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# Synthesis of Deuterated Clenbuterol

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## SUMMARY

The synthesis of $D_9$-clenbuterol (I) and $D_3$-clenbuterol (II) is described. $D_9$-clenbuterol (I) was prepared from 4-amino-α-bromo-3,5-dichloroacetophenone by reaction with $D_9$-*tert*-butylamine followed by reduction of the keto group with $NaBH_4$. $D_3$-clenbuterol (II) was prepared from 4-amino-α-*tert*-butylamino-3,5-dichloroacetophenone by an exchange reaction of the α-hydrogens with deuterium followed by reduction of the keto group with $NaBD_4$. The eventual products were characterized by mass spectrometry and NMR.

**Key words:** $D_3$-clenbuterol, $D_9$-clenbuterol, deuterium labelling, mass spectrometry.

## INTRODUCTION

Clenbuterol is a ß-agonist which (illegally) can be used as a growth promoter in farm animals [1]. When illegally used to increase the yield of meat the normal therapeutic dose is raised by a factor of between 5-10, resulting in an accumulation of clenbuterol in various tissues and organs [2,3]. Epidemiological surveillance has revealed food poisoning related to the consumption of clenbuterol tainted bovine liver [4].

Analytical methods ensuring a fast, sensitive and specific determination of residues of ß-agonists in food are demanded by the authorities. Mass spectrometry in combination with appropriate chromatographic methods represents well established strategies [5]. The use of isotopically labelled compounds as internal standards is preferred as the requirement to recovery in the work-up is highly reduced. Deuterium labelled standards have previously been used in the analysis of clenbuterol. Thus, Girault et al. have reported the use of $D_9$-clenbuterol [3,6] and Blanchflower et al. on the application of $D_6$-clenbuterol [7].

The Danish research programme FØTEK contained a subprogramme addressing fast analytical methods for the determination of residuals in foodstuffs. As part of this

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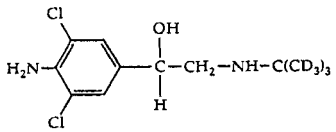
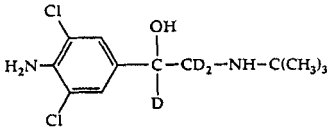
programme it was demonstrated that a number of common ß-agonists can be simultaneously analyzed using advanced mass spectrometric scan modes. A detailed analysis revealed that the sensitivity may be significantly increased due to a lower cycling time of the mass spectrometer, if specifically labelled internal standards, e.g. $D_3$- or $D_5$-clenbuterol, were used [8]. On this background we report the synthesis of the $D_9$-clenbuterol (I) and $D_3$-clenbuterol (II).



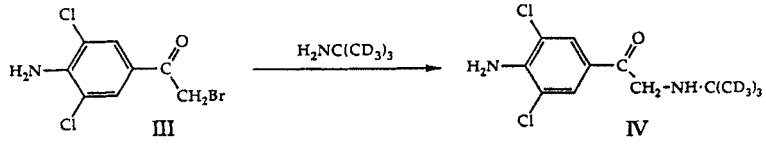

I




II

## RESULTS and DISCUSSION

The $D_9$-clenbuterol (I) was synthesized in four steps following the procedures previously reported for clenbuterol [9,10]. Thus, the commercially available 4-aminoacetophenone was chlorinated in concentrated acetic acid to give 4-amino-3,5-dichloroacetophenone [9]. The 4-amino-3,5-dichloroacetophenone was brominated in chloroform to give 4-amino-α-bromo-3,5-dichloroacetophenone (**III**). The reaction between $D_9$-*tert*-butylamine and **III** in chloroform afforded the 4-amino-α-$D_9$-*tert*-butylamino--3,5-dichloroacetophenone (**IV**) [10]. Finally, the keto group of **IV** was reduced with $NaBH_4$ in methanol. After crystallization of the free base the hydrochloride of $D_9$-clenbuterol was prepared [10]. In Scheme 1 the reaction for the introduction of the deuterium label is given.

