DEUTERIUM EXCHANGE LABELLING OF BIOLOGICALLY IMPORTANT PHENOLS, INDOLES AND STEROIDS.

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SUMMARY

Deuterated analogues of various phenolic steroids, catechols and indole derivatives were prepared in high chemical yield by heating the relevant compound in D_2O at 190°C for 24 hours.

Keywords: Deuterated steroids, deuterated catechols, deuterated indoles, isotopic labelling, deuterium labelling.

INTRODUCTION

Combined gas chromatography/mass spectrometry (GC/MS) is a highly specific and sensitive method for the determination of many biological compounds. Optimum use of GC/MS as an analytical tool requires the use of stable isotope labelled analogues of the compounds of interest. However, labelled analogues are often not commercially available and their custom synthesis may be a laborious and expensive exercise.

The preparation and use of deuterium labelled steroids (1-7), catechol derivatives (8), indoles (9) and other biologically relevant compounds (10) have recently been described, however most of these techniques involve either complex synthetic methods followed by rigorous purification or are catalytic methods involving the use of expensive catalysts and/or large quantities of deuterated solvents.

This paper reports that deuterated analogues of many thermally stable phenolic or keto steroids, catechols, indoles and their metabolites may be prepared by simply heating the

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compound for a day with D_2O. The more complicated catalytic and synthetic procedures often adopted are not necessary.

EXPERIMENTAL

Cautionary Note: The heating of sealed glass vessels containing volatile liquids involves a considerable risk of explosion. We have found that with carefully constructed and annealed pyrex glass reaction vessels, used in the manner described, the risk of explosion at 190°C is very low. The tubes should be placed in a metal box if it is necessary to move them while they are at an elevated temperature. The handling and opening of sealed glass reaction tubes which have been heated should be done with heavy leather gloves and an impact resistant face shield since it is possible that catalytic decomposition of the water with consequent explosion risk may have occurred at the elevated temperature, although we have not observed this phenomenon. The reactions which were carried out at temperatures in excess of 190°C were undertaken inside a certified steel pressure vessel.

Steroids were obtained from Steraloids, Makor and Sigma; catecholamines and indoles from Sigma; deuterated water from the Australian Institute of Nuclear Sciences and Engineering; trifluoroacetic anhydride from Sigma and 2,2,2-trifluoroethanol from Merck. The reaction tubes used for the labelling experiments were constructed from pyrex glass tubing (10mm OD, 8mm ID) and had a heavy walled constriction approximately 8cm from the bottom.

In a typical labelling experiment, 10mg of the compound of interest was placed in the bottom of the reaction tube and 0.5ml of D_2O added. The bottom of the reaction tube (containing D_2O) was then chilled in liquid nitrogen and the top of the tube connected to a vacuum line. The tube was evacuated to a

pressure of < 0.5 torr and the heavy walled constriction of the tube heated with a gas torch so that it collapsed, sealing the lower part of the tube which was then drawn off (by further heating) from the top section. The sealed tube was heated to approximately 190°C for 24 hours. The tube was then broken open and placed in a vacuum desiccator overnight after which the reaction product could be dissolved in a suitable solvent and was ready for analysis.

The deuterium content of the compounds was determined after conversion to the trifluoroacetyl derivatives, in the case of compounds having only amine or phenolic hydroxyl substituents, and the 2,2,2-trifluoroethyl esters of the trifluoroacyl derivatives in the case of compounds which also had a carboxylic acid group. The chemical recovery of each compound was estimated by spiking the deuterated product with a known amount of the starting material. Inspection of the GC/MS trace also enabled an estimate to be made of the nature and concentration of any impurities. The products were analysed with a Hewlett-Packard 5993A GC/MS data system utilising a glass GC column packed with 3% OV-101 on Chromosorb W (Applied Science Labs Inc.), helium carrier gas and a membrane separator at the GC/MS interface.

RESULTS AND DISCUSSION

All of the compounds studied were derivatised to increase their volatility before GC/MS analysis. The derivatisation process results in a loss of the labile hydrogen atoms attached to amine, hydroxyl and carboxylic acid groups. Thus the presence or absence of deuterium in these positions has not been considered in the presentation of the data for this communication.

The assignment of the position of labelling in the molecule

has been based on the mass spectral pattern exhibited by the molecule, together with its labelling pattern in other catalytic systems, and the behaviour of similar molecules in this system. NMR analysis was also used for some compounds.

