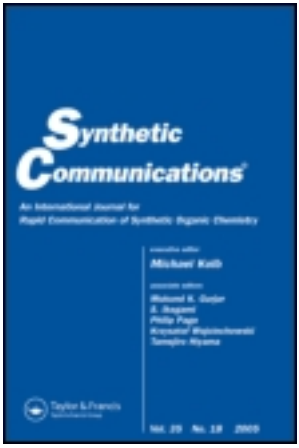

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PREPARATION AND ACYLATION OF A NEW BIOTINYLATION REAGENT

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ABSTRACT: Biotinylamidopropylammonium trifluoroacetate was obtained by acylation of 4-(tert-butyloxycarbonylamino)propylamine, followed by removal of the protective group. Consequently, it was found to be useful for the development of effective immunoassays.

Biotin-avidin (-streptavidin) complexes are currently used in many analytical procedures.[1] In particular, biotin conjugates are useful for the determination of various compounds in body fluids by non-isotopic immunoassays. As compared to radioimmunoassays, non-isotopic immunoassays have many advantages, including the reduced cost of the tracers and reagents, non-elimination of radioelements and absence of repetitive (every 2-3 months) isotopic labelling when ^{125}I is the isotope. The biotin conjugates required in these applications are in most cases obtained by acylation with biotin-N-hydroxysuccinimide.[2] Very few reagents can be used when an amino derivative of biotin is required. Biotinylhydrazide is the main nucleophilic derivative of biotin. However, the nucleophilicity of the free nitrogen is low and the

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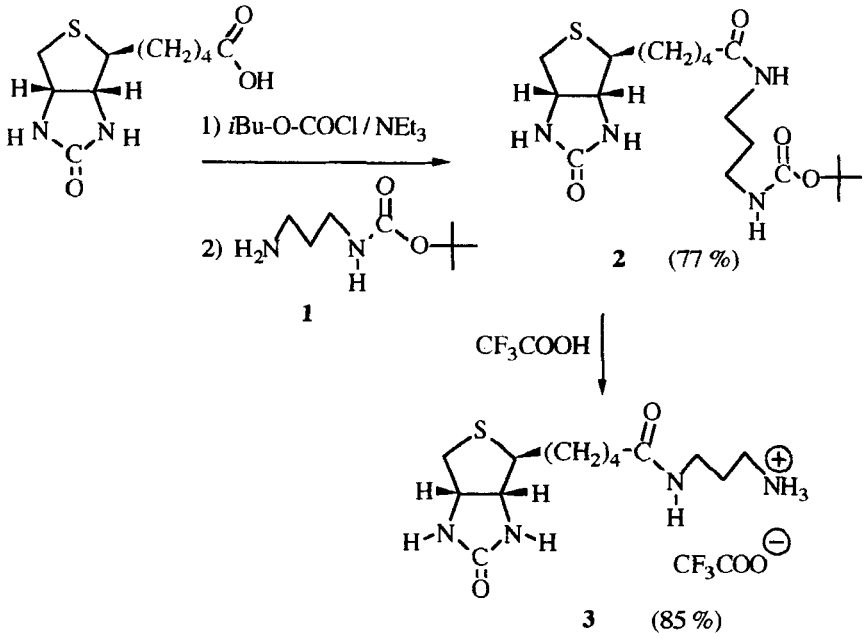
acylated compounds are formed in poor yield. The crude reaction mixture is usually used without isolation of the conjugates which are almost never characterized.[3]

We wish to report the synthesis of a new effective nucleophilic biotinylation reagent: 3-(biotinylamido)propylammonium trifluoroacetate **3**, intermediate in the preparation of biotin labeled derivatives.



Product **3** was obtained in a two-step procedure as depicted in the scheme, from (*tert*-butyloxycarbonylamino)propylamine **1**, which was condensed with biotin to give compound **2**, subsequently deprotected by trifluoroacetic acid to yield compound **3**. The problem encountered of biotin insolubility in most organic solvents could be circumvented by the use of dimethysulfoxide as a solvent for condensation. The mixed anhydride method allowed the isolation of the condensation product **2** by simple dilution of the reaction mixture with water.

In order to test the practical value of this new biotinylation reagent, three steroids were labeled. The conjugates were obtained by acylation of compound **3** under mixed anhydride conditions by the carboxymethyloxime (CMO) derivatives of testosterone, 21-deoxycortisol and 17β-hydroxyprogesterone. Immunoassays were performed for the determination of these compounds in plasma. In all the cases studied, the sensitivity and cross reactions of the analysis were at least equivalent to the best RIA[5] of these steroids. The cost of the analysis was ten-fold less than that of classical RIA methods.

The proposed biotinylation reagent could be useful for a wide range of applications.

