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Research Article

High specific activity tritium labeling of vitamin D derivative RO275646

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Summary

The octahydroindenone intermediate was tritium labeled using T_2O isotope exchange labeling, and then elaborated to the vitamin D derivative RO275646. Though this method of labeling was expected to give the minor isomer, it was the shortest and most convenient route to the high specific activity metabolically stable labeled sites. A total of 34 mCi (from two separate runs) at 64 Ci/mmol of [^{3}H]-RO275646 were prepared by this method. Copyright © 2005 John Wiley & Sons, Ltd.

Key Words: tritium; T_2O; exchange labeling; minor isomer; vitamin D

Introduction

Vitamin D derivatives have been shown to exhibit a much broader spectrum of biological activity than originally thought. These steroid hormone analogs have been used or have high potential for application as drugs in treating a diverse range of human diseases such as rickets, renal osteodystrophy, psoriasis, leukemia, breast cancer, prostate cancer, AIDS, and Alzheimer's disease.[1] These and other bio-medically important applications have stimulated our current research.

Biologically stable, high specific activity, tritium labeled vitamin D derivative RO275646 was required for pharmaco-kinetics studies for an osteoporosis program. Usually for DMPK studies ^{14}C-labeled compounds are prepared.[2] However, in our case high specific activity was necessary because of the low dosage requirement for this compound. Even if a double ^{14}C-labeled compound would have been used, the mass equivalent to the radioactive dose

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would have been higher than the toxic threshold. Thus, preparation of a ^{3}H-labeled compound with the label at a non-exchangeable site was requested.

A variety of approaches for stable high specific activity tritium labeling of vitamin D derivatives has appeared in the literature.[†,2] Each of the potential labeling sites was examined for ease of labeling, multiple labeling, existing available routes, number of total steps required, number of hot steps required, and expected liability/metabolic stability.

One of the sited approaches was an enolic exchange for deuterium (for a different vitamin D derivative) that was potentially applicable to tritium exchange.[2] Although the amount of D_2O is not of concern for deuterium labeling, the amount of T_2O must be kept to a minimum for practical and safety considerations. Thus, this crucial step required further development. We expected that the approach for tritium labeling would afford an isomer ratio similar to that reported for proton exchange at analogous positions in the vitamin D derivative. The reported synthesis[3] gave 1:3.6 ratio of the desired isomer. Necessary for this approach is a coupling step tethering the A-ring to the carbonyl carbon of the C-ring. As precedent for this step, a Horner-Wittig Olefination as applied to a non-labeled vitamin D synthesis [3] was sited. Also, a similar attachment step had been developed by our Process Chemistry Group for our particular compound, lending more support for this approach. Based on the above evaluation, we chose tritium exchange labeling at the [5,5,3aα-^{3}H] positions (9,9,14α-^{3}H in the vitamin D numbering system).[1]

Of particular advantage to tritium exchange labeling at this site was the high specific activity attainable by the simultaneous enolic exchange of three protons for three tritium atoms. Once these tritium atoms are locked in place via Horner-Wittig Olefination they become chemically and metabolically stable as exemplified by comparison with the corresponding ^{14}C-labeled vitamin D derivative in initial metabolic studies.[4] Other advantages to labeling at the [5,5,3aα-^{3}H] positions were our experience with T_2O exchange labeling, the known synthesis of the required precursor, and the known relatively few hot steps necessary for the remainder of the total synthesis.[5,§]

However, there were two major disadvantages to tritium exchange labeling at this site. The first major disadvantage was the Curie amounts of T_2O required. Fortunately, we had ready access to the National Tritium Labeling Facility (NTLF) at UC Berkeley where these amounts of activity could be readily handled. The second major disadvantage was the adverse isomer ratio

[†] An excellent review with 540 references by Zhu and Okamura (reference 1) describes a number of approaches for the synthesis and isotopic labeling of vitamin D derivatives.
[§] The synthesis of phosphine oxide **7** is in analogy to the chemistry developed by DeLuca (reference 9). The Ring A precursor was technically developed by the process research group of Roche-Basel with further work done at Roche Palo Alto.

expected resulting at the isotope exchange step. The desired isomer was expected to be the minor isomer.

