Synthesis of a Dihydroxy-Substituted Aminoxyl Spin Label and its Crown Ether and Cyclic Diester Derivatives

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4-Oxo-2,2,6,6-tetramethylpiperidine, Aminoxyl Radical, 4-Hydroxy-3-(3'-hydroxypropyl)-2,2,6,6-tetramethylpiperidine-1-oxo, Spin Label, Crown Ether

A novel dihydroxy-substituted aminoxyl spin label, 4-hydroxy-3-(3'-hydroxypropyl)-2,2,6,6-tetramethylpiperidine-1-oxo (5), has been prepared. From this aminoxyl radical, a spin-labeled ether, 16,16,18,18-tetramethyl-2,5,8,11-tetraoxa-17-azabicyclo[13,4,0]nonadecane-17-oxo (12), and a spin-labeled cyclic diester, 16,16,18,18-tetramethyl-3,10-dioxo-2,5,8,11-tetraoxa-17-azabicyclo[13,4,0]nonadecane-17-oxo (13), have been acquired. Other four new stable aminoxyl radicals 8, 9, 10, and 11 have also been obtained within the synthetic approach. It has been demonstrated that the cyclic ether compound (7) is generated by the reduction of its corresponding lactone which is sidely derived from the oxo-reduced species of 3 by intramolecular cyclization.

Introduction

The discovery around the mid-60’s by Rozantsev et al. [1–3] that a nitroxyl N–O' moiety its nitrogen atom linked to two tertiary carbons can maintain its paramagnetic radical property within many organic synthetic modification approaches makes it accessible for chemists and biochemists to prepare the modified aminoxyl compounds containing the desired functional group(s) needed for aminoxyl spin labeling [4]. Currently, aminoxyl spin-labeling method has been developed to many fields such as spin-labeled medical compounds for the study of the nature of drug-receptor interaction [4] and drug-metabolizing mechanism, spin-labeled transitional metal complexes for the study of interactions between the radical electron and the metal ion [5], spin-labeled NMR contrast-enhancing agents [6], and spin-labeled crown ethers [7]. One of the most popular aminoxyl spin labels in use today is 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxo (HTMPO):

\[
\text{HTMPO} \quad \text{OH}
\]

The disadvantage of this aminoxyl radical might be that spin labeling with HTMPO is mostly limited to the use of only acylation reaction and the nitroxyl N–O' moiety is far away from the desired system, e.g., in the case of the spin-labeled crown ether [7c]. Introducing of an extra hydroxy group to HTMPO skeleton may extend the use of the spin label and improve its advantages in some aspects. The spin label 5 involves two hydroxy groups which may be used in the construction of a crown ether that cavity is quite close to the N–O' moiety. Different properties of the secondary hydroxy and the primary hydroxy groups in 5 provide opportunities to convert 5 into a ketone–alcohol aminoxyl, a alcohol–aldehyde aminoxyl or a ketone–aldehyde species depending on the purpose of the research. The two hydroxy groups may also be used to connect to two different desired molecular species.

Aminoxyl spin-labeled crown ethers have attracted much attention and substantial synthetic efforts have been made [7]. A successful example is that the N–O' moiety is exactly involved in the construction of its monoazacrown ring and the sodium cation in the cavity can strongly interact...
with the spin label exhibiting a very large metal atom splitting \( a_N = 2.47 \) G in dichloromethane [7e]. Many aminoxyl spin-labeled crown ethers with the N–O' group toward the cavity do not achieve significant interaction with potassium cation, sodium cation, or lithium cation [7a]. It is curious to us that whether or not the dihydroxy aminoxyl \( 5 \) can build up some wonderful spin label crown ethers making the N–O' moiety interact with metal ions. Therefore, the synthesis of the crown ether \( 12 \) and its analogue \( 13 \) from \( 5 \) has been tried in our laboratory.

**Results and Discussion**

The route for the synthesis of the dihydroxy aminoxyl radical \( 5 \) is illustrated in Scheme 1. 4-Oxo-2,2,6,6-tetramethylpiperidine reacts with pyrrolidine to supply the enamine \( 1 \) which is treated with methyl acrylate or ethyl acrylate followed by hydrolysis providing the adduct \( 2 \) or \( 3 \). From the oxo-ester piperidine \( 3 \), two approaches have been tried to reach \( 5 \). The first way is the oxidation of \( 3 \) with hydrogen peroxide catalyzed with sodium tungstate and then reduction of \( 4 \) with lithium aluminum hydride. The second route is the reduction of \( 3 \) by sodium borohydride with heating to give \( 6 \) which is oxidized giving the desired aminoxyl \( 5 \). The comparatively convenient procedure and higher yield suggest that the second is a better way for the synthesis of \( 5 \).

