Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXIII, No. 7

Synthesis of [^{3}H]Proadifen for Studying Binding to Liver Membranes

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Summary

Tritium-labelled proadifen (1, SKF 525A, 2-(N,N-diethylamino)-ethyl-2,2-di-diphenylpentanoate hydrochloride), a potent inhibitor of microsomal drug metabolism in the liver, was synthesized by hydrogenation of the corresponding allylic derivative with tritium gas. The specific activity of the compound obtained was 65 Ci/mmol. [^{3}H]Proadifen was found to bind to washed rat liver membranes with high affinity (K_D 1.3 nM) and large capacity B_{max} 1300 pmol/g wet weight tissue.

Key Words: Proadifen, rat liver, high affinity binding, tritium.

Introduction

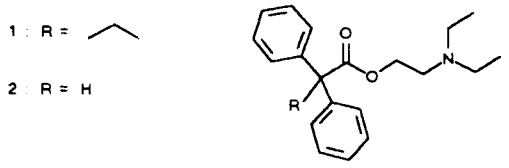
Proadifen (1, SKF 525A, 2-(N,N-diethylamino)ethyl 2,2-diphenylpentanoate hydrochloride) is an inhibitor of microsomal drug metabolism in the liver by some subforms of cytochrome P450 (1). In previous studies (2-4) it was observed that proadifen was very potent in inhibiting the high affinity binding of [^{3}H]alaproclate, a selective inhibitor of 5-hydroxytryptamine uptake (5), to rat liver and brain membranes, suggesting the possibility that at least a part of this binding occurred to cytochrome P450 forms. In order to further study those binding sites in the rat liver we now report the synthesis of tritium-labelled proadifen and preliminary experiments of the binding of this ligand to rat liver membranes.

0362-4803/93/070627-05$07.50

Received 1 February, 1993
Revised 3 March, 1993

Results and Discussion

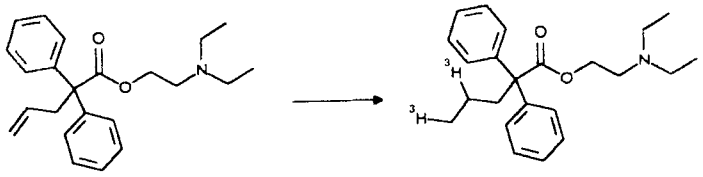
Figure 1.



Chemistry.

The labelling of [^{3}H]proadifen was performed with the allylic derivative 3 as starting material (Scheme 1). This compound was obtained by alkylation of the anion of 2-(N,N-diethylamino)ethyl diphenylacetate 2 (Fig. 1) with 3-bromopropene. The subsequent hydrogenation of the alkene 3 with tritium gas, using PdO as catalyst, produced the desired compound 4 with a specific activity of 65 Ci/mmol, as measured by quantitative HPLC analysis. Since the specific activity was higher than expected, the distribution of tritium atoms was elucidated by ^{3}H NMR.

Scheme 1.



3 4

The spectrum showed two signals at 0.8 and 1.0 ppm, signifying tritium present in the methyl and methylene positions, respectively. A notable feature of the spectrum was the uneven distribution of tritium across the original olefinic bond. Integration of the signals indicated an incorporation of tritium in a ratio of 2.8 to 1.0, which offers an explanation to the high specific activity obtained.

Pharmacology.

[^{3}H]Proadifen bound with high affinity to washed rat liver membranes (Fig. 2). This binding was inhibited by alaproclate and the specific, alaproclate-sensitive binding was at 1 nM [^{3}H]proadifen about 90% of the total binding at the membranal dilution used (1:5000, w/v). The B_{max} value obtained from the experiment shown in Fig. 2 was 1300 pmol/g tissue and the K_D value was 1.3 nM. Thus, [^{3}H]proadifen can be used as a suitable ligand in attempts to identify these binding sites in the rat liver that also binds alaproclate with high affinity (2,4).

