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Synthesis of Tritium-Labeled Selank

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Abstract—A scheme was developed for the synthesis of Selank (Thr–Lys–Pro–Arg–Pro–Gly–Pro) and its precursor (Thr–Lys–Pro–Arg–Pro–Gly). Condensation of the *tert*-butyloxycarbonyl derivative of Thr–Lys–Pro–Arg–Pro–Gly with labeled proline yielded Selank labeled in the C-terminal proline fragment. The molar radioactivity of tritium-labeled Selank was 56 Ci mmol^{-1}, which corresponded to the molar radioactivity of proline.

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Development of novel effective antianxiety drugs that would be free of side effects characteristic of benzodiazepine tranquilizers is an urgent problem. Such drugs are used not only for treatment of diseases, but also for correction of psychoemotional disorders of actively working humans. One of such agents if a seven-memberd analogh of taftsin, Selank (Thr–Lys–Pro–Arg–Pro–Gly–Pro). This compound not only reduces manifestations of anxiety but also inhibits encephalin-degrading enzymes, i.e., prolongs the lifetime of opioids in a living body [2].

The goal of this study was preparation of labeled Selank, required for more detailed examination of the biological effect of this peptide.

EXPERIMENTAL

In the synthesis we used *L*-amino acid derivatives. The *tert*-butyloxycarbonyl derivative of Thr–Lys–Pro–Arg–Pro–Gly was synthesized in the Russian Cardiological Research and Production Complex, Ministry of the Public Health of the Russian Federation.

All the solvents were dehydrated. The melting points were determined on a Boëtius heating block and are given without correction. Elemental analysis of the peptides was performed on a Carlo Erba Model 1106 analyzer; consistent results were obtained. The optical rotation was measured on an A1-EPO digital polarimeter. The chromatographic plates were developed by UV light, with ninhydrin, with Barton, Pauli, and Reindel–Hoppe reagents, and with *o*-tolidine in a chlorine atmosphere. The labeled peptide was purified and analyzed by HPLC.

The purity of nonlabeled compounds was checked by TLC on Silufol plates. The following solvent systems were used: **1**, acetone–benzene–acetic acid (50 : 100 : 1); **2**, chloroform–methanol (9 : 1); **3**, hexane–acetone (3 : 2); **4**, butanol–acetic acid–water (4 : 1 : 1); **5**, butanol–acetic acid–pyridine–water (30 : 6 : 20 : 24); **6**, chloroform–methanol–ammonia (6 : 4 : 1); **7**, benzene–ethanol (8 : 2); **8**, ethyl acetate–acetone–50% acetic acid (2 : 1 : 1); **9**, chlroform–methanol (14 : 1); **10**, chloroform–methanol–ammonia (8 : 1.75 : 0.25); and **11**, chloroform–methanol–ammonia (6.5 : 3.0 : 0.5).

We use the following abbreviations: Arg, arginine; Gly, glycine; Lys, lysine; Pro, proline; Thr, threonine; Z, benzyloxycarbonyl group (Cbz); DMF, dimethylformamide; DCU, dicyclohexylurea; DCC, dicyclohexylcarbodiimide; HOBT, hydroxybenzotriazole; TEA, triethylamine; OPfp, pentafluorophenyl ester; EA, ethyl acetate; Boc, *tert*-butyloxycarbonyl; TLC, thin-layer chromatography; PivCl, pivalyl chloride; TFA, trifluoroacetate; HTFA, trifluoroacetic acid; OBzl, benzyl ester; and THF, tetrahydrofuran.

I. Boc–Pro–Gly–OH. A 10.75-g portion of Boc–Pro (50 mmol) was dissolved 150 ml of acetonitrile. The solution was cooled to –5°C, and 7.7 ml of TEA (50 mmol) was added with stirring, after which the solution was cooled to –20°C. To the cooled solution, 6.8 ml of PivCl (55 mmol) was added with stirring over a period of 20 min at –10°C. The resulting solution was cooled to –30°C, and a precooled solution of Gly was added. The Gly solution was prepared as follows: 4.5 g of Gly (60 mmol, 1.2-fold excess) was dissolved in 35 ml of water and 60 ml of acetonitrile,

8.4 ml of TEA (60 mmol) was added, and the solution was cooled at –10°C for 20 min.

