SYNTHESIS OF TRITIUM LABELED RENIN INHIBITOR DITEKIREN

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SUMMARY

In the search for a radioactive form of the peptidomimetic renin inhibitor, ditekiren, with a metabolically suitable radiolabel for conducting drug disposition studies, we prepared [^{3}H]ditekiren with tritium labels in the N-methyl-histidine moiety and in the leu-val alcohol transition-state insert. [His-^{3}H]ditekiren was obtained by first introducing two iodine substituents into the N-methyl-histidine moiety of the parent drug, followed by catalytic hydrodehalogenation with tritium gas. Administration of this labeled drug to monkeys, however, resulted in prolonged retention of radioactivity in the test animals, even though little or no tritiated water was detected in urine. This suggested *in vivo* production of a labeled fragment from the drug, e.g., N-methyl-[^{3}H]histidine, which became incorporated into the endogenous amino acid pool. These results, together with similar earlier findings after administration of [^{3}H]ditekiren labeled in the proline moiety of the drug, led us to synthesize [^{3}H]ditekiren labeled in the "unnatural" leu-val alcohol (LVA) portion of the molecule. [LVA-^{3}H]ditekiren was obtained by first oxidizing the parent drug to produce an LVA-keto analog, which was then reduced with sodium borotritide to give a mixture of tritium labeled ditekiren and its LVA-epimer. The two epimeric labeled materials were separated and purified by means of preparative high performance liquid chromatography (HPLC). The tritium label in [LVA-^{3}H]ditekiren was found to be metabolically suitable for conducting drug disposition studies, with no liability for tritiated water production or prolonged retention of radioactivity in tissues of test animals.

Keywords: iodination, hydrodehalogenation, oxidation, sodium borotritide reduction, transition-state insert, epimers

INTRODUCTION

Inhibition of renin in the renin-angiotensin cascade is an important approach in the treatment of hypertension. Ditekiren (BOC-Pro-Phe-N-Me-His-Leuψ[CHOHCH$_2$]Val-Ile-2-aminomethylpyridine, also referred to in the literature as U-71038) is a potent and specific inhibitor of renin with *in vitro* resistance to hydrolysis by proteolytic enzymes (1) and prolonged hypotensive activity in animal models (2). A suitable radioactive form of the compound was needed for conducting drug disposition studies in support of developing

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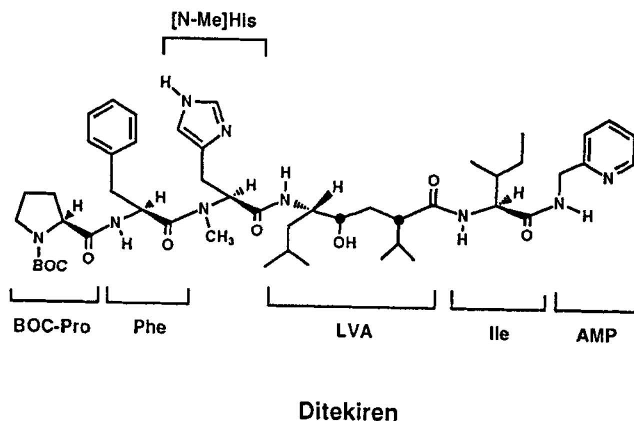
ditekiren as a potential new drug. Because of the very low dose anticipated for this drug, it was decided at the outset that tritium would be used as the radiolabel, so that the necessary high specific activity could be achieved. The first labeled drug chosen for this purpose was [^{3}H]ditekiren with labels in the proline moiety, since readily available [^{3}H]proline could be conveniently incorporated into the product as the terminal amino acid unit (3). After intravenous administration of [Pro-^{3}H]ditekiren to rats, there was high recovery (>95%) of radioactivity with absence of tritiated water in urine (4). However, there was also prolonged retention of radioactivity after oral administration of the labeled drug to cynomolgus monkeys (5), indicating that the [^{3}H]proline unit was being cleaved from the parent drug and distributed into the endogenous amino acid pool. This report describes the search for a form of radioactive ditekiren with labels which would be more suitable for carrying out drug disposition studies.