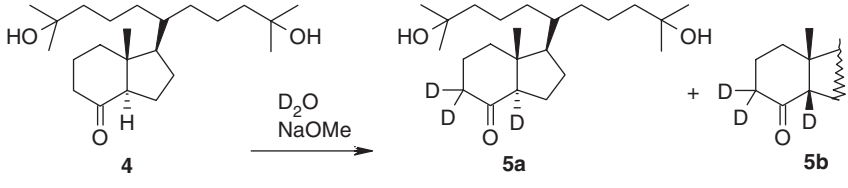
Though at first glance this approach does not appear to be very elegant, in reality the cost of labeling the major or the minor isomer is essentially the same since a large excess of T_2O is required in either case. The increased difficulty in separating the minor isomer is far off-set by the fewer number of required synthetic steps when compared to the other schemes that we evaluated.

Results and discussion

Deuterium experiments

The exchange reaction conditions for the conversion of **4** to the labeled isomers **5a** and **5b** were worked out using D_2O (Scheme 1).

Referring to Table 1, the best results obtained using D_2O gave an average incorporation of about 1.5 deuterium atoms per molecule, and an isomer content of 20–25% desired **5a**. The isomer content could be adjusted to about 45% desired (by reducing the reaction time, experiment 12), but then the average incorporation of deuterium was only about 0.3 deuterium atoms per



Scheme 1. Deuterium exchange reactions

Table 1. Results from the deuterium exchange reactions

Expt.	Substrate (mg)	Substrate (umol)	Solvent	D_2O (ul)	NaOMe (mg)	NaOMe (umol)	Time (h)	Temp (°C)	Isomer % desired	Isotope Equiv.
1	20	55	DMF	200	10.9	200	14	80	14	1.7
2	2	5.5	DMF	10	0.9	16	4	RT	25	1.1
3	2	5.5	DMF	10	0.9	16	1	50	15	1.6
4	2	5.5	DMF	10	0.9	16	4	50	17	1.5
5	2	5.5	DMF	10	0.9	16	1	80	14	1.5
6	2	5.5	DMF	10	0.9	16	4	80	14	1.7
7	1	2.7	THF	1.25	0.16	3	3	RT	90	0
8	1	2.7	1:1 THF:DMF	1.25	0.16	3	3	RT	21	0.8
9	3.5	9.5	1:4 THF:DMF	6	1	18	4	RT	26	1.2
10	1.2	3.3	DMF	12	0.5	9	1	RT	26	1.6
11	1.2	3.3	DMF	12	0.5	9	0.2	RT	21	1.4
12	1	2.7	DMF	12	0.5	9	0.02	RT	45	0.3

molecule. This may simply be a reflection of incomplete exchange and incomplete isomer distribution at short times. In fact, there appears to be a trend of higher ratio of desired isomer correlated to lower percent isotope incorporation. The efficiency of deuterium incorporation and isomer distribution were measured using GC/MS of the dehydration product (the upper side chain tertiary alcohols both dehydrate under GC conditions).

Referring to Table 1, the first experiment was performed at a relatively large scale to provide material for analysis and as a comparison for the scaled-down reactions. For example, comparing trial experiments 1, 5 and 6, the isomer ratio and deuterium incorporation is relatively insensitive to the reaction time (1, 4, and 14 h) and insensitive to an order of magnitude reduction in scale. Comparison of experiments 3 and 4 with experiments 5 and 6 shows that the isomer ratio and deuterium incorporation is also relatively insensitive to the temperature (50° vs 80°) and overall the reaction is quite reproducible at the 10 µl scale. Experiment 2 performed at ambient temperature affords an increase in the desired isomer at the expense of deuterium incorporation. The set of experiments 7–8 were aliquots from a stock solution to test THF as solvent. The relatively high desired isomer ratio in 7 was presumed to be simply a reflection of incomplete enolization and minimal (if any) deuterium incorporation in the absence of DMF. Experiment 9 compared with 2 showed that there was no advantage to incorporating THF as a co-solvent. Experiments 10–11 show that a reaction time of 12 min at ambient temperature is sufficient, while experiment 12 shows that 1 min at ambient temperature is insufficient for reasonable deuterium incorporation. Deuterium experiments where the ratio of deuterated water was decreased to give $>4\,M$ concentrations of $NaOD/D_2O$ resulted in the formation of an impurity.

