Synthesis of 4-Aminobenzoic Acid Esters of Polyethylene Glycol and Their Use for Pegylation of Therapeutic Proteins

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Abstract. As the majority of reagents commonly used for protein pegylation have certain disadvantages concerning their reactivity, stability, convenience and selectivity of pegylation reaction, etc., the search of new pegylation reagents is of current interest. In the present paper, 4-aminobenzoic acid esters of polyethylene glycol are considered as promising pegylation reagents for chemical modification of molecules of biologically active proteins to prepare their conjugates characterized by improved therapeutic properties. These reagents are highly reactive and stable and make it possible to perform the histidine- and tyrosine-targeted pegylation of the protein chain. The convenient technique for the synthesis of 4-aminobenzoic acid esters of polyethylene glycol was optimized to prepare these pegylation reagents with actually quantitative loading of polymer with functional group (the residue of 4-aminobenzoic acid). The efficiency of synthesized compounds was shown in pegylation of such proteins as interferon α-2b and erythropoietin β and it was found that the activity of prepared interferon α-2b conjugate is comparable with those of its commercial analogs such as Pegintron® and Pegasys®.

Keywords: pegylation via azo coupling; interferon α-2b; erythropoietin β.

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Materials and Methods

Commercially available mPEGs **1a,b** (JenChem Technology, USA) and concentrated solutions of recombinant human INF α-2b (LLC "Pharmapark", Russia) and EPO β (Shandong Kexing Bioproducts Co., Ltd., China) were used. Ethyl 4-[(2-hydroxynaphthyl)diazenyl]benzoate [1] and 4-aminobenzoic acid [2] were synthesized by known methods.

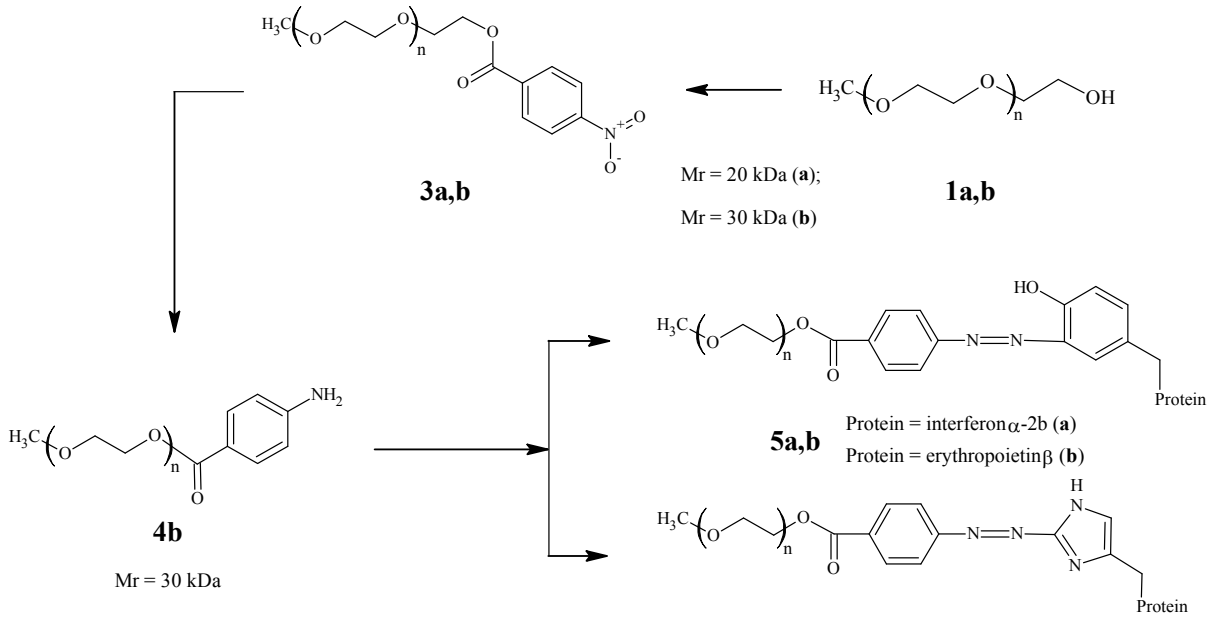
^{1}H NMR spectra were recorded with the use of a Bruker AM-300 instrument at a frequency of 300 MHz and a temperature of 25°C. To record UV and visible spectra, a Genesys 6 spectrophotometer (Thermoscientific, USA) was used. Reversed-phase HPLC was performed on a LC-20AD chromatograph (Shimadzu, Japan) equipped with a parallel double piston pump, an CTO-20A column oven, and an SPD-M20A diode array spectrophotometric detector at 30°C with the use of gradients prepared by mixtures of water and acetonitrile (Table). Luna 5μ C18 (2) 100A (for compounds **3** and 4-nitrobenzoic acid) and Jupiter 5μ C18 300A (for compounds **4** and 4-aminobenzoic acid) HPLC columns (Phenomenex, USA) were used at a rate of elution of 1.0 mL/min. Size-exclusion chromatography was carried out on a Smartline chromatograph (Knauer, Germany) equipped with a Smartline 1000 pump with a Manager 5000 gradient forming device and a Smartline 2550 spectrophotometric detector with 0.15 M NaCl solution in 0.05 M phosphate buffer containing 3 mM sodium azide used as an eluent. A Superdex 200 10/300 GL (GE Healthcare, Sweden) size-exclusion column was employed at a flow rate of 0.5 mL/min. Isolation and purification of the protein conjugates **5a**,**b** were performed on CM-Sepharose FF and Q-Sepharose FF ion exchangers (GE Healthcare), respectively, with the elution of fractions from the column being controlled with the use of a Smartline UV Detector 200 (Knauer, Germany).

