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TRITIUM LABELLING OF CHOLEST-4-EN-3-ONE BY CATALYTIC DEBROMINATION OF 2,2-6ß-TRIBROMO-CHOLEST-4-EN-3-ONE

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SUMMARY: The catalytic debromination of 2,2,6ß-tribromo-cholest-4-en-3-one with tritium gas (T_2) yields tritium-labelled cholest-4-en-3-one of high specific activity. The most favourable reaction parameters are reported. Using tritium-hydrogen (TH) gas mixtures the labelling reactions are accompanied by three kinetic isotope effects the importance of which is discussed.

KEY WORDS: Tritium labelling, steroids, catalytic debromination, high specific activity, kinetic isotope effects.

INTRODUCTION

The catalytic debromination of bromo derivatives of cholesterol with T_2 are used for preparing specifically labelled cholesterol in one step. With 2-bromocholesterols, [2α-^{3}H]- and [2ß-^{3}H]cholesterol with specific activities up to 18.5 Ci/mmol have been prepared [1]. The attempts to synthesize dibromocholesterol as precursor for higher speciic activity were unsuccessful.

Alternative other advantageous precursors for catalyzed debromination reactions are the numerous bromo derivatives of the cholest-4-en-3-one [2]. Labelled cholest-4-en-3one can be converted into labelled cholesterol [3] without difficulty using the procedure of Collins and Hobbs [4].

2,2,6ß-Tribromo-cholest-4-en-3-one (1) is especially suitable as a precursor both because of its simple synthesis and the fact that in crystallized form it is a compound with good stability on storage.