The reaction mixture was stirred for 1 h at –10°C and 2 h at 18–20°C. Then the mixture was evaporated, and ~50 ml of water was added to the residue. The aqueous solution was acidified with a threefold excess of $NaHSO_4$ (24.84 g) to pH 3 and extracted with EA (5 × 100 ml). The combined EA solution was washed with H_2O (50 ml), 10% $KHSO_4$ solution (50 ml), H_2O (50 ml), and saturated NaCl solution (50 ml), after which it was dried over $MgSO_4$. The dried solution was filtered, evaporated to a volume of 50 ml, and diluted with dry ether. The precipitate that formed on adding ether was filtered off and washed on the filter with dry ether. The resulting product was dried in a vacuum desiccator over KOH, P_2O_5, and paraffin. Yield 5.97 g (21.74 mmol, 43%), mp 70°C, R_f 0.863 (**1**), 0.903 (**2**), 0.746 (**7**), 0.847 (**8**).

II. Boc–Pro–Gly–Pro–OBzl. A 5.97-g portion of Boc–Pro–Gly–OH (21.74 mmol) was dissolved in 100 ml of acetonitrile; the solution was cooled to –5°C. A 1.1-fold excess of TEA (3.35 ml, 23.9 mmol) was added with stirring, and the solution was cooled to –20°C. To the cooled solution, a 1.1-fold excess of PivCl (2.34 ml, 23.9 mmol) was added with stirring over a period of 20 min at –10°C. The solution was cooled to –30°C, and a precooled solution of HCl·Pro–OBzl was added. This solution was prepared as follows: 6.3 g of HCl·Pro–OBzl (26.1 mmol, 1.2-fold excess) was dissolved in 50 ml of acetonitrile, 4.0 ml of TEA (28.71 mmol, 1.1-fold excess) was added, and the solution was cooled at –10°C for 20 min.

The reaction mixture was stirred for 1 h at –10°C and for 2 h at 18–20°C, after which it was evaporated, and 300 ml of EA was added to the residue. The EA solution was washed with H_2O (3 × 25 ml), 10% $KHSO_4$ solution (3 × 25 ml), H_2O (3 × 25 ml), 5% $NaHCO_3$ solution (3 × 25 ml), H_2O (3 × 25 ml), and saturated NaCl solution (25 ml). The EA solution was dried over $MgSO_4$. The dried EA solution was filtered and evaporated to an oily residue, which was treated with ~100 ml of dry ether. The precipitate that formed on adding ether was filtered off, washed on the filter with dry ether, and dried in a vacuum desiccator over KOH, P_2O_5, and paraffin. Yield 8.12 g (17.66 mmol, 81%), mp 125–126°C, R_f 0.326 (**1**), 0.947 (**2**), 0.390 (**3**), 0.620 (**7**).

III. TFA·Pro–Gly–Pro–OBzl. A 5.358-g portion of Boc–Pro–Gly–Pro–OBzl (11.65 mmol) was dissolved in 29.13 ml of CH_2Cl_2, 29.13 ml of HTFA was added, and the mixture was allowed to stand for 45 min at room temperature, after which it was evaporated two times with absolute ethanol, two times with benzene, and two times with ether. The residue was dissolved in benzene, and the product was precipitated with hexane. The supernatant was decanted, and the precipitated oil was dried in a desiccator over P_2O_5/KOH and paraffin. Yield 4.63 g (9.7 mmol, 98%), R_f 0.043 (**1**), 0.247 (**2**), 0.018 (**3**).

IV. Boc–Arg(NO$_2$)–Pro–Gly–Pro–OBzl. A 3.09-g portion of Boc–Arg(NO_2)–OH (9.7 mmol) was dissolved in 50 ml of THF and 10 ml of DMF, 2.07 g (10.67 mmol) of DCC was added, the mixture was cooled to 0°C and stirred for 40 min, after which a solution of TFA–Pro–Gly–Pro–OBzl in 50 ml of THF and 4.46 ml of TEA (9.7 mmol) were added. The mixture was stirred for 3 days. The DCU precipitate was filtered off, the solution was vacuum-evaporated, and the residue was treated with 200 ml of hexane; in so doing, the target product precipitated as an oil. This oil was dissolved in 500 ml of EA, and the solution was washed with 0.1 M HCl (3 × 25 ml), H_2O (3 × 25 ml), and saturated NaCl solution (25 ml). The organic layer was dried over $MgSO_4$, filtered, and evaporated. The residue was dissolved in EA and precipitated with dry ether. The precipitate was filtered off and vaccum-dried over P_2O_5/KOH and paraffin. Yield 4.76 g (7.9 mmol, 85%), mp 108–110°C, $[\alpha]_D^{22}$ = –77.8° (c = 0.5; CH_3COOH), R_f 0.44 (**4**), 0.8 (**5**).

