Preparation of the N-(4′-hydroxy-[3′-^{125}I]iodophenethyl)-6-(4-O-diethylstilbestryl)hexanamide for diethylstilbestrol radioimmunoassays

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The synthesis of a radioiodinated diethylstilbestrol (DES) derivative is described. This derivative was prepared by coupling the previously synthesized active ester of 6-(4-O-diethylstilbestryl)hexanoic acid with mono-[^{125}I]iodotyramine in dry tetrahydrofuran (20 to 22 C, 16 hours). The mono-[^{125}I]iodotyramine was prepared using a chloramine-T method and purified by paper electrophoresis. The final product, N-(4′-hydroxy-[3′-^{125}I]iodophenethyl)-6-(4-O-diethylstilbestryl)hexanamide, was separated by thin-layer chromatography (cyclohexane/ethanol/NH_4OH 2.5 N/acetone; 40:50:5:20, v/v/v/v); it was stable for 2 months in ethanol at 4 C and had a specific activity higher than 540 Ci/mmol. The [^{125}I]DES amide synthesized was found to retain the immunoreactivity of DES, since it competed with [^{3}H]DES or DES in an in vitro radioimmunoassay system for the binding sites of a rabbit anti-DES antibody; thus, it seems to be capable of replacing the tritiated tracer used so far in DES radioimmunoassays. (*Steroids* **57**:27–31, 1992)

Keywords: steroids; diethylstilbestrol, RIA; radioiodination, for DES RIA; radioimmunoassay, DES; N-(4′-hydroxy-[3′-^{125}I]iodophenethyl)-6-(4-O-diethylstilbestryl)hexanamide

Introduction

Diethylstilbestrol (DES), a synthetic nonsteroidal estrogen, has been widely used, mainly as an antiandrogenic agent for the treatment of patients with prostatic carcinoma[1,2] and as a growth promoter in cattle.[3]

The tritium-labeled tracers commonly used so far in all of the proposed DES radioimmunoassays[4–7] have some intrinsic problems mainly related to their instability,[4,6] low specific activity,[6,7] and detection difficulties.[8] On the other hand, the radioiodinated histamine amide of the DES derivative 4-(4-O-diethylstilbestryl)butanoic acid reported in the literature[9] does not look promising for utilization as a tracer in a DES radioimmunoassay due to its low specific activity and limited immunoreactivity.

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We describe the synthesis of the N-(4′-hydroxy-[3′-^{125}I]iodophenethyl)-6-(4-O-diethylstilbestryl)hexanamide. This radioiodinated derivative, which was prepared by coupling the previously synthesized active ester of 6-(4-O-diethylstilbestryl)hexanoic acid with mono-[^{125}I]iodotyramine, was obtained in very good yield and seemed to retain the DES-related immunoreactivity, which is necessary for its utilization as a tracer in DES radioimmunoassays.

Experimental

Materials

All reagents were analytic grade. The water used was doubly distilled. Gelatin was obtained from BDH Chemicals Ltd. (Poole, England). Diethylstilbestrol, tyramine, bovine serum albumin (BSA), and N-hydroxysuccinimide (NHS) were all obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dicyclohexylcarbodiimide (DCC) was obtained from Ferak-Berlin (West Berlin, Germany), and 6-bromohexanoic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). The carrier-free Na ^{125}I solution (17 kCi/g; radiochemical purity,

