Synthesis and NMR Studies of ^{13}C-Labeled Vitamin D Metabolites[1]

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Received November 21, 2001

Isotope-labeled drug molecules may be useful for probing by NMR spectroscopy the conformation of ligand associated with biological hosts such as membranes and proteins. Triple-labeled [7,9,19-$^{13}C_3$]-vitamin D_3 (**56**), its 25-hydroxylated and 1α,25-dihydroxylated metabolites (**58** and **68**, respectively), and other labeled materials have been synthesized via coupling of [9-^{13}C]-Grundmann's ketone **39** or its protected 25-hydroxy derivative **43** with labeled A ring enyne fragments **25** or **26**. The labeled CD-ring fragment **39** was prepared by a sequence involving Grignard addition of [^{13}C]-methylmagnesium iodide to Grundmann's enone **28**, oxidative cleavage, functional group modifications leading to seco-iodide **38**, and finally a kinetic enolate S_N2 cycloalkylation. The C-7,19 double labeling of the A-ring enyne was achieved by the Corey−Fuchs/Wittig processes on keto aldehyde **11**. By employing these labeled fragments in the Wilson−Mazur route, the C-7,9,19 triple-^{13}C-labeled metabolites **56**, **58**, and **68** as well as other ^{13}C-labeled metabolites have been prepared. In an initial NMR investigation of one of the labeled metabolites prepared in this study, namely [7,9,19-$^{13}C_3$]-25-hydroxyvitamin D_3 (**58**), the three ^{13}C-labeled carbons of the otherwise water insoluble steroid could be clearly detected by ^{13}C NMR analysis at 0.1 mM in a mixture of CD_3OD/D_2O (60/40) or in aqueous dimethylcyclodextrin solution and at 2 mM in 20 mM sodium dodecyl sulfate (SDS) aqueous micellar solution. In the SDS micellar solution, a double half-filter NOESY experiment revealed that the distance between the H_{19Z} and H_7 protons is significantly shorter than that of the corresponding distance calculated from the solid state (X-ray) structure of the free ligand. The NMR data in micelles reveals that **58** exists essentially completely in the α-conformer with the 3β-hydroxyl equatorially oriented, just as in the solid state. The shortened distance (H_{19Z}−H_7) in micellar solutions as compared to that in the solid state is most easily rationalized on the basis that the 5(10)-torsion angle in **58** is decreased in micellar solutions as compared to that in the solid state.

Introduction

It has been found in recent years that the vitamin D metabolic system exhibits a broad spectrum of biological activities, well beyond its classical functions in regulating calcium and phosphorus metabolism.[2] This steroid hormone system and its analogues have been used or have high potential in treating a diverse range of human diseases such as cancer, skin diseases, and diseases associated with aberrant immunological responses or calcium metabolism.[2a,d] An understanding of the detailed mechanism of vitamin D action at the molecular level remains incomplete, but recent progress in the X-ray crystallographic studies of the ligand bound vitamin D nuclear receptor (n-VDR)[3a−e] and plasma vitamin D binding protein (DBP)[3f,g] have been major steps forward in this regard.

The seco-steroid vitamin D in its principal metabolic forms [Scheme 1: vitamin D_3 (**1**, D3), 25-hydroxyvitamin D_3 (**2**, 25-D3) and the hormonally active form 1α,25-dihydroxyvitamin D_3 (**3**, 1,25-D3)] involve association with a variety of host systems including membranes and proteins during the course of mediating their biological actions. To gain insight into ligand design, extensive effort has gone into solid-state, solution, and computational studies of vitamin D ligands (**1**−**3**).[2b,4−7] X-ray crystallographic results for a number of vitamin D structures including **1**−**3** reveal that the $\Delta^{5,7}$-diene is 6-s-trans and that this diene subunit deviates from planarity within a range of only ±8.1°.[2b,8] For crystalline **3**, the deviation from planarity is less than 3°.[8c] The $\Delta^{10(19)}$ double bond deviates from the $\Delta^{5,7}$-diene plane, however, wherein the 5,10-s-cis bond deviates from planarity within the range ±52.3°.[2b] The near planarity of the $\Delta^{5,7}$-diene as well as the 6-s-trans orientation in **1**−**3** is also apparent from solution NMR Karplus relationship studies.[9] The only completed structures of protein-bound

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(1) (a) This study was supported by grants from the National Institutes of Health (NIH Grant No. DK-16595), the National Science Foundation facilities programs (NSF-MRI-CHE-9724392, NSF-ARI-9602708, NSF-ARI-9601831, and NSF-ARI-ST-193-13519), the Cambridge Isotopes Laboratories (CIL Research Grant Program), and the Committee on Research of UC Riverside. Selected chemicals were also provided by Solvay, Hoffmann-LaRoche, and Leo Pharmaceuticals. (b) Department of Chemstry. (c) Also, the Analytical Chemistry Instrumentation Facility. (d) Department of Biochemistry.

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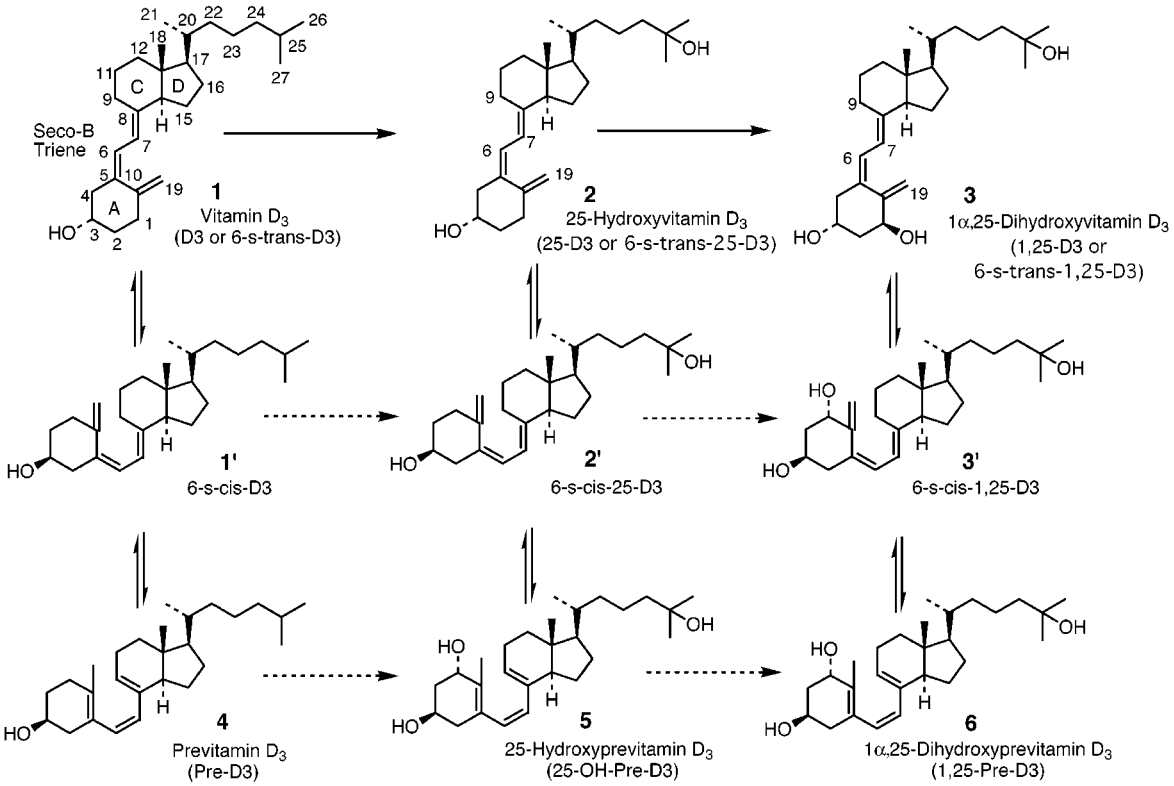
10.1021/jo011096y CCC: $22.00
Published on Web 02/02/2002

Scheme 1



vitamin D compounds are the X-ray studies of **3** and several analogues bound to a doubly truncated n-VDR-LBD (n-VDR-ligand binding domain).[3a,c,d] In these recently reported studies by the Moras Laboratory, amino acids 1−117 and 165−215 were deleted from the full length, wild-type human n-VDR (427 amino acids) in order to achieve good crystallinity. Interestingly, there is in the protein bound ligand **3** an out-of-plane 31.4° twist of the s-trans 6,7-single bond (as compared to only 2.5° for the free ligand[8c]). The cisoid 5(10)-single bond of the same bound ligand is twisted by 52.8° out of plane (as compared to 48.3° in the free ligand[8c]).

Thus far, there is no direct structural evidence for 6-s-cis forms **1′**−**3′** of metabolites **1**−**3**, respectively.[2b,4] It is known, however, that these metabolites interequilibrate with previtamin structures **4**−**6**, respectively, via antarafacial [1,7]-sigmatropic hydrogen shifts between the C_9 and C_{19} positions.[10] To undergo this intramolecular valence tautomerism, the 6-s-cis forms **1′**−**3′** must be available at kinetically competent concentrations. While the equilibrium concentrations of 6-s-trans vitamins **1**−**3** (∼90%) relative to the previtamins **4**−**6** (∼10%) are well documented, information concerning the concentrations of 6-s-cis vitamins **1′**−**3′**, respectively, at equilibrium remains elusive.

In view of the conformational flexibility of vitamin D and the ability of vitamin D to equilibrate tautomerically with the previtamins (Scheme 1), a pertinent question is whether these higher energy forms are in fact involved in native protein binding. That this may indeed be the case first emerged from our finding that 1,25-pre-D3 (**6**) is as active as the parent hormone 1,25-D3 (**3**) in stimulating at least one of its biological responses, namely the rapidly acting calcium transport response known as transcaltachia.[11−13] This response is thought to be initiated through binding to a membrane receptor. The 6-s-cis locked previtamin **6** was 5% as effective as the parent hormone **3**, however, in binding to the n-VDR.

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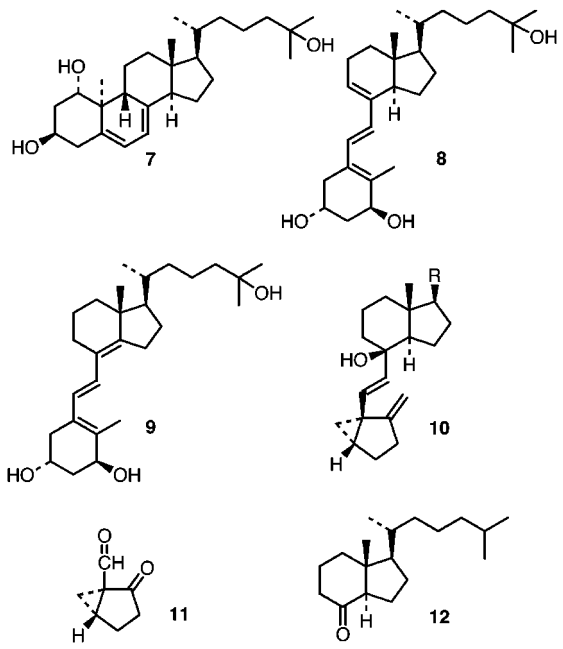
Chart 1



