SYNTHESIS OF 2,7-BIS [2-(DIETHYLAMINO) ETHOXY] FLUOREN-9 ^{14}C-ONE. 2HCL; (TILORONE-9^{14}C HYDROCHLORIDE*; BIS-DEAE-FLUORENONE-9^{14}C).

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SUMMARY

Tilorone hydrochloride labelled with carbon-14 in position 9 was synthesized in 8 steps on 0.5 millimolar scale. Starting from biphenyl-2-carboxylic-^{14}C-acid (I), fluorenone-9^{14}C (II) and fluoren-9^{14}C (III) were prepared by established radiochemical procedures [1,2,3]. Sulfonation of III gave fluoren-9^{14}C-2,7-disulfonic acid (IV), which was converted to its potassium salt and oxidized with $KMnO_4$ to obtain 9-oxo-9^{14}C-disulfonic acid dipotassium salt (V) in yields of 60% and 80% respectively. This was fused with NaOH at 325°C to get 4,4'-dihydroxy-biphenyl-2-carboxylic-^{14}C-acid (IV) in 67% yield. VI on dehydration with $ZnCl_2$ gave 2,7-dihydroxy-9-oxo-fluoren-9^{14}C (VII) with 82% yield.

Condensation of VII with 2-diethylaminoethyl chloride in presence of KOH resulted in the synthesis of the title compound in overall yield of 15% based on the amount of I used.

Characterisation data to establish the chemical and radiochemical purity are included. The labelled preparation showed the same biological activity [16] as an authentic specimen [23] of the inactive compound.

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*Tilorone hydrochloride is the nonproprietary name of the title compound adopted in the United States.

INTRODUCTION

Tilorone hydrochloride, the water soluble dihydrochloride salt of 2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one, is a broad-spectrum antiviral-agent [6], effective in vivo against viruses of both RNA and DNA groups [7].It is,in addition, the first recognized synthetic small molecular weight substance that is an orally active inducer of interferon [8,9].

Anti-tumour activity of tilorone hydrochloride against Walker carcinosarcoma 256, the reticulum cell sarcoma A-RCS [10], Ehrlich carcinoma solid tumour, and Friend Leukemia Virus [11] has been reported.

According to Munson et al [11] tilorone hydrochloride showed antibacterial activity in vivo against S.aureus,enhanced immunologic response to sheep red blood cells as measured by Jerne plaque technique and produced marked stimulation of phagocytic activity of the reticuloendothelial system as measured by the vascular clearance of colloidal carbon and phagocytic uptake of ^{51}Cr sheep erythrocytes into the major reticuloendothelial organs.

Rohovsky et al [12] have reported that tilorone hydrochloride, on oral administration, caused vaculation and granuolation of peripheral leukocytes in the mouse, rat, dog and monkey accompanied by the accumulation of a basophilic material in the Kupffer cells of the liver and macrophages of the spleen and lymph nodes in the mouse, rat, and dog.

Even though the effectiveness of tilorone hydrochloride as an antiviral agent in man is still controversial [13,14,15], the knowledge of its mode of action in mice [16] is of fundamental importance in the search of similar compounds suitable for application to human virus therapy and prophylaxis.

All attempts made uptodate to investigate the mode of action of tilorone hydrochloride [13-20], carried out with unlabelled compound, appear to suggest [17] that tilorone hydrochloride must be converted in vivo to some more active molecular species before it may be effective as inducer of interferon in cell cultures in vitro.

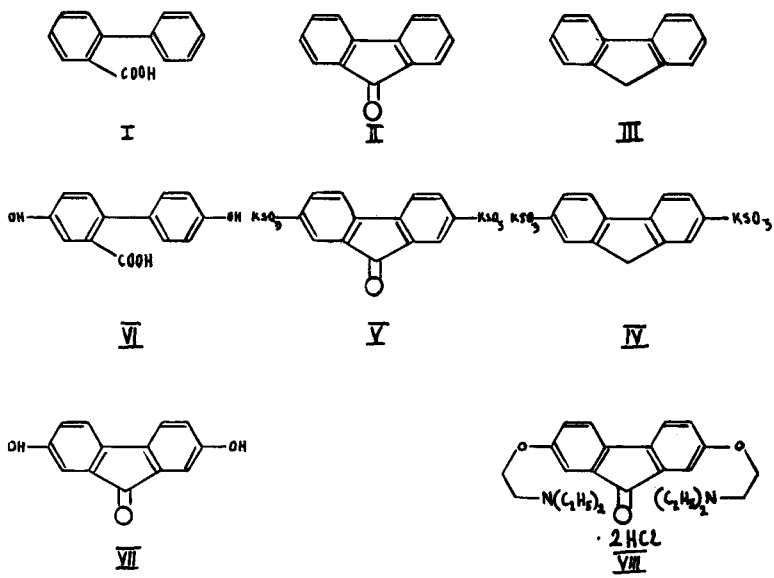
To investigate some of these molecular aspects of mechanism of antiviral activity of tilorone hydrochloride, synthesis of carbon-14 labelled preparation was undertaken.

