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SYNTHESIS OF TRITIUM LABELLED THYMIDINE AND URIDINE

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Abstract:

This study present tritium labeling methods for uridine and thymidine in the methyl position. The synthesis of tritiated thymidine has been performed using two different methods: catalytic hydrogenation of brominated derivative and respectively of hydroxymethyl deoxiuridine.
The tritiated uridine has been synthesized by catalytic hydrogenation of 5-I Uridine.
The labelled compounds were purified by Thin Layer Chromatography and characterized by determination of radioactive concentration, chemical concentration and radiochemical purities.
The obtained tritiated thymidine and uridine have been used for in vitro studies for evaluation of radiation effects in cell cultures.

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1. INTRODUCTION

The main objective of the IFA ELI 09/2020 project is to enlarge the portfolio of advanced biological investigations addressing the interaction of cells with radiation, relevant for astrobiology and FLASH radiotherapy studies at ELI-NP (Manda et al., 2020).

The post irradiation cellular functional investigations will be realized by: (a) cellular proliferation (flow cytometry with CFDA-SE and cell cycle analysis with Vybrant Dye Cycle Orange stain, the tritium-labelled thymidine assay) and (b) RNA synthesis using uridine labelled with tritium (Matei et al., 2010).

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Thymidine and uridine can be labelled with tritium by two methods: isotopic exchange and chemical synthesis.

The isotopic exchange method is much easier (Postolache et al., 2010), but it has the following disadvantages: (a) the distribution of tritium in the labelled molecule is not controlled and (b) the specific activities and implicitly the detection limits are lower.

The method of labelling by chemical synthesis is more laborious, involving the microsynthesis of specific precursors. The advantages of the method are: control of the distribution of the radioactive isotope in the labelled molecule and specific activities with an order of magnitude larger than in the case of isotopic exchange technique (Matei et al., 2008).

In radiometric molecular biology researches, the use of specifically labelled compoundsobtained by chemical synthesis is recommended.

In this paper, tritium labelling of thymidine and uridine obtained by catalytic hydrogenation of halogenated or hydroxyl precursors is described.

2. EXPERIMENTAL

2.1. Materials

The thymidine (Sigma Aldrich $\geq$ 99% purity), 5-I-uridine (Sigma Aldrich, $\geq$ 99% purity) and 5-(Hydroxymethyl)-2'-deoxyuridine or HMDU (Cayman Chemical, 98% purity) were dehydrated by storage in vacuum desiccators in the presence of $CaCl_2$ sic. for at least 24 h.

N-Bromosuccinimide or NBS (Merck, < 95% purity) and Azobisisobutyronitrile or AIBN (Sigma Aldrich, < 98% purity) were dehydrated by storage desiccators in nitrogen atmosphere and presence of $CaCl_2$ sic. for at least 48 h.

Sodium methoxide solution was purchased from Sigma Aldrich.

Acetic anhydride (Merck < 95% purity) was purified by distillation in presence of P2O5. 1,4-dioxane scintillation grade (Loba Chemie 99.5% purity) was purified by storage ontoalumina, distillation in nitrogen atmosphere and presence of KOH.

Chloroform and Dimethyl sulfoxide or DMSO were purified by distillation in presence ofP_2O_5.

Pd/C 10% and Rh/Al_2O_3 5% (provided by Sigma Aldrich) were used as hydrogenationcatalysts.

Tritium gas (T_2 mixed with ^3He) was obtained by recovering gas from expired beta lightsources (Matei et al., 2008).

2.2. Obtaining of Br Methyl Thymidine-3',5'-diacetate precursor

The synthesis of brominate thymidine was made according to the scheme shown in figure 1.

