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C-14 Labelling of NVP RAD001 - A New Rapamycin Derivative

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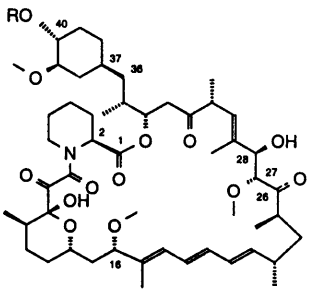
Summary

An optimized fermentation process is described for the carbon-14 labelling of rapamycin. It is characterized by good reproducibility, high radiochemical purity and excellent yields. L-[$^{14}C_2$]Pipecolic acid and (1R,3R,4R)-3,4-dihydroxy[1,7-$^{14}C_2$]cyclohexane carboxylic acid ([$^{14}C_2$]DHCCA) synthesized in an effective stereoselective approach proved to be highly suitable precursors for this purpose. In a final step [$^{14}C_4$]rapamycin was converted by selective C(40)-O-alkylation to **[$^{14}C_4$]NVP RAD001**, a highly interesting immunosuppressant.

Keywords: [$^{14}C_2$]BABS, [$^{14}C_2$]DPMGBS, [$^{14}C_2$]PABS, L-[$^{14}C_2$]Pipecolic Acid, (1R,3R,4R)-3,4-Dihydroxy[1,7-$^{14}C_2$]cyclohexane carboxylic acid, NVP RAD001, Rapamycin, Fermentation, Stereoselective Synthesis

Introduction

There is increasing interest in the immunosuppressant rapamycin **1a**, a macrolide produced by *Streptomyces hygroscopicus* [1,2]. Compounds of this class differ in their mode of pharmacological action from cyclosporine, thus providing a rationale for potential synergism between these two potent immunosuppressants [3]. A major complication in the drug development of rapamycin, however, is the lack of a formulation that can overcome the considerable inter-individual variation in pharmocokinetic properties seen with oral administration in both animals and humans [4,5]. In a preclinical program **NVP RAD001 1b** (C(40)-O-(2-hydroxyethyl)-rapamycin) was designed to overcome these difficulties [6].



R = H **1a** (Rapamycin)

R = HO-CH_2-CH_2- **1b** **(NVP RAD001)**

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To provide a suitably radiolabelled isotopomer for the evaluation of ADME-data, tritium labelling was expected to be the fastest approach, because of the highly complex nature of the molecule. The two methods of choice were tritiation of the ethylhydroxy side chain and tritiation of the rapamycin molecule, adapting Curran's procedure of an intramolecular radical translocation [7] to tritium. Curran's approach proved to be very well suited also for tritium labelling of molecules of the ascomycin and fujimycin-type. Experimental details will be reported elsewhere [8].

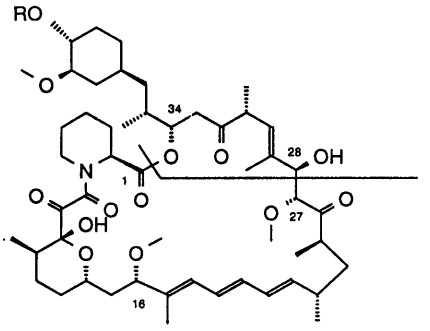
Although most of the ADME-studies in animals have been performed using this material, it was decided to change to carbon-14 labelled material for the human ADME study to avoid any metabolic instability of the label and thereby loss of metabolic information. For this reason also, the readily accessible carbon-14 labelling of the 16-methoxy moiety resulting from an acid-catalysed exchange reaction with [^{14}C]methanol [9] was not a satisfactory approach.

The requirements for the ^{14}C-labelled isotopomer were defined as follows:

- metabolic stability of the carbon-14 label

- specific activity in the range of 100 mCi/mmol

- labelling of the upper and the lower part of the molecule. Initial pilot biotransformation studies indicated metabolically induced splitting (lactone cleavage combined with retroaldol cleavage of the ring between position 27 and 28 - as outlined in the figure below) [10]. It was therefore essential to label both parts of the molecule simultaneously in order to trace all possible metabolites.



Two principal alternatives were open to us for achieving this ambitious goal - either total synthesis or fermentation process using radiolabelled precursors. We opted in favor of the biological approach; in particular, we expected the know-how of the fermentation process to be of advantage also for the labelling of structure-related macrolides such as ascomycin and fujimycin.

