SYNTHESIS OF RADIOLABELLED CLENBUTEROL ANALOGUES

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Summary

Oxidation of clenbuterol 1a with pyridinium chlorochromate yielded 4-amino-3,5-dichloro-α-tert.-butylaminoacetophenone 5 . Tritiated clenbuterol 1b was produced by reduction of 5 with sodium [^{3}H]borohydride. Radioiodination of the clenbuterol precursor [2-tert.-butylamino-1-(4-aminophenyl)-ethanol] 2 yielded [2-tert.-butylamino-1-(4-amino-3-[^{125}I] iodophenyl)-ethanol] 3b.

Key words : Synthesis, clenbuterol, tritiated clenbuterol, iodoclenbuterol analogues

Introduction

The ß-adrenoceptor agonist clenbuterol [2-*tert.*-butylamino-1-(4-amino-3,5-dichlorophenyl)-ethanol] 1a stimulates muscle growth and also attenuates muscle atrophy caused by denervation. The drug may be useful clinically in treating muscle injury, or certain muscle wasting disorders (1). Clenbuterol stimulates growth in meat producing animals and this anabolic effect is accompanied by significant repartioning of carcass fat and muscle (2). The pharmacokinetic properties of clenbuterol labelled with ^{14}C at the benzylic carbon have been studied in small animals (3), and in humans (4).

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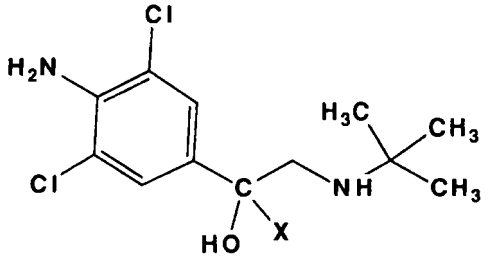
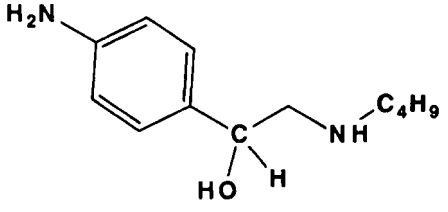


1a X=H

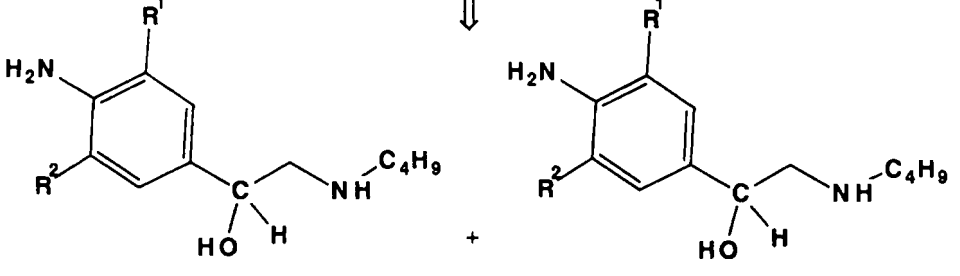
1b X=^{3}H

As part of our investigations (5) aimed at understanding the mode of action of clenbuterol on muscle, we required radiolabelled clenbuterol analogues to determine if either high affinity or atypical binding sites could be detected for these radioligands in muscle membrane preparations. A number of approaches for the synthesis of clenbuterol and related compounds have been described (2, 6) and at the outset it seemed that iodination of [2-*tert.*-butylamino-1-(4-aminophenyl)-ethanol] 2 might offer the most straightforward approach to the preparation of [^{125}I]-labelled analogues 3b and 4b (Equation 1).



2

Equation 1.



