 1.85 (6H, m, $CH_2CH_2CH_2$), 2.00 - 2.20 (2H, m, CH_2CO), 2.30 (2H, m, CH_2S), 3.16 (1H, m, CHS), 4.10 (2H, m, $PhCH_2$), 4.20 - 4.50 (2H, m, 2 x NH), 6.20 (2H, m, $PhNH_2$), 6.60 (2H, d, Ph), 7.03 (2H, d, Ph), 7.90 (1H, t, CONH). Anal. Calcd. for $C_{17}H_{24}N_4SO_2$: %C 58.62; %H 6.90; %N 16.09. Found: %C 58.50; %H 6.81; %N 16.23. The hydrochloride salt of 2 (mp 247-250°C) precipitated from the acidified ethanolic solution of 2 upon the slow addition of diethyl ether.

***N*-[4-(3',3'-Dimethyltriazenyl)benzyl]biotin Amide (3).** A suspension of 2 (0.55 g, 1.58 mmol) in 15 ml of water and 0.7 ml of concentrated HCl was heated at 60°C until 2 dissolved. The mixture was cooled to 0°C with vigorous stirring and then

sodium nitrite (0.1 g, 1.45 mmol) dissolved in 0.3 ml of water was added over a period of 10 min. Stirring was continued for 30 min at 0°C, and 1.5 ml of dimethylamine (40% aqueous solution) was added dropwise. The reaction mixture was kept at room temperature for 10 hr and then evaporated to one-half of its original volume to remove the excess dimethylamine. After the pH was adjusted to 8 with diluted HCl, the mixture was extracted with chloroform (3 x 30 ml). Combined extracts were dried ($MgSO_4$) and evaporated again. The crude product was purified on a silica gel column (EtOAc/CH_3OH, 10:1, v/v) to give 0.43 g (67%) of the triazene 3 as a polymorphous solid; mp 180-184°C. This material was subjected to preparative HPLC purification on a Maxsil 5 (250 x 10 mm) column using EtOAc as solvent; flow rate 2.5 ml min^{-1}. A white solid (0.37 g) was recovered from collected fractions after crystallization from CH_2Cl_2 with CH_3OH; mp 185-186°C. HPLC analysis on an analytical silica column showed a peak with R_t = 19 min; eluant A EtOAc, eluant B CH_3OH; flow rate 1 ml min^{-1}; initial composition 5% B with a 1% min^{-1} linear gradient of B. HPLC (C_{18} column) gave a single peak with R_t = 26 min; eluant 50% CH_3OH; flow rate 1.5 ml min^{-1}. ^{1}HNMR ($CDCl_3$) δ 1.12 - 2.05 (4H, m, CH_2CH_2), 2.15 - 2.25 (2H, m, CH_2CH), 2.90 (2H, m, CH_2CO), 3.15 (2H, m, CH_2S), 3.27 (2H, m, 2 x NH), 3.40 (6H, s, 2 x CH_3N), 4.17 (1H, m, CHS), 4.64 (2H, d, CH_2Ph), 6.92 (1H, t, $NHCH_2$), 7.25 (4H, m, Ph), 8.12 (2H, m, HNCONH). Anal. Calcd. for $C_{19}H_{28}N_6SO_2$: %C 56.44; %H 6.93; %N 20.79. Found: %C 56.32; %H 6.79; %N 20.93.

***N*-(4-[^{127}I]Iodobenzyl)biotin Amide (4a).** Compound 3 (0.11 g, 0.27 mmol) was dissolved in 1 ml of anhydrous CH_3CN and cooled on an ice bath. Trifluoroacetic acid (20 μl) was added followed by NaI (0.08 g, 0.27 mmol). The reaction mixture was removed from the ice bath and left at room temperature for 15 min. Vigorous gas evolution was evident and the solution turned yellow. The mixture was neutralized with 0.1 N aqueous sodium hydroxide and partitioned between chloroform and water.

