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Tritium Labelling of RAD001- A New Rapamycin Derivative

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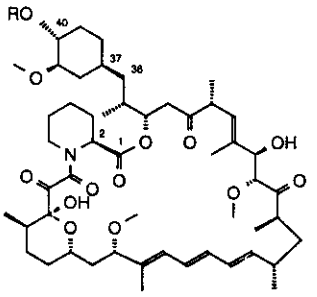
Summary

RAD001 2, a new immunosuppressant, was labelled in its hydroxyethyl side chain as well as in the rapamycin skeleton using carrier-free lithium triethylborotritide and tributyltin tritide (TBTT), respectively. The tritium labelling of the rapamycin moiety followed Curran's strategy of intramolecular hydrogen transfer. Our studies demonstrated that base-free TBTT is essential for a successful reaction.

Keywords: Tritium labelling, Rapamycin, **RAD001**, carrier-free lithium triethylborotritide, base-free tributyltin hydride

Introduction

The macrolide rapamycin **1**, a secondary metabolite of *Streptomyces hygroscopicus*, suppresses the rejection of transplanted allogenic solid organs in experimental animals [1,2]. **1** is of particular interest as a new immunosuppressant because its mode of action differs from that of cyclosporine A (CsA) and FK506. In a preclinical program **RAD001 2** (C(40)-O-(2-hydroxyethyl)-rapamycin) was designed to provide a macrolide with improved immunosupressive properties.



R = H — **1** (Rapamycin)

R = HO-CH_2-CH_2 — **2** (**RAD001**)

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Recently we described [3] the synthesis of carbon-14 labelled **1** and **2** essential for the human ADME studies envisaged. [$^{14}C_2$]Pipecolic acid and (1R,3R,4R)-3,4-dihydroxy[1,7-$^{14}C_2$]cyclohexane carboxylic acid, synthesized in a stereoselective approach, turned out to be highly suitable precursors for an effective fermentation process.

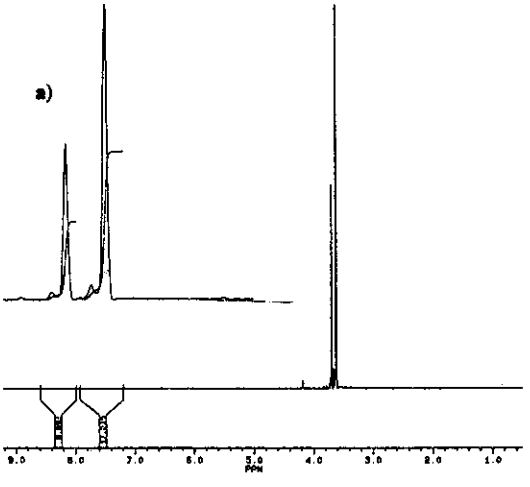
The optimization of this process required considerable capacity and time. Modern drug development, however, calls for fast access to radiolabelled isotopomers, which may be achieved using tritium-chemistry. In order to get the maximum information on the metabolic fate of **2** in previous animal studies we decided to label **RAD001 2** in its hydroxyethyl side chain as well as in the rapamycin skeleton. This communication describes the ^3H labelling of both compounds.

Results and Discussion

Synthesis of C(40)-O-(2-hydroxy[$^3H_2$]ethyl)-rapamycin 2a/b [4]