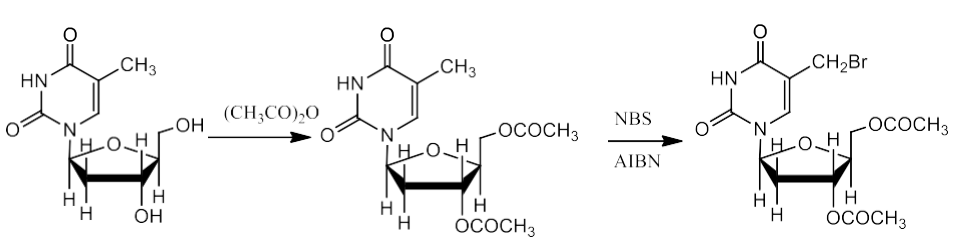

Figure 1. Brominate thymidine synthesis pathway

In the first stage, hydroxyl groups of thymidine were protected by esterification using acetic anhydride.

The reaction was carried out using a mini-reflux plant consisting of a 3-necked flask, an ascending double-jacketed coiled condenser provided with a desiccation cartridge and a drip funnel with a Teflon stopcock.

The ratio between thymidine and acetic anhydride was 1:10.

The obtained ester was purified by recrystallization from ethyl acetate and characterised by melting point determination using a Boetius microscope with thermostatic plate and Thin Layer Chromatography (TLC). The TLC system was: Support: Silicagel plate GF254 0.5 mm Merck, Eluent: N-butanol: Water (86:14, v/v), chemical identification of spots using a UV254 lamp.

In secondary step, brominated product was obtained using a similar glass facility.

The thymidine-3',5'-diacetate, NBS and AIBN were introduced into the reaction flask in molar ratio 1:2:1. The chloroform, used as solvent, was introduced in the flask through the drip funnel, and the halogenation reaction was performed at reflux temperature. At the end of reaction, chloroform solution was concentrated at a rotary evaporator Laborota Heidolph, Germany.

The synthesis of Br-Methyl-thymidine-3',5'-diacetate could not be controlled because the final product is unstable. In this situation, a rapid TLC separation was chosen. The TLC system was: Support-Silicagel plate GF254 0.5 mm Merck activated at 105^0C, Eluent: Chloroform: Methanol (9: 1, v/v). The product was extracted from the chromatographic plate with anhydrous Dimethyl sulfoxide. The DMSO extract was used in the labelling step.

2.3. Labelling with tritium of thymidine

Thymidine labelled with tritium at methyl group was obtained using two synthesis routes:

- Hydrogenation of HMDU using as catalyst Rh/Al_2O_3 5% (Figure 2) and
- Hydro-dehydrobromination of Br Methyl Thymidine-3',5'-diacetate using Pd/C 10%followed by hydrolysis of ester (see Figure 3)

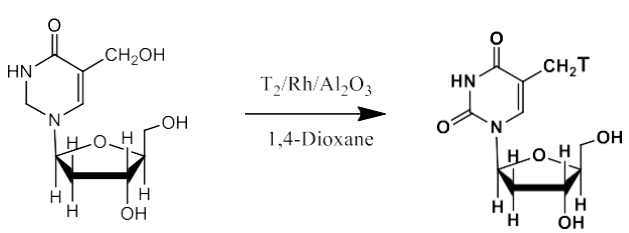

Figure 2. Labelling methods for obtaining of Thymidine-Methyl-T using HMDU precursor

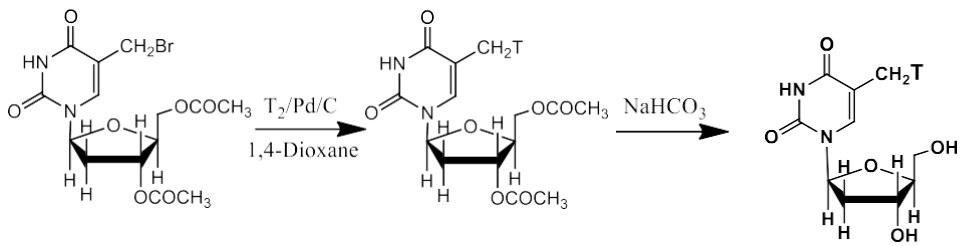

Figure 3. Labelling methods for obtaining of Thymidine-Methyl-T using Br MethylThymidine-3',5'-diacetate precursor

Labelling conditions are presented in table 1.

