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Article

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Identification of Buctopamine and Mebuctopamine, a β_2 Receptor Agonist and Its Metabolite, in Swine Hair and Feed Additives

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Abstract

4-[2-(*tert*-Butylamino)-1-hydroxyethyl]phenol (buctopamine, **4**), a new β_2 receptor agonist (β_2-agonist), was found to be an adulterant in feed additives for swine in Taiwan, where using β_2-agonists in food-production animals is prohibited. Buctopamine and its metabolite, 4-[2-(*tert*-butylamino)-1-hydroxyethyl]-2-methoxyphenol (mebuctopamine, **2**), were detected in swine hair specimens. Authentic compounds **2** and **4** were synthesized with 98.6% and 97.7% purity, respectively, as reference standards for analysis, and both compounds were more hydrophilic than ractopamine and clenbuterol. In a preliminary pharmacological evaluation, compounds **2** and **4** exhibited moderate human β_2 receptor binding affinity and did not show significant affinities for the human α_1, α_2, β_1, and β_3 receptors. After adding compounds **2**-**4** into the β_2-agonist library, a multi-residue analysis of 26 β_2-agonists by using triple quadrupole LC/MS/MS for routine screening conducted by regulatory authorities was established, in which the common limits of quantification for the 26 β_2-agonists in swine feed and hair are 10 and 25 ng/g, respectively. In addition, the illegal use of buctopamine (**4**) has been effectively prevented. The results of this study are also useful for controlling the illegal use of new β_2-agonists in food-production animals.

Key words: β_2-agonist, hair, feed additive, metabolite, identification

INTRODUCTION

β_2 receptor agonists (β_2-agonists) are sympathomimetic agents that are commonly used as bronchodilators for the clinical treatment of asthma. When used as feed additives for food-production animals, β_2-agonists can promote growth rates, increase muscle mass, and reduce fat deposition, feed consumption, and therefore, breeding costs.[1] However, β_2-agonists produce several undesirable behavioral side effects in livestock, especially pigs, such as agitation, restlessness, and aggressive behavior.

The β_2-agonist residues that accumulate in animal tissues can cause acute poisoning symptoms in humans.[1-3] For instance, several outbreaks of food poisoning in humans involving clenbuterol have occurred in Europe.[2,3] In 2006, reports from Shanghai, China, involved 300 cases of people who were poisoned by eating pork products contaminated by clenbuterol. Although no deaths occurred,[4] these incidents were substantial enough to create public resistance to using β_2-agonists as feed additives for food-production animals. In the United States, both ractopamine and zilpaterol (Chart 1) are used as feed additives for cattle and swine, whereas, in Australia, ractopamine can only be used in swine, and the use of zilpaterol is prohibited. In consideration of their potential hazards to human health, the use of β_2-agonists as growth promoters in animal breeding has been banned in more than 120 countries, such as those within the European Union (EU), China, and Russia.[1-3]

In Taiwan, the Food and Drug Administration, Ministry of Health and Welfare, collaborates with the Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ), Council of Agriculture (COA), closely to ensure food safety. The use of β_2-agonists as feed additives is prohibited, and seven β_2-agonists are officially listed in the law, including ractopamine, zilpaterol, clenbuterol, cimaterol, salbutamol, terbutaline, and tulobuterol (Chart 1). Several new β_2-agonists have recently been found to be used in

feedstuffs to elude official control.[5-8] In the spring of 2010, an outbreak of illicit new synthetic β_2-agonist use occurred in Taiwan. Rapid screening of swine hair samples by using a salbutamol (**1**, Chart 1) ELISA kit demonstrated strong positive results.[9,10] However, the unknown ingredient was not one of the seven β_2-agonists in the prohibited list. Therefore, the routine inspection conducted by the local authorities utilizing standard LC/MS/MS screening methods for multiple β_2-agonists was incapable of identifying this novel β_2-agonist in the feed and tissue specimens.[11] The suspicious material was named "Garlic Essential" and had been sold to local farmers as a new feed additive for swine. The salesmen claimed it contained the "No.8 lean meat agent", which could escape the regular screening control of the local government.

Here, we report the identification of 4-[2-(*tert*-butylamino)-1-hydroxyethyl]-2-methoxyphenol (**2**, mebuctopamine), a β_2-agonist metabolite found in swine hairs, followed by the identification of the novel β_2-agonist 4-[2-(*tert*-butylamino)-1-hydroxyethyl]phenol (**4**, buctopamine) in feed additives and swine hairs. In addition, we have synthesized authentic samples as reference standards for analysis, established an analytical method for the legal inspection of the residues of multiple β_2-agonists, including **2**, colterol (**3**) and **4** (Chart 1), and determined the lipophilicity and receptor binding affinities of compounds **2**-**4**.

MATERIALS AND METHODS

Chemicals and Samples. Absolute ethanol was purchased from J. T. Baker (Center Valley, PA, USA). Acetovanillone, sodium heptanesulfonate, and bromine were purchased from Acros (Geel, Belgium). 4'-Hydroxyacetophenone, *N*,*N*-dimethylformamide (DMF), EtOAc, MeCN, MeOH, and silica gel 60 (40–63 μm) for flash column chromatography were

purchased from Merck (Darmstadt, Germany). 4-(Chloroacetyl)catechol was purchased from TCI-JP (Tokyo, Japan). *t*-Butylamine and 10% Pd/C were purchased from Alfa Aesar (Lancashire, UK). 2-Propanol (IPA) and $MgSO_4$ were purchased from Fisher (Leicestershire, UK). $CHCl_3$ was purchased from Seedchem (Melbourne, Australia). Na_2CO_3 was purchased from Sigma-Aldrich (Saint Louis, USA). Hydrochloric acid and glacial acetic acid were purchased from Scharlau (Barcelona, Spain). DMSO-d_6 was purchased from Cambridge Isotope Laboratory, Inc. (Tewksbury, MA, USA). Deionized water was prepared with a Milli-Q Plus system at 18.2 MΩ (MilliPore, Bedford, MA, USA). Swine hair and feed additive samples were collected as part of the official control by BAPHIQ, COA, in Taiwan from local livestock farms.

Synthesis of Authentic Standards. General procedures ─ Melting points were determined on a MEL-TEMP II melting point apparatus (Barnstead/ Thermolyne Corp., Dubuque, IA, USA) and are uncorrected. NMR spectra were recorded on a Bruker DPX-200 FT-NMR spectrometer (Bruker, Billerica, MA, USA). Chemical shifts are expressed in parts per million (ppm) on the δ scale relative to a tetramethylsilane (TMS) internal standard. Electrospray ionization (ESI) mass spectrometry and high-resolution mass spectrometry (HRMS) spectra of authentic standards **2**-**4** were obtained with Esquire 2000 and Bruker Daltonik micrOTOF mass spectrometers (Bruker Daltonics Inc., Billerica, MA, USA), respectively. No attempt was made to optimize yields. Treatment of acetovanillone (**5a**) and 4'-hydroxyacetophenone (**5b**) with benzyl bromide and K_2CO_3 in DMF yielded compounds **6a** and **6b** as light yellow solids in 99% and 97% yields, respectively (Scheme 1).