Experimental

The NMR spectra were recorded on a Bruker 270 MHz spectrometer. Melting points were determined on a Buchi apparatus and are uncorrected. The thin layer chromatographies (tlc) were done using Merk Silica gel plates 60H. They were developed by ninhydrin. The following eluents were used: A: AcOEt-NEt_3 (10:0.1); B: AcOEt-MeOH (9:1); C: CH_2Cl_2-MeOH (9:1). 3-(*Tert*-butyloxycarbonyl-amino)propylamine **1** was prepared by acylation of 1,3-diaminopropane using di-*tert*-butyldicarbonate, $(Boc)_2O$, according to a described procedure.[4]

3-(*Tert*-butyloxycarbonylamino)propyl-1-biotinylamide 2.

To a cold (5 °C) solution of D(+)-biotin (5.0 g, 20.5 mmol) in DMSO (20 mL), triethylamine (3.3 mL, 25 mmol) was added followed after 5 mn stirring by *iso*-butylchloroformate (2.6 mL, 20 mmol) in 5 mL dioxane. The viscous mixture was then stirred at 5 °C for 30 min. A solution of 3-(*tert*-butyloxycarbonyl-amino)propylamine **1** (3.50 g, 20 mmol) and triethylamine (2.7 mL, 20 mmol) in 10 mL dioxane was then added dropwise to the mixture. The cooling bath was removed and stirring continued for 4 h. During this process, the solidification of the reaction medium was gradually observed. Cold water (50 mL) was added and

compound **2** was isolated by filtration, washed twice with cold water (5 mL), recrystallized from H_2O and dried. Rf(eluent A) = 0.15; Mp(H_2O) = 98-100 °C; ^{1}H-NMR ($CDCl_3$, TMS) δ: 1.40 (s, 9H, *tert*-butyl), 1.45-1.70 (m, 8H, 4 CH_2), 2.15 (t, 2H, J = 7 Hz, CH_2-CO), 2.65 and 2.80 (d and dd, 2H, J = 13 Hz, J = 4 Hz, CH_2-S), 3.12 (q, 2H, J = 5 Hz, Boc-NH-C$\underline{H_2}$), 3.15 (m, 1H, CH-S), 3.2 (q, 2H, Biotinyl-NH-C$\underline{H_2}$), 4.25 and 4.45 (2m, 2H, 2CH-N), 4.90 and 6.60 (2 broad t, 2H, J = 5 Hz, N$\underline{H}$-CH_2), 5.10 and 5.95 (2s, broad, 2H, NH-CO-NH), 6.20 (s,broad, 1H), 6.50 (broad t, 1H, NH); Calcd for $C_{18}H_{32}N_4O_4S$: C, 54.00; H, 8.00; N, 14.00. Found: C, 53.89; H, 7.91; N, 14.32.

3-Biotinylamidopropylammonium trifluoroacetate 3.

To a cold (5 °C) solution of **2** (4 g, 10 mmol) in 20 mL methylene chloride trifluoroacetic acid (5 mL) was added and the solution was vigorously stirred for 2 h at rt (monitoring by tlc). After completion, the solution was evaporated *in vacuo* and triturated in Et_2O until crystallization occured. Rf(eluent B) = 0.05; Mp(iPrOH) = 126-130 °C. ^{1}H-NMR ($CDCl_3$, TMS) δ: 1.40-1.80 (m, 6H, 3 CH_2), 1.90 (m, 2H, CH_2), 2.40 (t, 2H, CH_2-CO), 2.70 and 2.90 (d and dd, 2H, J = 13 Hz, J = 4 Hz CH_2-S), 2.95 (t, 2H, J = 6.5 Hz, C$\underline{H_2}$-NH_3^+), 3.10 (q, 2H, $\underline{CH_2}$-NHCO), 3.20 (m, 1H, CH-S), 4.35 and 4.55 (2m, 2H, 2CH-N), 6.00 and 6.10 (2s, 2H, NH-CO-NH), 7.90 (t, 1H, J = 5 Hz, CH_2-N$\underline{H}$CO); Calcd for $C_{14}H_{25}N_4O_4F_3S$: C, 41.79; H, 6.22; N, 13.93. Found: C, 41.65; H, 6.15; N, 14.09.

Acylation of 3 by 3-carboxymethyloxime derivatives of steroids.

To a cold (5 °C) solution of steroid-3-carboxymethyloxime (1,1 mmol) and triethylamine (0.2 mL) in dioxane (3 mL), was added on stirring *iso*-butylchloroformate (0.14 mL, 1.1 mmol). Stirring was continued at 5 °C for 30 min. To this mixture was added a solution of **3** (0.37g 1 mmol) and triethylamine (0.3 mL) in DMSO (0.5 mL). After 4h stirring at rt the mixture was diluted with cold water (20 mL). The solid which precipited was washed with water, dried *in vacuo* and the conjugates were purified by column chromatography using eluent C.

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