Column chromatography on silica gel of the crude product of \( 5 \) derived from the crude dihydroxy piperidine \( 6 \) gains a by-product which is assigned to the cyclic ether aminoxyl \( 8 \) based on its IR spectrum, MS spectrum, microanalysis data and \(^1\)H NMR spectrum of its hydroxylamine analogue resulted from the reduction of \( 8 \) with PhNHNH\(_2\). It is considered that \( 8 \) must be come from its amine analogue \( 7 \) which should be generated in the reduction reaction of \( 3 \) with sodium borohydride. The mechanism for the generation of \( 7 \) is proposed as those illustrated in Scheme 2. According to the mechanism, the lactone piperidine is resulted and the further reduction of this compound by sodium borohydride with heating affords \( 7 \). In order to confirm this mechanism, the reduction procedure is conducted at lower temperature. After oxidation of the reduced product, a new stable aminoxyl \( 9 \) (Scheme 3) is separated which is apparently from its corresponding amine. Therefore, the lactone piperidine is exactly gener-

![Scheme 1.](image-url)
cates that the $^{14}$N-hyperfine splitting constant (hfsc) of 12 is not sensitive to the metal cation Li$^+$, Na$^+$, or K$^+$ in methanol. The $^{14}$N-hfsc is only slightly increasing with the increase of the concentration of the metal ions, that is attributed to the polarity increase of the solution resulted from the addition of the metal salt. Acylation of 5 with ClCO(CH$_2$OCH$_2$)$_3$COCl in the presence of triethylamine produces a crown-liking diester aminoxyl 13. IR, MS spectra and elemental analysis confirm the structure.

**Experimental**

IR spectra were recorded on a Perkin-Elmer 683 spectrometer and $^1$H NMR spectra on a Varian EM-360L (60 MHz) spectrometer. Mass spectra were obtained on an AEI MS-50/DS-30 instrument and EPR spectra on a Varian E-109 spectrometer. Microanalysis of new compounds were performed by the Elemental Analysis Group of the Institute of Chemistry, Academia Sinica.
2,2,6,6-Tetramethyl-4-(1'-pyrrolidinyl)piperidine-3-ene (1)

To a single-necked flask (250 ml) were added 4-oxo-2,2,6,6-tetramethylpiperidine (77.5 g, 500 mmol), pyrrolidine (50 ml, 600 mmol) and dry benzene (100 ml). The mixture was refluxed for 8 h to remove water resulted. After rotary evaporation removing benzene and excess pyrrolidine, low pressure distillation gave 97 g (86% yield) of slightly yellowish liquid 1, b.p. 110–112 °C/2 mmHg. Part of the product (20 g) was re-distilled to prepare the sample for microanalysis. The product crystallized in a refrigerator and became a liquid at room temperature.

IR (neat): \( \nu_{\text{max}} = 3320 \) (N–H), 1640 cm\(^{-1}\) (C=O); MS: \( m/e = 208 \) (M\(^+\)); \(^1\)H NMR (CDCl\(_3\)/TMS): \( \delta = 0.81 \) (s, 1H, NH), 1.20 (s, 12H, \( 4 \times \text{CH}_2 \)), 1.83 (t, 4H, \( \text{CH}_2\text{CH}_2 \)), 1.98 (s, 2H, CH\(_3\)), 2.97 (t, 4H, \( \text{CH}_2\text{NCH}_3 \)), 4.15 ppm (s, 1H, C=CH).

\( \text{C}_{13}\text{H}_{24}\text{N}_2 \) (208.3)

Calcd C 74.95 H 11.61 N 13.44%,

Found C 74.28 H 11.65 N 13.61%.

2,2,6,6-Tetramethyl-4-oxo-3-(2'-methoxycarbonyl-ethyl)piperidine (2)

To a three-necked flask (100 ml) equipped with a thermometer and a condenser were added the enamine 1 (13.5 g, 65 mmol), absolute ethanol (30 ml) and methyl acrylate (16 ml, 180 mmol). To a single-necked flask (250 ml) were added distilled water (10 ml) and methyl acrylate (16 ml, 180 mmol). The mixture was refluxed for 4 h in an atmosphere of nitrogen. Addition of distilled water (10 ml) was followed by refluxing for an additional 1 h. After rotary evaporation, twice chromatographies on silica gel eluted with ethyl acetate supplied 8.3 g (53% yield) of yellowish liquid 2.