Table. Gradients used in HPLC of 4-nitro-, 4-aminobenzoic acids as well as polymers **3** and **4**

Time, min	For compound 3b (τ^* = 14.3 min) and 4-nitrobenzoic acid (τ = 13.4 min)		For compounds 4 (τ= 18.0 min) and 4-aminobenzoic acid (τ = 4.4 min)	
	Mobile phase *A* (H_2O-0.1% TFA), vol. %	Mobile phase *B* (CH_3CN-0.1% TFA), vol. %	Mobile phase *A* (H_2O-0.1% TFA) vol. %	Mobile phase *B* (CH_3CN-0.1% TFA), vol. %
0	80	20	90	10
20	20	80	30	70
22	20	80	30	70
25	80	20	90	10
30	80	20	90	10

* - retention time of compound in the chromatogram.

The antiviral activity of pegylated INF α-2b **5a** was examined by estimating its effect to protect the Madin-Darby line of bovine kidney cells against a vesicular stomatitis virus (VSV, Indiana strain). For all samples, 50% cytopathic effect was determined visually [3].



Scheme. Pegylation of therapeutic proteins (INF α-2b and EPO β) with 4-aminobenzoic acid ester of polyethylene glycol **4b**.

Determination of the Loading of Polymers **3** *and* **4**

The loading of polymers **3** and **4** with the residues of 4-nitro- and 4-aminobenzoic acids, respectively, was determined with the use of the calibration curves via chromatographic and spectrophotometric methods.

Chromatographic determination of the loading of nitro derivative 3b. ***Method A.*** 30 mg of compound **3b** were placed into a 5 mL volumetric flask, dissolved in water (2 mL), the volume was adjusted with water, and 0.5 mL of this solution were placed into a microtube. 1 M solution of NaOH in water (0.5 mL) was added into the microtube, the mixture was kept at 90 °C for 30 min, 1 M HCl in water (0.5 mL) was added followed by 0.1% solution of TFA in water (0.5 mL), and the resulting solution was analyzed by HPLC. The loading of polymer **3b** was determined via the calibration dependence of the peak area of 4-nitrobenzoic acid on its concentration.

Method B. As the extinction coefficients of nitro compound **3b** and 4-nitrobenzoic acid at the wavelength of the absorption maxima (λ^{max} = 261 nm) were shown to be approximately equal, the loading of polymer **3b** may be also determined directly (without hydrolysis of ester bond) via the calibration dependence of the peak area of 4-nitrobenzoic acid on its concentration. The determined loading value coincided with that obtained via the method A.

Chromatographic determination of the loading of amino derivatives 4a,b. 45 mg of corresponding compounds **4a**,**b** were placed into a 10 mL volumetric flask, dissolved in water (2 mL), the volume was adjusted with water, and 0.5 mL of this solution were placed into a microtube. 1 M solution of NaOH in water (0.5 mL) was added into the microtube, the mixture was kept at 90°C for 30 min, 1 M HCl in water (0.5 mL) was added followed by 0.1% solution of TFA in water (0.5 mL), and the resulting solution was analyzed by HPLC. The loadings of polymers were determined via the calibration dependence of the peak area of 4-aminobenzoic acid on its concentration.