1-(4-(Benzyloxy)-3-methoxyphenyl)-2-(*tert*-butylamino)ethanone (7a). Bromine (0.31 mL, 6.00 mmol) was added to a solution of compound **6a** (1.28 g, 5.00 mmol) in ethanol (30

mL) under nitrogen at room temperature and stirred for 1 h. The reaction mixture was cooled in an ice bath, and the white crystalline solid was collected by filtration (1.23 g, 3.68 mmol, 74%). This product was directly used in the following reaction without further purification. *t*-Butylamine (1.0 mL, 696 mg, 9.52 mmol) was added to a solution of the bromide (1.00 g, 2.98 mmol) in DMF (5 mL) and stirred at room temperature for 1 h under N_2. The mixture was poured into H_2O (30 mL), and the precipitate was collected and dried to obtain compound **7a** as a light yellow solid (920 mg, 2.81 mmol, 94%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.15 (s, 9, CH_3), 2.27 (bs, 1, NH), 3.95 (s, 3, OCH_3), 4.08 (s, 2, $COCH_2$), 5.23 (s, 2, OCH_2), 6.89 (d, 1, J = 8.3 Hz, aromatic), 7.29-7.58 (m, 7, aromatic); ^{13}C NMR (50 MHz, $CDCl_3$) δ 29.0 (3C), 49.0, 50.4, 56.3, 70.9, 110.6, 112.4, 122.0, 127.3 (2C), 128.3, 128.8 (2C), 129.1, 136.3, 149.8, 152.7, 196.7; ESI-HRMS calcd for $C_{20}H_{25}NO_3Na$ $[M+Na]^+$, 350.1732; found, 350.1733.

1-(4-(Benzyloxy)phenyl)-2-(*tert*-butylamino)ethanone (7b). Compound **7b** was synthesized from **6b** following the procedures for the preparation of compound **7a** to afford a white solid (75%, two steps). ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.16 (s, 9, CH_3), 2.09 (bs, 1, NH),4.10 (s, 2, $COCH_2$), 5.13 (s, 2, OCH_2), 6.93-7.07 (m, 2, aromatic), 7.28-7.52 (m, 5, aromatic), 7.88-8.00 (m, 2, aromatic); ^{13}C NMR (50 MHz, $CDCl_3$) δ 29.0 (3C), 49.0, 50.4, 70.3, 114.8 (2C), 127.6 (2C), 128.4, 128.8 (2C), 130.1 (2C), 136.2, 162.9, 196.5; ESI-HRMS calcd for $C_{19}H_{24}NO_2$ $[M+H]^+$, 298.1802; found, 298.1805.

4-[2-(*tert*-Butylamino)-1-hydroxyethyl]-2-methoxyphenol (2, mebuctopamine). An excess amount of HCl in CH_2Cl_2 was added to a solution of **7a** (450 mg, 1.37 mmol) in CH_2Cl_2 (5 mL), and the resulting mixture was evaporated to afford **7a**•HCl as a white solid. Catalytic hydrogenation of **7a**•HCl in ethanol (5 mL) using 10% Pd/C (251 mg) under H_2 (120 psi) for 2 h followed by filtration through celite and evaporation provided the HCl salt of the crude product. The crude solid was dissolved in a mixture of $NaHCO_{3(sat)}$ (35 mL) and EtOAc (22 mL), and then the organic layer was washed with brine, dried over $MgSO_4$,

filtered, and evaporated. The solid residue was chromatographed (silica gel, MeOH/CH_2Cl_2 = 1/50) to afford compound **2** as a light yellow solid (256 mg, 1.07 mmol, 78%). Purity (HPLC) 98.6%; mp 144-145°C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.11 (s, 9, CH_3), 2.61 (dd, 1, *J* = 11.7, 8.9 Hz, CHC$\underline{H}_2$), 2.85 (dd, 1, *J* = 11.7, 3.7 Hz, CHC$\underline{H}_2$), 3.88 (s, 3, OCH_3), 4.57 (dd, 1, *J* = 8.9, 3.7 Hz, C$\underline{H}$CH$_2$), 6.75-6.84 (m, 1, aromatic), 6.87 (d, 1, *J* = 8.0 Hz, aromatic), 6.94 (d, 1, *J* = 1.7 Hz, aromatic) (Figure 1A); ^{13}C NMR (50 MHz, $CDCl_3$) δ 28.9 (3C), 50.4, 50.7, 55.9, 72.2, 108.6, 114.5, 118.7, 134.8, 145.2, 146.9 (Figure 1B); ESI-HRMS calcd for $C_{13}H_{21}NO_3Na$ $[M+Na]^+$, 262.1414; found, 262.1420.

4-[2-(*tert*-Butylamino)-1-hydroxyethyl]benzene-1,2-diol (3, colterol). Compound **3** was prepared from 4-(chloroacetyl)catechol following the literature procedure to afford a light yellow solid.[12] ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.01 (s, 9, CH_3), 2.52-2.55 (m, 2, CHC$\underline{H}_2$), 4.33 (t, 1, *J* = 6.3 Hz, C$\underline{H}$CH$_2$), 6.55 (d, 1, *J* = 8.0 Hz, aromatic), 6.65 (d, 1, *J* = 8.0 Hz, aromatic), 6.69-6.78 (m, 1, aromatic); ^{13}C NMR (100 MHz, DMSO-d_6) δ 28.8 (3C), 49.7, 50.7, 72.2, 113.5, 115.1, 116.7, 135.6, 144.2, 145.0; ESI-HRMS calcd for $C_{12}H_{20}NO_3$ $[M+H]^+$, 226.1438; found, 226.1435.

4-[2-(*tert*-Butylamino)-1-hydroxyethyl]phenol (4, buctopamine). Compound **4** was synthesized from **7b** following the procedures for the preparation of compound **2** to afford a yellow solid (66%). An analytical sample was obtained by recrystallization (EtOAc) to grant an ivory white solid. Purity (HPLC) 97.7%; mp 166.5-167.3°C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.00 (s, 9, CH_3), 2.48-2.54 (m, 3, NH and CHC$\underline{H}_2$), 3.34 (bs, 1, OH), 4.39 (t, 1, *J* = 6.3 Hz, C$\underline{H}$CH$_2$), 6.65-6.72 (m, 2, aromatic), 7.12 (dd, 2, *J* = 8.9, 2.3 Hz, aromatic) (Figure 2A); ^{13}C NMR (50 MHz, DMSO-d_6) δ 29.0 (3C), 49.5, 50.8, 72.2, 114.7 (2C), 127.0 (2C), 134.9, 156.2 (Figure 2B); ESI-HRMS calcd for $C_{12}H_{20}NO_2$ $[M+H]^+$, 210.1494; found, 210.1488.

159

160 **Analysis of Authentic Standards.** The chemical purities of authentic standards **2-4** were
161 determined with a Shimadzu high-performance liquid chromatography (HPLC) system
162 (LC-10AT, Shimadzu Corp., Kyoto, Japan) coupled with a diode array detector (Shimadzu®,
163 SPD-M10Avp). The buffer containing the ion-pairing reagent (4.23 mM) was prepared by
164 dissolving sodium 1-heptanesulfonate (565 mg) and glacial acetic acid (6 mL) in deionized
165 water (600 mL). The buffer was filtered through a 0.45-μm membrane filter (PALL Corp.,
166 Port Washington, NY, USA) and degassed. A C_{18} column (Gemini® C18 Column, 5μ, 110 Å,
167 250 x 4.6 mm, Phenomenex, Torrance, CA, USA) was used, and the temperature was
168 maintained at 30°C using a column oven (COLBOX®, Hipoint Corp., Kaohsiung, Taiwan).
169 The flow rate was 1.0 mL/min and the detected wavelength was 276 nm. The initial mobile
170 phase was MeCN/buffer (3:7) held for 15 min before linearly increasing to 9:1 at 25 min,
171 where it was then held for another 5 min.