IR (neat): \( \nu_{\text{max}} = 3325 \) (N–H), 1740 (ester C=O), 1715 cm\(^{-1}\) (C=O); MS: \( m/e = 241 \) (M\(^+\)); \(^1\)H NMR (CDCl\(_3\)/TMS): \( \delta = 1.24 \) (s, 3H, CH\(_3\)), 1.29 (s, 3H, CH\(_3\)), 1.27 (s, 3H, CH\(_3\)), 1.57 (s, 1H, NH), 1.60–2.50 (m, 5H, \( \text{CH}_2\text{CH}_2\text{CH}_2 \)), 2.29 (s, 2H, CH\(_2\text{CO} \)), 3.63 ppm (s, 3H, \( \text{OCH}_3 \)).

\( \text{C}_{13}\text{H}_{22}\text{NO}_3 \) (241.3)

Calcd C 64.70 H 9.61 N 5.80%,

Found C 64.36 H 9.65 N 6.32%.

2,2,6,6-Tetramethyl-4-oxo-3-(2'-ethoxycarbonyl-ethyl)piperidine (3)

The compound 3 was prepared by the same procedure as that used for the production of 2; yield 59%.

IR (neat): \( \nu_{\text{max}} = 3345 \) (N–H), 1735 (ester C=O), 1705 cm\(^{-1}\) (C=O); MS: \( m/e = 255 \) (M\(^+\)); \(^1\)H NMR (CDCl\(_3\)/TMS): \( \delta = 1.02 \) (s, 3H, CH\(_3\)), 1.14 (s, 3H, CH\(_3\)), 1.24 (s, 3H, CH\(_3\)), 1.28 (s, 3H, CH\(_3\)), 1.70 (s, 1H, NH), 1.67–2.50 (m, 5H, CH\(_2\text{CH}_2\text{CH}_2 \)), 2.30 (s, 2H, CH\(_2\text{CO} \)), 4.08 ppm (q, 2H, COCH\(_3 \)).

\( \text{C}_{14}\text{H}_{23}\text{N}_2\text{O}_3 \) (255.3)

Calcd C 65.85 H 9.87 N 5.49%,

Found C 65.52 H 9.75 N 6.01%.

2,2,6,6-Tetramethyl-4-oxo-3-(2'-ethoxycarbonyl-ethyl)piperidine-1-0xy (4)

The mixture of crude 3 (11.5 g, 45 mmol), 30% hydrogen peroxide (15 ml), sodium tungstate dihydrate (0.5 g, 1.5 mmol) and water (100 ml) was stirred for 3 h at room temperature and then left overnight. The solution was extracted with ethyl acetate (150 ml), dried over anhydrous sodium sulfate, filtered. After evaporation, chromatography on silica gel eluted with chloroform gave 8 g (65% yield) of red oil.

EPR (\( \text{C}_6\text{H}_5\rangle \): (broad, C=O); MS: \( m/e = 270 \) (M\(^+\)).

4-Hydroxy-3-(3'-hydroxypropyl)-2,2,6,6-tetramethylpiperidine-1-0xy (5)

Method I. The reduction procedure of an ester compound containing aminoxyl radical refers to the previous literature [8]. To a solution of 4 (12.9 g, 48 mmol) in absolute ether (30 ml) was added dropwise for 20 min a solution of lithium aluminum hydride (0.9 g, 23.7 mmol) in absolute ether (70 ml) at 0–2 °C with a stirring. After stirring for additional 30 min, addition of water (10 ml) was followed by stirring for 1 h more. The mixture was filtered, evaporated, chromatographed on silica gel with ethyl acetate as an eluent to give 4.0 g (30% yield) of red oil 5.

EPR (\( \text{C}_6\text{H}_5\rangle \): \( a_N = 15.2 \) G; IR (neat): \( \nu_{\text{max}} = 3390 \) (O–H), 1050 cm\(^{-1}\) (C–O); MS: \( m/e = 230 \) (M\(^+\)).

\( \text{C}_{12}\text{H}_{24}\text{NO}_3 \) (230.3)

Calcd C 62.58 H 10.50 N 6.08%,

Found C 61.84 H 10.44 N 6.34%.

