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Research Article

M + 4 stable isotope labeling of levovirin and M + 7 and carbon-14 labeling of levovirin valinate pro-drug

Steve A. de Keczer[1,*], Mohammad R. Masjedizadeh[1], Shao-Yong Wu[1], Teresa Lara-Jaime[1], Kate Comstock[1], Charles Dvorak[1], Yu-Ying Liu[2] and Walter Berger[2]

[1] *Roche Palo Alto, 3431 Hillview Avenue, Palo Alto, CA 94304, USA*
[2] *Hoffmann- La Roche, 340 Kingsland Street, Nutley, NJ 07110, USA*

Summary

[M + 4]-labeled levovirin **5** (231 mg) was synthesized as an MS reference compound from [M + 4] triazole ester **2**. [M + 7]-labeled levovirin valinate **6** (127 mg) was synthesized as a comparison MS reference compound from [M + 6] triazole ester **3**. [^{14}C]-Levovirin **7** and [^{14}C]-levovirin valinate **8** were synthesized to support metabolism studies. The synthesis of **7** was accomplished in 33% overall yield (35.4 mCi, 57 mCi/mmol) from $Ba^{14}CO_3$ and **8** was synthesized in 41% yield (12.5 mCi, 57 mCi/mmol) from **7**. An efficient metallation/carbonation reaction was developed to synthesize [^{14}C]-triazole ester **4**.

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Key Words: carbon-13; carbon-14; nitrogen-15; stable isotope; levovirin; levovirin valinate; pro-drug

Introduction

Levovirin is the *L*-enantiomer of ribovirin. Like ribovirin, levovirin is intended for use in the treatment of patients with chronic hepatitis C. The immunomodulatory activity of levovirin is thought to be the underlying mechanism of action. Levovirin has similar potency to ribovirin in pre-clinical immunomodulation models, but has the advantage of a potentially more specific mode of action. However, because of a reduced clearance time, levovirin has reduced bioavailability. The problem of relatively low bioavailability of levovirin was ameliorated by the development of an appropriate

*Correspondence to: Steve A. de Keczer, Roche Palo Alto, 3431 Hillview Avenue, Palo Alto, CA 94304, USA. E-mail: Steve.de_keczer@roche.com

pro-drug. The increased bioavailability would ultimately result in a lower required dose, less frequent administration of the drug, lower costs of treatment, and higher exposures of the active drug component enabling enhanced antiviral response.[1] To test the bioavailability of the levovirin metabolically released from the pro-drug, levovirin valinate, compared to levovirin in its native form in the same individual, two isotopically labeled (M + 4 **5** and M + 7 **23**) differentiable forms of levovirin were synthesized. The M + 7 isotopic form was further elaborated to the M + 7 pro-drug levovirin valinate **6**. The reason for the relatively large mass displacements was to enhance the mass spectral signature of the labeled drugs. The ^{14}C-labeled forms of levovirin and levovirin valinate were synthesized for ADME studies.

Results and discussion

To simplify our syntheses, we chose to prepare both the M + 4 and M + 7 stable labeled and the ^{14}C-radiolabeled versions of levovirin via the common 1*H*-[1,2,4]-triazole-3-carboxylic acid methyl ester intermediates (**2**, **3**, and **4**).[2–6]

The condensation of these intermediates with 1,2,3,5-tetra-*O*-acetyl-L-ribose **1** was based on the modification of a method reported by Ramasamy.[7] Further elaboration afforded: [$^{13}C_3$,^{15}N]-levovirin **5**, [$^{13}C_3$,$^{15}N_4$]-levovirin valinate **6**, [^{14}C]-levovirin **7**, and [^{14}C]-levovirin valinate **8** (Figure 1).