Discussion and Results

Biosynthesis of Rapamycin

The immunsuppressants rapamycin, ascomycin and FK 506 are secondary metabolites of the polyketide type. Their biosynthesis starts from an unusual shikimate-derived (1R,3R,4R)-3,4-dihydroxycyclohexane carboxylic acid (DHCCA) starter unit (C(36) to C(42)) [11-13]. In rapamycin five acetate and six propionate units are condensed to this starter in a 'head to tail' fashion that is characteristic for polyketide biosynthesis [14]. The biosynthetic origin of C(12) and C(13) is unknown. C(11), C(10), C(9) and C(8) are again derived from a propionate and an

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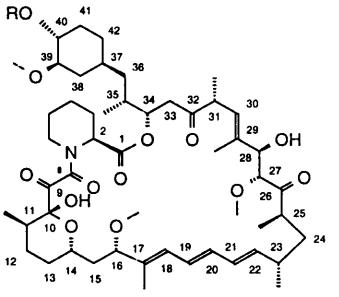
acetate unit, respectively. After condensation of one molecule of L-pipecolic acid (the biosynthetic origin is L-lysine [15]) to the chain, the lactone ring is enzymatically closed between position C(1) and C(34). The O-methyl-groups in position C(16), C(27) and C(39) are derived from methionine.



This information presented the possibility that, in contrast to published results with FK 506 [16], incorporation of more complex precursors such as L-[$^{14}C_n$]pipecolic acid and (1R,3R,4R)-3,4-dihydroxy[$^{14}C_n$]cyclohexanecarboxylic acid might represent a practicable approach to carbon-14 labelled rapamycin. To meet the specific activity of about 100 mCi/mmol, however, four-fold labelling of rapamycin seemed essential; this called for simultaneous addition of doubly labelled precursors (n = 2).

The following describes the synthesis of the carbon-13,14 labelled precursors.

Synthesis of L-[$^{13,14}C_1$] - and D-[$^{13,14}C_1$] Pipecolic acid

Monolabelled pipecolic acid was expected to be sufficient for optimizing fermentation conditions. Because the direct spermidine-mediated enantioselective approach via carboxylation of the achiral lithiated piperidine moiety ("Hoppe-approach") is not applicable to 6-membered rings [17], we synthesized racemic [^{13,14}C]pipecolic acid by carboxylation of lithiated N-(*tert*-butoxycarbonyl)piperidine following the procedure described for the synthesis of 2-formyl-piperidine [18]. It is worth noting that the subsequent preparative separation of both antipodes was easily possible by preparative HPLC using a chiral Teicoplanin phase [19].

Synthesis of L-[$^{14}C_2$]Pipecolic acid 7

In the past few years Voges [20] has developed several sultam derivatives as highly valuable building blocks for the synthesis of enantiomerically pure singly/multiply labelled materials. (-)-Diphenylmethylen[$^{14}C_2$]glycinyl bornane-10,2-sultam **2** ([$^{14}C_2$]DPMGBS) [21], a synthon for enantiomerically pure α-amino acids readily available in a one step by reaction of bromo[$^{14}C_2$]acetyl bornane-10,2-sultam **8a** ([$^{14}C_2$]BABS) with benzophenone imine, was demonstrated to be applicable also to the synthesis of multiply labelled e.p. cyclic amino acids. Reaction of lithiated (-)-[$^{14}C_2$]DPMGBS **2** with 4-chlorobutyl iodide **3** and hydrolytic cleavage of the diphenylmethylene group with 2N HCl-THF resulted in α-amino-ω-chlorohexanoylbornane-10,2-sultam hydrochloride **5**, which was easily purified by trituration in dry ether. Heating of **5** at 80 °C in the presence of ethyldiisopropylamine and tetrabutylammonium iodide promoted cyclisation and, after basic cleavage, resulted in the free L-[$^{14}C_2$]pipecolic acid **7** with an overall yield of > 46%. The enantiomeric excess was determined to be > 98% ee (+/- 1 % ee) by HPLC.

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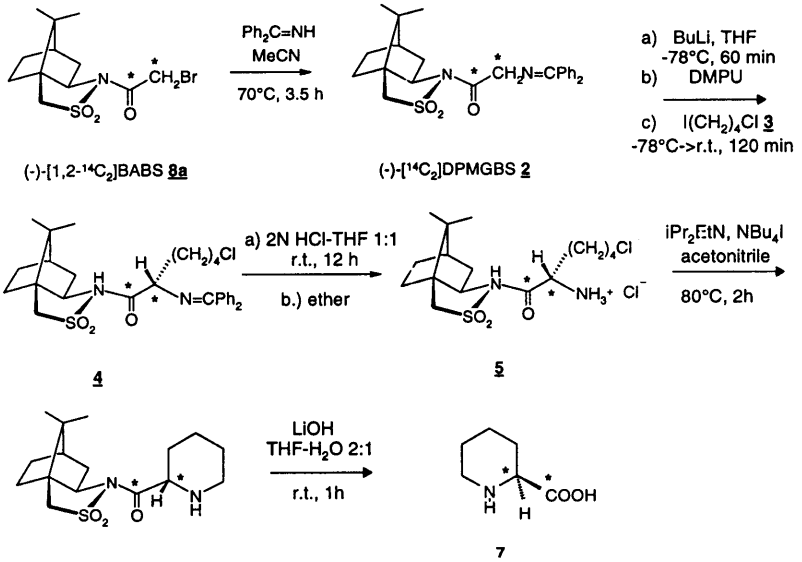
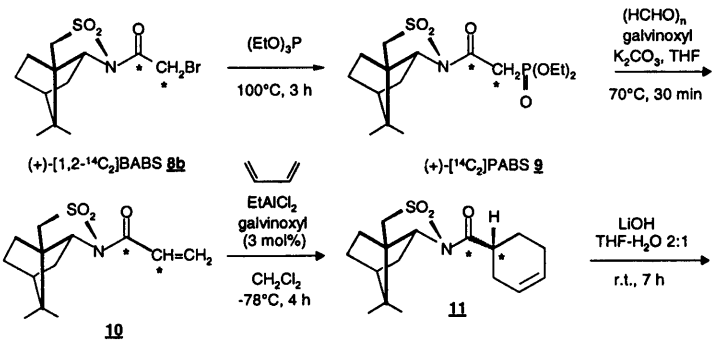
Scheme 1: Stereoselective synthesis of L-[$^{14}C_2$]pipecolic acid **7**