Exchange into the aromatic ring using the method proposed requires the presence of a substituent or substituents (such as an hydroxyl group) directly attached to the ring, which is capable of strongly activating the ring towards electrophilic substitution. Thus compounds such as benzylamine and phenyl-ethylamine are not labelled to any detectable extent.

Table 1. Hydrogen atom exchange between D_2O and 17β-estradiol after heating for 24 hours[a]

Temperature (°C)	Relative abundance of deuterated analogues				Chemical Recovery (%)
	d_0	d_1	d_2	d_3	
103	100	< 1	--	--	>95
128	100	< 3	--	--	>95
150	100	9	--	--	>90
154	100	16	19	<5	>90
190	< 0.5	< 5	100	<4	>90
250	< 0.5	< 0.5	100	<1	>90
290	< 0.5	< 0.5	100	38	>90
190[b]	< 4	< 4	100	<5	20

a - all reaction tubes had air evacuated before sealing, except as noted.

b - not evacuated before sealing.

The results of an examination of the effects of various reaction temperatures upon the rate of exchange of hydrogen atoms between D_2O and 17β-estradiol is displayed in Table 1. Clearly no exchange was observed at temperatures less than 150°C whereas by 190°C the molecule had been deuterated in positions 2 and 4. An increase in reaction temperature to 290°C resulted in further (but incomplete) deuteration of the

molecule. If the reaction tube was sealed without evacuating the air, and then heated to 190°C, the exchange still occurred but there was extensive degradation of the molecule. Thus, all further studies were carried out in vacuo.

Table 2. Deuterium labelling of phenolic steroids after exchange with D_2O at 190°C.

Compound	Relative abundance of deuterated analogues						Chemical Recovery (%)	Position of Labelling
	d_0	d_1	d_2	d_3	d_4	d_5		
17β-estradiol	< 0.5	< 5	100	< 4	-	-	>90	2,4
17α-estradiol	< 1	< 1	100	< 2	-	-	>95	2,4
16α-estriol	< 5	< 1	100	< 1	-	-	>90	2,4
2-hydroxy-estradiol	< 5	< 5	100	< 2	-	-	>80	1,4
2-hydroxy-estriol	< 3	< 1	100	< 3	-	-	>90	1,4
estrone	< 2	< 2	< 2	< 2	100	< 5	>90	2,4,16,16
2-hydroxy-estrone	< 0.2	< 0.2	< 1	< 1	100	< 2	>90	1,4,16,16
4-hydroxy-estrone	< 1	< 1	< 1	< 2	100	< 2	>90	1,2,16,16
2-methoxy-estradiol	< 2	100	< 2	< 2	-	-	>95	4
estradiol-3-benzoate	-	-	-	-	-	-	< 5[a]	-

a = hydrolysed to estradiol

The results of the exchange between D_2O and various steroids at 190°C are displayed in Table 2. In each case deuterium labelling occurred in positions ortho or para to the phenolic hydroxyl group or adjacent to a carbonyl group. The compounds were recovered with high chemical yield and no by-products were detected. Attempts to prepare labelled 17β-estradiol-3-benzoate, by heating to 190°C, resulted in complete hydrolysis of the ester linkage, to yield 17β E2 (d_2), deuterated at positions 2 and 4 and recovered in high yield. No deuterium exchange was detected in a number of steroids

which contained neither a carbonyl group nor a phenolic "A" ring. It is particularly noteworthy that the catechol derivatives of the estrogens studied, while very sensitive to oxidative degradation were all very effectively labelled with high chemical recovery and no obvious by-product formation.

The mechanism of the exchange reaction at the aromatic ring probably involves mild electrophilic attack by D^+ from the D_2O upon the activated hydrogen atom situated ortho (or para) to the hydroxyl group, the reaction being facilitated by the very high temperature used. The exchange of hydrogen atoms on the carbon atom adjacent to a carbonyl group would result from keto-enol tautomerism.

Table 3. Deuterium labelling of catechol derivatives after exchange with D_2O at 190°C.