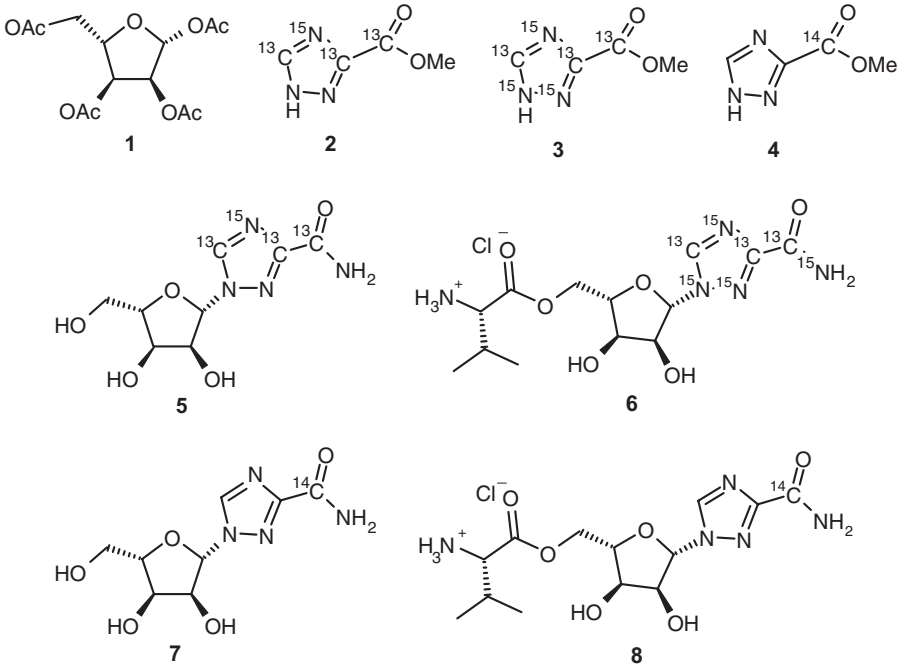

Figure 1. Key products and intermediates

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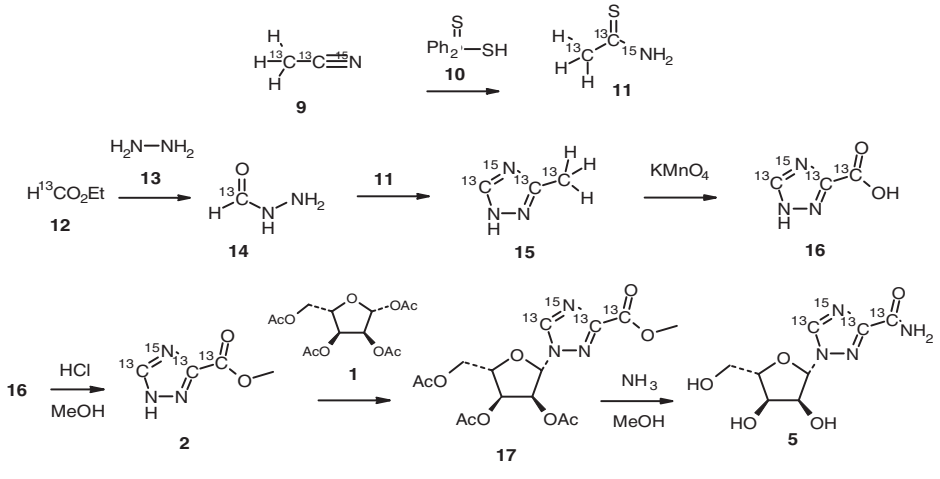
The synthesis of the M + 4 stable labeled methyltriazole involved the preparation of [$^{13}C_2$,^{15}N]-thioacetamide **11** from commercially available [$^{13}C_2$,^{15}N]-acetonitrile **9**. A potentially unreliable route (40–70%) requiring use of H_2S was avoided by using 2 equivalents of odorless diphenyldithiophosphonic acid **10**[2], affording pure labeled thioacetamide **11** in 93% yield. [^{13}C]-Formic hydrazine **14** was prepared by treating commercially available ethyl [^{13}C]-formate **12** with excess hydrazine hydrate **13** in toluene for 16 h at ambient temperature. Removal of volatiles afforded pure [^{13}C]-formic hydrazine **14** in 96% yield. The two labeled compounds **11** and **14** were coupled and cyclized by heating in toluene for 16 h at 88°C.[4] The crude [$^{13}C_3$,^{15}N]-methyltriazole **15** obtained by aqueous work-up was oxidized by the overnight reaction with aqueous $KMnO_4$ at 90°C.[5] Solids were removed by centrifuge, and the supernatant was acidified with conc. HCl to precipitate the [$^{13}C_3$,^{15}N]-triazole carboxylic acid **16** in 71% yield from **11**. The labeled acid **16** was stirred in HCl-saturated methanol at ambient temperature for 64 h, and the HCl salt of [$^{13}C_3$,^{15}N]-methyltriazole-carboxylate **2** was obtained by crystallization from the reaction mixture in 72% yield (300 mg).

Similarly, the M + 6 stable labeled methyltriazole (leading to M + 7 labeled levovirin) was prepared via the [$^{13}C_2$,^{15}N]-thioacetamide **11**. However, to increase the isotopic substitution, ethyl [^{13}C]-formate **12** was treated with [$^{15}N_2$]-hydrazine, converted to the free base from [$^{15}N_2$]-hydrazine sulfate. The elaboration to [$^{13}C_3$,$^{15}N_3$]-methyltriazole carboxylate **3** was accomplished in a similar fashion as described above, except that the intermediate [M + 6]-methyltriazole was purified before the oxidation step. This additional purification step resulted in an improved isotopic distribution as further discussed below.