Synthesis of [$^{14}C_2$]DHCCA **14**

(+)-Diethyl phosphono[$^{14}C_2$]acetyl bornane-10,2-sultam **9** ([$^{14}C_2$]PABS), derived from (+)-bromo[$^{14}C_2$]acetyl bornane-10,2-sultam **8b** ([$^{14}C_2$]BABS) by Arbuzov-reaction (triethyl phosphite, 100 °C, 3 h, >95%), was reacted with paraformaldehyde to give the (+)-[1,2-$^{14}C_2$]acroyl sultam **10**. This proved to be unexpectedly stable in respect of radiation-induced side reactions. Subsequent treatment with excess butadiene at -78 °C in dichloromethane in the presence of $EtAlCl_2$ gave diastereomerically pure (+)-(1R)-[$^{14}C_2$]cyclohexenoyl sultam **11** in a 50% overall yield. To suppress potential polymerization reactions, 0.1 equivalents of galvinoxyl were added as free radical scavenger [22]. Hydrolytic cleavage of the auxiliary (**11** -> **12**), and epoxidation of the free acid with MCPBA gave the corresponding epoxy-derivative, which was subject to intramolecular lactone formation when heated with NEt_3 [23]. Spectroscopic analysis of the lactone **13** revealed a diasteromeric (i.e. enantiomeric) excess of > 95% ee. Acid-catalysed hydrolysis provided the final precursor **14** in 15% overall yield.

Scheme 2: Stereoselective synthesis of [$^{14}C_2$]DHCCA **14**



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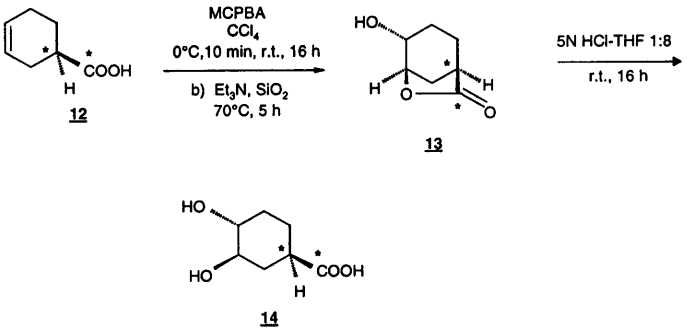


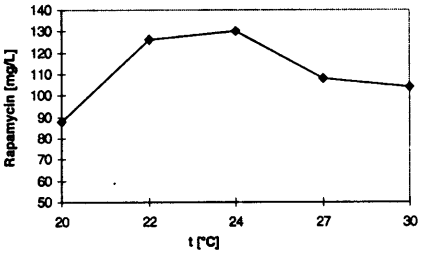
Based on the methods described above, the precursors required were readily obtainable on a scale of several hundred mCi.

Optimization of the process conditions

In a batch fermentation process, rapamycin was produced by *Streptomyces hygroscopicus* mutant RSH 1701. In contrast to other authors [14], who used an almost synthetic medium (enriched with 5 g/l yeast extract as sole complex ingredient), we performed all labelling experiments in a complex production medium. Neither L-pipecolic acid nor e.p. DHCCA were present in the complex medium components in order to avoid any exogenous dilution.

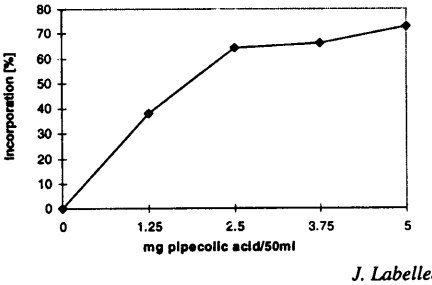
As anticipated, the productivity was significantly dependent on the incubation temperature. Optimum productivity, with 130 mg/l, was observed at 24 °C (see figure 1).

