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Biotin Reagents for Antibody Pretargeting. 2. Synthesis and *in Vitro* Evaluation of Biotin Dimers and Trimers for Cross-Linking of Streptavidin

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Polymerization and/or cross-linking of recombinant streptavidin (r-SAv) with biotin derivatives containing two biotin moieties (biotin dimers) or three biotin moieties (biotin trimers) has been investigated as a model for reagents to be used to increase the amount of radioactivity on cancer cells in tumor pretargeting protocols. In the investigation, six biotin dimers and three biotin trimers were synthesized. Most biotin derivatives synthesized had ether containing linker molecules incorporated to improve their aqueous solubility. The synthesized biotin dimers contained linker moieties which provided distances (when fully extended) of 13−49 Å between biotin carboxylate carbon atoms, and the biotin trimers contained linker moieties which provided distances of 31−53 Å between any two biotin carboxylate atoms. All of the biotin derivatives were evaluated for their ability to polymerize r-SAv in solution. When the biotin derivatives were mixed with r-SAv, none of the biotin dimers caused polymerization, but all of the biotin trimers resulted in complete polymerization. Some of the biotin dimers did cross-link r-SAv (to form r-SAv dimers, trimers, etc.), but the percentage of cross-linking was low (≤40%). The length of the linker molecule was important in cross-linking of biotin dimers. While linkers which provided distances of 13 and 19 Å between biotin carboxylate carbon atoms did not result in cross-linking, a linker which provided a 17 Å distance resulted in a small (≤10%) amount of cross-linking. Also, cross-linking was increased in biotin dimers with linkers which provided distances between biotin carboxylate carbon atoms of ≥23 Å. Cross-linking of streptavidin bound in polystyrene wells with biotin dimers and trimers was also examined. In those experiments, an excess of each biotin derivative was incubated at 37 °C for 10−30 min in polystyrene wells containing bound SAv. After the excess biotin derivative was rinsed from the wells, an excess of r-[^{125}I]SAv was incubated for another 10−30 min. The amount of r-[^{125}I]SAv bound after rinsing the excess from the wells was an indicator of the extent of cross-linking that occurred. The process of alternating additions of reagents was repeated four times to demonstrate that bound radioactivity could be increased with each addition of [^{125}I]SAv. The results of cross-linking r-SAv in polystyrene wells paralleled results from cross-linking in solution.

INTRODUCTION

An alternative method to using radiolabeled monoclonal antibodies for cancer diagnosis and therapy, termed "tumor pretargeting", is under investigation by a number of research groups, including our own (*1*−*14*). Similar to directly labeled antibodies, pretargeting protocols use monoclonal antibodies as tumor selective binding agents. However, pretargeting protocols involve "two-step" or "three-step" methods to deliver the radioactivity to the cancer cells (*3*, *14*). This multistep approach has an advantage in the fact that the delivery of radioactivity is separated from the pharmacokinetics of the monoclonal antibody (*15*), making it unnecessary to match the biological half-life of the antibody and the physical half-life of the radionuclide. The antibody pretargeting approach is made possible by the formation of antibody conjugates which bind strongly with another molecule that is used to carry the radionuclide. Because of the very strong binding with biotin (*16*), antibody conjugates of the proteins avidin (*17*) and streptavidin (*18*) have received the most attention in the development of pretargeting methods.

Our interest in the use of biotin/avidin or biotin/streptavidin in pretargeting extends further than the very high binding affinities of these compounds. Avidin (Av)[1] and streptavidin (SAv) are proteins which contain four identical subunits and bind four biotin molecules. The tetravalent biotin binding property of Av and SAv introduces the possibility of increasing the amount of radioactivity localized on cancer cells by increasing the number of (radiolabeled) biotin binding sites available. Indeed, it can be envisioned that an amplification of the number of biotin binding sites can be obtained in a two-step protocol by binding more than one Av or SAv molecule with a poly-biotinylated antibody bound with a cancer cell, or alternatively, an antibody−Av or antibody−SAv conjugate localized at a tumor could potentially bind up to 4 molar equiv of radiolabeled biotin per conjugated Av or SAv (*19*). In a multistep protocol, an increase in bound radioactivity might be achieved by binding a radiolabeled polybiotin molecule to an antibody−Av or antibody−SAv conjugate on a cancer cell, followed by administration of Av or SAv, and then repeat admin-

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[1] Abbreviations: Av, avidin; BSA, bovine serum albumin; ChT, chloramine-T; cpm, counts per minute; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; 2 HEDS, 2-hydroxyethyl sulfide; *nca*, no-carrier-added; NCS, *N*-chlorosuccinimide; PBS, phosphate-buffered saline; r-SAv, recombinant streptavidin; rt, room temperature; SAv, streptavidin; TFP, tetrafluorophenyl; TFP-OH, tetrafluorophenol.

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istration of the radiolabeled polybiotin molecule (*13*, *14*). The multistep approach appeared to be particularly interesting to us as it introduced the possibility of increasing the quantity of radioactivity on cancer cells in a fractionated dose regime and would, perhaps, allow the use of lower-specific activity radionuclide preparations in therapy.

Although the potential for increasing the amount of radioactivity at the tumor was apparent, it was also apparent that new biotin reagents had to be developed for the cross-linking step as no biotin reagents were available which could be used for this task. Because it was desired that the radiolabeled biotin molecules be excreted via renal clearance, the smallest biotin derivatives possible, biotin dimers and trimers, were of the highest interest. Unfortunately, very little information on the synthesis and properties of compounds containing more than one biotin moiety could be found in the literature. The most relevant studies were those of Green et al. (*20*), in which it was demonstrated that biotin dimers could be used to polymerize Av. However, the biotin reagents used in their investigation were quite insoluble in water and did not present a means by which radionuclides could be attached. It was apparent that the design of new biotin dimers and trimers for application to human disease would require incorporation of water-solubilizing moieties into the molecules and that the distance between the biotin moieties was very important.

To limit the scope of this investigation, only nonionic compounds containing two or three biotin moieties were synthesized and evaluated for their ability to polymerize recombinant SAv (r-SAv), the protein we have chosen for use in our tumor pretargeting studies (*14*). The biotin compounds prepared were designed to provide information pertaining to (1) the effect of distances between the biotin moieties on the efficiency of r-SAv polymerization and (2) the use of water-solubilizing linking moieties to improve the water solubility of the new compounds. The biotin compounds prepared in this study did not contain a radiolabeling moiety, as the syntheses of such compounds would be more difficult and the information sought did not require it. In the investigation, six biotin dimers and three biotin trimers were synthesized in which the distances between the biotin moieties ranged from 13 to 54 Å.[2] Linking moieties which contained two or three ether functionalities were incorporated into most of the molecules to improve their water solubility. Once the molecules were synthesized, the propensity for polymerization of r-SAv by the biotin dimers and trimers was evaluated in solution using size exclusion HPLC and on a SAv-bound surface using commercially available SAv-coated polystyrene wells. Reported here are the results of that investigation.

EXPERIMENTAL PROCEDURES

General. All purchased chemicals were analytical grade or better and were used without further purification. Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μm) prior to use. Biotin-*d*, 2,2′-(ethylenedioxy)bis(ethylamine) (**2**), 4,7,10-trioxa-1,-13-tridecanediamine **(3)**, trimesic acid chloride (**9**), 3,5-diaminobenzoic acid (**15**), 2,3,5,6-tetrafluorophenol, 3-aminobutyric acid, chloramine-T, and most other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). 2,3,5,6-Tetrafluorophenyl trifluoroacetate was prepared as previously described (*21*). Recombinant streptavidin was obtained as previously described (*22*). Silica gel chromatography was conducted with 70−230 mesh 60 Å silica gel (Aldrich Chemical Co.). Sephadex G-25 (NAP-10) columns were obtained from Pharmacia Biotech AB (Uppsala, Sweden). Avidin and Reacti-Bind streptavidin-coated polystyrene 96-well plates were obtained from Pierce (Rockford, IL). Melting points were obtained in open capillary tubes on a Mel-Temp II apparatus with a Fluke 51 K/J electronic thermometer and are uncorrected.

Spectral Analyses. ^{1}H NMR spectra were obtained on a Bruker AC-200 (200 MHz) instrument, and chemical shifts are expressed as parts per million using tetramethylsilane as an internal standard ($\delta = 0.0$ ppm). IR data were obtained on a Perkin-Elmer 1420 infrared spectrophotometer. Mass spectral data (both low- and high-resolution) were obtained on a VG Analytical (Manchester, England) VG-70SEQ mass spectrometer with an associated 11250J Data System. Mass spectral data were obtained by fast atom bombardment (FAB$^+$) at 8 keV using a matrix of MeOH/DMIX (thioglycerol/DMSO/TFAA, 90/9/1). Attempts to obtain mass spectral data for compounds **10** and **20** which contain the TFP esters were unsuccessful using several different techniques. The products from reactions involving these compounds had satisfactory HRMS data for identification. ^{1}H NMR and HPLC were used to assess compound identity and purity (these data are provided in the Supporting Information).

Chromatography. HPLC separations of biotin derivatives were obtained on a Hewlett-Packard quaternary 1050 gradient pumping system with a variable-wavelength UV detector (254 nm) and a Varex ELSD MKIII evaporative light-scattering detector. Analyses of the HPLC data were conducted on Hewlett-Packard HPLC ChemStation software. All reactions were monitored by HPLC. Reverse-phase HPLC was carried out using an Alltech Altima C-18 column (5 μm, 250 mm × 4.6 mm) with a gradient solvent system at a flow rate of 1 mL/min. The gradient mixture was composed of MeOH (solvent A) and 1% aqueous HOAc (solvent B). Starting with 40% MeOH, the initial solvent mixture was held for 2 min, and then the gradient was increased to 100% MeOH over the next 10 min and then held at 100% MeOH for 5 min. Retention times (t_R) under these conditions for biotin conjugates were as follows: **1**, 12.9 min; **4**, 7.4 min; **5**, 8.5 min; **6**, 11.6 min; **7**, 7.5 min; **8**, 8.4 min; **10**, 16.6 min; **11**, 2.5 min; **12**, 2.5 min; **13**, 9.2 min; **14**, 10.4 min; **16**, 8.6 min; **17**, 3.8 min; **18**, 9.3 min; **20**, 12.7 min; and **21**, 10.4 min.

HPLC separations of r-SAv and mixtures of r-SAv and biotin derivatives were conducted on a Rainin Dynamax variable-wavelength detector, an isocratic pump, and a Waters Protein Pak Glass 300SW size exclusion column (10 μm, 8.0 × 300 mm; Waters Corp., Milford, MA). The mobile phase consisted of 50 mM potassium phosphate (pH 6.8), 300 mM NaCl, 1 mM EDTA, and 1 mM NaN_3 in deionized water. A flow rate of 0.75 mL/min was used. The retention time of r-SAv under these conditions was 13.7 min. It should be noted that the total peak area for each injection was monitored, and when the area dropped significantly, a 200 μL bolus of 6 N guanidine hydrochloride was injected to clear polymerized SAv from the column. This procedure was particularly important when large excesses of the trimers were being studied.

Estimation of Aqueous Solubility of Biotin Derivatives. The aqueous solubility of biotin compounds was estimated by HPLC. To obtain aqueous concentra-

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[2] Measurements of distances in biotin derivatives were obtained from the computer program ChemDraw3D (CambridgeSoft Corp., Cambridge, MA) after structural and energy minimization of fully extended, planar conformations of the compounds.

tions, a standard curve for the biotin derivative was first obtained by dissolving a weighed quantity of compound in MeOH, making two dilutions of that solution (to obtain three points), and injecting a 20 μL volume of the diluted samples to obtain the peak area. Linear regression analysis of the peak areas vs quantity of biotin derivative provided an equation for calculating the concentration in water. The aqueous solubility was measured by adding an excess of biotin derivative to 1 mL of water, mixing occasionally on a vortex mixer while at rt for 10 min, filtering the aqueous solution from the solid present through a 0.45 μm syringe filter, and injecting 20 μL of the aqueous solution to obtain the peak area.

Biotin Tetrafluorophenyl Ester (1). This compound was prepared by reaction of biotin with 2,3,5,6-tetrafluorophenyl trifluoroacetate as previously described (*14*).