The recent X-ray structure noted above of the hormone 1,25-D3 bound to the n-VDR-LBD[3a−c] suggests that a 30° twisted s-trans form of **3** is required for binding, but it is difficult to explain why **6** shows even a 5% binding affinity. Moreover, additional evidence has accrued[14] suggesting that this same hormone binds to the membrane receptor in its spectroscopically invisible s-cis form **3′**, the rationale being that the latter is mimicked effectively by the s-cis-locked 1,25-pre-D3 (**6**). A study of 1α,25-dihydroxylumisterol (**7**, "lumi") shown in Chart 1 supported this s-cis interpretation in that the "lumi" analogue bound equally as well as **6** and **3**/**3′** to the membrane receptor. Moreover, the lumi analogue **7**, like **6**, bound weakly to the nuclear receptor. It was also shown[14] that the 6,7-trans-locked analogues 1α,25-dihydroxytachysterol (**8**) and 1α,25-dihydroxyisotachysterol (**9**) failed to bind effectively to either the nuclear or membrane receptors.[14] It was suggested therefore that an out-of-plane 6,7-twisted s-cis conformer of **3** or **3′** might be involved in nuclear receptor binding. However, on the basis of the recent protein X-ray result, the Moras group has logically suggested[3a] that the inability of **8** to bind to the n-VDR is that **8** is unable to twist (by 30°) about its 6,7-single bond because of the presence of the double bond. It is clear that there is a 360° continuum of topologies available to the hormone 1,25-D3 by rotation about its 6,7-single bond.[5] Determination of the topology of 1,25-D3 as well as the other metabolites shown in Scheme 1 by 6,7-single bond rotation in the active site of protein would provide insight into drug design. To evaluate this concept for this particular series of flexible ligands, we describe herein a strategy for determining the conformation of the seco-B region of vitamin D metabolites and analogues in ligand-protein complexes. The essential feature of the strategy is the synthesis of C-9,19 and C-7,19 double ^{13}C and C-7,9,19 triple ^{13}C labeled vitamin D compounds since the NMR techniques for measuring the distance between protons attached to pairs of ^{13}C atoms, or directly between pairs of ^{13}C atoms themselves, are available. Thus, a combination of C-9,19 and C-7,19 double ^{13}C labeled vitamin D metabolites would nicely provide a means of distinguishing 6-s-trans vitamin D from 6-s-cis vitamin D in the protein−ligand complex. For example, in solution, the NOESY spectrum containing exclusively NOEs of ^{13}C-bound protons could be achieved with use of a heteronuclear-resolved double half-filter experiment.[15,16] Utilizing these solution tech-

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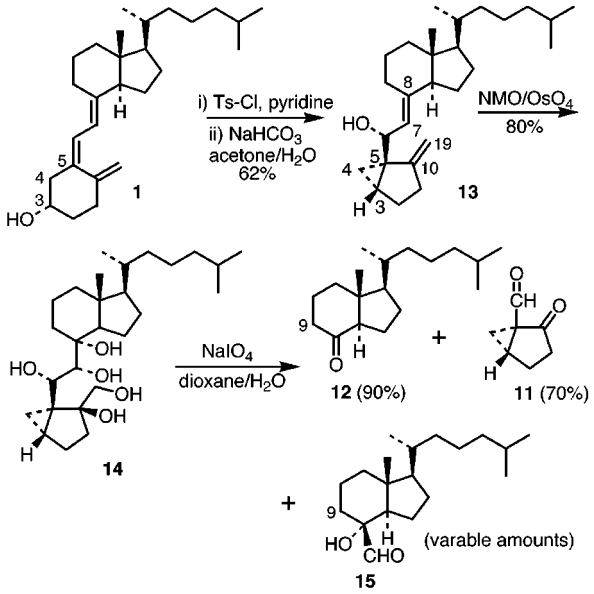
niques, it is anticipated that a significant NOE between H_9 and H_{19} would implicate the steroidal s-cis conformation (**1′**, **2′**, and **3′**) while an enhanced NOE between H_7 and H_{19} would suggest an s-trans conformation (**1**, **2**, and **3**). The use of triple-labeled (C-7,9,19) material rather than pairs of double-labeled materials (C-7,19 and C-9,19) could be more economical in probing for both possibilities in a single experiment. Alternatively, using solid-state NMR spectroscopy, the distance between pairs of ^{13}C-atoms can be obtained through rotationally resonant magnetization exchange with high accuracy (±0.1 Å up to 6.5 Å).[17,18] To demonstrate the feasibility of this approach, it is first necessary to synthesize doubly and triply ^{13}C-labeled vitamin D compounds[19] and to demonstrate that ^{13}C NMR studies of these steroids in water are feasible. We report herein the synthesis of multiply labeled D3, 25-D3, and 1,25-D3 and an initial NMR experiment on a model micellar system.

Results and Discussion

A number of general approaches toward construction of the vitamin D skeleton have been developed,[2c] but because of the central seco-B location of the desired labels in the targeted steroids, the Wilson−Mazur coupling protocol[20−25] entailing sovolysis of intermediates of type **10** (Chart 1) seemed best to suit our NMR requirements.

Scheme 2



This route employs keto aldehyde **11** to which Wittig methodology was anticipated to allow introduction of one or two ^{13}C labels into the triene/A-ring at a reasonably late stage. Several useful syntheses of derivatives of the keto aldehyde **11** have been reported[20a,22−23] and the synthesis reported herein extends these results. A number of total synthesis routes to derivatives of an appropriate CD fragment of the type **12** have been reported.[2c] Because the reported total syntheses including label incorporation appeared to require far too many steps, it was anticipated that proper transformations of Grundmann's ketone (**12**) would better allow introduction of ^{13}C label α to the carbonyl group at position-9. The easily available, parent vitamin D_3 would nicely provide by oxidative degradation the key keto aldehyde **11** as well as Grundmann's ketone (**12**).

Solvolysis of cholecalciferyl tosylate (aqueous acetone, $NaHCO_3$), which was synthesized from vitamin D_3 and *p*-toluenesufonyl chloride, furnished the known, starting cyclovitamin **13** (Scheme 2).[24] Hydroxylation of the cyclovitamin **13** with stoichiometric osmium tetraoxide in pyridine afforded pentaol **14** in poor yield (20−30%), whereas the catalytic version (NMO/OsO_4/acetone/H_2O/pyridine) gave this material in very good yield (80%). This interesting difference in reactivity can be rationalized on the basis that initial osmylation occurs at the $\Delta^{10,19}$ double bond, presumably to afford an osmate ester (with the stereochemistry corresponding to that in **14**).[25] Under stoichiometric conditions, it is suggested that the sterically bulky osmate ester hinders further osmylation across the $\Delta^{7,8}$ double bond. Under catalytic conditions, the less hindered 10,19-diol is produced in situ, permitting further oxidation of the $\Delta^{7,8}$-double bond. It is known that the corresponding methyl ether of **13** is selectively osmylated across the $\Delta^{10,19}$ double bond under stoichiometric conditions.[19,25] Under even our catalytic conditions (NMO, OsO_4/acetone/H_2O/pyridine) using excess reagent

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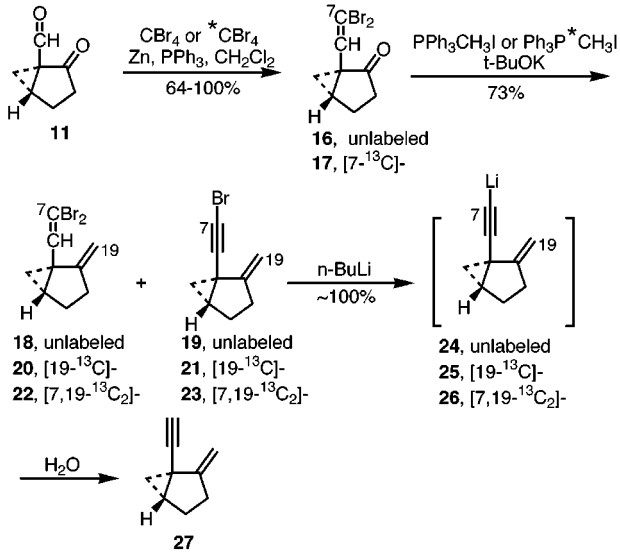
(25) Paaren, H. E.; Schnoes, H. K.; DeLuca, H. F. Electrophilic Addition of OsO_4 to 25-Hydroxycholecaliferol and Its 3,5-Cyclo Derivative. *J. Org. Chem.* **1983**, *48*, 3819−3820.

Scheme 3



described above, we have found that this same methyl ether undergoes osmylation only across the $\Delta^{10,19}$ double bond (unpublished observation). From our experience, the polarity of the desired product **14** is less than that expected of a pentanol ($R_f = 0.53$ in 1:10 ^{i}PrOH/CH_2Cl_2); its surprisingly moderate solubility in the aqueous phase facilitated the work up. Oxidative cleavage of the pentanol **14** with sodium periodate readily afforded Grundmann's ketone **12** and keto aldehyde **11**, the latter being the key intermediate for preparing the ^{13}C-labeled A-ring fragment. Variable amounts of hydroxyaldehyde **15** were also isolated from the reaction mixture and can be further transformed to Grundmann's ketone using prolonged reaction times. However, more optimal yields of keto aldehyde **11** (together with higher percentages of **15** relative to **12**) are obtained at shorter reaction times.

The unlabeled and labeled A-ring fragments were prepared as outlined in Scheme 3. Keto aldehyde **11** was transformed into enyne **27** by the Corey−Fuchs procedure[26] superimposed on a normal Wittig process. Treatment of **11** with at least 3.5 equiv of CBr_4-based reagent afforded a quantitative yield of **16**. The use of less reagent (2.5 equiv) afforded only a 64% yield of product, but there is the potential of more economically utilizing [^{13}C]-CBr_4. The second Wittig reaction using unlabeled reagent on **16** then afforded an 80% yield of products, which upon HPLC separation afforded diene dibromide **18** (56%) and bromoenyne **19** (17%). Treatment of either **18** or **19** or a mixture with butyllithium afforded **24**, which upon quenching afforded the previously reported, highly volatile enyne **27**.[22] The losses incurred because of this volatility prompted the direct use of the labeled lithium salts **25** and **26** in the coupling reactions described below.

The [19-^{13}C] monolabeled alkynyllithium salt **25** was prepared directly from a crude, dry mixture of **20** plus **21**, which were in turn prepared from unlabeled **16**. The [7,19-$^{13}C_2$] di-labeled alkynyllithium salt **26** was likewise prepared from a mixture of bromides **22** and **23**. In an analogous sequence, the latter mixture was prepared from the crude monolabeled ketone **17**.

The synthesis of the requisite ^{13}C labeled CD-ring moiety is summarized in Scheme 4. The known Grundmann's enone **28** was synthesized from Grundmann's ketone (**12**) in 50−60% yield as previously described.[27] Addition of [^{13}C]-methylmagnesium iodide to the enone in the presence of a 2-fold excess of cerium(III) chloride gave **29** in high yield. The allylic alcohol **29** was not stable to column chromatography (silica gel) but could be purified on a small scale by HPLC. The crude product was directly ozonized in methanol followed by $NaBH_4$ work up to afford triol **31**. Reduction of the methoxyhydroperoxide intermediate **30** (^{1}H NMR analysis) with sodium borohydride is very slow, requiring a large excess of sodium borohydride with overnight stirring at room temperature. Oxidative cleavage of the triol **31** with sodium periodate led to keto alcohol **32** but an attempt to directly derivatize the latter with tosyl chloride failed. Selective monotosylation of **31** at the C-11 hydroxyl followed by oxidative glycol cleavage was then considered since tosylation of the 8,9-glycol moiety might be expected to be significantly slower than the isolated primary alcohol. Such an approach would have shortened the eventually successful sequence leading to **37**. Surprisingly, no reaction was detected when **31** was treated with a large excess of tosyl chloride (5 equiv) in pyridine, even at room temperature for 20 h. Under stronger conditions (Et_3N, DMAP, CH_2Cl_2) monotosylation appeared to have occurred, but apparently not selectively at the C-11 hydroxyl. The putative monotosylate under oxidative cleavage conditions ($NaIO_4$/aqueous dioxane) did not afford **37**. This approach was abandoned.

The crude oxidation product **32** earlier obtained from **31** was subjected to direct sodium borohydride reduction, affording a mixture of two diastereomeric diols **33** and **34** (2:1 ratio). The stereochemistry of the two isomers follows from the subsequent transformation and analysis. Tosylation of the mixture of **33** and **34** with tosyl chloride at −25 °C afforded an easily separable mixture of monotosylate **35** and pyran **36**. In a separate experiment, **33** was transformed only to tosylate **35** and **34** only to cyclized product **36** under the tosylation conditions. In the latter case, the initially formed monotosylate is apparently not stable and is considered to be displaced by the hydroxyl group in an intramolecular S_N2 manner. The stereochemistry of **34** was correlated to that of **36**, which was assigned on the basis of an observation of a large trans-diaxial coupling (9.8 Hz) between H-8 and H-14 (while decoupling the ^{13}C and H's of the *CH_3 group). According to a Felkin−Ahn analysis (Chart 2), (8*S*) product **34** should be the major isomer. Here, however, a remote hydroxyl group chelation effect might be involved in the reduction of **32**, leading to a slight preference for the observed **33** rather than the expected **34**. As to why **33** affords the normal hydroxy-tosylate **35**, an intramolecular S_N2 process involving the latter would suffer a serious 1,3-diaxial interaction between the 18-methyl group and the isotopically labeled methyl group (i.e., the resulting product would be the C_8 epimer of **36**).