Scheme 1. [$^{13}C_3$, ^{15}N]-levovirin

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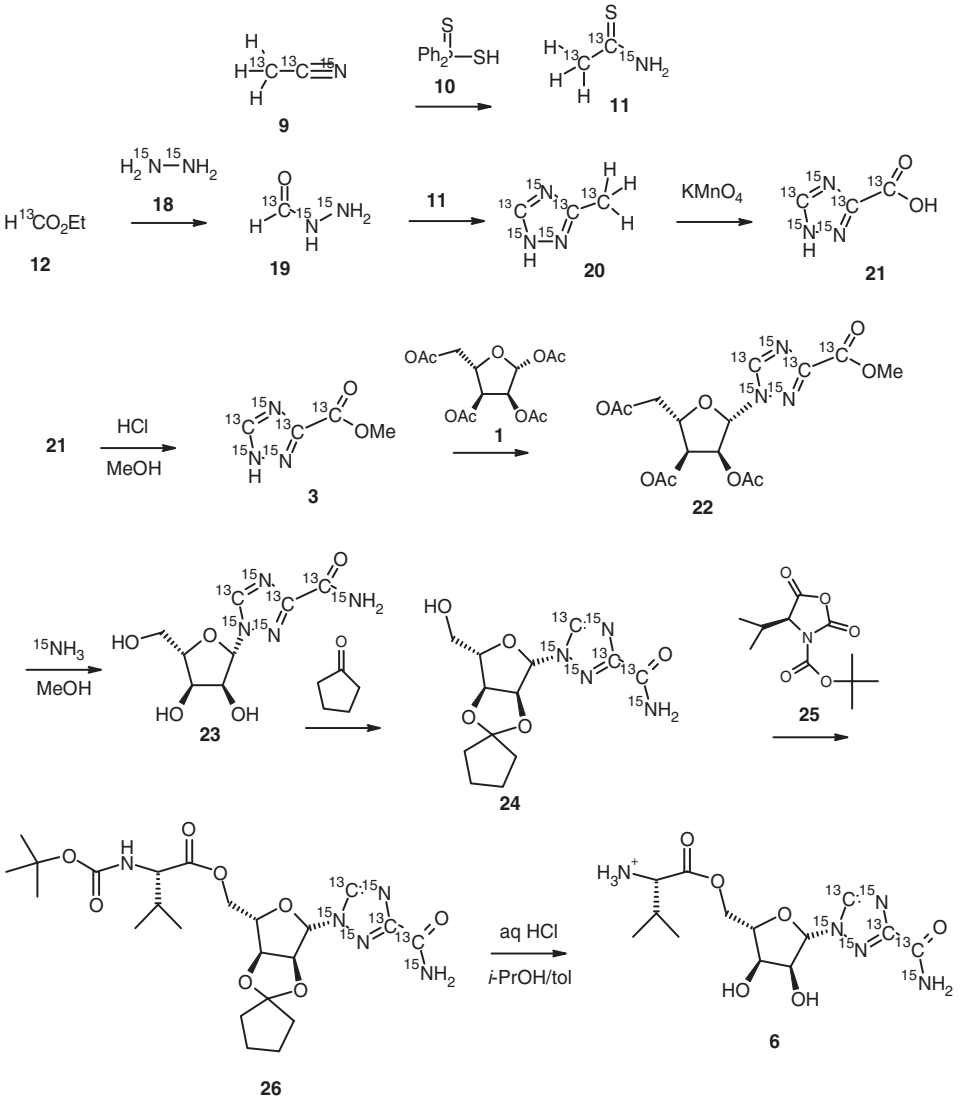
The ^{14}C-labeled triazole carboxylic acid methyl ester **4** was prepared in a three step, 1-pot procedure from readily prepared 1-pyrrolidin-1-ylmethyl-1*H*-[1,2,4]-triazole, **27**.[8] A THF solution of protected triazole **27** was treated first with *n*-BuLi followed by $^{14}CO_2$ (from $Ba^{14}CO_3$) to afford the protected triazole ^{14}C-carboxylate. Volatiles were removed by vacuum transfer and methanolic HCl was injected onto the residue. After stirring overnight, volatiles were again removed and the crude de-protected methyl ester paste was neutralized with aqueous NH_4OH. Chromatography afforded 168 mCi (80% yield) of 99.9% radio-TLC pure **4**.



Scheme 2. [$^{13}C_3$,$^{15}N_4$]-levovirin and [$^{13}C_3$,$^{15}N_4$]-levovirin valinate and 3-methyltriazole-[$^{13}C_3$,$^{15}N_3$]

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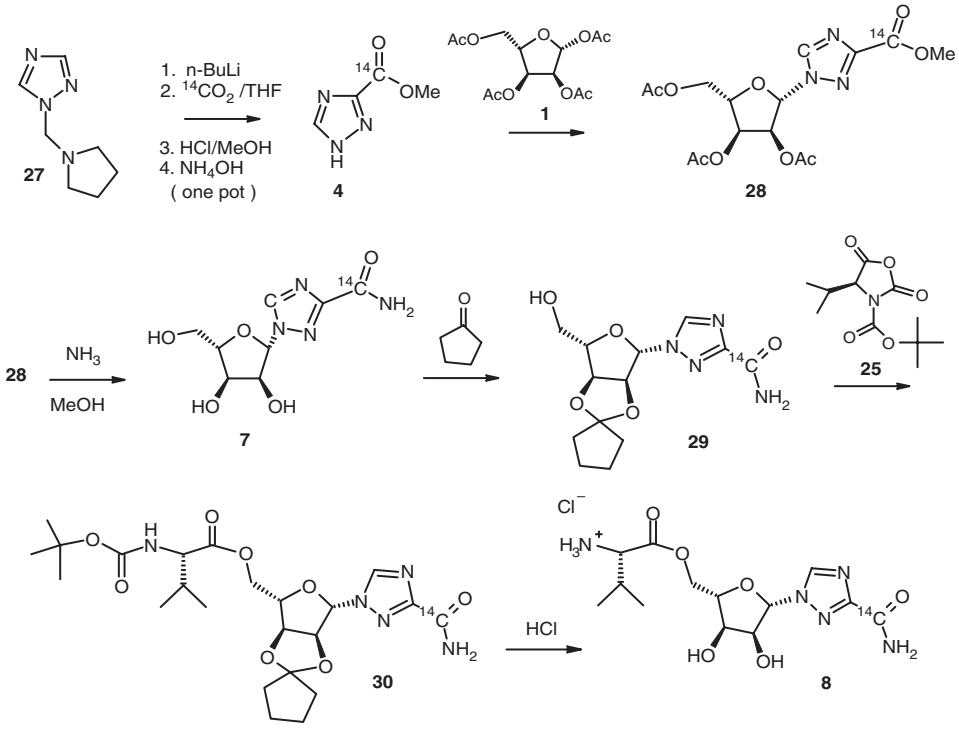
The remainders of the syntheses to labeled levovirin, **5** and **7**, and labeled levovirin valinate, **6** and **8**, were formally similar from all three of the labeled methyltriazole carboxylates, **2**, **3**, or **4**. However, in the case of [$^{13}C_3$,$^{15}N_4$]-levovirin valinate **6** the isomer substitution was increased from M + 6 to M + 7 by substituting commercially available [^{15}N]-ammonia (Scheme 2) in place of unlabeled ammonia (Scheme 1) at the amination step.