172

173 **Multi-residue Analysis of β_2-agonists in Swine Hairs.** A method[3,13-15] has been
174 established for the simultaneous determination of residues of 26 β_2-agonists (including the
175 seven prohibited β_2-agonists and compounds **2-4**) in swine hair and feed additives by HPLC
176 coupled with a triple quadrupole mass spectrometer (LC/MS/MS, Agilent 6460 Triple Quad
177 LC/MS, Santa Clara, CA, USA). ESI positive ion scan mode was used. Briefly, the collected
178 swine hair samples were washed with water and 95% alcohol, dried at 50°C, and then
179 pulverized in liquid nitrogen using a cryogenic grinder (6870 Freezer/Mill®, Thomas
180 Scientific, Swedesboro, NJ, USA) before use. $NaOH_{(aq)}$ (1 mol/L, 4 mL) was added to the
181 accurately weighted and powdered swine hairs (2.0 g), and the system was heated in a water
182 bath at 65°C for 2 h. After cooling to room temperature, HCl (1 mol/L, 3.5 mL) and NaOAc

183 buffer (0.25 mol/L, pH = 4.8, 5 mL) were added and mixed well. The mixture was centrifuged
184 at 4°C (20,000 rpm, 10 min), and the supernatant was then collected for purification. The
185 resulting solution was passed through an SCX solid-phase extraction cartridge (Varian, Inc.,
186 Palo Alto, CA, USA), and the cartridge was washed with acetic acid (1 mol/L, 1 mL) and
187 water (5 mL × 2). The Varian® SCX solid-phase extraction cartridge was dried for 30 min and
188 eluted with a solution of $NH_4OH_{(conc)}$, MeCN, and EtOAc (1:9:10, 5 mL). The eluent was
189 dried under a nitrogen flow at 45°C, and the residue was dissolved in a solution of MeCN and
190 7 mmol/L $NH_4OAc_{(aq)}$ (1:9, 1 mL) and then filtered (0.22 μm, PVDF membrane) to afford the
191 test solution. The test solution (20 μL) was injected into the LC/MS/MS instrument using
192 multiple reaction monitoring (MRM) mode for analysis. HPLC conditions—The NH_4OAc
193 buffer (7 mmol/L) was prepared by dissolving NH_4OAc (270 mg) in deionized water (200 mL)
194 in a 500-mL volumetric flask. Formic acid (0.5 mL) was then added, followed by adding
195 deionized water to the volume. Both the MeCN and NH_4OAc buffer were filtered through a
196 0.45-μm membrane filter and degassed. An SB-C_{18} column (Zorbex® SB-C18 Column, 5μ,
197 150 x 3.0 mm, Agilent, Santa Clara, CA, USA) was used under ambient temperature for the
198 analysis. The mobile phase was initially set at 1% MeCN and then linearly increased to 90%
199 MeCN over 5 min and held for 3 min. The flow rate was 0.8 mL/min. ESI-HRMS analysis
200 was conducted by injection of the test solution (5.0 μL) into the quadrupole time-of-flight
201 (QTOF) LC/MS/MS instrument (Agilent 6500 series, Agilent, Santa Clara, CA, USA).

202

203 **Multi-residue Analysis of β_2-agonists in Feed Additives.** Briefly, specimens of swine
204 feed additive (2.0 g) were accurately weighed, treated with a solution of 0.2 mol/L phosphoric
205 acid/MeOH (1:1; 10 mL) for 30 min, and centrifuged (3,000 rpm, 10 min). NaOAc buffer
206 (0.25 mol/L, pH = 4.8, 5 mL) was added to the supernatant (2 mL), and the solution was

mixed for 30 seconds. The resulting solution was passed through an SCX solid-phase extraction cartridge, and the cartridge was then washed with acetic acid (1 mol/L, 1 mL) and water (5 mL × 2). The SCX cartridge was dried for 30 min and eluted with a solution of $NH_4OH_{(conc)}$, MeCN, and EtOAc (1:9:10, 5 mL). The eluent was dried under a nitrogen flow, and the residue was dissolved by a solution of MeCN and 7 mmol/L $NH_4OAc_{(aq)}$ (1:9, 1 mL) and then filtered (0.22 μm, PVDF membrane) to afford the test solution. The test solution (10 μL) was injected into the triple quadrupole LC/MS/MS instrument using MRM mode. An additional 5.0 μL of the test solution was injected into the QTOF LC/MS/MS instrument using neutral loss mode. Both studies used the same HPLC conditions described in **Multi-residue Analysis of β_2-agonists in Swine Hairs** for analysis.

Pharmacological Activity and Physicochemical Properties. The human α_1, α_2, β_1, β_2, and β_3 receptor binding affinities of compounds **2-4** were determined by Eurofins Panlabs Taiwan Ltd., Eurofins Pharma Discovery Services (http://www.eurofins.com/biopharma-services/discovery/), based on literature methods.[16-20] The calculated LogP values (CLogP) were obtained directly from ChemDraw Ultra version 11.0 (PerkinElmer Informatics, Waltham, MA, USA). The experimental $LogD_{7.4}$ were determined by using the shake flask method following the OECD guideline.[21]

RESULTS AND DISCUSSION

Identification of Mebuctopamine (2) in Swine Hairs. Initially, the LC/MS/MS reconstructed chromatograms and spectra of the swine hair samples from the livestock farms demonstrated a suspicious ion peak, which had the same *m*/*z* as that of the molecular ion of salbutamol (**1**) but a different retention time and fragment ion profile (Figure 3). After

ESI-HRMS analysis, the exact mass of this unknown ion was determined to be 240.1605, and therefore, there were five most possible molecular ion formulas for the unknown component: $[C_8H_{20}F_2N_5O^+]$, $[C_{11}H_{19}FN_5^+]$, $[C_{10}H_{23}FNO_4^+]$, $[C_{13}H_{22}NO_3^+]$, and $[C_{11}H_{24}F_2NS^+]$. Based on the chemical structures of the seven prohibited β_2-agonists and the well-known structure−activity relationships of β_2-agonists,[16,17] the *N*-isopropyl-2-hydroxy-2-phenylethylamine scaffold **X** should be the common core structure and essential pharmacophore of various β_2-agonists (Chart 1). Therefore, the unknown compound should possess at least 11 carbon, one nitrogen, and one oxygen atoms to fulfill the structural requirements of the β_2-agonist pharmacophore. Formulas $[C_8H_{20}F_2N_5O^+]$ and $[C_{10}H_{23}FNO_4^+]$ were ruled out because they have only 8 and 10 carbon atoms, respectively, in their molecular formulas. There are no oxygen atoms in the molecular ion formulas $[C_{11}H_{19}FN_5^+]$ and $[C_{11}H_{24}F_2NS^+]$. As a result, only $[C_{13}H_{22}NO_3^+]$ could be the molecular ion formula of the unknown component. In addition, the molecular ion formula of compound **1** was also $[C_{13}H_{22}NO_3^+]$, and therefore, the unknown compound should be an isomer of compound **1**.