3,6-Dioxaoctane-1,8-*N*-dibiotinamide (4). To a solution containing 1.00 g (2.54 mmol) of biotin TFP ester (**1**) in 150 mL of CH_3CN at 55 °C was added 0.18 g (1.27 mmol) of **2** with stirring. The mixture was cooled to 0 °C, and the precipitate was filtered. Crystallization from CH_3CN gave 0.61 g (1.01 mmol, 40%) of the dibiotin **4** as white crystals: mp 200−203 °C; ^1H NMR (MeOH-d_4) δ 4.49 (m, 2H), 4.31 (m, 2H), 3.62 (s, NH, 4H), 3.35 (t, J = 5.6 Hz, 4H), 3.35 (m, 4H), 3.31 (m, 4H), 2.93 (dd, J = 12.9, 4.9 Hz, 2H), 2.69 (d, J = 12.9 Hz, 2H), 2.22 (t, J = 7.5 Hz, 4H), 1.63 (m, 8H), 1.44 (m, 4H); IR (KBr, cm^{-1}) 3270, 2920, 2840, 1690, 1640, 1550, 1460, 1320, 1260, 1140; HRMS calcd for $C_{26}H_{45}N_6O_6S_2$ $(M + H)^+$ 601.2842, found 601.2819.

4,7,10-Trioxatridecane-1,13-*N*-dibiotinamide (5). To a solution containing 1.00 g (2.54 mmol) of **1** in 150 mL of CH_3CN at 55 °C was added 0.28 g (1.27 mmol) of **3** with stirring. The mixture was cooled to 0 °C, and the precipitate was filtered. Crystallization from CH_3CN gave 0.65 g (0.96 mmol, 38%) of the dibiotin **5** as white crystals: mp 139−141 °C; ^1H NMR (MeOH-d_4) δ 4.48 (m, 2H), 4.30 (m, 2H), 3.64 (m, 4H), 3.62 (m, 4H), 3.54 (t, J = 6.2 Hz, 4H), 3.36 (m, 8H), 3.27 (m, 4H), 3.21 (m, 2H), 2.95 (dd, J = 12.3, 4.9 Hz, 2H), 2.72 (d, J = 12.3 Hz, 2H), 2.21 (t, J = 7.2 Hz, 4H), 1.77 (m, 8H), 1.66 (m, 8H), 1.47 (m, 4H); IR (KBr, cm^{-1}) 3270, 2920, 2840, 1690, 1640, 1550, 1460, 1320, 1260, 1140; HRMS calcd for $C_{30}H_{53}N_6O_7S_2$ $(M)^+$ 673.3417, found 673.3397.

3-(Biotinamido)butyrate Tetrafluorophenyl Ester (6). This compound was prepared in three steps as previously described (*23*). Briefly, reaction of biotin TFP ester with 3-aminobutyric acid and Et_3N in DMF provided the adduct, which was esterified with 2,3,5,6-tetrafluorophenyl trifluoroacetate to provide **6**.

3,6-Dioxaoctane-1,8-*N*-di-(3′-biotinamido)butyrate (7). To a solution containing 0.025 g (0.167 mmol) of **2** in 15 mL of DMF was added Et_3N followed by 0.16 g (0.335 mmol) of **6**. The reaction mixture was stirred at rt for 0.5 h (monitored by HPLC), and the solvent was removed under vacuum. The residue was loaded onto a silica column (2.5 cm × 35 cm). The column was first eluted with ethyl acetate and then with a mixture of ethyl acetate/methanol. The solvent was removed from eluted fractions containing **7** to give 0.09 g (70%) of colorless solid: mp 186−188 °C; ^1H NMR (MeOH-d_4) δ 4.5 (m, 6H), 4.2 (m, 4H), 3.1−3.5 (m, 16H), 2.8 (dd, J = 4.6, 12.9 Hz, 2H), 2.6 (d, J = 12.6 Hz, 2H), 2.1−2.4 (m, 8H), 1.0−1.5 (m, 20H); IR (KBr, cm^{-1}) 3280, 3060, 2920, 2840, 1695, 1640, 1540, 1260, 1100; HRMS calcd for $C_{34}H_{59}N_8O_8S_2$ $(M + H)^+$ 771.3897, found 771.3894.

4,7,10-Trioxatridecane-1,13-*N*-di-(3′-biotinamido)butyrate (8). To a solution containing 0.1 g (0.45 mmol) of **3** in 15 mL of DMF was added Et_3N followed by 0.43 g (0.9 mmol) of **6**. The reaction mixture was stirred at rt for 0.5 h (monitored by HPLC), and the solvent was removed under vacuum. The product was triturated in 50 mL of CH_3CN and was filtered. The isolated product was dried under vacuum to yield 0.31 g (82%) of **8** as a colorless solid: mp 175−177 °C; ^1H NMR (MeOH-d_4) δ 4.4 (m, 2H), 4.2 (m, 4H), 3.4−3.6 (m, 14H), 3.1−3.2 (m, 8H), 2.7−2.9 (m, 3H), 2.6 (d, J = 12.6 Hz, 2H), 2.1−2.4 (m, 9H), 1.2−1.6 (m, 18H), 1.0 (d, J = 6.6 Hz, 6H); IR (KBr, cm^{-1}) 3280, 3060, 2920, 2840, 1695, 1640, 1540, 1260, 1100; HRMS calcd for $C_{38}H_{67}N_8O_9S_2$ $(M + H)^+$ 843.4472, found 843.4457.

1,3,5-Benzene Tricarboxylic Acid Tris-(2,3,5,6-tetrafluorophenyl) Ester (10). To a solution containing 12.0 g (45.2 mmol) of trimesic acid trichloride (**9**) in 100 mL of CH_2Cl_2 was added 23.26 g (140.1 mmol) of 2,3,5,6-tetrafluorophenol in 150 mL of CH_2Cl_2 and 20 mL (247.2 mmol) of pyridine at 0 °C. The mixture was stirred for 4 h at rt, washed with (2 × 100 mL) 10% HCl and 3 × 100 mL of water, dried over anhydrous Na_2SO_4, and evaporated to give 28.0 g (42.8 mmol, 94%) of the triester **15** as a solid. Crystallization from CH_3CN gave a white crystalline solid: mp 156−158 °C; ^1H NMR ($CDCl_3$) δ 9.31 (s, 3H), 7.10 (m, 3H); IR (KBr, cm^{-1}) 3080, 1760, 1520, 1480, 1270, 1190, 950.

***N*-(8-Amino-3,6-dioxaoctanyl)biotinamide (11).** A 2.80 g (7.13 mmol) quantity of biotin TFP ester (**1**) was dissolved in 300 mL of warm CH_3CN, cooled to rt, and added dropwise over 45 min to a well-stirred solution containing 21.1 g (142 mmol) of diamine **2** in 200 mL of CH_3CN. The reaction mixture was stirred for 4 h, and the solvent was removed under reduced pressure. The resultant oil was triturated in 300 mL of diethyl ether and filtered to give a white solid. The crude product was purified on a silica gel column, eluting with methanol/ethyl acetate (8/2) to give 1.81 g (4.83 mmol) of **11** as a white solid: mp 106−107 °C; ^1H NMR (MeOH-d_4) δ 4.50 (m, 1H), 4.31 (m, 1H), 3.62 (m, 4H), 3.52 (m, 4H), 3.36 (m, 2H), 3.2 (m, 1H), 2.92 (dd, J = 12.6, 4.9 Hz, 1H), 2.79 (m, 2H), 2.70 (d, J = 12.6 Hz, 1H), 2.22 (m, 2H), 1.67 (m, 4H), 1.44 (m, 2H); IR (KBr, cm^{-1}) 3280, 3060, 2920, 2840, 1695, 1640, 1540, 1260, 1100; HRMS calcd for $C_{16}H_{31}N_4O_4S$ $(M + H)^+$ 375.2066, found 375.2056.[3]

***N*-(13-Amino-4,7,10-trioxatridecanyl)biotinamide (12).** This compound was prepared from biotin TFP ester (**1**) and 4,7,10-trioxa-1,13-tridecanediamine (**3**) as previously reported (*14*).

***N*,*N*′,*N*″-Tris(8-*N*-biotinamide-3,6-dioxaoctanyl)-benzene-1,3,5-tricarboxamide (13).** To a solution containing 0.15 g (0.21 mmol) of **10** in 5 mL of DMF was added 0.27 g (0.72 mmol) of **11** in 5 mL of DMF at rt. The reaction mixture was stirred at rt for 12 h, and the DMF was removed under vacuum. The solid obtained was purified on silica gel, eluting with methanol/ethyl acetate (7/3). The solvent was removed from eluted fractions containing **13** to give 0.18 g (0.14 mmol, 57%) of a colorless solid: mp 103−105 °C; ^1H NMR (MeOH-d_4) δ 8.48 (s, 3H), 4.49 (m, 3H), 4.30 (m, 3H), 3.65 (m, 24H), 3.55 (m, 6H), 3.35 (m, 6H), 3.16 (m, 1H), 2.91 (dd, J = 12.8, 4.9 Hz, 3H), 2.69 (d, J = 12.9 Hz, 3H), 2.16 (m, 6H), 1.60 (m, 12H), 1.40 (m, 6H); IR (KBr, cm^{-1}) 3280, 3060, 2920, 2840, 1695, 1640, 1540, 1260, 1100; HRMS calcd for $C_{57}H_{91}N_{12}O_{15}S_3$ $(M + H)^+$ 1279.5889, found 1279.5874.

***N*,*N*′,*N*″-Tris(13-*N*-biotinamide-4,7,10-trioxatridecanyl)benzene-1,3,5-tricarboxamide (14).** Compound **14** was prepared from reaction of 0.31 g (0.70 mmol) of

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[3] This compound (*N*-biotinyl-1,8-diamino-3,6-dioxaoctane) has been available from Boehringer Mannheim (*24*).

12 with 0.15 g (0.21 mmol) of **10** according to the procedure used for **13**. After column chromatography, 0.22 g (0.15 mmol, 71%) of **14** was obtained as a colorless solid: mp 106−108 °C; ^{1}H NMR (MeOH-d_4) δ 8.44 (s, 3H), 4.49 (m, 3H), 4.31 (m, 3H), 3.60 (m, 36H), 3.23 (m, 12H), 2.91 (m, 3H), 2.71 (d, J = 12.7 Hz, 3H), 2.15 (m, 12H), 1.91 (m, 6H), 1.65 (m, 12H), 1.40 (m, 6H); IR (KBr, cm^{-1}) 3280, 3060, 2920, 2840, 1695, 1640, 1540, 1260, 1100; HRMS calcd for $C_{69}H_{115}N_{12}O_{18}S_3$ $(M + H)^+$ 1495.7614, found 1495.7638.

***N,N′*-Bis(3′-biotinamidio)butyryl-3,5-diamidobenzoic acid (16).** To a solution containing 0.076 g (0.34 mmol) of **15** in 20 mL of DMF was added Et_3N followed by 0.325 g (0.68 mmol) of **6** in 5 mL of DMF. The reaction mixture was stirred at 60 °C for 15 h (monitored by HPLC), and the solvent was removed under vacuum. The solid obtained was purified on silica gel, eluting with methanol/ethyl acetate (8/2). The solvent was removed from eluted fractions containing **16** to give 0.1 g (40%) of a colorless solid: mp 252−254 °C; ^{1}H NMR (DMSO-d_6) δ 7.9−8.2 (m, 3H), 7.0−7.5 (3s, 1H), 6.6 (d, J = 12 Hz, 3H), 2.8−3.3 (m, 4H), 2.0-2.7 (m, 18H), 1.1−1.9 (m, 19H); IR (KBr, cm^{-1}) 3280, 3060, 2920, 2840, 1695, 1640, 1540, 1260, 1100; HRMS calcd for $C_{35}H_{51}N_8O_8S_2$ $(M + H)^+$ 775.3271, found 775.3241.

1-*N′*-(13-Amino-4,7,10-trioxatridecanyl)-3′-(biotinamido)butyramide (17). This compound was prepared by reaction of **6** with an excess of the diamine **3** as previously described (*23*).