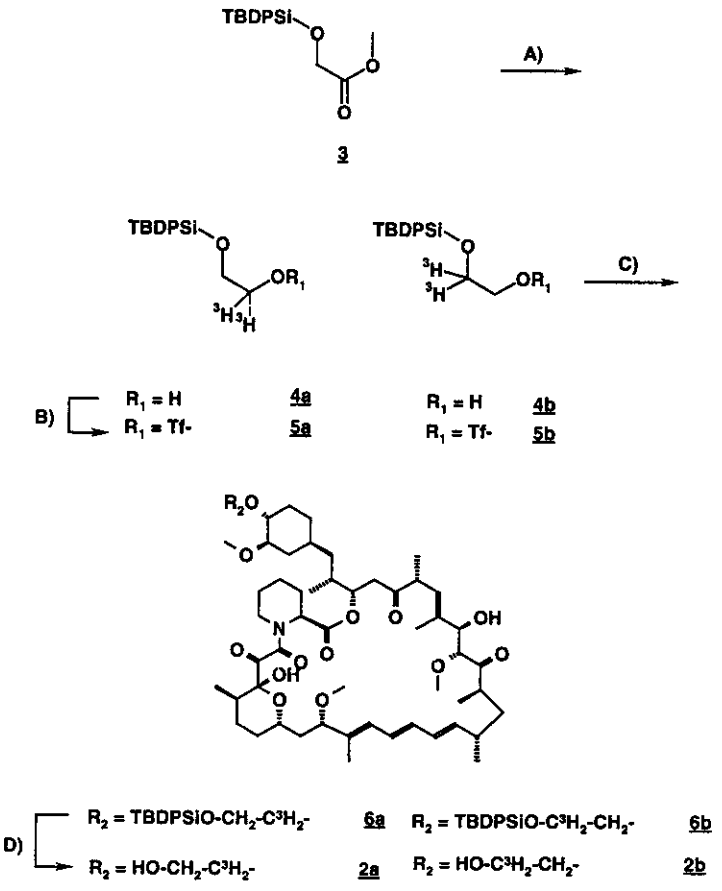
Methyl O-*tert*-butyldiphenylsilyl glycolate **3** was reduced with carrier-free lithium triethylborotritide [5]. The corresponding alcohol **4** was isolated after flash chromatography on silica gel in 48 % yield. The ^3H-NMR spectrum of **4** showed two triplets in a ratio of 66:34. This doubling of the peaks corresponded to the isotopomeric entities (**4a/4b**), which resulted from a silica mediated migration of the TBDPS-group [6]. The additional small peaks (8%) detectable in the proton-decoupled ^3H-NMR spectrum (figure 1) resulted from the monolabelled C^{1}H^{3}H-group (isotope-shift).

Figure 1: Proton-decoupled ^3H-NMR spectrum of **4** indicating a mixture of the isotopomeric entities **4a**/b (a) enlargement of the region of interest)



Conversion of **4a/b** into their triflates (Tf_2O, N,N-diisopropylethylamine, CH_2Cl_2, 75%) and their employment for regioselective alkylation [7] of rapamycin **1** gave **6a/b**. Final deprotection (HF, pyridine : acetonitrile 1:10, -45°C/30 min., r.t./3 h) provided [^{3}H]**RAD001 2a/b** at a specific activity of 2.05 TBq/mmol (55.6 Ci/mmol).

Scheme 1: Synthesis of C(40)-O-(2-hydroxy[$^3H_2$]ethyl)-rapamycin **2a/b**

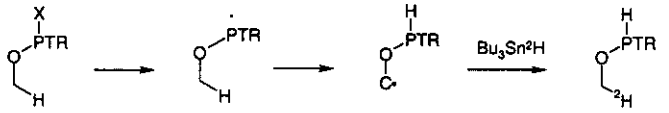


Reaction conditions: **A)** n-BuLi (1.6 M in n-hexane), T_2, TMEDA, THF, BEt_3, **3**, flash chromatography, 48%; **B)** Tf_2O, N,N-diisopropylethylamine, CH_2Cl_2, -20°C / 20 min., r.t. / 60 min., flash chromatography, 76%; **C)** **1**, N,N-diisopropylethylamine, toluene : dimethoxyethane 1:1, 50°C, 16 h, repeated chromatography, 6%; **D)** HF / pyridine : acetonitrile 1 : 10, -45°C / 30 min., r.t. / 3 h, HPLC-purification, 21%;

Synthesis of C(40)-O-(2-hydroxyethyl)-[^{3}H]rapamycin 2c

The *ortho*-(bromophenyl)dimethylsilyl group was introduced by Curran [8] as a reactive protecting group for alcohols enabling intramolecular hydrogen transfer reactions from adjacent positions (Protecting Radical Translocating = PRT-group). Generation of a radical within the protecting group is followed by an intramolecular hydrogen transfer leading to a new radical, which can then participate in standard radical reactions (Scheme 2). Based on this strategy Curran described [9] the preparation of $^2H_1$-rapamycin: C(40)-O-(*ortho*-(bromophenylsilyl)-rapamycin **8** was reacted with Bu_3SnD in the presence of catalytic amounts of AIBN at 80°C. Deprotection with acetic acid gave [$^2H_1$]-rapamycin. It was our intention to adapt this procedure for the H-3 labelling of **RAD001 2**.

Scheme 2: Strategy of ^{2}H-Labelling using Intramolecular Hydrogen Transfer



The starting material for this approach is Bu_3Sn^3H (TBTT). Therefore, in order to improve the radiochemical yield we optimized an internally developed procedure [4].

First experiments using Bu_3Sn^2H, prepared according to this procedure, resulted in a nearly complete decomposition of the rapamycin derivative. Identification of the decomposition products gave evidence that due to the extreme sensitivity of rapamycin to basic conditions even small traces of lithium hydroxide resulting from the hydrolysis of n-BuLi were sufficient to destroy the rapamycin molecule. Therefore, purification of $Bu_3Sn^2H/^3H$ by flash chromatography (silica gel) was essential for a successful reaction.

