GENERAL METHODS FOR THE SYNTHESIS OF METHYL ISOTOPE LABELLED CATECHOLAMINE METABOLITES. PREPARATION OF 4-HYDROXY-3-METHOXY-d_3-(MANDELIC ACID, PHENYLACETIC ACID, AND PHENYLETHYLENE GLYCOL)

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SUMMARY

Two general schemes for the synthesis of isotope labelled monomethyl catecholamine metabolites are described. Catechol was converted to 2-methoxy-d_3-phenol (2) with deuteromethyl iodide and reacted with sodium glyoxylate to form 4-hydroxy-3-methoxy-d_3-mandelic acid (3). Hydrogenolysis of the methyl ester-diacetate of 3 with excess sodium borohydride in water yielded 4-hydroxy-3-methoxy-d_3-phenylacetic acid (4). Reduction of 3 with diborane produced 4-hydroxy-3-methoxy-d_3-phenylethylene glycol (5). An alternative route was to alkylate 3,4-dihydroxybenzaldehyde (6) and produce 3 or its 3-hydroxy-4-methoxy isomer via the nitrile mandelic acid synthesis. Suitability of these isotopic and isomeric variants as internal standards for quantitation by gas chromatography-mass spectrometry is discussed.

Key Words: 2-methoxyphenol, vanillin, isovanillin, homovanillic acid, 4-hydroxy-3-methoxy-phenylethylene glycol

0362-4803/80/0117-0103$01.00

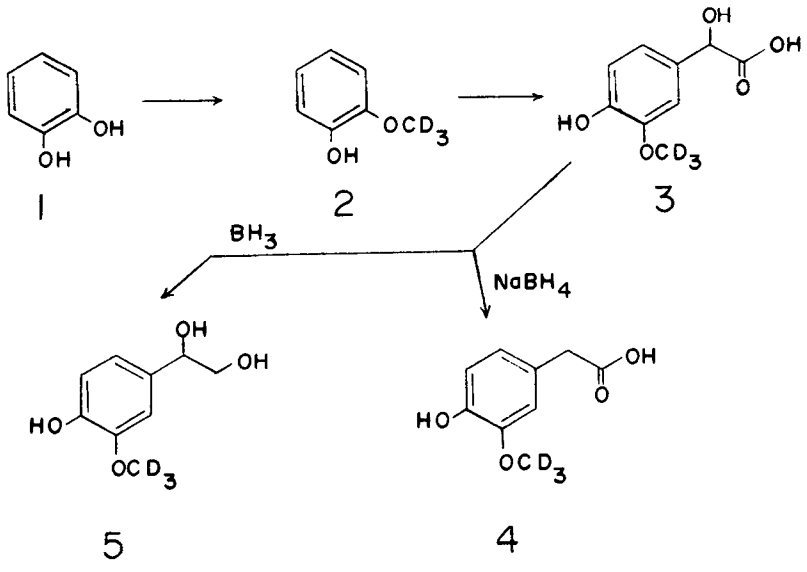
Received November 6, 1978
Revised February 16, 1979

INTRODUCTION

Human metabolic studies of endogenous catecholamine metabolites require the availability of suitable stable isotopic variants both as internal standards for mass spectral analytical procedures (1), and for the administration of the labelled metabolite in order to perform in vivo kinetic studies (2). The quantities of material required (mg/patient) preclude the use of enzyme mediated synthesis for the production of high purity methylated catecholamine metabolites. Catalyzed exchange of aromatic or benzylic protons in D_2O is readily achieved and results in useful analytical internal standards (3,4), but is of questionable value for compounds to be employed in vivo. A general scheme for deuteromethyl catecholamine metabolites was desired. From the trideuterated methyl analogs, heavier variants could be prepared by exchange, so that the assessment of sample recovery (endogenous and administered deuterated) could be made by selected ion monitoring using gas chromatography-mass spectrometry (GC-MS).

General synthetic schemes for the preparation of various monomethylated catechols invariably utilize readily available starting materials such as vanillin (5-8). Thus a suitable method was sought for the preparation of a common isotope labelled intermediate which could be used for preparation of the labelled norepinephrine metabolites 4-hydroxy-3-methoxy-d_3-mandelic acid, 3, 4-hydroxy-3-methoxy-d_3-phenylethylene glycol, 5 and the labelled dopamine metabolite 4-hydroxy-3-methoxy-d_3-phenylacetic acid (homovanillic-d_3 acid 4).