Method II. To a solution of crude 6 (2.6 g, 12 mmol), which will be described latter, in water (25 ml) was added sodium tungstate dihydrate (0.06 g, 0.18 mmol) and 30% hydrogen peroxide (3.5 ml). The solution was stirred for 8 h at room temperature and left overnight. Ammonium carbonate (5 g) was added and the mixture was extracted with dichloromethane (100 ml). The organic layer was dried over anhydrous sodium sulfate, filtered, evaporated. Chromatography on silica gel eluted with ethyl acetate produced two
aminoxy radicals ($R = 0.80$ and 0.45). The compound of which $R$ is equal to 0.45 is the dihydroxy aminoxyl 5; yield 64%.

EPR ($C_6H_6$): $a_N = 15.25$ G; IR (neat): $v_{max} = 3390$ (O–H), 1050 cm$^{-1}$ (C–O), exactly same as the spectrum for the sample from Method I; MS: $m/e = 230$ (M$^+$).

$C_{18}H_{32}N_06$ (358.4)
Calcd C 60.31 H 9.00 N 3.91 %,
Found C 61.06 H 9.11 N 4.68%.

4-Hydroxy-3-[(3'-hydroxypropyl)-2,6,6-tetramethylpiperidine (6)

The reduction procedure follows that reported by the previous literature [9]. To cooled methanol (30 ml) at $0–5^\circ$C was added powdered sodium borohydride (1.5 g, 39 mmol), 3 (3.3 g, 13 mmol). The reaction mixture was stirred for 12 h. The solvent was removed, ace tone (50 ml) was added and the mixture was refluxed again for 1.5 h. After evaporation, aqueous potassium carbonate solution (50 ml) was added and then the solution was refluxed for 1.5 h. The solution was concentrated to 30 ml which was extracted with $4 \times 30$ ml of chloroform. After drying and filtration, the remaining product 6 (2.6 g, 92% yield) of which IR spectrum showed the absence of ketone and ester function groups was distilled for the preparation of the elemental analytic sample; b.p. 126 °C/2–3 mmHg.

IR (neat): $v_{max} = 3350$ (N–H, O–H), 1050 cm$^{-1}$ (C–O); MS: $m/e = 215$ (M$^+$); $^1H$ NMR (CD$_2$COCD$_3$/TMS): $\delta = 1.06$ (s, 3H, CH$_3$), 1.10 (s, 3H, CH$_3$), 1.16 (s, 3H, CH$_3$), 1.20 (s, 3H, CH$_3$), 1.45 (m, 3H, CHCH$_3$), 2.03 (m, 4H, 2 x CH$_2$), 3.50 (m, 2H, CH$_2$), 4.12 ppm (217.6)

$C_{12}H_{23}NO_3 \times 1/4H_2O$ (217.6)
Calcd C 66.25 H 11.70 N 6.44%,
Found C 66.17 H 11.70 N 5.86%.

7,7,9,9-Tetramethyl-3-oxo-2-oxa-8-azabicyclo[4,4,0]decane-8-0xy (9)

To a solution of 3 (1.20 g, 5 mmol) in methanol (100 ml) was added sodium borohydride (0.25 g, 6 mmol) at 0–2 °C. The mixture was stirred for 1.5 h at 0–2 °C and for 20 h at room temperature. After rotary evaporation, the residue was dissolved in water (40 ml) and extracted with n-butanol (2 x 30 ml). The combination was dried over anhydrous sodium sulfate, filtered and evaporated to give 1.17 g of slightly yellowish oil. Part of the residue (0.55 g) was dissolved in ethanol (20 ml) and the benzene solution of perbenzoic acid (ca. 0.4 g, 2.7 mmol) was added dropwise at 15 °C. After stirring for 4 h, the solution was twicely washed with 3% of aqueous sodium carbonate, dried, filtered and evaporated. Twice column chromatographies on silica gel provided 70 mg (13% yield) of orange needle crystals of 9; m.p. 149–151 °C.

EPR ($C_6H_6$): $a_N = 15.05$ G; IR (KBr): $v_{max} = 1735$ cm$^{-1}$ (C=O); MS: $m/e = 226$ (M$^+$).

$C_{12}H_{20}NO_3$ (226.3)
Calcd C 63.69 H 8.91 N 6.19%,
Found C 63.65 H 8.74 N 6.21%.

2,2,6,6-Tetramethyl-4-acetoxy-3-/(6'-acetoxy-4-oxahe xy)piperidine-1-0xy (10)

To the solution of the aminoxyl 5 (0.65 g, 2.8 mmol) in completely dried tetrahydrofuran (THF, 15 ml) was added dropwise at −15 °C n-butyl lithium solution (3.5 ml, 5.6 mmol) in hexane (15%) and stirred for 20 min. This solution was added for 25 min at 15 °C to a solution of 2-bromoethyl acetate (1.87 g, 11.2 mmol) in THF (10 ml) and refluxed for 10 h. After evaporation, the residue was chromatographed on silica gel eluted with a mixed solvent of chloroform and ethyl acetate (50/50, v/v) and the first main red band was collected to give 0.35 g (35% yield) of red oil 10. EPR ($C_6H_6$): $a_N = 15.4$ G; IR (neat): $v_{max} = 1740$ cm$^{-1}$ (ester C=O); MS: $m/e = 358$ (M$^+$).