Table 1. Parameters used in the labelling of thymidine

Parameters	Using Brominated thymidine	Using HMDU
3-He:T_2 pressure	160 mbar/N_2 liq.	150 mbar/N_2 liq.
Solvent	DMSO & Sodium methoxide solution	1,4-Dioxane
Catalyst	Pd/C 10%	Rh/Al_2O_3 5%
Ratio catalyst/precursor	15	10
Reaction time	1.5 h	2 h

2.4. Labelling with tritium of uridine

The uridine has been labelled by catalytic hydrogenation (Postolache et al., 2007) of 5-I-uridine following the reaction showed in Figure 4.

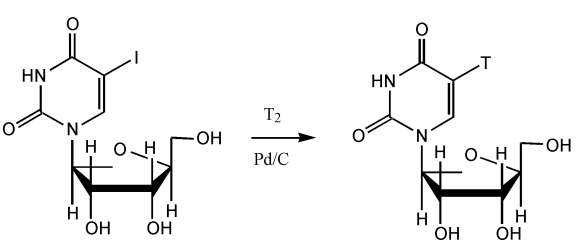

Figure 4. Synthesis of Uridine 5-T

The parameters used in labelling step was:

- T_2 pressure: 100 mbar/N_2 liq.
- Solvent: 1,4-Dioxane (1 ml) and 0.05 ml NaOH 0.1 N methanol solution
- Catalyst: Pd/C 10%
- Ratio catalyst/precursor: 12
- Reaction time: 2 h

2.5. Removal of labile tritium and purification of tritium labelled compounds

After labelling steps, the catalysts were removed from the reaction mixtures by filtration usingMillipore filters with 0.2 µm pore size.

The labile tritium was removed from the labelled thymidine and uridine by multiple dissolution in ethanol/evaporation at vacuum using a dedicated rotary evaporator (Tuta et al., 2015).The number of dissolution-evaporation steps was 10.

The crude labelled compounds were purified by preparative TLC using as separation system Silicagel plates GF254 0.5 mm and as eluents: N-butanol: Water (86:14, v/v) for thymidine and N-butanol: Acetic acid: Water (4:1:1, v/v/v) for uridine.

The chemical distribution was determined using a UV lamp 254 nm. The radioactive distribution was identified using a Beta-TLC Rita Star, Raytest, Germany with open window gas flow proportional counter.

2.6. Characterization of labelled compounds

After purification, the labelled compounds was characterised in radioactive concentration, chemical concentration and radiochemical purity point of view.

The radioactive concentration was determined by sampling of solutions, using a 50 µl fixed volume Eppendorf pipette, 400 times dilution using ethanol, putting the sample in the glass vial which contained 10 ml liquid scintillator ULTIMA GOLD M type, and measurement using Liquid Scintillation Counter TRICARB 2800TR from Canberra Packard.

The chemical concentrations were determined using UV VIS Spectrometer SPECORD 210 UV Vis Analytic Jena with 10 mm standard quartz UV cuvette.

The chemical concentrations were obtained by determining the absorbance at 264 nm (for thymidine) or 262 nm (for uridine) and intercomparing with the previously obtained calibration curves (Figure 5).

The radiochemical purity was determined by radio-TLC using unlabelled referential solution,the separation system above shown and Beta-TLC Gina Star, Raytest, Germany.