Spectrophotometric determination of the loading of nitro derivative 3b. 30 mg of nitro compound **3b** were placed into a 25 mL volumetric flask, dissolved in 0.1 M acetic acid water solution (1 mL) and 13 mg of sodium acetate was added followed by 60 mg of a 20% titanium trichloride solution in 3% hydrochloric acid. The mixture was kept at room temperature for 20 min for complete oxidation of excess titanium trichloride (blue-green color of the solution disappears), 0.1 M solution of HBr in water (1 mL) was added followed by 0.01 M solution of $NaNO_2$ in water (1 mL), and the reaction was kept at room temperature for 15 min. 0.01 M solution of sulfamic acid in water (1 mL) was added, the reaction was kept at room temperature for 5 min, 14 mg of 2-naphthol were added followed by 0.1 M borate buffer solution (5.0 mL), and the volume was adjusted with ethanol. The solution was centrifuged (at 7000 min^{-1} for 10 min) to get rid of insoluble titanium salts and the optical density of supernatant was determined at 487 nm relative to the reference solution. The loading of polymer **3b** was found via the calibration dependence of the optical density of a solution of ethyl 4-[(2-hydroxynaphthyl)diazenyl]benzoate (dissolved in a mixture of ethanol (16 mL), 0.1 M borate buffer solution (5 mL), and water (4 mL)) on its concentration.

Spectrophotometric determination of the loading of amino derivatives 4a,b. 15 mg of corresponding compounds **4a**,**b** were placed into a 25 mL volumetric flask, dissolved in water (2 mL), 0.1 M solution of HBr in water (1 mL) was added followed by 0.01 M solution of $NaNO_2$ in water (1 mL) and the reaction was kept at room temperature for 15 min. 0.01 M solution of sulfamic acid in water (1 mL) was added, the reaction was kept at room temperature for 5 min, 14 mg of 2-naphthol were added followed by 0.1 M borate buffer solution (4.5 mL), and the volume was adjusted with ethanol. The optical density of the final solution was determined at 487 nm relative to the reference solution (Fig. 2). The loadings of polymers were found via the calibration dependence of the optical density of a solution of ethyl 4-[(2-hydroxynaphthyl)diazenyl]benzoate (dissolved in a mixture of ethanol (15 mL), 0.1 M borate buffer solution (5 mL), and water (5 mL)) on its concentration.