$C_{18}H_{37}NO_6$ (358.4)
Calcd C 60.31 H 9.00 N 3.91%,
Found C 61.06 H 9.11 N 4.68%.
To a solution of 5 (0.5 g, 2.17 mmol) in dry benzene (10 ml) was added dropwise a solution of n-butyllithium (7 ml, 11.5 mmol) in hexane (15%) for 20 min at -15 °C. After stirring for 15 min, the solution was added to a solution of 1,8-dibromo-3,6-dioxoacetic acid (1.5 g, 5.4 mmol), prepared by bromination of triethylene glycol dichloride (0.51 g, 2.1 mmol), prepared from triethylene glycol by oxidation and chlorination [10], in chloroform (50 ml). The mixture was stirred for 7 h at room temperature and then evaporated. The residue was dissolved in benzene (40 ml), filtered and evaporated. Column chromatography on silica gel elution with a mixed solvent of chloroform and ethyl acetate (50/50, v/v) and collection of the first red band gave 0.26 g (30% yield) of red oil 13.

EPR (C6H5)2: aN = 15.4 G; IR (neat): νmax = 1750 cm⁻¹ (ester C = O); MS: m/e = 372 (M⁺).

C16H30NO7 (372.4)
Calcd C 58.05 H 8.12 N 3.76%.
Found C 57.77 H 8.48 N 3.59%.

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Synthesis and Use of Pentafluorophenyl 6-(Biotinylamido)hexanoate
An Alternative Reagent for Labelling of Proteins with Biotin Moiety

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Antibody, Biotin-Active Ester, Biotinylation, ELISA, Protein

The protein biotinylating agent, pentafluorophenyl 6-(biotinylamido)hexanoate (3) was synthesized. A straightforward synthetic route, biotin, biotin pentafluorophenyl ester (1), 6-(biotinylamido)hexanoate (2) and pentafluorophenyl 6-(biotinylamido)hexanoate (3) was followed. This agent was used for biotin-labelling of different proteins, bovine serum albumin, immunoglobulin, horseradish peroxidase and ß-galactosidase. Optimal conditions for the labelling were determined, as well as the influence of biotin-tagging to the biological activity of the proteins. An optimal ratio of the biotinylating agent to protein resulted in effective biotinylation without a significant decrease in the activity of the labelled enzyme. The labelled proteins proved to be suitable for immunodetection of proteins in both microplate and Western blot immunohistochemistry, as well as in a competitive steroid ELISA of testosterone and 20-hydroxyecdysone.

Introduction

Biotin has a very strong and selective interaction with avidin and streptavidin [1]. This interaction is widely exploited in biochemical and immunological experiments if biotin is used as a tag on antigens, antibodies, proteins etc., which then can be utilized for detection or separation with avidin or streptavidin. The key to build a separation or a detection system of such a kind is biotinylation of the protein, peptide, antigen and antibody of interest. 6-(Biotinylamido)hexanoate is preferentially used [2] because it keeps the biotin moiety six carbon atoms separated from the reacting amino group permitting the biotin residue to react with the binding site of avidin. The strategy to couple biotin to a protein or a peptide-requiring mild reaction conditions- is to use the autocatalytic reaction of active esters of biotin. Up to now the preparation of p-nitrophenyl and N-hydroxysuccinimide esters of biotin were reported [3, 4], however, only the latter one was used for this purpose [5, 6].

The chemical synthesis of the N-hydroxysuccinimide ester of biotin has some disadvantages to achieve a reasonable yields. To eliminate these difficulties we present here an alternative reagent for biotinylation of proteins. The highly reactive pentafluorophenyl esters of protected amino acids [7] are widely used for peptide bond formation offering an excellent coupling method in peptide chemistry [8, 9]. In this paper we explore the synthesis and the use of the pentafluorophenyl ester of biotin. The synthesis of our reagent, pentafluorophenyl 6-(biotinylamido)hexanoate (3) follows a direct route with very good yields. The reagent can be stored without decomposition (standing in fridge at −5 °C) and allows to attach biotin to proteins under mild conditions, furthermore, the reagent is at least as effective as the N-hydroxysuccinimide ester of 6-(biotinylamido)hexanoate.