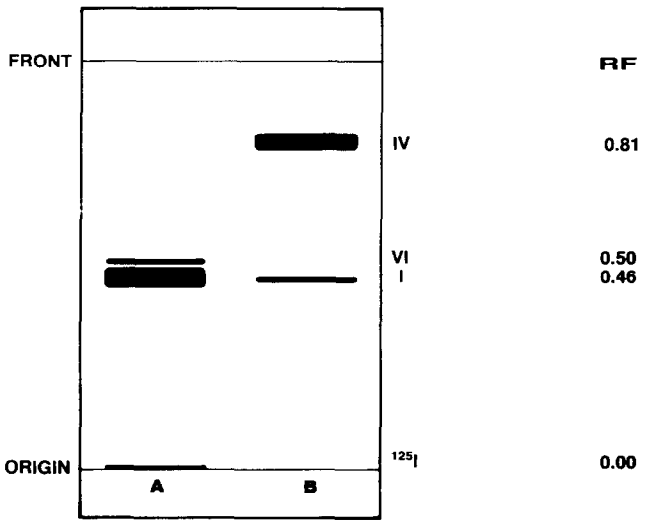

Figure 1 Autoradiograph of TLC plate. (A) Aliquot of the reaction mixture for the preparation of mono-[^{125}I]iodotyramine. (B) Aliquot of the coupling reaction mixture. Mono-[^{125}I]iodotyramine **(I)**, di-[^{125}I]iodotyramine **(VI)**, N-(4'-hydroxy-[3'-^{125}I] iodophenethyl)-6-(4-O-diethylstilbestryl)hexanamide **(IV)**.

99.9%, iodates, <2%) was obtained from Nordion, Atomic Energy of Canada (Ottawa, Canada). The anti-DES antiserum was kindly offered by Roussel-Uclaf (Seine-St. Denis, France). [^{3}H]Monoethyldiethylstilbestrol ([^{3}H]DES) was purchased from Radiochemical Centre (Amersham, UK). All of the other reagents were obtained from Merck-Schuchardt (Darmstadt, Germany), except as otherwise indicated.

Mono-[^{125}I]iodotyramine (I)

Carrier-free Na ^{125}I was diluted in 0.25 mol/L phosphate buffer, pH 7.5, to obtain 250 Ci/L. A 10-μl aliquot of diluted Na ^{125}I and 5 μl of a chloramine-T solution (0.2 mg/ml in 0.25 mol/L phosphate buffer, pH 7.5) was added to a 3-ml polystyrene test tube containing 10 μl of tyramine solution (0.1 mg/ml). After a 68-second incubation, the reaction was stopped with 5 μl of a sodium metabisulfite solution (0.2 mg/ml in 0.25 mol/L phosphate buffer, pH 7.5). The mono-[^{125}I]iodotyramine synthesized was isolated from the reaction solution, which contained a mixture of mono- and di-radioiodinated tyramines (Figure 1A, spots **I** and **VI**, respectively), unreacted tyramine, and ^{125}I, by paper electrophoresis (3M Whatman, 500 V, 175 minutes, in barbital buffer 0.2 mol/L, pH 8.4). The product was eluted from a band located approximately 5 cm from the point of origin (Figure 2). The elution was performed using 3 × 0.5 ml of a methanol/ethanol (1 : 1, v/v) mixture, the solvent was evaporated under a stream of nitrogen, and the resultant residue of mono-[^{125}I]iodotyramine was redissolved in 300 μl of dry tetrahydrofuran.

The radioiodination of tyramine was properly timed so that the product would be available for immediate use in the preparation of derivative **IV**.

6-(4-O-diethylstilbestryl)hexanoic acid (II)