Oxidation of **35** with PCC afforded ketotosylate **37**, which upon treatment with either lithium diisopropylamide (LDA) or lithium or sodium bistrimethylsilylamide

(26) Corey, E. J.; Fuchs, P. L. A Synthetic Method for the Formyl to Ethynyl Conversion. *Tetrahedron Lett.* **1972**, 3769−3772.

(27) (a) Okamura, W. H.; Aurrecoechea, J. M.; Gibbs, R. A.; Norman, A. W. Synthesis and Biological Activity of 9,11-Dehydrovitamin D_3 Analogues: Stereoselective Preparation of 6β-Vitamin D Vinylallenes and a Concise Enynol Synthesis for Preparing the A-Ring. *J. Org. Chem.* **1989**, *54*, 4072−4083. (b) Daniel, D.; Middleton, R.; Henry, H. L.; Okamura, W. H. Inhibitors of 25-Hydroxyvitamin D_3-1α-Hydroxylase: A-Ring Oxa Analogs of 25-Hydroxyvitamin D_3. *J. Org. Chem.* **1996**, *61*, 5617−5625.

Scheme 4

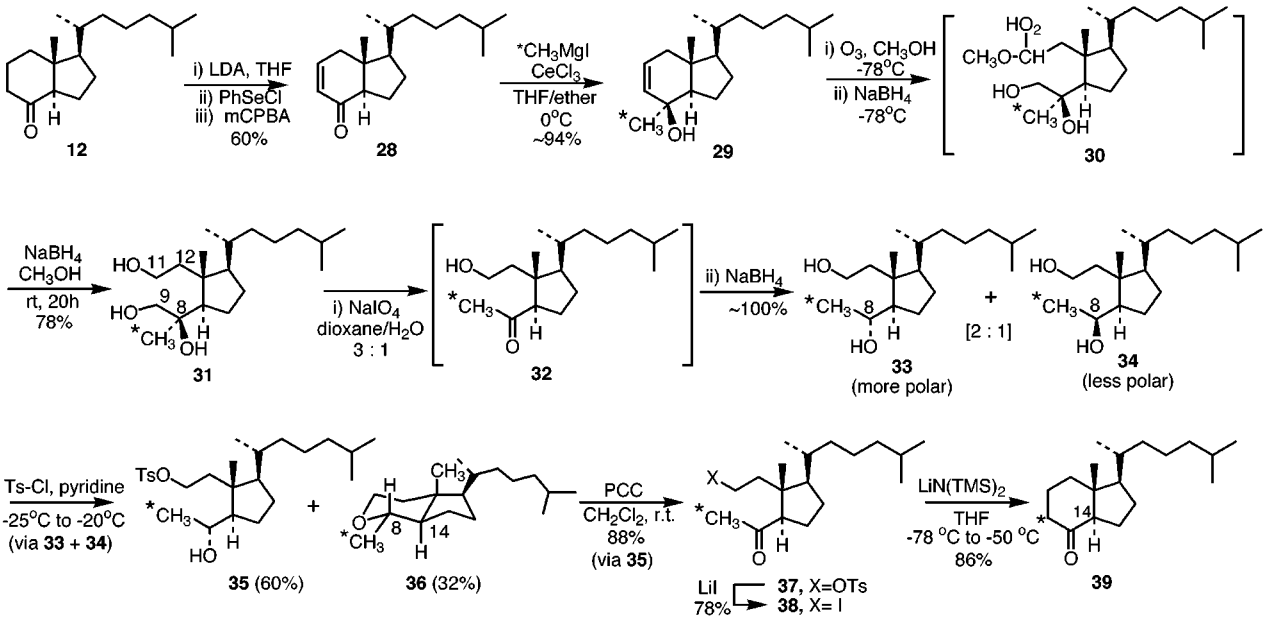
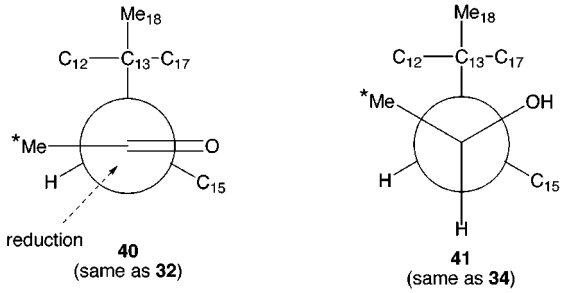


Chart 2



[LiN(TMS)$_2$ or NaN(TMS)$_2$] failed to give cyclized product, leading only to tosylate cleavage product **32**. It should be noted that the tosylate **37** is relatively stable in THF, hexane, ethyl acetate and benzene, but in chloroform it readily produces **32**. When **37** was transformed into iodide **38**, a highly labile compound, cyclization by treatment with LiN(TMS)$_2$ at −78 °C with quenching at −50 °C afforded 86% of the desired [9-^{13}C]-Grundmann's ketone (**39**) and no detectable epimerization at C-14. The use of NaN(TMS)$_2$ led to small amounts of C-14 epimer. Finally, it should be mentioned that several alternative schemes directed toward preparing **39**, including a more direct method reported by DeNinno[28] using intermediates such as **29**, were unsuccessful.

Coupling of the CD-ring fragment **39** with the doubly labeled A-ring enyne anion **26** gave a nearly quantitative yield of triple labeled adduct **44**. Reduction of propargyl alcohol **44** with a 1:1 mixture of lithium aluminum hydride and sodium methoxide resulted in formation of trans-$\Delta^{6,7}$ olefin **50** in 84% yield. In the absence of sodium methoxide, lower yields were obtained. Acid-catalyzed solvolysis of **50**[20,22] provided the desired triply ^{13}C labeled vitamin D$_3$ **56** as well as its 5,6-trans isomer **57**. The latter could be transformed into the former by photosensitized irradiation (9-acetylanthracene, *tert*-butyl methyl ether, Pyrex, Hanovia 450 W medium-pressure Hg lamp)[22] in satisfactory overall yield. An analogous sequence starting from the protected 25-hydroxy CD fragment **43**, which was derived from direct 25-hydroxylation of **39**[29,30] followed by TMS protection, and double labeled A-ring fragment **26** produced the triply ^{13}C labeled 25-hydroxy metabolite **58** and 5,6-trans isomer **59**. Likewise, the latter could be converted into the former by photosensitized irradiation. In repeated trials, 25-hydroxylation of Grundmann's ketone by RuCl$_3$ proved erratic, but acceptable results were obtained(**39** to **42**: 40−45% yield; 52−56% based on recycled starting material) by using increased amounts of RuCl$_3$ catalyst (up to 20 mol %) and shorter reaction times (1−2 days) when compared to that previously reported.[29] In a second more efficient procedure 25-hydroxylation was carried out with methyl-(trifluoromethyl)dioxirane (**39** to **42**: 71−90% yield).[30] Dimethyldioxirane oxidation[30] was slower and gave yields similar to those obtained using RuCl$_3$.[29]

The double-labeled vitamin D metabolites **60** and **62** and their 5,6-trans isomers **61** and **63**, respectively, were prepared according to the same protocol as described for the triple-labeled materials except that mono-^{13}C-labeled A-ring fragment **25** was used.

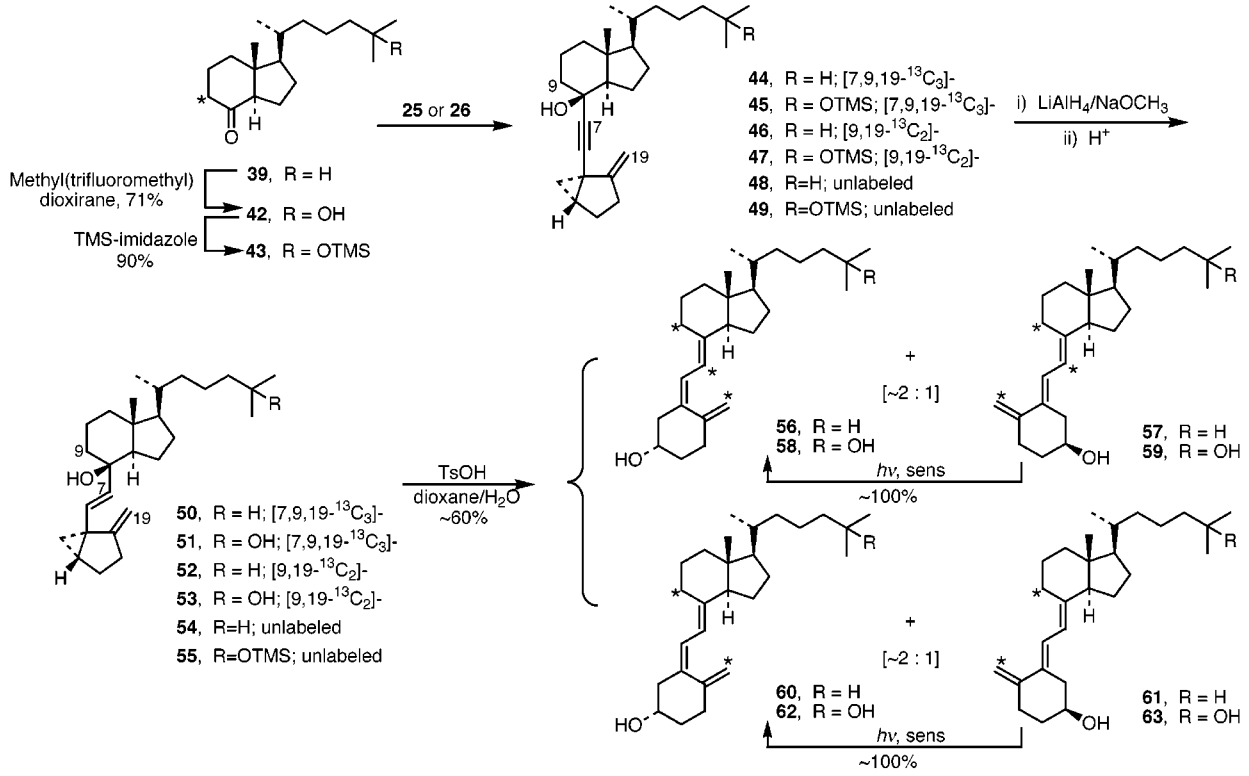
The triple-labeled form of the hormone **3**, namely **68**, was synthesized from **45** in a manner parallel to that described by Wilson[20] as modified by the Roche group[22] (Scheme 6). Allylic oxidation followed by TBDPS protec-

(28) (a) DeNinno, M. P. "Anomalous" Ozonolysis of Cyclic Allylic Alcohols: Mechanism and Synthetic Utility. *J. Am. Chem. Soc.* **1995**, *117*, 9927−9928. (b) DeNinno, M. P.; McCarthy, K. E. The ^{14}C Radiolabelled Synthesis of the Cholesterol Absorption Inhibitor CP-148, 623. A Novel Method for the Incorporation of a ^{14}C Label in Enones. *Tetrahedron* **1997**, *53*, 11007−11020.