Experimental

Abbreviations used: BSA = Bovine serum albumin; DCC = 1,3-Dicyclohexylcarbodiimide; DCU = 1,3-Dicyclohexylurea; DMF = N,N-Dimethylformamide; DMSO = Dimethyl sulfoxide; ELISA = Enzyme Linked Immuno Sorbent Assay; HABA = 2-(4'-hydroxyazobenzene)-benzoic acid; HOPfp = Pentafluorophenol; HRPO = Horseradish Peroxidase; IgG = Immunoglobulin; ONPG = 4-Nitrophenyl-ß-D-galactopyranosidase; OPD = 1,2-Phenylenediamine; PAGE = Polyacrylamide Gel Electrophoresis; PBS = Sodium phosphate and Sodium
chloride; Tris = 2-Amino-2-hydroxymethyl-1,3-propanediol.

**Enzymes:** β-Galactosidase from recombinant E. coli strain carrying the E. coli gene on a vector. – β-D-Galactosidase galactohydrolase, EC 3.2.1.23. – Peroxidase (POD) from horseradish. – Donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7.

**Materials and methods**

The chemicals and solvents were obtained from Reanal, Hungary. Melting points were determined in a PAMK VEB apparatus and are uncorrected. Optical rotations were measured in a Zeiss Polamat (A) polarimeter at 25 °C. TLC was carried out on silica gel precoated glass plates 60 F254 (Merck) using CHCl3/MeOH (9:1) as solvent system. Streptavidin was purchased from Apvel, England, and horseradish peroxidase from Reanal, Hungary (specific activity: 60–80 U/mg in pirogallol units). β-Galactosidase was of recombinant origin and obtained from Richter, Hungary (specific activity: 300 000 U/mg in ONPG units, HABA and pirogallol were from Aldrich. The protein molecular weight standard was purchased from Pharmacia.

**Antibodies**

The anti-mouse and anti-rabbit sheep and goat IgGs were purified from sera obtained from the Animal Breeding Station of Mezőhegyes, Hungary. The anti-mouse rabbit IgG was from Dakopatts. Anti-testosterone rabbit serum was a generous gift of Dr. V. Csernus, Medical School, Pécs, Hungary. Anti-ecdysteroid sera were risen against an immunogen prepared according to the method of Borst and O’Connor [10] coupling 20-hydroxyecdysone-6-carboxymethylxime to carrier thyroglobulin.

**Buffers**

The coating of ELISA plates with conjugates and antibodies were performed in PBS (10 mmol sodium phosphate, 145 mmol sodium chloride, pH = 7.2).

The Z buffer is a mixture of 0.1 M sodium phosphate (pH = 8.0), 0.1 M KCl, 1 mM MgCl₂ and 50 mmol β-mercaptoethanol.

**Syntheses**

**Pentafluorophenyl ester of biotin (1)**

Pentafluorophenol (1 g, 5.4 mmol) and DCC (1.2 g, 6 mmol) were added to biotin (1 g, 4.1 mmol) dissolved in DMF (12 ml) at 50–60 °C, and then the reaction mixture was stirred at room temperature for 3 h. DCU was filtered off and the solution evaporated to dryness in vacuum. The solid residue was crystallized from MeOH to give a white crystalline product (1.6 g, 94%), m.p.: 182–186 °C, [α]D = +33.2° (c = 0.5, MeOH), Rf = 0.53.

**6-(Biotinylamido)hexanoate (2)**

Biotin pentafluorophenyl ester (1, 1.2 g, 2.9 mmol), dissolved in DMF (6 ml), was added to the solution of ε-aminocaproic acid (400 mg; 3.1 mmol in 4 ml 1 M NaOH). After stirring for 1 h at room temperature, the reaction mixture was acidified to pH = 2 using 1 N HCl. The precipitated product was collected by filtration, washed with distilled water and dried. Yield: 900 mg (85%), m.p.: 221–222 °C, [α]D = +23.8° (c = 0.5, 1 N NaOH), Rf = 0.06.

**Pentafluorophenyl 6-(biotinylamido)hexanoate (3)**

Pentafluorophenol (460 mg, 2.5 mmol) and DCC (570 mg, 2.75 mmol) were added to the solution of 6-(biotinylamido)hexanoate (2, 800 mg, 2.23 mmol in 30–35 ml DMF). After stirring for 3 h at room temperature, DCU was removed by filtration and the filtrate was evaporated to dryness in vacuum. The residue was crystallized from MeOH to give 900 mg (80%) of the product, m.p.: 161–164 °C, [α]D = +18.7° (c = 1, DMF), Rf = 0.24.