Tritium synthesis

The tritium exchange reaction was run at the Lawrence Berkeley National Labs National Tritium Labeling Facility because of the large amount of tritiated water used ($\sim$40 Ci). The tritium exchange reaction required the transformation of tritium gas on a platinum oxide catalyst to tritiated water. The tritiated water ($\sim$15 µl) was vacuum transferred onto 3.7 mg NaOMe to give a $\sim 4\,M$ solution of $NaOT/T_2O$; then a DMF solution of 9.5 mg substrate was injected. Based on deuterium experiments 10 and 11, the reaction was presumed complete in 30 min at ambient temperature. Upon quenching the reaction mixture with HOAc, the labile tritiums were removed by methanol exchange affording 1.3 Ci of the crude mixed isomers. Isomer separation could only be accomplished after the protection step. Thus, unlike the deuterium experiments the desired isomer contents were based on the two steps. TLC-radioscan showed only about 6% desired isomer.

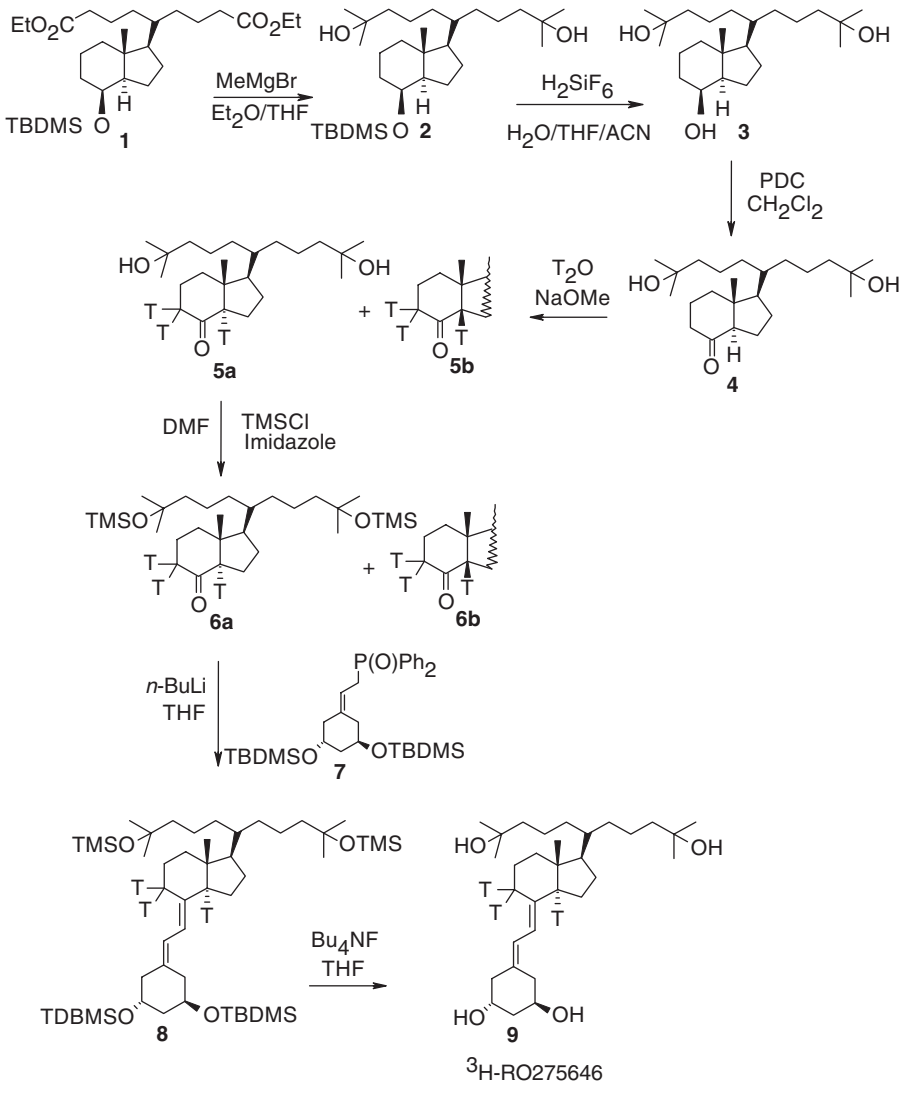
Tritium incorporation (ca. $\geqslant$50 Ci/mmol based on total activity/starting mass) was better than expected as compared to the GC/MS deuterium experiments. The mixed isomer products were TMS protected and the desired isomer **6a** was isolated by a combination of flash column chromatography and preparative HPLC. Following procedures that were worked out using the deuterium analog, the pure single isomer **6a** was coupled to the anion of the extended A-ring phosphene oxide **7** using Horner-Wittig Olefination (0.4 μmol scale). The coupling product **8** was deprotected to afford 22 mCi [9,9,14α-^{3}H]-RO275646 **9** in >99% purity by HPLC and having a specific activity of 64 Ci/mmol. The measured specific activity of the final product **9** was determined by HPLC/liquid scintillation corresponding to 2.2 tritium atoms incorporated per molecule of the desired isomer of the final product. This was higher than expected based on the highest (1.7 deuterium atoms per molecule of desired isomer) GC/MS. This discrepancy may be explained because of the different modes of analysis. The specific activity result for the final product **9** was obtained by HPLC/liquid scintillation. The deuterium incorporation result was obtained by GC/MS on intermediate **5a**. The former method is presumed to be more accurate than the latter. This is because the tritium is non-exchangeable in the final product, while the deuterium is in an exchangeable form in the intermediate, and is thought to partially exchange under GC conditions as discussed above (Scheme 2).