Scheme 3: Synthesis of base-free Bu_3Sn^3H

$$^3H_2 + \text{n-BuLi} \xrightarrow{\text{TMEDA}} Li^3H + Bu^3H \xrightarrow[\text{purification}]{Bu_3SnCl} Bu_3Sn^3H$$

A second drawback of this procedure was that using AIBN as a radical initiator required reaction temperatures > 70°C leading to a significant release of radioactivity when working on a multi Ci-scale. In order to suppress potential decomposition of TBTT we decided to develop conditions at ambient temperature. BEt_3/O_2 as a radical initiator is efficient [10] at and below room temperature. Additionally, in hot pilot studies these reaction conditions gave evidence of a significantly improved selectivity.

Without any protection of other functionalities (2-bromophenyl)dimethylchlorosilane **7** [11] reacted selectively with **1** to give C(40)-O-(*ortho*-bromophenyldimethylsilyl)-rapamycin **8** (imidazole, DMF, -10°C, 30 min., 85%). Subsequently **8** was reacted with 1.2 equivalents of base-free Bu_3Sn^3H in the presence of BEt_3 and O_2. The reaction stopped (even addition of BEt_3 and O_2 did not initiate further reaction) without complete conversion. Early non optimized chromatographic separation techniques failed to separate labelled product **9** from unlabelled starting material **8**. The subsequent deprotection of the reaction mixture (**8** and **9**) resulted in a significant decrease in the specific activity. Final purification afforded [^{3}H]rapamycin **1c** at a specific activity of 70 - 200 GBq/mmol (1.9-5.4 Ci/mmol) sufficient for the ADME-studies envisaged. Further improvement of the separation technique identified HPLC conditions, which separated **8** from **9**. Thereby radiochemically pure [^{3}H]rapamycin **1c** was obtained at significantly higher specific activities of up to 370 GBq/mmol (10 Ci/mmol). Mass spectroscopy of **1c** revealed that 37% of the material was monolabelled and 63% of the material unlabelled.

The synthesis of Bu_3Sn^3H and the conversion of **8** into **9** was routinely carried out by TRITEC (CH-Teufen). It is interesting to note that working on a TBq-scale less than 0.5% of the radioactivity was released.

The final conversion of [^{3}H]rapamycin **1c** into **[^{3}H]RAD001 2c** was performed according to the procedure described in scheme 4.

The Tritium-NMR analysis of **2c** revealed the distribution pattern of the label as given in Table 1.

Table 1: Distribution of the label of **[^{3}H]NVP RAD001 2c**

position	2	CH_3O-(39)	39, 40	41 equ.	41 ax.
incorporation [%]	0.5	8.5	80.4	1.8	8.8

The labelling of the positions 39, 40 and 41 is explained by intramolecular 1,5- and 1,6-hydrogen transfer with subsequent reduction of the generated radical by Bu_3Sn^3H. Labelling of the positions 2 and CH_3O-(39) required immediate proximity of both centers, which might be explained by appropriate folding of the molecule.

This strategy has also been applied to other macrolides [11]. Therefore, it seems to be a general approach for the ^{3}H labelling of structurally complex molecules containing free hydroxy groups.