There were thousands of compounds in the Reaxys database possessing the same molecular formula as compound **1**. After screening with the essential pharmacophore of β_2-agonist **X**, the number of candidate compounds was reduced to 13. Further analysis of the LC/MS/MS data of the swine hair specimens and **1** disclosed additional structural information about the unknown component in the swine hair. There are three major fragment ions in the mass spectrum of **1**, which are formed by i) dehydration to afford ion **1-II** (m/z = 222), ii) dehydration and elimination of isobutene to afford ion **1-III** (m/z = 166), and iii) additional dehydration of **1-III** to yield ion **1-IV** (m/z = 148) (Figure 4). However, there are only two major fragment ions for the unknown compound, m/z = 222 and 166, which could be produced by dehydration and elimination of isobutene, and there is no fragment ion with a

mass of 148. This implied the existence of an *N*-*tert*-butyl substitution and the absence of the benzylic hydroxyl group at the *meta*-position of the phenyl group in compound **1**, which is essential for the additional dehydration. In consideration of the above information about the unknown component, compound **2** was chosen for synthesis as a potential candidate.

The authentic compound **2** was prepared as shown in Scheme 1 in 98.6% purity and analyzed by LC/MS/MS using MRM mode (parent ion: m/z = 240; daughter ions: m/z = 222, 166, 148). There are only two major fragment ions in the mass spectrum of **2**, which are formed by i) dehydration to afford ion **2-II** (m/z = 222) and ii) dehydration and elimination of isobutene to yield ion **2-III** (m/z = 166). The hydroxymethyl group at the *meta*-position of compound **1** was substituted with the methoxy group in **2**, and therefore, the additional dehydration in compound **1** to form the fragment ion, i.e., m/z = 148, was impossible for compound **2**. In addition, the authentic **2** demonstrated the same retention time and mass fragmentation pattern as the unknown component in the swine hair samples (Figures 3b and 3c), and therefore, compound **2** was confirmed to be the unknown component in swine hairs.

Identification of Buctopamine (4) in Feed Additives and Swine Hairs.

Interestingly, no compound **2** was detected in the "Garlic Essential" feed additive when using authentic **2** as the reference standard. Furthermore, compound **2** exhibited only weak β_2 receptor affinity (discussed later). Thus, compound **2** was not the active ingredient adulterating the swine feed, and it may be a metabolite of another, more potent, β_2-agonist. In the literatures, compound **2** has been reported as a metabolite of colterol (**3**, Th 1206, *t*-butylarterenol, tBA),[24,25] transformed by catechol-*O*-methyl transferase (COMT, EC 2.1.1.6) (Scheme 2).[26-28] Since compound **3** is a known potent β_2-agonist, it may be the active compound added to the "Garlic Essential". The authentic standard of **3** was then prepared for

LC/MS/MS analysis of the feed additive samples. However, compound **3** was not detected in the "Garlic Essential" samples, and therefore, the unknown β_2-agonist in the feed additive was not compound **3**。

After carefully examining the chemical structures and MS/MS data of compounds **1** and **2**, the [M+H-74] fragment ions resulting from dehydration and elimination of isobutene were determined to reflect characteristic fragmentation of the pharmacophore of these β_2-agonists. Therefore, the unknown β_2-agonist should also demonstrate a similar fragmentation pattern in its MS/MS spectra. Therefore, the LC/MS/MS analysis of the feed additive specimens using "neutral loss" mode (M→M-74) was conducted to screen the potential β_2-agonist-related substances in the feed additives. A strong parent ion signal with a molecular mass of 210 was identified, which was 30 and 16 less than those of compounds **2** and **3**, respectively (Figure 5). This difference means that the molecular formula of the unknown component should be a "CH_2O" fragment and one "O" atom less than those of compounds **2** and **3**, respectively.

In a metabolism study, tulobuterol, a β_2-agonist possessing pharmacophore **X**, was oxidized by aromatic hydroxylase to produce the corresponding catechol metabolite in rat.[29] The meta-hydroxyl group of compound **3** might also be introduced by the same enzyme, and therefore, compound **4** (buctopamine, *N*-*tert*-butyl-norsynephrine, KWD2066) was proposed to be the unknown component in the "Garlic Essential". Compound **4** could be metabolized by aromatic hydroxylase in swine to yield compound **3** (Scheme 2). Compound **4** was first synthesized in 1945 and exhibited marked depressor action.[30] The authentic standard of **4** was prepared as shown in Scheme 1 in a purity of 97.7%. After LC/MS/MS analysis of the feed additive samples using MRM mode (parent ion: m/z = 210; daughter ions: m/z = 192, 136, 91), compound **4** was confirmed to be the unknown β_2-agonist in the "Garlic Essential" (Figure 6). Further analysis of the swine hair samples using **4** as the reference standard disclosed that

304 compound **4** also existed in the swine hairs (Figure 6c).

305

306 **Multi-residue Analysis of β_2-agonists**

307 An analytical method has been established for the routine simultaneous determination of
308 residues of 26 β_2-agonists, including compounds **2**-**4** and the seven prohibited β_2-agonists, in
309 swine hair and feed additives using LC/MS/MS. ESI positive ion scan mode was used. All 26
310 β_2-agonists showed good linear regression in the range of 5.0−30.0 ng/mL, and the correlation
311 coefficients (r) were not less than 0.995. The intraday and interday recoveries were in the
312 range of 80.7-110.8%, with average relative standard deviations (RSD) of 1.06-10.8%.
313 Because β_2-agonists are not allowed in either swine feed or hair in Taiwan, the detection and
314 quantitative limits for the β_2-agonists in this multi-residue analysis method were therefore not
315 determined individually. For practical high-throughput screening, common limits of
316 quantification for the 26 β_2-agonists in swine feed and hair are set at 10 and 25 ng/g,
317 respectively. More than 2000 specimens were collected and analyzed by local authorities in
318 2010-2011, with compounds **2** and **4** detected in approximately 6% and 2% of the swine hair
319 samples, respectively. The concentrations of compound **2** in the positive hair samples were
320 between 30- and >100 ng/g. The contents of compound **4** in the feed additive "Garlic
321 Essential" were in the range of 5.0-10 mg/g. Currently, this method is used for the analysis of
322 2000-3000 swine hair and feed additive specimens each year.

323

324 **Pharmacological Activity and Physicochemical Property**

325 The binding affinities of β_2-agonists for the human β_1-, β_2- and β_3-adrenoceptors and the
326 bovine β_2-adrenoceptor in the literature are shown in Table 1.[25,31,32] All β_2-agonists have
327 demonstrated high binding affinity and potent agonistic activity toward the β_2 receptor.

Although ractopamine, clenbuterol, and cimaterol have also shown high β_1-receptor affinity, ractopamine is only an agonist of the porcine β_2 receptor and possesses no agonistic activity toward the porcine β_1 receptor.[33] Interestingly, these β_2-agonists are full agonists of the human β_1 receptor,[31] and therefore, their residues in swine meat and tissue may cause potential adverse effects that did not exist in pigs.

