

SYNTHESIS OF ^3H - AND ^{14}C -LABELLED DOISYNOLIC AND MARRIANOLIC ACIDS, AND STABILITY OF TRITIUM LABEL.

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SUMMARY

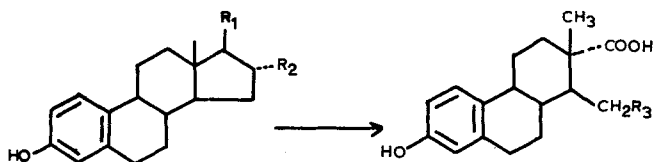
^3H - and ^{14}C -Labelled doisynolic acid was prepared from 6,7- ^3H -, 2,4,6,7- ^3H - and 4- ^{14}C -labelled oestradiol and oestrone by a regulated alkali fusion. Labelled marrianolic acid was similarly prepared from labelled oestriol. Unstability of regiospecific tritium labels in alkali was observed by use of double labelled compounds.

INTRODUCTION

Among approximately one thousand artificial oestrogens so far tested, ⁽¹⁾ doisynolic acid derivatives ⁽²⁾ (16 seco-oestrogen carboxylic acids) are the only type found to surpass oestradiol in oestrogenic activity evaluated either by the Allen-Doisy test or by uterine weight increase in rats. ^(1b) This type of oestrogen is also unique in that the mode of administration, subcutaneous or oral, or methylation of the phenolic group does not affect its oestrogenic activity. There is evidence that the major metabolism of oestradiol occurs at the 16 α ⁽³⁾ and 2 ⁽⁴⁾ positions. The possibility of ring cleavage at C16/17 in the metabolism of oestrogens has not been excluded. For studies of the metabolism of D-seco oestrogens and possible identification of the unknown polar metabolites of oestradiol, the synthesis of labelled compounds with sufficiently high specific activities was desired. Labelling of marrianolic

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acid derivatives with ^{14}C or ^3H at the acidic side chain has been reported.⁽⁵⁻⁷⁾ Our method of synthesis is a modification of the



I. $\text{R}_1 = \text{OH}$, $\text{R}_2 = \text{H}$; oestradiol

IV. $\text{R}_3 = \text{CH}_3$; doisyonic acid

II. $\text{R}_1 = \text{O}$, $\text{R}_2 = \text{H}$; oestrone

V. $\text{R}_3 = \text{COOH}$; marrianolic acid

III. $\text{R}_1 = \text{R}_2 = \text{OH}$; oestriol

classical method of potassium hydroxide fusion of oestrogens^(8,9) since the original procedures failed to give labelled products with specific activities high enough for metabolic studies. The loss of tritium at different positions in the oestrogens during alkali fusion was observed by mixing ^3H - and $4\text{-}^{14}\text{C}$ -labelled compounds and measuring the $^3\text{H}/^{14}\text{C}$ ratios of the purified products.

MATERIALS AND METHODS

Oestrone, oestradiol and oestriol were purchased from Searle Chemicals, Inc. Oestrone- $4\text{-}^{14}\text{C}$ (51.0mCi/mmole), oestradiol- $4\text{-}^{14}\text{C}$ (51.4mCi/mmole), oestriol- $4\text{-}^{14}\text{C}$ (53.0mCi/mmole) and oestradiol-2,4,6,7- ^3H (100Ci/mmole) were obtained from Amersham Searle, Inc. Oestradiol-6,7- ^3H (48Ci/mmole) and oestriol-6,7- ^3H (14.1Ci/mmole) were purchased from New England Nuclear Corp. Radioactivity was measured using a Packard Tri-Carb model 3375 as described previously⁽¹⁰⁾. The conversion to dpm was made using quenched standards.

Thin-layer chromatography (TLC).

The plates were made with silica gel GF, E. Merck, Germany (0.25mm thickness). The solvent systems used for purification and identification are as follows: for doisyonic acid, chloroform:acetone 80:20 (System I), R_f 0.75, and chloroform:isooctane:ethyl alcohol 80:20:6 (System II), R_f 0.31; for marrianolic acid, chloroform:acetone:formic acid 80:30:1 (System III), R_f 0.54, and chloroform:acetic acid 90:10 (System IV), R_f 0.56. Both the acids were visualized in UV light as well as developed by Pauly's diazotized reagent.

Preparation of non-radioactive marrianolic acid.