Attempts were made to improve the coupling step with each individual isotopic synthesis. In the first coupling, tetra-*O*-acetyl-L-ribose **1** and labeled triazole methyl ester **2** were combined with a catalytic amount of bis(4-nitrophenyl)phosphate.[7] This neat mixture was heated for 35 min at 150°C under vacuum and the evolved acetic acid was trapped. Following an aqueous $NaHCO_3$ workup and chromatography, a 35% yield of coupling product **17** was obtained. In the second coupling, excess tetra-*O*-acetyl-L-ribose **1** and a catalytic amount of bis(4-nitrophenyl)phosphate were added in two portions to labeled triazole methyl ester **3** contained in a rounded bottom flask to avoid 'bumping'. This neat mixture was heated for 1 h at 105–110°C under vacuum and the evolved acetic acid was trapped. The melt was directly purified by chromatography affording a 77% yield of nearly pure coupling product. Further purification by crystallization from methanol afforded a 57% yield of pure product **22**. In the coupling of ^{14}C-labeled triazole methyl ester **4**, an



Scheme 3. [^{14}C]-levovirin and [^{14}C]-levovirin valinate

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attempt to improve the yield by mixing the components in methanol followed by removal of the methanol as the toluene azeotrope did not increase the yield (Scheme 3).

The isotopic distribution of the M + 4 labeled levovirin was unexpected. Based on the purities of the isotopically labeled intermediates, we expected ⩾ 90% M + 4 isotopic substitution. Instead, we obtained 71.2% M + 4, and 27.8% M + 3. Though this distribution was adequate for our purposes, we attempted to discover the source of the apparent isotope dilution to M + 3. Mass spectral distributions were run of each of the intermediates to determine the source of the dilution. The dilution was found to occur during the $KMnO_4$ oxidation step. We could not rationalize this based on the chemistry at that step. We attributed the dilution related to the impure intermediates used at that step. In the subsequent M + 6 synthesis a purification was performed on compound **20** to give ~80% purity by NMR. After the $KMnO_4$ oxidation the isotopic distribution was somewhat improved to 87.2% M + 7 and 12.6% M + 6 for [$^{13}C_3$,$^{15}N_4$]-levovirin valinate **6**. The dilution of isotopic incorporation is still not fully understood.

Experimental

Proton spectra were run on a Brucker 300 MHz NMR spectrometer. Mass spectrometric analyses were carried out on a Finnegan LCQ ion trap mass spectrometer equipped with an electrospray ionization (ESI) source. The operating conditions for the ESI source were as follows: capillary temperature: 220°C, capillary voltage: 32 V. The sample was flow injected via a HP model 1100 HPLC system with 30% solvent 'A' (H_2O with 0.5% acetic acid) and 70% solvent 'B' (Acetonitrile) at a flow rate of 200 µl/min. LC/MS data were recorded from an Agilent 1100 Series LC/MS. The identity of intermediates was confirmed by MS and ^{1}H-NMR. All spectral data were consistent with the proposed structures. The specific activity was determined using a Beckman LS 6000IC scintillation counter and confirmed by MS. TLC plates (silica gel 250 µm) were obtained from Analtech and scanned using a Bioscan System 200 Imaging Scanner. The small molecules were reagent-grade, used as is from various sources. [$^{13}C_2$,^{15}N]-acetonitrile and ethyl [^{13}C]-formate were acquired from Isotec. [$^{15}N_2$]-hydrazine sulfate was obtained from Cambridge Isotope Laboratories, Inc. Anhydrous ammonia 99.9% and [^{15}N]-ammonia were purchased from Aldrich. Tetra-*O*-acetyl-L-ribose **1** was obtained from Hoffman-La Roche, Nutley, NJ. For the preparation of **1** from L-ribose see Liu and Burger[3].

[1,2-^{13}C,^{15}N]-thioacetamide **11**

The labeled acetonitrile **9** (440 mg, 10.0 mmol) was combined in a 250 cm^3 stopcock flask with 6.37 g (25.5 mmol) diphenyldithiophosphonic acid **10** in

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50 ml anhydrous isopropanol. The sealed flask was heated and stirred in a 58°C bath for 38 h. After cooling to ambient temperature, the suspension was filtered, and the filtrate was cooled to −20°C and decanted from the bulk of the remaining phosphonic anhydride by-product. This mixture was concentrated to an oil and the [1,2-^{13}C,^{15}N]-thioacetamide **11** (725 mg, 93% yield) was isolated by flash chromatography (silica gel; 1% NH_4OH, 9% MeOH in CH_2Cl_2). A second batch of [1,2-^{13}C,^{15}N]-thioacetamide **11** was prepared for the M + 7 synthesis by the method described here for the M + 4 synthesis.

[^{13}C]-formic acid hydrazide **14**

The ^{13}C-labeled ethyl formate (1.0 g, 13 mmol) **12** was added dropwise to a solution of 1.5 ml (26.5 mmol) hydrazine hydrate in 10 ml toluene. The biphasic mixture was stirred for 14 h at ambient temperature, then volatiles were removed under vacuum for 2 h at 50°C. After cooling, the residual white crystals (0.758 g, 96% yield) were identified as pure [^{13}C]-formic acid hydrazide **14**.