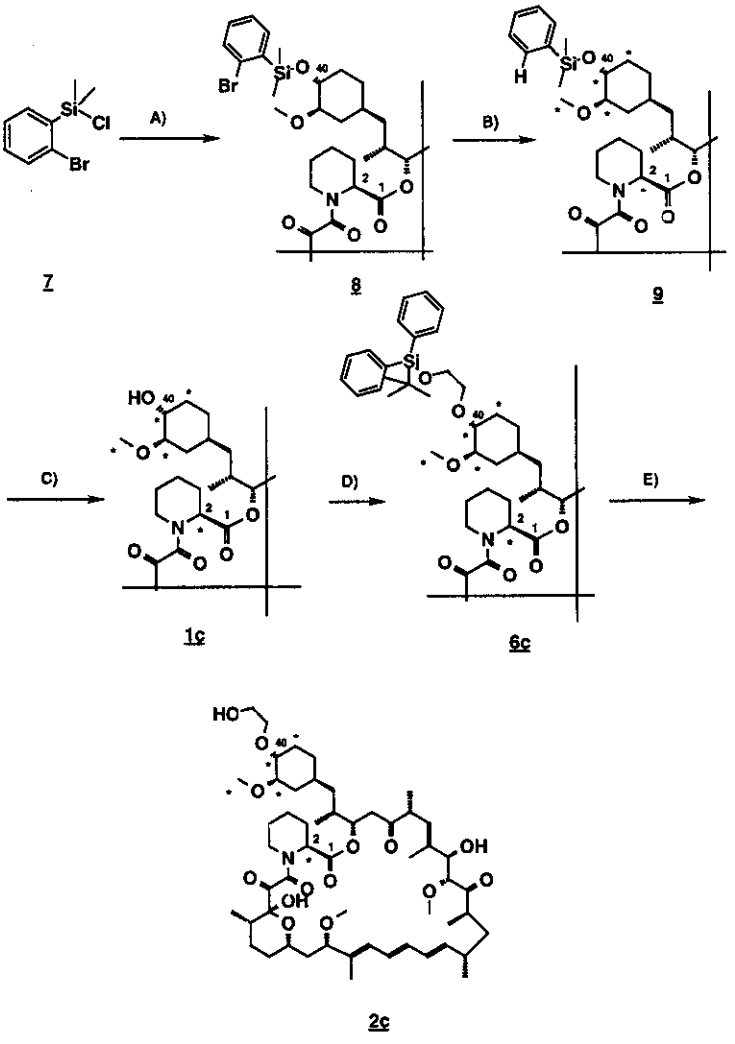
Experimental details

Unless otherwise mentioned, chemicals and radiochemicals were purchased from commercial suppliers. The identity of intermediates and precursors were identified by either chromatographic and/or spectroscopic methods.

Synthesis of O-*tert*-butyldiphenylsilyl [^{3}H]ethylene glycol **4a/4b**

A solution of n-butyllithium in n-hexane (2 mmol) was stirred at room temperature in an atmosphere of carrier-free tritium gas at 700 hPa. Tetramethylethylenediamine (290 mg, 377 µl, 2.5 mmol) was added to the reaction vessel through a rubber septum of the side-arm, whereupon the Li^{3}H started to precipitate immediately. The tritium pressure was kept at 700 hPa by occasional addition of tritium from the tritium line. After 1 hour the uptake of tritium was completed. A 1-molar solution of triethylborane (2.0 mmol) in THF was added to the suspension to form a solution of lithium triethylborotritide. 230 mg (0.7 mmol) of the methyl glycolate **3** dissolved in 300 µl of THF were slowly added by syringe. The mixture was stirred for 30 minutes and then the excess of reagent was destroyed with 100 µl of methanol. The mixture was cooled to - 195°C and the excess of tritium gas in the reaction vessel was reabsorbed on a uranium-trap for waste-tritium. The solvents were lyophilized off and labile tritium was removed by lyophilization with methanol (2 x 1 ml). Extractive work-up with toluene and water, drying of the combined organic phases with $MgSO_4$ and evaporation of the solvent afforded crude reaction product **4a**. Purification by flash chromatography (silica gel: hexane - ethyl acetate 80:20) produced a mixture of **4a/4b** in 48.6% chemical and radiochemical yield (135.5 mg, 1.0 TBq (27 Ci), specific activity: 2.05 TBq/mmol (55.6 Ci/mmol)).

Scheme 4: Synthesis of C(40)-O-(2-hydroxyethyl)-[^{3}H]rapamycin **2c**



Reaction conditions: A) 1, imidazole, DMF, -10°C / 10 min., 10°C / 30 min., flash chromatography, 85%; B) Bu_3SnT: n-BuLi, TMEDA, T_2, r.t., 45 min., THF, Et_3B, Bu_3SnCl, r.t., 30 min., flash chromatography; 8, THF, Et_3B, 23°C, 120 min.; C) THF, CH_3COOH, -5 - 0°C, 60 min., 50%; D) 4, N,N-diisopropylethylamine, Tf_2O, CH_2Cl_2, -18°C / 20 min, r.t. / 60 min., flash chromatography; 1c, N,N-diisopropylethylamine, toluene /dimethoxyethane 1 : 0.05, 50°C, 3 h, purification; E) CH_3COOH, n-heptane, water, ethyl acetate, r.t., 60 min. 20%;

Synthesis of C(40)-O-(*ortho*-bromophenyldimethylsilyl)-rapamycin **8**

To a solution of rapamycin (5000 mg, 5.48 mmol) and imidazole (2200 mg, 31.3 mmol) in anhydrous DMF (180 ml) *ortho*-bromodimethylsilylphenyl chloride 7 dissolved in anhydrous DMF was added at -10°C within 10 min. After stirring for 30 minutes the reaction was completed (TLC: silica gel, n-hexane : 2-propanol : N,N-diisopropylamine 85 : 15 : 0.1). The

reaction mixture was poured onto water, and thoroughly extracted with ether. The combined organic phases were dried over Na_2SO_4, filtered off and evaporated. The residue was purified by flash chromatography to obtain 5.21 g (4.62 mmol) of **8** (84.4%).