***N,N′,N″*-Tris(13-*N*-4,7,10-trioxatridecanyl)-3′-[(biotinamido)butyryl]benzene-1,3,5-tricarboxamide (18).** To a solution containing 0.123 g (0.188 mmol) of **10** in 15 mL of DMF was added Et_3N, followed by 0.3 g (0.565 mmol) of **17**. The reaction mixture was stirred at room temperature for 16 h (monitored by HPLC), and the solvent was removed under vacuum. The residue was loaded onto a silica column (2.5 cm × 35 cm). The column was first eluted with ethyl acetate and then with a mixture of ethyl acetate/methanol. The solvent was removed from eluted fractions containing **18** to give 0.13 g (40%) of colorless solid: mp 152−154 °C; ^{1}H NMR (MeOH-d_4) δ 8.4 (s, 3H), 4.5 (m, 3H), 4.1−4.3 (m, 6H), 3.4−3.7 (m, 46H), 3.1−3.3 (m, 16H), 2.9 (dd, J = 4.6, 12.8 Hz, 3H), 2.7 (d, J = 12.6 Hz, 3H), 2.1−2.4 (m, 13H), 1.4-2.0 (m, 33H), 1.0 (d, J = 6.8 Hz, 9H); IR (KBr, cm^{-1}) 3280, 3060, 2920, 2840, 1695, 1640, 1540, 1260, 1100; HRMS calcd for $C_{81}H_{136}N_{15}O_{21}S_3$ $(M + H)^+$ 1750.9197, found 1750.9265.

3,6,9-Trioxaundecanedioate Bis-tetrafluorophenyl Ester (20). A 10 g (45 mmol) quantity of 3,6,9-trioxaundecanedioic acid (**19**) was dissolved in 150 mL of anhydrous DMF, and 19 g (115 mmol) of tetrafluorophenol was added, followed by 21 g (108 mmol) of EDC. The reaction mixture was stirred overnight at rt, and the solvent was removed under vacuum. The residue was extracted with 300 mL of ether, and the ether was washed with a saturated $NaHCO_3$ solution (3 × 25 mL) and then with water (2 × 25 mL). The ether solution was dried over anhydrous Na_2SO_4. Then the ether was removed under vacuum. The resultant product was dried under vacuum at 50−55 °C to yield 15.8 g (76%) of **20** as an oil: ^{1}H NMR ($CDCl_3$) δ 3.6−3.8 (m, 8H), 4.46 (s, 4H), 6.9 (m, 2H); IR (neat, cm^{-1}) 1795, 1635, 1520, 1480, 1370, 1270, 1100, 950.

1,11-Bis-*N*-(13-*N*-biotinamide-4,7,10-trioxatridecanyl)-3,6,9-trioxaundecanediamide (21). To a solution of 0.232 g (0.448 mmol) of **20** in 25 mL of DMF was added 0.14 μL of Et_3N, followed by 0.4 g (0.897 mmol) of **12**. The reaction mixture was stirred at rt for 4 h (monitored by HPLC), and the solvent was removed under vacuum. The residue was loaded onto a silica column (2.5 cm × 35 cm). The column was first eluted with ethyl acetate and then with a mixture of ethyl acetate/methanol. The solvent was removed from eluted fractions containing **21** to give 0.2 g (42%) of colorless solid: mp 96−98 °C; ^{1}H NMR (MeOH-d_4) δ 4.8 (s, 4H), 4.5 (m, 2H), 4.3 (m, 2H), 4.0 (s, 4H), 3.4−3.7 (m, 36H), 3.1−3.3 (m, 11H), 2.9 (dd, J = 4.6, 12.8 Hz, 2H), 2.7 (d, J = 12.6 Hz, 2H), 2.2 (t, J = 7 Hz, 4H), 1.3−1.8 (m, 22H); IR (KBr, cm^{-1}) 3280, 3060, 2920, 2840, 1695, 1640, 1540, 1260, 1100; HRMS calcd for $C_{48}H_{87}N_8O_{15}S_2$ $(M + H)^+$ 1079.5732, found 1079.5714.

***N*-[13-(*p*-Tri-*n*-butylstannylbenzamido)-4,7,10-trioxatridecanyl]biotinamide (22).** This compound was synthesized as previously described (*23*).

***N*-[13-(*p*-Iodobenzamido)-4,7,10-trioxatridecanyl]-biotinamide (23a).** This compound was synthesized as previously described (*23*).

***N*-[13-(*p*-[^{125}I]Iodobenzamido)-4,7,10-trioxatridecanyl]biotinamide (23b).** This compound was prepared as previously described (*23*). Briefly, to 50 μL of a 1 mg/mL solution of **22** was added 1−5 μL of Na^{125}I in 0.1 N NaOH, followed by 10 μL of a 1 mg/mL solution of NCS in MeOH. After 2 min, 10 μL of a 1 mg/mL aqueous sodium metabisulfite solution was added. The reaction mixture was then drawn into a syringe for purification by HPLC. The purified **23b** was obtained from the HPLC effluent. A small aliquot of **23b** in HPLC effluent was mixed with a weighted amount of **23a** to obtain the specific activity desired.

Radioiodination of Streptavidin. To 25 μL of sodium phosphate buffer (0.5 M, pH 7.4) were added 4 μL (1.19 mCi) of Na^{125}I in 0.1 N NaOH and 167 μL of a 6 mg/mL solution of r-SAv. This was followed by the addition of 19 μL of a 1 mg/mL solution of chloramine-T in water. After 30 s at rt, the reaction was quenched with 1.9 μL of a 10 mg/mL solution of $Na_2S_2O_5$ in water. The entire reaction mixture was placed on a NAP-10 column and eluted with PBS. The fractions containing protein were combined for 1.05 mCi (88%) with a specific activity of 1.07 μCi/μg. A second labeling conducted in the same manner provided 0.45 mCi (79%) r-[^{125}I]SAv with a specific activity of 0.82 μCi/μg. These preparations were used in the binding in coated wells study for experiments 1 and 2, respectively.

Streptavidin Polymerization in Solution. *Procedure A.* A 100 μL aliquot of r-SAv (0.5 mg/mL in PBS) was placed in a microcentrifuge tube. The dimer or trimer was diluted appropriately in 5% DMSO/water. While vortexing, 10 μL of the diluted biotin dimer or trimer was added rapidly. After incubating at rt for 30 min, the entire reaction mixture was injected onto a size exclusion HPLC column and eluted.

Procedure B. This was conducted as was procedure A, except the order of addition was reversed as follows. To a microcentrifuge tube containing 40 μL of PBS was added 10 μL of the appropriately diluted biotin dimer or trimer in 5% DMSO. While vortexing, 100 μL of r-SAv (0.5 mg/mL in PBS) was added slowly.

Streptavidin Cross-Linking Streptavidin-Coated Wells. *Experiment 1.* A Reacti-Bind Streptavidin Coated Polystyrene Strip Plate was washed two times with 100 mM PBS (100 μL/well) prior to use. To lane 1 (12 wells) was added 100 μL of PBS. To lanes 2−7 was added 100 pmol of biotin dimer (**4**, **5**, or **8**) or trimer (**13**, **14**, or **18**) to be tested in 100 μL of PBS. Lane 8 was treated in the same manner with 100 pmol of biotin monomer (**23a**). The plate was then incubated at 37 °C with shaking for 10 min. All the wells were emptied and rinsed with 100 μL of PBS. The PBS was removed, and 25 pmol of [^{125}I]-SAv (specific activity of 1 μCi/μg) was added to each well

Table 1. Estimated Distances between Two Biotin Carboxylate Carbon Atoms in the Synthesized Biotin Dimers and Trimers[a]

compd no.	distance (Å)	compd no.	distance (Å)
4	13	**21**	49
16	17	**13**	31
5	19	**14**	41
7	23	**18**	53
8	29		

[a] Listed in order of increasing distance.

in 100 μL of PBS. The plate was incubated at 37 °C with shaking for 10 min. The nonbound [^{125}I]SAv was removed, and all wells were washed with 100 μL of PBS. After removal of the wash PBS, the first three wells of each lane were again filled with 100 μL of PBS. The remaining wells were filled with 100 μL of their respective biotin dimer or trimer (as in the first step). The plate was incubated for 10 min at 37 °C, and all wells were rinsed with PBS as before. Another 100 μL of the [^{125}I]SAv was added to all wells and incubated for 10 min at 37 °C. After removal of the unbound SAv by rinsing of the wells, 100 μL of PBS was added to the first six wells of each lane. The remaining six wells in each lane were filled with 100 μL of their respective biotin dimer or trimer and again incubated for 10 min at 37 °C. After removing the liquid in the wells and washing with PBS, 100 μL of the [^{125}I]SAv was again added to each well followed by incubation. The [^{125}I]SAv was removed, and the wells were washed with PBS. The first nine wells in each lane were then filled with 100 μL of PBS, and the remaining three wells in each lane were filled with 100 μL of their respective biotin dimer or trimer. After incubating and washing, 100 μL of [^{125}I]SAv was again added to each well. After incubating for 10 min at 37 °C, each well was washed with 100 μL of PBS and all liquid removed. The wells were then separated, and each well was placed in a γ counter and counted. Results of the r-[^{125}I]SAv binding data are shown in Figure 5A.

Experiment 2. This experiment was conducted exactly as described in experiment 1, except 100 pmol of biotin dimers (**5**, **8**, **16**, or **21**) and biotin trimers (**13**, **14**, or **18**) was added to wells in lanes 2−8, respectively, and the mixtures were incubated for 30 min at 37 °C. Results of the binding of r-[^{125}I]SAv are shown in Figure 5B.

RESULTS

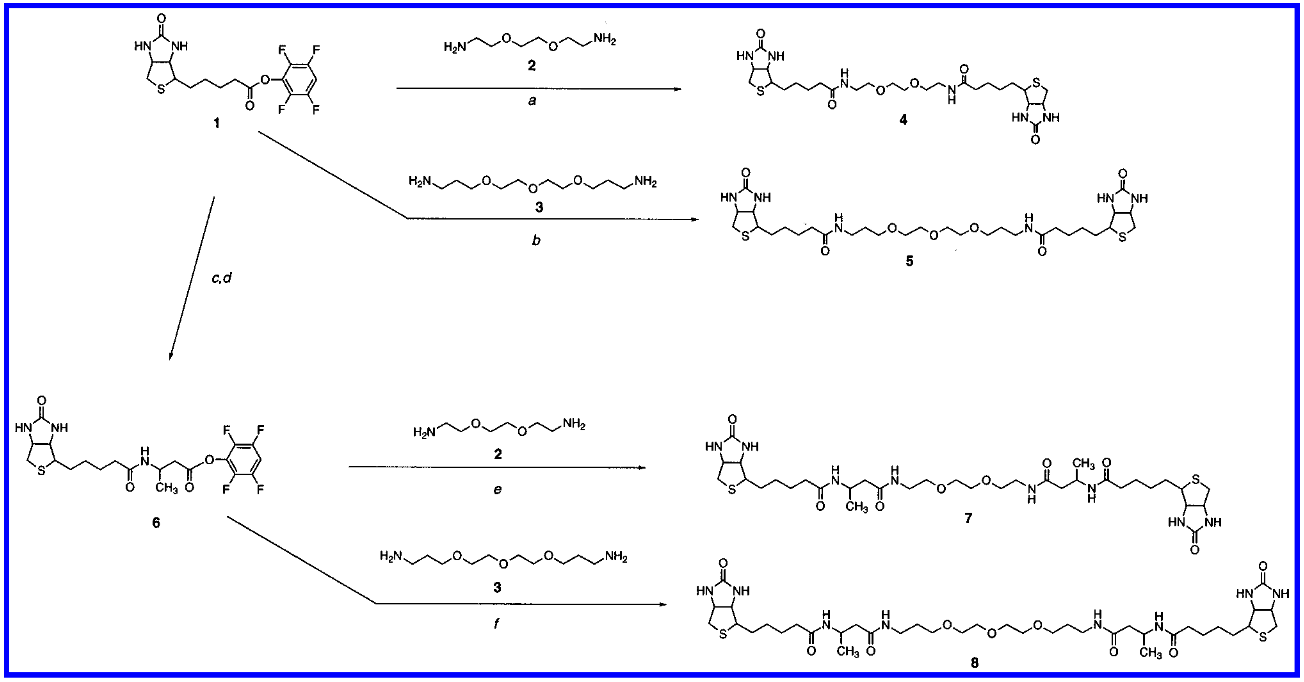
Synthesis of Biotin Derivatives. Six biotin derivatives containing two biotin moieties each (biotin dimers) and three biotin derivatives containing three biotin moieties each (biotin trimers) which have varying distances between biotin carboxylate carbons (Table 1) were synthesized. Biotin dimers **4**, **5**, **7**, and **8** were readily synthesized (Scheme 1) by reaction of 2 molar equiv of biotin TFP ester (**1**) or 3-(biotinamido)butyrate TFP ester (**6**) with 1 molar equiv of the oxadiamines **2** or **3** in DMF. Synthesis of biotin dimer **16** (Scheme 3) was accomplished by reaction of 2 equiv of **6** with 3,5-diamiobenzoic acid (**15**). The coupling of **6** with **15** was conducted under conditions that resulted in a slow reaction, but elevating the reaction temperature to 50 °C resulted in obtaining a reasonable yield of **16**. Synthesis of biotin dimer **21** (Scheme 4) was also readily accomplished by reaction of the cross-linking agent 3,6,9-trioxaundecanedioate bis-tetrafluorophenyl ester (**20**) with *N*-(13-amino-4,7,10-trioxatridecanyl)biotinamide (**12**). Syntheses of biotin trimers **13**, **14** (Scheme 2), and **18** (Scheme 3) were readily accomplished by reaction of 3 molar equiv of *N*-(8-amino-3,6-dioxaoctanyl)biotinamide (**11**), *N*-(13-amino-4,7,10-trioxatridecanyl)biotinamide (**12**), or 1-*N*′-(13-amino-4,7,10-trioxatridecanyl)-3-(biotinamido)butyramide (**17**) with benzenetricarboxylate tri-TFP ester (**10**) at room temperature in DMF.