3-[^{13}C]-methyl-[3,5-^{13}C, 4-^{15}N]-1H-[1,2,4]-triazole **15**

[1,2-^{13}C,^{15}N]-thioacetamide **11** (302.3 mg, 3.87 mmol) and [^{13}C]-formic acid hydrazide **14** (300.9 mg, 4.93 mmol) were combined with 5 ml anhydrous toluene in a 20 cm^3 tube connected to a pair of traps, each containing 10 ml 10% NaOH solutions to capture the evolved H_2S gas. The tube was heated for 26 h at 98°C, then cooled to ambient temperature and the toluene extracted with a total of 5 ml water. The combined aqueous phases, containing [M + 4]-methyltriazole **15**, were used as is in the next step.

[3,5-^{13}C, 4-^{15}N]-1H-[1,2,4]-triazole-3-[^{13}C]-carboxylic acid **16**

The aqueous solution from the previous step (4.9 of 5.0 ml, ca. 3.8 mmol from **3**) was added dropwise via an addition funnel to a 40 cm^3 tube containing a 90°C stirred solution of 1.9 g (12 mmol) $KMnO_4$ in 14 ml water. The addition funnel was replaced with a condenser and the deep purple solution was stirred for 15 h at 90°C. Following removal of the spin bar from the deep brown suspension, the tube was centrifuged at 2 K rpm. The clear colorless aqueous phase was removed and the brown pellet was washed twice with 15 ml portions of water at 2.5 K rpm. The water was removed from the combined aqueous solutions by rotary evaporation and the white crystalline solid was re-dissolved and rinsed into a 20 cm^3 tube using a total of 2 ml water. This solution was titrated to pH 1–2, while cooled in an ice bath, with a total of 0.45 ml conc. HCl. The creamy suspension was centrifuged, decanted and the pellet washed twice by centrifugation with 1.0 ml portions of water. The solid **16** was dried to constant weight as a snow white powder (317 mg, 81% yield based on **11**).

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[3,5-^{13}C, 4-^{15}N]-1H-[1,2,4]-triazole-3-[^{13}C]-carboxylic acid methyl ester **2**

The labeled triazole acid **16** (303.4 mg, 2.62 mmol) was added to an ice bath cooled solution of HCl (2.4 g) in 3 ml MeOH. The suspension was stirred for 62 h and was re-cooled in a 0°C refrigerator for 2 h. The supernatant was removed and the fine white powder was washed once with −20°C MeOH affording a first crop 216 mg HCl salt of the methyl ester. After concentration of the mother liquor, a second crop (88 mg) and a third crop (18 mg) were similarly obtained. The combined yield of [3,5-^{13}C, 4-^{15}N]-1*H*-[1,2,4]-triazole-3-[^{13}C]-carboxylic acid methyl ester **2** HCl salt was 322 mg (72%), as a fine, snow-white powder.

The free base was obtained by neutralizing 154 mg (0.92 mmol) of **2**, to pH ~7 with 100 µl conc. NH_4OH, and then adding 4 ml of a solution of 1% NH_4OH/20% MeOH/CH_2Cl_2 to a final pH 8–9. The clear colorless supernatant was removed and was concentrated to dryness giving 96 mg of **2** (80% yield from the HCl salt).

1-(3,4-diacetoxy-5-acetoxymethyl-tetrahydro-furan-2-yl)-[3,5-^{13}C,4-^{15}N]-1H-1,2,4]triazole-3-[^{13}C]-carboxylic Acid methyl ester **17**

Labeled triazole methyl ester **2** (81 mg, 0.62 mmol) and tetra-*O*-acetyl-L-ribose **1** (203 mg, 0.64 mmol) were combined with a catalytic amount of bis(4-nitrophenyl)phosphate (4.8 mg, 0.014 mmol) in a 20 cm^3 tube. An ice bath cooled trap was attached to contain the HOAc evolved and the system was evacuated to 30 mmHg. The evacuated reaction flask was lowered into a 150°C bath. The initial vigorous effervescence diminished and completely ceased after 35 min, at which time the brown melt was allowed to cool to ambient temperature. The brown solid was completely dissolved in 2 ml EtOAc by heating at reflux. After cooling, 1.0 ml saturated aqueous $NaHCO_3$ was added, and the organic phase was removed and the aqueous phase was washed with 2 ml EtOAc. The combined organic phase was sequentially extracted with 2.0 ml $NaHCO_3$, 1.0 ml water, and 2.0 ml brine, and then dried with Na_2SO_4, filtered, and concentrated to a brown oil. The product was isolated by Chromatotron chromatography (2 mm silica gel rotor; 0.33% NH_4OH, 33% MeOH, CH_2Cl_2), affording 85 mg (35% yield) of coupling product **17** as a clear, pale, nearly colorless film.

1-(3,4-dihydroxy-5-hydroxymethyl-tetrahydro-furan-2-yl)-[3,5-^{13}C,4-^{15}N]-1H-[1,2,4]-triazole-3-[^{13}C]-carboxylic acid amide **5**, *(levovirin)*

The labeled methyl ester **17** (79 mg, 0.20 mmol) was rinsed with a total of 2 ml of 20% MeOH/CH_2Cl_2 into a 5 cm^3 stopcock flask, concentrated with a nitrogen sweep, and then pumped to dryness. The partially crystallized glass was re-dissolved in 1.5 ml refluxing methanol, cooled with an ice bath, and

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immediately (before crystallization of **17** from the cold methanol could begin) bubbled with ammonia gas. Moderate bubbling was continued for 10 min, and then the flask was sealed. After warming to ambient temperature, the stopcock was 'cracked' to allow minor degassing to reduce the pressure. The sealed reaction was stirred for 18 h at ambient temperature, and then the stopcock was carefully opened allowing the reaction to degas ($\sim$15 s). The crude product was purified by Chromatotron chromatography (2 mm silica gel rotor; 20–33% $MeOH/CH_2Cl_2$) affording 41.3 mg (83% yield) of a clear brittle colorless glass which appeared to be pure by TLC. In order to transfer the product, it was re-dissolved in 2 ml MeOH at reflux and transferred to a 4 cm^3 vial. On cooling to ambient temperature, the bulk of the levovirin **5** crystallized from solution. This first crop was pumped to constant weight (23.7 mg), the mother liquor was concentrated and a second crop (15.0 mg) was obtained. The isotopic distribution by MS was 68.3% M + 4, 28.3% M + 3, and 1.4% M + 2.

