Bioconjugate
Chemistry

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Thin Layer Chromatography. Plastic sheets, precoated with 0.2 mm silica gel 60 without fluorescent indicator (Merck) were used. Eluents I, II and III contained chloroform/methanol/acetic acid (70/30/5, 70/26/4, and 100/30/2, respectively), eluent IV contained chloroform/methanol/water (70/26/4), and eluents V and VI contained chloroform/methanol/ammonia (70/26/4 and 100/30/2, respectively). Besides nonspecific staining with iodine vapor, amines were specifically detected with ninhydrin (Schuurmans-Stekhoven et al., 1992) and biotin derivatives were visualized by color reaction with DACA (McCormick & Roth, 1970). Boc-protected amines responded to ninhydrin after ~1 min delay at 100 °C, due to thermal deprotection.

Syntheses. General Precautions. Autoxidation of the primary amines used in this study had to be avoided by rigorous exclusion of ambient air and/or use of acidic conditions whenever possible. The second problem regularly encountered was hydrolysis of the amide bond between PEG and fluorophore residues as previously observed between PEG and the 3-(4-pyridyldithio)propionyl group (see manuscript #1). Paradoxically amide cleavage did not occur in water at pH ~7 but was very significant in chloroform solutions or in films dried from this solvent during storage at -25 °C. Pre-treatment of *all* chloroform quantities with basic alumina cured the problem. For long term storage, however, the biotin-PEG-fluorophore conjugates were lyophilized from dilute aqueous solutions. Fluorophores were protected from light as much as possible.

Synthesis of N-Boc-4,9-dioxa-1,12-diaminododecane·CH_3COOH. 6 g 4,9-dioxa-1,12-diaminododecane (29.4 mmol) were dissolved in 50 ml methanol and a solution of 6.2 g (28.4 mmol) Boc_2O in 20 ml methanol was slowly added while stirring (see above for general precautions). After overnight stirring under Ar 5 ml acetic acid and 100 ml toluene were added. The solution was concentrated to ~50 ml at 25 °C bath temperature, and toluene addition was repeated twice before the mixture was taken to dryness. Only in this way was cleavage of the Boc group completely suppressed. Yield: 10.5 g of crude product. 3 g of crude product were purified by chromatography on silica 60 (50 g), using chloroform/methanol/acetic acid mixtures for elution (250 ml 90/10/0.1 and 400 ml 70/30/5). Product fractions were taken to dryness by the "toluene procedure" as described above. In order to remove silica, the residue was redissolved in 15 ml water, saturated with NaCl, and extracted with chloroform (3 x 15 ml). The organic layers were dried with Na_2SO_4 and the solvent was removed. Yield 1.09 g (3.20 mmol), pure by TLC (R_f^I= 0.41, ninhydrin, iodine). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 1.41 (9H, s, t-butyl) 1.59 (4H, t, $\mathrm{OCH_2}\mathit{CH_2CH_2}\mathrm{CH_2O}$) 1.65-1.78 (2H, m, $\mathit{CH_2}\mathrm{CH_2NH}$-Boc) 1.94-2.07 (2H, m, $\mathit{CH_2}\mathrm{CH_2NH_3^+}$) 3.10-3.23 (2H, m, $\mathit{CH_2}\mathrm{NH}$-Boc) 3.38-3.48 (8H, m, 2 x $\mathit{CH_2}\mathrm{O}\mathit{CH_2}$) 3.56 (2H, t, $\mathit{CH_2}\mathrm{NH_3^+}$) 4.95 (1H, broad s, N*H*-Boc) 6.7 (3H, broad s, $\mathrm{N}\mathit{H_3}^+$).