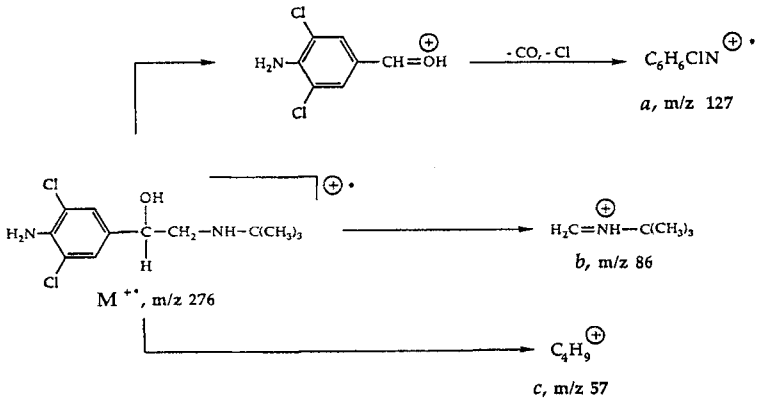



III IV

**Scheme 1**

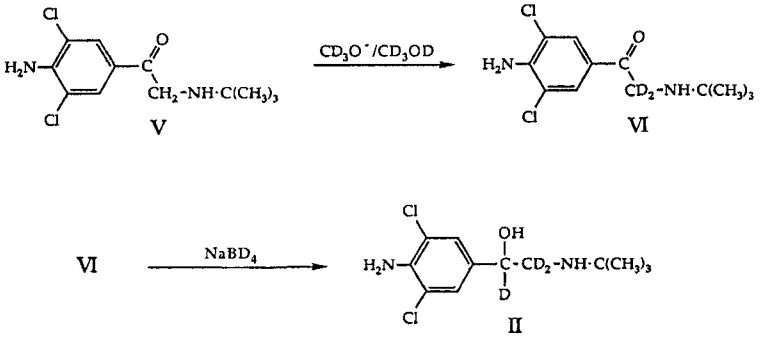
The $D_9$-clenbuterol (I) was characterized by mass spectrometry. The electron impact mass spectrum of clenbuterol contains three abundant and characteristic fragment ions at m/z 57, 86 and 127 corresponding to the $C_4H_9^+$ cation, the amino functionality and aromatic moiety of the molecule as rationalized in Scheme 2. The molecular ion (m/z 276) is of low abundance.

The mass spectrum of $D_9$-clenbuterol revealed the expected isotope shifts. Thus, the electron impact mass spectrum of $D_9$-clenbuterol exhibited a molecular ion ($M^{+\bullet}$ = 285) nine mass units higher than that of clenbuterol ($M^{+\bullet}$ = 276). The fragment ions at m/z 57 and m/z 86 cleanly changed to m/z 66 and m/z 95, whereas the fragment ion m/z 127 remained unchanged. This indicates that the label is completely retained in the *tert*-butyl group. The isotopic purity was calculated as 98% corresponding to that of the used $D_9$-*tert*-butylamine.




**Scheme 2**

The $D_3$-clenbuterol was synthesized in five steps, where the first three steps followed the methods previously described for clenbuterol [9,10]. Thus, the first three steps were identical to the preparation of $D_9$-clenbuterol except that *tert*-butylamine was used instead of $D_9$-*tert*-butylamine. The α-hydrogens of 4-amino-α-*tert*-butylamino-3,5-dichloroacetophenone (V) were exchanged with deuterium using methoxide as catalyst in perdeutero methanol (Scheme 3). It may be mentioned that the exchange apparently requires a fairly strong base since no reaction was observed using $Na_2CO_3$ in $D_2O$. Finally, the keto group was reduced with $NaBD_4$. After recrystallization in isopropopanol the hydrochloride of $D_3$-clenbuterol was prepared.




**Scheme 3**

The $D_3$-clenbuterol (**II**) was characterized by electron impact and electrospray mass spectrometry and NMR. The electron impact mass spectra of $D_3$-clenbuterol carries significant information on the degree and position of the deuterium labels, since the two fragment ions ***a*** and ***b*** (cf. Scheme 2) directly reflect the labels in the CHOH and $CH_2$ groups, respectively. Thus, the clean change of m/z 127 (***a***) of clenbuterol to m/z 128 in II obviously supports the arguments that the CHOH group retains the deuterium atom introduced by the reduction with $NaBD_4$. The fragment ion ***b*** reveals a more

complex pattern as the m/z 86 change to m/z 88, however, is accompanied by ions at m/z 86 and 87 (cf. Fig. 1). This may imply that the exchange reaction of the α-hydrogens is not complete. The ion pattern was simulated using the appropriate generating functions for the elements [11]. The measured and calculated ion patterns are shown in Fig. 1. The calculations reveal a deuterium content of 68% in the methylene group.