Figure 1: Correlation of the rapamycin production and temperature



Under these conditions the incorporation rates of both precursors were studied using carbon-13 labelled material synthesized by identical routes as described above. Addition of varying amounts of labelled L-pipecolic acid revealed incorporation rates up to 73%. Even at only 4-fold molar excess of L-pipecolic acid (compared to the amount of rapamycin formed) significant incorporation rates of 60% were observed.

Figure 2: Incorporation of L-[^{13}C]pipecolic acid into rapamycin [% labeled]



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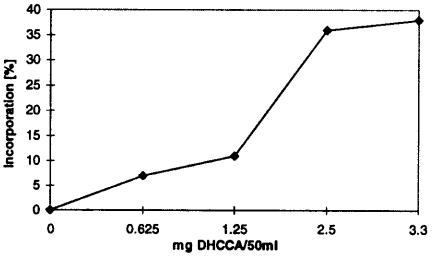
Similar studies demonstrated lower but nevertheless acceptable incorporation rates for e.p. [^{13}C]DHCCA. The maximum rate achievable under the conditions chosen was 37%. This result is consistent with the values published by Staunton et.al. [11].

Figure 3: Incorporation of e.p.[^{13}C]DHCCA into rapamycin [% labeled]



On the basis of these incorporation rates the maximum specific acitivity of four-fold labelled rapamycin could be expected to be in the range of 130 mCi/mmol. Pilot studies with radiolabelled precursors were performed to demonstrate the compatibility of the microorganisms with radioactivity and to provide the opportunity for achieving a balance between the radioactivity which was employed and that which was isolated. Since productivity was practically unchanged, the β-radiation of C-14 would not seem to have significant impact on the biological activity of the strain. The recovery rate of activity was found to be in the range of 80-95%, which demonstrates that no significant amounts of activity were lost in the form of volatile metabolites (i.e. CO_2).

Process

Nearly 150 fermentation batches were run, divided into five operations. Despite the relatively high number of individual batches the time required for the complete preparative fermentation process (excl. pilot studies) was only about 25 working days. Table 1 displays the results:

Table 1: Data for the preparative fermentation process (operations 1-5)

Run	Addition L-Pip. [mg/50ml]	Addition DHCCA [mg/50ml]	No. batches	Yield crude [mCi]	Purity crude [%]	Yield purified [mCi]	Purity purified [%]	Yield radioch. [%]	Re-covery [%]
1	3.8 (3.6mCi)	4 (2.9mCi)	12	13.5	60	7.2	>95	9.2	>85
2	2.3 (2.05mCi)	3.4 (2.34mCi)	30	30.5	71	17.5	>98	13.3	>90
3	2.3 (2.05mCi)	3.7 (2.54mCi)	30	32.8	68	17.2	>98	12.5	>95
4	2.3 (2.05mCi)	-	30	21.4	80	12.4	>97	20.2	>95
5	2.3 (2.05mCi)	4.45 (3.06mCi)	30	34.8	58	17.1	>98	11.2	>92

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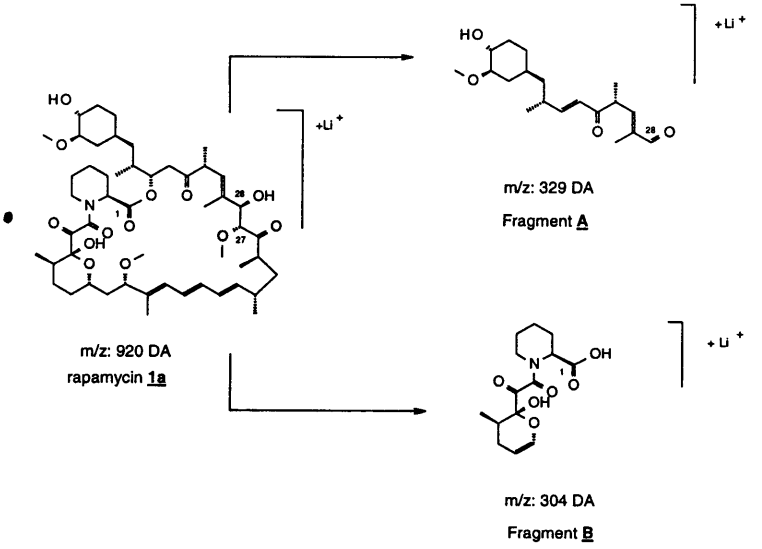
The data in Table 1 show the good reproducibility of this convenient process. An important aspect is the high radiochemical purity, which facilitates the subsequent HPLC-purification step. The radiochemical yield was consistently higher than 10%. In view of the complex structure, this is an excellent result. Of practical relevance was the nearly complete recovery of the radioactivity employed.

Distribution of the Label

Based on the method described [$^{14}C_4$]rapamycin **1a** was labelled in the positions 1, 2 (derived from L-[$^{14}C_2$]pipecolic acid **7**), 36 and 37 (derived from e.p.[$^{14}C_2$]DHCCA **14**). The specific activities of **1a** (run 1,2,3 and 5) were determined to be in the range 100 - 110 mCi/mmol by mass spectroscopy.