**Biotin labelling of proteins**

A solution of pentafluorophenyl 6-(biotinylamido)hexanoate (3) (1 mg/ml in DMSO) was brought into a protein solution (1 mg/ml in 0.1 M NaHCO₃, pH = 9.0, unless otherwise specified) in a ratio of 1/5 volumes and incubated for 2 h. The unreacted biotin was removed by extensive dialysis against PBS. The dialyzed protein solution was supplemented with BSA to 1% and stored at 4 °C or lyophilized.

**Colorimetric determination of biotin**

The amount of 6-(biotinylamido)hexanoate (2) moieties bound to a protein was determined by specific displacement with the dye HABA of biotin on avidin and was monitored colorimetrically in the following way: the biotinylated protein sample was digested with proteinase K in 10 mmol Tris (pH = 8.0) for 1 h at 37 °C. Then the enzyme was inactivated by heating the sample to 67 °C. 5–10 µl of protein digest was given to HABA (1 ml) and the avidin complex (0.25 mmol and
400 µg/ml, respectively in PBS, pH = 7.2) and the absorption was measured at 500 nm. The biotin content of the sample was then read from a calibration curve.

**Competitive ELISA**

Solutions of BSA-20-hydroxyecdysone-6-carboxymethylxime or BSA-testosterone-3-carboxymethylxime conjugates (prepared as described in [10], 16 or 25 ng/100 µl PBS, respectively) were coated to ELISA plates three times for 2 h at 37 °C. The plates were rinsed three times with PBS containing 0.05% Tween 20, then “blocked” by incubation with 0.5% BSA in PBS for 1 h. The plates were rinsed three times with PBS/0.05% Tween 20.

Standard solutions of the appropriate hormones (competitor), samples and diluted steroid antibody were added in 100 µl volume and incubated at 37 °C for 2 h. The excess Ig was removed by rinsing with PBS/Tween 20 (three times) and biotinylated anti-rabbit sheep Ig (1000×dil.). After an incubation time of 1 h and subsequent washing biotinylated HRPO/streptavidin complex was added (1000×dil.) and incubated for another hour. The plates were then rinsed with PBS/Tween 20 and 80 µl of the substrate mixture (0.003% H₂O₂ and 0.8 mg/ml OPD in citrate buffer, pH = 5.0) were added. The reaction was allowed to proceed for some minutes and then was stopped with the same volume of 4 M H₂SO₄. Plates were read (OD₄₉₂ nm) with an ELISA plate reader (CLS 962, Cambridge Life Sciences).

**Results and Discussion**

The biotin pentafluorophenyl ester (Fig. 1, 1) was obtained directly upon reacting biotin with pentafluorophenol in the presence of DCC in DMF (yield 94%, crystalline product). In the following reaction with ε-aminocaproic acid 6-(biotinylamido)hexanoate (Fig. 1, 2) can be collected in a very good yield (85%) which was used without further purification for the next synthetic step. Pentafluorophenyl 6-(biotinylamido)hexanoate (Fig. 1, 3) can be easily prepared from 2 using pentafluorophenol in DMF in the presence of DCC (yield 80%, crystalline product).

The pentafluorophenyl 6-(biotinylamido)hexanoate (3) is expected to be a rapidly reacting reagent under alkaline pH conditions. Indeed, when antimouse rabbit immunoglobulin was treated with this reagent, the reaction reached completion after 120 min (Fig. 2). The carbonate buffer (pH = 9.0) proved to be optimal compared to the neutral phosphate (pH = 7.2) and the slightly acidic citrate (pH = 5.0) buffers investigated (see Table).

Bovine serum albumin (BSA) and immunoglobulins (anti-mouse and anti-rabbit Ig) were exposed to different doses of pentafluorophenyl 6-(biotinylamido)hexanoate (3). After dialysis, the protein was immobilized on a microtitrator plate for ELISA measurements. Using excess streptavidin and the biotinylated horseradish peroxidase com-

**Table. Dependence of biotinylating of anti-mouse anti-rabbit immunoglobulin upon pH.**

<table>
<thead>
<tr>
<th>Buffer pH</th>
<th>Dilution 1000</th>
<th>2000</th>
<th>3000</th>
<th>4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>0.48</td>
<td>0.17</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>7.2</td>
<td>1.85</td>
<td>1.02</td>
<td>0.46</td>
<td>0.22</td>
</tr>
<tr>
<td>9.0</td>
<td>&gt;2.00</td>
<td>1.72</td>
<td>0.87</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Data are given in optical density (OD₄₉₂).