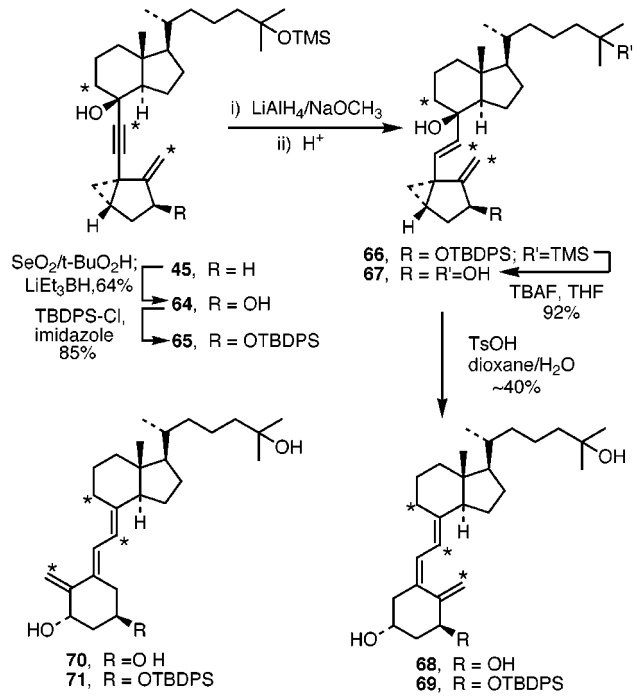
(29) (a) Kiegiel, J.; Wovkulich, P. M.; Uskokovic, M. R. Chemical Conversion of Vitamin D$_3$ to Its 1, 25-Dihydroxy Metabolite. *Tetrahedron Lett.* **1991**, *32*, 6057−6060. (b) Sicinski, R. R.; DeLuca, H. F. Ruthenium Tetroxide Oxidation of Grundmann's Ketone Derived from Vitamin D$_3$. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 159−162.

(30) (a) Bovicelli, P.; Lupattelli, P.; Mincione, E. Oxidation of Natural Targets by Dioxiranes. 2. Direct Hydroxylation at the Side-Chain C-25 of Cholestane Derivatives and of Vitamin D$_3$ Windaus-Grundmann Ketone. *J. Org. Chem.* **1992**, *57*, 5052−5054. (b) Curci, R.; Detomaso, A.; Prencipe, T.; Carpenter, G. B. Oxidation of Natural Targets by Dioxiranes. 3. Stereoselective Synthesis of (all-*R*)-Vitamin D$_3$ Triepoxide and of Its 25-Hydroxy Derivative. *J. Am. Chem. Soc.* **1994**, *116*, 8112−8115. (c) Rossella, M.; Fiorentino, M.; Fusco, C.; Curci, R. Oxidations by Methyl(trifluoromethyl)dioxirane. Oxyfunctionalization of Saturated Hydrocarbons. 2. *J. Am. Chem. Soc.* **1989**, *111*, 6749−6757.

Scheme 5

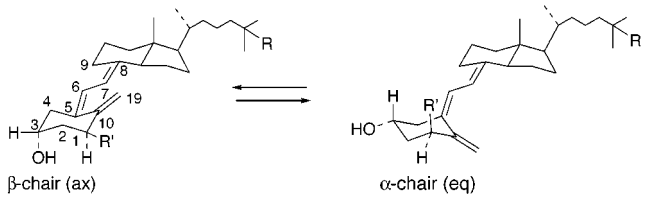


Scheme 6



tion of **45** afforded **65**, which was then reduced and then deprotected to the triol **67**. Solvolysis of **67** (to afford **68**) or its protected precursor **66** (to afford **69**) proceeded poorly and somewhat irreproducibly. Under our best conditions, the triple-labeled hormone **68** was prepared in ~40% yield via the procedure described in the Experimental Section. In our hands, only trace amounts of the 5,6-trans isomers (**70** or **71**) could be detected upon sovolysis of **67** or **69**, respectively. In the Roche report[22] (unlabeled series), sovolysis of **67** carried out on a larger scale and for a prolonged reaction time afforded a 75% yield of a 7:3 mixture of **68** and **70**. It is noteworthy that in Wilson's original report, this same sovolysis carried out under conditions similar to ours, only **68** was reported although in higher yields (67%).

NMR Studies of Triple-Labeled 25-Hydroxyvitamin D_3 (25-D3). It is well-known from NMR studies that the A-ring of vitamin D is a dynamic mixture of two rapidly equilibrating chair conformers, the α- and *β*-forms shown in Figure 1. For D3 (**1**) and 25-D3 (**2**), the equilibrium shifts toward the more stable α-chair (**73**) with its equatorially oriented hydroxyl as the solvent polarity increases from cyclohexane (α/*β* = 52/48) to chloroform (α/*β* = 58/42) to methanol (α/*β* = 76/24).[2b,7,9e] These conformational ratios were calculated using a standard Karplus correlation based on the ^3J trans couplings ($J_{a,a}$~11.1 Hz and $J_{e,e}$~2.7 Hz)[9] between H-3α and H-4*β* of 7.1, 7.6 and 9.1 Hz, respectively. The analogous chair/chair ratio of ~1:1 for the hormone 1,25-



72a, R = R' = H
72b, R = OH; R' = H
72c, R = R' = OH

73a, R = R' = H
73b, R = OH; R' = H
73c, R = R' = OH

Figure 1. Chair conformers of vitamin D. In solution, the A-ring of vitamin D rapidly equilibrates between the α-chair (**73**) with an equatorial 3*β*-OH and the *β*-chair (**72**) possessing an axial 3*β*-OH. The figure depicts this equilibrium for **1** (D3, **72a** and **73a**), **2** (25-D3, **72b** and **73b**), and the hormone 1,25-D3 (**3** or **72c** and **73c**). In each case, the intercyclic diene (C-5,6,7,8) possesses a near-planar 6-s-trans conformation with the exocyclic 10(19) double bond twisted out of the C-6,5,10 plane by ~±50°.

D3 (**3**) is less solvent dependent (the ^3J trans couplings ranged from 6.7 to 6.8 Hz for methanol and chloroform), a not surprising result since its A-ring possesses a *trans*-1,3-diol moiety. In the solid state (X-ray), **2** and **3** crystallize in opposite chair forms, the α-(**73b**) and *β*-(**72c**) chair forms, respectively, but the parent D3 (**1**) crystallizes as a 1:1 complex of both chair forms, *β*-(**72a**) and α-(**73a**).[8] Interestingly, the X-ray structure of the hormone 1,25-D3 (**3**) bound to the ligand binding domain of its nuclear receptor as discussed in the Introduction has revealed that this hormone, as in the crystal structure of the free ligand,[8c] resides as the *β*-conformer **72c**.[8a−e] It was of some interest therefore that the prohormone 25-D3 (**2**), according to a conformational analysis of its A-ring (by a standard Karplus correlation based on the ^3J trans coupling of H-3α to H-4*β*), exists strictly in the opposite α-conformer **73b** when dissolved in SDS-micelles, a membrane mimic.[2b,7] With labeled material in hand, NMR studies were initiated to illustrate the feasibility of obtaining more detailed structural information regarding ligand in aqueous micellar solutions.

The ^{13}C NMR studies described herein were conducted on the relatively more accessible triple labeled 25-D3 (**58**) and the results follow. The chemical shifts of the C-7, -9, and -19 signals of **58** in chloroform-*d* were virtually invariant (*δ* 117.5, 29.0, and 112.4 ppm, respectively) at concentrations between 0.1 and 40 mM. Saturated solutions of **58** in D_2O exhibited no detectable ^{13}C NMR signals, but it was determined that 0.1 mM aqueous solutions of triple- labeled **58** in a solvent mixture of 60% methanol-d_4 and 40% D_2O did exhibit easily detectable signals at *δ* 117.9, 29.6, and 112.8 ppm. In light of the low solubility of cholesterol in water,[31] a medium in which **58** would be expected to be only slightly more soluble, attention was next directed toward the use of solubilizing agents. Experiments also revealed that 0.1 mM aqueous solutions of **58** containing dimethylcyclodextrin[32] (DMCD) afforded weak, but acceptable signals at *δ* 118.0, 29.7, and 112.6 ppm.

Triple-labeled 25-D3 (**58**, 0.4 mM) in SDS (sodium dodecyl sulfate, 40 mM) micelles (*δ* 117.4, 28.9 and 112.4 ppm) afforded excellent ^{13}C NMR spectra.[33] The 5,6-trans isomer **59** under the latter conditions (0.4 mM in ligand and 40 mM in SDS) afforded similar results with the corresponding signals appearing at *δ* 116.4, 28.8, and 107.4 ppm (in chloroform-*d*, the corresponding signals appear at *δ* 115.8, 29.0, and 108.1 ppm). Thus, these labeled steroids exhibit only small differences in ^{13}C NMR chemical shifts of the labeled carbons in various media.

Internuclear distances (r_{ij}) within a molecule can be evaluated from the NOE intensity (I_{ij}) between two nuclei i and j using the equation $r_{ij} = r_{ref}(I_{ref}/I_{ij})^{1/6}$.[34] The precision of the distance measurement is strongly dependent on the reliability of the intensity measurements (I_{ij} and I_{ref}), best achieved by carrying out the NOESY experiment at short mixing times. The reference distance (r_{ref}) is often that between a pair of protons whose internuclear distance is fixed and can be determined fairly precisely (e.g., that between the geminal protons of a methylene group). A series of NOESY NMR experiments were conducted using a modified version of the double half-filter pulse sequence described by Wider et al.[15] In the double half-filter variant of the standard NOESY experiment, only those cross-peaks of the protons attached to pairs of labeled carbons (on ^{13}C−^{13}C pairs, namely the five protons attached to C-7, -9, and -19) appear, thus eliminating cross-peaks resulting from all other proton pair combinations (those attached to ^{12}C−^{12}C and ^{12}C−^{13}C pairs). Thus, background NOESY cross-peaks (due to protons on natural abundance ^{13}C nuclei) are minimized so that accurate cross-peak intensity measurements can be made at very short mixing times.

The NOE cross-peaks of proton pairs 9α−9*β*, 19*E*−19*Z*, 7−19*Z*, and 7−19*E* were screened at mixing times of 25, 50, 100, 200, 300, 600, and 800 ms to determine the mixing time that provided optimal volume cross-peaks for the distance calculations. The NOESY spectrum for a 50 ms mixing time, optimized for the 9α−9*β* pair, is given in Figure 2 and additional details are presented in the Experimental Section and in the Supporting Information section. Using the 9α−9*β* pair as r_{ref} [1.778 Å from molecular mechanics optimization (PC Model, Serena Software) of published X-ray crystallographic data[8b]] and the experimental I_{ref} value of 8.39 (from Figure 2), the 19*Z*−7 and 19*Z*−19*E* distances are calculated to be 2.2 and 1.9 Å, respectively. The latter 19*Z*−19*E* distance is in excellent agreement with that calculated from the X-ray crystallographic data (1.896 Å).[8b] It should be noted that the absence of an H_{19E}−H_7 cross-peak at short mixing times precluded a reliable distance measurement of this pair while cross-peaks involving $H_{9\alpha}$ and $H_{9\beta}$, except for the geminal pair, were altogether absent.

Alternatively, using the 19*Z*−19*E* I_{ref} value of 5.66 at the same 50 ms mixing time and the 19*Z*−19*E* calculated X-ray distance of 1.896 Å (r_{ref}), the 9α−9*β* and 7−19*Z* distances are calculated to be 1.8 (calculated 9α−9*β* X-ray distance = 1.778 Å[8b]) and 2.2 Å, respectively. Finally, using the initial slopes of the build up curves over the first 100 ms mixing times rather than the optimal volume intensities at 50 ms, the corresponding calculated distances proved to be in excellent agreement (within <0.03 Å) with those given above.

Thus, the double half-filter NOESY data for **58** (2 mM) in 20 mM SDS micellar solutions, which was evaluated in four different ways using two different reference distances (9α−9*β* or 19*Z*−19*E*), leads to what we believe is a reliable 19*Z*−7 H−H distance of 2.2 Å. This distance is shorter than the calculated X-ray distance of 2.54 Å. The published X-ray crystallographic data[8b] for 25-D3 **(58)** reveals torsion angles of −176.9 and −56.7° for C-5,6,7,8 and C-6,5,10,19, respectively. PC Model energy minimization of the X-ray structure leads to torsion angles of +169.2 and −48.2° for these same rotatable

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(33) (a) The CMC (critical micelle concentration) for SDS is quoted as being in the range 8.0−8.4 mM. See Mukerjee, P.; Mysels, K. J. Critical Micelle Concentrations of Aqueous Surfactant Systems. *NSRDS−NBS 36*; U. S. Government Printing Office: Washington D. C., 1977; 222 pp. (b) For leading references, see also: Mittal, K. L.; Fendler, E. J., Eds.; *Solution Behavior of Surfactants−Theoretical and Applied Aspects;* Plenum Press: New York, 1982; 739 pp.

