THE PREPARATION OF ^{3}H-LABELED ACYCLIC NUCLEOSIDE PHOSPHONATES AND STUDY OF THEIR STABILITY

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9-(2-Phosphonomethoxyethyl)-2,6-diamino-[8-^{3}H]purine (**4**), 9-(2-phosphonomethoxyethyl)-[8-^{3}H]guanine (**6**) and (*R*)-9-(2-phosphonomethoxypropyl)-[8-^{3}H]adenine (**11**) with specific activities of 10.9, 7.9 and 16 Ci/mmol, respectively, were prepared by a catalytic dehalogenation of the corresponding 8-bromo derivatives **1**, **2** and **9**. The rate of the exchange of the tritium label on C-8 of the purine ring in title compounds with the hydrogen of water under physiological pH at 20 °C was studied using ^{3}H NMR. The loss of ^{3}H-label attained 7% in [8-^{3}H]tenofovir (**11**), 10% in [8-^{3}H]PMEDAP (**4**) and 12% in [8-^{3}H]PMEG (**6**) after the period of 3 weeks. Storage at a temperature of –196 °C in liquid nitrogen ensured a better than 97% radiochemical purity of the prepared labeled compounds even after a six-month period.
Keywords: Tritium; ^{3}H NMR; Acyclic nucleotide analogues; ^{3}H-label stability; Tenofovir; Adefovir; Radioactive labelling.

Acyclic nucleoside phosphonates (ANPs) exhibit a broad spectrum of antiviral and cytostatic activities[1]. Among them, (*R*)-9-(2-phosphonomethoxypropyl)adenine ((*R*)-PMPA, tenofovir) (**10**) is active against retroviruses. Its prodrug tenofovir disoproxil fumarate was approved for the treatment of the human immunodeficiency virus (HIV) infection (Viread®) as well as its fixed combinations with emtricitabine (Truvada®), emtricitabine and efavirenz[2] (Atripla®). Tenofovir was also approved for the treatment of the hepatitis B virus (HBV) infection[3] in 2008. 9-(2-Phosphonomethoxyethyl)-2,6-diaminopurine (PMEDAP) (**3**) shows potent *in vitro* and *in vivo* antiviral activity against both DNA viruses and retroviruses[4]. 9-(2-Phosphonomethoxyethyl)guanine (PMEG) (**5**) has also antiviral activity, but the interest in PMEG has focused on its anticancer activity. It is an active compound of GS-9219, a potent antineoplastic agent, targeting preferentially lymphoid cells[5].

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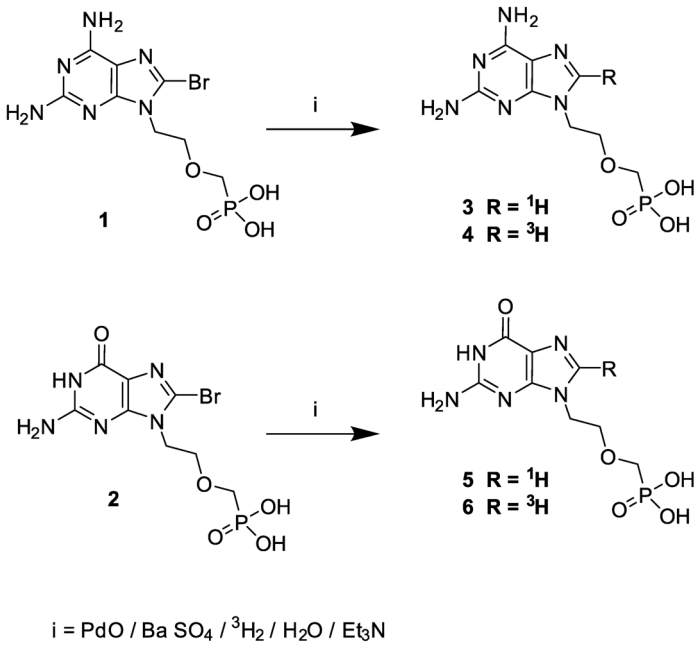
Because of the importance of these compounds their biological activities are still being further explored, the high biological potency is dependent on their transport across the cellular membrane and on their stability inside the cells[6]. The above mentioned ANPs were labeled by the ^{3}H radionuclide at position 8 of the purine ring to facilitate their detection in the biochemical assays monitoring their cellular uptake and metabolism in the cell. It has previously been reported in the literature[7] that the tritium label at position 8 of the purine ring can under certain conditions undergo an exchange of the hydrogen with the solvent. We have thus studied the stability of the tritium label in water and phosphate buffer (pH 7.4) solutions by ^{3}H NMR.