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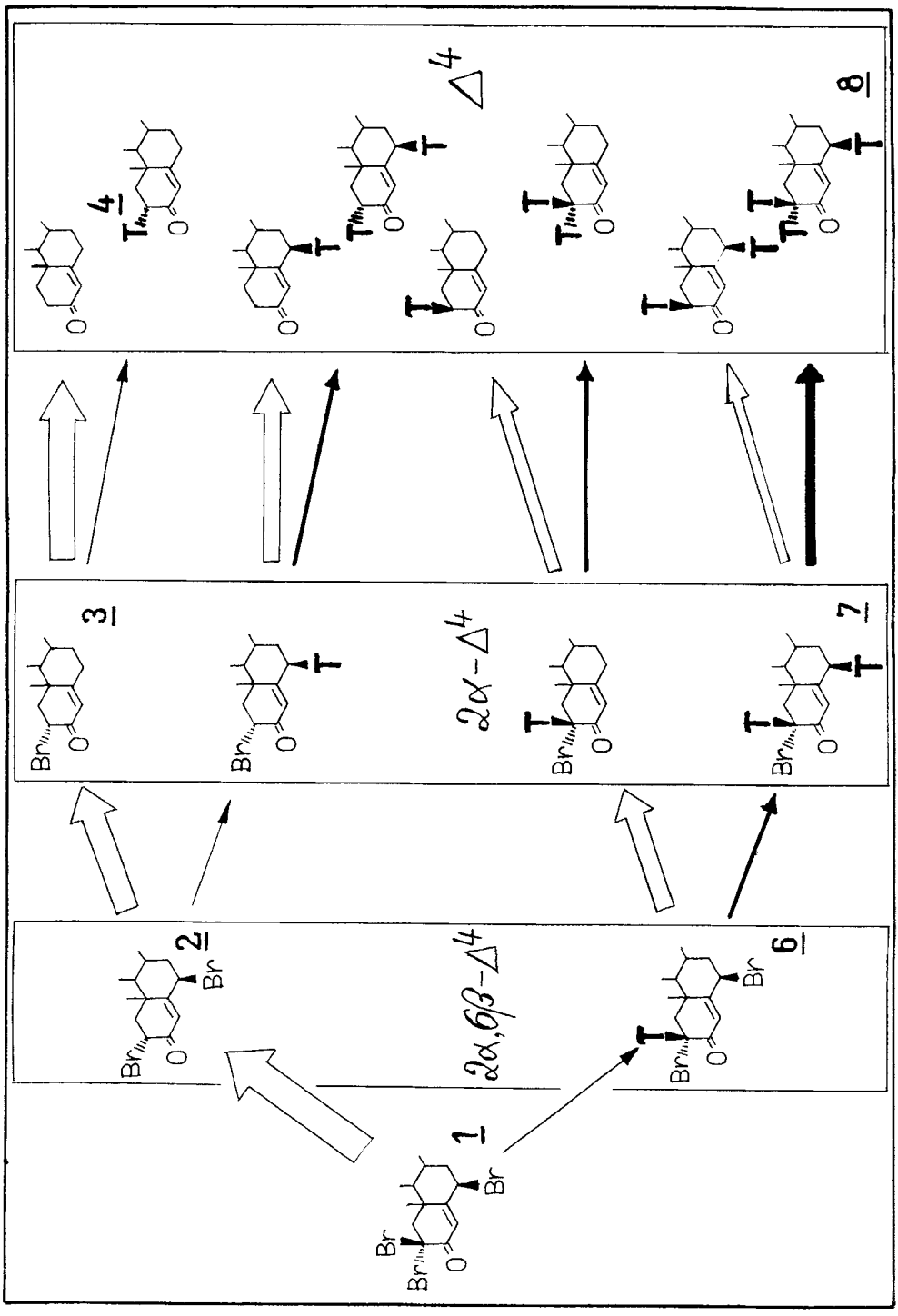
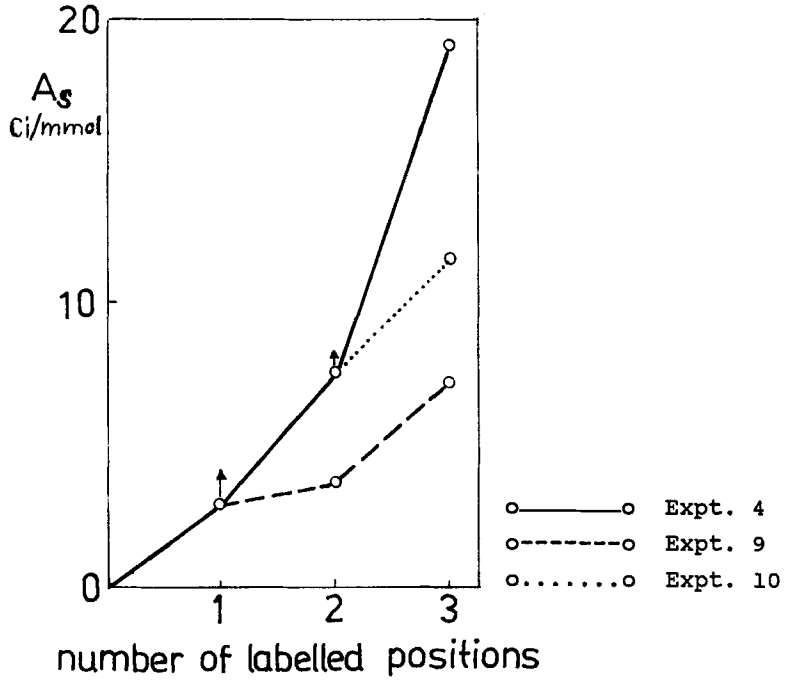
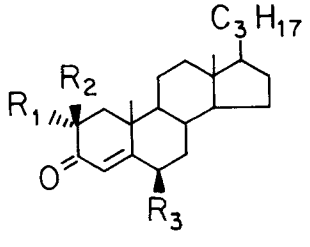


F I G U R E 1: Complex reaction course if 2,2,6ß-tribromo-cholest-4-en-3-one (1) is debrominated catalytically with diluted tritium gas (further explanations in text)



F I G U R E 2: Stepwise increase of specific activity of $2\alpha,6\beta$-Δ^4, 2α-Δ^4, and Δ^4 if diluted tritium gas is used for the catalyzed debromination of 2,2,6ß-tribromo-cholest-4-en-3-one (1)

Inactive experiments and the following ^{13}C-NMR spectroscopic characterization of the reaction products [5] showed that in presence of $Pd/CaCO_3$ the tribromide 1 can be conveniently debrominated via $2\alpha,6\beta$-dibromo-cholest-4-en-3-one (2) and 2α-bromo-cholest-4-en-3-one (3) to cholest-4-en-3-one (4). Reaction rate and turnover depend on the catalyst amount. The bromides 2, and 3 can be isolated as products. 6ß-Bromo-cholest-4-en-3-one (5) does not form in this reaction.



1 $R_1=R_2=R_3=Br$
2 $R_1=R_3=Br;R_2=H$
3 $R_1=Br;R_2=R_3=H$
4 $R_1=R_2=R_3=H$
5 $R_1=R_2=H;R_3=Br$
6 $R_1=R_3=Br;R_2=T$
7 $R_1=Br;R_2=R_3=T$
8 $R_1=R_2=R_3=T$

The favourable conditions of the inactive tests were then extended to experiments with T_2, in order to synthesize cholestenone with a high specific activity. The results, and a discussion of their significance are reported below.