Experimental

Proton spectra were run on a Bruker 300 MHz NMR spectrometer. LC/MS data were recorded from an Agilent 1100 Series LC/MSD. Preparative HPLC was run on a Beckman System 32 Karat Gold using a 125 Solvent Module and a 166 Detector; activity was measured using an INUS System β-Ram. TLC scans were run on a Bioscan System 200 Imaging Scanner; TLC plates (silica gel 250 microns) were obtained from Analtech. ^{1}H NMR (300 MHz) and MS ES+ and GCMS were consistent with the each of the non-labeled products.

*6-[(1R,3aR,4S,7aR)-4-(tert-Butyl-dimethyl-silanyloxy)-7a-methyl-octahydro-inden-1-yl]-2,10-dimethyl-undecane-2,10-diol (**2**)*

Dry THF (10 ml) was injected into a dry septum sealed 3-neck 50 ml flask containing 994 mg of substrate **1** (1.95 mmol). Methyl magnesium bromide (6.0 ml 3 *M* in ether) was added dropwise to the solution stirred at −30°C under nitrogen, and the reaction was allowed to slowly warm to ambient temperature overnight. After cooling to 0 °C, the reaction was quenched by slow addition of 15 ml saturated aqueous ammonium chloride. The product was extracted three times with a total of 45 ml ethyl acetate. The organic phase was dried with brine, and anhydrous sodium sulfate, then filtered and concentrated under pump vacuum. Flash chromatography (silica gel: 40%



Scheme 2. Preparation of [^{3}H]-RO275646

ethyl acetate/hexane) afforded 720 mg (1.49 mmol, 76% yield) of compound **2** as a brittle white foam.

*6-((1R,3aR,4S,7aR)-4-Hydroxy-7a-methyl-octahydro-inden-1-yl)-2,10-dimethyl-undecane-2,10-diol (***3***)*

A 700 mg (1.45 mmol) portion of product **2** was rinsed with 4 ml THF and 4 ml acetonitrile into a 30 ml Teflon bottle. A 25% aqueous solution of fluorosilicic acid (5 ml) was added and the reaction was stirred at ambient temperature for 69 h, and then at 37° for 20 h until nearly all of compound **2** had been

deprotected. The reaction was quenched with 10 ml saturated aqueous sodium bicarbonate, and then extracted three times with 20 ml portions of ethyl acetate. The combined organics were dried with an equal volume of brine, and then with anhydrous sodium sulfate. The solution was filtered and concentrated to a clear, colorless glass. Flash chromatography (silica gel; 50% ethyl acetate/hexane) followed by the concentration of pure product fractions afforded 399 mg (1.08 mmol, 74% yield) of triol **3**.