RESULTS AND DISCUSSION

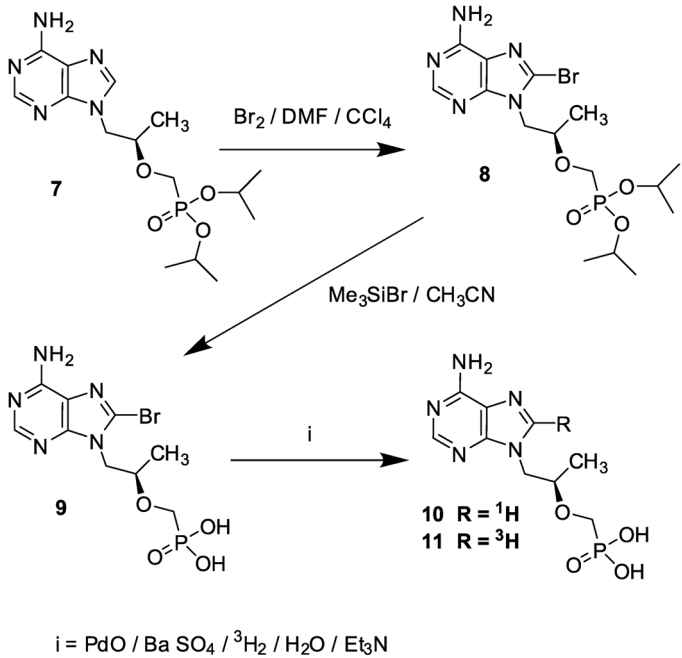
Syntheses of ^{3}H-labeled Acyclic Nucleoside Phosphonates

As the 8-bromo derivatives of PMEDAP (**1**) and PMEG (**2**) have recently been prepared[8], a reductive catalytic dehalogenation has been chosen as the method of the introduction of tritium into the title compounds (Scheme 1).



SCHEME 1

8-Bromo tenofovir (**9**) was prepared using the same procedure as has been published for derivatives **1** and **2**, i.e. the bis(2-propyl) (*R*)-9-(2-phosphonomethoxypropyl)adenine (**7**) was first brominated to yield bis(2-propyl) 8-bromo-(*R*)-9-(2-phosphonomethoxypropyl)adenine (**8**). The deprotection of the phosphonate group of derivative **8** was then accomplished by a bromotrimethylsilane procedure (Scheme 2).



SCHEME 2

The 5% palladium oxide on the barium sulfate was used as a catalyst, triethyl amine was used as the base and the dehalogenation was performed in water. In Table I, the yields of the crude products, the radiochemical purities after the purification on radio-HPLC and the specific activities assayed by two independent methods have been summarized.

The low yield of crude [8-^{3}H]PMEDAP (**4**) was caused by 1 ml of acetic acid having been added to the reaction mixture in an attempt to prevent the exchange of the tritium label in alkaline milieu. Unfortunately, the solubility of the product was suppressed and it remained absorbed on the catalyst. During the preparation of [8-^{3}H]PMEG (**6**), the addition of acetic acid was omitted, which improved the yield. However, even though the attained specific activities for [8-^{3}H]PMEDAP (**4**) and [8-^{3}H]PMEG (**6**) were sufficient

for the planned biochemical experiments, it seemed that higher specific activities should have been obtained. We evaluated the weak spots of the experimental procedure. The reaction of the 8-bromo derivatives **1** and **2** with gaseous tritium was performed by the service laboratory. The removal of the catalyst by centrifugation was preceded by the removal of the labile activity (by successive evaporations of the three 3-ml water portions from the reaction mixture). We concluded that there is probably a backward exchange of the tritium with the water in the presence of the catalyst during the procedure of the removal of the labile activity. During the preparation of [8-^{3}H]tenofovir, the catalyst was removed by filtration through a 0.45-μ teflon syringe filter, prior to the labile activity removal. The much higher specific activity of [8-^{3}H]tenofovir (**11**) (see Table I) in comparison with [8-^{3}H]PMEDAP (**4**) and [8-^{3}H]PMEG (**6**) supports the above-mentioned idea of the backward exchange with water in the presence of the catalyst. The structures of the tritiated compounds **4**, **6** and **11** were confirmed by their ^{1}H NMR spectra. The distinctive position of the label at C-8 of the purine ring in all the three compounds was confirmed by ^{3}H NMR spectra.