Synthesis of tributyltin tritide (TBTT)

Lithium tritide was prepared from n-BuLi (1.5 mmol) as described above. After cessation of the tritium-uptake the mixture was cooled to -195°C and unconsumed tritium was reabsorbed on the uranium-trap. The solvent and TMEDA were lyophilized off and the remaining Li^3H was dried at r. t. and high vacuum for 30 minutes. Protection gas (H_2, He or Ar) was admitted to a pressure of 500 hPa. Subsequently 1.5 ml of dry THF was injected via syringe followed by 200 µl of 1M triethylborane in THF. Under intensive stirring 360 µl of tri-n-butyltin chloride was injected as rapidly as possible. A short evolution of tritium gas and a corresponding increase of pressure* was observed (**slow addition of Bu_3SnCl leads to almost complete liberation of $^3H_2$**). After a short dissolution the lithium chloride formed precipitated. After stirring for 30 minutes the mixture was cooled to -195°C and excess tritium was reabsorbed on the uranium-trap (in case of He or Ar as protection gas the gas mixture had to be circulated over the uranium-trap). The solvent was lyophilized off and replaced by 2 ml of n-hexane or n-heptane. The resulting slurry was filtered over 2 g of silica gel with 10 x 2 ml of n-hexane or n-heptane. The filtrate was evaporated on a rotary evaporator and the residue dried at room temperature under high vacuum to obtain 365 mg (83 %) of pure and base-free tri n-butyltin tritide.

* In additional experiments the liberated tritium was determined volumetrically: 10 - 15 % of the theoretical amount was found in the gas phase.

Synthesis of C(40)-O-(phenyldimethylsilyl)-[^{3}H]rapamycin **9**

The rapamycin-derivative **8** (500 mg, 0.55 mmol, 0.8 mol equivalents) and BEt_3 (150 µl, 0.2 equivalents) were added to the TBTT. The reaction mixture was stirred for 2 h at 23°C (RTLC-check). Despite an incomplete conversion the solvent was lyophilized and the residue separated from unreacted TBTT by flash chromatography. Eluting with n-hexane removed unreacted TBTT from the column, while the product came off with n-hexane : 2-propanol 80:20. Evaporation of the solvent afforded a mixture of labelled product **9** and unreacted starting material **8** (226 GBq (6.1 Ci)).

Separation of both compounds, however, is possible by preparative HPLC (Hypersil-ODS, 5 µm, 25 x 2.2 cm, solvent A H_2O : MeOH 500 : 500, solvent B H_2O : MeOH 50 : 950, 0 min. to 24 min. 30 % B to 90 %B, 24 min. to 27 min. 90 %B, 27 min. to 27.5 min. 90 %B to 30%B)

Synthesis of [^{3}H]rapamycin **1c**

A solution of unpurified **9** (226 GBq (6.1 Ci)) dissolved in THF (15 ml) was treated with H_2O (4.5 ml) and acetic acid (4.5 ml) at 0°C. After completion of the reaction (2.5 h, 0°C; RTLC-control: silica gel: n-hexane : 2-propanol 9 : 3) the reaction mixture was extracted with diethyl ether. The organic layer was washed with water, with $NaHCO_3$-solution (2x) and finally with water until pH 7. The organic phase was dried with Na_2SO_4, filtered off and finally evaporated to obtain crude rapamycin **1c** (221.6 GBq). Purification was accomplished by combined flash chromatography (silica gel: n-hexane : 2-propanol 9 : 4) and preparative HPLC (Hypersil-ODS (Bischoff), 5 µm, 25 x 2.2 cm, solvent A H_2O, solvent B MeOH, 0 min. to 24 min. : 70 %B to 80 %B, 24 min to 25 min. : 80 %B to 95 %B, 25 min. to 29 min. : 95 %B, 29 min. to 30 min. : 95 %B to 70 %B) Due to the sensitivity of rapamycin to methanol the HPLC-fraction of interest was collected in methylene chloride. After separation of the phases and

extraction of the aqueous phase, the combined organic phases were dried over Na_2SO_4, filtered off and completely evaporated. For storage the residue was redissolved in toluene to obtain 26.6 GBq (718 mCi) of **1c** with a radiochemical purity > 98%. The specific activity was determined to be 200 GBq/mmol by ms-spectroscopy.

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experimental details will be reported elsewhere

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