Compound	Relative abundance of deuterated analogues						Chemical recovery %	Position of labelling
	d_0	d_1	d_2	d_3	d_4	d_5		
Dopamine	< 0.1	0.4	7	100	-	-	>95	2,5,6
Vanillin	< 1	100	-	-	-	-	>95	5
3-methoxy-tryamine	< 5	100	-	-	-	-	>95	5
Dopa	< 1	< 1	< 1	8	100	-	>95	2,5,6,β
DOPAC[a]	< 0.1	< 0.1	< 1	7	46	100	>95	2,5,6,α,α
HVA[b]	< 0.1	< 0.1	< 0.1	< 0.5	5	100	>95	2,5,6,α,α

a - DOPAC - 3,4 dihydroxyphenylacetic acid
b - HVA - 3-methoxy-4-hydroxyphenylacetic acid

The results of applying the labelling procedure to a number of catechol derivatives are listed in Table 3. In each of the cases listed the compound was recovered in very high chemical yield and usually with undetectable quantities of the d_0 species remaining. Exchange occurred in the aromatic ring in positions ortho and para to phenolic hydroxyl groups and in side chain positions adjacent to carbonyl groups. This may result in a loss of sterospecificity in compounds such as dopa.

3-Methoxy-4-hydroxyphenylacetic acid (HVA) (d_5) was the only O-methylated catechol derivative to exchange more than one aromatic hydrogen atom and it would appear that the acyl substituent of this molecule activates the aromatic ring to enable exchange of all three available positions.

An attempt was made to apply the labelling method to a number of compounds containing an hydroxyl group attached to the α carbon of the side chain, e.g. metanephrine, normetanephrine and 3-methoxy-4-hydroxy-mandelic acid (VMA). However, in each case the side chain was oxidised with only a -CHO group remaining and thus the product was either vanillin (d_1) or 3,4-dihydroxybenzaldehyde (d_2).

Table 4. Deuterium labelling of indole derivatives after exchange with D_2O at 190°C.

Compound	Relative abundance of deuterated analogues						Chemical Recovery %	Position of Labelling
	d_0	d_1	d_2	d_3	d_4	d_5		
Indole	< 2	100	-	-	-	-	>90	3
Tryptamine	20	100	-	-	-	-	>95	2
Tryptophan	5	24	100	-	-	-	>95	2,β
5-hydroxy-tryptophan	< 1	< 1	< 5	50	100	-	>95	2,4,6,β
5-hydroxy-tryptamine (serotonin)	< 0.5	< 2	12	100	-	-	50	2,4,6
5-hydroxyindole acetic acid	< 0.5	< 1	3	11	46	100	>95	2,4,6,α,α

The extent of labelling achieved in the basic indole molecule and with a number of biologically important indole derivatives are listed in Table 4. Of the six compounds investigated five were recovered in high yield and with no by-products detected; however, only 50% of the 5-hydroxytryptamine (serotonin) was recovered and there were obvious by-products

detectable in the GC/MS analysis. The deuterium atom incorporated into indole (d_1) was shown by NMR analysis to be attached to carbon 3. 5-Hydroxyindole acetic acid was labelled in 5 positions. Clearly these would include the two positions ortho to the phenolic hydroxyl group and the two hydrogens attached to the α carbon of the side chain, with the fifth deuterium being attached to either the 2 or 7 position. Exchange at carbon 7 seems unlikely since it did not exchange in indole and because it typically exhibits a low reactivity in electrophilic substitution reactions (11). Thus the hydrogen attached to carbon 2, although not exchangeable in indole itself, is probably activated towards exchange by the presence of the side chain on carbon 3. All of the indole derivatives studied had a side chain attached to carbon 3 and it appears that they each underwent exchange of the hydrogen on carbon 2. The labelling observed in the indole derivatives follows the same rules of electrophilic substitution and keto-enol tautmerism which have governed the earlier results.

The use of reflux conditions with D_2O or D_2O/MeOD mixtures (usually acidified with DCl to increase the rate of exchange) has been used by many workers in the preparation of deuterium labelled compounds. However, the use of such conditions often requires the use of large volumes of deuterated solvent because of solubility problems and the difficulty of refluxing very small volumes. Furthermore, many catechol derivatives are particularly sensitive to oxidative degradation and low chemical yields may result from using a refluxing technique.

The method we have described in this communication provides an extremely simple and convenient, but effective, method for preparing in high chemical yield a large number of deuterated analogues of such compounds as phenolic steroids, keto steroids, catechol derivatives and indole derivatives. It

should also be applicable to many other compounds which have hydrogen atoms which are easily replaced in electrophilic substitution reactions. Since the technique involves simply heating the compound, in a small quantity of deuterated water at 190°C in a sealed glass tube from which the air has been evacuated, only a minimal amount of both equipment and deuterated solvents are required. Virtually no side reactions are evident and thus the need for post labelling purification is minimised.

Many of the compounds labelled using this technique have already been used in the development of novel assay systems (12,13) and the purity of the products makes them eminently suitable for administration to humans in clinical studies of turnover or metabolism.

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