Diethylstilbestrol (268 mg, 1 mmol) was dissolved in a mixture of 2 ml ethanol and 0.5 ml methanol; 200 μl of NaOH 5 N and 200 μl of distilled water were then added. In a separate test tube, an amount of 6-bromohexanoic acid (195.6 mg, 1 mmol) was dissolved in 200 μl of NaOH 5 N and 200 μl of distilled water. The two solutions were mixed in an acid-washed 10-ml glass tube, a stream of nitrogen was bubbled through for 1 minute, and the tube was stoppered, sealed with parafilm, and placed in a 100 C oil bath for 45 minutes. A mixture of 100 μl of NaOH 5 N and 200 μl of distilled water were then added, the reaction mixture was degassed with nitrogen, the tube was sealed, and the mixture was incubated at 100 C for 45 minutes. The pH was adjusted to 8.5 to 9.0 with HCl 1 N, and the reaction solution was washed four times with 0.5 ml of chloroform. The aqueous phase was then adjusted to pH 3.0 with HCl 1 N and extracted three times with 0.5 ml of water-saturated ethyl acetate. The organic solvent of the combined extracts was evaporated under a stream of nitrogen, leading to a viscous oily crude product, which was expected to contain the derivative **II**, unreacted DES, 6-bromohexanoic acid, and 6-hydroxyhexanoic acid. Pure derivative **II** was obtained by adding 0.5 ml methanol to the oily residue and separating on preparative-layer chromatography (20 × 20 cm silica-gel plates, 2 mm thick, cyclohexane/ethanol/NH_4OH 2.5 N/acetone; 40 : 50 : 5 : 20, v/v/v/v). The product band with Rf = 0.18 to 0.32 (visible under ultraviolet light) was scratched off and eluted three times with ethanol. The combined eluates were dried, leaving 196 mg of a white amorphous powder; this powder was dissolved in an appropriate amount of methanol to obtain a 10 mg/ml solution. When stored in the dark at 4 C, this methanolic solution remained stable for at least 1 year.

Succinimidyl 6-(4-O-diethylstilbestryl)hexanoate (III)

A portion (50 μl) of the methanolic solution of 6-(4-O-diethylstilbestryl)hexanoic acid (10 mg/ml) was added to a 3-ml glass tube and the solvent was evaporated under a stream of nitrogen. Equivolume aliquots (200 μl) of NHS (2.99 mg/ml) and DCC (2.68 mg/ml) solutions in dry tetrahydrofuran were added to the resultant residue. The mixture was incubated at 20 to 22 C for 4

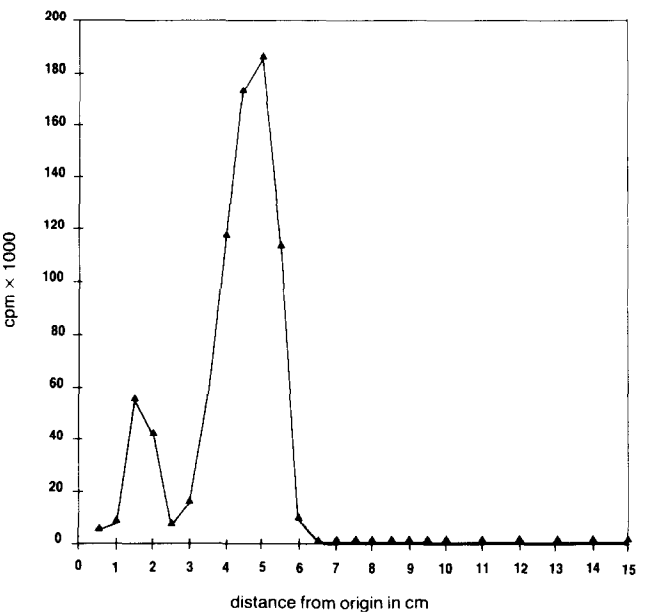

Figure 2 Paper electrophoresis of the radioiodination reaction mixture. Di-[^{125}I]iodotyramine at a distance of 1.0 to 2.5 cm, mono-[^{125}I]iodotyramine at a distance of 3.0 to 5.5 cm from the point of origin. ^{125}I was moved out of the electrophoresis paper strip.

hours. A white precipitate of dicyclohexylurea was removed by filtration and the liquid phase, containing the derivative **III**, was immediately used in the coupling reaction with mono-[^{125}I]iodotyramine.