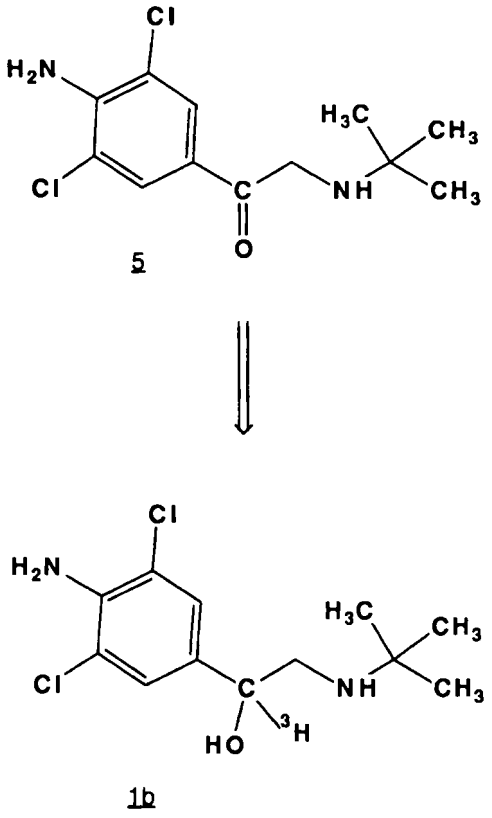
3a $R^1 = I$, $R^2 = H$

3b $R^1 = {}^{125}I$, $R^2 = H$

4a $R^1 = I$, $R^2 = I$

4b $R^1 = {}^{125}I$, $R^2 = {}^{125}I$

We wished also to prepare the tritiated clenbuterol congener 1b through sodium [^{3}H]borohydride reduction of 4-amino-3,5-dichloro-∝-*tert.*-butylaminoacetophenone 5 (Equation 2).



Equation 2.

Results and Discussion

Pri-Bar and Buchman (7) have described the preparation of tritiated clenbuterol. Those authors reported difficulty in effecting the direct oxidative conversion of 1 to 5 using a variety of common oxidation methodologies, necessitating the development of a more lengthy alternative approach. We find however that clenbuterol is cleanly oxidised to the required acetophenone derivative 5 using pyridinium chlorochromate (8) in dichloromethane solvent at room temperature. The ketone 5 was found to be acid labile and appeared to deaminate upon attempted conversion to the hydrochloride salt through reaction with dry hydrogen chloride gas in ethanol. Under neutral or slightly basic conditions, the free amine 5 was found to be relatively stable. Sodium borohydride

or sodium [^{3}H]borohydride reduction of 5 at 0 °C yielded 1a and 1b respectively. These products were then purified by h.p.l.c. or t.l.c. prior to being used in ligand binding studies.

A number of different approaches were investigated for the conversion of 2 to 3a and 4a (Equation 1). With chloramine-T and 2 mole equivalents of sodium iodide, the precursor 2 was found to be converted to the mono-iodo analogue 3a. None of the diiodo analogue 4a was recovered from these reactions. For iodinations using sodium [^{125}I]iodide, an excess of the precursor 2 was always employed, favouring formation of 3b exclusively. The labelled product was separated chromatographically from unreacted starting material. For small scale conversions, facilitation of the reaction with Pierce iodo-beads (9) in place of chloramine-T also resulted in conversion of 2 to 3a or 3b. Preparative scale non-labelled conversion of 2 to either 3a or 4a was found to be achieved most conveniently with molecular iodine and peracetic acid (10).

Experimental

General

^{1}H and ^{13}C n.m.r. spectra were recorded either with Varian EM-360 or Bruker AMX-300 spectrometers as appropriate. Chemical shifts are quoted as δ values relative to tetramethylsilane ($SiMe_4$). Infrared spectra were recorded with a Perkin-Elmer 1600 Series FT-IR. UV spectra were obtained using a Varian DMS-100 spectrophotometer. Radioactivity of [^{125}I]- and [^{3}H]-labelled compounds and precursors was measured using a Kontron Gamma Counter (84% efficiency) and a LKB Wallac ß-Scintillation Counter (55% efficiency) respectively. Sodium [^{3}H]borohydride and sodium [^{125}I]iodide were purchased from Du Pont Radiochemicals (North Ryde, NSW, Australia). Speedy column chromatography was accomplished through dry-packed Merck Kieselgel G type 60 absorbent, and was vacuum assisted. Analytical and semi-preparative thin-layer chromatography (t.l.c.) (ascending) employed Merck Kieselgel G 60F 254 plates. Autoradiographs of thin layer plates were prepared with Kodak X-ray film using a Kodak X-Omatic cassette. Preparative thin layer chromatography using 2mm thickness silica absorbent (Merck 7749 Kieselgel 60PF 254) was accomplished using 24 cm