The material was prepared according to the method described by MacCorquodale *et al.*⁽⁸⁾ Oestriol (1g) was fused with potassium hydroxide (20g) and water (3ml) in a nickel crucible, heated in a metal-bath at 280-290°C for one hour with occasional stirring with a nickel spatula. The yellow colored melt was dissolved in water and filtered from precipitated nickel oxide. The filtrate was then saturated with carbon dioxide gas to precipitate out unreacted starting material as a colorless flocculant precipitate which was filtered off. The pale yellow filtrate was then acidified in the cold with 18N sulfuric acid to yield an almost colorless precipitate. It was filtered, washed thoroughly with cold water and dried in air. The crude material was dissolved in absolute alcohol and filtered. The yellow solution was treated with alcoholic potassium hydroxide solution; a sandy-colored potassium salt of the desired acid precipitated out. The precipitate was filtered, washed with alcohol and dried in air. It was dissolved in minimum quantity of water and the solution was acidified with 6N hydrochloric acid and an almost colorless precipitate was obtained. The precipitate was treated with Norite in aqueous acetone (1:1) and recrystallized at least twice, giving 300 mg of marrianolic acid, mp 202-205 C (lit.⁽⁸⁾ mp 206-208 C).

Preparation of non-radioactive doisyonic acid.

This was prepared from oestradiol and purified as described above. For final purification it was recrystallized from aqueous methanol to give 315 mg, mp 198-199 C (lit.⁽⁸⁾ mp 193-195 C).

Preparation of radioactive marrianolic and doisyonic acids.

Fusion experiment with oestrone-4-¹⁴C: Isolation of doisyonic acid-¹⁴C - Oestrone-4-¹⁴C (1μCi) was fused with potassium hydroxide (4g) in a nickel crucible for 3-4 min at 275-285 C. The melt was dissolved in water (30 ml), washed with ether (3 x 20 ml) to remove any neutral product, then saturated with carbon dioxide gas followed by extraction with ether (3 x 25 ml) to remove unreacted oestrone. The solution was then acidified with 18N sulfuric acid and extracted with ether (3 x 25 ml) to isolate the acidic material. The ethereal solution was washed with saturated sodium chloride solution and dried (Na₂SO₄). The residue, after evaporation of the ether, was then subjected to TLC (System I). The radioactive peak corresponding to doisyonic acid was eluted with methanol, and its radiochemical purity was

checked by TLC using systems I and II as well as by recrystallization to constant specific activity after addition of carrier (Table 2a). The other fusion experiments (Table 2b-g) were carried out essentially as described as above. The starting materials, TLC system used and the products of the fusion experiments are summarized in Table 1.

Table 1. Potassium hydroxide fusion experiments with labelled oestrogens.

Starting Material	TLC System	Product
(a) $\text{OE}_1\text{-4-}^{14}\text{C}$; $1\mu\text{Ci}$ ($0.187\mu\text{Ci}/\mu\text{g}$)	I and II	Doisyolic acid-4- ^{14}C
(b) $\text{OE}_2\text{-6,7-}^3\text{H}$; $20\mu\text{Ci}$ ($175\mu\text{Ci}/\mu\text{g}$)	I	Doisyolic acid-7- ^3H
(c) $\text{OE}_3\text{-4-}^{14}\text{C}$; $1\mu\text{Ci}$ ($0.183\mu\text{Ci}/\mu\text{g}$)	III	Marrianolic acid-4- ^{14}C
(d) $\text{OE}_3\text{-6,7-}^3\text{H}$; $500\mu\text{Ci}$ ($52.6\mu\text{Ci}/\mu\text{g}$)	III	Marrianolic acid-7- ^3H
(e) $\text{OE}_2\text{-6,7-}^3\text{H}$; $15\mu\text{Ci}$ + $\text{OE}_2\text{-4-}^{14}\text{C}$; $1\mu\text{Ci}$ ($2.77\mu\text{Ci}^3\text{H}$ and $0.18\mu\text{Ci}^{14}\text{C}/\mu\text{g}$)	I	Doisyolic acid-7- ^3H -4- ^{14}C
(f) $\text{OE}_3\text{-6,7-}^3\text{H}$; $72.7\mu\text{Ci}$ + $\text{OE}_3\text{-4-}^{14}\text{C}$; $2\mu\text{Ci}$ ($5.91\mu\text{Ci}^3\text{H}$ and $0.16\mu\text{Ci}^{14}\text{C}/\mu\text{g}$)	III	Marrianolic acid-7- ^3H -4- ^{14}C
(g) $\text{OE}_2\text{-2,4,6,7-}^3\text{H}$; $36\mu\text{Ci}$ + $\text{OE}_2\text{-4-}^{14}\text{C}$; $1\mu\text{Ci}$ ($6.64\mu\text{Ci}^3\text{H}$ and $0.18\mu\text{Ci}^{14}\text{C}/\mu\text{g}$)	I	Doisyolic acid-7- ^3H -4- ^{14}C

Recrystallization to constant specific activity.