N-(4'-hydroxy-[3'-^{125}I]iodophenethyl) -6-(4-O-diethylstilbestryl)hexanamide (IV)

The N-(4'-hydroxy-[3'-^{125}I]iodophenethyl)-6-(4-O-diethylstilbestryl)hexanamide ([^{125}I]DES) was synthesized by adding 15 μl (0.02 μmol) of the succinimidyl 6-(4-O-diethylstilbestryl)hexanoate **(III)** solution to the solution of mono-[^{125}I]iodotyramine **(I)** and incubating at 20 to 22 C for 16 hours. The final [^{125}I]DES amide **(IV)** formed was isolated by thin-layer chromatography (TLC) (cyclohexane/ethanol/NH_4OH 2.5 N/acetone; 40 : 50 : 5 : 20, v/v/v/v), scratching off the spot of the product, and eluting with ethanol. Visual localization of the spots was accomplished by exposing the TLC plate to iodine fumes, spraying it with color detection reagent (Folin-Ciocalteu), or exposing x-ray film to the plate for 5 minutes. After elution of the TLC spot, the desired storage radioactivity (828 nCi/ml) was obtained by diluting with ethanol. The final radiotracer stock solution was divided into 10-ml aliquots and stored for 2 months in the dark at 4 C. Before use, an aliquot of the radiotracer stock solution was evaporated under a stream of nitrogen and redissolved in 0.01 mol/L phosphate-buffered saline, pH 7.4, containing 0.1% NaN_3 and 0.05% BSA (radiotracer working solution, 828 nCi/ml).

Competition of diethylstilbestrol and [^{125}I]DES amide for antidiethylstilbestrol antibody sites

A 50-μl aliquot of the radiotracer working solution, 50 μl of serially diluted DES (0.25 to 32 μg/L) in phosphate-buffered saline (0.01 mol/L, pH 7.4, 0.1% NaN_3, 0.05% gelatin), 100 μl of anti-DES antiserum (1 : 2,500), and the appropriate amount of doubly distilled water to obtain a 500 μl final incubation volume were pipetted into 3-ml polystyrene test tubes. The final mixture was incubated at 37 C for 4 hours. A portion (500 μl) of goat anti-rabbit IgG-antiserum solution (1 : 50 in Tris buffer 20 mmol/L, pH 8.5, containing 80 mg/L normal rabbit IgG, 20 g/L polyethylene glycol 6000, and 2 g/L NaN_3) was then added and the mixture was incubated for 15 minutes at 20 to 22 C and centrifuged at 3,000 × *g* for 15 minutes. The supernatant was discarded and the radioactivity of the precipitate was measured. The percentage of the radioiodinated DES amide bound to anti-DES antibody in the presence of increasing concentration of DES was determined by plotting percent Bx/Bo versus the concentration of DES, where Bx represented the fraction of radioactivity bound to anti-DES antibody and Bo was the fraction of radioactivity bound to anti-DES antibody when no DES was added.

Results

The radioiodination of tyramine was performed by a previously described chloramine-T method[10] and led to a mixture of approximately 90% mono- and 10% di-radioiodinated products. As indicated in Figure 1A, the mono- and di-^{125}I–labeled products could be separated by TLC (spots **I** and **VI**, respectively). However, an essentially better separation was achieved by paper electrophoresis[11] at 3.0 to 5.5 cm (mono-[^{125}I]iodotyramine) or 1.0 to 2.5 cm (di-[^{125}I]iodotyramine) from the point of origin (Figure 2).