TABLE I
The yields, radiochemical purities, specific activities and stability data of the tritiated compounds

Parameter	[^{3}H]PMEDAP (**4**)	[^{3}H]PMEG (**6**)	[^{3}H]Tenofovir (**11**)
Yield of the crude product, mCi	25.3	112.3	226
Radiochemical purity after HPLC, %	>99	>99	>98
Speccific activity (UV), Ci/mmol	10.9	7.9	16
Specific activity (^{1}H NMR), Ci/mmol	10.7	7	15.7
Radiochemical purity after 5 months of storage[a], %	97.1	97.5	97.7
Radiochemical purity after 11 months of storage[a], %	96.1	96.33	97.1

[a] The tritiated compounds were stored at a concentration of 2 mCi/ml of water at –196 °C in liquid nitrogen.

The Stability of ^{3}H-labeled Acyclic Nucleoside Phosphonates

To mimic the physiological conditions, the stability of the tritium label on C-8 of the purine ring of [^{3}H]PMEDAP (**4**), [^{3}H]PMEG (**6**) and [^{3}H]tenofovir (**11**) was studied in 50 mM phosphate buffer, pH 7.4. For comparison, the loss of tritium was followed in neutral water solution for all three ^{3}H-labeled derivatives and in alkaline solution for [^{3}H]tenofovir (**11**). The percentage of tritium label on C-8 was calculated from integrals of intensity of NMR signals of ^{3}H on C-8 and in water (no other ^{3}H signals appeared in spectra during a 60-day period). The results are summarized in Fig. 1. Under neutral conditions, the tritium label on C-8 was reasonably stable and after 3 weeks there was still more than 95% of the tritium bound in the purine ring. At pH 7.4, the exchange rate was higher and after 3 weeks the content of ^{3}H-label in guanosine derivative **6** dropped by 12%, in diaminopurine derivative **4** and in adenine derivative **11** by 10 and 7%, respectively. The highest rate of exchange in guanosine derivative **6** is in accordance with the literature data[7a,7b]. On the other hand, our results suggest higher dependence of the exchange rate on the pH in nucleotide analogues **6**, **4** and **11** in comparison with the free bases and the nucleosides. The increase in the rate of the exchange with increasing pH supports the idea of abstraction of tritium from C-8 by hydroxyl anion as a rate determining step.

The results of the radiochemical purity checks of the samples kept in liquid nitrogen are summarized in Table II. In the ^{3}H NMR spectra of all three of the labeled compounds stored in liquid nitrogen for periods indicated in Table II, no ^{3}H signal of water was detected.

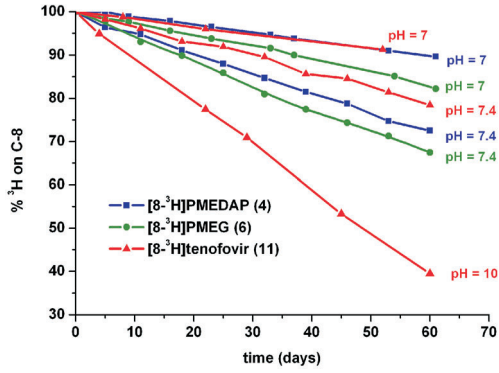


FIG. 1
The exchange of ^{3}H-label on C-8 with water at different pH and 20 °C (the detailed conditions are given in Experimental)

TABLE II
Analytical HPLC retention times

Parameter	8-Br-PMEDAP (**1**)	PMEDAP (**3**)	8-Br-PMEG (**2**)	PMEG (**5**)	8-Br-Tenofovir (**9**)	Tenofovir (**10**)
Retention time, min	15	8.5	17	8	17	8
UV detector, nm	287		253		260	

Column: Synergi 4μ Fusion-RP 80, 250 × 3 mm (Phenomenex); flow: 0.5 ml/min; mobile phase: A – water with 0.25% (v/v) acetic acid, B – acetonitrile with 0.25% (v/v) acetic acid; elution: linear gradient from 0 to 40% B within 10 min