Ditekiren

DISCUSSION AND RESULTS

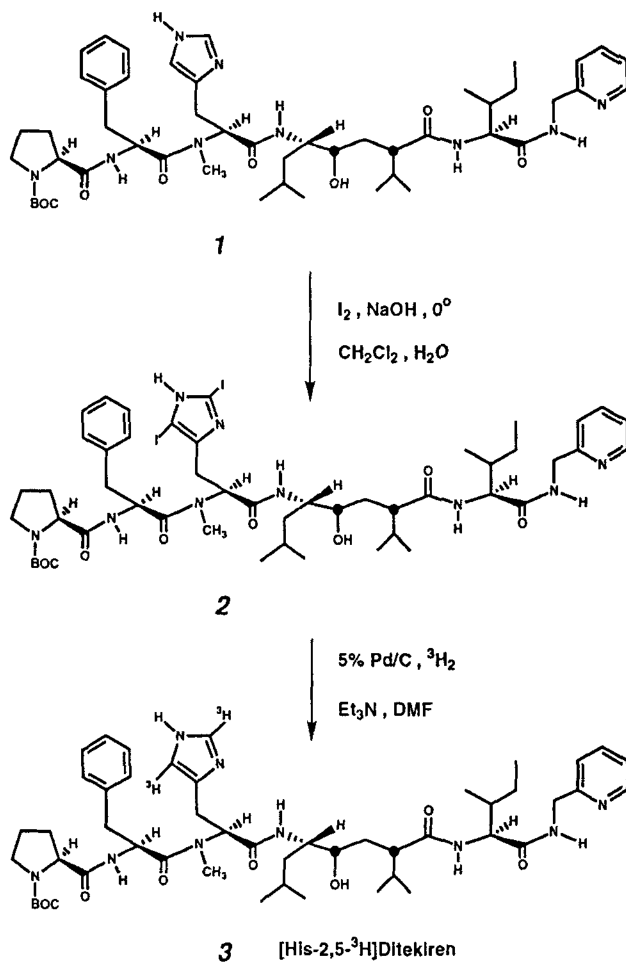
Although ditekiren had been shown to resist *in vitro* protease mediated hydrolysis (1), prolonged tissue retention of radioactivity after oral dosing of monkeys with [Pro-^{3}H]ditekiren indicated that cleavage of the peptide bond did occur *in vivo*, at least at the

proline end of the pseudopeptide. In the hope that relocation of the radiolabel further into the interior in the molecule might alleviate the problem, we next synthesized [^{3}H]ditekiren with tritium labels in the N-methyl histidine moiety. The procedures are shown in Scheme 1. Ditekiren (*1*) underwent facile iodination exclusively in the imidazole ring of N-methyl histidine. The resulting diiodo derivative ***2*** was readily reduced with tritium gas at ambient temperature in the presence of 5% palladium on charcoal catalyst to give [N-Me-His-^{3}H]ditekiren (***3***) with a specific activity of 35 Ci/mmol. Administration of this labeled drug to monkeys, however, once again resulted in prolonged tissue retention of radioactivity, albeit again without significant production of tritiated water (5).