The chloroform layer was repeatedly washed with water, dried ($MgSO_4$) and evaporated to give a yellow solid. This was purified on a silica gel column (EtOAc/CH_3OH, 10:1, v/v) to give 90 mg (72%) of 4a as white crystals; mp 199-201°C. TLC (EtOAc/ CH_3OH, 10:1, v/v) R_f = 0.40. HPLC on a silica gel column gave a single peak with R_t = 36 min; eluant A EtOAc, eluant B CH_3OH; flow rate 1 ml min^{-1}; initial composition 7% B with a 1% min^{-1} linear gradient of B. HPLC performed on a C_{18} reverse phase column gave a peak with R_t = 38 min; eluant 50% CH_3OH; flow rate 1.5 ml min^{-1}. ^{1}HNMR (DMSO-d_6) δ 1.15 - 2.12 (1H, m, C*H*$_2$C*H*$_2$C*H*$_2$), 2.20 - 2.35 (2H, m, C*H*$_2$CONH), 2.96 (1H, m, SC*H*), 3.20 - 3.30 (3H, m, SC*H*$_2$C*H*), 4.20 - 4.40 (3H, m, PhC*H*$_2$, C*H*CH$_2$), 4.80 - 4.90 (1H, d/d, CON*H*CH$_2$), 7.05 - 7.65 (4H, m, Ph), 8.10 - 8.25 (2H, m, *H*NCON*H*). Anal. Calcd. for $C_{17}H_{22}N_3ISO_2$: %C 44.45; %H 4.79; %N 9.15. Found: %C 44.38; %H 4.84; %N 9.21.

***N*-(4-[^{125}I]Iodobenzyl)biotin Amide (4b).** Into the open end of a plastic syringe (2 cc) fitted with a 0.2-μm Millex filter, 5 μl of phosphate buffer (0.25 M), pH 7.5, was added followed by 0.1 to 1.0 mCi of Na^{125}I and 150 μl of CH_3CN. The syringe was capped and vortexed. The cap was removed and 20 mg of anhydrous, finely powdered $MgSO_4$ was added. The syringe was capped again, vortexed and left for 5 min. The suspension was filtered, the syringe and the filter were washed with anhydrous CH_3CN (3 x 100 μl), and the combined filtrates were evaporated with a stream of dry nitrogen. Approximately 90% of the radioactivity was in the filtrate. The residue was dissolved in 100 μl of dry CH_3CN and placed on an ice bath. Compound 3 (0.4 mg, 1 μmol) in 200 μl of CH_3CN was treated with 10 μl of cold CF_3COOH (1% solution in CH_3CN) and immediately transferred to the iodide solution. The reaction mixture was kept at room temperature for 10 min and chromatographed on a short silica gel column (EtOAc/CH_3OH, 10:1, v/v). Collected fractions were examined on TLC and the radioactive content of each fraction was determined. The radiochemical yield of crude 4b (R_f = 0.40 in the same solvent system) was over

50%. Sodium [^{125}I]iodide and free [^{125}I]iodine accounted for 81% and 11% of the remaining radioactivity, respectively. Combined fractions containing 4b were evaporated with nitrogen and the residue, dissolved in 250 μl of CH_3OH/H_2O (1:1, v/v), was injected on a C_{18} analytical column with 50% CH_3OH as eluant; 1.5 ml min^{-1} flow rate. The major radioactive peak with R_t = 38 min was collected in four fractions which were evaporated with dry nitrogen. Based on eleven experiments an average yield of the pure isolated 4b was 33%. However only 7 to 12% yield of 4b was consistently obtained when the procedure described above for drying the commercial aqueous Na^{125}I solution was omitted.

***N*-(4-[^{123}I]Iodobenzyl)biotin Amide (4c).** When the aqueous solution of sodium [^{123}I]iodide was used, the course of the synthesis was identical to that described for the [^{125}I]iodo-analogue 4b. If dry Na^{123}I could be obtained, 100 μl of anhydrous CH_3CN was added directly to a vial containing sodium iodide and the mixture was sonicated. An adequate amount of CF_3COOH (1% solution in CH_3CN) was added to neutralize the NaOH. The cold solution of 3 in CH_3CN was added and the reaction mixture was separated and purified as described for 4b. An average yield of 30% for no-carrier-added iodination was obtained.