CONCLUSIONS

[8-^{3}H]PMEDAP (**4**), [8-^{3}H]PMEG (**6**) and [8-^{3}H]tenofovir (**11**) with high specific activities and excellent radiochemical purities were prepared. Using ^{3}H NMR spectroscopy, we observed the exchange of the ^{3}H-label on C-8 of purine ring of these labeled compounds with water at room temperature. The rate of the exchange increased with increasing pH of the solution. At pH 7.4, i.e. under the conditions of biochemical experiment on plant cells, the loss of ^{3}H-label attained 7% in [8-^{3}H]tenofovir (**11**), 10% in [8-^{3}H]PMEDAP (**4**) and 12% in [8-^{3}H]PMEG (**6**) after the period of 3 weeks. This exchange must be taken into account when interpreting the results of biochemical experiments with cultivation periods longer than 1 week. The storage of the compounds labeled by tritium at the temperature –196 °C in liquid nitrogen proved to be a very efficient method of conserving sufficient radiochemical purity for more than 6 months.

EXPERIMENTAL

The NMR spectra (δ, ppm) were recorded with a Bruker Avance 400 spectrometer (^{1}H at 400, ^{13}C at 100.6 MHz) in DMSO-d_6 or D_2O. Chemical shifts in DMSO-d_6 were referenced to the solvent signal for ^{1}H δ 2.5 and for ^{13}C δ 39.7. Chemical shifts in D_2O were referenced to 1,4-dioxane for ^{1}H δ 3.75 and for ^{13}C δ 67.19. The ^{3}H and ^{1}H NMR spectra of the labeled compounds were measured on a Bruker Avance II 300 MHz spectrometer in D_2O, with the signal of water at 4.7 ppm taken as an internal standard. Mass spectra were measured on a LCQ classic spectrometer using electrospray ionization (ESI). HPLC was performed on a system consisting of a WATERS Delta 600 Pump and Controller, a WATERS 2487 UV detector and a RAMONA radio chromatographic detector from Raytest (Germany) with interchangeable fluid cells. For preparative runs, the cell with a single small crystal of solid scintillator was used; for analytical runs, the column effluent was mixed with a Zinsser Quickszint Flow 302 cocktail at a 1:3 ratio. The data were collected and processed using Empower 2.0 software. Radioactivities were measured on a Perkin–Elmer Tri-Carb 2900 liquid scintillation counter (LSC) in a Zinsser Quicksafe A cocktail. The evaporations were done using a CentriVap concentrator from Labconco. The 5% PdO on the $BaSO_4$ catalyst and the triethyl amine were purchased from Sigma–Aldrich.

Bis(2-propyl) 8-Bromo-(*R*)-9-(2-phosphonomethoxypropyl)adenine (**8**)

Bis(2-propyl) (*R*)-9-(2-phosphonomethoxypropyl)adenine (**7**) prepared as described in ref.[9] (1.6 g, 3.6 mmol) in DMF (10 ml) was treated with 0.7 M bromine solution in CCl_4 at room temperature for 48 h. The resulting mixture was diluted with $CHCl_3$ and washed with saturated $Na_2S_2O_3$ and brine, with the organic extract subsequently dried over $MgSO_4$. Flash chromatography ($CHCl_3$/MeOH) afforded diester **8** as a white solid (1.08 g, 56%), m.p. 111 °C. ESI MS: 450.1 (85) $[M + H]^+$; 472.1 (100) $[M + Na]^+$. ^{1}H NMR (DMSO-d_6): 8.11 s, 1 H (H-2); 7.39 bs, 2 H (NH_2); 4.45 dh, J(H-C-O-P) = 7.6, J(CH-CH_3) = 6.2, 1 H and 4.36 dh, J(H-C-O-P) = 7.7, J(CH-CH_3) = 6.2, 1 H (CH ipr.); 4.05–4.19 m, 3 H (H-1′, H-2′); 3.75 dd,

*J*gem = 13.9, *J*(H-C-P) = 8.9, 1 H and 3.56 dd, *J*gem = 13.9, *J*(H-C-P) = 9.3, 1 H (PCH_2O); 1.17 d, *J*(3′-2′) = 6.4, 3 H (H-3′); 1.16 d, 3 H, 1.11 d, 3 H, 1.08 d, 3 H, 1.03 d, 3 H, *J*(CH_3-CH) = 6.2 (CH_3 ipr.). ^{13}C NMR (DMSO-d_6): 154.99 (C-6); 152.94 (C-2); 151.19 (C-4); 127.17 (C-8); 119.17 (C-5); 74.98 d, *J*(2′-P) = 12.0 (C-2′); 70.35 d, *J*(C-O-P) = 6.4 and 70.22 d, *J*(C-O-P) = 6.3 (CH ipr.); 62.72 d, *J*(C-P) = 164.4 (PCH_2O); 48.69 (C-1′); 23.65–23.99 m (CH_3 ipr.); 16.94 (C-3′).