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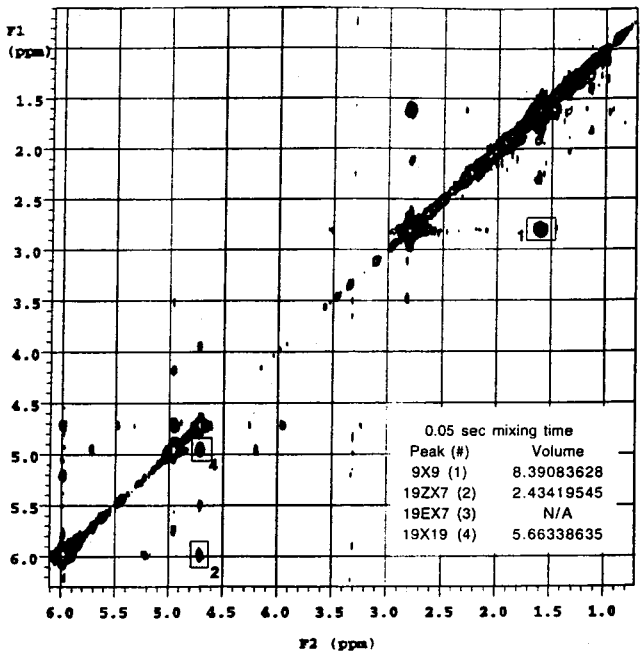

Figure 2. Double half-filter NOESY NMR spectrum of [7,9,19-$^{13}C_3$]-25-hydroxyvitamin D_3 (**58**) in sodium dodecyl sulfate (SDS) micelles. A typical two-dimensional double half-filter NOESY spectrum is shown. Using a modified version of the pulse sequence reported by Wider et al.,[15] this spectrum was recorded at the optimum mixing time of 50 ms for a 2 mM sample of **58** in 20 mM SDS-d_{25} in D_2O at 25 °C. The bracketed H−H cross-peaks are for 9α−9β (1), 19*Z*−7 (2) 19*E*−7 (3) and 19*Z*−19*E* (4) pairs; the 19*E*−7 cross-peak (3), which does not appear in this spectrum, emerges only at longer mixing times. The data for the other mixing times (25, 100, 200, 300, 600, and 800 ms) as well as distance calculations are summarized in the Supporting Information. The latter also contains the one-dimensional ^{1}H NMR spectrum of the same sample used in this NOESY experiment. Additional details can be found in the text. In another experiment, a sample for recording the ^{13}C NMR spectrum was prepared from 7,9,19-($^{13}C)_3$-25-D3 (**58**, 0.4 mg) and SDS (29 mg) in D_2O (2.5 mL) in a 10 mm NMR tube (26 °C, GN-500 spectrometer, 8,000 scans and LB = 3 Hz). Chemical shift data for this sample as well as for **58** in other media are discussed in the text.

bonds, including the19*Z*−7 H−H distance of 2.54 Å mentioned above. It is apparent that simply manually changing the C-5,6,7,8 angle from +169.2 to −165° can easily account for the shortened 19*Z*−7 H−H distance of 2.2 Å determined from the NOESY experiment. On the other hand, constraint of the 19*Z*−7 H−H distance to 2.2 Å followed by energy minimization leads to torsion angles of +177.0 and −44.6°, respectively, of the C-5,6,7,8 and C-6,5,10,19 bonds. Thus, rotation of either of the latter torsion angles in an appropriate manner can offer a simple explanation for the shortening of the H_7−H_{19Z} distance. The UV maximum of the micellar solutions of 25-D3 reveals an absorption at 265 nm, essentially the same as that of the free ligand in methanol or hexane. Thus, we cannot detect any unusual flattening or twisting of the conjugated triene portion of the molecule from its electronic spectrum. The ^3J coupling for 25D3 between H_6 and H_7 remains at ∼11.1 Hz both in micelles and in chloroform, suggesting that the C-5,6,7,8 diene portion of the triene is nearly planar.

Finally, as mentioned earlier, the ^3J coupling between the 3α and 4β protons of ligand in micellar solutions is characteristic of the presence of only the α-chair **73b**. This same ^3J coupling is observed for the steroid without the side-chain hydroxyl (namely **73a**), so presumably the equatorial chair cyclohexanol portion of the steroid is oriented with the polar hydroxyl oriented toward the outer, polar surface of the micellar environment. At this juncture, it is unclear whether the micellar environment mechanically suppresses the chair−chair equilibrium (Figure 1) so that the steroid A-ring is locked into only the α-chair conformation **73b**. Alternatively, the A-ring may be rapidly equilibrating between both chair forms (**72b** and **73b**) with a strong bias toward the α-chair. As indicated earlier, solvents of increasing polarity, especially those with hydrogen bonding ability, increases the proportion of the equatorial hydroxyl conformer (**73b**) at the expense of the axial form (**72b**). This question remains unresolved.

In summary, the synthetic sequence developed for the target isotopically labeled compounds is reasonably practical. The addition of isotopically enriched Grignard reagent to an enone, oxidative cleavage and enolate cyclization contributes a general procedure for C-9 isotope labeling of the CD fragment. The requirement for *CBr_4, an expensive reagent for introducing label at C-7, is a shortcoming in the synthesis. Some improvement utilizing a cheaper reagent would be desirable here. Using the Wuthrich-type double half-filter pulse sequence, the NOESY NMR experiment provided new steroid solution structural information of a ligand associated with a biological host system, namely in an SDS micellar system, for the first time. The recent finding that LDL (low-density lipoprotein) is a delivery system for certain vitamin D metabolites renders the results described herein of particular interest.[35] Moreover, the micellar constraining of the 25-D3 (**2**) molecule has recently had practical value in studies of the photoisomerization of the parent D3 (**1**).[7] It remains for future studies to demonstrate the use of the labeled ligands in biological host systems, particularly proteins.

Experimental Section

General Methods. Spectral data are given in the Supporting Information unless otherwise noted. The purity of all major isomers or intermediates was estimated by a combination of HPLC and NMR analysis. The level of purity is indicated by the inclusion of copies of ^{1}H NMR spectra and ^{13}C NMR spectra in the Supporting Information.

Transformation of Vitamin D_3 (1) to Cyclovitamin 13.[24] To a solution of vitamin D_3 (50 g, 0.13 mol) in pyridine (300 mL) were added *p*-toluenesulfonyl chloride (recrystallized from hexane, 49.5 g, 0.26 mol) and DMAP (2.0 g) at 0 °C under argon. The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 15 h. An additional portion of *p*-toluenesulfonyl chloride (10 g) was added at room temperature. After being stirred for 8 h, the reaction mixture was poured into a mixture of saturated aqueous $NaHCO_3$ solution and ice. The mixture was stirred at room temperature for 30 min to quench the excess *p*-toluenesulfonyl chloride and then extracted with ether. The combined ether solution was washed

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with 5% HCl and water and dried over anhydrous magnesium sulfate. Flash chromatography of the crude material (10% EtOAc in hexane) afforded cholecalciferyl tosylate (68.4 g, 98%)[21] as a white solid, which was used directly in the solvolysis reaction described next. To a solution of the tosylate (34.2 g, 0.063 mol) in acetone (2.8 L) and water (280 mL) was added powdered sodium bicarbonate (350 g) at room temperature. The reaction mixture was refluxed with vigorous mechanical stirring for 28 h and then was filtered. The filtrate was concentrated under vacuum to about 300−500 mL. The residual mixture was extracted with ether. The white solid from the filtration was dissolved in water and extracted with ether. The combined ether solution was washed with water and dried over anhydrous magnesium sulfate. The crude material was separated by flash chromatography (4% EtOAc in hexane) to give the cyclovitamin **13** (15.0 g, 62%) as a colorless oil.

(3*R*,5*S*,6*S*,7*R*,8*R*,10*S*)-3,5-Didehydro-6,7,8,10,19-pentahydroxy-9,10-secocholestane (14). To a solution of cyclovitamin **13** (19.8 g, 51.5 mmol) in a mixture of acetone (408 mL), pyridine (20.4 mL), and water (51 mL) was added osmium tetraoxide (2.0 g, 7.8 mmol) and NMO (24.2 g, 0.206 mol) at room temperature. The brown solution was purged with argon and was then refluxed with stirring for 3.5 h. The black reaction mixture was filtered through a short column of silica gel covered with Celite (acetone). The filtrate was concentrated under vacuum, and then a saturated aqueous NaCl solution was added to the black residue. The mixture was extracted with ether (4 × 400 mL), and then the combined ether solution was dried over anhydrous $MgSO_4$ and filtered through a short silica gel column (EtOAc). Concentration of the filtrate afforded pentaol **14** (light yellow foam, 18.7 g, 80%), which was used directly in the following reaction. A sample for spectral analysis was obtained by further purification by HPLC (silica gel, 1:20 CH_3OH/CH_2Cl_2).

(1*S*,5*R*)-1-Formylbicyclo[3.1.0]hexan-2-one (11), Grundmann's Ketone (12), and 8*β*-Formyl-de-A,B-cholestan-8α-ol (15). To a solution of pentaol **14** (10.41 g, 23 mmol) in dioxane (162 mL) and water (54 mL) was added sodium periodate (25.0 g, 116 mmol) at room temperature. The milky white reaction mixture was stirred vigorously at room temperature for 2.5 h and was then quenched by addition of saturated aqueous NaCl solution. The mixture was extracted with ether (4 × 500 mL), and then the combined ether solution was dried over $MgSO_4$. The solids were filtered off, and the filtrate was concentrated carefully by fractional distillation under a slight vacuum. The crude residue was separated by flash chromatography (silica gel, 1:3 ether/pentane) to give in order of elution Grundmann's ketone **12** (6.0 g) and then keto aldehyde **11** (2.8 g). The former was further purified by flash chromatography (silica gel, 1:20 EtOAc/hexane) to give pure **12** (4.86 g, 90%, oil), and the latter was further purified by HPLC (2:1 ether/hexane) to give **11** (2.0 g, 70%, oil). Small amounts of residual solvent signals were invariably present in ^1H and ^{13}C NMR spectra of the keto aldehyde, but considerable loss of material due to its instability was encountered in attempts at further purification. When reactions were carried out at shorter reaction times, variable amounts (20−50%) of the 8*β*-hydroxy-8α-carbaldehyde **15** (which eluted between ketone **12** and keto aldehyde **11**) were also isolated.

(1*S*,5*R*)-1-(2′,2′-Dibromovinyl)bicyclo[3.1.0]hexan-2-one (16). To a suspension of zinc dust (780 mg, 12 mmol) and triphenylphosphine (3.14 g, 12 mmol) in dry CH_2Cl_2 (15 mL) was added a solution of carbon tetrabromide (3.98 g, 12 mmol) in dry CH_2Cl_2 (7 mL) via cannula at room temperature. The resulting yellow suspension was stirred at room temperature for 22 h. The keto aldehyde **11** (350 mg, 2.82 mmol) in CH_2Cl_2 (6 mL) was then added to the red reaction mixture via cannula at 0 °C. The reaction mixture was stirred at 0 °C for 1.5 h. Pentane (200 mL) was slowly added to the reaction mixture with stirring, and then the mixture was filtered through Celite. The solid was dissolved in CH_2Cl_2 (30 mL) and was then precipitated with pentane (200 mL). The solids were filtered off through Celite. The combined filtrate was concentrated under vacuum, and the residual oil was separated by flash chromatography (1:4 EtOAc/hexane) to give **16** (790 mg, ∼100%, colorless oil).