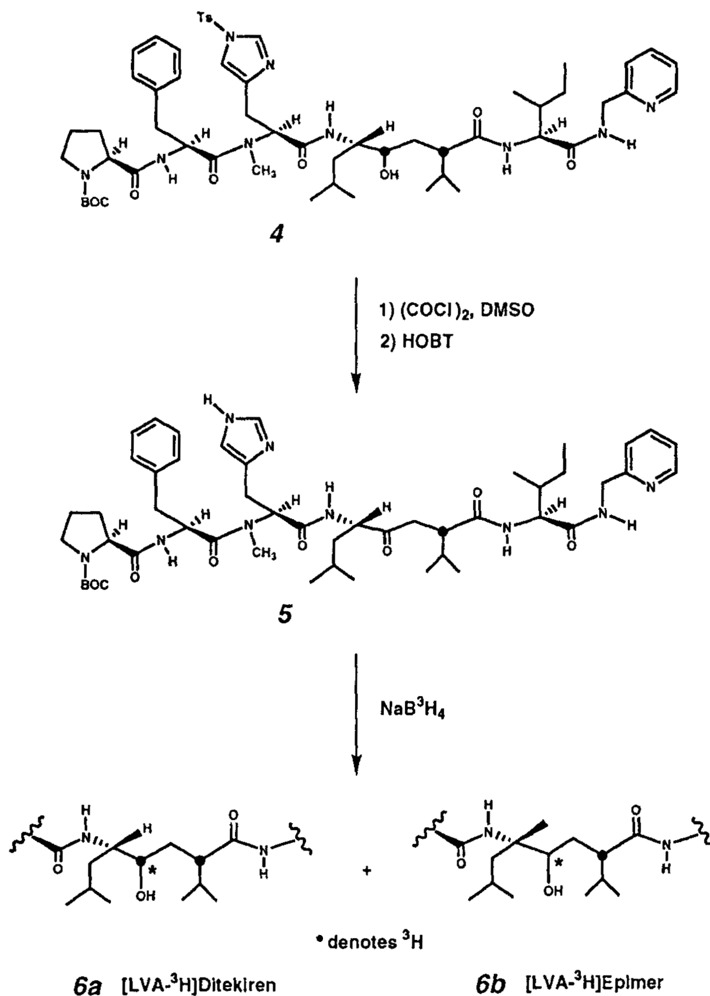
The results of disposition studies with proline-labeled and N-methylhistidine-labeled ditekiren indicated that in order to produce a metabolically suitable labeled drug, the tritium label would have to be placed in the "unnatural" transition insert, the leu-val alcohol (LVA) portion of the ditekiren molecule, so that the fragment bearing the label, when released from the drug molecule, would not be assimilated into endogenous pools. One possible approach to achieve this was to synthesize tritium labeled LVA for subsequent incorporation into ditekiren. However, the lengthy synthesis of this high specific activity tritium labeled intermediate would have been a formidable task. Fortunately, it was discovered that the hydroxy function of the LVA moiety in the N-tosyl (in the imidazole ring of N-Me-histidine) derivative of ditekiren could be cleanly oxidized to the corresponding N-tosyl ketone without affecting any other portions of the ditekiren molecule. Removal of the protecting tosyl group produced the ketone ***5*** as shown in Scheme 2. Furthermore, the ketone ***5*** could be readily reduced with sodium borohydride ($NaBH_4$) back to ditekiren. Substituting sodium borotritide for the borohydride would then lead to [^{3}H]ditekiren ***6a*** labeled in the LVA moiety. The reduction produced a pair of diastereomers, ***6a*** and ***6b***, with opposing stereochemistry at the carbon atom bearing the hydroxy function. However, the two isomers proved easily separable by means of reversed phase HPLC.

The borohydride reduction of ketone ***5*** afforded the epimeric alcohols in the ratio of ~3.5:1 in favor of the unwanted isomer. Nevertheless, the simplicity and ease of execution of this route, especially in view of the unattractive alternative of a long synthesis, made the

Scheme 1



oxidation-reduction sequence our method of choice. A series of studies were carried out to optimize the reduction conditions. Reduction of *5* was first carried out in methanol. Because $NaBH_4$ reacts rapidly with methanol, we investigated substituting ethanol as the solvent, in which the reducing agent would be more stable. It was found that reduction of *5* in ethanol led to the same mix of epimers, albeit the reaction was slower. To determine the optimum reaction time, the reduction was monitored by HPLC for 48 h. In order to define conditions for maximizing both product specific activity and utilization of valuable sodium borotritide, the reduction was carried out at the outset with a substrate to reducing agent molar ratio of