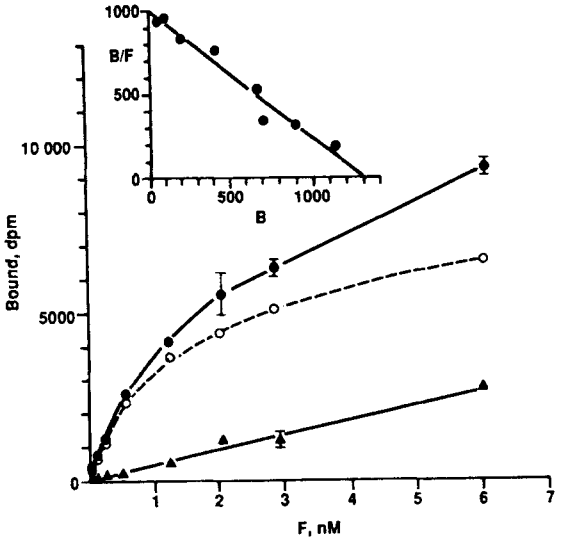


Figure 2. Saturation curve of the binding of [^{3}H]proadifen to membranes from rat liver. The experimental conditions are given in the Experimental section. The incubation was performed in the absence (●) and the precence (▲) of 10 µM alaproclate; o denotes the difference. The values are means ± SEM (vertical bars) of triplicate determinations. Insert: Scatchard plot of the same experiment where B is specifically bound [^{3}H]proadifen expressed in pmol/g tissue and F is the free concentration.

Experimental

Chemistry

Melting points were obtained on a Mettler FP 61 apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a Varian Gemini 300 MHz spectrometer and ^{3}H NMR spectra were obtained on a Brucker 500 MHz spectrometer with $CDCl_3$ and CD_3OD as solvents using Me_4Si as internal standard. Mass spectra (EI, 70 eV) were recorded on an Automas Delsi Nermag mass spectrometer using GLC inlet technique. Elemental analyses were performed by Mikro Kemi AB, Uppsala, Sweden and were within ± 0.4 % of the theoretical values unless otherwise noted. Tritium gas was purchased from ICI, Billingham, England. Radiochemical purity was determined by TLC using a Berthold LB 283 Linear Analyzer. Specific radioactivity was determined by quantitative HPLC analysis using a 4 x 120 mm, Nucleocil C-18 column eluted with phosphate buffer pH 2/MeOH (35:65) + DMEOA 0.1 g/L. The eluate was monitored by UV using a Linear 100 UV-spectrometer. Radioactivity was measured in a Packard 2200 CA liquid scintillation spectrometer using Packard Ultima Gold as counting medium.

2-(N,N-Diethylamino)ethyl 2,2-diphenylpent-4-enoate (3). To a solution of 2-(N,N-diethylamino)ethyl diphenylacetate 2 (6) (1.0 g, 3.21 mmol) in THF (13 mL), lithium bis(trimethylsilyl)amide (1 M solution in THF, 5 mL) was added at -40 °C during 15 min. After stirring for 30 min at -30 °C and 90 min at -5 °C the reaction mixture was cooled to -25 °C and 3-bromopropene (430 µL, 5.0 mmol) was introduced to the reaction mixture. The cooling bath was removed and the reaction mixture was stirred at room temperature for 1 h, then treated with saturated ammonium chloride and diethyl ether. The organic phase was separated, washed with water and extracted with 2 N HCl. The aqueous phase was washed with diethyl ether and thereafter made alkaline with 5 M NaOH (pH≈12) and extracted with diethyl ether. The ether extract was washed with water, dried over Na_2SO_4 and the solvent removed in vacuo giving 980 mg of crude material obtained as an oil.
This oil (500 mg) was purified using preparative TLC (SiO_2, 2 mm thickness/diethyl ether-hexane-ammonia; 20:5:0.5) affording 174 mg of the pure base 3 (GLC purity 98 %). ^1H NMR ($CDCl_3$): δ 7.27 (10 H, s), 5.70-5.40 (1 H, m), 4.95-4.90 (2 H, m), 4.18 (2 H, t, J 6 Hz), 3.17-3.15 (2 H, m), 2.60 (2 H, t, J 6 Hz), 2.44 (4 H, q, J 7 Hz), 0.93 (6 H, t, J 7 Hz). MS (m/z, rel. int. %): 309(3), 165(6), 129(3), 100(5), 99(26), 87(6), 86(100). The base 3 (105 mg) was converted to the hydrochloride salt and recrystallized from ethyl acetate (1.2 ml) giving 55 mg of colourless crystals (mp. 113-114 °C).
Anal. ($C_{23}H_{30}Cl_2NO_2$) C, H, N, Cl.