(1*S*,5*R*)-1-(2′,2′-Dibromovinyl)-2-methylenebicyclo[3.1.0]-hexane (18) and (1*R*,5*R*)-1-(2′-Bromoethynyl)-2-methylenebicyclo[3.1.0]hexane (19). To a suspension of methyltriphenylphosphonium iodide (978 mg, 2.42 mmol) in THF (8 mL) was added solid potassium *tert*-butoxide (271 mg, 2.42 mmol) at room temperature. The yellow reaction mixture was stirred at room temperature for 1 h, and a solution of **16** (340 mg, 1.21 mmol) in THF (6 mL) was then added. The reaction mixture was stirred at room temperature for 2 h and was quenched by addition of water. The mixture was extracted with hexane (2 × 100 mL), and then the combined organic phase was dried over $MgSO_4$ and concentrated under vacuum. The residue was dissolved in CH_2Cl_2 (10 mL), and pentane (200 mL) was introduced slowly. The precipitates were removed by filtration through a short column (silica gel covered with Celite, pentane). The filtrate was concentrated under vacuum, and the residual liquid was separated by flash chromatography (silica gel, hexane) (263 mg, colorless oil). This material was normally used directly for the following coupling reaction without further separation. Separation of the material by HPLC (silica gel, hexane) afforded the less polar dibromide **A** (**18**, 192.1 mg, 56%, colorless liquid) and the more polar bromoenyne **B** (**19**, 41.1 mg, 17%, colorless liquid). Treatment of either component **A** or **B** or a mixture with BuLi followed by quenching gave the volatile enyne **27** as the only product as determined by ^1H NMR analysis.

^{13}C-Labeled A-Ring Fragments 20−23. The monolabeled A-ring enyne for introduction of ^{13}C label at the C_{19} position of the vitamin D metabolite was prepared from dibromoketone **16** (790 mg, 2.82 mmol in 8 mL of THF), [$^{13}CH_3$]-methyltriphenylphosphonium iodide (1.72 g, 4.23 mmol), and potassium *tert*-butoxide (1 M in THF, 4.23 mL, 4.23 mmol) in 20 mL of THF as described above for the unlabeled material. This afforded a mixture (524 mg, 66%) of the less polar dibromide **A** (**20**) and the more polar alkynyl bromide **B** (**21**) in a 4:1 ratio (^1H NMR analysis), which was used directly in the next step as indicated at the end of this section.

The double-labeled A-ring enyne for introduction of ^{13}C label at both the C_7 and C_{19} positions of the vitamin D metabolite was prepared as described for the unlabeled compound starting from keto aldehyde **11** (300 mg, 2.42 mmol) in 8 mL of CH_2Cl_2 and labeled dibromo-Wittig reagent [prepared from [^{13}C]-CBr_4 (2.61 g, 7.89 mmol), triphenylphosphine (2.06 g, 7.89 mmol), and Zn dust (511 mg, 7.89 mmol)]. There was obtained labeled dibromoketone **17** (635 mg, 94%), which was used in the next step without further examination. The Wittig reaction of the crude labeled dibromoketone **17** (635 mg, 2.26 mmol) with [$^{13}CH_3$]-methyltriphenylphosphonium iodide (1.83 g, 4.52 mmol) and potassium *tert*-butoxide (507 mg, 4.52 mmol) in 25 mL of THF as described above for the unlabeled material afforded a mixture (442 mg, 70%) of the less polar dibromide **A** (**22**) and the more polar alkynyl bromide **B** (**23**) in a 4:1 ratio (isolated yield). The latter mixture was used directly in the next step without further purification. Analytical samples of double labeled materials were prepared by HPLC separation (silica gel, hexane).

No attempts were made to isolate and characterize the labeled forms of hydrocarbon **27** because of volatility problems encountered in handling **27** itself. The crude lithium salts **25** and **26** were used directly. For preparative purposes, crude dry mixtures of **20** plus **21** or **22** plus **23** were transformed directly to **25** and **26**, respectively. A detailed analysis of NMR data for separated and purified **22** and **23** (with a side by side comparison to unlabeled materials **18** and **19**, respectively), but not for **20** and **21**, is presented in the Supporting Information.

De-A,B-cholest-9(11)-en-8-one (28). This material was prepared exactly as previously described by this laboratory.[27] In a typical sequence, reaction of Grundmann's ketone (**12**, 5.30 g, 18.9 mmol) with LDA [from 2.49 g (24.6 mmol) of diisopropylamine and 24.6 mmol of *n*-BuLi] in THF (100 mL) followed by PhSeCl (4.95 g, 25.8 mmol) treatment and then

m-CPBA oxidation [10.4 g (49.2 mmol) in CH_2Cl_2 (60 mL)] afforded 3.18 g (60%) of enone **28**.

[8α-^{13}C]-9,11-Didehydro-8α-methyl-de-A,B-cholestan-8*β*-ol (29). To magnesium turnings (360 mg, 15 mmol) covered with ether (3 mL) was added a solution of [^{13}C]-iodomethane (1.95 g, 13.6 mmol, 99% isotopic purity, Cambridge Isotope Laboratory) in ether (8 mL) at a rate slow enough to maintain a gentle reflux of the reaction mixture. After the addition, the reaction mixture was refluxed for 30 min. The Grignard reagent was then transferred via cannula to a suspension of $CeCl_3$ (dried at 160 °C for 5 h in vacuo, 6.65 g, 27 mmol) in THF (45 mL) at 0 °C. The slurry was stirred at 0 °C for 1 h, and then a solution of enone **28** (1.42 g, 5.40 mmol) in THF (3 mL) was added. The reaction mixture was then stirred at 0 °C for 2 h and at room temperature for 1 h. Ether (100 mL) was added to the reaction mixture followed by saturated NH_4-Cl. The separated aqueous phase was extracted with ether (2 × 150 mL) and then the combined ether solution was dried over $MgSO_4$ and concentrated in vacuo to give **29** (1.43 g, 94%) as a colorless oil, sufficiently pure for the following reaction. The analytical sample was prepared by further purification by HPLC (1:10 EtOAc/hexane), but the product is unstable to silica gel column chromatography.

[8α-^{13}C]-9,11-Seco-8α-methyl-de-A,B-cholestane-8*β*,9,11-triol (31). A solution of allylic alcohol **29** (1.42 g, 5.09 mmol) dissolved in methanol (250 mL) and pyridine (8 mL) was purged with nitrogen through a gas dispersion tube. The solution cooled to −78 °C was then purged with ozone until the solution turned blue (1.5−2 h). The ozone flow was stopped and the reaction mixture was purged with N_2 at −78 °C for 1 h. Sodium borohydride (2 g) was added to the solution at −78 °C in one portion and the resulting mixture was stirred at −78 °C for 1 h while a gentle flow of N_2 was maintained. After addition of another 2 g of $NaBH_4$, the cooling bath was removed. The mixture was stirred at room temperature for 1 h followed by slow addition of 2 g of $NaBH_4$. After another 1 h stirring, a final portion of $NaBH_4$ (2 g) was added and the reaction mixture was allowed to stir at room temperature for 24 h. The mixture was concentrated in vacuo, saturated NaCl solution was added to the remaining residue, and the mixture was extracted with ether (4 × 150 mL). The combined ether solution was dried over $MgSO_4$ and concentrated in vacuo. The crude product was separated by flash chromatography and then subjected to HPLC (1:10 CH_3OH/CH_2Cl_2) to give pure **31** (1.25 g, 78%).

[9-^{13}C]-9,11-Seco-de-A,B-cholestane-8,11-diols (33 and 34). To a solution of **31** (1.20 g, 3.8 mmol) in a mixture of dioxane (30 mL) and water (15 mL) was added sodium periodate (1.63 g, 7.6 mmol) at room temperature. The reaction mixture was stirred at room temperature for 3 h and was then cooled to 0 °C. Sodium borohydride (2.0 g) was added slowly, and then after the mixture was stirred at 0 °C for 1 h, the ice bath was removed. The mixture was allowed to stir at room temperature overnight, saturated aqueous NaCl solution was added to the mixture, and then the latter was extracted with ether. The combined ether solution was dried over $MgSO_4$ and concentrated in vacuo. The residual oil was purified by flash chromatography (silica gel, 1:2 EtOAc/hexane) to give an epimeric mixture of diols (1.11 g, ∼quantitative). This mixture was used directly in the following reaction without further separation. Analytical samples were prepared by HPLC separation (silica gel, 1:1.2 EtOAc/hexanes): **A** (less polar, minor isomer **34**) and **B** (more polar, major isomer **33**) in a ∼1:2 ratio.

[9-^{13}C]-11-Tosyloxy-9,11-seco-de-A,B-cholestan-8α-ol (35) and [8-^{13}C]- 8α-Methyl-9-oxa-de-A,B-cholestane (36). To a solution of the epimeric mixture of diols **34** and **33** (4.87 g, 17.1 mmol; ∼1:2 mixture of minor, less polar diol **A** and major, more polar diol **B**) in pyridine (50 mL) was added *p*-toluenesulfonyl chloride (3.91 g, 20.5 mmol) at −40 °C. The reaction mixture was stirred at −25 °C for 15 h and then at −20 °C for 6 h. Water (250 mL) was added at −20 °C, and then the mixture at ambient temperature was extracted with ether (4 × 300 mL). The combined ether solution was dried over $MgSO_4$ and filtered through a short column (silica gel, ether). The crude product was separated by flash chromatography (silica gel) to give a minor nonpolar product **36** (1.45 g, 32%) and hydroxy tosylate **35** (4.49 g, 60%). The analytical samples of ether **36** and hydroxy-tosylate **35** were prepared by further HPLC purification (silica gel, 2.5% EtOAc in hexane and 1:2 EtOAc/hexane, respectively).

[9-^{13}C]-9,11-Seco-11-tosyloxy-de-A,B-cholestan-8-one (37). To a solution of hydroxy-tosylate **35** (980 mg, 2.23 mmol) in CH_2Cl_2 (30 mL) was added pyridinium chlorochromate (960 mg, 4.46 mmol) at room temperature. The dark brown mixture was stirred at room temperature for 15 h and was then directly loaded onto a short column and eluted (silica gel, 1:4.5 EtOAc/hexane). Further purification by HPLC (1:4 EtOAc/hexane) afforded ketone **37** as a liquid (860 mg, 88%).

[9-^{13}C]-11-Iodo-9,11-seco-de-A,B-cholestan-8-one (38). To a solution of keto-tosylate **37** (458 mg, 1.05 mmol) in THF (20 mL) was added lithium iodide (421 mg, 3.14 mmol) at room temperature. The reaction mixture was heated at 50 °C with stirring for 2 h. Water (100 mL) was added and then the mixture was extracted with ether (2 × 150 mL). The combined ether solution was dried over anhydrous $MgSO_4$ and concentrated under vacuum. The residual oil was separated by HPLC (silica gel, 1:20 EtOAc/hexane) to give iodoketone **38** (319 mg, 78%) as a slightly yellow liquid, which was used immediately without purification in the next step because of its instability even at room temperature. Its crude ^{1}H NMR spectrum was consistent with the assigned structure.

[9-^{13}C]-De-A,B-cholestan-8-one (39). To a solution of LiN(TMS)$_2$ (LHMDS, 1 M solution in THF, 1.12 mL, 1.12 mmol) in THF (2 mL) was added a solution of iodoketone **38** (220 mg, 0.56 mmol) in THF (2 mL) by cannula at −78 °C. The reaction mixture was stirred at −78 °C for 1 h at which time ether (10 mL) was added. After stirring for 5 min at −78 °C, H_2O (5 mL) was then added to the mixture and the latter was removed from the cold bath. The ether was decanted and the remaining ice slush was washed with ether (1 × 10 mL). The combined ether solutions were dried over $MgSO_4$, filtered, and concentrated. The residual oil was separated by flash chromatography (1:10 EtOAc/hexane) and was further purified by HPLC (1:20 EtOAc/hexane) to give 9-^{13}C-Grundmann's ketone **39** (127 mg, 86%, colorless oil). Similar reaction conditions using NaN(TMS)$_2$ (NHMDS) resulted in a reduced yield of the desired [9-^{13}C]-Grundmann's ketone **39** with varying amounts of the C_{14} epimer. LDA was even less effective.