RESULTS and DISCUSSION

Experiments showed that pure tritium gas is the most essential prerequisite for the attainment of cholestenone at high specific activity. Using TH mixtures, tritium labelled cholestenone with moderate specific activity was obtained, because H_2 appears to be strongly favoured against T_2 in all three labelling reactions, due supposedly to a large primary kinetic isotope effect (KIE).

Hydrogen (or deuterium) in small amounts is often present in commercially available tritium gas, and therefore it is impossible to exclude the primary KIE. Moreover, catalyzed dehalogenation reactions with Pd as a catalyst are accompanied by hydrogen isotope exchange reactions [6] which also dilute the tritium gas. Using catalyzed dehalogenation it seems, therefore, to be difficult to attain the maximum specific activity (29 Ci/mmol) per labelling position in practice.

When even small amounts of hydrogen are favoured by the primary KIE, then synthesizing pure [2,2,6ß-^{3}H]cholestenone (8) must be assumed to be impracticable. Additionally, a complex reaction course is presumed to take place, as illustrated in figure 1. If H_2 is present at the outset, or is formed by fast exchange reactions, a mixture of 2 and 6 ($2\alpha,6ß$-Δ^4) is produced in the first debromination step, followed by a mixture of 3 and 7, and two further mono-tritiated (2α-Δ^4) in the second step and, finally, there is a mixture consisting of 4 and 8 and three mono-tritiated and three double tritiated species (Δ^4) at the end of the catalytic debromination. In figure 1, bold hollow arrows are intended to express the preference of the inactive reactions.

The best possible conditions for synthesis of cholestenone at high specific activity are as follows:

1) a large excess of gaseous tritium G (= µmol gas per µval Br bound in 1)

2) a high substrate concentration c (= μval Br bound in 1 per ml solvent), and

3) a large amount of catalyst m (= mg 2%Pd/$CaCO_3$ per μval Br bound in 1).

These conditions minimise the influence of exchange reactions (large G), cause fast conversion (large c, large m) and avoid premature cessation of the reaction caused by catalyst poisoning (large m). the product c.m. represents the number of available reactive centres at the catalyst surface per ml solvent. The product G.c.m. is an expression for the reaction probability at these reactive centres.

Three further reaction parameters (reaction time, stirring and tritium gas-pressure at start of reaction) were kept constant. All the experiments were started at a gas-pressure between 600 and 650 torr and carried out 60 min at r.t. under vigorous stirring.

The results obtained at different values for G, c and m are summarized in Table 1. These results were attained in single experiments except for expts. 5 and 6 which had been intentionally carried out under comparable conditions.

Table 1: Results of the threefold catalytic debromination of 2,2,6ß-tribromo-cholest-4-en-3-one (1) with tritium

Expt.	G a)	c a)	m a)	c.m.	G.c.m.	%T_2	A_s [Ci/mmol]
1	7.31	19.3	1.83	35.3	258	77	16.0
2 b)	18.70	11.6	1.72	19.9	372	77	34.0
3	8.06	31.5	0.65	20.5	165	86	25.8
4 c)	5.60	72.4	0.69	50.0	280	86	19.1
5	14.50	16.1	2.07	33.3	482	85	52.1
6	13.90	16.6	2.07	33.3	463	85	60.0
7 d)	8.53	21.0	1.03	21.6	185	96	62.5
8 d)	8.84	18.3	1.50	27.5	243	96	93.9

a) See text for explanation of the dimension
b) There were small residua of 2α,6ß-Δ^4 and 2α-Δ^4
c) Reaction was halted intentionally after a short time
d) Fresh gas from an ampoule showed 96%T_2

In addition to the tritium content of the synthesis gas it would appear that G is the most important parameter. The larger the G (see expts. 3 - 6) the higher was the specific activity of Δ^4 ($A_S(\Delta^4)$). Furthermore, the influence of G on the $A_S(\Delta^4)$ is dependent on the tritium content of the gas used. For instance, at 77% T_2 (expts. 1 and 2) a 2.5 fold G value increased $A_S(\Delta^4)$ from 16 to 34 Ci/mmol; at 85% T_2 (expts. 4 and 5) from 19.1 to 52.1 Ci/mmol. Using highest purity T_2 the influence of G on $A_S(\Delta^4)$ will be even greater. However, the present two examples (expts. 7 and 8) permit no statement on this dependence.