To an aliquot of labelled product purified by TLC was added 20-50 mg of carrier and recrystallized several times (doisy~~no~~lic acid from aqueous methanol, marrianolic acid from aqueous acetone). One to 4 mg of dried crystals were counted by the liquid scintillation method⁽¹⁰⁾.

Table 2. Crystallization of doisy~~no~~lic and marrianolic acids obtained from labelled oestrogens

Product	No. of crystallization	Specific activity dpm/mg		
		³ H	¹⁴ C	³ H/ ¹⁴ C *
a. Doisy no lic acid- ¹⁴ C	TLC **		225	
	1		211	
	2		235	
	3		217	
	4		198	
b. Doisy no lic acid- ³ H	TLC	1040		
	1	1000		
	2	1030		
	3	1020		
c. Marrianolic acid- ¹⁴ C	TLC		750	
	1		760	
	2		640	
	3		750	
d. Marrianolic acid- ³ H	TLC	3040		
	1	3000		
	2	2940		
	3	3130		
e. Doisy no lic acid- ³ H- ¹⁴ C	TLC	346	60	5.8
	1	332	36	9.2
	2	358	49	7.3
	3	359	50	7.2
f. Marrianolic acid- ³ H- ¹⁴ C	TLC	12500	825	15.2
	1	14500	870	16.7
	2	15200	920	16.5
	3	14900	900	16.5
g. Doisy no lic acid- ³ H- ¹⁴ C	TLC	2500	250	10.0
	1	2400	250	9.6
	2	2390	250	9.6
	3	2530	280	9.0

* Initial ratios of starting materials were 15 for e, 36 for f, and 35 for g.

** Calculated as radioactivity in an aliquot of TLC eluant used/weight of authentic acid added to it.

RESULTS AND DISCUSSIONS.

The results of identification of the labelled products by recrystallization to constant specific activity are in Table 2a-f. The yields of the syntheses were rather poor, varying from 0.1 to 2.0 percent. The previously reported method for the preparation of doisynolic⁽⁸⁾ and marrianolic acid⁽⁹⁾ by fusion with alkali at high temperature is a drastic treatment. It is applicable for the preparation of non-radioactive acids initiated from large amounts of starting materials. The identical procedure, adopted in the case of radioactive material, where the mass of the material is negligible, produced no desired product; almost all the tritium being exchanged to form radioactive water in the case of ^3H -oestradiol. In the case of ^{14}C -oestriol, a radioactive peak corresponding to marrianolic acid was obtained along with several adjoining peaks from which pure product could not be isolated. The best condition for the preparation of the D-seco acids was found to be fusion with alkali for a very short period of time, usually 3-4 min; then the by-products were less and the desired product could be sufficiently purified by repeated TLC.

From the reaction mixture, negligible amounts of neutral and phenolic product (starting material) were obtained. The bulk of the radioactivity (about 50%) remained in the aqueous phase which could not be extracted with organic solvent. Doisynolic and marrianolic acids were isolated and purified from the acidic fraction by repeated TLC. In the preparation of doisynolic acid, 2-3 sharp radioactive peaks were obtained during TLC, one of which corresponded to doisynolic acid. In the preparation of marrianolic acid, three radioactive peaks were always obtained by TLC, one of which corresponded to marrianolic acid.

Fusion experiments with double labelled oestrogens indicated a regio-specificity of stability of the tritium labels. The $^3\text{H}/^{14}\text{C}$ of the purified doisynolic and marrianolic acids were one-half that of the starting 6,7- ^3H -4- ^{14}C -oestrogens (Table 2e and 2f). The $^3\text{H}/^{14}\text{C}$ of doisynolic acid isolated from 2,4,6,7- ^3H -4- ^{14}C -oestradiol fusion became one-quarter that of the starting ratio (Table 2g). These results are consistent with a hypothesis that the tritium at the aromatic and benzylic positions are exchanged and only the homobenzylic tritium at the 7-position remained.

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2. Abbreviations used are: Marrianolic acid, 7-hydroxy-2-carboxy-1,2,3,4,4a,9,10,10a-octahydro-2-methylphenanthrene acetic acid; doisyolic acid, 7-hydroxy-1,2,3,4,4a,9,10,10a-octahydro-2-methyl-1-ethylphenanthrene 2-carboxylic acid; oestradiol, OE₂, 1,3,5(10)-oestratriene-3-17 β -diol; oestriol, OE₃, 1,3,5(10)-oestratriene-3,16 α ,17 β -triol; oestrone, OE₁, 17 β -hydroxy-1,3,5(10)-oestratrien-17-one.
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