V. TFA·Arg(NO$_2$)–Pro–Gly–Pro–OBzl. A 4.76-g portion of Boc–Arg(NO_2)–Pro–Gly–Pro–OBzl (7.9 mmol) was dissolved in 20 ml of CH_2Cl_2, 20 ml of HTFA was added, and the mixture was allowed to stand for 45 min at room temperature, after which it was evaporated two times with absolute ethanol, two times with benzene, and two times with ether. The residue was dissolved in benzene, and the product was precipitated with hexane. The supernatant was decanted, and the precipitated oil was dried in a desiccator over P_2O_5/KOH and paraffin. Quantitative yield; R_f 0.16 (**4**), 0.27 (**5**).

VI. Boc–Pro–Arg(NO$_2$)–Pro–Gly–Pro–OBzl. A 1.7-g portion of Boc–Pro (7.9 mmol) was dissolved in 20 ml of THF, 1.07 g of HOBT (7.9 mmol) was added, the mixture was cooled to 0°C, and a solution of 1.8 g of DCC in 50 ml of THF was added. The mixture was allowed to stand for 40 min, after which a solution of TFA·Arg(NO_2)–Pro–Gly–Pro–OBzl (7.9 mmol) in 50 ml of THF and 1.1 ml of TEA (7.9 mmol) were added. The mixture was stirred for 2 h at 0°C and 2 days at room temperature, after which DCU was filtered off, the solution was vacuum-evaporated, and the residue was dissolved in 500 ml of EA and worked up as described above for

Boc–Arg(NO_2)–Pro–Gly–Pro–OBzl. Yield 4.07 g (6.5 mmol, 68%), mp 147–148°C, R_f 0.42 (**4**), 0.72 (**5**), 0.31 (**9**).

VII. TFA · Pro–Arg(NO_2)–Pro–Gly–Pro–OBzl. A 4.07-g portion of Boc–Pro–Arg(NO_2)–Pro–Gly–Pro–OBzl (5.3 mmol) was dissolved in 14 ml of CH_2Cl_2, 14 ml of HTFA was added, and the mixture was allowed to stand for 45 min at room temperature, after which it was evaporated two times with absolute ethanol, two times with benzene, and two times with ether. The residue was dissolved in benzene, and the product was precipitated with hexane. The supernatant was decanted, and the precipitated oil was dried in a desiccator over P_2O_5/KOH and paraffin. Yield 3.94 g (5.2 mmol, 97%), R_f 0.25 (**2**), 0.10 (**4**), 0.20 (**10**).

VIII. Boc–Lys(Z)–Pro–Arg(NO_2)–Pro–Gly–Pro–OBzl. To 3.8 g of Boc–Lys(Z)–OH (10 mmol) in 25 ml of absolute EA, we added 2.26 g of DCC and 1.87 g of pentafluorophenol in 50 ml of THF; the mixture was cooled to 0°C. Then the mixture was stirred for 2 h at 0°C, DCU was filtered off, and the solution was vacuum-evaporated; 25 ml of absolute ether and 75 ml of hexane were added to the residue. A precipitate gradually formed on cooling; it was filtered off and vacuum-dried. Yield 2.8 g (87%), mp 117–120°C, R_f 0.84 (**4**), 0.91 (**9**).

To a solution of 2.27 g of TFA · Pro–Arg(NO_2)–Pro–Gly–Pro–OBzl (5.2 mmol) in 50 ml of THF, cooled to 0°C, we added with stirring 0.75 ml of TEA and then 2.8 g of Boc–Lys(Z)–OPfp (5.2 mmol) in four portions over a period of 2 h. The mixture was stirred for 24 h at room temperature, after which it was vacuum-evaporated, the residue was dissolved in 300 ml of EA, and the solution was worked up as described above for Boc–Arg(NO_2)–Pro–Gly–Pro–OBzl. Yield 3 g (3 mmol, 58%), mp 130–132°C, $[\alpha]_D^{22}$ = –86.2° (c = 0.5, CH_3COOH), R_f 0.38 (**4**), 0.75 (**5**).