[^{13}C,$^{15}N_2$]-formic hydrazide **19**

[$^{15}N_2$]-hydrazine sulfate (5 g, 37.9 mmol) was added to a solution prepared from $Ba(OH)_2 \cdot 8H_2O$ (11.95 g, 37.9 mmol) in H_2O (60 ml). The mixture was stirred at room temperature for 2.5 h, filtered and the filtrate, containing [$^{15}N_2$]-hydrazine **18** as a free base, was mixed with toluene (30 ml). To this mixture, [^{13}C]-ethyl formate (**12**, 2.84 g, 37.9 mmol) was added dropwise and the mixture stirred at room temperature overnight. The solvent was removed under vacuum and the residue was purified by passing through a short silica gel cartridge eluting with a mixture of $NH_4OH/MeOH/CH_2Cl_2$ at an initial ratio of 0.1:1:9, slowly changing to a final ratio of 0.3:3:7. After removal of solvent, the product was obtained as a wax-like solid. 2.24 g, 94% yield. TLC showed the same R_f as that of a non-labeled reference sample.

[$^{13}C_3$,$^{15}N_3$]-3-methyltriazole **20**

The preparation of [$^{13}C_3$,$^{15}N_3$]-3-methyltriazole **20** was similar to that described for the synthesis of [$^{13}C_3$,^{15}N]-3-methyltriazole **15**, except stoichiometric amounts of the reagents were used, the toluene phase was discarded, and the crude solid product was purified by silica gel chromatography ($MeOH/CH_2Cl_2$) in 70% yield. The structure was confirmed by LCMS (M + 84.2).

[1,2,4-^{15}N-3,5-^{13}C]-1H-[1,2,4]-Triazole-3-[^{13}C]-carboxylic acid **21**

The same method was followed as described above for the synthesis of [M + 4]-triazole-carboxylic acid **16** except purified [$^{13}C_3$,$^{15}N_3$]-3-methyl triazole **20** was

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used instead of crude [$^{13}C_3$,$^{15}N_3$]-3-methyltriazole **15**, affording a 67% crude yield of [M + 6]-triazole carboxylic acid **21**.

[1,2,4-^{15}N-3,5-^{13}C]-1H-[1,2,4]-triazole-3-[^{13}C]-carboxylic acid methyl ester **3**

The same method as described for the synthesis of [M + 4]-triazole-carboxylic acid methyl ester **2** was followed. The product was further purified by silica gel chromatography (1% NH_4OH, 15% MeOH/CH_2Cl_2) to afford 291 mg (54% yield) of white solid methyl ester **3**.

1-(3,4-diacetoxy-5-acetoxymethyl-tetrahydro-furan-2-yl)-[1,2,4-^{15}N-3,5-^{13}C]-1H-[1,2,4]-triazole-3-[^{13}C]-carboxylic acid methyl ester **22**

The method described above was somewhat improved and used for the synthesis of the acetoxytetrahydrofuranyl [M + 4]-triazole-carboxylic acid methyl ester **17**. The reagents were added in two portions and the reaction was heated for 50 min at 105–110°C, (instead of 35 min at 150°C). Also, the eluent used for the silica gel chromatography was changed to 5–10% acetone/CH_2Cl_2. These changes resulted in a 77% yield of product which was further purified by crystallization from methanol to afford 422.6 mg (57% yield) of pure **22**.

1-(3,4-dihydroxy-5-hydroxymethyl-tetrahydro-furan-2-yl)-[1,2,4-^{15}N-3,5-^{13}C]-1H-[1,2,4]-triazole-3-[^{13}C]-carboxylic acid [^{15}N]-amide **23***, (levovirin)*

The same method was followed as described above for the synthesis of the [M + 4]-levovirin **5** with [^{15}N]-ammonia gas being used as described below.

Product **22** (397.9 mg, 1.017 mmol) was transferred using 5 ml warm methanol to a 30 cm^3 centrifuge tube attached to a wishbone on the vacuum line. After evacuation at −78°C the clear colorless solution was equilibrated with 335 mg (18.6 mmol) [^{15}N]-ammonia gas contained in a 500 cm^3 stopcock flask. The reaction was warmed to ambient temperature, and stirred overnight. Chromatography afforded 230.9 mg (90.5% yield) of [M + 7]-levovirin **23** having a purity of 99.3% by HPLC. The isotopic distribution by MS was 87.8% M + 7, and 10.5% M + 6.