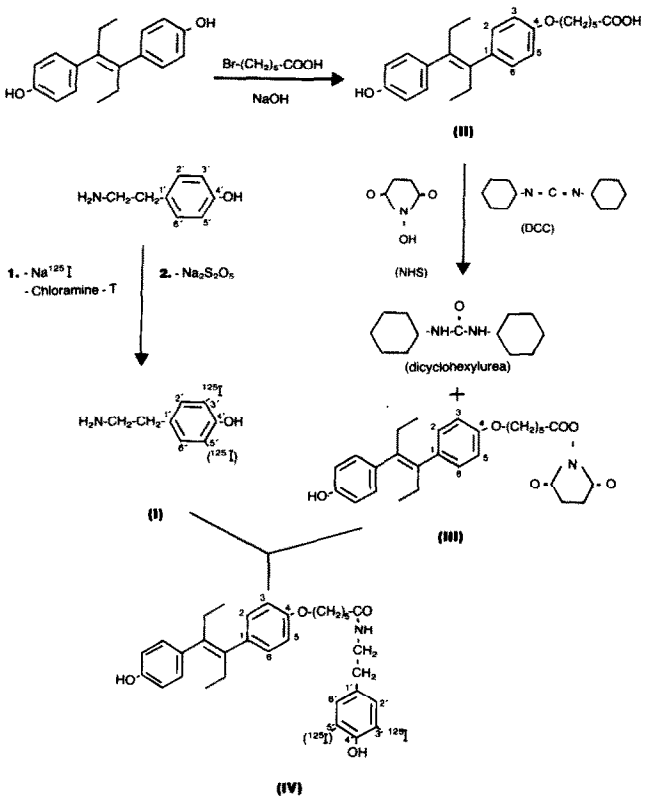

Figure 3 Synthesis of N-(4'-hydroxy-[3'-^{125}I]iodophenethyl)-6-(4-O-diethylstilbestryl)hexanamide **(IV)**. Mono-[^{125}I]iodotyramine **(I)**, 6-(4-O-diethylstilbestryl)hexanoic acid **(II)**, succinimidyl 6-(4-O-diethylstilbestryl)hexanoate **(III)**.

The [^{125}I]DES amide **(IV)** was synthesized in three steps with intermediate products **II** and **III**, after the reaction of **III** with mono-[^{125}I]iodotyramine **(I)**, as shown in Figure 3.

Using equimolar amounts of DES and 6-bromohexanoic acid, we obtained the monoether DES derivative **(II)** in a 51% yield. By increasing the relative amount of DES, the percent yield of the reaction decreased to less than 25% of the totally added DES. On the other hand, by increasing the relative amount of 6-bromohexanoic acid, the di-ether derivative was also produced. For instance, when the molar ratio of DES to 6-bromohexanoic acid was 1 : 3, the reaction led to a mixture of approximately 70% mono- and 30% di-ether DES derivatives. Figure 4A shows TLC analysis of the reaction mixture under optimum conditions, i.e., using equimolar amounts (1 mmol) of DES and 6-bromohexanoic acid on 0.25-mm thick silica-gel plates in cyclohexane/ethanol/NH_4OH 2.5 N/acetone (40 : 50 : 5 : 20, v/v/v/v). The derivative **II**, Rf = 0.4, showed strong fluorescence quenching under ultraviolet light and was finally isolated by preparative-layer chromatography.

The succinimidyl 6-(4-O-diethylstilbestryl)hexanoate **(III)** was synthesized, under the conditions described, in 40% yield, and was obtained in the supernatant of the reaction mixture after filtration of the precipitated dicyclohexylurea without any further

purification. The reaction yield was quite adequate for the requirements of the coupling step with mono-[^{125}I]iodotyramine; nevertheless, by increasing the incubation time from 4 to 16 hours, the yield increased from 40% to approximately 60%. Figure 4B shows TLC analysis of an aliquot of the reaction mixture supernatant using the same developing system as for derivative **II.** It should be noted that on the plates, product **III** is transformed into the 6-(4-O-diethylstilbestryl)hexanamide derivative (Figure 4B, spot **V**; Rf = 0.77) due to its coupling with the NH_4OH present in the developing system.

Optimum conditions for the preparation of N-(4'-hydroxy - [3' - ^{125}I]iodophenethyl) - 6 - (4 - O - diethylstilbestryl)hexanamide required a 1 : 42 molar ratio of mono-[^{125}I]iodotyramine to derivative **III** and approximately 16 hours' incubation time. Under these conditions, 80% yield was obtained, as calculated from TLC data. The yield decreased considerably when the relative amount of the derivative **III** decreased. On the other hand, when this amount increased, a lower specific activity of the final product was obtained (approximately 170 Ci/mmol using a 1 : 120 molar ratio). The final product was isolated using a TLC protocol similar to that described for product **III** and had Rf = 0.81, as determined by autoradiography (Figure 1B, spot **IV**). The apparent specific activity, as calculated by comparison of the self-displacing ability of increasing amounts of the radiotracer with a standard curve of the noniodinated DES,[12] was found to be higher than 540 Ci/mmol. The [^{125}I]DES amide antagonized the binding of [^{3}H]DES or varying amounts of DES to anti-DES antibody in a radioimmunoassay system (Figure 5). The binding, 55% with 1 : 2500 diluted anti-DES antiserum, remained stable for a 2-month period when the radiotracer stock solution was stored in the dark at 4 C.