To quantify the different metabolites, however, it is also necessary to know exactly how the label is distributed. Although the relative incorporation rates in the upper and the lower part of the molecule could be controlled by varying the ratio of the added precursors, an analytical method was imperative for final demonstration of proof. Under controlled MS-conditions Moser and Hauck [10] observed different fragments containing the respective labelling positions.

Scheme 3: MS-fragmentation of [$^{14}C_4$]rapamycin **1a** (ESI)



Extremely small amounts of [$^{14}C_4$]rapamycin **1a** (run 2,3 and 5) were continously injected and analysed on-line using ESI-source fragmentation conditions. The incorporation rates emerged from an interpretation of the molecular ion and the different fragmentation ion clusters [24]. The results are summarized in the following table.

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Table 2: Approximative distribution of the label between fragment A and B determined by mass spectroscopy

Run	Spec.Act. Fragm. A [mCi/mmol]	Incorp. Fragm. A [%]	Spec.Act A to Spec.Act 1a [%]	Spec.Act. Fragm. B [mCi/mmol]	Incorp. Fragm.B [%]	Spec.Act. B to Spec.Act. 1a [%]	Specific Activity 1a [mCi/mmol]
2	38	28	38	62.4	50	62	100.4 (1)
							107 (2)
3	38	28	38	62.4	50	62	100.4 (1)
							107 (2)
5	38.7	29	39	60.5	48	61	99.2 (1)
							109 (2)

(1) calculated as sum of the specific activities of the fragments A and B; (2) calculated by interpretation of the molecular ion cluster;

Method: ESI: solvent CH_3CN : H_2O 1:1, flow 5 µl/min, acquisition 910-955 amu (parents, 10 scans accumulated) and 290-340 amu (fragments, 20 scans accumulated) in 3 sec., source temp. 80 °C, nebulizing gas 20 l/min., drying gas 300 l/min., U_{ion} +3.8 kV, $U_{cone\ voltage}$ +100V (parents), +80V (fragments), injected amount: 2 µg of [$^{14}C_4$]rapamycin (equivalent to 240 nCi dissolved in 40 µl of acetonitrile)

Cross-checking of the results defines the margin of error to be about +/- 10. The precision of the results is expected to improve significantly, when increasing the amount of material to be analysed. Nevertheless, the incorporation rates observed are in fairly good agreement with those expected by the pilot studies (see figure 2 and 3). The approximate distribution of the activities on the fragments A and B is represented by the ratio 1.5 to 1.

Conversion of [$^{14}C_4$]Rapamycin 1a into [$^{14}C_4$]NVP RAD001 1b

Using the procedures elaborated and optimized by our Preclinical Department and Chemical Development [25] carbon-14 labelled rapamycin **1a** was selectively alkylated at position C(40).

Monosilylated ethylenglycol is converted into its triflate **15**, which alkylates rapamycin **1a** in a mixture of toluene/dimethoxyethane within 3h at 50°C. After extractive separation from unreacted rapamycin **16** was deprotected by treatment with acetic acid. Final HPLC-purification afforded **[$^{14}C_4$]NVP RAD001 1b** in 25% overall yield. Experimental details will be reported elsewhere.

Conclusion

L-[$^{14}C_2$]Pipecolic acid **7** and (1R,3R,4R)-[$^{14}C_2$]DHCCA **14**, which were made available by stereoselective syntheses, proved to be highly suitable precursors for the biological labelling of rapamycin. The resulting fermentation process is characterized by good reproducibility, high radiochemical purity of the crude product and excellent radiochemical yields. The almost complete recovery of the activity employed avoided any radiation safety problems. In a final regioselective alkylation step the rapamycin **1a** was converted to **[$^{14}C_4$]NVP RAD001 1b**.

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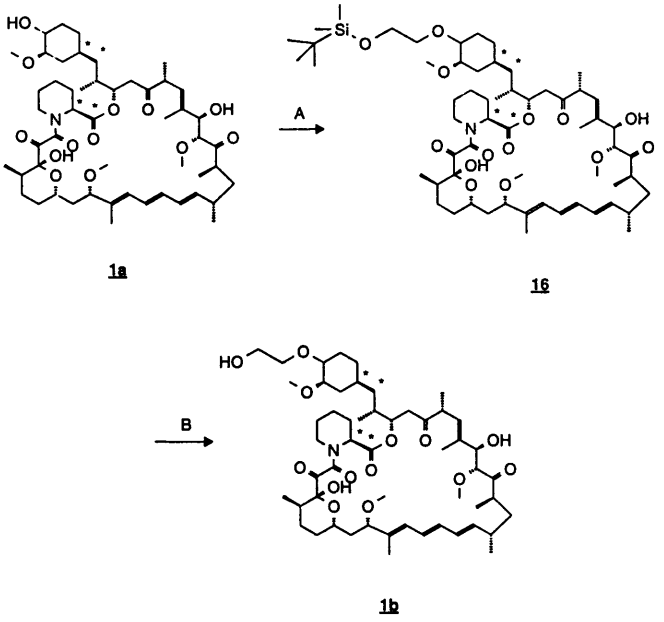
Scheme 4: Synthesis of [$^{14}C_4$]NVP RAD001 1b