IX. HCl · Lys(Z)–Pro–Arg(NO_2)–Pro–Gly–Pro–OBzl. To 4.68 g (5.3 mmol) of the protected hexapeptide Boc–Lys(Z)–Pro–Arg(NO_2)–Pro–Gly–Pro–OBzl we added 7.95 ml of a 1 M solution of HCl in EA. The mixture was allowed to stand for 45 min at room temperature, and the product was precipitated with hexane. The supernatant was decanted, and the precipitated oil was dried in a desiccator over P_2O_5/KOH and paraffin. The dried oil was reprecipitated from absolute methanol with dry ether, and the precipitate was filtered off and vacuum-dried. Yield 4.03 g (4.5 mmol, 86%), R_f 0.12 (**4**), 0.45 (**5**), 0.293 (**10**), 0.492 (**11**).

X. Boc–Thr–Lys(Z)–Pro–Arg(NO_2)–Pro–Gly–Pro–OBzl. To a solution of 3 g of HCl · Lys(Z)–Pro–Arg(NO_2)–Pro–Gly–Pro–OBzl (3 mmol) in 15 ml of THF, cooled to 0°C, we added 0.42 ml of TEA (pH of the reaction mixture should be 6–7) and 1.15 g of Boc–Thr–OPfp (3 mmol) prepared similarly to Boc–Lys(Z)–OPfp as described above. Further procedure was similar to that described above for Boc–Lys(Z)–Pro–Arg(NO_2)–Pro–Gly–Pro–OBzl. Yield 3.08 g (80%), mp 155–156°C, R_f 0.39 (**4**), 0.8 (**5**), 0.96 (**6**), 0.145 (**9**).

XI. Boc–Thr–Lys–Pro–Arg–Pro–Gly–Pro–OH. A 0.85-g portion of Boc–Thr–Lys(Z)–Pro–Arg(NO_2)–Pro–Gly–Pro–OBzl (0.75 mmol) was dissolved in 50 ml of methanol, and 0.5 ml of 1 M HCl and 0.85 g of a catalyst, 10% Pd/Al_2O_3, were added. The mixture was hydrogenated in an H_2 flow at room temperature and a pressure of 1 atm for 6 h. Then the catalyst was filtered off and washed on the filter with methanol. The combined filtrate was evaporated to dryness. The residue was reprecipitated from absolute CH_3OH with ether and vacuum-dried. Yield 0.56 g (0.67 mmol, 89%), R_f 0.125 (**4**), 0.37 (**5**), 0.57 (**6**).

XII. Thr–Lys–Pro–Arg–Pro–Gly–Pro (Selank). A 0.56-g portion of Boc–Thr–Lys–Pro–Arg–Pro–Gly–Pro (0.67 mmol) was suspended in 10 ml of a 2 M solution of HCl in dioxane; the mixture was allowed to stand at room temperature for 45 min. The product was precipitated with hexane. The precipitate was filtered off and repeatedly washed on the filter with hexane to remove excess HCl. The washed precipitate was vacuum-dried, reprecipitated from absolute methanol with dry ether, filtered off, vacuum-dried, and analyzed by HPLC [4.6 × 150-mm column, Kromasil 100 C18, 6 μm; eluent CH_3OH–50 mM $(NH_4)H_2PO_4$, pH 2.8 (5 : 95), flow rate 1 ml min^{-1}, retention time 8.53 min]. Yield 0.40 g (75%), mp 134–136°C, $[\alpha]_D^{20}$ = –62.8° (c = 0.25; CH_3COOH), R_f 0.263 (**5**), 0.22 (**6**). Amino acid composition relative to arginine: Thr 0.9 (1), Lys 0.98 (1), Pro 2.78 (3), Gly 1.1 (1).

