BIOTINYLATION OF AMINES WITH THE PENTAFLUOROPHENYL ESTER OF D-BIOTIN

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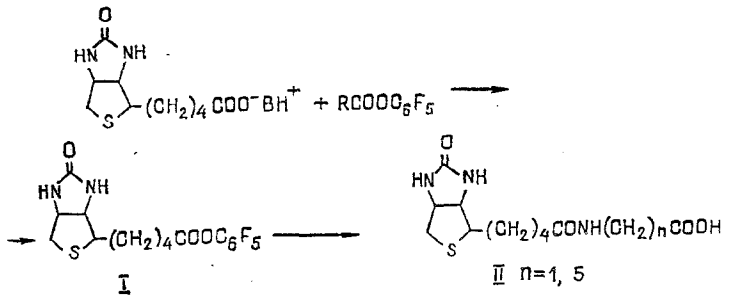
The synthesis has been effected of the pentafluorophenyl ester of D-biotin by the reaction of D-biotin with bispentafluorophenyl carbonate or pentafluorophenyl trifluoroacetate. The possibility has been shown of using the ester obtained for the biotinylization of amino acids and NH_2-containing macromolecules, and D-biotinyl-ε-aminocaproic acid and -[^{14}C]glycine have been synthesized. It is proposed to use the biotinyl-[^{14}C]glycine for measuring the activity of avidin.

The avidin-biotin system has come into ever wider use in recent years in various fields of molecular biology, biotechnology, and laboratory diagnosis. Its use has proved to be particularly fruitful in affinity chromatography and immunoenzyme assay and as a nonisotopic label in the hybridization analysis of nucleic acids. The functioning of the system is based on the extremely high affinity of the water-soluble vitamin biotin for a protein from hens' eggs, avidin (K_d 10^{-15} M). The introduction of biotin residues into macromolecules permits their specific and strong binding through avidin (a tetramer with four identical binding centers) fulfilling the role of a bridge.

At the present time, a wide variety of biotin derivatives is being used which permits the biotinylation of proteins at various groups of the biopolymer: NH_2—, aldehyde, —COOH, —SH—, etc. [1, 2]. The most common is biotinylation at NH_2 groups for which the N-hydroxysuccinimide and p-nitrophenyl esters are mainly used. However, their preparation has a number of disadvantages connected with the use of dicyclohexylcarbodiimide as condensing agent [1, 3]. Furthermore, it is known that N-hydroxysuccinimide esters are insufficiently stable on storage, and when amines are isolated with them side reactions are sometimes observed.

In the last decade, wide use in the chemistry of peptides has been found by the highly reactive pentafluorophenyl esters, the synthesis of which is possible without the use of dicyclohexylcarbodiimide with the aid of bispentafluorophenyl carbonate (BPPC) [4] or pentafluorophenyl trifluoroacetate (PPTA). An important advantage of these reagents in comparison with the use of the carbodiimide method consists in the fact that no by-products are formed in the reaction. It was therefore desirable to study the possibility of using these reagents for obtaining the pentafluorophenyl ester of biotin and its subsequent use as a biotinylating agent. The reactions of biotin with BPPC and PPTA were performed under the conditions recommended for obtaining N-pentafluorophenyl-substituted amino acids with the aid of these reagents [4].



B = pyridine or N-methylmorpholine; R = CF_6 or C_6F_4O

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The ester (I) obtained consisted of a high-melting crystalline compound which crystallized from dimethylformamide even during the process of its preparation and was readily freed from by-products. The structure of the ester (I) was confirmed by IR spectroscopy and elementary analysis, and its individuality by TLC results. The yields of the ester (I) using BPPC and PPTA amounted to 94 and 74%, respectively. The ester (I) reacted with the triethylammonium salts of amino acids without complications and, in particular, N-biotinylglycine and biotinyl-ε-aminocaproic acid (II, n = 1 and 5) were isolated in high yield (85%) and were identical with the substances obtained with the use of the N-hydroxysuccinimide ester. The ester (I) synthesized has also been used to obtain a biotin-containing support – biotinyl-6-aminohexyl-Sepharose, which was then used to isolate avidin from hen's egg proteins by the method of [5]. For this purpose, a solution of the fraction from 65-100% saturation with $(NH_4)_2SO_4$ was deposited on a column with the support mentioned and elution was performed with 6 M guanidine–HCl, pH 1.5.