During the course of these studies, two general routes were investigated. The method of choice is described in Scheme 1. Catechol (1) was monomethylated with methyl-d_3 iodide in acetone with potassium carbonate. Monomethylation of catechol with dimethyl sulfate in nitrobenzene was tried repeatedly in order to obtain the high yield of 2-methoxy-d_3-phenol (2) reported by Bredereck et al. (9)



Scheme 1

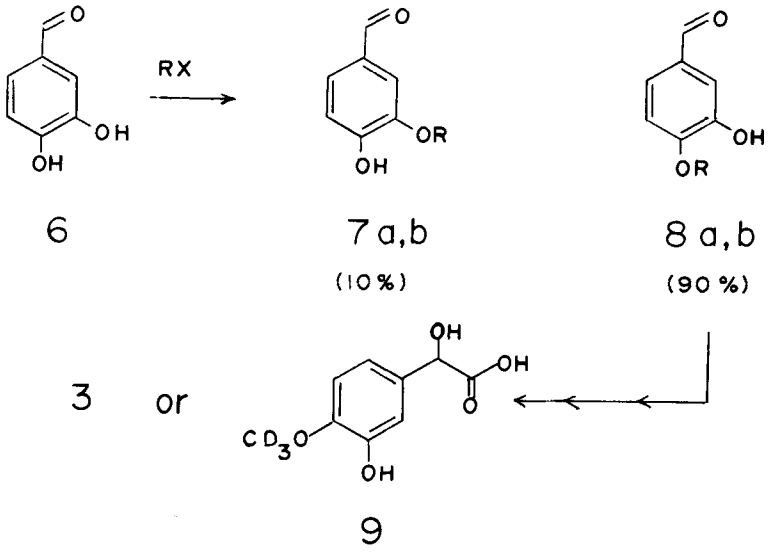
and consistently referenced in the literature as an example of selective conditions which influence product formation (10). In our hands, the product 2 was difficult to separate from nitrobenzene with which it co-distills, and which probably is responsible for the reported high yields.

The intermediate 2 was reacted with sodium glyoxylate to form 3 in 34% yield (11,12). Attempted hydrogenolyses using various catalysts (Pd/C, $Pd/BaSO_4$), solvents (alcohol, acids), and derivatives of 3 failed to produce 4 under conditions which successfully reduced other benzylic alcohols. However, from the ethyl ester-diacetate of 3, aqueous borohydride reduction gave the acid 4 in 54% yield (13). Anhydrous borohydride conditions recommended by Quick and Crelling (14) for the reduction of a benzylic alcohol *para* to a phenol resulted in a mixture of products, with ester reduction to 5 predominating

over 4. Reduction of 3 to 5 was performed with diborane, but the formation of intermediate borate salts necessitated long (30-40 hr) reaction times to obtain complete reduction (15). Diborane-d_6 was used to prepare a small quantity of a second isotopic (d_5) variant of 5.

A second route (Scheme 2) is based upon the synthetic routes described by Shaw, McMillan and Armstrong (5) for the preparation of catecholamine metabolites starting with vanillin, a route which has also been employed by La Manna and Ghislandi (16) to synthesize iso-derivatives by starting with isovanillin. Due to the ready availability of vanillins from natural sources, methods for their preparation by selective alkylation of 3,4-dihydroxybenzaldehyde (6) have not been well characterized. The greater acidity of the para hydroxyl can be used to obtain 90-95% reaction at this site with either benzyl bromide or methyl iodide in acetone with potassium carbonate. The vanillin/isovanillin or 3-/4-benzyl isomeric mixtures can be separated by repeated solvent extractions in buffered solutions, utilizing the greater acidity of the 4-hydroxy isomer, and monitoring the separation by gas chromatography. After solvent extraction, the 4-benzyl isomer 8b was methylated with methyl-d_3 iodide and deblocked with acid to yield labelled vanillin 7a in 18% yield from 6. From purified vanillin-d_3 7a or isovanillin-d_3 8a, the respective mandelic acids 3 and 9 were prepared via nitrile intermediates (5). Using these starting materials, Shaw et al. have also described the preparation of 4 and 3-methoxytyrosine through intermediate oxazolones.