[9-^{13}C]-25-Hydroxy-de-A,B-cholestan-8-one (42). To a solution of [^{13}C]-Grundmann's ketone (**39**,128 mg, 0.483 mmol) in carbon tetrachloride (3.6 mL) and acetonitrile (3.6 mL) in a 25 mL round-bottom flask was added a solution of a 1/1 mixture of 0.5 M KH_2PO_4/0.5 M NaOH (4.8 mL) and sodium periodate (368 mg, 1.72 mmol). The mixture was stirred at room temperature for 5 min, and then $RuCl_3{-}xH_2O$ (12 mg) was added.[29] The resulting brown solution was purged with argon, and after 10 min, the reaction mixture became yellow. After the mixture was stirred under argon at 50 °C for 52 h, the reaction was quenched by the addition of saturated aqueous NaCl solution (30 mL). The mixture was extracted with ether (3 × 120 mL), and then the combined ether solution was dried over $MgSO_4$ and concentrated. The crude residue was separated by flash chromatography and was further purified by HPLC (silica gel, 1:2 EtOAc/hexane) to give **42** (55 mg, 41%; 54% based on recovered starting material) and starting material **39** (31 mg).

In a second more efficient procedure, a distilled solution of methyl(trifluoromethyl)dioxirane (MTD) in 1,1,1-trifluoropropanone (TFP) was prepared according to the method of Curci[30a−c] by oxidation of a solution of TFP (10 mL, 13.3 g, 119 mmol), $NaHCO_3$ (10 g, 119 mmol), ethylenediaminetetraacetic acid−disodium salt dihydrate (0.33 g, 1 mmol, Aldrich), and doubly distilled water (15 mL) with potasssium peroxomonosulfate (oxone, 16.7 g, 0.054 mmol). To the solution of MTD at 0 °C was added a solution of labeled Grundmann's ketone **39** (150 mg, 0.56 mmol) in 2 mL of dry CH_2Cl_2 and then the solution was stirred at this same temperature for 1 h. The reaction was quenched by addition of 10 mL of H_2O followed by extraction with ether (2 × 15 mL). The combined organic layers were dried with $MgSO_4$, filtered, and concen-

trated under reduced pressure. The resulting crude oil was separated by flash chromatography (silica gel, 1:2, EtOAc/hexane) and further purified by HPLC (1:2 EtOAc/hexane) to give **42** (111 mg, 0.40 mmol, 71%).

[7,9,19-$^{13}C_3$]-(3*R*,5*R*,8*R*)-3,5-Didehydro-8-hydroxy-9,10-secocholest-10(19)-en-6-yne (44). To a solution of a mixture of the double-labeled dibromide **22** and the alkynyl bromide **23** (160 mg, 0.57 mmol) in THF (8 mL) was added BuLi (2.5 M solution in hexane, 460 μL, 1.14 mmol) at −78 °C. The solution was stirred at −78 °C for 0.5 h, and the cooling bath was removed. The solution of **26** was stirred at room temperature for 1 h and was then again cooled to −78 °C. A solution of ketone **39** (100.7 mg, 0.38 mmol) in THF (4 mL) was then added via cannula, and the reaction mixture was stirred at −78 °C for 0.5 h and at room temperature for 0.5 h. After addition of water, the mixture was extracted with ether (2 × 100 mL). The combined ether solution was dried over $MgSO_4$ and concentrated under vacuum. The crude product was separated by flash chromatography (1:25 EtoAc/hexane) and was further purified by HPLC (1:25 EtOAc/hexane) to afford triple-labeled **44** (142.4 mg, 97%, oil).

[7,9,19-$^{13}C_3$]-(3*R*,5*R*,8*R*)-3,5-Didehydro-8-hydroxy-9,10-seco-25-(trimethylsilyloxy)cholest-10(19)-en-6-yne (45) from 42 via 43 and 22−23. To a solution of hydroxyketone **42** (125.3 mg, 0.445 mmol) in CH_2Cl_2 (15 mL) was added trimethylsilylimidazole (287 mg, 2.0 mmol) at room temperature. The reaction mixture was stirred at room temperature for 30 h and was directly subjected to a column chromatography (silica gel, 1:10 EtOAc/hexane). The crude product was further purified by HPLC (1:12 EtOAc/hexane) to give **43** (142.3 mg, 90%, colorless oil). The unlabeled **43** has been previously reported.[36,37]

To a solution of a mixture of the dibromide **22** and the alkynyl bromide **23** (181 mg, 0.64 mmol) in THF (8 mL) was added BuLi (2.48 M solution in hexane, 520 μL, 1.28 mmol) at −78 °C. The solution of **26** was stirred at −78 °C for 0.5 h, and then the cooling bath was removed. The solution was stirred at room temperature for 1 h and was then again cooled to −78 °C. A solution of ketone **43** (134 mg, 0.38 mmol) in THF (4 mL) was added via cannula. The reaction mixture was stirred at −78 °C for 0.5 h and at room temperature for 0.5 h. After addition of water, the mixture was extracted with ether (2 × 100 mL). The combined ether solution was dried over $MgSO_4$ and concentrated under vacuum. The crude product was separated by flash chromatography (1:20 EtOAc/hexane) and was further purified by HPLC (1:20 EtOAc/hexane) to afford **45** (161.9 mg, 90%, oil).

[9,19-$^{13}C_2$]-(3*R*,5*R*,8*R*)-3,5-Didehydro-8-hydroxy-9,10-secocholest-10(19)-en-6-yne (46). To a solution of a mixture of the monolabeled dibromide **20** and alkynyl bromide **21** (100 mg, 0.36 mmol) in THF (6 mL) was added *n*-BuLi (2.45 M solution in hexane, 290 μL, 0.72 mmol) at −78 °C. The solution was stirred at −78 °C for 0.5 h, and then the cooling bath was removed. The resulting solution of **25** was stirred at room temperature for 1 h and was then again cooled to −78 °C. A solution of ketone **39** (61.2 mg, 0.23 mmol) in THF (2 mL) was added via cannula. The reaction mixture was stirred at −78 °C for 0.5 h and at room temperature for 0.5 h. After addition of water, the mixture was extracted with ether (2 × 100 mL). The combined ether solution was dried over $MgSO_4$ and concentrated under vacuum. The crude product was separated by flash chromatography (1:20 EtOAc/hexane) and was further purified by HPLC (1:20 EtOAc/hexane) to afford pure **46** (74.8 mg, 84%, oil).

[9,19-$^{13}C_2$]-(3*R*,5*R*,8*R*)-3,5-Didehydro-8-hydroxy-9,10-seco-25-(trimethylsilyloxy)cholest-10(19)-en-6-yne (47). To a solution of a mixture of monolabeled dibromide **20** and alkynyl bromide **21** (142 mg, 0.51 mmol) in THF (6 mL) was added *n*-BuLi (2.45 M solution in hexane, 420 μL, 1.02 mmol) at −78 °C. The solution was stirred at −78 °C for 0.5 h, and the cooling bath was removed. The solution of **25** was stirred at room temperature for 1 h and was then again cooled to −78 °C. A solution of ketone **43** (64 mg, 0.18 mmol) in THF (4 mL) was added via cannula. The reaction mixture was stirred at −78 °C for 0.5 h and at room temperature for 1 h. After addition of water, the mixture was extracted with ether (2 × 100 mL). The combined ether solution was dried over $MgSO_4$ and concentrated under vacuum. The crude product was separated by flash chromatography (1:20 EtOAc/hexane) and was further purified by HPLC (1:20 EtOAc/hexane) to afford **47** (76.1 mg, 89%, oil).

[7,9,19-$^{13}C_3$]-(3*R*,5*R*,8*R*)-(6*E*)-3,5-Didehydro-9,10-secocholesta-6,10(19)-dien-8-ol (50). To a solution of propargyl alcohol **44** (140 mg, 0.364 mmol) in THF (18 mL) was added $LiAlH_4$ (50 mg) and CH_3ONa (50 mg) successively at room temperature. The reaction mixture was refluxed for 1 h and was then quenched at room temperature by addition of 0.1 M aqueous HCl solution. The mixture was extracted with 1:1 ether/hexane, and then the combined organic phase was dried over $MgSO_4$ and concentrated under vacuum. The residual oil was separated by flash chromatography (1:40 EtOAc/hexane) and was further purified by HPLC (1:40 EtOAc/hexane) to give **50** (118 mg, 84%, oil).

[7,9,19-$^{13}C_3$]-(3*R*,5*R*,8*R*)-(6*E*)-3,5-Didehydro-8,25-dihydroxy-9,10-secocholesta-6,10(19)-diene (51). To a solution of propargyl alcohol **45** (160 mg, 0.34 mmol) in THF (18 mL) were added $LiAlH_4$ (50 mg) and CH_3ONa (50 mg) successively with stirring at room temperature. The reaction mixture was refluxed for 1 h and was then quenched at room temperature by addition of saturated aqueous NH_4Cl solution. The mixture was extracted with 1:1 ether/hexane. The combined organic phase was dried over $MgSO_4$ and concentrated under vacuum. The residual oil was separated by flash chromatography (1:20 EtOAc/hexane and then 1:4 EtOAc/hexane) to give the less polar silyl ether of **51** (from 1:20 EtOAc/hexane elution) and the more polar trimethylsilyl cleavage compound **51** (from 1:4 EtOAc/hexane elution). The former was dissolved in CH_2Cl_2 (2 mL), and pyridinium *p*-toluenesulfonate (20 mg) was added to the solution. The solution was stirred at room temperature for 5 h and was directly filtered through a column (silica gel, 1:4 EtOAc/hexane). The resulting alcohol was combined with the earlier polar trimethylsilyl cleavage compound **51**. The combined alcohol fractions were further purified by HPLC (1:3 EtOAc/hexane) to afford a colorless oil (109.8 mg, 80%).

[9,19-$^{13}C_2$]-(3*R*,5*R*,8*R*)-(6*E*)-3,5-Didehydro-9,10-secocholesta-6,10(19)-dien-8-ol (52). To a solution of propargyl alcohol **46** (73 mg, 0.19 mmol) in THF (10 mL) were added $LiAlH_4$ (25 mg) and CH_3ONa (25 mg) successively at room temperature. The reaction mixture was refluxed for 1 h and was then quenched at room temperature by addition of 0.1 M aqueous HCl solution. The mixture was extracted with 1:1 ether/hexane, and then the combined organic phase was dried over $MgSO_4$ and concentrated under vacuum. The residual oil was separated by flash chromatography (1:20 EtOAc/hexane) and was further purified by HPLC (1:50 EtOAc/hexane) to give **52** (64.9 mg, 88%, oil).

[9,19-$^{13}C_2$]-(3*R*,5*R*,8*R*)-(6E)-3,5-Didehydro-8,25-dihydroxy-9,10-secocholesta-6,10(19)-diene (53). The same procedure as for the preparation of triple-labeled material **51** was used. Starting from **47** (75 mg, 0.16 mmol), 59.4 mg (92%) of **53** was obtained after the deprotection of the intermediate 25-trimethylsilyloxy derivative of **53**.

[7,9,19-$^{13}C_3$]-Vitamin D_3 (56) and [7,9,19-$^{13}C_3$]-5,6-*trans*-Vitamin D_3 (57). To a solution of **50** (117 mg, 0.30 mmol) in dioxane (15 mL) and H_2O (5 mL) was added *p*-toluenesulfonic acid monohydrate (20 mg) at room temperature. The solution was purged with argon then heated at 60 °C with stirring for 3 h. After cooling and the introduction of 1:1 ether/hexane (150 mL), the solution was washed with saturated aqueous $NaHCO_3$ solution. The aqueous phase was extracted with 1:1 ether/hexane (2 × 150 mL), and the combined organic phase was dried over $MgSO_4$ and concentrated under vacuum. The residual material was separated by HPLC (1:5 EtOAc/hexane): the first major fraction (white foam, 30.2 mg, 26%) was characterized as triply ^{13}C-labeled 5,6-trans-vitamin D_3 (**57**); the second major peak was triply ^{13}C-labeled vitamin D_3 (**56**, white foam, 39 mg, 34%).