8-Bromo-(*R*)-9-(2-phosphonomethoxypropyl)adenine (**9**)

Diester **8** (540 mg, 1.2 mmol) in acetonitrile (10 ml) was treated with Me_3SiBr (1 ml) at room temperature overnight. The volatiles were removed under reduced pressure and the residue was co-distilled with water. The crude product was purified by the preparative HPLC (a gradient of MeOH (0–100%) in H_2O). Crystallization from ethanol afforded a white crystalline product (150 mg, 35%), whose m.p. is not reached below 250 °C, decomposition. ESI MS: 364.1 (38) $[M - H]^-$. ^1H NMR (D_2O, ref. dioxane): 8.37 s, 1 H (H-2); 4.37 m, 2 H (H-1′); 4.05–4.12 m, 1 H (H-2′); 3.62 dd, 1 H, *J*(H-C-P) = 8.90, *J*gem = 13.48 and 3.43 dd, 1 H, *J*(H-C-P) = 8.90, *J*gem = 13.47 (OCH_2P); 1.28 d, 3 H, *J*(3′-2′) = 6.29 (H-3′). ^{13}C NMR (D_2O, ref. dioxane): 150.92 (C-6); 150.09 (C-4); 145.95 (C-2); 132.53 (C-8); 119.44 (C-5); 76.63 d, *J*(2′-P) = 10.27 (C-2′); 65.62 d, *J*(CH_2-P) = 157.73 (OCH_2P); 50.31 (C-1′); 17.14 (C-3′). For $C_9H_{13}BrN_5O_4P$ (366.11) calculated: 29.53% C, 3.58% H, 21.83% Br, 19.13% N, 8.46% P; found: 29.36% C, 3.62% H, 21.57% Br, 18.90% N, 8.00% P.

9-(2-Phosphonomethoxyethyl)-2,6-diamino-[8-^{3}H]purine (**4**)

To 8-bromo PMEDAP **1** (6 mg, 16.3 μmol) in the presence of 27 mg 5% PdO on $BaSO_4$ in a small flask equipped with a magnetic stirrer, water (1.2 ml) and triethyl amine (0.13 ml) were added. The reaction mixture was degassed by three successive freeze-thaw cycles under vacuum. 5 Ci of carrier-free $^3H_2$ were transferred to the reaction flask (with a starting pressure of 640 torr) and the reaction was left to proceed under vigorous agitation at room temperature for 1.5 h. The residual tritium gas was pumped off and the reaction mixture was transferred to a heart-shaped flask (the reaction flask was washed 3 times with 1 ml of water, added to the reaction mixture). Acetic acid (0.5 ml) was added and the solvents were evaporated in a closed system under vacuum (the solvent trap was cooled by liquid nitrogen). The residue was dispersed in 3 ml of water and the mixture was evaporated to dryness. This procedure was repeated once more. After the last evaporation, the residue was dispersed in 3 ml of water and the catalyst was separated by centrifugation. The supernatant was taken off and the catalyst was washed three times with 2 ml of water. The volume of the unified supernatants was adjusted to 10 ml with water. The total activity of the crude product was 25.3 mCi. According to the HPLC analysis of the crude product, there was no starting compound and more than 95% of the activity was in desired product **4**. The crude product was purified on a semi-preparative Synergi 4μ Fusion-RP 80, 250 × 10 mm column (Phenomenex, USA) in the isocratic mode with water containing 0.25% (v/v) of acetic acid as the mobile phase. The yield was 17.65 mCi of the product with a radiochemical purity of >99% (radio HPLC). The ^3H NMR (D_2O, ref. TDO) was 7.98 s (^{3}H-8). The ^1H NMR was identical to that of the standard with the exception of the integration of the H-8 signal. From the decrease of the intensity of the ^{1}H-8 signal, the specific activity was calculated as 10.7 Ci/mmol. The UV method of specific activity assay consisted of a measurement of the volume activity by LSC and an assay of the mass concentration by HPLC at 284 nm. The re-

sult was 10.9 Ci/mmol. Before the solution was stored in liquid nitrogen, the volume activity was adjusted to 2 mCi/ml.