Synthesis of Tritium-Labeled Selank

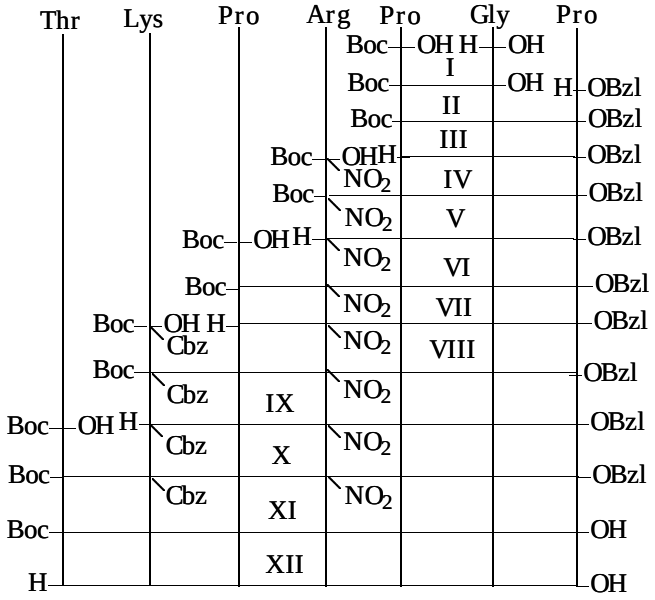
Synthesis of *L*-proline highly labeled with tritium. A mixture of 100 mg of 5% PdO/$BaSO_4$ and 10 mg of $RhCl_3$ in 0.1 ml of water was evaporated on a rotary evaporator, and a solution of 10 mg of pyrrole-2-carboxylic acid in 0.15 ml of aqueous methanol (1 : 1) was added. The resulting mixture was evaporated on a rotary evaporator and freeze-dried. A 50-mg portion of the residue was taken for the reaction. The hydrogenation with tritium was performed at 135°C

for 10 min. Excess tritium was pumped off. After cooling, the reaction products were extracted with 10% aqueous methanol (6 × 1 ml), the catalyst was filtered off, and the filtrates were evaporated several times as a solution in 10% methanol (3 × 3 ml) to remove labile tritium. The primary purification was performed on a C18 cartridge in 0.1% HTFA, analysis in the system isopropanol–methanol–water–heptafluorobutyric acid (10 : 30 : 959 : 1). After HPLC purification [Kromasil 100 C18, 7 μm, 4 × 150 mm, v 1.0 ml min^{-1}, system isopropanol–methanol–water–heptafluorobutyric acid (10 : 20 : 969 : 1), retention time 4.28 min), the yield of the labeled product was 15–20%, molar radioactivity 50 – 60 Ci $mmol^{-1}$. Racemic proline was separated into optical isomers on a column packed with polystyrene sorbent containing *L*-hydroxyproline groups [3, 4]. The stationary phase was saturated with 0.6 mM $CuCl_2$, racemic proline dissolved in a minimal volume of water was applied, and the isomers were eluted with 0.1 M NH_4OH (enantioselectivity of ligand-exchange chromatography 3.95, retention factor of *L*-proline 14.4); molar radioactivity of *L*-proline 50 – 60 Ci $mmol^{-1}$.

Condensation of *tert*-butyloxycarbonyl derivative of Thr–Lys–Pro–Arg–Pro–Gly with labeled proline. A solution of 10 mCi of *L*-[^{3}H]proline in 0.5 ml of methanol was treated with 10 μl of TEA; the solution was evaporated on a rotary evaporator, and the residue was freeze-dried. A solution of 1 mg of the protected hexapeptide and 2 mg of carbonylditriazole in 0.4 ml of DMF was stirred at room temperature for 80 min and transferred into an ampule with labeled proline. The ampule was sealed, and the mixture was stirred at room temperature for 1.5 h and at 50°C for 15 min. The solvent was removed by freeze drying, and the residue was treated with 0.4 ml of HCl-saturated dioxane. Labeled Selank was purified by HPLC [Kromasil 100 C18, 6 μm, 4.6 × 150 mm, precolumn: 4.0 × 4 mm; eluent **A**: methanol–ammonium phosphate buffer, pH 2.8 (5 : 95); eluent **B**: methanol; linear gradient from 0 to 100% eluent **B** in the course of 30 min, flow rate 1 ml min^{-1}, Selank retention time 8.93 min]. The yield reached 15%; molar radioactivity 56 Ci $mmol^{-1}$.