EXPERIMENTAL PROCEDURES

The sequence of reactions used to synthesize tilorone hydrochloride are outlined in Figure 1.

Synthesis of fluoren-9^{14}C (III) has been reported [1,2,3]. Reaction rout to obtain 2,7-dihydroxy-9-oxo-fluoren (VII) from fluoren (III) has been described by courtot in 193o for the cold synthesis [4]. These procedures had to be considerably modified by us to meet our requirements of higher yields at micropreparative scale. Condensation of VII with 2-chlor-triethylamin to obtain tilorone were initially conducted by us with sodium ethylate in absolute ethanol by heating under reflux for 24 hours. But since in subsequent experiments the condensation carried out in toluene [5] in presence of potassium hydroxide proved definitely superior in yields the procedures were accordingly modified.

Fig.1: Sequence of reactions for the synthesis of tilorone-9^{14}C.HCl



Fluoren-9^{14}C-one (II)[1].

99 mg (0.5 mM,100 µCi) of biphenyl-2-carboxylic-^{14}C-acid (New England Nuclear,Boston,Mass.) were mixed with 330 µl of a solution of 2.2 ml of concentrated sulfuric acid and 0.4 ml of water. The mixture was heated at 85°C for 10 minutes, then poured into a mixture of ice and water, and the crude product was filtered off. Yield 87.2 mg, 96%.

Fluoren-9^{14}C (III)[2,3].

A mixture of 90 mg of II, 65 mg of potassium hydroxide, 1 ml of diethylenglycol and 0.2 ml of 85% hydrazine hydrat was refluxed for about 3 hours. The nearly colorless solution was cooled and poured into water and the product was filtered off. Yield 73.97 mg, 90%.

2,7-disulfonic acid dipotassium salt of fluoren-9^{14}C (IV).

In a standard-joint microflask of 1 ml vol., 73 mg of IV were mixed with 150 µl of conc. sulfuric acid. The flask was attached to a rotary evaporator and immersed in a water bath preheated to 85°C. Under suction with a water-aspirator, the flask was rotated in the water-bath for about 7 minutes, then allowed to cool to room temperature (The reaction mixture solidified into a grey-white hard mass), treated with ice to a vol. of about 0.8 ml and the solution filtered off. 200 mg of KCl were added and the mixture heated on a steam bath for 30 minutes. After letting the mixture stand overnight in an ice-box, the precipitated material was collected on a glass-sintered filter plate and resuspended in 3 ml of water. The suspension was heated on a steam-bath, made alkaline with potassium carbonate and filtered. By adding equal vol. of saturated potassium chloride solution the salt IV was allowed to precipitate in ice-box and the product filtered off. The air-dried product thus obtained weighed 139 mg and proved to be only 73% in purity when analyzed with the help of UV-spectroscopy (Extinction of 20 µg/ml of pure IV in water at 279 nm = 1.54 in 1 cm thick cuvett). The product was recrystallized by dissolving in 1 ml of 20% KCl solution and letting it stand at 5°C overnight.94.7 mg of IV with a purity of above 95% were obtained. This represented a yield of 60 %.

2,7-disulfonic acid dipotassium salt of fluoren-9^{14}C-one (V).

94.7 mg of IV were dissolved in 2 ml of water by heating. A solution of 50 mg of potassium permanganate in 1 ml of water was added in such a manner that the temperature of the mixture remained between 22 and 25°C. The mixture was let stand overnight at r.t. and filtered. The filter cake of MnO_2 was washed with hot water, the filtrate and the wash-water were combined and evaporated to a vol. of 1 ml, and the concentrated solution let stand at 5°C for several hours. The precipitated product was filtered off and air-dried. 88.7 mg of V with a purity of about 85% were obtained. Based on the content of pure substance this represents a yield of 80%. The product so obtained was dried in vacuum and used for the next step without further purification.

4,4'-dihydroxybiphenyl-2-carboxylic-^{14}C-acid (VI)

88.7 mg of crude vI were added in small portions to 200 mg of molten NaOH preheated to a temperature of 300°C. The temperature was slowly raised to 325°C. The fusion mixture was constantly stirred with a nickle spatula to ensure thorough fusion and to controll the foamimg. After about 7 minutes, the mixture was taken out of the metal-bath and let cool to room temperature. The cooled mixture was dissolved in 1.2 ml of water and at 0°C made strongly acidic with 0.4 ml of conc. HCl. The precipitated product was let stand, filtered, washed with little cold water and thoroughly dried in vacuum exicator. The crude product, 31.8 mg, which proved to be about 90% in purity was used for the next step without further purification. Yield of Vi was 67.5%.