4:1. After the initial supply of $NaBH_4$ was consumed, as indicated by stabilization of the ketone to alcohols ratio in the product mix, an excess of additional $NaBH_4$ was added to consume remaining ketone. The reaction mixture was analyzed by HPLC to determine the ketone and epimeric alcohols contents at selected time intervals. The results (Table 1) showed that reduction with 1/4 molar equivalent of $NaBH_4$ had reached an optimum at ~24 h, and the prolonged reaction had no apparent deleterious effects on the product. Interestingly, the ratio of the two epimers remained constant throughout the reaction period, indicating that they were produced at essentially the same rate. Accordingly, reduction of *5* with

nominally 2 Ci of $NaBT_4$ was carried out with ¼ molar equivalent of the tritiated reducing agent for 26 h, followed by 1.0 molar equivalent of $NaBH_4$ to complete the reaction. The crude product mixture was then purified by preparative HPLC to afford 362 mCi of [LVA-^{3}H]ditekiren (*6a*) sp. act. 19.5 mCi/mg (35.2 Ci/mmol) with 99% radiochemical purity (RCP). There was also obtained 1.448 Ci of the LVA-epimer *6b* with 97.5% RCP. The total radiochemical yield was 1.81 Ci, or 90.5%.

The LVA-labeled *6a* was administered intravenously to cynomolgus monkeys. Excellent recovery of total radioactivity showed that the tritium label in the LVA moiety was metabolically stable. There was no prolonged tissue retention of radioactivity, and only a trace (<0.01% of dose) of radioactivity in the urine was found to be tritiated water (5). Therefore this labeled drug should be suitable for conducting drug disposition studies.

Table 1.

Reduction of Ketone *4* with 0.25 Molar Equivalent of $NaBH_4$.

Time hr	Ketone %	Alcohols %	Ditekiren %	Epimer %	Epimer/Ditekiren
0.5	50.6	46.6	9.6	37.0	3.9
1.75	31.9	64.4	14.1	50.3	3.6
4	20.2	75.4	16.7	58.7	3.5
7	15.0	79.4	17.9	61.5	3.4
12	11.5	83.8	18.8	65.0	3.5
24	8.7	87.9	19.8	68.1	3.4
48	8.1	88.9	20.0	68.9	3.4

EXPERIMENTAL

Thin layer chromatographic (TLC) analysis was done on 2.5 x 10 cm glass plates precoated with a 250 µm layer of silica gel GF (Analtech). Developed zones were visualized by UV light (254 nm). Radioactive zones were detected with a Bioscan System 200 Imaging

Scanner. ^{1}H-NMR spectra were obtained from a Brucker AM 300 spectrometer. The ^{3}H-NMR spectra was obtained on an IBM AF-300 spectrometer. Mass spectral analyses were performed on a Finnigan Model TSQ-70 spectrometer. Radioactivity determinations were carried out with a Pharmacia Wallac 1410 liquid scintillation spectrometer, using the external standard method with Ultima Gold as the scintillation cocktail. HPLC analyses were carried out using a Spectra Physics Model 8700 Solvent Deliver System and a Zorbax C-8 6 μ (250 x 4.6 mm I.D.) analytical column. Mobile phases used were:
A) 500:500:4:7.2 v/v methanol:H_2O:TFA:TEA[a] (apparent pH 3.0) and B) 400:600:4:7 v/v acetonitrile:water:TFA:TEA (apparent pH 3.0). The eluate was analyzed with an LDC/Milton Roy SpectroMonitor-D variable wavelength detector set at 232 nm or at 264 nm, and with a Radiomatic model Flo-One/Beta CR radioactivity detector.