diameter glass plates on a Chromatotron (Professional Technology). High performance liquid chromatography (h.p.l.c.) employed Waters Associates μBondapack C-18 reversed phase analytical columns (200 x 4.6 mm) with a Perkin-Elmer Series 2 Liquid Chromatograph and LC-75 spectrophotometric detector. A flow rate of 1 ml/minute of 70% methanol/ 30% 10mM K_2HPO_4 with detection at 240 nm was used for all analyses. Elemental analyses were performed by the Canadian Microanalytical Service, Delta, B.C., Canada.

Preparation of clenbuterol 1a from 4-aminoacetophenone

The synthesis of clenbuterol 1a was achieved by an approach derived from a number of earlier literature reports (6,7). Starting from 4-aminoacetophenone, introduction of the 3,5-dichloro substituents was effected by reaction with N-chlorosuccinimide in refluxing toluene solvent. Selective monobromination - to the carbonyl group was then achieved by reaction with bromine in chloroform at 0 °C. The crude product was reacted with *tert.*-butylamine in ethanol to yield the hydrobromide salt of the key acetophenone analogue 5. Purification of 5 was achieved by exploiting the low solubility of the hydrobromide salt in dichloromethane. Virtually all of the UV absorbing impurities in the crude product were found to be extractable into dichloromethane, allowing the sparingly soluble product 5 and *tert.*-butylamine hydrobromide to be recovered by filtration. Conversion to the respective free amines was accomplished by treatment with aqueous base followed by extraction into chloroform. Removal of the solvent and *tert.*-butylamine *in vacuo* afforded 5 as a yellow solid. (See spectral details below). Sodium borohydride reduction of 5 was achieved at low temperature in methanol to yield clenbuterol 1a (25% yield from 4-aminoacetophenone), which was subsequently converted to the water soluble hydrochloride salt by reaction with dry hydrogen chloride gas in absolute ethanol. This material gave spectral and chromatographic characteristics identical to an authentic clenbuterol sample supplied to us by Boehringer Ingelheim.

Pyridinium chlorochromate oxidation of 1a to 5

Pyridinium chlorochromate (110 mg, 0.5 mmol) was added in one portion to a solution of clenbuterol.HCl 1a (100 mg, 0.32 mmol) in dry dichloromethane (4 ml) maintained

under a dry nitrogen atmosphere . The reaction mixture was stirred at room temperature overnight, after which analytical t.l.c. (15% methanol, 1% aqueous ammonia, 84% dichloromethane) revealed that the starting material (R_F 0.71) had been converted to a more mobile product (R_F 0.93) . The dark reaction mixture was then filtered, and after removal of the solvent *in vacuo*, the crude product was subjected to silica column chromatography, with a mixture of 5% methanol, 1% ammonia, 94% dichloromethane as the eluant. The product 5 was isolated as the free amine in three fractions. Removal of the solvent *in vacuo* yielded a yellow solid : 4-amino-3,5-dichloro-α-*tert*.-butylaminoacetophenone (65 mg, 72 %). ^{1}H n.m.r. (d_6-acetone, 300 MHz) δ 1.31, s, $C(CH_3)_3$; 2.87, s, CH_2; 6.04, bs, Ar-NH_2; 7.89, s, Ar-H; 8.19, s, Ar-H. ^{13}C n.m.r. (d_6-acetone, 75 MHz) δ 29.14, $C(CH_3)_3$; 59.49, CH_2; 118.46, arom C-Cl; 131.73, arom. C-H; 155.55, arom. C-C=O; 187.54, arom. C-NH_2; 206.18, C=O; ($C(CH_3)_3$ not distinguishable from baseline noise). I.r. (KBr disc) 1610 cm^{-1} (C=O, sharp, strong).