RESULTS AND DISCUSSION

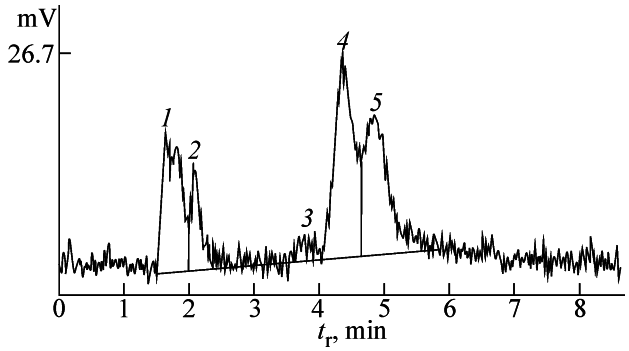
To prepare Selank and its precursor (with which tritium-labeled proline was condensed to obtain Selank labeled in the C-terminal proline fragment), we used the following scheme:



Selank was prepared by stepwise lenghthening of the peptide chain by classical methods of the peptide chemistry in solution. We used the methods of mixed anhydrides and activated esters. *L*-Amino acid derivatives were taken.

To prepare the protected precursor (Thr–Lys–Pro–Arg–Pro–Gly), this scheme was modified. Firstly, as the starting compound we used Boc–Pro–Gly–OBzl instead of Boc–Pro–Gly–Pro–OBzl. Secondly, Pro–Arg(NO_2)–Pro–Gly–OBzl was condensed with Boc–Thr–Lys(Boc)–OH and not with Boc–Lys(Z)–OH; by so doing, we prepared Boc–Thr–Lys(Boc)–Pro–Arg(NO_2)–Pro–Gly–OBzl. The latter modification was caused by the fact that the hydrogenation of Boc–Thr–Lys(Z)–Pro–Arg(NO_2)–Pro–Gly–OBzl prepared by the initial scheme yields the peptide Boc–Thr–Lys–Pro–Arg–Pro–Gly with a free ε-NH_2 group in lysine, which does not allow its use in the condensation with labeled proline.

The condensation of Boc–Thr–Lys(Boc)–Pro–Arg–Pro–Gly with tritium-labeled *L*-proline was performed using carbonylditriazole as condensing agent, at varied ratio of the Selank precursor and proline. We found that with increased amounts of proline the yield of Selank was higher. This fact may be due to partial consumption of proline in side reactions. For example, at the proline concentration increased by a factor of 3, the yield of Selank increased by a factor of 2.5. The relatively low yield of labeled Selank (~15%) may be caused by the presence of the unprotected guanidine group in the arginine fragment of the precursor. This fact is apparently responsible for the formation of a number of by-products in the condensation of Boc–



Radioactivity distribution in HPLC analysis of the reaction mixture after the condensation of labeled proline with the Selank precursor [Kromasil 100 C18, 4.6 × 150 mm, 6 μm, precolumn: 4.0 × 4 mm; 60% methanol + 0.1% HTFA and methanol, gradient from 0 to 100% in the course of 30 min, v 1 ml min^{-1}, retention time of the *tert*-butyloxycarbonyl derivative of Selank (peak *4*) 4.41 min]. (*1*–*3*, *5*) Impurity peaks.

Thr–Lys(Boc)–Pro–Arg–Pro–Gly with labeled proline (see figure). However, an increase in the amount of tritium-labeled *L*-proline is economically unfeasible because of its high cost.

The labeled compounds giving peaks *4* and *5* were isolated, the *tert*-butyloxycarbonyl protecting group was removed by acid hydrolysis, and the products were analyzed by HPLC using as references Selank and Thr–Lys–Pro–Arg–Pro–Gly synthesized by the above scheme. We found that the major amount of the labeled compound was present in the first fraction (labeled compound corresponding in the chromatographic mobility to peak *4*). This fact indicates that the protected Selank only slightly differs from Boc–Thr–Lys(Boc)–Pro–Arg–Pro–Gly in the chromatographic mobility, and the by-products are less polar substances. After the deprotection, the differences in the chromatographic mobility between Selank, on the one hand, and the starting compounds and by-products, on the other hand, become more pronounced, which allowed preparation of the labeled product with a high radiochemical purity.

Thus, we prepared labeled Selank with a molar radioactivity of 56 Ci mmol^{-1}.

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