2,7-dihydroxy-9^{14}C-9-fluorenone (VII)

In an aluminium oxide crucible, 31.8 mg of VI were added in bulk to about 70 mg of anhydrous ZnCl preheated to a temperature of 120°C With the help of a glass rod the components were thoroughly mixed and the temperature slowly raised to 225°C and held at this temp. for about an hour. During this period the fused mass was constantly scratched with the glass rod and homogenized to ensure quantitative reaction. The reaction mixture which after a brief spell of sofetening turns into a deep red dry mass is taken up in 10 ml of

water, heated shortly to boiling,let stand at room temperature, and the product filtered off. Yield 23.2 mg of VII with a purity of about 90% as judged by ultraviolet analysis.This represents a calculated yield of 80% based on the amount of pure content of VI used. The crude product was employed for the next condensation without further purification.

2,7-bis[2-(diethylamino)ethoxy]fluoren-9-^{14}C-one dihydrochloride (Tilorone-9-^{14}C hydrochloride (VIII) [5].

23.2 mg of crude VII were added to a solution of 47 mg of KOH in 120 µl of water. 72.2 mg of 2-diethylaminoethyl chloride hydrochloride with 210 µl of toluene were also mixed. The mixture was refluxed overnight, under stirring, over a sand-bath with a temp. of 120°C. The next morning, the hot mixture was brought to room temp. by the addition of 0.2 ml of ice-water. The toluene-phase containing the major portion of the desired product was separated. Water phase was extracted with 2 ml portions of toluene as long as the toluene extract showed significant amount of extractable radioactivity. The toluene-phases were all combined,evaporated to a vol. of 2 ml. and washed twice with 2 ml portions of saturated NaCl solution, dried with anhydrous $MgSO_4$, filtered and evaporated to dryness with mild heat. The residual oil (tilorone-9-^{14}C free base) was dried at 60°C under vacuum for 30 minutes, dissolved in 0.2 ml of absolute methanol and ethereal hydrogen chloride added untill the solution was strongly acidic. The hydrochloride salt of tilorone separated out as a voluminous precipitate of orange color, or as a thick yellowish-brown syrup. Dry ether was added and the colorless ethereal supernatent was decanted off. The process was repeated 3 or 4 times to ensure complete removal of any excess amount of ethereal hydrogen chloride. The crude product was dissolved in 0.5-1.0 ml of isopropanol and minimum amount of absolute methanol, and poured in a beaker containing absolute ether. The hydrochloride salt of tilorone separated out as an orange-colored flocculent precipitate. It was collected by filtration,washed with anhydrous ether and dried overnight in a vacuum oven at 70°C. 38.1 mg of VIII, m.p. 218-220°C were obtained.Based on the amount of VII used this represented a yield of about 80%. The product was further purified and characterized as follows.

PURIFICATION AND CHARACTERIZATION OF TILORONE-9^{14}C HYDROCHLORIDE.

The crude tilorone-9^{14}C hydrochloride was characterized to determine its chemical and radiochemical purity. The chemical purity as indicated by infrared and ultraviolet analysis (Fig.2) appeared to be indistinguishable from an authentic reference standard, although the melting point was 6-8 centigrade degrees lower.

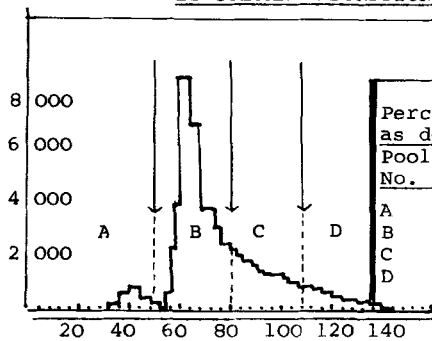
Radiochemically, the product behaved the same as authentic tilorone hydrochloride in six thin layer chromatography (TLC) systems (Appendix-systems A,B,C,D,E, and F), in paper electrophoresis (PE), and in paper chromatography (PC) in systems C and E. However, the radiochemical purity was estimated at only 93%. In high-voltage PE a radiochemical impurity amounting to approximately 1.5% of the amount of sample applied was detected with an electrophoretic mobility almost twice that of the major component. Two additional components containing 1.6% and 2.3% of the total radioactivity and with electrophoretic mobilities lesser than that of tilorone hydrochloride were also detected.