Evaluation of r-SAv Polymerization in Solution. Biotin dimers **4**, **5**, **7**, **8**, **16**, and **21** and biotin trimers **13**, **14**, and **18** were evaluated for their ability to polymerize r-SAv in aqueous solution. To affect polymerization, varying molar quantities of the biotin dimers and trimers were mixed with solutions containing r-SAv in PBS, incubated at rt, and then evaluated by size exclusion HPLC. The chromatograms for mixtures containing 1/1 molar quantities of r-SAv-to-biotin dimer **4**, **5**, **7**, **8**, **16**, or **21** are provided in panels A−F of Figure 1. The chromatograms indicate that only a small percentage of the r-SAv formed what may be r-SAv dimers or trimers, but it is clear that no polymerization occurred.

Contrary to the biotin dimer results, all of the biotin trimers (**13**, **14**, and **18**) dramatically changed the retention time for r-SAv observed by size exclusion chromatography (Figure 2A−C). Indeed, only very high-molecular weight species, eluting at the solvent front, were observed, indicating that complete polymerization had occurred at a 1/1 molar ratio of r-SAv and biotin trimers **13**, **14**, or **18**. Changes in the molar ratio of r-SAv and biotin trimer and in the order of mixing of the components led to very dramatic differences in the product composition as observed for **14** by size exclusion HPLC (Figure 3A−F). Rapid addition of 0.5 molar equiv of the biotin trimer **14** into a solution of r-SAv led to a 1/1 mixture of a high-molecular weight streptavidin polymer and unaltered r-SAv (Figure 3A). Rapid addition of 10 molar equiv of **14** to r-SAv led to a mixture containing approximately 50% polymer, 30% dimer, trimer, tetramer, etc., and 20% r-SAv (Figure 3C). It seems likely that the r-SAv species observed by HPLC ($t_R = 14$ min) was saturated with biotin (i.e. two trimers bound). As one might expect, reversing the additions of reagents by slow addition of 1 equiv of r-SAv to an excess (10×) of **14** led to a mixture that had 65% of the (perbiotinylated) r-SAv, and the remaining peaks were higher-molecular weight species, with no polymer observed (Figure 3D). Repeating the foregoing additions with 100 equiv of **14** resulted in a larger shift from polymerization (Figure 3E), with 80% of the (perbiotinylated) r-SAv being observed when the r-SAv was added to the excess **14** (Figure 3F). It was interesting to note that column retention of r-SAv increased slightly as perbiotinylation of r-SAv was achieved (i.e. t_R has increased from 13.7 to 14.1 min).

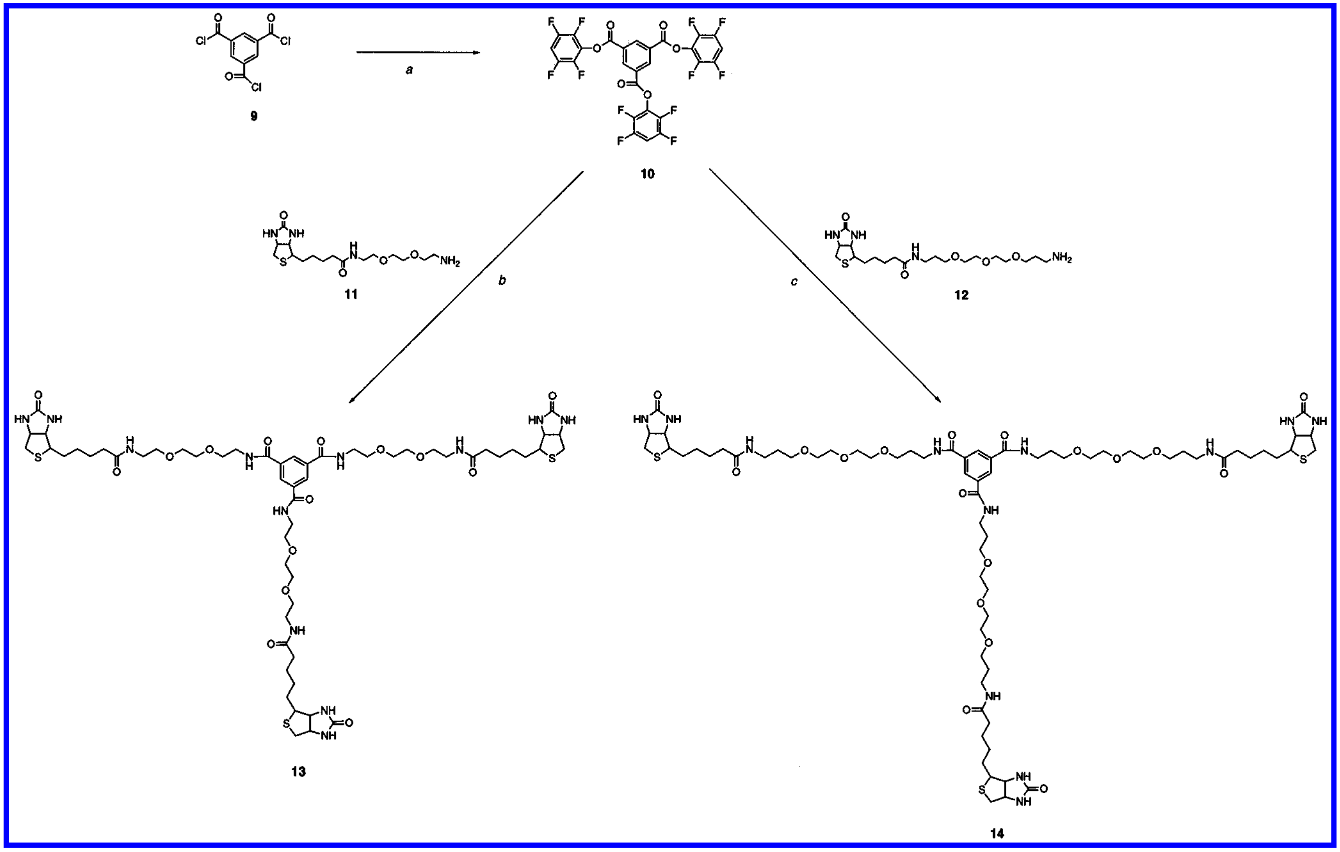
Biotin Cross-Linking of r-SAv with Immobilized SAv. To model cross-linking of SAv on cancer cells, SAv bound to the surface of polystyrene wells was used. Rather than using radiolabeled biotin derivatives, cross-linking was measured by the quantity of [^{125}I]SAv retained in the wells after treatment of the bound SAv with biotin derivatives. An initial experiment evaluated triplicate runs having one to four additions of a large excess (100 pmol) of biotin derivatives **4**, **5**, **8**, **13**, **14**, and **18**, alternating with one to four additions of a large excess (25 pmol) of [^{125}I]SAv. As controls, triplicate wells were examined in which no biotin was added and in which biotin monomer **23a** was added. In that experiment, the samples were incubated at 37 °C for 10 min after each reagent was added. The picomoles of [^{125}I]SAv bound vs additions of biotin derivative and [^{125}I]SAv is plotted in Figure 5A. Column 1 of Figure 5A had no biotin added and therefore shows the amount of nonspecific binding occurring. Figure 5A shows that only a very small amount (if any) of cross-linking with [^{125}I]SAv was obtained with biotin dimers. Contrary to that, all three biotin trimers provided significant cross-linking under

Scheme 1. Synthesis of Oxadiamine-Linked Biotin Dimers[a]



[a] (a) **2**, 55 °C, 16−24 h, CH_3CN, 40%; (b) **3**, 55 °C, 16−24 h, CH_3CN, 38%; (c) 3-aminobutyric acid, Et_3N, DMF, 24 h, 98%; (d) Et_3N, TFP-OTFA, 81%; (e) **2**, DMF, rt, 0.5 h, 70%; (f) **3**, DMF, rt, 0.5 h, 82%.

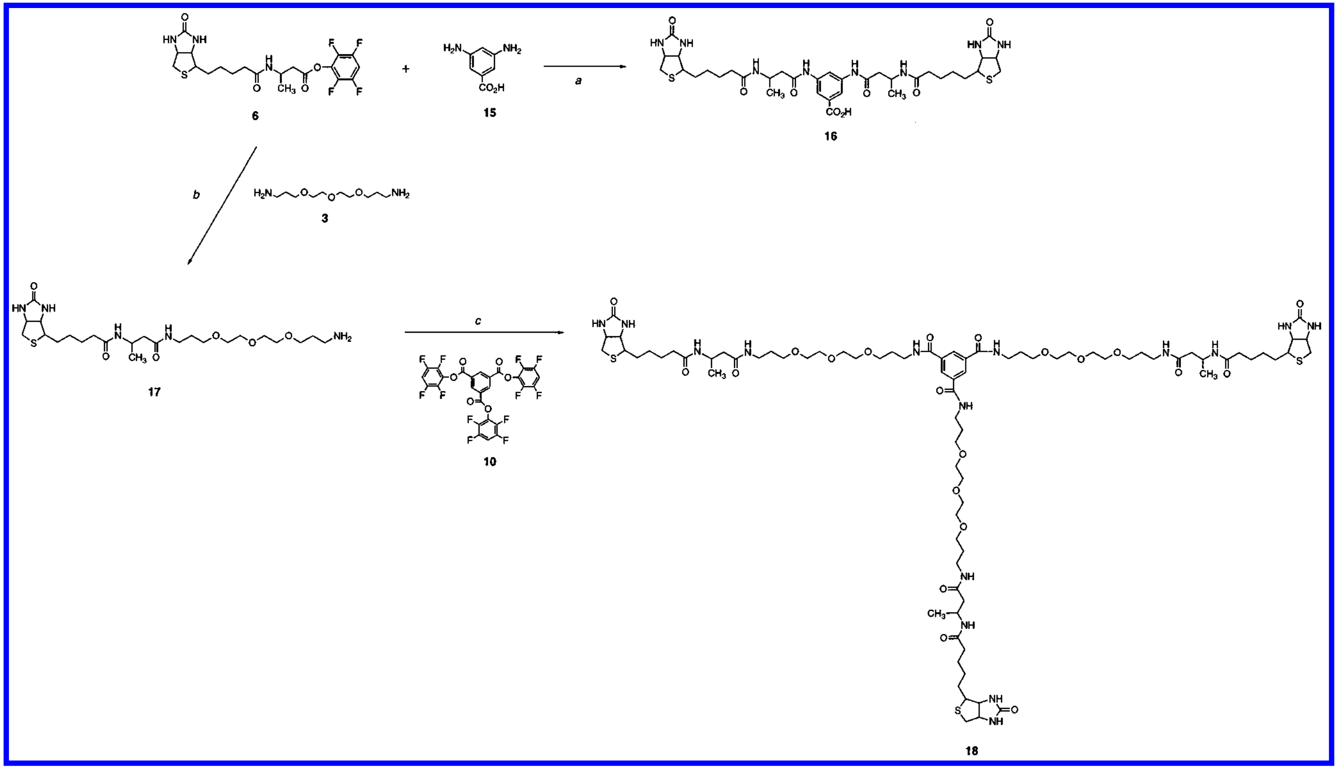
Scheme 2. Synthesis of Oxadiamine-Linked Biotin Trimers[a]



[a] (a) **9**, CH_2Cl_2, TFP-OH, pyridine, rt, 4 h, 94%; (b) **11**, DMF, rt, 12 h, 57%; (c) **12**, DMF, rt, 12 h, 71%.