Partial mass spectra of the trifluoroacetyl-ethyl ester (TFA-Et) derivatives of the three synthesized isomeric and isotopic variants of 4-hydroxy-3-methoxymandelic acid are shown in Figure 1. There are significant intensity differences for the major fragment ions and the molecular ion, but the mass shifts are



Scheme 2. R=CD_3 for 7a, 8a; R=benzyl for 7b, 8b

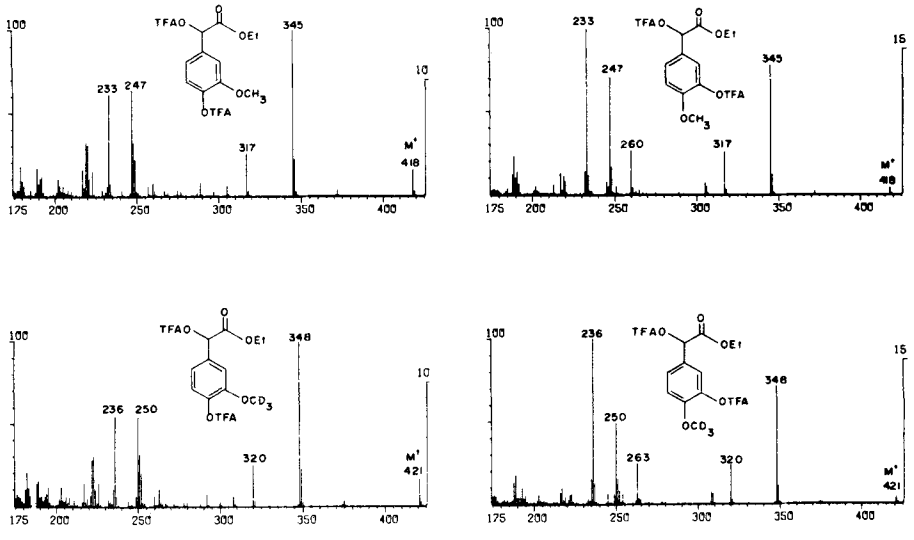


Figure 1. Electron impact mass spectra of the trifluoroacetyl (TFA)-ethyl esters (Et) of isotopic and isomeric variants of hydroxymethoxymandelic acid.

consistent with proposed fragments. Major ions are observed for the losses of CO_2Et (m/e 345, 348), CO_2Et+CO (317, 320), CF_3CO_2+OEt (260, 263), $CF_3COH+CO_2Et$ (247, 250) and CF_3CO+CO_2Et+H (233, 236). The 3- and 4-methoxy isomers can be separated on OV-22 liquid phase as TFA-Et derivatives, and consequently 3, 9, or non-deuterated 9 are possible internal standards for mass spectrometric assays. All three fulfill the criteria for useable standards--they chemically react with similar rates so that a constant proportion of standard to compound exists during the course of analysis, and standard curves over the range of greatest biological interest (0-100 ng) are linear. Over a wider range (0-1000 ng) the 4-methoxy isomers show a distinct deviation from linearity which may be due to different extraction efficiencies (17).

Experimental Section

Electron ionization mass spectra were recorded with a Finnigan 3200 gas chromatograph-mass spectrometer with a model 6000 data system. Infrared spectra were obtained with a Beckman Acculab 4. Melting points were observed on a Koeffler hot stafe apparatus and are uncorrected. Methyl-d_3 iodide (99 + atom percent) and THF-BH_3 complex were purchased from Aldrich. TLC was used to monitor the purification of 3, 4, 5, and 9 using silica gel plates eluted with benzene-acetic acid-water (62:35:1.5).

2-Methoxy-d_3-phenol (2). In a 250 ml round bottom flask fitted with a reflux condenser and calcium chloride drying tube, catechol (8.36 g, 75 mmol) was dissolved in 100 ml acetone. Powdered potassium carbonate (10.4 g, 75 mmol) and methyl-d_3 iodide (6.35 ml, 75 mmol) were added and the mixture refluxed for six hr. After cooling, water was added to dissolve the salt, and acetone was removed by distillation. The pH was adjusted to 10 and the mixture rinsed with three 50 ml

portions of hexane to remove dimethylated biproduct. The combined hexane layers were backwashed with three 50 ml portions of dilute base and the aqueous portions combined. Following acidification, 2 was extracted into ethyl acetate with three 50 ml portions. The combined ethyl acetate extracts were dried over sodium sulfate, reduced to an oil under vacuum and distilled at 205°C to yield 4.96 g, 36 mmol (49.2% yield) of 2. Spectroscopic data for 2: IR (film) 3500-3400, 3050, 2250, 2223, 2125, 2160, 1990, 1590, 1485, 1455, 1360, 1255, 1225, 1095, 1025, 980, 730 cm^{-1}. Mass spectrum m/e (% of base peak) M+127 (80), 109 (100), 81 (77), 54 (29), 53 (17), 52 (15).