Reaction conditions: synthesis of triflate 15 : ethylene glycol mono-*tert*-butyldimethylsilyl ether, N,N-diisopropylethylamine, trifluoromethanesulfonic acid, CH_2Cl_2, -18°C/20', RT/60'; A: N,N-diisopropylethylamine, toluene/dimethoxyethane, 50°C, 3h; B: CH_3COOH, n-heptane, water, ethyl acetate, RT, 60';

Experimental details:

Materials

Unless otherwise mentioned, chemicals and radiochemicals were purchased from commercial suppliers. *Streptomyces hygroscopicus* mutant RSH 1701 was obtained from Biochemie Kundl, Austria. The identity of intermediates and precursors was verified by either chromatographic and/or spectroscopic methods.

Synthesis of L-[$^{13,14}C_1$]- or D-[$^{13,14}C_1$]Pipecolic acid ($C_6H_{11}NO_2$, MW. 129.16)

TMEDA (2.25 ml, 15 mmol) and *sec*-butyllithium (0.95M in cyclohexane, 15.8 ml, 15 mmol) were consecutively added at -60°C to a solution of N-(*tert*-butoxycarbonyl)piperidine (2279 mg, 15 mmol) in anhydrous Et_2O. The reaction mixture was stirred at 20 °C for 10 min. and finally reacted with [$^{13,14}C$]CO_2 , which was released from [$^{13,14}C$]$BaCO_3$ (280 mCi, 987 mg, 5 mmol). After 2 h at -15 °C the reaction mixture was quenched by H_2O/THF 1:1 (3 ml), basified with 2N NaOH to pH 12. The aqueous phase was extracted with Et_2O and subsequently acidified with citric acid (20%) to pH 4. The acidic aqueous phase was extracted with Et_2O (4x), and the combined organic phases dried (Na_2SO_4) and evaporated to afford crude N-(tert.butoxycarbonyl)pipecolic acid (709 mg, 62%). Without further purification the residue was treated with 6N HCl (23 ml) in THF (23 ml) at room temperature for 2 h. The reaction mixture was freed from THF and finally purified by ion-exchange chromatography (Amberlite IR-120). Preparative chromatography (Chirobiotic T, ASTEC, H_2O : EtOH 84 : 16, 30°C, 20 ml/min.) succeeded in separating the antipodes.

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Synthesis of (-)-Diphenylmethylen[$^{14}C_2$]glycinyl bornane-10,2-sultam **2**

((-)-[$^{14}C_2$]DPMGBS, $C_{25}H_{28}N_2O_3S$, MW. 436.57)

N,N-Diisopropylethylamine (2.75 ml, 15.8 mmol), benzophenone imine (5.05 ml, 30.1 mmol), and molecular sieve (3 A°, 1.8 g) were consecutively added to a solution of carefully dried (-)-bromo[$^{14}C_2$]acetyl bornane-10,2-sultam ((-)-[$^{14}C_2$] BABS) (429 mCi, 1264 mg, 3.76 mmol) in anhydrous acetonitrile (20 ml). The mixture was stirred under argon at 70 °C for 3.5 h. After cooling to room temperature, the reaction mixture was quenched with cold saturated aqueous $NaHCO_3$ solution and repeatedly extracted (5x) with ethyl acetate. The combined organic extracts were washed with brine, dried (Na_2SO_4), and evaporated to give an orange oil. Purification by flash chromatography (silica gel 0.04-0.063 mm, hexane - ethyl acetate - triethylamine 85 : 15 : 0.05; remark: in order to avoid hydrolysis of the product it is necessary to pretreat the silica gel with mobile phase at least 10 h before use) yielded 1.4 g (3.2 mmol, 85%) of (-)-[$^{14}C_2$]DPMGBS as a yellowish foam.

Synthesis of (2S)-(4-chlorobutyl)-(-)-[$^{14}C_2$]DPMGBS **4** ($C_{29}H_{35}ClN_2O_3S$, MW. 527.13)

A solution of (-)-[$^{14}C_2$]DPMGBS (326 mCi, 1250 mg, 2.86 mmol) in anhydrous THF (15 ml) was cooled to -78 °C under argon. *n*-Butyllithium in hexane (1.5M, 2.06 ml, 3.09 mmol) was added via a syringe, and the solution was kept at -78 °C for another hour. Then DMPU (4.6 ml) and 1-chloro-4-iodobutane (3120 mg, 14.3 mmol) were consecutively added dropwise, the cooling stopped, and the reaction mixture stirred at room temperature for another 2 h. Then it was quenched with cold water, and repeatedly extracted (5x) with ethyl acetate. The combined organic phases were washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure yielding a dark orange oil. Purification by flash chromatography (silica gel 0.04-0.063 mm, hexane - ethyl acetate - triethylamine 80 : 20 :0.5) yielded 977 mg (1.85 mmol, 65%) of product as a colourless oil.