Synthesis of N-Boc-N'-biotin-4,9-dioxa-1,12-diaminododecane. 627 mg N-Boc-4,9-dioxa-1,12-diaminododecane·HCl (1.8 mmol) were dissolved in 10 ml absolute DMF, 882 mg biotin-NHS (2.7 mmol) and 240 µl Et_3N were added (see above for general precautions). After overnight stirring 10 ml water were added and stirring was continued for 3 h. The solvent was removed at 1-10 Pa and the dry product was subjected to chromatography on silica 60 (eluent chloroform/methanol/acetic acid 120/30/0.5). Product fractions were taken to dryness by the "toluene procedure" (see above). Dry product was redissolved in chloroform and silica was removed by centrifugation. Yield: 590 mg (1.11 mmol), pure by TLC (R_f^I = 0.64, DACA, iodine, ninhydrin response after ~1min delay at 100 °C). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 1.41 (9H, s, t-butyl) 1.60-1.77 (12H, m, $\mathrm{OCH_2}\mathit{CH_2CH_2}\mathrm{CH_2O}$ + $\mathit{CH_2}\mathrm{CH_2NH}$-Boc + $\mathit{CH_2CH_2CH_2}\mathrm{CH_2CONH}$ from biotin side chain) 2.18 (2H, t, $\mathit{CH_2}\mathrm{CONH}$, biotin side chain) 2.72 and 2.9 (2H, d and q, J_{gem} = 12 Hz, J_{vic} = 5 Hz, $\mathrm{S}\mathit{CH_2}$) 3.12-3.18 (1H, m, $\mathrm{S}\mathit{CH}$) 3.28-3.51 (12H, m, 2 x $\mathit{CH_2}\mathrm{N}$ and 2 x $\mathit{CH_2}\mathrm{O}\mathit{CH_2}$) 4.30-4.49 (2H, m, 2 x C*H*NH in biotin) 5.01 (1H, broad s, N*H* amide) 6.29 and 6.54 (2H, broad s, N*H* in biotin).

Synthesis of N-biotin-4,9-dioxa-1,12-diaminododecane·HCl. 590 mg N-Boc-N'-biotin-4,9-dioxa-1,12-diaminododecane (1.11 mmol) were dissolved in 6 ml 98 % formic acid and stirred at RT for 8 h. Formic acid was removed by azeotroping with several portions of toluene. The crude product was dissolved in 2 ml water, clarified by centrifugation and loaded on a 1.5 x 14 cm column of SP-Sephadex C-25 (Na-form) at 0.5 ml/min. After washing with 15 ml water the product was eluted with 500 mM ammonium acetate. Pooled product fractions were adjusted to pH = 12 by addition of NaOH while bubbling with Ar (no stirring) and extracted with chloroform (3 x 15 ml) under Ar. The organic layers were taken to dryness, the residue was dissolved in 0.1 M HCl and lyophilized. Yield 300 mg (0.64 mmol), pure by TLC (R_f^{I} = 0.15, positive with iodine, DACA, and ninhydrin). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 1.60-1.77 (12H, m, $\mathrm{OCH_2}\mathit{CH_2CH_2}\mathrm{CH_2O}$ + $\mathit{CH_2}\mathrm{CH_2NH_3^+}$ + $\mathit{CH_2CH_2CH_2}\mathrm{CH_2CONH}$, biotin side chain) 2.18 (2H, t, $\mathrm{C}\mathit{H_2}\mathrm{CONH}$, biotin side chain) 2.72 and 2.90 (2H, d and q, J_{gem} = 12 Hz, J_{vic} = 5 Hz, $\mathrm{SC}\mathit{H_2}$) 3.12-3.18 (1H, m, $\mathrm{SC}\mathit{H}$) 3.28-3.51 (12H, m, 2 x $\mathrm{C}\mathit{H_2}\mathrm{N}$ and 2 x $\mathrm{C}\mathit{H_2}\mathrm{OC}\mathit{H_2}$) 4.30-4.49 (2H, m, 2 x $\mathrm{C}\mathit{H}\mathrm{NH}$ in biotin) 5.01 (1H, broad s, $\mathrm{N}\mathit{H}$, amide) 6.29 and 6.54 (2H, broad s, $\mathrm{N}\mathit{H}\mathrm{CON}\mathit{H}$ in biotin).