4-Hydroxy-3-methoxy-d_3-mandelic Acid (3). To 1.27 g, 10 mmol of 2 in 50 ml water was added 0.92 g, 10 mmol of 80% glyoxalic acid and 10 mg of ascorbic acid. The pH was adjusted to 9.5 with base, the vessel shielded from light, and stirred at 70°C for three hr. The pH was brought to 2 with acid and the reaction mixture extracted with three 50 ml portions of ethyl acetate, dried over sodium sulfate and reduced to dryness. White crystals (0.66 g, 33%) were obtained from diethylether in the first crop, and an additional 0.15 g of product could be obtained from the supernatant. Mp 127-129°C [lit. (18) (undeuterated) mp 133°C] IR (KBr): 3300-2800, 2200, 1700, 1590, 1480, 1420, 1200 (multiplet), 1035, 840, 805, 670. Mass spectrum-Figure 1.

4-Hydroxy-3-methoxy-d_3-phenylacetic Acid (Homovanillic-d_3 Acid,4). Compound 3 (0.6 g, 3 mmol) was esterified with 3% hydrochloric acid in ethanol (10 ml, anhydrous) at 25°C, for 30 min. The product was dried *in vacuo*, acetylated in 10 ml pyridine-acetic anhydride (1:1) at 25°C for 2 hr, and concentrated to dryness. The product was transferred to a 250 ml round bottom flask to which was added 50 ml water, 0.3 ml 10N NaOH, and 1.36 g $NaBH_4$ (36 mmol), and the contents heated with stirring to reflux for 1 hr. The mixture was cooled;

acidified with 6N HCl to pH 2; extracted 3 times with equal volumes of ethyl acetate; and the extract dried over Na_2SO_4. The crude product was dissolved in water; the pH was adjusted to 6.7; and applied to a 20 mm x 250 mm column packed with Sephadex DEAE A-25-120. Rinsing with water eluted a neutral fraction (44 mg) identified by mass spectrometry as 2-(4-hydroxy-3-methoxyphenyl) ethanol. Elution with 0.2N HCl produced 4 which was extracted with ethyl acetate, concentrated and crystalized from 1,2-dichloroethane in 54% yield. Mp 144°C [lit (19) (undeuterated) 143°C] Mass spectrum (trimethylsilyl derivative) m/e (% base peak) M+ 329 (35), 314 (35), 296 (18), 267 (40), 212 (65), 179 (45), 75 (90), 73 (100).

4-Hydroxy-3-methoxy-d_3-phenylethylene glycol (5). Freshly distilled tetrahydrofuran (THF, 100 ml) was added to 3 (2.0 g, 10 mmol) in an ice cooled 3-neck round bottom flask fitted with an addition funnel containing 100 ml BH_3-THF (100 mmol BH_3), an N_2 inlet, and mercury bubbler outlet. Addition of reagent proceeded with magnetic stirring over 1 hr, after which time the solution was allowed to warm to room temperature. Aliquots were trimethylsilylated and assayed by gas chromatography over the next 40 hr. The mixture was cooled in an ice bath and quenched by the dropwise addition of methanol (200 ml). The product was dried on a rotary evaporator with repeated additions of methanol. The residue was dissolved in dilute ascorbic acid solution. The pH was adjusted to 4 with 6N HCl and the product was extracted into ethyl acetate (4 times, total 250 ml), dried over Na_2SO_4. It was reduced to dryness and chromatographed on 20 g silica gel column with ethyl acetate. Pure 5 eluted between 120-180 ml ethyl acetate (20 ml fractions assayed by TLC) to produce 490 mg colorless oil. The product was dissolved in ethanol and crystalized from benzene-hexane as the piperazine salt. Yield 0.484 g (21% from 3). Mp 117-117.5°C [lit (8)

(undeuterated) 116-118°C]. Mass spectrum (trimethylsilyl derivative) m/e (% base peak) M+403 (.5), 388 (2), 300 (100), 226 (25), 212 (15), 194 (30), 179 (21), 147 (85), 103 (25), 73 (92).