2-(N,N-Diethylamino)ethyl 2,2-diphenyl-[4,5-^{3}H]pentanoate (4). A solution of 3 (5.02 mg, 14.3 µmol) in DMF (500 µL) was stirred at room temperature under carrier-free tritium gas (10 Ci) in the presence of PdO (5.23 mg). After 20 h the reaction mixture was freeze-degaussed, filtered and the solvent distilled in vacuo. Labile tritium was removed by repeated lyophilization with ethanol. The residue was purified by column chromatography (SiO_2/diethyl ether-hexane; 20:5). The chromatographic procedure was monitored by TLC (SiO_2/diethyl ether-hexane-ammonia; 20:5:0.5) and fractions with a radiochemical purity of about 90 % were pooled giving 301 mCi of labelled material. The chromatographic procedure was repeated once with a part of the material above (about 100 mCi) affording 34 mCi of the desired product 4 with a radiochemical purity of 94 % (TLC) and a specific activity of 65 Ci/mmol.

Pharmacology.

Male Sprague-Dawley rats (B & K Universal AB, Sollentuna, Sweden) weighing 200 to 250 g were housed in plastic cages with saw-dust in groups of 5 under constant temperature and lighting (6 a.m. - 6 p.m.) and were allowed free access of food and water. The rats were killed by decapitation and the livers were removed. Pieces of the liver were

homogenized in 100 volumes (w/v) of ice-cold 0.05 M Tris-HCl buffer, pH 7.4 in an Ultra-Turrax for 5 sec. The homogenizer was washed with the same volume of the buffer solution, which was added to the homogenate. The homogenate was centrifuged for 10 min at 39,000 g and 4 ^{o}C in a Beckman centrifuge model J2-21 M/E. The pellet was re-homogenized in the Tris buffer at a dilution of 1:5000 (w/v) of the original tissue weight.

The binding assay was performed as described previously (2). The incubation mixture in 10 mL plastic tube consisted of 200 µL membrane suspension (corresponding to 40 µg wet weight tissue), 25 µL [^{3}H]-proadifen in appropriate dilution giving final concentrations between 0.05 and 6 nM, 25 µL distilled water or alaproclate (10 µM final concentration). The binding was equilibrated at 0 ^{o}C (ice bath) for 2 h. The membranes were collected on Whatman GF/B glass filters soaked in 0.3% polyethylenimine solution for 2 h or more in order to minimize binding to the filters which was large in non-treated filters. The filtration was performed in a 24-channel Brandel Cell Harvester (Gaitersburg, MA, U.S.A.). The radioactivity in the punched filters was counted after addition of 5 mL Ultima Gold^{TM} (Packard) scintillation liquid in a Beckman liquid scintillation spectrometer model LS-5801 at 48 % efficiency. The binding parameters (B_{max} and K_D) were determined by linear regression analysis of Scatchard plots.

Acknowledgement

We thank Ingemar Nilsson and Roger Simonsson, Astra Hässle, for their assistance in obtaining the ^{3}H NMR data.

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