N$^\alpha$-Boc-Pro-Phe-N$^\alpha$-methyl-[2,5-diiodo]His-Leuψ(CHOH-CH_2)Val-Ile-2-aminomethylpyridine, (*2*)

A solution of 1.61 mmol of iodine in 73 mL of methylene chlroide (CH_2Cl_2) was added with stirring in 5 min to an ice cold two-phased mixture of 750 mg of ditekiren (*1*, 0.81 mmol) in 25 mL of CH_2Cl_2 and 50 mL of water. The yellow-brown solution was basified with the dropwise addition of 17.7 mL of 0.1N sodium hydroxide (1.77 mmol) over a 10 min period. After stirring for 20 min, the light yellow reaction mixture was partitioned with 40 mL of CH_2Cl_2 and 50 mL of water. The aqueous phase (pH = 9.5) was extracted with 50 mL CH_2Cl_2. The combined extract was washed with 50 mL of brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure at 20°C to give 958 mg of crude *2*. The crude material was chromatographed on a column of 80 g of silica gel packed in and eluted with 5% v/v MeOH in CH_2Cl_2. The fractions containing *2*, as monitored by TLC (silica gel, 10% v/v MeOH in CH_2Cl_2, Rf = 0.6), were pooled and concentrated to give 848 mg (89% yield) of *2*, shown to be >99% pure by HPLC analysis (mobile phase A, UV 232 nm), mass spectrum, FAB (argon) positive ion mode, m/z:1204 M^+ + 23 (sodium aduct), 1182 M^{+1}, ms/ms

[a] TFA is trifluoroacetic acid and TEA is triethylamine.

daughter spectrum of m/z 1182 gave fragmentation m/z:838 (N-methyl-[2,5-diiodo]His-Leuψ(CHOH-CH_2)Val-Ile-AMP^+), 435 (Leuψ(CHOH-CH_2) Val-Ile-AMP^+, 376 (N-methyl-[2,5-diiodo]His^+), 222 (Ile-AMP^+); ^{1}H-NMR, 300 MHz δ (MeOH-d_4) identical to spectrum of *1* with the noted absence of signals at 7.55, 7.47 (2s[b], 1H, histidine ring proton at C-2) and 6.91, 6.82, 6.80 (3s[b], 1H, histidine ring proton at C-5).

N^α-Boc-Pro-Phe-N^α-methyl-[2,5-^{3}H]His-Leuψ(CHOH-CH_2)Val-Ile-AMP, [N-Me-His-2,5-^{3}H]Ditekiren (*3*)

A solution of 25 mg of *2* (0.021 mmol) in 2 mL of dry DMF containing 6.4 μL of TEA (0.046 mmol) was placed in a hydrogenation apparatus containing 10 mg of 5% Pd/C degassed under high vacuum for 16 h) suspended in a spoon above the solution[c]. The solution was frozen in liquid nitrogen and the apparatus was evacuated. Three successive freeze-thaw cycles were used to completely degas the solution. The catalyst was added to the solution, and the mixture was stirred under 130 curies of tritium gas for 2.1 h. The excess tritium gas was evacuated and the reaction mixture was diluted with 2 x 2 mL of methanol. The mixture was concentrated each time under high vacuum to remove residual labile tritium. The reaction mixture was filtered through a bed of Celite, which was washed with 50 mL of ethanol. The combined filtrate and wash were lyophilized under high vacuum overnight. The total activity found in the residue was 1.1 Ci. This material was dissolved in 5 mL of CH_2Cl_2 and the yellow solution was washed with 7 mL of 1.2 M sodium bicarbonate, followed by 8 mL of water and 10 mL of brine in that order, and dried over anhydrous sodium sulfate. The dry solution was filtered, concentrated under a stream of nitrogen, and dissolved in 0.5 mL of methanol-d_4 to give 772 mCi of *3*, sp. act. 37.9 mCi/mg (35.2 Ci/mmol). The sample was analyzed by ^{3}H-NMR (320 MHz) which showed tritium only in the imidazole ring of histidine: δ 7.6, 7.5 (H-2, 35% ^{3}H by integration), 6.9, 6.85, 6.8 (H-5, 65% ^{3}H by

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[b] Each of the histidine ring protons in *1* presented itself as more than one siglet because of stable conformations assumed by the pseudopeptide molecule.