Fig. 1. Scheme for the synthesis of pentafluorophenyl 6-(biotinylamido)-hexanoate (3).
Fig. 2. Kinetics of biotin-labelling of immunoglobulin. Purified rabbit Ig was reacted with the reagent (3, see Materials) for different lengths of time. The wells of an ELISA plate were coated with labelled Ig (100 ng/well) for 1 h, then washed and blocked with 0.5% BSA and subsequently reacted with the biotinylated peroxidase/streptavidin complex. After washing with PBS/Tween, the enzyme activity was developed with 80 μl of 0.003% H2O2 and 0.8 mg/ml of OPD in citrate buffer (pH = 5.0) for 5 min. The optical density was determined after addition of 80 μl 4 M sulfuric acid.

As the molar ratio of the reagent (3) to protein is increased the number of the biotin moieties coupled to the protein is increased up to saturation. The proteins become saturated with biotin if the excess of the reagent (3) exceeds a 50-fold value in the case of BSA and an 150-fold in the case of Ig. 80–85 biotin residues were found to be attached on an Ig molecule as determined by means of HABA in a colorimetric assay [11] from an enzymatic proteolysate. Extensive biotin labelling resulted in a decrease of the enzymatic activity if HRPO and β-galactosidase were labelled. The two enzymes exhibit different sensitivity. With increasing biotin content the number of the enzyme molecules bound to streptavidin is also enlarged as indicated by the higher enzyme activity on the plate. However a too high degree of biotinylation resulted in a significant decrease of the enzyme activity in the complex (Fig. 4). This change was quite sharp in case of peroxidase, where 70-fold excess of the reagent (3) resulted in a complete elimination of the enzyme activity. β-Galactosidase proved to be much more resistant to this treatment, even a 1000-fold molecular excess of the reagent (3) caused only a 20% decrease of the enzymatic activity if HRPO and β-galactosidase were incubated with biotinylated BSA (50 ng/well). After washing, 100 μl of substrate solutions (0.003% H2O2, 0.8 mg/ml OPD in citrate buffer, pH = 5.0 and 200 μg/ml ONPG in phosphate buffer respectively) were given. The plates were incubated for 5 min. The HRPO reaction was stopped with 4 M sulfuric acid, and then the optical density (420 nm) recorded.
enzyme activity. For optimal biotin labelling a 1:25 molar ratio was chosen for horseradish peroxidase and 1:460 for \( \beta \)-galactosidase. Comparing the enzyme activity before and after the optimal biotinylation a 20\% decrease of the activity was found for HRPO and only 7.5\% for \( \beta \)-galactosidase.

For ELISA measurements the optimal ratio of biotinylated enzyme and streptavidin can only be determined empirically by titration against the biotinylated model protein immobilized on an ELISA plate. Fig. 5 shows the result of such a typical experiment using biotinylated BSA. For the optimal signal a 1:0.6 HRPO:streptavidin weight ratio was found. A similar titration in the case of \( \beta \)-galactosidase yielded an optimum around 1:2.5 weight ratio (data not shown). The successful biotinylation of proteins with the reagent (3) allowed their suitability for a quantitative ELISA. Plates coated with biotinylated BSA or Ig were incubated for one hour with the biotinylated HRPO streptavidin complex and developed according to the standard procedure [12]. The sensitivity of the detection was found to be 1.5 ng for BSA and 5 ng for Ig. Longer incubation time lowers the sensitivi-

Fig. 5. Optimization of the biotinylated HRPO/streptavidin complex. Biotinylated horseradish peroxidase (1 \( \mu \)g/ml) was incubated with 0.2–1.4 \( \mu \)g/ml streptavidin for 1 h. 100 \( \mu \)l aliquots of the complex were introduced into the wells of the ELISA plates, precoated with 50 ng/well biotinylated BSA and blocked with 0.5\% BSA. After incubation for 1 h and subsequent washing, the enzyme activity was developed as described in the legend of Fig. 2.
and sensitivities of 0.1 respectively 0.5 ng were achieved.

In summary, our new biotinylating reagent, the pentafluorophenyl 6-(biotinylamido)hexanoate (3) can be prepared by a very convenient way with good yield and proved to be an appropriate agent for effective biotin-labelling of proteins. This reagent is quite stable during storage over a year period. The specific Ig-s labelled by the reagent were suitable tools to use them in specific and selective steroid ELISA test.