Preparation of 4b-Avidin Complex. The complex of avidin with 4b was prepared by incubating the protein with the radiotracer at room temperature for 5 hr. The extent of biotin-avidin binding was determined using instant TLC (ITLC, silica impregnated glass fiber, Gelman Sciences, Ann Arbor, MI). About 2 μl of the incubated mixture was spotted on a 2 x 20 cm ITLC strip, dried briefly, and eluted with saline/CH_3OH (80:20, v/v). Virtually all the radioactivity (>95%) was found associated with avidin at the origin.

Binding Studies: Equilibrium Dialysis. Into a series of glass vials containing various concentrations of the 4b/4a mixture (0.006 to 120 nM) in 18.4 ml PBS (pH 7.4) and 0.4 ml methanol were immersed dialysis bags filled with 1 ml of the avidin

solution (30 nM) in the same buffer. Two control vials contained 4b/4a and dialysis bags with PBS only. These were used to determine any binding to membranes and glass. After a 24-hr incubation at 25°C the mixtures were analyzed for free and total (bound plus free) biotin by taking aliquots of PBS solution from the outside (free) and the inside (total) of dialysis bags and measuring their radioactive content. The data were analyzed assuming noncooperative single-site binding per molecule of avidin.

Dissociation Kinetics for *N*-(4-[^{125}I]Iodobenzyl)biotin Amide (4b)-Avidin Complex. One ml of avidin in 0.2 M phosphate buffer, pH 7.4 (0.01 mg/ml; 1.5 x 10^{-4} μmol; 650-fold molar excess over radiolabeled biotin derivative) was placed in triplicate dialysis bags (molecular weight cut-off 12,000 daltons; Spectrapor) for each time point. Each bag was placed in a separate vial containing 49 ml of the same buffer and 0.5 μCi of *N*-(4-[^{125}I]iodobenzyl)biotin amide (4b). Following a 10-hr incubation at 37°C, the distribution of radioactivity was analyzed in one set of three vials (time point = 0 hr). Two-ml aliquots from the outside of the dialysis bag (to determine the concentration of free biotin) and 0.8 ml aliquots from within the dialysis bag diluted with 1.2 ml of 0.2 M phosphate buffer, pH 7.4 (to determine the total concentration, i.e. free and avidin-bound biotin) were counted in a gamma counter. The dialysis bags were emptied, rinsed with buffer, and their radioactive content was assayed separately. To the remaining vials, 1 ml of biotin (0.15 mg/ml, 0.6 μmol) was added and the equilibration was allowed to proceed for 1, 2, 4, 6, 12 and 24 hr. At indicated time intervals the radioactive content was analyzed as described above. The concentration of bound radiolabeled biotin was calculated as dpm/ml inside dialysis bags minus dpm/ml outside dialysis bags.

Blood Clearance and Tissue Distribution of *N*-(4-[^{125}I]Iodobenzyl)biotin Amide (4b). Groups of 21-day-old Balb/c male mice maintained on normal diet and water *ad libitum* were injected via a tail vein with either 2.5 μCi of 4b or 5 μCi of its complex with avidin (9 μg per animal) in 100 to 300 μl physiologic saline. Blood (5 to 20 μl)

was collected from the tail vein punctured about 1 cm above the injection site (n = 5 for each time point) and the radioactive content measured in a gamma counter. At selected times (0.05, 0.25, 1, 1.5, 2, 2.5, 3.5, 4, 5, 22, 24 hr), two animals were sacrificed and dissected (each experiment was done in duplicate). Liver, thyroid with surrounding tissue, and kidneys were collected, washed in saline, blotted dry, and their radioactive content was also measured in a gamma counter. To determine the absolute value of the injected dose, tails were collected to assign the radioactivity associated with the injection site. In all cases less than 0.9% of the injected dose was found in the tail. Nevertheless these counts were subtracted from the counts in injectates. The data were analysed using Pharmkin (a generous gift from A. Johnston and R. Woollard) and SigmaPlot 5.0 (Jandel Scientific) computer curve-fitting programs.

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