The β_3-receptor affinities of these β_2-agonists are much weaker than their β_1- and β_2-receptor affinities. In addition, selective β_3-agonists lack efficacy for protein synthesis, which is crucial for muscle formation, and therefore, the β_3 receptor should not play a significant role in livestock production.[1]

Previously, compounds **3** and **4** were shown to demonstrate moderate to potent bovine β_2-receptor binding affinity and agonistic efficacy (-log K_D = 6.20-6.58, Table 1).[24,25] In this study, the binding affinities of compounds **2**-**4** for the human α and β receptor subtypes were evaluated. Compounds **2**-**4** had negligible binding to the α_1 and α_2 receptors (Table 2), while they showed moderate to high β_2 receptor binding inhibition in the rank order of **3** > **4** > **2**. Compound **3** also possessed high binding affinity for the β_1 receptor, whereas compounds **2** and **4** did not demonstrate significant binding to the β_1 and β_3 receptors.

The calculated CLogP and experimental $LogD_{7.4}$ values of the β_2-agonists were determined for the quantitative evaluation of their lipophilicity.[21] Both CLogP and $LogD_{7.4}$ values demonstrate similar trends for these β_2-agonists (Table 2). The differences between the values from the two methods may be due the protonation of the basic amino groups of the β_2-agonists at physiological conditions (pH = 7.4). Of the compounds in this study, ractopamine and clenbuterol were the most lipophilic, while salbutamol (**1**) was the most hydrophilic. The lipophilicities of compounds **2**-**4** were higher than that of **1** and lower than those of ractopamine and clenbuterol. This can explain why the residues of compounds **2** and

352 **4** existed in the swine hair specimens, while the more hydrophilic **3** was not detected.

353 In this study, compound **4**, a novel β_2-agonist adulterating swine feed additives, was
354 identified. Both compound **4** and its metabolite **2** existed in the swine hair specimens.
355 Authentic **2** and **4** were synthesized and added to the library containing of 26 β_2-agonists for
356 screenings of multiple residues of β_2-agonists using LC/MS/MS conducted by regulatory
357 authorities. The illegal use of compound **4** as a feed additive has been successfully prevented.
358 No specimen of swine hair or feed additive has been found to contain either **2** or **4** in Taiwan
359 since 2013. In comparison to ractopamine and clenbuterol, compounds **2**-**4** possess lower β_2
360 receptor affinity and higher hydrophilicity. This work and experience should also be useful
361 for other countries in preventing the illegal use of new β_2-agonists in animal breeding.

362

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Notes

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Figure captions

Figure 1A. The ^{1}H NMR spectrum (200 MHz, $CDCl_3$) of mebuctopamine (**2**).

Figure 1B. The ^{13}C NMR spectrum (50 MHz, $CDCl_3$) of mebuctopamine (**2**).

Figure 2A. The ^{1}H NMR spectrum (200 MHz, DMSO-d_6) of buctopamine (**4**).

Figure 2B. The ^{1}H NMR spectrum (50 MHz, DMSO-d_6) of buctopamine (**4**).

Figure 3. The LC/MS/MS reconstructed chromatograms and spectra using MRM mode (multiple reaction monitoring mode; parent ion: *m*/*z* 240; daughter ions: *m*/*z* 222, 166, 148) of (a) salbutamol in a standard mixture of 10 β_2-agonists; (b) unknown component in swine hair samples; (c) authentic standard of mebuctopamine (**2**).

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Table 1. The β-receptor binding affinities and agonistic activities of β_2-agonists in the literature.

	β_1		β_2		β_2		β_3
Compounds	-log K_D[a]	-log EC_{50}[b]	-log K_D[a]	-log EC_{50}[b]	-log K_D[c]	cAMP[c] production	-log K_D[a]
salbutamol (**1**)	4.68	6.21	6.01	7.72	6.58	122	3.98
colterol **(3)**					6.55	83	
buctopamine (**4**)					6.20	97	
ractopamine	6.97	8.74	6.93	7.63			5.82
zilpaterol			6.24[d]				
clenbuterol	6.62	7.29	7.90	9.18	7.74	64	5.35
cimaterol	6.57	8.42	7.26	8.94			5.28
terbutaline	3.90	5.76	5.51	7.29	5.73	50	3.68
tulobuterol	5.62	6.59	6.83	7.60	7.18	61	4.72

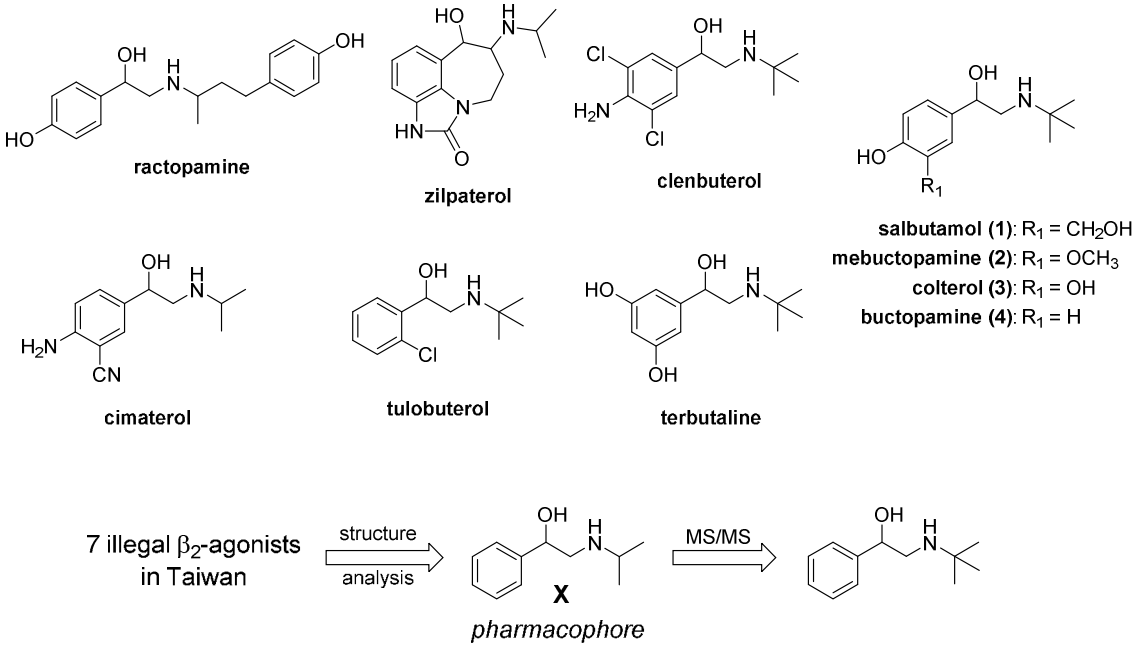
[a]Human β_1-, β_2- and β_3-adrenoceptor binding affinities.[32] [b]Data obtained from [^{3}H]-cAMP accumulation for cells expressing the human β_1- and β_2-adrenoceptors.[32] [c]β_2-Adrenoceptor affinities and intrinsic activities using a bovine skeletal muscle preparation. The cAMP production values are in pmol (mg of protein)$^{-1}$ min^{-1}.[25] [d]The binding affinity of zilpaterol is presented as -log K_i.[33]

Table 2. ClogP, $LogD_{7.4}$, and receptor binding affinities of β_2-agonists.