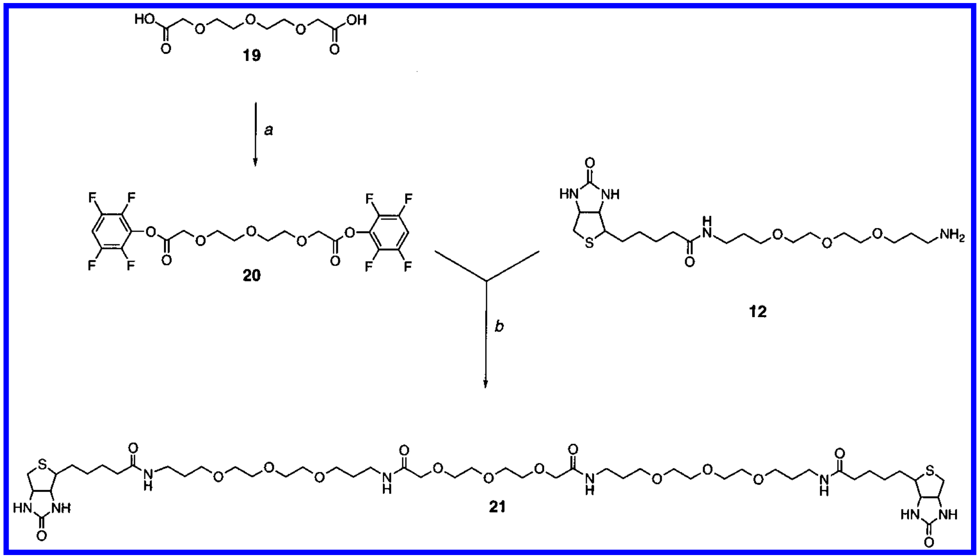
the conditions studied. A second experiment involving [^{125}I]SAv binding in SAv-coated wells was conducted with biotin dimers **5**, **8**, **16**, and **21** and biotin trimers **13**, **14**, and **18**. That experiment was conducted to examine cross-linking of dimers **16** and **21**, which had not been evaluated in the first experiment, and to determine if higher quantities of [^{125}I]SAv would bind when incubated for 30 min at 37 °C with some of the previously tested biotin derivatives. The data obtained in that experiment are plotted in Figure 5B. Biotin dimers **16** and **21** did cross-link r-[^{125}I]SAv (Figure 5B, first addition), but the amount is quite small and is mostly obscured by a high nonspecific binding of r-[^{125}I]SAv brought about by the longer incubation time. More importantly, the picomoles of [^{125}I]SAv bound by cross-linking with biotin trimers increased significantly. While optimization of the poly-

Scheme 3. Synthesis of 3-Aminobutyric Acid-Derivatized Biotin Dimer and Trimer[a]



[a](a) **15**, DMF, Et_3N, 60 °C, 15 h, 40%; (b) **3**, DMF, Et_3N, rt, 30 min, 90%; (c) **10**, DMF, Et_3N, 40%.

Scheme 4. Synthesis of a Long Oxadiamine Biotin Dimer[a]



[a] (a) DMF, TFP-OH, EDC, rt, 16−24 h, 76%; (b) **20**, DMF, Et_3N, rt, 4 h, 42%.

styrene well binding assay was not a focus of these studies, it seems likely that the nonspecific binding of [^{125}I]SAv could be decreased by including a small amount (i.e. 0.1%) of BSA in the plate washing solution (as is done in antibody-based applications of the Reacti-Bind plates).

The quantities of biotin derivatives and [^{125}I]SAv used in the SAv-coated well assay were based on having an excess (i.e. 4×) of the 25 pmol of biotin binding capacity per well that the manufacturer's literature listed. If 25 pmol of biotin could bind, there should be the equivalent of 6.1 pmol of streptavidin in each well. Since binding with a biotin trimer could potentially produce twice the picomoles of bound SAv (assuming two biotins from each trimer bound), then there should be about 12.2 pmol of [^{125}I]SAv binding capacity in the wells. In our initial binding experiment (Figure 5A), only about 2.5 pmol of [^{125}I]SAv bound in the first addition, and the quantity per addition decreased with subsequent additions. When the plates were incubated for a longer period of time, the quantity of the first addition doubled to approximately 5.5 pmol of [^{125}I]SAv bound, but this was still less than expected. These results were perplexing since a large excess of biotin derivative and [^{125}I]SAv had been added in each step. Therefore, some assays were conducted to examine the number of biotin binding sites available per well using a radioiodinated biotin derivative, [^{125}I]**23b**. In one assay, 100 pmol of [^{125}I]**23b** was added to each of eight SAv coated wells, incubated at 37 °C for 10 min, then rinsed, and counted. The value obtained was 4.16 ± 0.19 pmol of biotin binding/well. Repeat of this assay at 37 °C for 30 min yielded a value of 4.33 ± 0.11 pmol/well, and conducting the assay at rt for 10 min yielded a

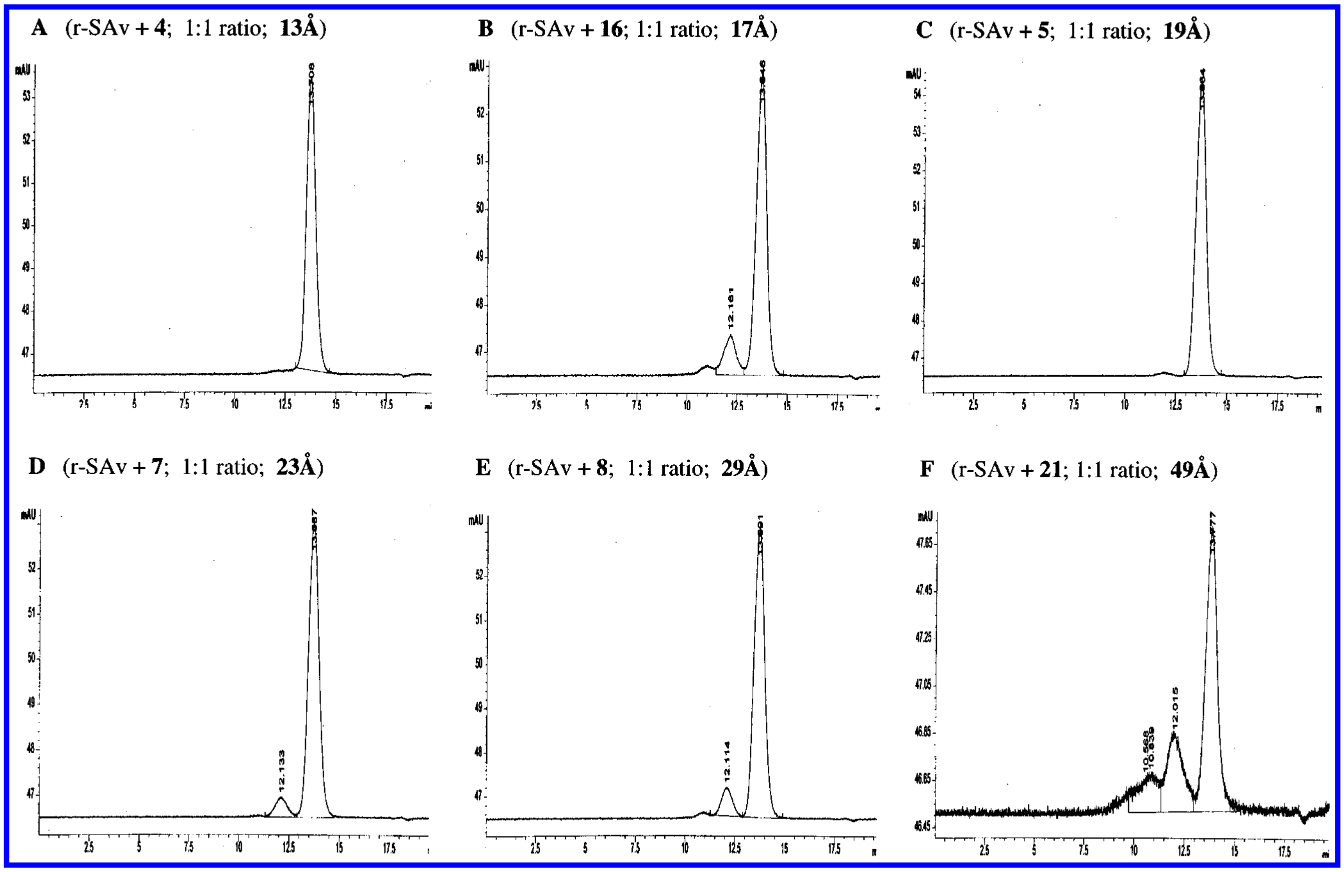


Figure 1. Size exclusion chromatograms of reaction mixtures after mixing r-SAv with biotin dimers **4**, **5**, **7**, **8**, **16**, and **21** at rt for 30 min. The chromatograms are shown in the order (A−F) of the length of the spacer between the biotin carboxylate carbon atoms (distances shown in parentheses). All chromatograms were obtained after addition of 1 molar equiv of the biotin dimer to 50 μg of r-SAv in 100 μL of PBS. A chromatogram (not shown) of r-SAv which had not been mixed with a biotin derivative was identical to that seen in panel A. See Chromatography in Experimental Procedures for details of the HPLC equipment and conditions used.

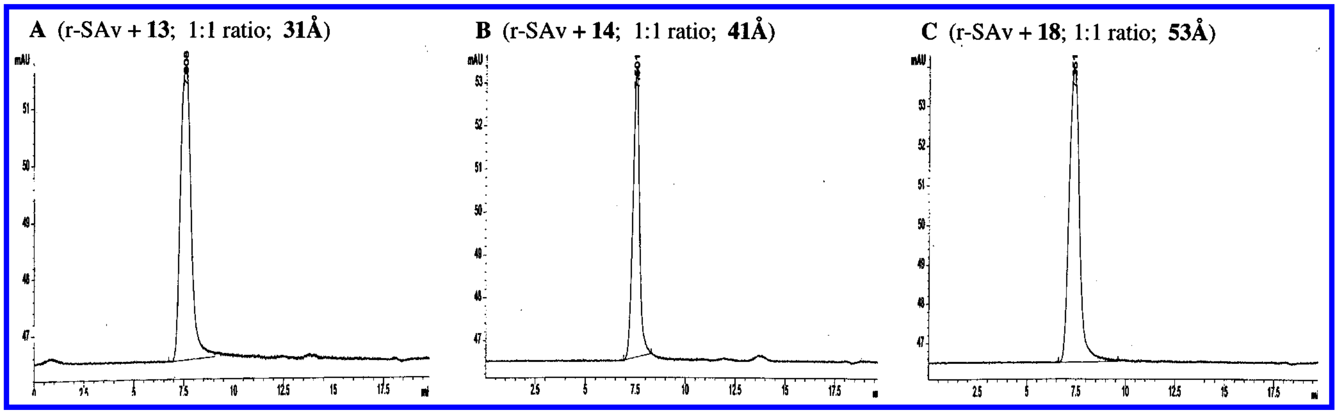


Figure 2. Size exclusion chromatograms of reaction mixtures after mixing r-SAv with biotin trimers **13**, **14**, and **18** at rt for 30 min. The chromatograms are shown in the order (A−C) of the length of the spacer between the biotin carboxylate carbon atoms (distances shown in parentheses). All chromatograms were obtained after addition of 1 molar equiv of the biotin trimer to 50 μg of r-SAv in 100 μL of PBS. The retention time (t_R) of monomeric r-SAv under the same HPLC conditions was 13.7 min. See Chromatography in Experimental Procedures for details of the HPLC equipment and conditions used.

value of 4.39 ± 0.16 pmol/well. From this data, a value of 4.2 pmol biotin binding/well was used to assess the results from the cross-linking experiments. Considering that half the amount of biotin trimers can bind as biotin monomers, one might expect 2.1 pmol of r-[^{125}I]SAv to saturate its binding, which is very close to the amount obtained with an incubation at 37 °C for 10 min (Figure 5A). It is difficult to explain the 2 times higher binding observed when the solution is incubated at 37 °C for 30 min. However, one explanation might be that an extended incubation period produced higher nonspecific binding of the r-[^{125}I]SAv and/or biotin derivative and that these residual materials could have resulted in cross-linking in excess of the picomoles of SAv bound. Nonspecific binding of [^{125}I]SAv is shown in Figure 5B (lane 1, after addition of biotin monomer **23a**). Nonspecific binding of [^{125}I]**23b** was measured as described above for assessment of picomoles of biotin binding/well, except the biotin binding sites were saturated by incubating the bound SAv with an excess of biotin at rt or at 37 °C for 10 or 30 min prior to incubating with [^{125}I]**23b**. This assay indicated that the amount of nonspecific biotin binding, as assessed with [^{125}I]**23b**, increased with temperature and time. When the incubation was conducted at rt for 10 min, there was approximately 14% [^{125}I]**23b** nonspecifically bound; at 37 °C for 10 min, 19% [^{125}I]**23b** was nonspecifically bound, and at 37 °C for 30 min, 25% [^{125}I]**23b** was nonspecifically bound.