Reduction of 5 to 1b with sodium [^{3}H]borohydride

A stock solution (5 mM) of the acetophenone derivative 5 was prepared by dissolving 6.9 mg of this material in 5 mL of demineralised water. Trial small-scale reductions of 1 mL aliquots of this stock solution were conducted with 20 µL of a 25 mM solution of sodium borohydride prepared in 0.001 M sodium hydroxide. The reaction mixtures were allowed to stir for 15 minutes at room temperature, then extracted three times with dichloromethane (1 mL) and the combined organic layers dried with sodium sulphate, prior to removal of the solvent *in vacuo*. The residue was dissolved in a small volume of methanol and the solution was analysed by h.p.l.c.. These experiments showed that approximately 15% of the starting ketone 5 was reduced to a product coincident with clenbuterol by h.p.l.c. In these experiments the molar ratio of 5 to reductant was approximately 10:1 whereas in the preparative labelled run described below this ratio was approximately 80:1. A higher amount of reductant was employed in the non-labelled run so that the progress of the reaction could be monitored conveniently by h.p.l.c. using U.V. detection.

When these experiments were repeated with the addition of a 20 µL spike of sodium [^{3}H]borohydride (9.25 MBq/L), the results were somewhat erratic. In some experiments

however, almost all of the counts introduced were recovered in the clenbuterol product fraction. It was found that best incorporation was obtained with short reaction times (5-10 minutes) at 0 °C. For the preparative conversion of 5 to tritiated clenbuterol 1b under these conditions, 185 MBq of a sodium [^{3}H]borohydride solution in 0.01 M sodium hydroxide (925 MBq/mL, 0.33 μmol/mL) was reacted with 5μmoles of the acetophenone 5. The [^{3}H]-labelled clenbuterol fraction was purified twice by thin layer chromatography to yield 370 KBq of [2-*tert.*-butylamino-1-(4-amino-3,5-dichlorophenyl)-1-[^{3}H]ethanol] 1b , having specific activity 0.7 TBq/mmol. By h.p.l.c. analysis, the radiochemical purity of this material was found to exceed 98%. The low yield obtained was attributed to the instability of the labelled reducing agent under the reaction conditions.

Preparation of [2-tert.-butylamino-1-(4-amino-3-iodophenyl)-ethanol] 3a. The precursor [2-*tert.*-butylamino-1-(4-aminophenyl)-ethanol] 2 was prepared from -bromo-4-nitroacetophenone as described by Keck and coworkers (6). A solution of 2 (4.2 mg, 0.02 mmol) in 150 μL of glacial acetic acid was added to a mixture of chloramine-T (4.3 mg, 0.02 mmol) and sodium iodide (6.0 mg, 0.04 mmol) maintained under a nitrogen atmosphere. After stirring for 1 hour at room temperature, analysis of a small aliquot of the reaction mixture by t.l.c. (15% methanol, 1% ammonia, 84% dichloromethane) revealed that the starting material (R_F 0.28) had been converted into a single product component (R_F 0.54). The reaction mixture was first basified by the dropwise addition of 10M sodium hydroxide solution and then extracted three times with dichloromethane (2 mL). The combined organic layers were first dried over sodium sulphate, the solvent was then removed *in vacuo* and the crude product subsequently purified by silica column chromatography (eluting with a mixture of 5% methanol, 1% ammonia, 94% dichloromethane) to yield [2-*tert.*-butylamino-1-(4-amino-3-iodophenyl)-ethanol] 3a (3.4 mg, 51%). H.p.l.c. single peak. T.l.c. (15% methanol, 1% ammonia, 84 % dichloromethane) single spot (R_F 0.54). ^{1}H n.m.r. ($CDCl_3$, 300 MHz) δ 1.10, s, $C(CH_3)_3$; 2.53, dd, J 9.1, 11.9 Hz, $CH(OH)CH_aH_b$; 2.84, dd, J 3.3, 11.9 Hz, $CH(OH)CH_aH_b$; 3.6-3.8, br NH and OH; 4.06, bs, $Ar\text{-}NH_2$; 4.45, dd, J 3.3, 9.1 Hz, Ar-CH(OH); 6.72, d, J 8.9 Hz and 7.13, dd, J 8.9, 2 Hz, *ortho* Ar-H's; 7.65, d, J 2 Hz, Ar-H. ^{13}C n.m.r. ($CDCl_3$, 75 MHz) δ 29.17, $C(CH_3)_3$; 50.16, CH_2; 71.28, CHOH; 114.47, arom. CH *ortho* to NH_2; 127.08, arom. CH

