Model Studies of the Biosynthesis of Non-Head-to-Tail Terpenes. Stereochemistry of the Head-to-Head Rearrangement[1]

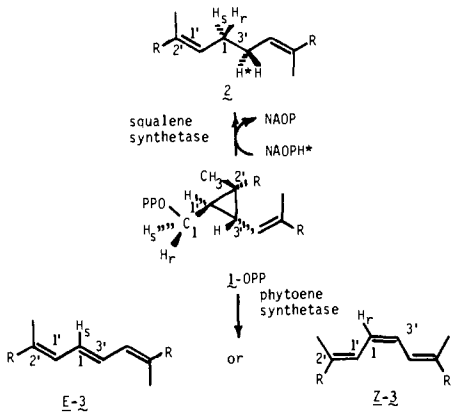
C. Dale Poulter*[2] and John M. Hughes

Contribution from the Department of Chemistry, University of Utah, Salt Lake City, Utah 84112. *Received August 10, 1976*

Abstract: The stereochemistry of the head-to-head rearrangement which occurs during biosynthesis of squalene from presqualene pyrophosphate was studied with a ten-carbon model system, *N*-methyl-4-[(1*S*,1′*R*,3′*R*)-[1-^{3}H]chrysanthemyloxy]pyridinium iodide, in a nonenzymatic reaction. A head-to-head product, [5-^{3}H]-2,7-dimethylocta-2,6-dien-4-ol, is isolated after hydrolysis. The absolute configuration at C(5) was determined by converting the alcohol to malic acid and measuring retention of tritium after treatment of the acid with fumarase. The chemical and biochemical transformations of the cyclopropylcarbinyl compounds both give a net inversion of configuration at C(1).

The stereochemistry of the head-to-head rearrangements of presqualene pyrophosphate and prephytoene pyrophosphate in the sterol and carotenoid biosynthetic pathways is known. In their classic work on the stereochemistry of the biosynthesis of squalene, Cornforth and Popjak demonstrated that C(1) of presqualene pyrophosphate (R = $C_{11}H_{19}$) is inverted during the reductive rearrangement catalyzed by squalene synthetase (Scheme I).[3] Stereochemical work with phytoene revealed that

Scheme I

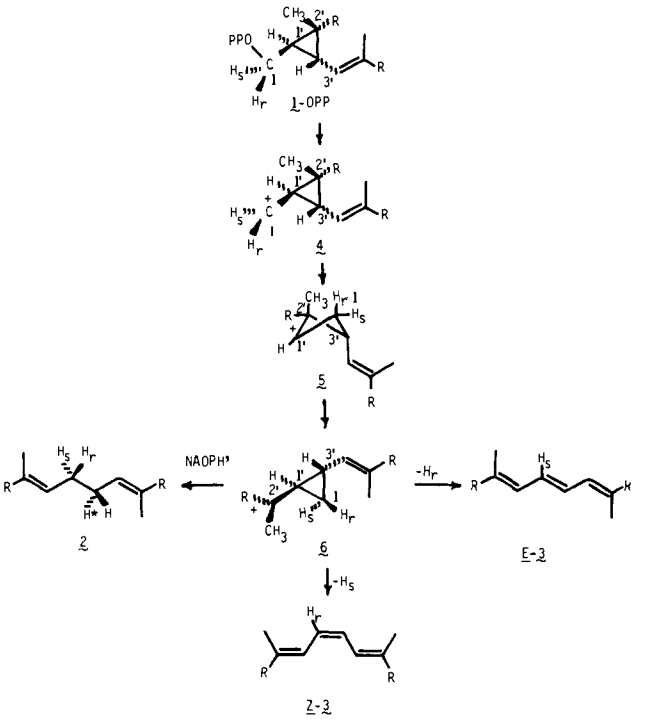


the *E* synthetase from *Mycobacterium* sp. removes the *pro-R* proton from the carbon atom that was originally C(1) of prephytoene pyrophosphate, while the *Z* synthetase from the C(5) mutant of *Phycomyces blakesleeanus* removes the *pro-S* proton.[4]

We have explained these observations with a general mechanism for the head-to-head rearrangement shown in Scheme II, where a tertiary cyclopropylcarbinyl cation is the immediate precursor of the 1,1′ hydrocarbons.[5] Capture of the tertiary intermediate by hydride from NADPH affords squalene (R = $C_{11}H_{19}$), while elimination of H_R or H_S provides phytoene. Stereoelectronic considerations dictate that elimination of H_R gives *E*-phytoene and elimination of H_S, *Z*-phytoene.[4,5b] The proton which is removed (H_R or H_S) should be determined by the location in the active site of the base which assists with the elimination. Thus, we proposed that the stereochemistry for C(1) during rearrangement of **1**-OPP to **6** is the same in both the sterol and carotenoid biosynthetic pathways and involves a net inversion of configuration.[5b]

C(1) undergoes two changes in bonding between **1**-OPP and **6**: the C(1)-oxygen bond is cleaved during ionization, and the C(1)-C(3′) bond is formed during rearrangement. Net inversion of configuration at C(1) can occur in one of two ways—ionization of **1**-OPP from a conformation where the

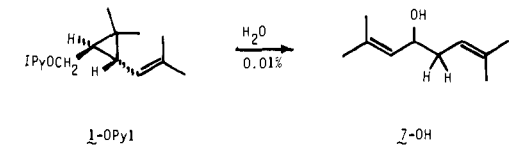
Scheme II. A Mechanism for the Head-to-Head Rearrangement



C(1)-oxygen bond is antiperiplanar to the C(1′)-C(3′) cyclopropane bond, followed by a suprafacial migration of the C(1′)-C(3′) bond to C(1) (**4** → **5**), or periplanar ionization, followed by antarafacial migration. In a recent study using a C_{10} model system (R = CH_3) for presqualene and prephytoene pyrophosphate, we found a 6.1 to 1 preference for ionization from the antiperiplanar conformation in a normal solvolysis reaction.[6] We now report experiments which demonstrate that rearrangement of **4** to **6** follows the suprafacial path.