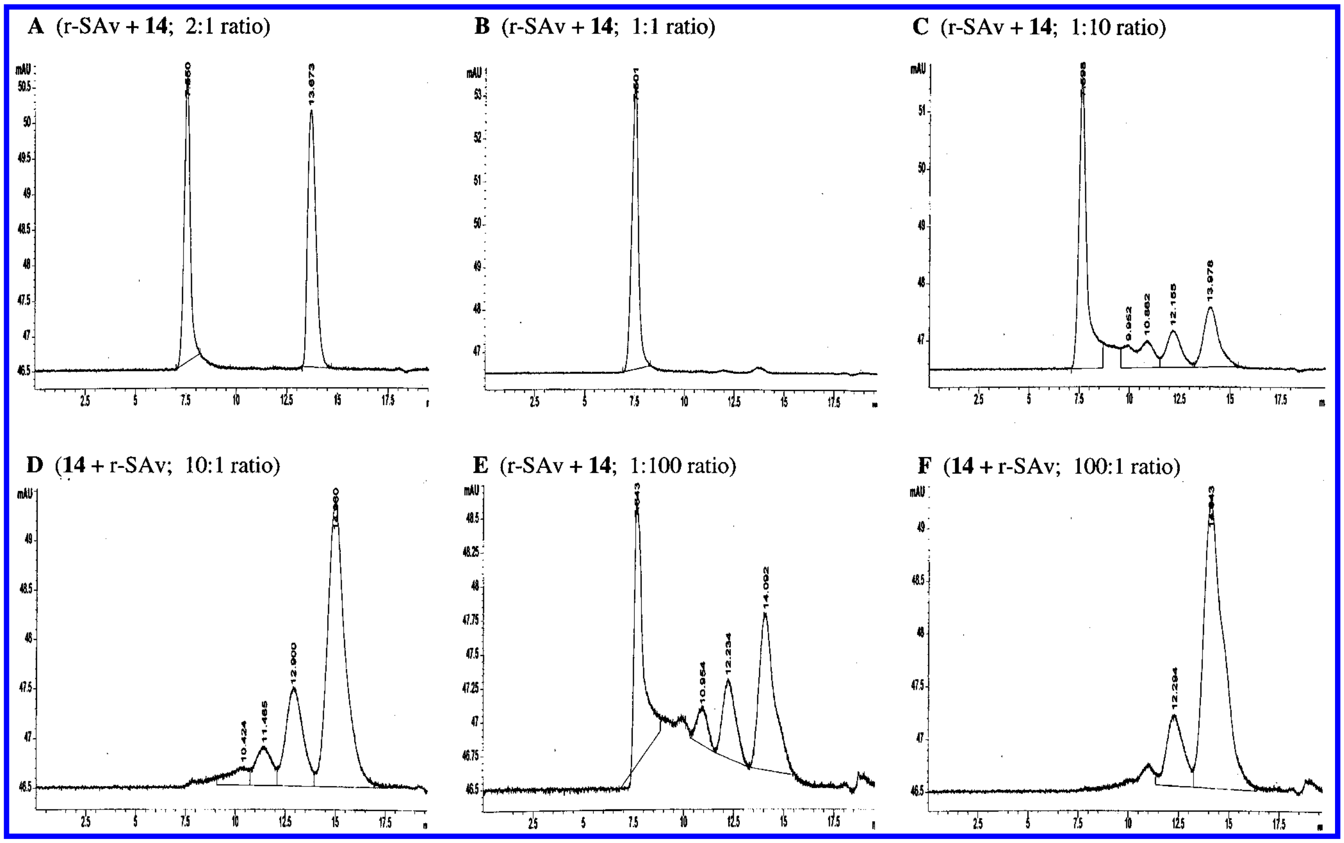


Figure 3. Size exclusion chromatograms of reaction mixtures after mixing r-SAv with varying molar quantities of biotin trimer **14** and reversing the order of addition of reagents. In chromatograms A–C and E, varying quantities of **14** were rapidly added to 50 μg of r-SAv in 100 μL of PBS. In chromatograms D and F, a solution containing 50 μg of r-SAv in 100 μL of PBS was slowly added to a 10× or 100× molar quantity of **14**. See Chromatography Experimental Procedures for details of the HPLC equipment and conditions used.

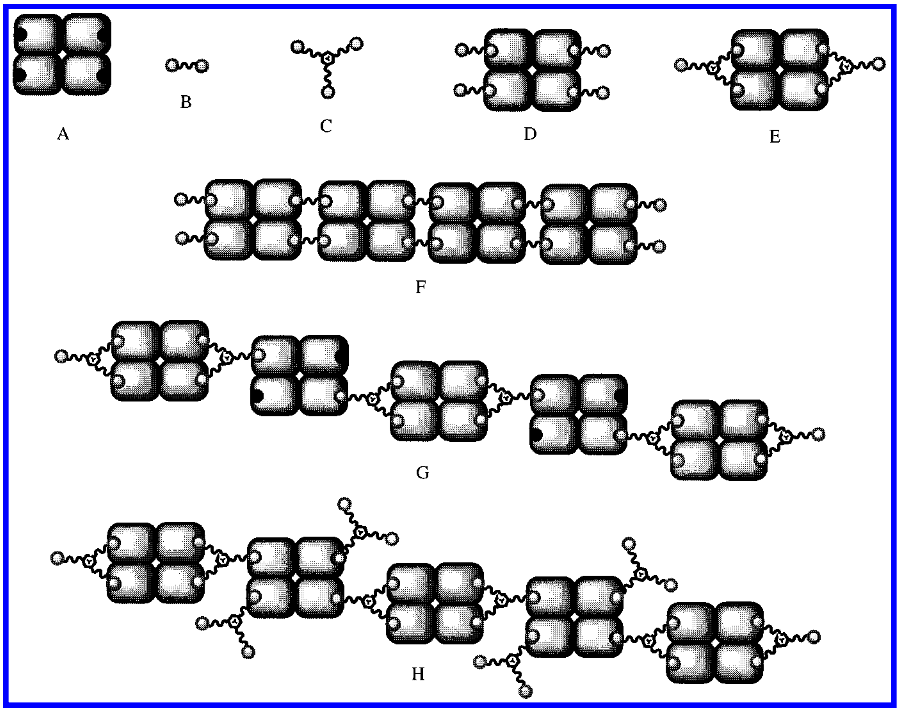


Figure 4. Schematic representations of biotinylated and cross-linked SAv or Av. Schematic A represents Av or SAv with four biotin binding sites (black circles). The representation does not try to show the 222 symmetry of these molecules. Schematic B represents a biotin dimer molecule. Schematic C represents a biotin trimer molecule. Schematic D represents perbiotinylation of Av or SAv with a biotin dimer which is too short to undergo intramolecular binding of the two biotin moieties. Schematic E represents perbiotinylation of Av or SAv with a biotin trimer. Schematic F represents cross-linking or polymerization of Av or SAv with a biotin dimer (having the correct spacing between biotin carboxylate carbons). Schematic G is a representation of cross-linking or polymerization of Av or SAv with 1 molar equiv of a biotin trimer. Schematic H illustrates the potential for branching when an excess of biotin trimer is used for polymerization (it is unclear that this is possible on the basis of molecular modeling).

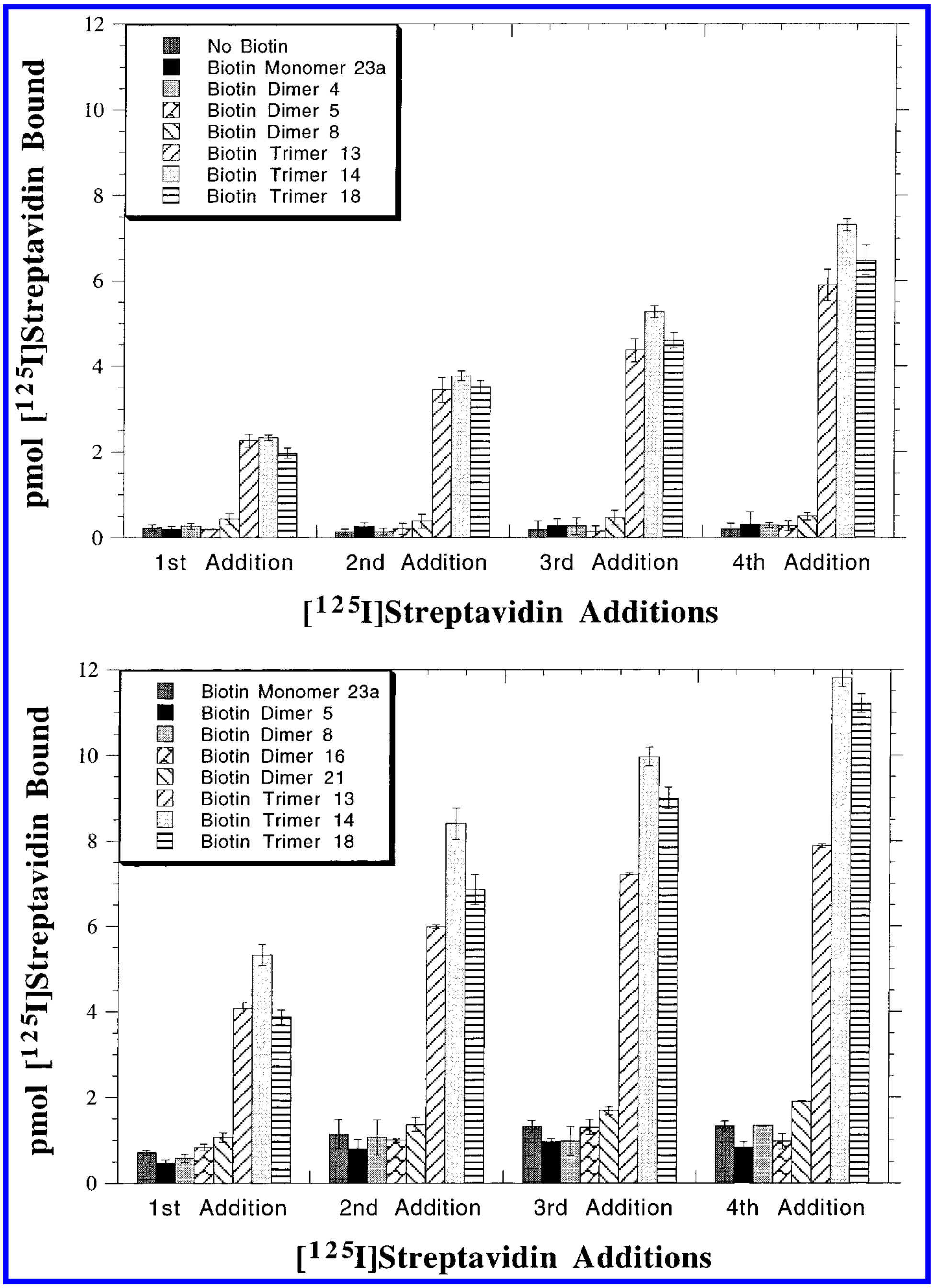


Figure 5. Graph of data obtained for cross-linking of polystyrene-bound SAv and r-[^{125}I]SAv with biotin derivatives at 37 °C for 10 min (top, A) and 30 min (bottom, B). Data were obtained from triplicate runs and are plotted as average ± standard deviation. The wells had an average ($n = 8$) of 4.2 pmol of biotin binding capacity (as measured by [^{125}I]**23b**) which equates to 1 pmol of SAv bound. An excess of biotin derivative was added and rinsed with PBS, and then an excess of [^{125}I]SAv was added and rinsed with PBS. This procedure was repeated four times, with the only change being the number of additions of the biotin derivative (i.e. all wells received four additions of [^{125}I]SAv). When one, two, or three additions of biotin derivative were made, 100 μL of PBS was added in the place of the biotin derivative to keep the number of additions and rinses constant. All additions and rinses were made in a volume of 100 μL.