Synthesis of Biotin-dode-TMR. 9 mg N-biotin-4,9-dioxa-1,12-diaminododecane·HCl (19 µmol) were dissolved 2 ml chloroform, 16 mg 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester and 10 µl Et_3N were added (see above for general precautions). TLC analysis proved quantitative conversion of the ninhydrin- and DACA-positive educt (R_f^{V} = 0.48, R_f^{IV} = 0.16, R_f^{II} = 0.25) into TMR-labeled product (R_f^{V} = 0.84, R_f^{IV} = 0.51, R_f^{II} = 0.64). The mixture was taken to dryness and chromatographed on silica 60 (chloroform/methanol/water 70/26/4), yielding 83 % pure product with a 17 % content of free TMR (see Experimental Procedures). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 1.25-1.70 (14H, m, $\mathrm{OCH_2}\mathit{CH_2CH_2}\mathrm{CH_2O}$ + 2 x $\mathit{CH_2}\mathrm{CH_2N}$ + $\mathit{CH_2CH_2CH_2}\mathrm{CH_2CONH}$, biotin side chain) 1.96 (12H, m, 4 x $\mathrm{NC}\mathit{H_3}$ in TMR) 2.12 (2H, t, J = 7 Hz, $\mathrm{C}\mathit{H_2}\mathrm{CONH}$, biotin side chain) 2.72 and 2.87 (2H, d and q,

J_{gem} = 12 Hz, J_{vic} = 5 Hz, SC*H2*) 3.10 (1H, m, SC*H*) 3.31-3.74 (13H, m, 2 x C*H2*N and 2 x C*H2*OC*H2*, biotinoyl-N*H*CH2) 4.27 and 4.48 (2H, m, C*H*NH in biotin) 5.59 and 5.65 (together 1H, broad s, N*H*-TMR, 5-and-6 isomer) 6.42-6.70 (8H, 6 H from TMR xanthene moiety and 2 H from N*H*CON*H* in biotin).

Synthesis of Biotin-PEG$_{800}$-TMR. 10 mg biotin-NH-PEG$_{800}$-NH$_2$·HCl (nominally 8.4 µmol) were dissolved in 0.5 ml chloroform, 12 mg 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester (23 µmol, dissolved in 1 ml chloroform) and 20 µl Et$_3$N were added (see above for general precautions). After 2 h of stirring TLC showed quantitative conversion of biotin-NH-PEG$_{800}$-NH$_2$·HCl into TMR-labeled product. The mixture was taken to dryness, redissolved in 3 ml water and loaded on a 1.5 x 48 cm column of Sephadex G-25 M at 1.0 ml/min. Elution with water resulted in complete separation of the PEG derivative (eluting shortly after the void volume) from all by-products. Yield: 4.8 µmol of Biotin-PEG$_{800}$-TMR according to biotin end group assay (see Experimental Procedures). TLC indicated near purity, besides the product (R_f^{IV} = 0.56-0.81) traces of biotin-NH-PEG$_{800}$-NH$_2$·HCl (R_f^{IV} = 0.85) and of free TMR (R_f^{IV} = 0.22) were visible. ^{1}H-NMR: (CDCl$_3$, 200 MHz) δ: 1.09 (6H, m, 2 x CH$_3$, aminopropyltermini of PEG) 1.17-1.63 (6H, m, C*H2*C*H2*C*H2*CH$_2$CONH, biotin side chain) 2.03 (12H, m, 4 x NC*H3* in TMR) 2.12 (2H, t, J = 7 Hz, C*H2*CONH, biotin side chain) 2.68 ppm (1H, d, J_{gem} = 13 Hz, SC*H2* (I), biotin) 2.84 (1H, q, J_{1gem} = 13 Hz, J_{2vic} = 5 Hz, SC*H2* (II), biotin) 3.08 (1H, m, SC*H*, biotin) 3.20-3.93 (~72H, m, OC*H2*C*H2*O, PEG chain) 3.38-3.47 (6H, m, 2 x OC*H2* and 2 x NC*H*, aminopropyltermini of PEG) 4.03 (1H, broad s, biotinoyl-N*H*) 4.28 (1H, m, NC*H*, biotin), 4.44 (1H, m, NC*H*, biotin) 4.83 and 5.46 (together 1H, broad s, N*H*-TMR, 5-and-6 isomer) 6.33, 6.42, 6.50 (each 2H, TMR xanthene moiety) 6.13-6-82 (2 H, N*H*CON*H* in biotin) 7.16, 7.53, 7.98, 8.16, 8.34 (together 3H, TMR phtalide residue, 5-and-6 isomer).