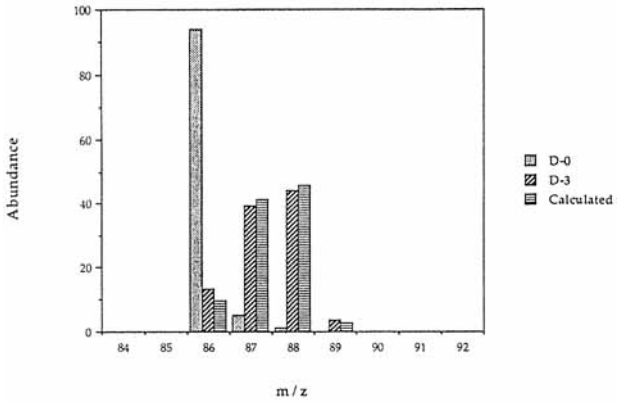



Figure 1. The electron impact histogram showing the abundance of the ion *b*; D-0), the measured abundance of unlabelled clenbuterol, D-3), the measured abundance of **II**, and the calculated abundance with 68 % deuterium in the methylene group.

Electrospray mass spectrometry of clenbuterol gives rise to an intense ion pattern due to protonated molecules. The ion pattern of the $MH^+$ ions of $D_3$-clenbuterol was simulated using the labels 1 H : 100% D and 2 H : 68% D. In Fig. 2 the measured and calculated ion patterns are shown.

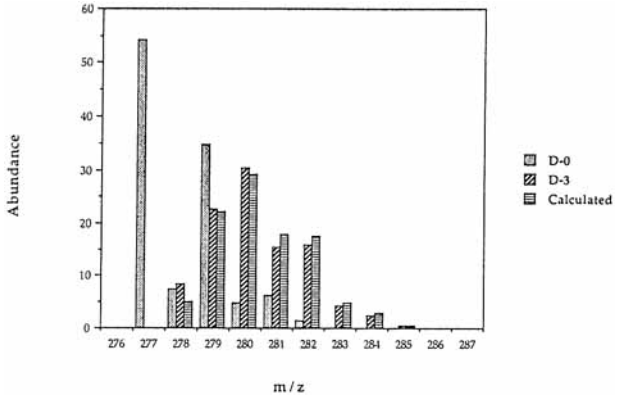



Figure 2. The electrospray histogram showing the abundance of the $MH^+$ ions; D-0), the measured abundance of unlabelled clenbuterol, D-3), the measured abundance of **II**, and the calculated abundance with 68 % deuterium in the methylene group.

The observed agreement between the measured and calculated ion patterns in the fragment ions *a* and *b* as well the $MH^+$ ion indicates that the isotopic labels retain the positional identity in the ionized state. In addition, the distribution of deuterium in the methylene group is purely statistical, in agreement with the preparation.

The $D_3$-clenbuterol was further characterized with $^1H$ and $^{13}C$ NMR. The $^{13}C$ chemical shifts are given in the experimental section and are in agreement with those calculated for the doubly protonated clenbuterol using the additivity contributions for the various groups. $^{13}C$ signals for the CDOH and $CD_2$ groups are not observed due to increased relaxation times for the deuterium substituted carbons. The isotopomer distribution can be obtained from Fig. 1. It is apparent that CDOH-CHD is an abundant sub-structure (42%) comparable in abundance to the fully labelled CDOH-$CD_2$ (46%), as visualized by the ions m/z 87 and m/z 88, respectively. Thus, the $^1H$ spectrum changes from an ABX pattern as observed for the CHOH-$CH_2$ sub-structure to a "doublet" reflecting the residual non-equivalent protons in the methylene group (CDOH-CHD) of II. The CDOH-$CH_2$ sub-structure (12%) remains just observable. The $^1H$ NMR measurements reveal the presence of approximately 0.02 H equivalents in the CH group and 0.66 H equivalents in the methylene group, respectively. The latter value corresponds to a label of 67% D and is in excellent agreement with the mass spectrometric analysis (68%).

## CONCLUSION

The synthesis of $D_9$-clenbuterol and $D_3$-clenbuterol has been accomplished with satisfactory yield and an isotopic purity sufficient for stable isotope dilution mass spectrometry.