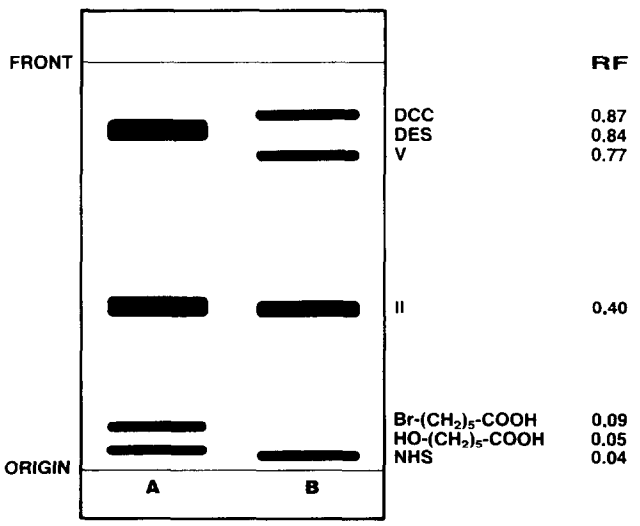

Figure 4 Analytic TLC plate after exposure to iodine fumes. (A) Aliquot of the reaction mixture for the preparation of 6-(4-O-diethylstilbestryl)hexanoic acid **(II)**. (B) Aliquot of the reaction mixture for the preparation of succinimidyl 6-(4-O-diethylstilbestryl)hexanoate **(III)**. On the TLC plates, product **III** is transformed into the 6-(4-O-diethylstilbestryl)hexanamide **(V)**.

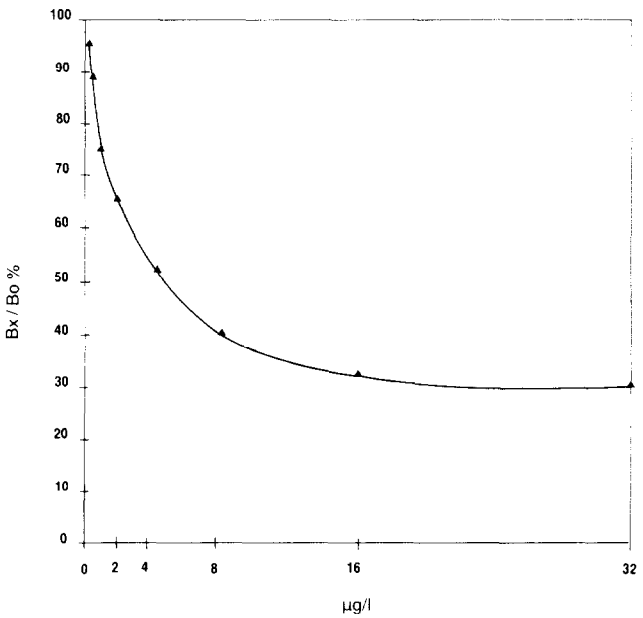

Figure 5 Competition of DES and [^{125}I]DES amide **(IV)** for the anti-DES antibody-binding sites.

Discussion

The present work deals with the synthesis of N-(4'-hydroxy - [3' - ^{125}I]iodophenethyl) - 6 - (4 - O - diethylstilbestryl)hexanamide, which is proposed for use in DES radioimmunoassays as a gamma-emitting tracer, replacing the beta-emitting tritium-labeled tracers used to date.