Synthesis of (2S)-(4-chlorobutyl)-(-)-[$^{14}C_2$]-glycinyl bornane-10,2-sultam hydrochloride **5** ($C_{16}H_{28}Cl_2N_2O_3S$, MW. 399.38)

A solution of (2S)-(4-chlorobutyl)-(-)-[$^{14}C_2$]DPMGBS (997 mg, 1.85 mmol) in 50 ml of 1N HCl-THF 1:1 was stirred at room temperature for 18 h. The solvent was lyophilized at room temperature, the residue was repeatedly triturated with dry ether (5x), the ether was decanted and the residual hydrochloride was dried at room temperature *in vacuo* to give 649 mg (1.63 mmol, 88%).

Synthesis of L-[$^{14}C_2$]pipecolic acid **7** ($C_6H_{11}NO_2$, MW. 129.16)

Tetrabutylammonium iodide (4318 mg, 11.7 mmol) and N,N-diisopropylethylamine (545 µl, 3.1 mmol) were consecutively added to a solution of (2S)-(4-chlorobutyl)-(-)-[$^{14}C_2$]glycinyl bornane-10,2-sultam hydrochloride (186 mCi, 649 mg, 1.63 mmol) in acetonitrile (150 ml). The reaction mixture was heated to 80°C and stirred at this temperature for another 2 h. After cooling to room temperature the solvent was replaced by THF-H_2O 2 : 1 (40 ml). Lithium hydroxide monohydrate (249 mg, 5.9 mmol) was added to the reaction mixture, which was stirred at room temperature for 3 h. The solvent was evaporated and the residue purified by flash chromatography (silica gel 0.04-0.063 mm, methanol - acetonitrile - toluene - aqu. ammonia 50 : 25 : 25 : 10) to afford L-[$^{14}C_2$]pipecolic acid (170 mg, 1.32 mmol, 190.7 mCi, 81%). The radiochemical and enantiomeric purity was determined by HPLC (Chirobiotic T (ASTEC), 220x4.6 mm, water - ethanol 86 : 14, RT, 1.0 ml/min., 210 nm) with > 98% and >98% ee, respectively. The specific activity was determined to be 114.2 mCi/mmol by MS (ESI, negative ion mode).

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Synthesis of diethylphosphono[^{14}C]acetyl (+)-bornane-10,2-sultam **9** ((+)-[^{14}C]PABS, $C_{16}H_{29}NO_6PS$ MW.394.45)

1597 mg (4.75 mmol, 513 mCi) of bromo[$^{14}C_2$]acetyl bornane-10,2-sultam **8** [=(+)-[$^{14}C_2$]BABS was reacted with 6 ml of triethyl phosphite under an atmosphere of argon at 100 °C for 3 h. The resulting reaction mixture was allowed to cool to room temperature and subsequently separated by column chromatography (silica gel H 0.063-0.04 mm Merck, hexane- ethyl acetate 1:1). 1866 mg (4.73 mmol, 99%) of (+)-[$^{14}C_2$]PABS of a colourless oil were isolated, which slowly crystallized overnight.

Synthesis of [1,2-$^{14}C_2$]acroyl (+)-bornane-10,2-sultam **10** ((+)-[1,2-$^{14}C_2$]ACBS, $C_{13}H_{19}NSO_3$ MW. 269.63)

A solution of 1866 mg (4.73 mmol, 511 mCi) of (+)-[$^{14}C_2$]PABS **9** in 20 ml of dry THF was sequentially treated with solid paraformaldehyde (565 mg, 18.8 mmol), 176 mg of galvinoxyl and 1324 mg of potassium carbonate. The reaction mixture was stirred at 70 °C for 30 min. Afterwards, the solution was almost completely removed under reduced pressure and the resulting crude material carefully triturated with dichloromethane. The combined extracts were filtered, the filtrate was evaporated and the residue separated by column chromatography (silica gel H 0.063-0.04 mm Merck, hexane- ethyl acetate 1:1) yielding 658mg (2.44 mmol, 264 mCi, 51.7%) of slightly brownish (+)-[$^{14}C_2$]ACBS.