para to I; 134.54, arom. C-CHOH; 136.37, arom. CH *ortho* to I; 146.8, arom. C-NH_2; ($C(CH_3)_3$ and arom. C-I not distinguishable from baseline noise). U.v. (methanol) λ_{max} 242, 298 nm. Found: C 43.12%, H 5.69%, N 8.16%. $C_{12}H_{19}IN_2O$ requires C 43.13%, H 5.73%, N 8.38%.

Preparation of [2-tert.-butylamino-1-(4-amino-3-[^{125}I]iodophenyl)-ethanol] 3b.

74 MBq (1nmol) of carrier free sodium [^{125}I]iodide was added to a 20 µL aliquot of [2-*tert.*-butylamino-1-(4-aminophenyl)-ethanol] 2 (5 µg, 24 nmol) in pH 7.5 phosphate buffer. A 20 µL aliquot of a chloramine-T solution in demineralised water (0.3 mg/mL, prepared freshly) was then added and the mixture allowed to stand for a further two minutes before the reaction was stopped by the addition of 300 µL of 6.3 mM sodium metabisulphite solution. After making the reaction mixture alkaline by adding 10 µL of 1 M sodium hydroxide solution, the organic components were extracted into ethyl acetate (3 x 300 µL). The solvent was then removed *in vacuo*, and the product separated from unreacted starting material 2 by t.l.c. (10 % methanol, 1% ammonia, 89 % dichloromethane). The radioiodinated product 3b was located on the t.l.c. plate by autoradiography and this band was then extracted from the silica absorbent into methanol. By this procedure, 22 MBq of [2-*tert.*-butylamino-1-(4-amino-3-[^{125}I]-iodophenyl)-ethanol] 3b was obtained. H.p.l.c. analysis revealed that this product eluted coincidently with authentic 3a, while radioactive purity exceeded 98%.

Preparation of [2-tert.-butylamino-1-(4-amino-3,5-diiodophenyl)-ethanol] 4a (2).

To a mixture of [2-*tert.*-butylamino-1-(4-aminophenyl)-ethanol] 2 (50 mg, 0.3 mmol) and iodine (80 mg, 0.32 mmol) in glacial acetic acid (5mL) was added 500µL of peracetic acid (3.5 M in acetic acid). The reaction mixture turned dark and after 1 hour, t.l.c. analysis (5% methanol in dichloromethane) revealed that the starting material had all been converted into a single product (R_F 0.25). The reaction mixture was made basic by the dropwise addition of concentrated ammonia solution, and then the organic components were extracted into chloroform (3 mL x 3). The combined organic layers were washed with sodium thiosulphate solution (1M) and saturated sodium chloride prior to drying over sodium sulphate. Removal of the solvent *in vacuo* yielded the crude

product which was subsequently purified using a chromatotron (eluting with 4% methanol in dichloromethane) to afford 4a (2) (40 mg, 36%). H.p.l.c. single peak. ^{1}H n.m.r. (60 MHz, $CDCl_3$) δ 1.10, s, $C(CH_3)_3$; 2.75, m, $CH(OH)CH_2$; 3.3, bs, OH; 4.45, dd, J 4, 9 Hz, Ar-CH(OH); 4.90, bs, Ar-NH_2; 7.72, s, Ar.

Acknowledgements

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