[c] Reduction with tritium gas was carried out at the National Tritium Labeling Facility, Lawrence Berkeley Laboratory, Berkeley, CA, in collaboration with H. Morimoto.

integration). The ^{1}H-NMR spectrum (300 MHz) was identical to that of a standard sample of ditekiren with the noted exception of diminished intensity for signals attributed to the protons at C-2 and C-5 of the imidazole ring in histidine. HPLC analysis (mobile phase B, UV 264 nm) of the sample, after concentration under a stream of nitrogen and dilution with 100 mL of methanol, showed 95.3% RCP. A 155 mCi sample of this solution was purified by preparative HPLC (mobile phase B, UV 264 nm). The sample was concentrated and divided into four injections, and the pooled collected fractions containing ***3*** were concentrated to remove acetonitrile. The aqueous remainder was basified (1.2 M $NaHCO_3$) and salted (brine) before extraction with methylene chloride. The extract was washed with 1.2 M sodium bicarbonate and brine, dried over anhydrous sodium sulfate, filtered, and concentrated to give 83.7 mCi (54% recovery) of ***3***, dissolved in 160 mL of 9:1 v/v ethanol:water, >99% RCP by HPLC (mobile phase D, UV 264 nm) and TLC (10% v/v MeOH in CH_2Cl_2).

Ketone ***5***

A 25 mL, two-necked round-bottomed flask fitted with a septum, magnetic stirrer and argon inlet was flame-dried, then cooled to room temperature in an atmosphere of argon. The flask was charged with 4 mL of methylene chloride, which was cooled to -78°C and treated with 0.11 mL of oxalyl chloride, followed two min later by 0.188 mL of dimethylsulfoxide added dropwise. After 10 min at -78°C, a solution of 1.084 g (1 mmole) of ***4*** in 4 mL of methylene chloride was added over about one min, and stirring was continued at -78°C for 30 min. Triethylamine (0.38 mL) was added, and the mixture was allowed to warm to 25°C over 15 min. The reaction mixture was then poured into half-saturated aqueous sodium bicarbonate and extracted with three 100 mL portions of methylene chloride. The combined extracts were washed with brine, dried over anhydrous sodium sulfate, and evaporated to dryness, thereby affording ketone ***5*** with tosyl protecting group at the imidazole nitrogen.

For removal of the histidine tosyl group, the residue from above was dissolved in 10 mL of methanol and treated with 500 mg of 1-hydroxybenzotriazole (HOBT) hydrate, and

the homogeneous mixture was allowed to stand at 25°C for 18 h. Following removal of the methanol *in vacuo*, the crude product was chromatographed on a 130 g column of 40-60 μm silica gel, packed and eluted with 95:5 v/v $CHCl_3$:4 M NH_3 in MeOH (13 mL fractions). Fractions 40-49 were combined and yielded 750 mg of pure ketone ***5***, an amorphous white solid (81% yield over the two steps); single component by TLC (92:8 v/v $CHCl_3$:4 M NH_3 in MeOH, Rf 0.24); NMR ($CDCl_3$; TMS): δ (ppm) 8.55-8.44 (m, 2H), 7.75-7.50 (m, 3H), 7.40-7.15 (m, 6H), 6.75-6.65 (m, 1H), 6.35-6.20 (m, 1H), 4.65-4.20 (m, 3H), 1.47 (9H); mass spectrum (FAB): $[M + H]^+$ observed at M/Z 928.5683; calc'd for $C_{50}H_{74}N_9O_8$, 928.5660; other ions at M/Z 582, 276, 124, 109, 92, 70, 57, 45.