Synthesis of (R)-[1,7-$^{14}C_2$]cyclohex-3-enylcarbonyl-(+)-bornane-10.2-sultam **11** ($C_{17}H_{25}NO_3S$ MW.323.46)

A 250 ml flame-dried, 3-necked, round-bottomed flask equipped with an argon balloon, a magnetic stirring bar, a septum and a gas inlet tube was charged with 658 mg (2.44 mmol, 264 mCi) of (+)-[1,2-$^{14}C_2$]ACBS **10**, 30mg of galvinoxyl and 20 ml of dry dichloromethane. The resulting solution was cooled to -78 °C and treated dropwise with 3.9 ml of 1M Et_2AlCl-solution in hexane. Then, 2 ml of condensed 1,3-butadiene were slowly transferred, and the reaction mixture was stirred at -78 °C for 4 h. Excess butadiene was allowed to slowly evaporate at room temperature for 20 min.. The reaction was quenched by addition of saturated aequeous Na_2SO_4-solution. The reaction product formed was repeatedly extracted with additional dichloromethane, the combined organic phases were dried over Na_2SO_4 and evaporated. The residue was separated by column chromatography (silica gel H 0.063-0.04 mm Merck, hexane- ethyl acetate 1:1), and the isolated material (790 mg) was crystallized from cyclohexane to give 417 mg (1.29 mmol, 139 mCi, 52.9%) of yellow solid material. ^{1}H-NMR spectroscopy showed the compound to have a diastereomeric purity of > 98%.

To avoid radiation induced side reactions the material was stored dissolved in 50 ml of toluene at -80 °C.

Synthesis of (R)-[1,7-$^{14}C_2$]cyclohex-3-ene carboxylic acid **12** ($C_7H_{10}O_2$ MW.126.16)

417 mg (1.29 mmol) of (R)-[1,7-$^{14}C_2$]cyclohex-3-enylcarbonyl (+)-bornane-10,2-sultam **11** in 15 ml of THF were treated with 25 ml of 1N aqueous LiOH-solution and stirred for 3 h at room temperature. Most of the solvent was removed *in vacuo*. The residue was taken up in ether and water, the phases separated, the aqueous alkaline solution was cooled to 0°C and acidified with conc. HCl to pH 0. The liberated acid was repeatedly extracted with ether, the combined ether phases dried over Na_2SO_4, evaporated under reduced pressure, and the isolated product dried *in vacuo* to give 164 mg (1.3 mmol, 140 mCi, 100%) of yellow cristalline solid.

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Synthesis of (1R, 4R, 5R)-4-hydroxy-6-oxa-[1,7-$^{14}C_2$]bicyclo[3,2,1]octan-7-one **13** ($C_7H_{10}O_3$ MW.142.16)

A solution of 328 mg (2.6 mmol, 280 mCi) of (R)-[1,7-$^{14}C_2$]cyclohex-3-enylcarboxylic acid in 20 ml of CCl_4 was treated with 588 mg (3.4 mmol) freshly recrystallized 3-chloroperbenzoic acid at room temperature overnight. Subsequently, 0.9 ml of triethylamine were added and the resulting reaction mixture was slowly warmed up and stirred for 4 h at 70 °C. After 2 h 0.9 ml of triethylamine were injected. Finally, the solvent was removed *in vacuo*, and the residue purified by column chromatography (silica gel H 0.063-0.04 mm Merck, hexane- ethyl acetate 1:1) to yield 228 mg (1.6 mmol, 173 mCi, 61.5%) of white crystalline lactone. The specific activity was determined to be 111.7 mCi/mmol by MS (FAB).

Synthesis of (1R,3R,4R)-3,4-dihydroxy[1,7-$^{14}C_2$]cyclohexane carboxylic acid **14**

(= (1R,3R,4R)-[1,7-$^{14}C_2$]DHCCA, $C_7H_{12}O_4$ MW. 160.17)

94 mg (0.66 mmol, 71 mCi) of the lactone were dissolved in 20 ml of THF. The solution was cooled to 0 °C, treated with 1.1 ml of 5N HCl, slowly warmed up and stirred at room temperature overnight. After completion of the hydrolysis (TLC-control: silica gel 60 F254, hexane-ethyl acetate 1:1) the solvent was removed under reduced pressure, the residue was twice taken up in ether, the solvent again removed to receive 98 mg (0.61 mmol, 66 mCi, 93%) of colourless oily (1R,3R,4R)-[1,7-$^{14}C_2$]DHCCA **14**.

Fermentation Process

4.5 ml of bacterial suspension from a preculture was added to 500 ml Erlenmeyer flasks filled with 50 ml of a complex production medium, which typically contained glucose, soybean meal, cottonseed flour and a mineral salt solution. The flasks were incubated on a rotary shaker at 230 rpm for four days at 24°C. Labelled precursors **7** and **14** were fed after 24 h (table 1). After an additional 72 h of incubation the broth was thorougly extracted with ethylacetate. The combined organic phases were evaporated and the residue was treated with n-hexane (1 h, r.t.). The solvent was decanted and the residue dried under HV was purified by preparative HPLC (C-18 250 x 21.4 mm, acetonitrile : water 55 : 45, 30 °C, 30 ml/min.). Yields of **1a** are listed in table 1.

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