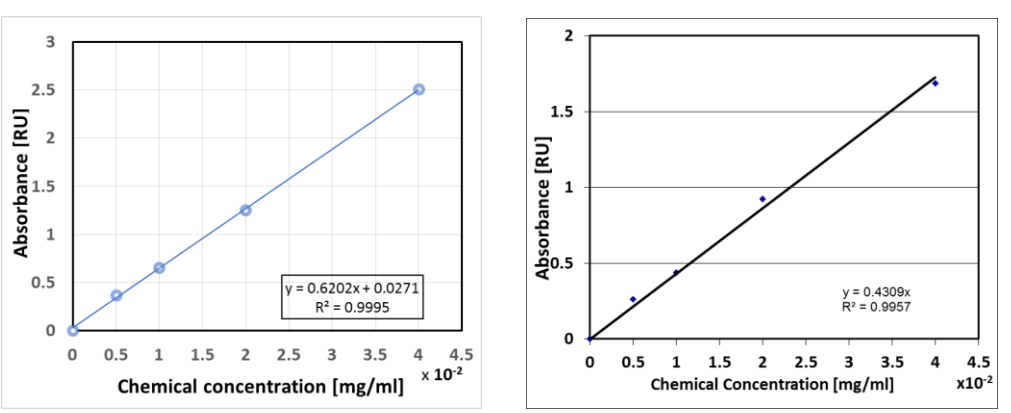

Figure 5. Absorbance curves as a function of chemical concentration for Thymidine at 267nm (left), and Uridine at 262 nm (right)

3. RESULTS

3.1. Obtaining of Br Methyl Thymidine-3',5'-diacetate precursor

The brominated methyl thymidine was synthesized by radical halogenation of thymidine with hydroxyl groups protected by esterification.

The obtained thymidine ester was purified by recrystallization and characterised by TLC and melting point determination. The TLC analysis revealed a unique product. The obtained melting point domain was 128-129ºC in accordance value from the Chemical Book site (126-128ºC- https://www.chemicalbook.com/ChemicalProductProperty_EN_CB0219554.htm).

The yield of purified obtained product was 95.4%.

3.2. Labelling with tritium of thymidine

The synthesis of thymidine has been performed using two different ways: catalytic hydrogenation of the methyl brominated and hydroxy methyl derivatives.

After removal of labile tritium (associated with ester hydrolysis in the case of brominated derivative) and preparative radio-TLC purification, the labelled compounds with characteristics presented in Figures 6, 7 and Table 2 have been obtained.

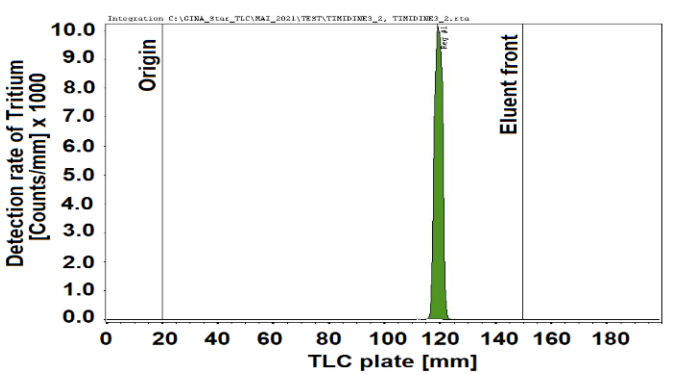

Figure 6. Radio chromatogram of purified Methyl-T-thymidine obtained by hydrogenation of HMDU

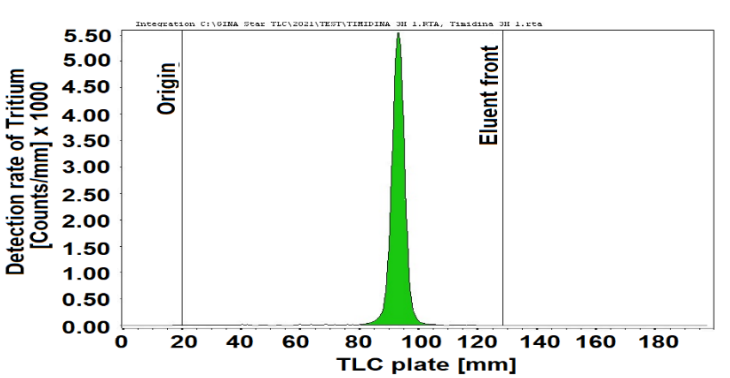

Figure 7. Radio chromatogram of purified Methyl-T-thymidine obtained by hydrogenation ofbrominate derivative

3.3. Labelling with tritium of uridine

The tritiated uridine in position 5 was obtained by catalytic hydrogenations of 5-I uridine, followed by removal of labile tritium. After purification by preparative radio-TLC, the labelled compound was characterised by radioactive and chemical concentrations, and radiochemical purities (Figure 8). The main characteristics of obtained 5-T-uridine are shown in Table 2.