Various methods have been developed for measuring the activity of avidin: radioligand, colorimetric, and fluorometric [6-8]. Of these, only the first, based on the interaction of the protein with [^{14}C]biotin, permits the direct measurement of the specific activity of avidin (maximum activity, 15 μg of biotin per 1 mg of protein, 15 AU/mg). On the basis of the idea of the key role of the ureide group of the heterocyclic fragment of the biotin molecule in binding with avidin and the unimportance of the aliphatic part of the molecule for measuring the activity of avidin, we used biotinyl-[^{14}C]glycine obtained by the reaction of the ester (I) with [^{14}C]glycine. We showed that this compound, like [^{14}C]biotin interacts quantitatively with avidin. The main advantage, distinguishing it from [^{14}C]biotin, is the simplicity of the synthesis of the labeled ligand for measuring avidin activity. Avidin activity was calculated from the formula

$$I = \frac{\alpha A \cdot M_b}{SA},$$

where I is the binding capacity (activity) of the avidin, μg of biotin;

α is the counting efficiency;

A is the radioactivity found experimentally, pulses/min;

SA is the specific activity of the biotinyl-[^{14}C]glycine (μCi/μmole); and

M_b is the molecular mass of biotin.

EXPERIMENTAL

We used D-biotin from Sigma, amino acids from Reanal, AH-Sepharose-4B from Pharmacia, CM-cellulose from Whatman, guanidine–HCl from Merck, [^{14}C]glycine from Izotop, PPTA kindly supplied by O. M. Galkin (Institute of Organometallic Compounds of the USSR Academy of Sciences, Moscow), and BPPC from Soyuzkhimreaktiv. The other reagents were freshly prepared or of os.ch. ["ultrapure"] grade. IR spectra were taken in KCl tablets on a Pye Unicam spectrophotometer. Angles of optical rotation were determined on a Perkin-Elmer 241 polarimeter. TLC was conducted on Silufol plates (Kavalier) in the following solvent systems: 1) benzene–acetone–acetic acid (100:50:1); and 2) chloroform–methanol–acetic acid (8:2:1). Melting points were determined in open capillaries on Boetius heated stages.

Pentafluorophenyl Ester of D-Biotin (I). A. A suspension of 130 mg (0.5 mmole) of D-biotin in 0.5 ml of dimethylformamide was treated with 0.08 ml of pyridine and 0.1 ml of PPTA. The mixture was stirred for 2 h, during which it gradually thickened. After the addition of 2 ml of dimethylformamide it was stirred for another 1 h. Then it was diluted with water to 10 ml and was stirred for 1 h, and the precipitate was filtered off, washed with water and with ether, and dried in vacuum. This gave 150 mg (74%) of a product with mp 186-189°C, R_f 0.31 (in system 1).

B. A suspension of 0.5 g (2 mmole) of D-biotin in 3 ml of dimethylformamide was treated with 0.3 ml of N-methylmorpholine. The mixture was stirred for 10 min, and 0.9 g (2.2 mmole) of BPPC was added. A vigorous reaction took place with the evolution of a gas, and the reaction mixture thickened. It was stirred for 1.5-2 h and was left in the cold for the completion of crystallization. Then it was diluted with ether to 20 ml and the precipitate was separated off, washed with ether, and dried. This gave 0.8 g (97%) of product with mp 186-187°C, $[\alpha]_D^{20}$ +64.0° (c 1; CH_3COOH), R_f 0.31 (in system 1). IR spectrum (in KCl),

ν, cm^{-1}: 3300 (NH), 1800, 1715 (C=O). $C_{16}H_{15}N_2O_3F_5S$. The results of analysis for N and F corresponded to the calculated figures.

D-Biotinyl-ε-aminocaproic Acid (II, n = 5). A solution of 0.2 g of ε-aminocaproic acid and 0.2 ml of triethylamine in 3 ml of water and 2 ml of acetone was treated with 500 mg of the ester (I). The mixture was stirred for 6 h and was then filtered and acidified with acetic acid. The resulting precipitate was filtered off, washed with water and with ether, and was dried in vacuum over P_4O_{10}. This gave 380 mg (95%) of the desired product with mp 219-220°C, $[\alpha]_D^{20}$ +53.0° (c 1; CH_3COOH). R_f 0.50 (in system 2), $C_{16}H_{27}N_3O_4S$. The results of analysis for C, H, and N corresponded to the calculated figures.

D-Biotinyl-[^{14}C]glycine (II, n = 1). A solution of 80 mg of glycine (1 mCi/mmole) in 0.4 ml of H_2O was treated with 0.2 ml of dimethylformamide and 0.15 ml of triethylamine. After the addition of 250 mg of the ester (I), it was brought into complete solution with 0.3 ml of acetone and the mixture was stirred for 16 h, after which it was diluted with H_2O to 20 ml and was acidified with 0.5 ml of glacial acetic acid. The resulting precipitate was filtered off, washed with H_2O, and dried in the air. About 250 mg of product was formed. After recrystallization from methanol and washing with ether, the substance was dried in vacuum. This gave 200 mg of the desired product in the homogeneous state. R_f 0.5 (in system 2). mp 242-243°C (decomp.). $C_{12}H_{19}N_3O_4S$. The results of analysis corresponded to the calculated figures.