Initial attempts to purify the preparation by recrystallization failed to remove the major impurities. However, the product was purified as follows: 30 g of aluminium oxide (acidic,activity I, Merck A.G,Darmstadt) was added slowly to a 50 cm 1 cm column equipped with a sintered glass base and a stop-cock and containing 22 ml of isopropanol as developing solvent. The adsorbent was allowed to settle overnight. Excess solvent drained from the column amounted to 1.5 ml. leaving a hold-up vol. of 20.5 ml. 38 mg of crude tilorone-9^{14}C hydrochloride dissolved in minimum amount of iso-propanol, or a mixture of methanol and isopropanol, was placed on the column and developed with isopropanol. Each of the column eluate fractions was analysed for carbon-14. The results, illustrated in a radiochromatogramm in Fig.2, show that the minor component in pool A containing 1.5% of the total radioactivity appeared almost immidiately after the collection of the hold-up vol. (elution Vol.30-45ml) and was followed closely by the major carbon-14 peak.

The eluate fractions of the major carbon-14 peak were pooled together separately in parts B, C, and D, the solvent evaporated, the residue taken up in 1 ml dry ether, treated with equal vol. of ethe-real hydrochloric acid, and the orange-colored precipitate filtered

off, washed well with dry ether and dried in vacuum oven at 70°C. 0.57 mg, 19.0 mg, 7.6 mg and 2.5 mg dry substance from pools A,B,C and D were obtained resp. These fractions were analysed for carbon^{14} yield and for radiochemical purity by TLC, PC and PE. The results summarized in a table in Fig.2 indicated that pool B contained tilorone-9^{14}C hydrochloride with radiochemical purity greater than 99%. Characterization data of this fraction are summarized in table 1. Attempts to separate pure tilorone hydrochloride from less pure fractions C and D are being continued.

Fig.2: RADIOCHROMATOGRAMM OF PURIFICATION OF TILORONE-9^{14}C.2 HCl BY COLUMN ADSORPTION CHROMATOGRAPHY



Percent Composition of pools as determined by TLC analysis

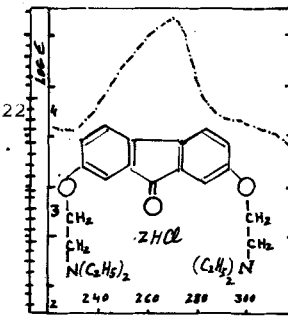
Pool No.	Percent of total CPM	Percent of Tilorone
A	1.5%	---
B	50.0%	99%
C	21.0%	90%
D	7.0%	70%

Table 2:SUMMARY OF CHARACTERIZATION OF TILORONE-9^{14}C HYDROCHLORIDE

	Parameter	
1.	Color	Orange
2.	Melting point	218-220°C.
3.	Mixed melting point	218-220°C.++
4.	UV-analysis.	H_2O λ_{max} 269 mμ, $E^{1\%}_{1cm}$=1622
5.	IR-analysis.	identical with refr.
6.	TLC-analysis.(Sys.C)	R_f: 0.68;Sys.D-0.46
7.	Paper chromatograph. and electrophoretic analysis.	identical with the authentic reference standard.PC/D:R_f.57

++ mixed with the inactive reference (1:1).

Ultraviolet spectrum of Tilorone hydrochloride.



APPENDIX: CHROMATOGRAPHIC METHODS

Tilorone-9^{14}C hydrochloride and the carbon-14 labelled intermediates VI and VII (Fig.I) were characterized to establish their chemical identity and radiochemical purity by using the following thin layer chromatography (TLC) systems shown in the table below. The compounds were chromatographed on thin layers of aluminium oxide (Merck, Art.5550/0025),cellulose (Carl Schleicher & Schüll, Al440) and silica gel (Schl.&Schüll) thin layers untill the solvent front was 15 cm from the start. The chromatogramms were scanned for carbon-14 by measuring CPM on each of the 1 cm sections cut along the chromatographic lane from the origin to the solvent front. Beckmann Model LS-100 liquid scintillation spectrometer was used to measure amounts of carbon-14 on each of the 1 cm sections using dioxan scintillator. Radio-chromatographic profiles were constructed and the radiochemical purity of the product was estimated.

THIN LAYER CHROMATOGRAPHIC SYSTEMS

System	Medium	Solvent composition
A	Al_2O_3-neutral	Chloroform; and Chloroform:Methanol (1:1)
B	Al_2O_3 ,,	Chloroform-Acetic acid-Acetone (5:1:1)
C	Al_2O_3 ,,	n-butanol-Acetic acid-water (7:1:2)
D	Cellulose	Solvent system C
E	Cellulose	n-butanol-water (86:14)
F	SILICA GEL	Solvent composition C

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