Compounds	CLogP[a]	$LogD_{7.4}$[b]	% Inhibition at 1 μM[c]				
			α_1	α_2	β_1	β_2	β_3
salbutamol (**1**)	0.06	−1.14					
mebuctopamine (**2**)	1.00	−0.23	−2	2	5	**19**	−5
colterol **(3)**	0.55		−8	1	**50**	**53**	15
buctopamine (**4**)	1.15	−0.45	−8	6	12	**28**	−8
ractopamine	2.03	0.23					
zilpaterol	0.95	−0.68					
clenbuterol	2.39	0.20					

[a]The calculated LogP values (CLogP) were obtained from ChemDraw Ultra version 11.0 (PerkinElmer Informatics, Waltham, USA). [b]The experimental $LogD_{7.4}$ were determined by using the shake flask method following the OECD guideline.[21] [c]The receptor binding assays were determined by measuring the binding inhibition using a single drug concentration (1 • M) by Eurofins Pharma Discovery Services.[16-20]

Chart 1


ractopamine
zilpaterol
clenbuterol
salbutamol (1): R1 = CH2OH
mebuctopamine (2): R1 = OCH3
colterol (3): R1 = OH
buctopamine (4): R1 = H
cimaterol
tulobuterol
terbutaline
7 illegal β2-agonists in Taiwan
structure analysis
X
pharmacophore
MS/MS

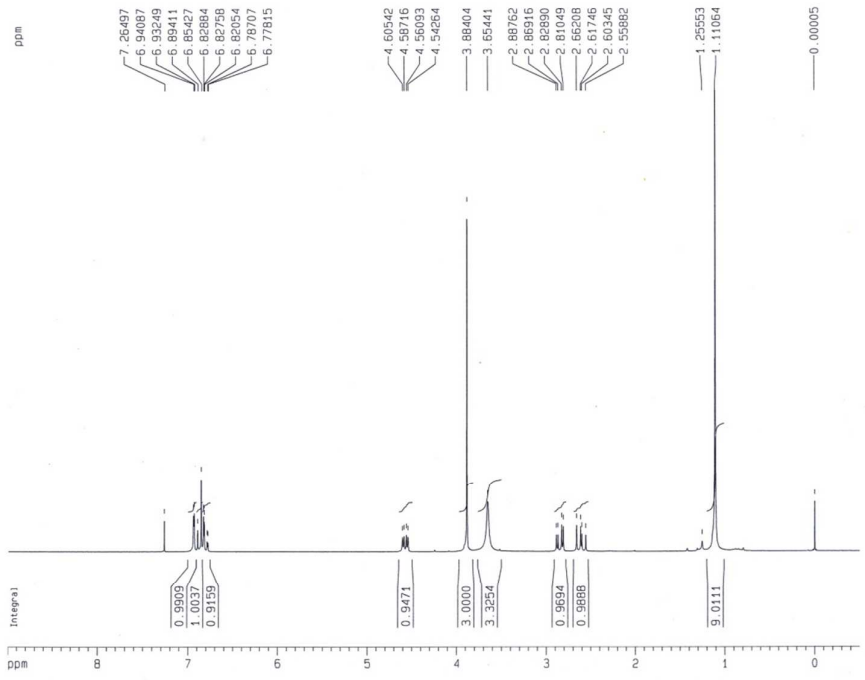


Figure 1A. The ^{1}H NMR spectrum (200 MHz, $CDCl_3$) of mebuctopamine (**2**).

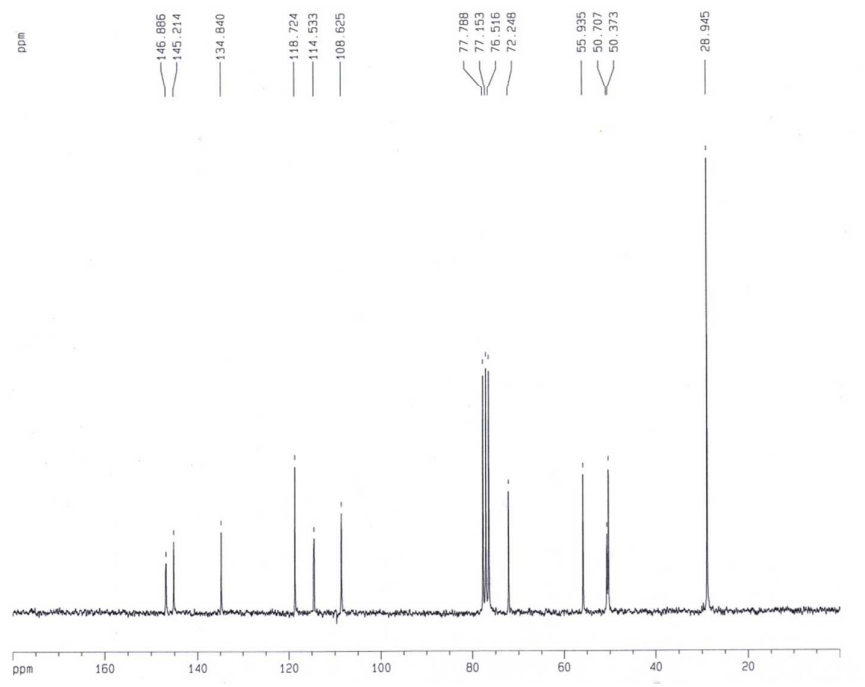


Figure 1B. The ^{13}C NMR spectrum (50 MHz, $CDCl_3$) of mebuctopamine (**2**).

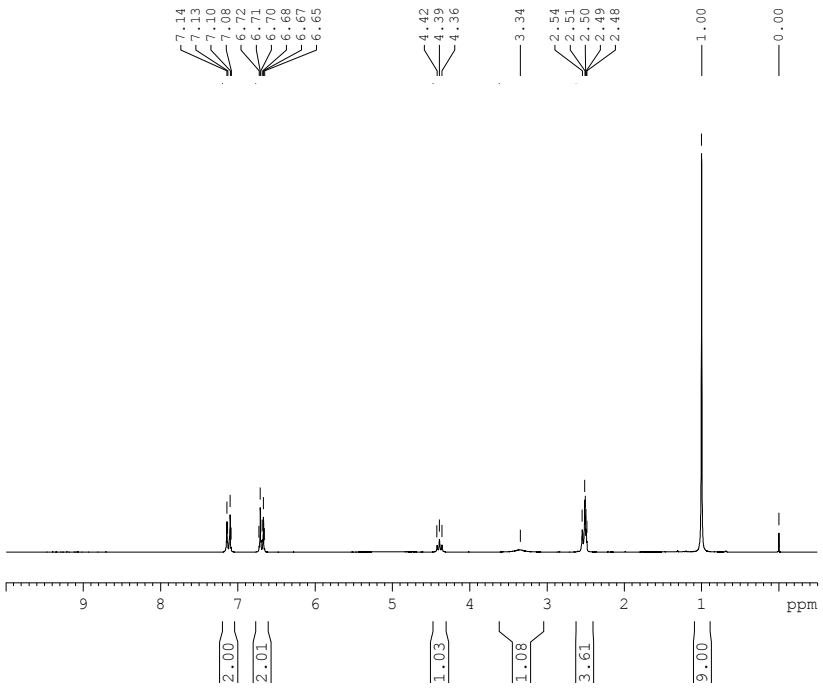
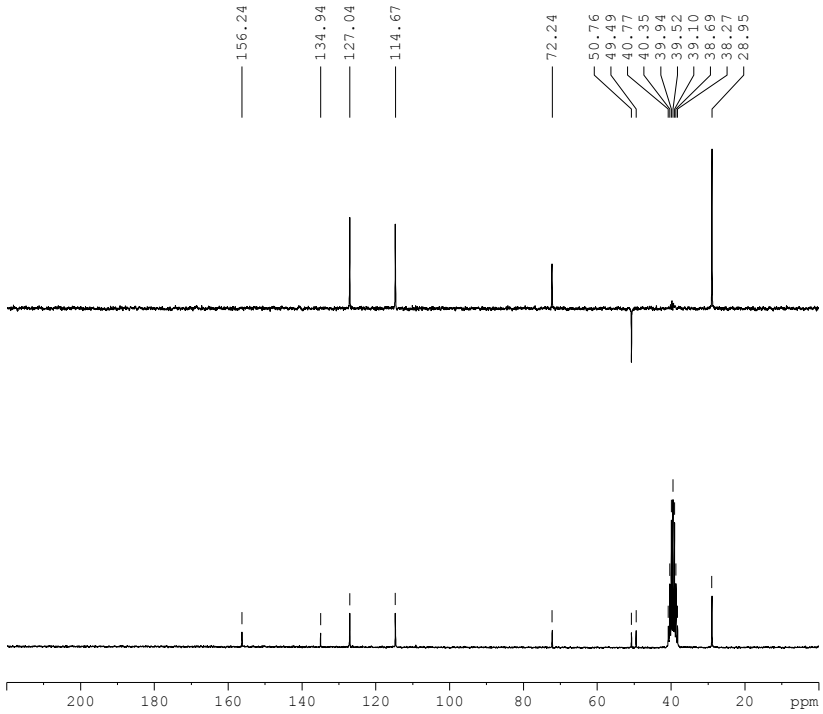