Reduction of ***5*** with $NaBH_4$

To a solution of 149 mg of ***5*** (0.16 mmol) in 4 mL of absolute ethanol was added dropwise with stirring in ~2 min 1 mL of 0.04 M $NaBH_4$ in the same solvent. The clear mixture was stirred at room temperature, and 100 μl aliquots were taken in 0.5, 1.75, 4, 7, 12, 24, and 48 h[d]. Each sample was treated with 100 μl of mobile phase B to quench the reaction, and the mixture was concentrated at reduced pressure and 30°C to remove ethanol. The aqueous residue was diluted with another 100 μl of mobile phase B. Ten μL samples were analyzed by HPLC with mobile phase B pumped isocratically at 1.5 mL/min. The contents of ***5***, ***6a*** and the LVA-epimer ***6b*** (all unlabel forms) at each time point were determined. The results are shown in Table 1.

The remaining reaction mixture at 26 hours was treated with 6.2 mg of additional $NaBH_4$ (0.16 mmol) for 4 hours to complete the reduction . The excess $NaBH_4$ was quenched with 1 mL of 0.4 N HCl. After evolution of gases had subsided, the mixture was concentrated at ~15 torr and 35°C. The aqueous residue, pH 3, was neutralized with 0.5 N NaOH to pH 7.0 and subjected to preparative HPLC (250 μL aliquots containing ~20 mg of products) on a 20 mm ID x 250 mm column packed with 6 μm Zorbax C-8, using the same mobile phase

[d] The 48 hr sample was taken at 26 hours but kept at room temperature for another 22 hours before workup to provide data for 48 hours.

B described above pumped at 20 mL/min (Waters Delta-Prep 3000). The pooled fractions containing ditekiren were concentrated at ~15 torr and 35°C to remove acetonitrile. The aqueous residue was basified with 4N NaOH and extracted with 3 x 50 mL of CH_2Cl_2. The combined extracts were washed with brine, dried over anhydrous sodium sulfate, and concentrated to give 21 mg of ditekiren which was shown to be pure by HPLC. Similarly, the combined epimer pool was worked up to afford 73 mg of the LVA-epimer. The epimer/ditekiren ratio of 3.47 agreed well with the 3.5 ratio found for the 48 hours sample of the reaction mixture (Table 1).

Reduction of *4* with $NaBT_4$

The $NaBT_4$, supplied by Amersham Corp., nominally 2.0 Ci at 89.0 Ci/mmol, ± 15%, was dissolved in 3 mL of absolute ethanol and added dropwise with stirring to a solution of 100 mg of *4* (0.108 mmol) in 1.5 mL of absolute ethanol. The mixture was stirred at room temperature under a N_2 atmosphere in a 25 mL 3-necked round bottom flask fitted with a septum and vented to a fume hood. After 26 h, $NaBH_4$ (4 mg, 0.105 mmol) was added to the reaction mixture to complete the reduction. After stirring for 3 more hours, 1 mL of 0.4 N HCl was added dropwise by syringe through the septum to quench the excess $NaBH_4$. After the evolution of gases had subsided[e], the mixture was adjusted to pH 7 with 0.6 mL of 0.5 N NaOH and concentrated at ~15 torr and 35°C. The oily aqueous residue was clarified with the addition of 1.5 mL acetonitrile. HPLC analysis showed absence of *4* and epimer:ditekiren ratio of ~4:1. The mixture was subjected to preparative HPLC purification as described above to afford 362 mCi of [LVA-^{3}H]ditekiren (*6a*) and 1.448 Ci of the [^{3}H]epimer ***6b***. The specific activity of ***6a*** was determined by relating the mass of an HPLC sample, as measured by the UV response against a standard curve, to the associated radioactivity as measured by liquid scintillation counting of the collected peak. The specific activity was found to be 19.5 mCi/mg, with 99.0% RCP. For lack of a pure standard sample of the LVA-epimer, the standard curve for ditekiren was used to estimate the specific activity of the labeled epimer *5b*, which was found to be 20.9 mCi/mg, with 97.5% RCP by HPLC.

[e] Throughout the reaction, the vessel was gently swept with N_2 which was vented to a fume hood.

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