[7,9,19-$^{13}C_3$]-25-Hydroxyvitamin D_3 (58) and [7,9,19-$^{13}C_3$]-25-Hydroxy-5,6-*trans*-vitamin D_3 (59). To a solution of **51** (109 mg, 0.27 mmol) in dioxane (15 mL) and water (5

mL) was added *p*-toluenesulfonic acid monohydrate (20 mg) at room temperature. The solution was purged with argon and was heated with stirring at 60 °C for 3 h. Water (50 mL) was added, and the mixture was extracted with ether (2 × 100 mL). The combined ether solution was dried over $MgSO_4$ and concentrated under vacuum. The residual material was separated by HPLC (silica gel, 1:1.5 EtOAc/hexane) to afford two major fractions: A, less polar (a mixture of triple labeled 25-hydroxyvitamin D_3 (95%) and 25-hydroxyprevitamin D_3 (5%); B, more polar [triple-labeled 5,6-*trans*-25-hydroxyvitamin D_3, (**59**, white powder, 23 mg, 21%)]. The vitamin−previtamin mixture (fraction A) was then further separated by HPLC (Microsorb Cyano column, 5 μm, 10 × 250 mm, Rainin Instrument Co., 98:2:0.3 hexane/^{i}PrOH/CH_3CN) to afford the desired labeled 25-hydroxyvitamin **58** (white powder, 37 mg, 34%).

[9,19-$^{13}C_2$]-Vitamin D_3 (60) and [9,19-$^{13}C_2$]-5,6-*trans*-Vitamin D_3 (61). Following the same procedure as for the preparation of triple-labeled **56**, 64 mg (0.165 mmol) of alcohol **52** afforded doubly ^{13}C-labeled vitamin D_3 **60** (21.5 mg, 34%, more polar) and 5,6-*trans*-vitamin **61** (17.4 mg, 27%, less polar).

[9,19-$^{13}C_2$]-25-Hydroxyvitamin D_3 (62) and [9,19-$^{13}C_2$]-25-Hydroxy-5,6-*trans*-vitamin D_3 (63). Following the same procedure for the preparation of triple-labeled **58**, 18 mg (0.045 mmol) of **53** afforded 7.3 mg of doubly ^{13}C-labeled vitamin D **62** (40%, less polar) and 4.4 mg of 5,6-*trans*-vitamin **63** (24%, more polar).

[7,9,19-$^{13}C_3$]-(1*S*,3*R*,5*R*,8*R*)-3,5-Didehydro-1,8-dihydroxy-9,10-seco-25-(trimethylsilyloxy)cholest-10(19)-en-6-yne (64). To a suspension of 7.1 mg (0.064 mmol) of selenium dioxide in 5 mL of dry CH_2Cl_2 was added 0.21 mL of a *tert*-butyl hydroperoxide solution (3.0 M in 2,2,4-trimethylpentane, 0.64 mmol), and the resulting mixture was stirred at room temperature for 1 h. The solution was then cooled to 0 °C, and 100 mg (0.21 mmol) of propargyl alcohol **45** in 2 mL of CH_2Cl_2 was added. The reaction mixture was kept at 4 °C for 20 h, whereupon 5 mL of 20% aqueous $Na_2S_2O_3$ was added. After the mixture was stirred vigorously at room temperature for 0.5 h, the separated aqueous phase was extracted with ether (2 × 100 mL). The organic extracts were combined, dried over magnesium sulfate, filtered, and concentrated under vacuum. The residue was dried under high vacuum for 2 h, dissolved in 8 mL of dry THF, and cooled to −50 °C. A lithium triethylborohydride solution (0.64 mL, 1 M in THF) was added dropwise over 5 min. The solution was then allowed to warm to 0 °C and quenched by the addition of 50 mL of H_2O. The mixture was extracted with 2 × 100 mL of ether, and then the organic extracts were combined, dried over magnesium sulfate, and concentrated under vacuum. The crude product was purified by silica gel chromatography (1:4 EtOAc/hexanes) to give 66 mg (64%) of allylic alcohol **64**.

[7,9,19-$^{13}C_3$]-(1*S*,3*R*,5*R*,8*R*)-1-(*tert*-Butyldiphenylsilyloxy)-3,5-didehydro-8-hydroxy-9,10-seco-25-(trimethylsilyloxy)cholest-10(19)-en-6-yne (65). A solution of 20 mg (0.04 mmol) of propargyl alcohol **64**, 7 mg (0.1 mmol) of imidazole and 21 mg (0.07 mmol) of TBDPS-Cl in 1 mL of CH_2Cl_2 was stirred under argon at room temperature for 12 h. After addition of 2 mL of H_2O, the mixture was extracted with 3 × 5 mL of hexanes. The combined organic extracts were dried over magnesium sulfate, filtered, and concentrated under vacuum. The residue was purified by preparative thin-layer chromatography, eluting with 10% EtOAc/hexanes to afford 25 mg (0.034 mmol, 85% yield) of **65** as a colorless oil.

[7,9,19-$^{13}C_3$]-(1*S*,3*R*,5*R*,8*R*)-1-(*tert*-Butyldiphenylsilyloxy)-3,5-didehydro-8-hydroxy-9,10-seco-25-(trimethylsilyloxy)-cholesta-6,10(19)-diene (66). To a solution of propargyl alcohol **65** (6 mg, 0.008 mmol) in 1 mL of THF was added NaOMe (3 μL of a 25% NaOMe in methanol solution) and $LiAlH_4$ (140 μL of a 1.0 M solution in THF). The reaction mixture was stirred under argon at room temperature for 2 h. The reaction mixture was then quenched by the addition of 5 mL of saturated NH_4Cl solution and extracted with 3 × 10 mL of diethyl ether. The organic extract was washed with brine, dried over magnesium sulfate, filtered, and concentrated under vacuum. The crude product was purified by HPLC (Whatman Partisil 10) using 10% EtOAc/hexanes to yield the pure trans alcohol **66** (4.3 mg, 72%), as a colorless oil.

[7,9,19-$^{13}C_3$]-(1*S*,3*R*,5*R*,8*R*)-3,5-Didehydro-1,8,25-trihydroxy-9,10-seco-25-cholesta-6,10(19)-diene (67). A solution of 2.9 mg (0.0039 mmol) of TBDPS protected alcohol **66** in 0.5 mL of THF was stirred under argon at room temperature. To this solution was added 12 μL of TBAF (1 M in THF, 0.012 mmol), and the solution was stirred in the dark at room temperature for 16 h. The mixture was then diluted with 5 mL of EtOAc and then washed with water (3 × 5 mL). The combined organic extracts were then dried over magnesium sulfate, filtered, and concentrated under vacuum. The crude product was purified by HPLC using a Whatman partisil 10 column eluting with 90:10 EtOAc/hexanes to afford 1.5 mg (92% yield) of spectroscopically pure triol **67** as a colorless oil.

[7,9,19-$^{13}C_3$]-1α,25-Dihydroxyvitamin D_3 (68). A solution of 0.7 mg (0.0016 mmol) of triple-labeled starting material **67**, 0.3 mg of *p*-TsOH, and 1 mL of dioxane−H_2O (2:1) was stirred at 75 °C for 1 h. The cooled solution was extracted with 5 mL of EtOAc, and then the combined organic extracts were washed successively with water (2 × 5 mL) and aqueous $NaHCO_3$. After drying over magnesium sulfate, the EtOAc solution was filtered and concentrated under vacuum. The crude product was purified by HPLC using EtOAc as the eluent on a Whatman Partisil 10 column to afford spectroscopically pure triple labeled hormone **68** (0.3 mg) as a colorless oil in 40% yield.

General Procedure for Photoisomerization of 5,6-*trans*-Vitamin D to the Corresponding Vitamin D. Argon was bubbled through a solution of the mixture of the 5,6-*cis*- and 5,6-*trans*-vitamin D compounds (20 mg) and 9-acetylanthracene (10 mg) in *tert*-butyl methyl ether (12 mL) in a $^3/_4$ × 5 in. Kimax test tube for 10 min. The solution was irradiated under argon using a Hanovia 450 W medium-pressure lamp filtered through a Pyrex, water jacketed condenser well for 1 h. The solvent was removed, and the residual oil was separated by flash chromatography to afford the corresponding vitamin (∼20 mg, ∼100%).

2D NOE Double-Half-Filtered NMR Study of [7,9,19-$^{13}C_3$]-25-D3 (58) in SDS-d_{25} Micelles. To a solution of sodium dodecyl sulfate-d_{25} (SDS-d_{25}, MW = 313.50, Cambridge Isotope Laboratories, 3.14 mg, 0.0100 mmol) in ethanol (0.75 mL), prepared by warming the mixture over a steam bath until it dissolved, was added an ethanolic solution of [7,9,19-$^{13}C_3$]-25-D3 (**58**, 0.890 mL, 0.001124 M, 0.0010 mmol). The concentration was established from its UV-based extinction coefficient of ϵ = 18 300 at λ_{max} 265 nm (a value of ϵ ∼12 000 in SDS was estimated experimentally, but the weight of steroid used was quite small). The solvent was then removed under reduced pressure on a rotary evaporator. The resulting white residue was dried under high vacuum with protection from light for 12 h at room temperature. To the white residue was added deuterium oxide (Cambridge Isotope Laboratories, 99.9%, 0.500 mL) and the resulting suspension subjected to sonication for 5 min (270 W, 43 kHz; Fisher Ultrasonic Cleaner, model no. FS14). The resulting clear colorless solution (20 mM in SDS-d_{25}; 2.0 mM in [7,9,19-$^{13}C_3$]-25-D3) was transferred to a Shigemi NMR tube and the plunger inserted so as to eliminate any bubbles present.

Using the above sample, NOESY NMR experiments were run using a variant of the pulse sequence described by Wider et al.[15] in which the phase cycling was modified to produce the desired sub-spectrum directly. A build up curve was created using the volumes for the cross-peaks observed for the proton pairs 9α−9β, 19*E*−19*Z*, 7−19*Z*, and 7−19*E* at mixing times of 25, 50, 100, 200, 300, 600, and 800 milliseconds to determine the appropriate mixing time that provides the optimal volume cross-peak intensities for the distance calculations. The results are discussed in the text (including Figure 2), and additional details are given in the Supporting Information.

In another experiment, a solution of sodium dodecyl sulfate-d_{25} (Cambridge Isotope Laboratories, 29 mg) in ethanol (10 mL), prepared by warming the mixture until dissolved, was

added a solution of [7,9,19-$^{13}C_3$]-25-D3 (**58**, 0.4 mg) in ethanol (400 μL). The solvent was removed under vacuum on a rotary evaporator. The resultant free flowing white powder was dried under high vacuum for 24 h at room temperature. Deuterium oxide (CIL, 99.9% D, 2.5 mL) was added and the resulting suspension was subjected to sonication for 5 min (270 W, 43 kHz; Fischer Ultrasonic Cleaner, model no. FS14). Solid material was removed by filtration (Millex-SR Millipore filter, 0.5 μm). The resulting clear solution was used for recording the ^{13}C NMR spectrum. Additional details for the NMR measurements are given in the caption to Figure 2.

NMR Sample Preparation for 58 in Aqueous Dimethylcyclodextrin (DMCD) Solution. To a solution of dimethylcyclodextrin (DMCD, 10 mg, Aldrich; also referred to as methyl-β-cyclodextrin) in D_2O (2.5 mL) was added a solution of triply labeled 25-D3 (0.3 mg) in CD_3OD (30 μL) with stirring. The clear solution was lyophilized (cooled with dry ice and evacuated with vacuum pumping) at room temperature overnight to give a white powder. Approximately 1 mg of the powder was dissolved in D_2O (0.7 mL) and used for the NMR study (5 mm tube).

Acknowledgment. This study was generously supported by grants from the NIH, NSF, the Cambridge Isotope Laboratories, and the Committee on Research of the University of California, Riverside. We also acknowledge generous supplies of starting materials from Solvay, Hoffmann-LaRoche, and Leo Pharmaceuticals. K.R. also acknowledges the DFG (Germany) for a postdoctoral stipend.

Supporting Information Available: Spectral and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

JO011096Y