1-(3,4-dihydroxycyclopentanoneacetal-5-hydroxymethyl-tetrahydro-furan-2-yl)-[1,2,4-^{15}N-3,5-^{13}C]-1H-[1,2,4]-triazole-3-[^{13}C]-carboxylic acid [^{15}N]-amide **24**

[M + 7]-Levovirin **23** (160.5 mg, 0.64 mmol) was dissolved in 2 ml CH_3CN in a 100 cm^3 pear flask. The sides were rinsed down by mild reflux. To this suspension was added a solution of 250 µl (2.8 mmol, 4.4 eq) cyclopentanone, 350 µl trimethylorthoformate (3.15 mmol, 4.9 eq.), and 20 mg *p*-TSA in 0.5 ml CH_3CN. The clear colorless solution was stirred at 35°C bath temperature for 3 h. The pH of the reaction mixture was adjusted to 7 with 150 µl 1 N NaOH

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and concentrated to dryness. The residue was re-dissolved in 2 ml warm 3% $MeOH/CH_2Cl_2$ and applied to a 1 mm Chromatotron rotor pre-equilibrated with 3% $MeOH/CH_2Cl_2$. Pure product containing fractions were combined and concentrated affording 149.8 mg (74% yield) 100% pure (by HPLC) of acetal **24**.

2-tert-butoxycarbonylamino-3-methyl-butyric acid 5-(3-[^{13}C,^{15}N]-carbamoyl)-[1,2,4-^{15}N-3,5-^{13}C]-[1,2,4]-triazol-1-yl)-3,4-dihydroxycyclo pentanoneacetal-tetrahydro-furan-2-yl methyl ester **26**

The [M + 7]-levovirin cyclopentylidene **24** (149.8 mg, 0.47 mmol), contained in a 100 cm^3 pear flask, was dissolved in 5 ml anhydrous THF at mild reflux. After addition of 5 ml of dry toluene the solvents were removed by rotary evaporation and the process repeated. The residue was re-dissolved in 5 ml THF, triethylamine (10 ml) and 4-isopropyl-2,5-dioxo-oxazolidine-3-carboxylic acid- *tert*-butyl ester **25** (0.54 g, 2.2 mmol, 4.7 eq.) were added and the solution was stirred overnight at ambient temperature. The crude reaction was concentrated to dryness and diluted with 2–3 ml CH_2Cl_2 and applied directly to a pre-equilibrated (3% $MeOH/CH_2Cl_2$) 1 mm silica gel Chromatotron rotor. Pure product fractions were combined to afford 267.2 mg of TLC pure BOC-valinate **26**, which was used directly in the next step.

1-[5-(3-[^{13}C,^{15}N]-carbamoyl-)-[1,2,4-^{15}N-3,5-^{13}C]-[1,2,4]-]-triazol-1-yl)-3,4-dihydroxy tetrahydro-furan-2-ylmethoxycarbonyl]-2-methyl-propyl-ammonium chloride **6**

The [M + 7]-BOC-levovirin valinate **26** (200 mg, 0.39 mmol) was dissolved in 1.2 ml toluene at 50°C. Isopropanol (0.24 ml) and 6 N aq. HCl (0.27 ml) were added and the two phase reaction was stirred at ambient temperature overnight. The reaction was allowed to settle and HPLC evaluation of each phase indicated about 30% of the starting material remained in the toluene phase and the product was in the aqueous phase. The aqueous phase was removed, and the toluene phase was rinsed with two 0.1 ml portions of water. The toluene layer was mixed with water (0.24 ml) and 3 N HCl (0.27 ml) and stirred overnight. HPLC of this second reaction showed complete conversion to nearly pure product **6**. The products from the first and second reactions were crystallized from *i*-$PrOH/H_2O$. The aqueous extracts were concentrated in a stream of nitrogen and then re-dissolved in water (0.15 ml) and *i*-PrOH (0.5 ml). The solution was then warmed to 50°C and treated with *i*-PrOH (1.5 ml), which was added dropwise over about 5 min. The clear colorless solution was stirred for about 5 min until the product began to crystallize and then 10 additional minutes. The fine white suspension was removed from the bath and stirred overnight at ambient temperature, compacted by centrifuge, and the clear colorless supernatant was removed by pipette. The snow white

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pellet was washed with 1 ml 4°C *i*-PrOH (0.5 ml for the second product) and dried for 2 h at 0.5 mm Hg. The total dry weight of the combined two pellets was 127.1 mg (~85% yield) of [M + 7]-levovirin valinate **6**, 99% pure by HPLC. The isotopic distribution by MS was 87.2% M + 7 and 12.6% M + 6.

1-pyrrolidin-1-ylmethyl-1H-[1,2,4]-triazole **27**

[1,2,4]-Triazole (10 g, 145 mmol) was dissolved in 50 ml EtOH. To this solution was added 13 ml (156 mmol) pyrrolidine and 11.5 ml (153 mmol) 37% aq HCHO and the solution was stirred under nitrogen at reflux for 6 h. The cooled reaction was diluted with 50 ml water, extracted three times with 50 ml portions of CH_2Cl_2, the combined organic phase was dried with Na_2SO_4 and concentrated. The residual oil was vacuum distilled using a Vigreux column (66–68°C head temp at 0.4 mm Hg) affording 16.3 g (74% yield) of NMR pure product **27**.