Figure 2A. The ^{1}H NMR spectrum (200 MHz, DMSO-d_6) of buctopamine (**4**).



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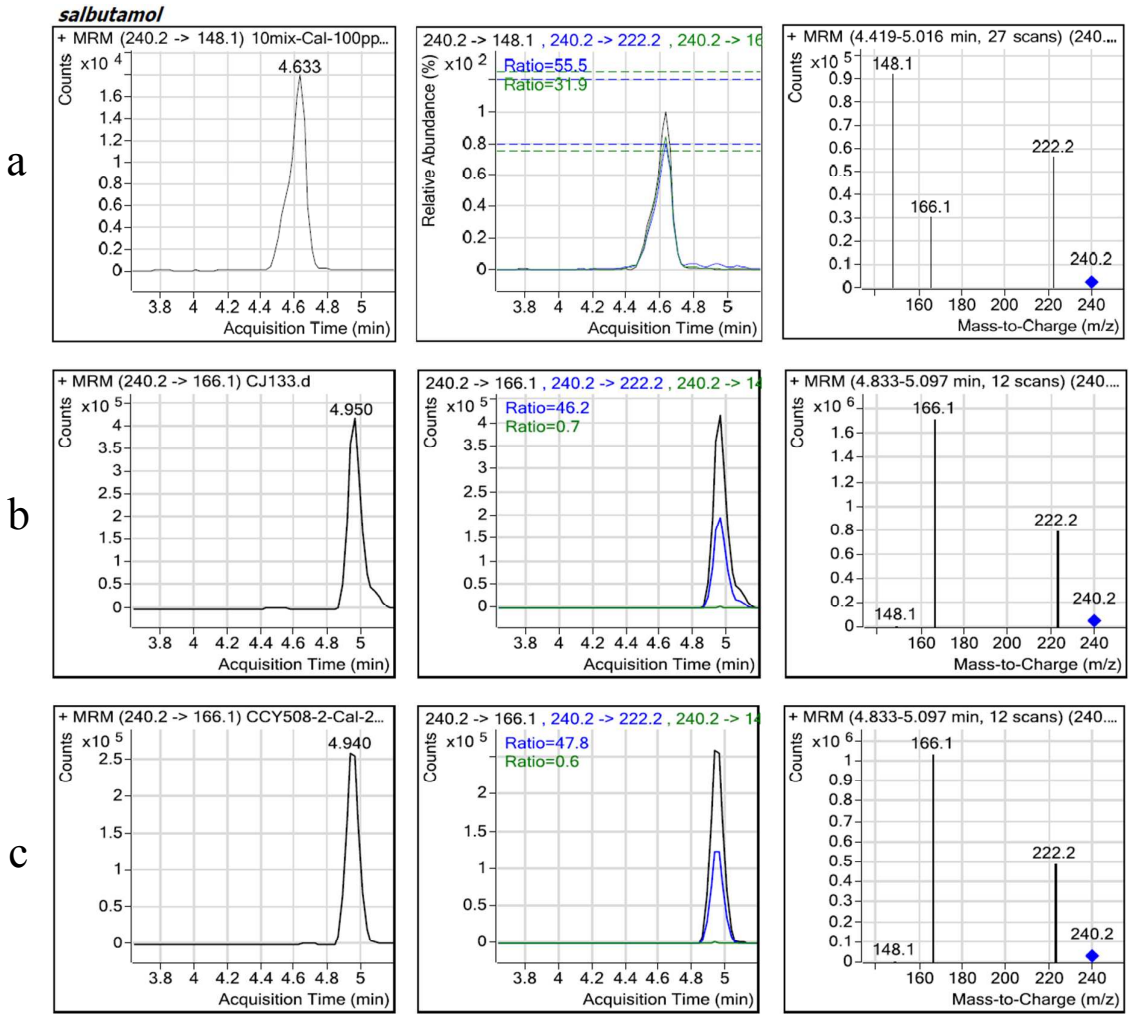

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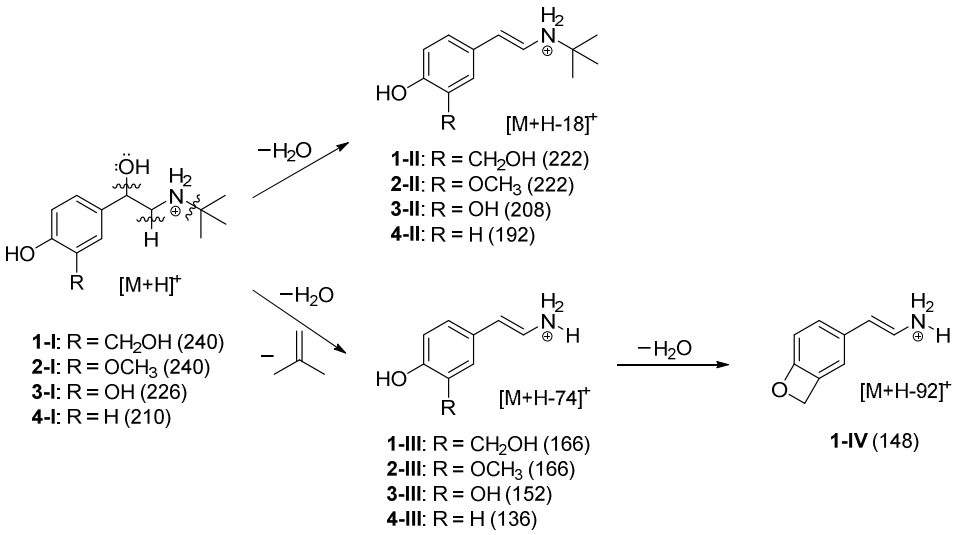