*(1R,3aR,7aR)-1-[5-Hydroxy-1-(4-hydroxy-4-methyl-pentyl)-5-methyl-hexyl]-7a-methyl-octahydro-inden-4-one (**4**)*

Triol **3** (93.8 mg, 0.255 mmol) was dissolved in 2.5 ml dichloromethane. Pyridinium dichromate (PDC, 389 mg, 1.01 mmol) was added and the deep brown solution was stirred at ambient temperature overnight. An additional 200 mg of PDC and 100 mg dry Celite were added and the reaction was stirred for a second night until the reaction was about 75% complete. The salts were precipitated by dilution with 25 ml ether. The mixture was filtered, and the filtrate was concentrated to dryness. Product **4** was isolated by flash chromatography (silica gel; 2.5% $MeOH/CH_2Cl_2$) affording 66.7 mg white foamy semi-solid (0.18 mmol, 71% yield).

*(1R,3aR,7aR)-[3a,5,5-^{3}H]-1-[5-hydroxy-1-(4-hydroxy-4-methyl-pentyl)-5-methyl-hexyl]-7a-methyl-octahydro-inden-4-one (**5a**)*

Cold preparation. All glassware was dried. The reaction apparatus consisted of a 'wishbone' equipped with three stopcocks arranged so that the apparatus and each of two 5 ml septum side-arm flasks could be isolated. To the flask on the left was added 200 mg PtO_2, and to the flask on the right was added ca. 3.7 mg NaOMe under nitrogen. Both flasks (each containing a micro-spin bar) were evacuated. Separately, the substrate **4** (9.5 mg, 0.026 mmol) was dissolved in 0.5 ml freshly degassed and distilled DMF.

Hot work. The tritium gas ($\sim$40 Ci) was transferred onto the PtO_2 catalyst at ambient temperature. After the flash, the T_2O generated was cooled to $-198\,^{\circ}C$, and the excess tritium gas was removed. The T_2O was then vacuum transferred on to the NaOMe. The mixture was warmed to ambient temperature, the vacuum was released with nitrogen and the NaOMe was dissolved in the T_2O by means of the internal micro spin bar. A 0.5 ml DMF solution of the substrate **4** was injected all at once, followed by a 0.25 ml DMF rinse. After stirring for 30 min, the reaction was neutralized by the injection of 50 µl 1:3 v/v HOAc:DMF. After stirring for 10 min, volatiles were removed by vacuum transfer, and chased with three 0.5 ml portions of MeOH to exchange off the tritium from the hydroxyl groups, and once with toluene to azeotrope off the excess HOAc. The residue was redissolved in 2 ml ether, and filtered

(0.2 μ Teflon filter, to remove NaOAc) with two 2 ml ether rinses into a 25 ml flask. The solution was concentrated to dryness by N_2 evaporation, and the residue was redissolved in freshly distilled THF. A 50 mCi portion was removed for tritium NMR and the solution was assayed for activity (1.3 Ci total) and transferred to two ampoules in a total of 2.7 ml THF each and sealed under nitrogen. ^{3}H NMR (300 MHz) δ 1.95 (2T, m), δ 2.22 (1T, m).

*(1R,3aR,7aR)-1-[5-(tert-Butyl-dimethyl-silanyloxy)-1-(4-dimethylsilanyloxy-4-methyl-pentyl)-5-methyl-hexyl]-[3a,5,5-^{3}H]-7a-methyl-octahydro-inden-4-one (**6a**)*

The 2.7 ml THF solution of mixed isomers was transferred to a dry 15 ml #7 O-ring stopcock flask containing a micro-spin bar. The THF was removed by vacuum transfer, and the residue was chased with 1 ml dry toluene. A 0.5 ml DMF solution of TMSCl (0.13 mmol) and imidazole (0.33 mmol) from a freshly prepared bulk solution was added and the mixture was stirred for 17 h at ambient temperature. The DMF was removed by vacuum transfer and the residue was triturated with 0.5 ml hexane. The hexane solution was applied directly to an 8 ml SiO_2 disposable flash column pre-equilibrated with 2.5% EtOAc/hexane + 0.25% TEA, rinsing with two 0.25 ml portions of hexane and two 0.5 ml portions of eluent. The hexane insoluble residue was dissolved in 1.0 ml DMF. Chromatography of the hexane soluble portion, combining all product containing fractions, purified the isomers and enriched the isomer ratio to ~20% correct isomer. A second re-purification gave about a ~50 mCi 1:1 mixture of isomers. The pure desired isomer **6a** (26 mCi) was isolated by preparative HPLC (4.6 × 250 mm Zorbax RX-Silica; 0.1% TEA in pet ether, 2 ml/min; dry β-Ram cell; compound **6b** 8 min, compound **6a** 12.5 min).