UV-VIS spectra

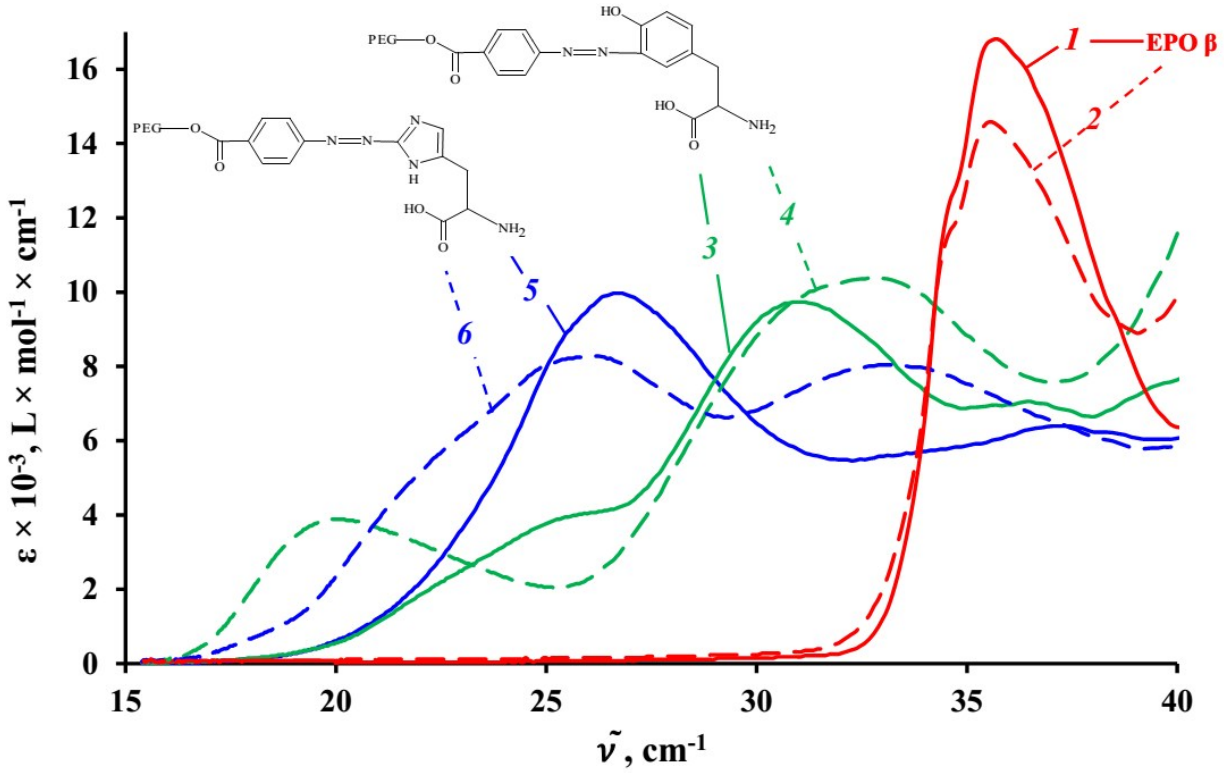


Figure 1. Absorption spectra of (*1*, *2*) EPO β and the products of azo coupling of pegylation reagent **4b** with (*3*, *4*) tyrosine and (*5*, *6*) histidine at pH 6.0 (curves *1*, *3*, and *5*) and pH 10.5 (curves *2*, *4*, and *6*).

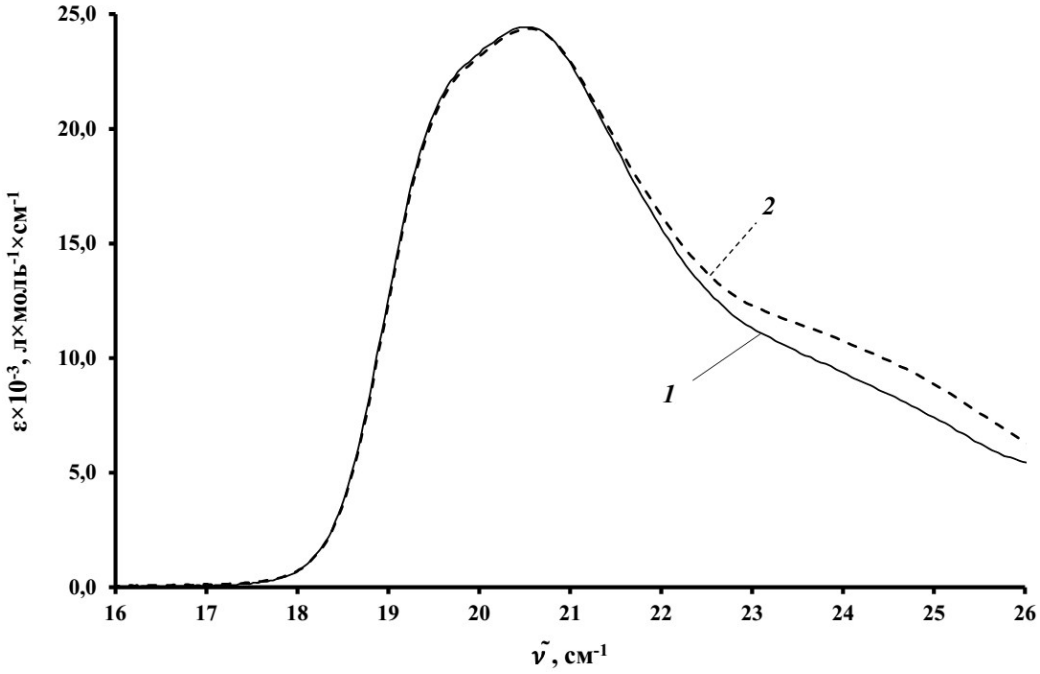


Figure 2. Absorption spectra of the products of azo coupling of diazotated (*1*) pegylation reagent **4a** and (*2*) ethyl 4-aminobenzoate with 2-naphthol under the conditions used for determination of the loading of polymer **4a**.

References

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