D-Biotinyl-6-aminohexyl-Sepharose. A solution of 500 mg (1.22 mmole) of the ester (I) in 50 ml of dimethylformamide was mixed with 50 ml of AH-Sepharose containing 8 μmole of amino groups in 1 ml. The mixture was stirred slowly at 4°C overnight. In the morning, the gel was washed with 1 liter of 1 M NaCl, 100 ml of 6 M guanidine–HCl, and 2 liters of water. The synthesis of biotinyl-6-aminohexyl-Sepharose was judged from the decrease in the number of NH_2 groups in the reaction with picric acid [9]. The results obtained indicated the inclusion of about 4 μmole of biotin per 1 ml of Sepharose.

Isolation of Avidin from Hens' Eggs [5]. The whites were separated from 50 eggs and were diluted with double-distilled water in a ratio of 1:1.5, after which fractionation with ammonium sulfate was carried out. The fraction from 65-100% was collected, dissolved in 1 M NaCl solution, and deposited on a column (3 × 10 cm) containing biotinyl-6-aminohexyl-Sepharose at the rate of 20 ml/h. The column was washed with 1 M NaCl and then with 3 M guanidine–HCl. The avidin was eluted with 6 M guanidine–HCl, pH 1.5. The fractions containing proteins were collected, combined, dialyzed, and lyophilized. The yield of avidin with a specific activity of 14-14.5 AU/mg of protein in the procedure described amounted to 50 mg.

Determination of the Activity of Avidin. An aliquot of an avidin solution was added to 1 ml of 0.05 M pyridine acetate buffer, pH 5.0, containing 12 mg of CM-cellulose and 2000-3000 pulses/min of biotinyl-[^{14}C]glycine, and, after periodic stirring up, the sample was left for the complete settling of the resin. The method is a modification of [6] based on determining the residual radioactivity in the supernatant after the absorption of the avidin-[^{14}C]biotin complex on CM-cellulose. This permits the calculation of the amount of biotin bound to unit mass of protein possessing avidin activity. Having calculated the specific radioactivity of the biotinyl-[^{14}C]glycine that had been synthesized with respect to an internal standard (in our case, it was $4 \cdot 10^6$ pulses/min·mg of substance) and having confirmed the formula given above for the calculation of activity

$$I = \frac{A \cdot 244.3}{4 \cdot 10^3 \cdot 318},$$

It was possible to determine the specific activity of the avidin preparation obtained.

SUMMARY

1. A method has been developed for obtaining a new activated pentafluorophenyl ester of biotin, and the possibility of its use for modifying the NH_2 groups of amino acids and macromolecules has been shown.

2. It is proposed to use biotinyl-[^{14}C]glycine for measuring the activity of avidin.

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QUANTITATIVE DETERMINATION OF ANTIBIOTICS IMMOBILIZED ON INSOLUBLE COLLAGENOUS MATERIALS

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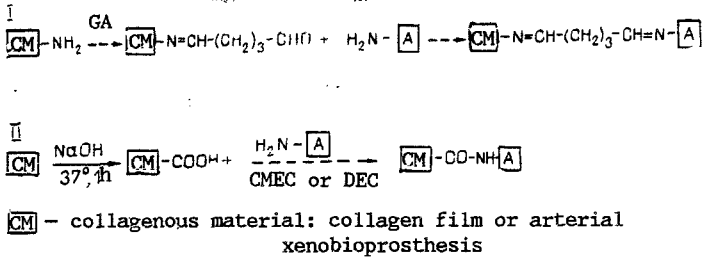
For the quantitative determination of antibiotic bases (ABs) immobilized on insoluble collagenous materials (CMs) the preparation is treated with TNBS in borate buffer, pH 9.1, at 37°C for 2 h, followed by the elimination of the excess of TNBS, the splitting out of the TNP groups in the form of TNP-NH_2 in 2 N NH_4OH at 37°C for 2 h, and measurement of the concentration of TNP-NH_2 at 409 nm. The method permits concentrations of ABs in the range of 2-50 μg/cm² of surface of the CM to be determined.

We have continued investigations of the covalent immobilization of antibiotics [1] and anticoagulants [2] on various collagenous supports.

In the course of the work, the problem arose of the quantitative determination of antibiotics immobilized on insoluble collagenous materials – films, arterial xenobioprostheses, etc. A number of methods of determining amounts of low-molecular-mass amino-containing biologically active substances fixed to a solid support is known. They are based on an analysis of the products of the complete hydrolysis of the complex [3] or on a determination of the residual amounts of dye after its binding by the biologically active substances immobilized on the polymer [4]. However, in our case, for the analysis of xenobioprostheses, in particular, these methods are inapplicable, since the percentage content of immobilized antibiotic is low, usually being less than 5-10%. This complicates the quantitative determination in amino acid analyzers of nonprotein amino acids or amino carbohydrates from an antibiotic in an acid hydrolysate of the complex, and also the real measurement of the change in the color of the solution after the sorption of a dye by the bound antibiotic.

Scheme 1



CM – collagenous material: collagen film or arterial xenobioprosthesis

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