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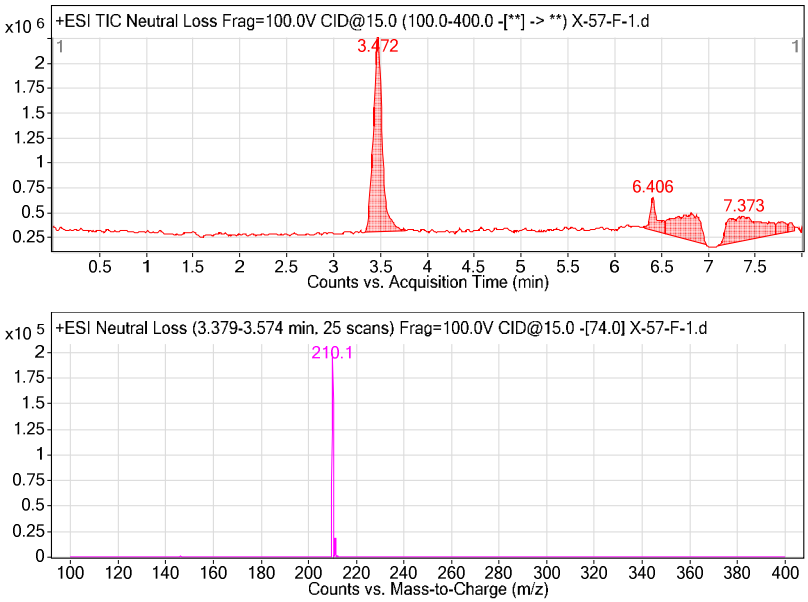

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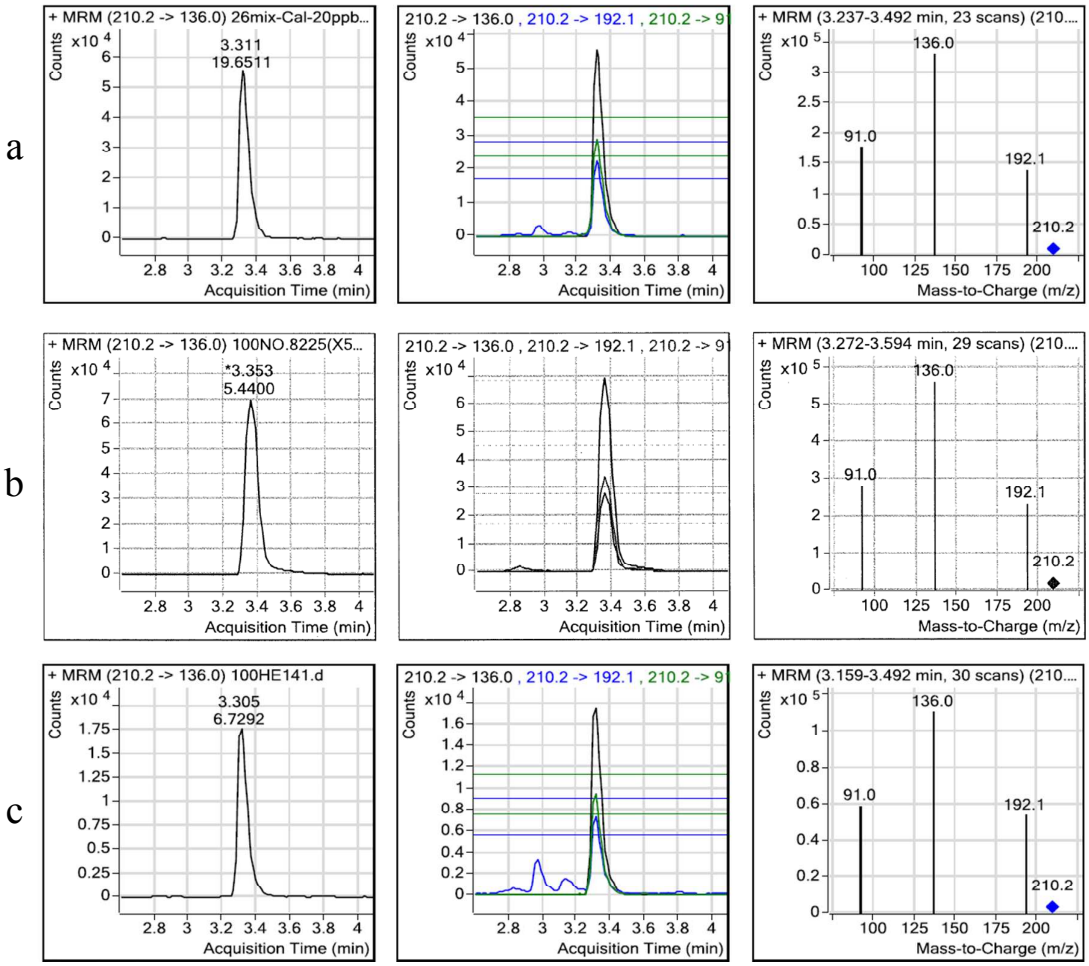

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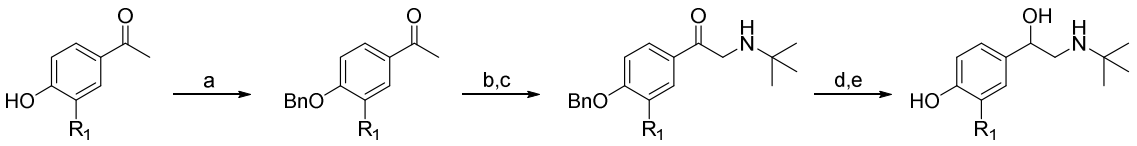
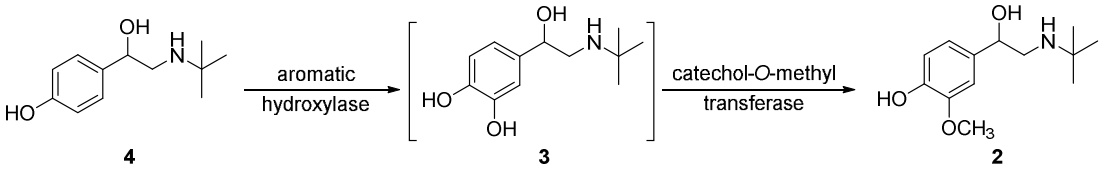
Scheme 1



5a: $R_1 = OCH_3$ **6a**: $R_1 = OCH_3$ **7a**: $R_1 = OCH_3$ **2**: $R_1 = OCH_3$
5b: $R_1 = H$ **6b**: $R_1 = H$ **7b**: $R_1 = H$ **4**: $R_1 = H$

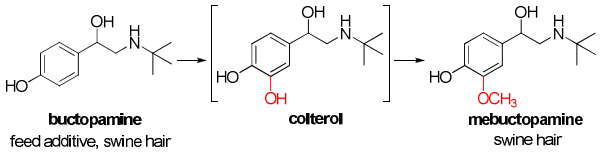
Reagents and conditions: a) benzyl bromide, K_2CO_3, rt, 1 h; b) Br_2, EtOH, rt, 1 h; c) *t*-butylamine, DMF, rt, 1 h; d) HCl, CH_2Cl_2, rt; e) 10%Pd/C, H_2 150 psi, EtOH, rt, 2 h.

Scheme 2



4 **3** **2**

Graphic for table of contents



buctopamine
feed additive, swine hair

colterol

mebuctopamine
swine hair