All the experiments listed in Table 1 show that there is no linear dependence of $A_S(\Delta^4)$ on the product G.c.m. Other parameters, supposedly, also play an important part. Nevertheless, it can be said that the conditions of expt. 8 belong to the optimum conditions which permit compensation of the negative effect of the small percentage of hydrogen in the tritium gas.

In the reaction mixture of expt. 2 noticeable amounts of $2\alpha,6\beta$-Δ^4 and 2α-Δ^4 had by chance been left over. These products were separated by thin-layer chromatography (TLC), and their specific activities measured. The reaction in expt. 4 was terminated before quantitative debromination had taken place, in order to determine also the specific activities of the intermediary products. The specific activities found are noted in Table 2.

Table 2: Determination of the specific activity of all the debromination products of 2,2,6ß-tribromo-cholest-4-en-3-one (1) after incomplete reaction of 1 (in the expts. 2 and 4 of Table 1) with diluted tritium gas.

Expt.	specific activity of the products [Ci/mmol]		
	$2\alpha,6\beta$-Δ^4	2α-Δ^4	Δ^4
2	7.1	19.3	34.0
4	2.8	7.5	19.1

The results of the expts. 2 and 4 are very important for our task of preparing cholestenone of high specific activity, because the intermittent increase of specific activity values in both experiments appears to be attributable to a considerable secondary KIE, due to tritium incorporated in molecules of the intermediary products. By this secondary KIE T_2 is favoured as the reactant of all the labelled molecules present. Therefore, using tritium gas as pure as possible and in such excess that exchange reactions do not play a significant part, the reactions series 1 6 7 8 occurs because of the secondary KIE now predominating. This fact is illustrated in figure 1 by full arrows.

In addition, the intermediary products 2α,6ß-Δ^4 with A_S = 2.8 Ci/mmol and 2α-Δ^4 with A_S = 7.5 Ci/mmol obtained in the expt.4 were catalytically debrominated with T_2 having a purity of approx. 86%. These additional catalyzed debromination reactions are to be named expt. 9 and expt. 10. In both experiments the conditions of expt. 4 (gas volume, solvent amount, m) were maintained. Because of the lower substrate amount than in expt. 4 the value of G was here, of course, greater, and c was correspondingly smaller, but the product G.c.m was the same as in expt. 4. Investigations were then conducted to determine whether specific activities such as those in expt. 4 could be obtained (7.5 Ci/mmol, and 19.1 Ci/mmol, see Table 2).

The results of the expts. 4, 9 and 10 are shown in figure 2. Although the same reaction conditions were ensured, the step-wise labelling did not yield the expected specific activities. The larger specific tritium amount was obviously over-compensated by the lower substrate concentration. As in expt. 4, however, the fact that a secondary KIE exists was confirmed by the intermittent increase of the specific activity values in expt. 9.

Because of the small catalyst amount used, and the low substrate concentration c, the reactions for expts. 9 and 10 were incomplete. for this reason it was possible to determine the specific activity of the unconverted substrate in both experiments. The specific activity of the substrate was found to increase during debromination, namely, from 2.8 Ci/mmol to 4.1 Ci/mmol in expt. 9 and from 7.5 Ci/mmol to 8.1 Ci/mmol in expt. 10.

The increase of the specific activity of the labelled substrate indicates a second secondary KIE. The inactive molecules are converted faster than the labelled ones, whereby the reactant (H_2 or T_2) is insignificant. In figure 2, the increase of specific activity of 2α,6ß-Δ^4 and 2α-Δ^4 is designated by small vertical arrows.

This second secondary KIE is not important if the three successive debromination reactions are quantitative; it is significant, however, at low substrate concentration c, and counteracts the first secondary KIE. In the expts 2, 5 and 6 in Table 1, the substrate concentration c was also low. Therefore, the debromination reactions were not quantitative and the yield of Δ^4 was small. Only 20.2% (expt. 2), 30.0% (expt. 5) and 49.2% (expt. 6) of the total activity was found to be cholestenone. A comparison with the data in Table 1 shows that the yield of Δ^4 is in good correlation with the specific activity of Δ^4.

For the purpose of synthesizing cholestenone with high specific activity, the most essential KIE is the first secondary KIE. This fact demonstrates the particular importance of 2ß-T for the further labelling. Only if 2ß-T is present in a substrate can a final product with high specific activity be attained. Therefore, even when using fresh tritium gas, 6ß-bromo-cholest-4-en-3-one (5) yielded [6ß-^{3}H]cholest-4-en-3-one with 6Ci/mmol only. Starting with 2α,6ß-dibromo-cholest-4-en-3-one (2) 18 Ci/mmol was attained as the highest specific activity.