3-Hydroxy-4-methoxy-d_3-benzaldehyde (Isovanillin-d_3, 8a). In a 250 ml round bottom flask fitted with a reflux condenser was placed 100 ml acetone, 3,4-dihydroxybenzaldehyde (2.76 g, 20 mmol), powdered potassium carbonate (2.76 g, 20 mmol) and methyl-d_3 iodide (2.9 g, 20 mmol). The mixture was refluxed for six hours, cooled, filtered, and reduced to dryness. GLC of crude product showed 97% 8a and 3% 7a from which 1.25 g (40% yield) of 8a was crystalized (methanol) containing less 0.1% 7a. Mp 110-11°C [lit (20) (undeuterated) 116-117°C].

4-Benzyloxy-3-hydroxybenzaldehyde (8b). Benzyl bromide (23.6 ml, 200 mmol) was added to 27.6 g, 200 mmol of 6 and 27.6 g, 200 mmol of powdered potassium carbonate in a 500 ml flask with 250 ml acetone and stirred at room temperature for 64 hr. The mixture was filtered, concentrated and redissolved in 200 ml ethyl acetate. GLC of crude product indicated a ratio of 8b:7b of 100:3. The mixture was extracted 5 times with 100 ml 1% sodium hydroxide to leave dibenzylated 4 in the ethyl acetate layer. The sodium hydroxide extracts were acidified with HCl and extracted back into ethyl acetate, charcoaled, evaporated to dryness and crystalized from methanol in several crops to yield 13.75 g (30% yield). Complete separation of 7b from 8b can be effected by washing an ethyl acetate solution of the mixture with a pH 9.9 phosphate buffer to extract the more acidic 7b. Mp 120-122°C [lit (21) (undeuterated) 122°C]. IR (KBr) 3100, 1660, 1590, 1560, 1495, 1440, 1375, 1330, 1270, 1100, 1000, 800, 775, 725 cm^{-1}. Mass spectrum (trimethylsilyl derivative) m/e (% base peak) M+300 (25), 285 (10), 257 (10), 195 (10), 194 (25), 193 (35), 179 (20), 150 (15), 148 (18), 91 (100), 73 (35).

4-Hydroxy-3-methoxy-d_3-benzaldehyde (Vanillin-d_3, 7a). Methylation of 8b (10.4 g, 45 mmol) with methyl-d_3 iodide was performed as above for 8a, and the crude product (9.79 g) hydrolyzed in 130 ml 3% hydrochloric acid in glacial acetic acid by refluxing for 16 hr. The mixture was diluted with 600 ml water and extracted four times with 400 ml ethyl acetate, dried with sodium sulfate and evaporated. Treatment with charcoal and crystallization yielded 5.62 g, 35 mmol (78% based on 8b) of 7a. Mp 75-77°C [lit (22) (undeuterated) 81-83°C] IR (KBr) 3150, 2900, 2200, 2050, 1640, 1565, 1485, 1415, 1275, 1245, 1228, 1135, 1095, 1075, 830, 690 cm^{-1}.

3-Hydroxy-4-methoxy-d_3-mandelic Acid (9). The procedure of Shaw et al. (5) was applied to deuterated isovanillin 8a. A solution of 8.8 ml containing 55 mmol KCN was added dropwise over 30 min to a solution of 2.12 g, 14 mmol of 8a in 17 ml containing 55 mmol bisulfite with continuous stirring. The temperature was maintained at 0°C with an ice salt bath, and the reaction continued for an additional 90 min. The mixture was extracted three times with 50 ml of diethyl ether, and the combined extracts backwashed with 4 N bisulfite solution to remove starting material. The organic extract was dried over sodium sulfate and concentrated to yield 2.0 g, 11 mmol of crude nitrile, which appeared free of contamination as determined by gc-ms of the trimethylsilyl derivative. The nitrile was suspended in diethyl ether and a 1.1 molar excess of 100 mM anhydrous ethanol/HCl added and let stand in a refrigerator for 45 hr. The imino ester was taken to dryness and stirred for four hr with 100 ml H_2O to produce 1.3 g, 5.7 mmol of ethyl ester of 9. An aliquot (10 mg) of the ethyl ester of 9 was hydrolyzed in 1 ml of 3N HCl at 75°C in a Teflon lined screw cap tube and extracted to produce 9. Mass spectrum in Figure 1. In a similar fashion, isovanillin (6 g, 40 mmol) was reacted to yield 1.89 g, 8.4 mmol (21% yield) of undeuterated 9. Mp 118-120 (lit (16) 122.5-123.5).

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