The DES molecule comprises elements that do not have radioisotopes suitable for use as gamma-emitting tracers in biochemical techniques, such as radioimmunoassays. Preliminary studies indicated that direct radioiodination of DES-phenyl rings[9,13] led to significant stereochemical modifications, which made the molecule unable to be bound to anti-DES antibody, probably due to the incorporation of a bulky iodine atom into the aromatic rings. Stereochemical hindrance from antibody binding was also noticed in case of a partial iodination of one of the phenyl rings, using a chloramine-T method (molar ratio of DES to Na^{125}I, 46 : 1). Thus, to prevent the loss of immunoreactivity usually observed in radioiodinated DES derivatives, it was necessary to design, through conjugation labeling, a molecule that would retain the unchanged main structure of DES and be attached through one of the phenolic hydroxyl groups to a previously radiolabeled moiety. Radioiodinated tyramine is a commonly used reagent for conjugation labeling and requires an activated carboxyl group to be attached. Thus, a plan was made to synthesize the succinimidyl 6-(4-O-diethylstilbestryl)hexanoate **(III)**, since it retains the main structure of DES and was expected to be easily cou-

pled with radioiodinated tyramine after a simple mixing. The five-carbon aliphatic chain was introduced to provide an adequately long spacer between the DES molecule and radioiodinated tyramine. In our case, it was necessary to couple DES with radioiodinated tyramine; nevertheless, when such a nondirect labeling procedure is applied to other steroids, it is advisable, for practical reasons, to first prepare and then radioiodinate the tyramine-steroid amide derivative whenever possible.

The synthesis of the radioiodinated DES derivative designed was performed in three steps (Figure 3). First, the 6-(4-O-diethylstilbestryl)hexanoic acid was synthesized by O-alkylation of DES with 6-bromohexanoic acid in alkaline pH.[14] Then, the reaction of the carboxyl derivative with DCC and NHS in dry tetrahydrofuran gave the succinimidyl 6-(4-O-diethylstilbestryl)hexanoate.[15] This intermediate product (derivative **III**) was immediately used in the coupling reaction with mono-[^{125}I]iodotyramine without any previous purification, except filtration of dicyclohexylurea, since the presence of the unreacted reagents did not affect either the coupling reaction or the immunoreactivity of the final product. Other relative protocols required at least the destruction of unreacted dicyclohexylcarbodiimide.[15,16]

In indirect conjugation labeling of steroids, radioiodinated histamine is usually used, because histamine forms a mono-iodinated derivative only.[8] In this work, mono-[^{125}I]iodotyramine was purified and used instead of a mixture of mono- and di-[^{125}I]iodotyramines, often applied in relevant radiolabeling techniques. Thus, it was possible to take advantage of the better labeling characteristics[13] of the tyramine molecule (higher rate and yield of iodination) and to also synthesize a chemically more specified labeled derivative.

The coupling reaction required an incubation time of at least 16 hours, a 42-fold excess of the reacting reagent **III**, and an environment of dry tetrahydrofuran (instead of other organic solvents, like dimethylformamide) to give optimal yield. The high molar ratio of **III** to **I** reagents is not well understood, but it might be attributed to the relative instability of the reagent **III** under the coupling reaction conditions. The final product was isolated from the reaction mixture by a TLC separation method (Figure 1B, spot **IV**; Rf = 0.81). Unreacted reagent **III** may introduce purity problems, since during the TLC procedure it is transformed into the 6-(4-O-diethylstilbestryl)hexanamide derivative (Figure 4B, spot **V**; Rf = 0.77), which is rather difficult to completely separate from the final product **IV**, thus affecting the specific activity of the product. Nevertheless, under the conditions described and by carefully scratching off the relevant TLC spot, we were able to obtain a tracer with an apparent specific activity higher than 540 Ci/mmol, as estimated by the method of Chiang,[12] and with proper immunoreactivity characteristics for use in DES radioimmunoassays.

The proposed radioiodinated tracer has physical characteristics for optimal assay utilization compared with other reagents reported to date.[9] On the other hand, our preliminary studies indicate that it is recognized by anti-DES antibody molecules in a manner similar to that of [^{3}H]DES. Thus, it can be a useful tracer tool in the in vitro measurements of DES in biologic fluids or in other biochemical applications.

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