Synthesis of Biotin-PEG$_{1900}$-TMR. The procedure was the same as for the PEG$_{800}$ derivative. 43 mg biotin-NH-PEG$_{1900}$-$NH_2 \cdot HCl$ (nominally 18.7 µmol, ~20% water content) were reacted with 19 mg 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester (36 µmol). The product eluted as sharp void peak from Sephadex G-25 M. Yield: 12.8 µmol of Biotin-PEG$_{1900}$-TMR according to biotin end group assay (see Experimental Procedures). TLC indicated near purity, besides the product (R_f^{IV} = 0.53-0.80) traces of biotin-NH-PEG$_{800}$-$NH_2 \cdot HCl$ (R_f^{IV} = 0.86) and of free TMR (R_f^{IV} = 0.21) were visible. ^{1}H-NMR: ($CDCl_3$, 200 MHz) δ: 1.11 (6H, m, 2 x CH_3, aminopropyltermini of PEG) 1.21-1.67 (6H, m, $CH_2CH_2CH_2CH_2CONH$, biotin side chain) 2.05 (12H, m, 4 x NCH_3 in TMR) 2.15 (2H, t, J = 7 Hz, CH_2CONH, biotin side chain) 2.69 ppm (1H, d, J_{gem} = 13 Hz, SCH_2 (I), biotin) 2.84 (1H, q, J_{1gem} = 13 Hz, J_{2vic} = 5 Hz, SCH_2 (II), biotin) 3.12 (1H, m, SC*H*, biotin) 3.22-3.95 (~172H, m, OCH_2CH_2O, PEG chain) 3.42-3.50 (6H, m, 2 x OCH_2 and 2 x NC*H*, aminopropyltermini of PEG) 4.07 (1H, broad s, biotinoyl-N*H*) 4.30 (1H, m, NC*H*, biotin), 4.45 (1H, m, NC*H*, biotin) 4.89 and 5.51 (together 1H, broad s, N*H*-TMR, 5-and-6 isomer) 6.35, 6.43, 6.53 (each 2H, TMR xanthene moiety) 6.20-6-90 (2 H, N*H*CON*H* in biotin) 7.18, 7.56, 8.00, 8.17, 8.36 (together 3H, TMR phtalide residue, 5-and-6 isomer).

Synthesis of Biotin-PEG$_{800}$-Flu. 50 mg biotin-NH-PEG$_{800}$-$NH_2 \cdot HCl$ (22 µmol) and 20 mg 5-(and-6)-carboxyfluoresceine succinimidyl ester (43 µmol) were dissolved in 1 ml DMF and 15 µl Et_3N were added (see above for general precautions). After 2 h of stirring the mixture was cooled and evaporated at 1-10 Pa with stirring. The residue was dissolved in 5 ml chloroform and washed with 20 ml 0.2 M Na_2CO_3 saturated with NaCl. The strongly colored aqueous phase was re-extracted 4 x with 10 ml chloroform. All 5 organic phases were pooled and washed with 10 ml of saturated NaCl solution containing 15 µl acetic acid (to remove traces of Et_3N). Drying of the chloroform solution with Na_2SO_4 and evaporation yielded 51 mg crude product which was dissolved in 2 ml water and

30 µl 0.2 M Na_2CO_3 and loaded on a 2.5 x 17 cm column of QAE-Sephadex A-25 (pre-equilibrated in buffer B) at 1 ml/min. After washing with 90 ml of buffer B the product was eluted with 200 ml 150 mM NaCl in buffer B. The volume was reduced to ~20 ml on a rotavap at ~10^2 Pa, saturated with NaCl, extracted 4 x with chloroform, the organic layers were dried with Na_2SO_4, taken to dryness and dried at 1-10 Pa for 1 h. Yield 27 mg (18 µmol) highly viscous oil. Pure by TLC: $R_f^{III} = 0.46\text{-}0.65$, $R_f^{VI} = 0.04\text{-}0.28$, typical PEG tails, fluorescent, positive stain with DACA. ^{1}H-NMR: ($CDCl_3$, 200 MHz) δ: 1.09 (6H, m, 2 x CH_3, aminopropyltermini of PEG) 1.27-1.73 (6H, m, $\mathit{CH_2CH_2CH_2}CH_2$CONH, biotin side chain) 2.14 (2H, t, J = 7 Hz, $\mathit{CH_2}$CONH, biotin side chain) 2.67 ppm (1H, d, J_{gem} = 13 Hz, S$\mathit{CH_2}$ (I), biotin) 2.81 (1H, q, J_{1gem} = 13 Hz, J_{2vic} = 5 Hz, S$\mathit{CH_2}$ (II), biotin) 3.07 (1H, m, SC*H*, biotin) 3.18-3.91 (~72H, m, O$\mathit{CH_2CH_2}$O, PEG chain) 3.31-3.47 (6H, m, 2 x O$\mathit{CH_2}$ and 2 x NC*H*, aminopropyltermini of PEG) 4.04 (1H, broad s, biotinoyl-N*H*) 4.24 (1H, m, NC*H*, biotin), 4.40 (1H, m, NC*H*, biotin) 4.75 and 5.64 (together 1H, broad s, N*H*-fluoresceine, 5-and-6 isomer) 6.52 (4H, fluoresceine xanthene moiety) 6.66 (2H, fluoresceine xanthene moiety) 6.10-6-85 (2 H, N*H*CON*H* in biotin) 7.64, 7.97, 8.07, 8.12, 8.43, 8.84 (together 3H, fluoresceine phtalide residue, 5-and-6 isomer).