## EXPERIMENTAL

### Synthesis

4-Amino-3,5-dichloroacetophenone, 4-amino-α-bromo-3,5-dichloroacetophenone (III) and 4-amino-α-*tert*-butylamino-3,5-dichloroacetophenone (V) were prepared based on procedures previously described [9,10]. The starting material was 4-amino-acetophenone (Fluka no. 06640).

4-Amino-α-$D_9$-*tert*-butylamino-3,5-dichloroacetophenone (IV)

4.8 g of 4-amino-α-bromo-3,5-dichloroacetophenone (III) were suspended in 40 ml chloroform. The suspension was heated to 50°C and 5 g $D_9$-*tert*-butylamine (MSD-Isotopes, 98% D) added during stirring. The mixture was refluxed for two hours. After cooling the reaction mixture was washed three times each with 25 ml water. The chloroform phase was dried with $Na_2SO_4$. After filtering, 15 ml absolute ethanol was added and the solution was acidified to pH 3 to 5 with isopropanolic hydrogen chloride resulting in precipitation of the dihydrochloride of IV. The mixture was stored overnight in a freezer to complete crystallization. The crystals were filtered off, washed with 10 ml absolute ethanol/chloroform (1/1) and dried under vacuum. The melting point was 261-263°C, litt. 252-257°C [10] for the unlabelled analogue. The yield was 3.7 g (61%).

$D_2$-clenbuterol: 1-(4-amino-3,5-dichloro-phenyl)-2-$D_2$-*tert*-butylamino-ethanol (I)
3.7 g of the dihydrochloride of 4-amino-α-$D_9$-*tert*-butylamino-3,5-dichloroacetophenone (IV) were suspended in a mixture of 20 ml methanol and 13 ml water. Under stirring, a solution of 0.68 g $NaBH_4$ in 1.3 ml water was added dropwise at 25-35°C maintaining the pH between 3 and 7 by the addition of 6 M HCl. Finally, the mixture was acidified to pH 1 and 0.75 g activated carbon was added. After 10 minutes the mixture was filtered and the filtrate cooled by icewater and 2 ml of a concentrated ammonia solution was added dropwise. Clenbuterol started to crystallize as the free base. After one hour on an ice-bath the crystals were filtered off, washed with 50 ml water and dried in vacuo at 50°C (200 mmHg). The yield was 2.3 g (70%), m. p. 108-115°C.

The clenbuterol was recrystallized several times to obtain a pure product. Thus, 2.3 g of the base were dissolved in 19 ml isopropyl alcohol under gentle warming. The hot solution was filtered through a silicone filter, and isopropanolic hydrogen chloride was added to the filtrate to pH 6-7. The solution was cooled on an ice-bath and stored overnight in a freezer to complete precipitation. The precipitate was filtered off and washed with 5 ml isopropanol and dried in vacuo at 50°C. The yield was 2.2 g, m.p. 176.5-179°C. The hydrochloride was redissolved in 24 ml water and 0.26 g activated carbon was added followed by stirring for 15 minutes. The mixture was filtered and the filtrate made alkaline with 0.5 ml of a concentrated ammonia solution. After one hour under stirring the clenbuterol was filtered off, washed with water and dried in vacuo. The yield was 1.5 g, m.p. 110-115°C. Finally, the free base was dissolved in 13.5 ml isopropanol under gentle warming, the hot solution was filtered through a silicone filter and the filtrate adjusted to pH 6-7 with isopropanolic hydrogen chloride. The solution was cooled on an ice-bath and stored overnight in a freezer to complete crystallisation. The hydrochloride of clenbuterol was filtered off, washed with isopropanol and dried in vacuo at 50°C. The yield of the hydrochloride of $D_9$-clenbuterol was 1.3 g (35 %). The melting point was 175-176.5°C, litt. 174-175.5°C for the unlabelled analogue [10].

α-$D_2$-4-amino-α-*tert*-butylamino-3,5-dichloroacetophenone (VI)
1.6 g sodium was dissolved in 100 ml deuterated methanol, $CD_3OD$, under a nitrogen atmosphere. 6.5 g of the dihydrochloride of 4-amino-α-*tert*-butylamino-3,5-dichloroacetophenone (V) was added and the mixture heated for three hours at 50°C while stirring under a nitrogen atmosphere. After cooling, ethanolic hydrogen chloride was added to pH 3 and the solution evaporated to dryness on a rotary evaporator at 30°C. The residue was dissolved in 50 ml water and made alkaline with a concentrated ammonia solution. The solution was extracted three times with 25 ml $CH_2Cl_2$. The collected $CH_2Cl_2$ phases were washed twice with 25 ml water and dried with $CaCl_2$. After filtering, ethanolic hydrogen chloride was added to pH 3 and the solution evaporated to dryness. Ether was added to the residue and the crystals filtered off, washed with ether and dried under vacuum. The yield of the dihydrochloride of VI was 5.2 g (75%).