*(1R,3aR,7aR)-4-[2-[(3R,5R)-3,5-Bis-(tert-butyl-dimethyl-silanyloxy)-cyclohexylidene]-eth-(Z)-ylidene]-[3a,5,5-^{3}H]-7a-methyl-1-[5-methyl-1-(4-methyl-4-trimethylsilanyloxy-pentyl)-5-trimethylsilanyloxy-hexyl]-octahydro-indene (**8**)*

The reaction was run on a vacuum line using a #7 O-ring 'Tribone' manifold containing four stopcocks. The stopcock at the top was used to isolate the manifold from a Firestone valve (that allows for vacuum or atmospheric nitrogen pressure), and stopcocks at each of three arms. One arm was used for the attachment of a 5 ml pear shaped septum side-arm reaction flask containing a spin bar. The second arm was used for the attachment of a 10 ml pear shaped septum side-arm phosphene oxide anion generation flask containing a spin bar. The third arm was used for attachment of either a distillate receiver or a THF/LAH reservoir. The system was dried by pump overnight. From this point on all products were treated as air and light sensitive. A 0.5 ml toluene solution of 18.5 mCi (ca. 0.4 μmol) of the labeled bis-TMS ketone substrate **6a** was transferred to the reaction flask. The toluene

was removed by vacuum transfer to a distillate receiver flask. Two 0.5 ml portions of dry toluene were used to rinse in the remainder of the substrate into the reaction flask, followed by removal of volatiles by vacuum transfer. The distillate receiver flask was replaced by the THF/LAH reservoir flask. About 0.5 ml THF was distilled into the reaction flask containing the semi-solid substrate residue. The residue was dissolved by use of an internal spin bar, and cooled to −72 °C. The phosphine oxide was dried by twice vacuum transfer of volatiles from 1 ml toluene solutions of the phosphine oxide **7** (18.8 mg, 0.033 mmol, containing 4% water) contained in the anion generation flask. The residue was redissolved in about 1 ml THF distilled into the flask. The anion (deep orange-red) was generated by adding 16 µl of 1.6 *N* *n*-BuLi/hexane (0.026 mmol) to the stirred −72 °C solution. With both flasks at −72 °C, the anion was transferred dropwise (by syringe injection of dry nitrogen into the sealed anion flask) by canula to the reaction flask until an orange color remained (9 drops), a further 9 drops was then added. The titration procedure was continued at 30–60 min increments until the orange color remained in the reaction flask for about 30 min (about 2.5 h total and about 15 drops total more anion added). The reaction was quenched by canula transfer from the reaction flask to a well stirred ambient temperature 2 ml 2 *M* pH 7 sodium phosphate buffer solution. After rinsing in with distilled THF (5 ml), the aqueous phase was extracted three times with hexane (2 ml ea.). The combined hexane/THF phase was dried with brine and anhydrous sodium sulfate, filtered, and concentrated. The residue was redissolved in 0.5 ml hexane and applied to 8 ml silica gel pre-equilibrated with the eluent (1.25% EtOAc/hexane + 0.125% TEA) in a 10 ml disposable column. Following flash chromatography, the product containing fractions were combined and concentrated to dryness and re-dissolved in hexane affording a nearly quantitative recovery of compound **8** (TLC: Silica gel: 5% EtOAc/hexane + 0.5% TEA, R_f 0.8).