Synthesis of Biotin-PEG_{1900}-Flu. 50 mg biotin-NH-PEG_{1900}-NH_2·HCl (22 µmol) were reacted with 15.5 mg 5-(and-6)-carboxyfluoresceine succinimidyl ester (33 µmol). The procedure was the same as for the PEG_{800} homologue, except that 50 mM NaCl in buffer B was used to elute the product from QAE-Sephadex A-25. Yield: 31.5 mg (12 µmol). Pure by TLC: ($R_f^{III} = 0.55\text{-}0.88$, $R_f^{VI} = 0.04\text{-}0.38$). ^{1}H-NMR: ($CDCl_3$, 200 MHz) δ: 1.07 (6H, m, 2 x CH_3, aminopropyltermini of PEG) 1.25-1.69 (6H, m, $\mathit{CH_2CH_2CH_2}CH_2$CONH, biotin side chain) 2.12 (2H, t, J = 7 Hz, $\mathit{CH_2}$CONH, biotin side chain) 2.67 ppm (1H, d, J_{gem} = 13 Hz, S$\mathit{CH_2}$ (I), biotin) 2.82 (1H, q, J_{1gem} = 13 Hz, J_{2vic} = 5 Hz, S$\mathit{CH_2}$ (II), biotin) 3.08 (1H, m, SC*H*, biotin) 3.21--3.91 (~172H, m, O$\mathit{CH_2CH_2}$O, PEG chain) 3.31-3.47 (6H, m, 2 x O$\mathit{CH_2}$ and 2 x NC*H*, aminopropyltermini of PEG) 4.04 (1H, broad s, biotinoyl-N*H*) 4.24 (1H, m, NC*H*, biotin), 4.42 (1H, m, NC*H*, biotin) 4.76 and 5.35 (together 1H, broad s, N*H*-fluoresceine, 5-and-6 isomer) 6.50 (4H, fluoresceine xanthene moiety) 6.69 (2H, fluoresceine xanthene moiety) 5.77-6-45 (2 H, N*H*CON*H* in biotin) 7.57, 7.68, 7.97, 8.07, 8.15, 8.38, 8.86 (together 3H, fluoresceine phtalide residue, 5-and-6 isomer).

Synthesis of Biotin-PEG$_{800}$-Cy3. One vial Cy3 monofunctional dye ("reactive dye to label 1 mg of antibody" according to Amersham) was dissolved in 1 ml absolute DMF. 2 mg biotin-NH-PEG$_{800}$-NH$_2$·HCl (1.7 µmol) and 5 µl Et$_3$N were added (see above for general precautions). After 2 h the mixture was cooled and evaporated at 1-10 Pa with stirring. TLC (eluent V) indicated that all of the succinimidyl ester component (~1/2 of the commercial reagent) had reacted with the primary amine while the other dye components (mostly free carboxylic acid of Cy3) remained unchanged. The dry residue was dissolved in 2 ml buffer B and loaded on a 1 x 10 cm column QAE-Sephadex A-25 at 0.16 ml/min. After washing with 30 ml of the same buffer the product band was eluted with 125 mM NaCl in buffer B. Yield: 63 nmol determined from A_{550} (ε_{550} = 150000 M^{-1} cm^{-1} for Cy3 according to Amersham) or 54 nmol according to biotin end group assay with ANS (see Experimental Procedures).

LITERATURE CITED in the Supplements only

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