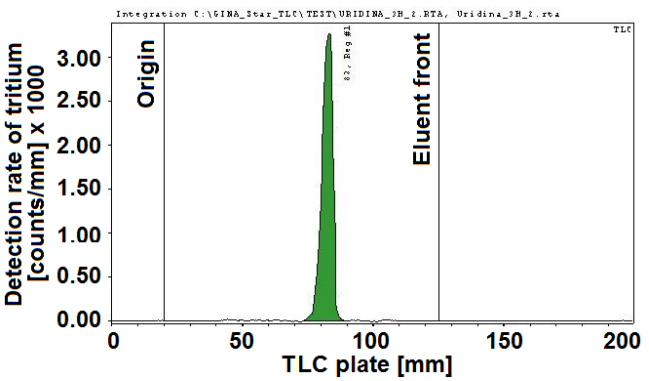

Figure 8. Radio chromatogram of purified 5-T-Uridine obtained by hydrogenation of 5-I-Uridine

Table 2. Characteristics of obtained tritiated thymidine and uridine

	Thymidine-Me-T		Uridine-5-T
	from Br derivative	from HMDU	
Raw product activity [MBq]	25086	10767	9805
Purified product activity [MBq]	13986	9472	7955
Raw product radiochemical purity [%]	59	92	85
Purified product radiochemical purity [%]	>95	>95	>95
Radioactive concentration [MBq/mL)	35	37	34
Chemical conc. [mg/mL]	0.008	0.011	0.010
Specific activity [GBq/mmol]	999	814	851

4. CONCLUSIONS

The labelling, in specific and stable positions, of Thymidine and Uridine was performed by chemical methods of incorporating tritium into molecules.

It was chosen as a technique for incorporating tritium, the hydrogenation of halogenated or hydroxyl (inserted in active positions like allylic/benzyl) derivatives.

Platinum group metals (Pd and Rh) deposited on a hydrophilic support (carbon or alumina) were used as hydrogenation catalysts.

A mixture of T: ^{3}He recovered from expired tritium gas sources was used as the labelling agent.

The Methyl-T-Thymidine was obtained by two methods: (a) Catalytic hydrogenation of 5- (Hydroxymethyl)-2'-deoxyuridine and (b) Catalytic hydrogenation of Br-Methyl-thymidine- 3',5'-diacetate followed by hydrolysis of ester in presence of $NaHCO_3$.

The precursor used in the first method was purchased from Cayman Chemical Company; the precursor used in the second method is not available on the market because it has low stability and has been synthesized from thymidine in two steps.

5-T-Uridine was synthetized by catalytic hydrogenation of 5-I-Uridine.

After the labelling step, the tritiated compounds were purified and stabilized in ethanol solutions with radioactive concentrations around 37 MBq/ml.

Finally, the compounds were characterized in terms of radioactive concentration, chemical concentration and radiochemical purity.

By relating the radioactive concentrations to the chemical ones, the mass activities were obtained and in agreement with the molecular masses, the specific activities were deduced.

The obtained specific activities are similar in case of 5-T-uridine and Methyl-T-thymidine synthetized by hydrogenation of 5-(Hydroxymethyl)-2'-deoxyuridine. In case of Methyl-T- thymidine obtained via Br-Methyl-thymidine-3',5'-diacetate, the specific activity is higher. This increase is explained by protection of labile position and presence of traces of dibrominated derivative.

The labelled compounds were used for in vitro studies for evaluation of radiation effects in cell cultures.

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Author contributions: C Postolache coordinated the synthesis of labelled compounds and wrote the original draft. The chemical and radiochemical characterization of the labelled compounds was performed by C. S. Tuta, G. Bubueanu and A. S. Cucoanes. All authors have formulated the conclusions.

Conflict of Interest Statement: The authors declare no conflicts of interest associated with this manuscript.

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