1H-[1,2,4]-triazole-3-[^{14}C]-carboxylic acid methyl ester **4**

Three 100 cm^3 septum-side arm stopcock flasks (A, B, and C) all equipped with spin bars were attached by a 'tri-bone' ($^{14}CO_2$ generator flask C via Drierite tube) to the vacuum line. The system was dried by heat gun under vacuum. $Ba^{14}CO_3$ (738.5 mg, 3.71 mmol, 57 mCi/mmol, 211 mCi) was weighed into flask C. Flask A was charged with about 30 ml THF/lithium aluminum hydride (LAH) as drying agent. Protected triazole **27** (651 mg, 4.28 mmol) was weighed into flask B. THF (15–20 ml) was vacuum transferred from LAH into flask B at −78°C. After warming to ambient temperature to dissolve the triazole in the THF, flask B was again cooled to −78°C and the vacuum was released with nitrogen. A solution of *n*-BuLi (2.70 ml of 1.6 N in hexane, 4.3 mmol) was added slowly, and the white suspension was stirred at −78°C for at least 30 min and then gradually warmed to −15°C over 1 h. The mixture was cooled first with dry ice and then with liquid N_2. Flasks B and C were evacuated and then isolated. Conc. H_2SO_4 (10 ml) was injected onto the $Ba^{14}CO_3$. After 15 min all effervescence had ceased and all of the activity had transferred to the reaction flask (B) as determined by β-probe. The reaction was warmed to −78°C and the milky suspension was allowed to warm to ambient temperature overnight. Volatiles were vacuum transferred from the reaction flask B and the white granular free flowing crude lithium carboxylate salt was used directly in the next step.

A 38% solution of HCl in methanol was freshly prepared by bubbling HCl gas into 10 ml methanol at ice bath temperature. All of this solution was added to the crude lithium carboxylate salt still in the same flask attached to the vacuum line (no exotherm was observed). The clear pale yellow solution was stirred overnight at ambient temperature. Formation of product (~80%)

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was confirmed by TLC (sample neutralized with NH_4OH before spotting). Volatiles were removed to a liquid N_2 trap. The cream colored paste was neutralized with 6 ml 5% aq. NH_4OH, and 6 ml methanol was added (pH = 7–8). The crude solution was concentrated and redissolved in a mixture of 5 ml MeOH, 5 ml CH_2Cl_2, and 1 ml water. This solution was chromatographed through a 100 cm^3 silica gel column pre-equilibrated with 1% aq. NH_4OH in 7% MeOH/CH_2Cl_2. Pure product fractions were combined and concentrated to a white solid. The solid was redissolved in 50 ml MeOH. Purity of **4** by TLC was 99.9%, and total activity was 168 mCi (80% yield).

1-(3,4-diacetoxy-5-acetoxymethyl-tetrahydro-furan-2-yl)-1H-[1,2,4]-triazole-3-[^{14}C]-carboxylic acid methyl ester **28**

The same method as described above for the preparation of acetoxytetrahydrofuranyl [M + 6]-triazole-carboxylic acid methyl ester **22** was followed except methanol was added to the reaction mixture to improve mixing and was then removed as the toluene azeotrope under vacuum. The reaction time was extended to 2 h with no improvement of yield. Chromatography afforded 48 mCi (57% yield) of coupling product **28** as a clear colorless glass.

1-(3,4-dihydroxy-5-hydroxymethyl-tetrahydro-furan-2-yl)-1H-[1,2,4]-triazole-3-[^{14}C]-carboxylic acid amide **7**

The same method was followed as described above for the synthesis of the [M + 4]-levovirin **5** to afford 35.4 mCi (74% yield) of deprotected product **7** having a radiochemical purity of 96% by HPLC.

1-(3,4-dihydroxycyclopentanoneacetal-5-hydroxymethyl-tetrahydro-furan-2-yl)-1H-[1,2,4]-triazole-3-[^{14}C]-carboxylic acid amide **29**

The same method was followed as described above for the [M + 7]-cyclopentanone adduct **24** except the number of equivalents of cyclopentanone and trimethylorthoformate were reduced to 2.8 and 3.0, respectively. However, this resulted in an incomplete reaction. To drive the reaction, a second portion of reagents was added. Following chromatography, this afforded 22.5 mCi (66% radiochemical yield) of 90% pure acetal **29** as a clear colorless glass.

2-tert-butoxycarbonylamino-3-methyl-butyric acid 5-(3-[^{14}C]-carbamoyl-[1,2,4]-triazol-1-yl)-3,4-dihydroxycyclopentanoneacetal-tetrahydro-furan-2-yl-methylester **30**

The same method as described above for the [M + 7]-BOC-valinate **26** was followed, affording 18.5 mCi (88% yield) of radiochemically pure [^{14}C]-BOC-valinate **30** which was used directly in the next step.

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1-[5-(3-[^{14}C]-carbamoyl-[1,2,4]triazol-1-yl)-3,4-dihydroxy-tetrahydro-furan-2-ylmethoxycarbonyl]-2-methyl-propyl-ammonium Chloride **8** *([^{14}C]-levovirin valinate*

The same method as described above for the [M + 7]-valinate **6** was followed, affording 12.5 mCi [^{14}C]-levovirin valinate **8** with a specific activity of 57 mCi/mmol. The radiochemical purity by AN-HPLC was 98.75%, with 1.25% [^{14}C]-levovirin **7**.

Conclusion

The seven step synthesis of [M + 4]-levovirin was accomplished in 12% overall yield from commercially available [$^{13}C_2$,^{15}N]-acetonitrile and ethyl [^{13}C]-formate. The similar seven step synthesis of [M + 7]-levovirin was accomplished in 14% overall yield from the same stable labeled commercial reagents and commercially available [$^{15}N_2$]-hydrazine sulfate and [^{15}N]-ammonia. The three step conversion to the [M + 7]-levovirin valinate pro-drug was accomplished in 69% overall yield from [M + 7]-levovirin. The synthesis of [^{14}C]-levovirin was achieved in 33% overall yield from barium [^{14}C]-carbonate. This included a one pot procedure for the synthesis of triazole-[^{14}C]-carboxylic acid methyl ester in 80% yield from barium [^{14}C]-carbonate. The three step conversion to the [^{14}C]-levovirin valinate pro-drug was accomplished in 41% overall yield from [^{14}C]-levovirin.

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