DISCUSSION

The rationale for developing tumor pretargeting methods is based on the fact that directly labeled monoclonal antibodies are limited in their application to cancer therapy due to some inherent problems (*15, 25*) and that an alternative approach which circumvents some of those problems might have broad application (*2*). Importantly, the tumor pretargeting approach to cancer therapy has been successfully demonstrated in preclinical studies and is now undergoing clinical evaluation. It should be noted that development of tumor pretargeting methods is more difficult as there are more reagents and more steps involved. However, this increased complexity also offers the advantage of being able to optimize the reagents and conditions used. Two important factors in the tumor pretargeting approach are the choice of the molecule to be used to carry the radionuclide and the choice of the antibody conjugate that it will bind. We, and other investigators, have chosen to employ the biotin/(strept)avidin technology (*26*−*28*) in tumor pretargeting, as biotin binds streptavidin and avidin with a very high affinity (e.g. 10^{-13}−10^{-15} M^{-1}), resulting in essentially irreversible binding.

In this investigation, we sought to gain information which would aid in the development of new biotin reagents designed to increase the amount of radionuclide (attached to a biotin moiety) on cancer cells. We hypothesized that it might be possible to increase the amount of radioactivity bound to cancer cells in antibody pretargeting protocols by utilizing the tetravalent binding nature of r-SAv (or Av). The hypothesis was that a radiolabeled molecule which contained at least two biotin moieties could be administered to bind with antibody−SAv conjugates prelocalized at tumors, leaving some biotin moieties unbound, and subsequent administration of nonlabeled SAv would provide new binding sites for the radiolabeled biotin derivative. Repeat administration of the radiolabeled biotin cross-linking reagent and SAv would again provide more binding sites for radiolabeled biotin, and this process could potentially be repeated many times.[4] Thus, each administration of radiolabeled biotin dimer or trimer could add radioactivity to the cells pretargeted with antibody. After consideration of the factors involved, it was apparent that investigation of our hypothesis could only be accomplished if the appropriate radiolabeled biotin derivatives were made.

Few investigations involving small molecules containing more than one biotin have been reported.[5] However, in one particularly relevant investigation, Green et al. described the use of bifunctional biotin derivatives to probe the nature of subunits in Av (*20*). The bifunctional biotin derivatives evaluated were dimers of biotin or biotinamidobutyric acid produced by reaction of biotin-mixed anhydride with diaminoalkane linkers of various lengths. From their study, it was concluded that (1) the dimensions of Av were 55 Å × 55 Å × 41 Å, (2) the biotin binding site was about 9 Å below the surface of Av, (3) linkers smaller than 15 Å did not permit the second biotin molecule to project far enough from the surface of Av to reach a biotin binding site on another Av molecule, (4) linkers of 15−17.5 Å between biotin moieties (carboxylate carbons) resulted in weak binding of the second biotin, and (5) a linker of 18.4 Å cross-linked Av tightly whereas linkers longer than 20 Å resulted in intramolecular binding of the second biotin. The linker lengths in biotin dimers reported by Green et al. were used as a guide for designing new biotin dimers.

Although it was apparent from the studies of Green et al. (*20*) that the biotin dimers had quite a low water solubility, we were not concerned initially about that parameter in the design of new biotin derivatives. However, it became apparent soon after our initial synthetic efforts that biotin dimers which were linked by aliphatic molecules were highly insoluble in many solvents, including water. For example, a biotin dimer synthesized by cross-linking 5-(iodobenzoyl)aminoisophthalate with 1,3-diaminopropane had a water solubility so low that it was barely detectable by HPLC (i.e. <1 μg/mL). Another biotin dimer, prepared by cross-linking two biotin moieties with 1,12-diaminododecane, had an even lower aqueous solubility. These water solubilities are much lower than biotin's aqueous solubility under the same conditions (e.g. 0.22 mg/mL) (*30*), presumably due to the fact that the ionization of biotin carboxylate aids in its solubilization. Since the biotin dimers and trimers were being developed for potential injection into patients, we decided to incorporate water-solubilizing functional groups into their design so that they could be administered in aqueous solutions. Previous studies by our group had shown that ether-containing linkers helped solubilize radiolabeled biotin derivatives (*23*) and biotinylation reagents (*14*, *29*) for use in aqueous media. Thus, 2,2′-(ethylenedioxy)bis(ethylamine) (**2**) and 4,7,10-trioxa-1,13-tridecanediamine (**3**) were used in the design of most biotin derivatives synthesized in the study. The use of the ether-containing linkers greatly improved the aqueous solubility of biotin dimers and trimers over the use of aliphatic linkers. For example, biotin dimer **5**, which has a linker length similar to that of the biotin dimer containing diaminododecane (i.e. 19 vs 18 Å), has an estimated solubility of approximately 9 mg/mL[6] at ambient temperature, and biotin trimer **14**, which uses the same linker molecule, has a water solubility of approximately 25 mg/mL.[6]

Biotin dimers **4** and **5** were the first water-solubilized biotin derivatives synthesized (Scheme 1) and evaluated for *in vitro* polymerization of r-SAv. On the basis of the previous studies by Green et al. (*20*), dimer **4**, which has a 13 Å distance between biotin carboxylate carbons, was not expected to polymerize SAv, but dimer **5**, which has a 19 Å linker, was expected to polymerize SAv. However, neither dimer polymerized SAv to any extent (Figure 1A,B). We felt that the length of the linker molecule might be too short; therefore, we prepared dimer **7** in which the distance between carboxylate carbons was 23 Å. We also prepared biotin dimer **8** to evaluate the effect of an even longer (29 Å) linker. The biotinamidobutyrate TFP ester (**6**) was chosen to extend the distance between the biotin carboxylates in **7** and **8** as it had been previously prepared for studies on stability toward biotinidase cleavage (*23*). Although neither dimer polymerized r-SAv, some cross-linking could be noted as small peaks at higher molecular weight (i.e. 12.1 and 10.6 min).

The failure to obtain polymerization of SAv with biotin dimers led to consideration of molecules which contained more than two biotin moieties. Initial consideration was to prepare molecules with four biotin moieties, but it was expected that this would produce linear polymers, limiting the amplification possibilities. However, consideration of molecules with three biotin moieties appeared attractive as one biotin binding site would be left open per cross-linking, and it was thought this might allow branching of the polymeric chain as depicted in Figure 4H. Since it was expected that two of the three biotin moieties in biotin trimers would bind with the same SAv molecule, it seemed that the distance between any two biotins might be >25 but <55 Å. Thus, biotin trimers **13** and **14** were synthesized (Scheme 2) and evaluated. Both of these compounds caused immediate polymerization of SAv in solution.

Although biotin trimers **13** and **14** readily polymerized SAv, we still wanted to find a biotin dimer which could polymerize SAv. Our desire to continue to look for biotin dimer was based on the facts that use of biotin trimers resulted in fewer radiolabeled biotin derivatives being bound per SAv molecule as three binding sites would be occupied and in biotin trimers the molecular weights are increased significantly over the dimers, potentially altering the desired renal clearance of biotin molecules to hepatobiliary. This prompted us to evaluate the distances between biotin carboxylates in SAv by molecular

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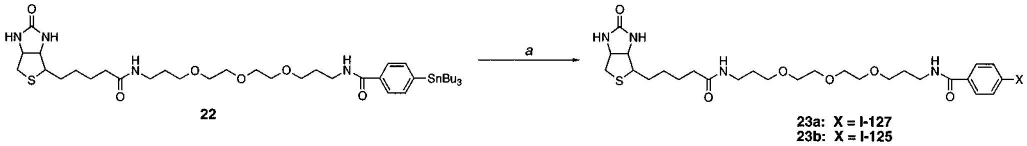
[4] A schematic representation of this multistep tumor pretargeting approach has been provided in ref 14.

[5] A biotin dimer used in antibody pretargeting (*1*), diethylenetriaminepentaacetic acid α,ω-bis(biocytinamide) (DTPA−biotin), is available from Sigma Chemical Co. This biotin dimer has a distance of approximately 32 Å between the biotin carboxylate carbons when fully extended. It would be expected to undergo intramolecular binding, resulting in both biotin moieties bound to the same SAv molecule (even when the distance between biotin moieties is reduced by metal complex formation).

[6] The aqueous solubility values must be considered crude estimates as the experiments were not conducted in a rigorous manner.

Scheme 5. Preparation of a Radioiodinated Biotin Monomer[a]



[a] (a) NCS, NaI, MeOH, 1−5% HOAc.

modeling methods. Molecular modeling of a SAv crystal structure which contains four bound biotin molecules indicated that the biotin carboxylate carbons for biotins on the same face were approximately 20 Å apart and that it is over 60 Å around the molecule to another biotin binding site.[7] Green et al. (*20*) reported that the Av molecule appeared to compress 2−3 Å on cross-linking. Although such a compression of Av has not been confirmed, the dynamic nature of the protein is likely to make it possible for the 19.4 Å distance in **5** to be long enough to bind intramolecularly. Therefore, we became interested in synthesizing a biotin dimer which had a distance between biotin carboxylate carbons >15 but <19 Å. Synthesis of a biotin dimer with a linker arm between 15 and 19 Å in length could not be readily achieved with an ether-containing linker. However, biotin dimer **16** which contained a carboxylate to aid in water solubilization was obtained by cross-linking **6** with 3,5-diaminobenzoic acid (Scheme 3). In **16**, the biotin carboxylate carbons are approximately 17 Å apart. Examination of a mixture of r-SAv and **16** in solution indicated that a higher percentage of cross-linking[8] was obtained with it than was obtained with the longer biotin dimer **5**, or the shorter biotin dimer **4**. However, the amount of cross-linking obtained was only about 10%, and no polymerization was noted (Figure 1E). Although there was little optimism for finding a biotin dimer that efficiently cross-linked r-SAv, it had been noted that the increasing length of biotin dimers **7** and **8** had shown a trend toward an increased percentage of cross-linking r-SAv (Figure 1C,D). Therefore, an additional biotin dimer, **21**, which had a much longer linker distance (49 Å) was prepared (Scheme 4) and evaluated. The percentage of r-SAv cross-linked[8] with **21** was found to be higher (40%), but no polymer was obtained. It seems that it will be difficult, if not impossible, to develop biotin dimers that have a long enough spacer to allow intermolecular binding of the second biotin moiety with another SAv molecule without also being long enough to permit the more facile intramolecular binding of the second biotin with the same SAv molecule. On the basis of the results obtained, we must conclude that biotin dimers will not be useful for rapid, facile polymerization of SAv and, thus, that further studies with biotin dimers for application to *in vivo* tumor targeting are not warranted.

A desire to examine the effect of an increased linker length on cross-linking led to the synthesis of a third biotin trimer, **18** (Scheme 3). This trimer was prepared to evaluate the effect on r-SAv polymerization with a distance between two biotin moieties of approximately 53 Å. This molecule was of interest as the biotin moieties were separated by a distance that seemed unlikely to result in all three biotin moieties binding with the same r-SAv molecule. Indeed, when a solution with a 1/1 ratio of **18**/r-SAv was mixed, only polymerization was observed (Figure 2C). This result indicates that the third (unbound) biotin moiety is not capable of binding on the opposite face of the same r-SAv molecule as our modeling predicted. However, the extra length did not appear to increase cross-linking of r-[^{125}I]SAv with polymer-bound SAv in wells as the percentages bound (Figure 5) were generally less than those observed for the biotin trimer **14**.