9-(2-Phosphonomethoxyethyl)-[8-^{3}H]guanine (**6**)

8-Bromo PMEG **2** (6.6 mg, 18 μmol) in the presence of 26.5 mg of 5% PdO on $BaSO_4$ and triethyl amine (0.13 ml) in water (1.2 ml) was treated with 5 Ci of carrier-free $^3H_2$ as described for 8-bromo PMEDAP **1** (see above). The work-up was the same with one exception, i.e. no acetic acid was added before the first evaporation of the reaction mixture. The yield of the crude product was 112.3 mCi. According to the radio HPLC, some unreacted 8-bromo PMEG **2** was present; more than 95% of activity was in desired product **6**. One half of the solution of the crude product was evaporated to dryness, dissolved in 1 ml of 0.25% (v/v) ammonium hydroxide and purified on a semi-preparative HPLC column under the same conditions as for [8-^{3}H]PMEDAP **4** (see above). The yield was 31.1 mCi of product with a radiochemical purity of >99% (radio HPLC). The ^{3}H NMR (D_2O, ref. TDO) was 7.93 s (^{3}H-8). The ^{1}H NMR was identical with that of the standard. The specific activity calculated from the ^{1}H NMR was 7 Ci/mmol, the UV assay yielded 7.9 Ci/mmol. Before the solution was stored in liquid nitrogen, the volume activity was adjusted to 2 mCi/ml.

(*R*)-9-(2-Phosphonomethoxypropyl)-[8-^{3}H]adenine (**11**)

8-Bromo (*R*)-PMPA **9** (25.4 mg, 69.4 μmol) in the presence of 26.2 mg of 5% PdO on $BaSO_4$ and triethyl amine (0.13 ml) in water (1 ml) was treated with 5 Ci of carrier-free $^3H_2$ as described for 8-bromo PMEDAP **1** (see above). The reaction mixture was left in contact with tritium gas for 3 h. The reaction flask was disconnected from the tritiation manifold and the reaction mixture was filtered through a 0.45-μ PTFE syringe filter. The reaction flask and filter were washed with three 1-ml portions of water. The clear colorless reaction mixture was evaporated to dryness. The solid white residue was dissolved in 4 ml of water and again evaporated to dryness. The residue was dissolved in 8 ml of water and the activity was verified. The total activity of the crude product was 226 mCi. More than 95% of the activity was in the desired product and no starting compound was present according to radio HPLC. Part of the crude product (56 mCi) was purified on a semi-preparative HPLC column under the same conditions as for [8-^{3}H]PMEDAP **4** (see above). The yield was 49.8 mCi of the product with a radiochemical purity of >99% (radio HPLC). The ^{3}H NMR (D_2O, NaOD, ref. TDO) was 8.35 s (^{3}H-8). The ^{1}H NMR was identical with that of the standard. The specific activity calculated from ^{1}H NMR was 15.7 Ci/mmol, the UV assay yielded 16 Ci/mmol. Before the solution was stored in liquid nitrogen, the volume activity was adjusted to 2 mCi/ml.

The Study of the Stability of the Tritiated Compounds **4**, **6** and **11**

a) The stability of ^{3}H-label at pH 7. The solution of 2 mCi of each of the labeled compounds in 0.6 ml of water containing 10% D_2O was placed in an NMR tube and the ^{3}H NMR spectra were taken at specified time intervals. The tubes were kept at 20 °C between the measurements.

b) The stability of ^{3}H-label at pH 7.4. The aliquotes of water solutions of labeled compounds (2 mCi) were evaporated on a CentriVap to dryness, each residue was dissolved in a mixture of 540 μl of 0.05 M phosphate buffer, pH 7.4, and 60 μl of deuterated water. The solutions were filtered through 0.45-μ teflone syringe filter to NMR tubes and ^{3}H NMR

spectra were taken at specified time intervals. The tubes were kept at 20 °C between the measurements.

c) The solutions of the labeled compounds **4**, **6** and **11** in water at a concentration of 2 mCi/ml were placed in 1.5 ml Nalgene® Cryovials protected by Nunc Cryoflex® foil and stored in liquid nitrogen. At the intervals indicated, the vials were withdrawn from the liquid nitrogen, the contents were left to thaw, and 2 μl of the solution were injected on the analytical radio-HPLC. D_2O was added to the concentration of 10% and the sample was checked by ^3H NMR.

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