$D_3$-clenbuterol: 1-(4-amino-3,5-dichloro-phenyl)-2-*tert*-butylamino-1,2,2-$D_3$-ethanol (II)
5.0 g of the dihydrochloride of α-$D_2$-4-amino-α-*tert*-butylamino-3,5-dichloroacetophenone (VI) were suspended in 25 ml $CD_3OD$ and 18 ml $D_2O$. A solution of 0.98 g $NaBD_4$

(Aldrich, 98% D) in 1.8 ml $D_2O$ was added dropwise under stirring at 25-35°C maintaining the pH between 3 and 7 by the addition of 6 M HCl. Finally, the mixture was acidified to pH 1, and 1 g of activated carbon was added. Stirring was continued for 5 minutes and the mixture was filtered. The filtrate was cooled by icewater and the solution made alkaline with the addition of a concentrated ammonia solution. Clenbuterol crystallizes as the free base. After one hour on an ice-bath the crystals were filtered off, washed with 75 ml water and dried in vacuo at 50°C (200 mmHg).

The clenbuterol was recrystallized several times to obtain a pure product. Thus, the free base was dissolved in 25 ml isopropyl alcohol under gentle warming and isopropanolic hydrogen chloride was added to pH 6. The solution was stored overnight in a freezer to complete precipitation. The precipitate was filtered off and washed with isopropanol followed by ether. The crystals were dried in vacuo at 50°C. The hydrochloride was redissolved in 35 ml water, and 0.4 g activated carbon was added. The mixture was filtered after stirring for 15 minutes and the iced filtrate was made alkaline with a concentrated ammonia solution. After one hour on an ice-bath the clenbuterol was filtered off, washed with 25 ml water and dried in vacuo at 50°C. Finally, the clenbuterol was redissolved in 20 ml isopropanol under gentle warming and isopropanolic hydrogen chloride was added to pH 6. The solution was kept for five hours in a freezer to complete precipitation. The compound was filtered off, washed with isopropanol followed by ether. The crystals were dried in vacuo at 50°C. The yield of $D_3$-clenbuterol hydrochloride was 2.6 g (58%). The melting point was 175.8-176.1°C, litt. 174-175.5°C for the unlabelled analogue [10]. $^{13}C$ NMR ($CD_3OD$): δ 142.1 (C4), 131.8 (C1), 126.8 (C2+C4), 120.2 (C3+C5), 58.2 & 25.8 ($C(CH_3)_3$). $^1H$ NMR (DMSO): 1.30 (9H, ($C(CH_3)_3$)), 3.01/2.91 (0.66H ($CH_2$)), 4.85 (0.02H (CHOH)), 7.29 (2H (arom.)). Non-labelled clenbuterol $^{13}C$ NMR ($CD_3OD$): δ 142.1 (C4), 132.0 (C1), 126.8 (C2+C4), 120.2 (C3+C5), 69.9 (CHOH), 49.5 (CH2), 58.2 & 25.8 ($C(CH_3)_3$). $^1H$ NMR (DMSO): 1.31 (9H, ($C(CH_3)_3$)), 3.00/2.92 (2H ($CH_2$)), 4.86 (1H (CHOH)), 7.30 (2H (arom.)).

## ANALYTICAL METHODS

### Mass spectrometry

Electron impact mass spectra were obtained on a Varian MAT CH 5D double-focussing instrument. The electron energy was 70 eV and the resolution approximately 1000. The samples were introduced in aluminium crucibles by the direct inlet system.

Electrospray mass spectra were obtained on a Finnigan TSQ 700 triple quadrupole instrument. The samples were introduced by HPLC (liquid phase: methanol-/water+1% acetic acid (8/2), flow rate: 100 ml/min) and electrospray ionization (5 kV). The HPLC-electrospray-MS measurements were performed by the Danish Meat Research Institute, Roskilde.

### NMR

The $^1H$ and $^{13}C$ NMR spectra were obtained on 250 MHz Bruker instruments at Roskilde University and Risø National Laboratory.

## ACKNOWLEDGEMENTS

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