Evaluation of the polymerization of SAv in solution was an important step to undertake as it clearly showed the effects of distances between biotin carboxylate carbons, dilution, and varying quantities of biotin compounds on the polymerization process. However, we felt that a better *in vitro* model for the application of the biotin dimers and trimers *in vivo* would be one that would demonstrate an increase in the amount of radioactivity localized to a surface containing bound SAv. The model chosen was SAv-coated polystyrene plates. In that system, the various biotin dimers and trimers (added in excess) could saturate the biotin binding sites, and then [^{125}I]SAv could be added to cross-link with available biotin moieties, resulting in a new layer of biotin binding sites. Indeed, SAv-bound wells were a good model as the cross-linking results correlated with the results obtained in solution, and it was easy to detect an increase of the amount of radioactivity bound with each cycle of reagents. The magnitude of increase was less than expected after an incubation for 10 min at 37 °C on the basis of the manufacturer's cited 25 pmol/well biotin binding capacity. However, after a biotin binding capacity of approximately 4.2 pmol/well was measured, the data obtained indicate that at least 1 equiv of r-[^{125}I]SAv was added in the first cross-linking step. Although less r-[^{125}I]SAv was added in each subsequent addition, the quantity that was added was significant. Assessing the results of the binding to SAv in polystyrene wells was complicated by issues of nonspecific binding of reagents. As our goal was simply to demonstrate that cross-linking can be achieved, optimization of the conditions to decrease the nonspecific binding has not been done. It is clear that increasing the incubation time to 30 min increased the percentage of biotin derivative and r-[^{125}I]SAv bound nonspecifically. Further, no difference in biotin binding was noted when the wells were incubated at room temperature or at 37 °C. Therefore, it seems likely that the nonspecific binding could be decreased by conducting the incubations at room temperature. Irrespective of the issues of absolute quantities bound and nonspecific binding, the fact that an increase of radioactivity bound was obtained in each step is very encouraging for continued studies toward *in vivo* application. To our knowledge, this is the first demonstration of a biotin-

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[7] This estimation was made by adding distances between atoms on the surface of the streptavidin molecule from a biotin carboxylate on one face to that of another biotin on the opposite face. Molecular modeling was conducted on a Silicon Graphics, IRIS INDIGO computer running Insight II software (version 2.3.7).

[8] The percentage of cross-linking refers to the area under peaks eluting between 8 and 12.5 min, presumably the SAv dimer and trimer, relative to the total peak area included in those peaks plus the monomeric SAv peak (t_R = 13.7 min) on size exclusion HPLC chromatograms (see Figure 1). The percentage of polymerization found refers to the percentage of very high-molecular weight species eluting with the exclusion volume at 7.6 min.

containing small molecule being able to increase the amount of SAv bound to a surface.

We undertook this study to gain information on the design of biotin derivatives that could be used with r-SAv to increase the amount of radioactivity bound to cancer cells in tumor pretargeting protocols. Although the radioactivity will be delivered to the cancer cells as a radiolabeled biotin derivative, the compounds in this study were not designed with iodine-containing moieties attached as it was felt that those compounds would be more difficult to synthesize, and likely be less water soluble than those investigated. Now that we have gained the information sought, studies are planned to prepare a radiolabeled biotin trimer and evaluate its *in vitro* and *in vivo* cross-linking with r-SAv. As shown in this study, the distance between biotin moieties is very important, but other molecular design features such as inclusion of functional groups to block the amide-cleaving action of serum biotinidase are also critical (*23*). Additional modifications such as preparing water-solubilized biotin derivatives that contain ionic charged functional groups are planned.

Summary. Six molecules containing two biotin moieties (biotin dimers **4**, **5**, **7**, **8**, **16**, and **21**) and three molecules containing three biotin moieties (biotin trimers **13**, **14**, and **18**) were synthesized and characterized. Investigation of the polymerization or cross-linking of r-SAv in solution and bonding to a solid support was carried out on each biotin derivative prepared. None of the biotin dimers polymerized r-SAv in solution, but some cross-linking to form small adducts (e.g. dimers, trimers, etc.) was noted with biotin dimers having linking moieties of 17 and ≥23 Å. Contrary to this, the three biotin trimers synthesized completely polymerized r-SAv in solution when a 1/1 molar ratio of reagents was used. Similar results were obtained in the surface-bound SAv cross-linking experiments as had been observed for polymerization of SAv in solution. On the basis of the results obtained in this study, it seems unlikely that biotin dimers will be found which effectively cross-link SAv, but biotin trimers should be useful for both *in vitro* and *in vivo* applications which require cross-linking of SAv or Av.

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Supporting Information Available: HPLC chromatograms of compounds **1**, **4**−**8**, **10**−**14**, **17**, **18**, **20**, and **21** and ^{1}H NMR spectra of the new compounds prepared in the research described in this paper (20 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

(1) Hnatowich, D. J., Virzi, F., and Rusckowski, M. (1987) Investigations of Avidin and Biotin for Imaging Applications. *J. Nucl. Med. 28*, 1294−1302.

(2) Goodwin, D. A. (1995) Tumor Pretargeting: Almost the Bottom Line. *J. Nucl. Med. 36*, 876−879.

(3) Paganelli, G., Malcovati, M., and Fazio, F. (1991) Monoclonal antibody pretargetting techniques for tumor localization: the avidin-biotin system. *Nucl. Med. Commun. 12*, 211−234.

(4) Axworthy, D. B., Fritzberg, A. R., Hylarides, M. D., Mallett, R. W., Theodore, L. J., Gustavson, L. M., Su, F., Beaumier, P. L., and Reno, J. M. (1994) Preclinical Evaluation of an Anti-tumor Monoclonal Antibody/Streptavidin Conjugate For Pretargeted ^{90}Y Radioimmunotherapy in a Mouse Xenograft Model. *J. Immunother. 16*, 158.

(5) del Rosario, R. B., and Wahl, R. L. (1993) Biotinylated Iodo-Polylysine for Pretargeted Radiation Delivery. *J. Nucl. Med. 34*, 1147−1151.

(6) Jeong, J. M., Kinuya, S., Paik, C. H., Saga, T., Sood, V. K., Carrasquillo, J. A., Neumann, R. D., and Reynolds, J. C. (1994) Application of High Affinity Binding Concept to Radiolabel Avidin with Tc-99m Labeled Biotin and the Effect of pI on Biodistribution. *Nucl. Med. Biol. 21*, 935−940.

(7) Oehr, P., Westermann, J., and Biersack, H. J. (1988) Streptavidin and Biotin as Potential Tumor Imaging Agents. *J. Nucl. Med. 29*, 728−729.

(8) Pimm, M. V., Fells, H. F., Perkins, A. C., and Baldwin, R. W. (1988) Iodine-131 and indium-111 labelled avidin and streptavidin for pre-targetted immunoscintigraphy with biotinylated anti-tumour monoclonal antibody. *Nucl. Med. Commun. 9*, 931−941.

(9) Hashmi, M., and Rosebrough, S. F. (1995) Synthesis, Pharmacokinetics, and Biodistribution of ^{67}Ga Deferoxamineacetyl-Cysteinylbiotin. *Drug Metabol. Dispos. 23*, 1362−1367.

(10) Shoup, T. M., Fischman, A. J., Jaywook, S., Babich, J. W., Strauss, H. W., and Elmaleh, D. R. (1994) Synthesis of Fluorine-18-Labeled Biotin Derivatives: Biodistribution and Infection Localization. *J. Nucl. Med. 35*, 1685−1690.

(11) Yao, Z., Zhang, M., Kobayashi, H., Sakahara, H., Nakada, H., Yamashina, I., and Konishi, J. (1995) Improved Targeting of Radiolabeled Streptavidin in Tumors Pretargeted with Biotinylated Monoclonal Antibodies through an Avidin Chase. *J. Nucl. Med. 36*, 837−841.

(12) Khawli, L. A., Alauddin, M. M., Miller, G. K., and Epstein, A. L. (1993) Improved Immunotargeting of Tumors with Biotinylated Monoclonal Antibodies and Radiolabeled Streptavidin. *Antibody, Immunoconjugates, Radiopharm. 6*, 13−27.

(13) Kassis, A. I., Jones, P. L., Matalka, K. Z., and Adelstein, S. J. (1996) Antibody-Dependent Signal Amplification in Tumor Xenografts after Pretreatment with Biotinylated Monoclonal Antibody and Avidin or Streptavidin. *J. Nucl. Med. 37*, 343−352.

(14) Wilbur, D. S., Hamlin, D. K., Vessella, R. L., Stray, J. E., Buhler, K. R., Stayton, P. S., Klumb, L. A., Pathare, P. M., and Weerawarna, S. A. (1996) Antibody Fragments in Tumor Pretargeting. Evaluation of Biotinylated Fab′ Colocalization with Recombinant Streptavidin and Avidin. *Bioconjugate Chem. 7*, 689−702.

(15) Fritzberg, A. R., Beaumier, P. L., Bottino, B. J., and Reno, J. M. (1994) Approaches to improved antibody- and peptide-mediated targeting for imaging and therapy of cancer. *J. Controlled Release 28*, 167−173.

(16) Green, N. M. (1963) Avidin. 3. The Nature of the Biotin-Binding Site. *Biochem. J. 89*, 599−609.

(17) Green, N. M. (1975) Avidin. *Adv. Protein Chem. 29*, 85−133.

(18) Green, N. M. (1990) Avidin and Streptavidin. *Methods Enzymol. 184*, 51−67.

(19) Paganelli, G., Riva, P., Deleide, G., Clivio, A., Chiolerio, F., Scassellati, G. A., Malcovati, M., and Siccardi, A. G. (1988) In Vivo Labelling of Biotinylated Monoclonal Antibodies by Radioactive Avidin: A Strategy to Increase Tumor Radiolocalization. *Int. J. Cancer 2*, 121−125.

(20) Green, N. M., Konieczny, L., Toms, E. J., and Valentine, R. C. (1971) The Use of Bifunctional Biotinyl Compounds to Determine the Arrangement of Subunits in Avidin. *Biochem. J. 125*, 781−791.

(21) Gamper, H. B., Reed, M. W., Cox, T., Virosco, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Meyer, R. B. (1993) Facile preparation of nuclease resistant 3′ modified oligodeoxynucleotides. *Nucleic Acids Res. 21*, 145−150.

(22) Chilkoti, A., Tan, P. H., and Stayton P. S. (1995) Site Directed Mutagenesis Studies of the High-Affinity Streptavidin-Biotin Complex: Contributions of Tryptophan Residues 79, 108 and 120. *Proc. Natl. Acad. Sci. U.S.A. 92*, 1754−1758.

(23) Wilbur, D. S., Hamlin, D. K., Pathare, P. M., and Weerawarna, S. A. (1997) Synthesis, Radioiodination and *In Vitro* Evaluation of Water Soluble, Biotinidase Resistant Biotin Derivatives. *Bioconjugate Chem. 8*, 572−584.

(24) Dressendorfer, R. A., Heim, J.-M., Gerzer, R., and Strasburger, C. J. (1995) A Non-Isotopic Immunoassay for Guanosine 3′,5′-Cyclic Monophosphate Using a Cyclic GMP-Biotin Conjugate as Tracer. *J. Immunoassay 16*, 37−53.

(25) Buchsbaum, D. J. (1995) Experimental Approaches to Increase Radiolabeled Antibody Localization in Tumors. *Cancer Res. 55*, 5729s−5732s.

(26) Wilchek, M., and Bayer, E. A. (1990) Introduction to Avidin-Biotin Technology. *Methods Enzymol. 184*, 5−45.

(27) Diamandis, E. P., and Christopoulos, T. K. (1991) The Biotin-(Strept)Avidin System: Principles and Applications in Biotechnology. *Clin. Chem. 37*, 625−636.

(28) Wilchek, M., and Bayer, E. A. (1988) The Avidin-Biotin Complex in Bioanalytical Applications. *Anal. Biochem. 171*, 1−32.

(29) Wilbur, D. S., Hamlin, D. K., Pathare, P. M., Vessella, R. L., Buhler, K. R., Stray, J. E., Stayton, P. S., and Klumb, L. A. (1996) Evaluation of the Effect of Serum Biotin and a Water Soluble, Biotinidase Stabilized, Biotinylation Reagent on the Co-Localization of Fab′ and Streptavidin in Tumor Xenografts. *Tumor Targeting 2*, 158.

(30) Budavari, S., O'Neil, M. J., and Smith, A., Eds. (1989) *The Merck Index*, Merck & Co., Inc., Rahway, NJ.

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