CONCLUSIONS

The results obtained can be summarized in five points:

1) 2,2,6ß-Tribromo-cholest-4-en-3-one (1) is a suitable precursor for synthesizing tritium-labelled cholest-4-en-3-one with high specific activity. Using Collins' procedure [4] specifically labelled cholesterol with high specific activity becomes available.

2) Pure T_2 and three variable parameters which were found in optimization experiments, and which must then be optimized to permit the attainment of maximum specific activity of cholest-4-en-one.

3) If hydrogen is present in the tritium gas a complex reaction course is induced due to three different kinetic isotope effects. The primary KIE favouring hydrogen as reactant is effective in all three reaction steps causing a decrease in the specific activity of the cholest-4-en-3-one.

4) The first secondary KIE due to tritium already incorporated (especially 2ß-T), favours the labelled molecules of the intermediary products which now prefer T_2 as reactant. Its effectiveness in two reaction steps is understood to permit the desirable reaction route 1 6 7 8.

5) A secondary KIE, due to incorporated tritium also, favours the inactive molecules of the intermediary compounds, these being converted faster than the labelled ones. This effect is not significant if the debromination reaction is quantitative.

MATERIAL and METHODS

The catalyzed debromination reactions with tritium gas (product from the USSR) were carried out in the tritium laboratory of the Institute of Nuclear Research Rossendorf [7]. The tritium gas quality was determined by a rough measurement in a home-made ionization chamber [8]. The activities of the labelled compounds were measured in an LS-233 liquid scintillation spectrometer (Beckman, USA). The activity on radio-TLCs was detected by a scanner LB 2723 (Berthold-Friesecke, FRG). Preparative radio-TLC was carried out on silica gel plates (20 cm x 20 cm, 2mm, Merck). In order to determine the activity distribution and the radiochemical purity of the purified products on radio-TLCs analytical silufol plates (15 cm x 15 cm, Kavalier, CSSR) were used. The mass of the purified products eluted from the chromatographic plates was measured in a spectrophotometer DK-2A (Beckman, USA). The following lgε values were determined: 1 (4.04), 2 (4.09), 3 (4.10), and 4 (4.22). Benzene (= solvent 1) and benzene/ether (19.:1) (= solvent 2) were used for developing TLCs. The following R_f values were measured:

Solvent 1.....1 (0.58) 2 (0.38) 3 (0.16) 4 (0.05)
Solvent 2.....1 (0.70) 2 (0.66) 3 (0.41) 4 (0.17)

EXPERIMENTAL

2,2,6ß-Tribromo-cholest-4-en-3-one (1): 2α,6ß-Dibromo-cholest-4-en-3-one (2) [9] (1 g = 1.85 mmol) was dissolved in glacial acetic acid (75ml) containing NaOAc (anhydrous, 2 g). Under stirring, a solution of Br_2 in glacial acetic acid (1M, 2ml) was added dropwise. Further agitation in a bath (90°C, some minutes) lightened the orange-coloured solution. On standing overnight at room temperature colourless crystals were precipitated which were filtered off, washed with cold ethanol and recrystallized from ethanol (99.9%) to give 200 mg pure 1 (= 0.32 mmol, yield 17.3%). M.p. (dec.) 170 - 180°C. UV spectrum λ max (lgε) 258nm (4.04). IR spectrum: 552, 693, 708, 738, 773, 852, 958, 1026, 1085, 1145, 1182, 1205, 1238, 1260, 1385, 1470, 1617 and 1692 cm^{-1}. ^{13}C-NMR spectrum [5]: δ(C-2) = 60.7 ppm (off-resonance singlet) because of the geminal bromine, and δ(C-19) = 24.3 ppm because of 2ß-Br and 6ß-Br as syn-diaxial substituents of the 10ß-methyl group.

[2,2,6ß-^{3}H]Cholest-4-en-3-one (8): 1 (15.2 mg = 24.4 µmol) dissolved in benzene (4 ml) was vigorously agitated (60 min) with T_2 in presence of 2%Pd/$CaCO_3$ (110mg). After measuring gas consumption (71 µmol) and reabsorbing excess T_2, the reaction mixture was filtered and the filtrate was lyophilized. The residue was separated on a Merck plate using solvent 2. The zone of 8 was visible under UV. fter extracting this zone (benzene/ether (1:1), 5 x 4 ml) 8 was obtained with a radiochemical purity >99%. Activity: 740 mCi. Spectrophotometric mass determination: 3.3 mg (= 7.88 µmol, yield 32.3%). This means A_S = 93.9 Ci/mmol.

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