*(1R,3R)-5-{2-[(1R,3aR,7aR)-[3a,5,5-^{3}H]-1-[5-hydroxy-1-(4-hydroxy-4-methyl-pentyl)-5-methyl-hexyl]-7a-methyl-octahydro-inden-(4Z)-ylidene]-ethylidene}-cyclohexane-1,3-diol (**9**)*

A portion (90%) of the hexane solution of ^{3}H-labeled tetra-silyl substrate **8** from the previous step was transferred to a 10 ml pear shaped flask and the volatiles were removed by vacuum transfer. The residue was treated with a large excess (1 ml) of tetra-butyl ammonium fluoride 1 *M* in THF. The solution (wrapped in foil to exclude light) was stirred for 15 h, and then partitioned between 5 ml ea. water and EtOAc. The aqueous phase was extracted twice with 5 ml portions of EtOAc, and the combined organic phase was dried with brine and anhydrous Na_2SO_4. After filtration, the filtrate was concentrated to dryness and immediately re-dissolved under nitrogen in 0.5 ml

EtOAc. This solution was applied to a 10 ml disposable flash column containing 8 ml silica gel in EtOAc. The product was eluted with 50 ml of EtOAc, then 50 ml 2% *i*-PrOH/EtOAc, and finally, 50 ml 5% *i*-PrOH/EtOAc. Product containing fractions were combined affording 12.5 mCi of **9**. A second run afforded 22 mCi of **9**.

Conclusion

The method that we have developed for T_2O exchange labeling was based on D_2O exchange labeling experiments. This method is quite robust based on the reproducibility of the tritiation run and trial deuterium incorporation experiments. The reactions run in DMF were relatively insensitive to time (12 min–14 h), temperature (22–80 °C), and scale (3–55 μmol substrate) as long as 2.5 equiv. of NaOMe and at least 0.5 μl of D_2O/μmol of substrate was used. The method was readily translated to T_2O exchange labeling at scale. Following procedures that were worked out using the deuterium analog, the pure single isomer **6a** was coupled to the anion of the extended A-ring phosphene oxide **7** using Horner-Wittig Olefination (0.4 μmol scale). The coupling product **8** was deprotected to afford 34 mCi [9,9,14α-^{3}H]-RO275646 **9** (from two runs) in >99% purity by HPLC and having a specific activity of 64 Ci/mmol.

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References

1. Zhu G-D, Okamura WH. *Chem Rev* 1995; **95**: 1877–1952.
2. Hartung T, Hilpert H, Mohr P. Synthesis of the carbon-14 labelled antipsoriatic vitamin D_3 derivative RO 65-2299. In *Synthesis and Applications of Isotopically Labelled Compounds*, Pleiss U, Voges R (ed). vol. 7. Wiley: New York, 2000; 189–192.
3. Napoli JL, Fivizzani MA, Hamstra AJ, Schnoes HK, DeLuca HF. *Anal Biochem* 1979; **96**: 481–488.
4. Perlman K, Schnoes HK, Tanaka Y, DeLuca HF, Kobayashi Y, Taguchi T. *Biochemistry* 1984; **23**: 5041–5048.
5. Yamada S, Schnoes HK, DeLuca HF. *Anal Biochem* 1978; **85**: 34–41.
6. DeLuca HF, Weller M, Blunt JW, Neville PF. *Arch Biochem Biophys* 1968; **124**: 122–128.
7. Hoeger CA, Okamura WH. *J Am Chem Soc* 1985; **107**: 268–270.
8. Neville PF, DeLuca HF. *Biochemistry* 1966; **5**: 2201–2207.

9. Holick SA, Holick MF, Frommer JE, Henley JW, Lenz JA. *Biochemistry* 1980; **97**: 1031–1037.
10. Holick MF, Tavela TE, Holick SA, Schnoes HK, DeLuca HF, Gallagher BM. *J Biol Chem* 1976; **251**: 1020–1024.
11. Okamura WH, Hoeger CA, Miller KJ, Reischl W. *J Am Chem Soc* 1988; **110**: 973–974.
12. Perlmam KL, Swenson RE, Paaren HE, DeLuca HF. *Tetrahedron Lett* 1991; **32**: 7663–7666.
13. Ahene AB, Chang FE, Masjedizadeh MR, Wu SY, de Keczer SA, Tarnowski T. *Poster presented at AAPS Meeting in Salt Lake City*, October 26–30, 2003.
14. Hilpert H, Wirz B. Roche Internal Report, 2000.