Results and Discussion

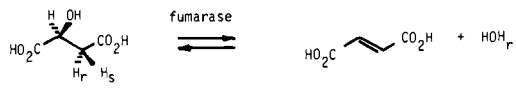
A. General Approach. Model studies with chrysanthemyl derivatives show that the head-to-head rearrangement is a minor pathway for cation **4** (R = CH_3) in the absence of an enzyme.[7] However, hydrolysis of *N*-methyl-4-chrysanthemyloxypyridinium iodide (**1**-OPyI) affords a small (ca. 0.01%) amount of a head-to-head alcohol, 2,7-dimethyl-2,6-octadien-4-ol (**7**-OH), which is a C_{10} analogue of squalene where hydroxyl replaces hydride as the entering nucleophile.[7d] Since we have determined the stereochemistry of ionization for **1**-OPyI during hydrolysis, it should be possible to deduce the



1-OPyl 7-OH

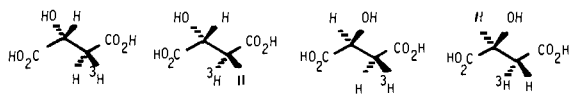
stereochemistry for C(1) during rearrangement of **4** to **6** if the absolute configuration of the methylene group in 7-OH can be determined.

Because low yields of 7-OH are obtained during solvolysis, we decided to use tritium as a stereochemical label at C(1) and to rely on an enzymatic assay of absolute configuration. Fumarase catalyzes the stereospecific exchange of the hydroxyl group and the *pro-R* methylene hydrogen of malic acid with water and appeared to be a logical method for our stereochemical assay, providing 7-OH could be degraded to malic



8-OH 9

acid. From previous work we knew the head-to-head alcohol and consequently malic acid obtained by degradation would be a mixture of four diastereomers.[5b,6] The configuration of the methylene group is partially randomized during ionization (86% antiperiplanar, 14% periplanar),[6] and the carbon bearing the hydroxyl group in 7-OH is partially randomized by a cyclopropylcarbinyl–allyl isomerization of **6** before reaction with

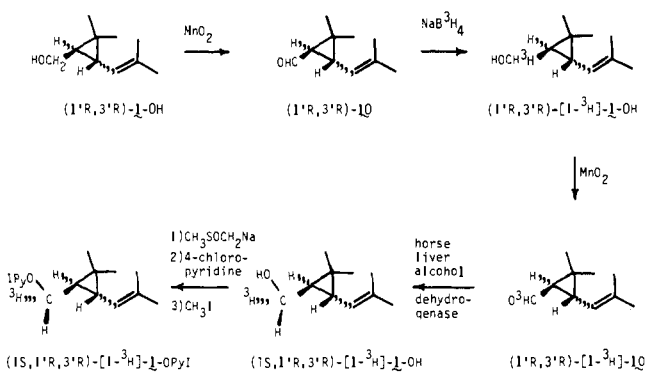


2R,3S 2R,3R 2S,3S 2S,3R

solvent.[5b] The assay with fumarase requires precise knowledge of the *R/S* ratio at the hydroxyl since the procedure can only distinguish (2*S*,3*R*)-[3-^{3}H]malic acid from the other three diastereomers.[8] Since hydrolysis of (1′*R*,3′*R*)-**1**-OPyl should give a predominance of the 2*R* diastereomers of 7-OH, and ultimately malic acid, we decided that it would be desirable to enrich the mixture in the 2*S*,3*S* and 2*S*,3*R* diastereomers and determine the *R/S* ratio at C(2) in the enriched material prior to the fumarase reaction. Here, the logical assumption is made that the *R/S* ratio at C(3) is the same for the 2*R* and 2*S* diastereomers and remains constant during a resolution step.

B. Synthesis and Solvolysis of *N*-Methyl-4-[(1*S*,1′*R*,3′*R*)-[1-^{3}H]chrysanthemyloxy]pyridinium Iodide. (1*S*,1′*R*,3′*R*)-[1-^{3}H]Chrysanthemol was prepared from (1′*R*,3′*R*)-chrysanthemol by the sequence of oxidation–reduction reactions shown in Scheme III. Tritium was removed from the

Scheme III. Synthesis of *N*-Methyl-4-[(1*S*,1′*R*,3′*R*)-[1-^{3}H]-chrysanthemyloxy]pyridinium Iodide

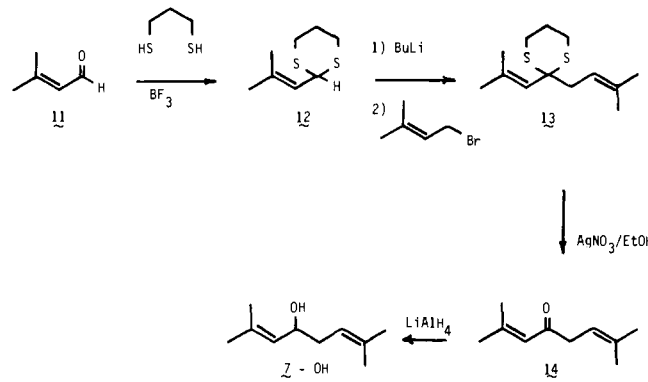


(1′R,3′R)-1-OH (1′R,3′R)-10 (1′R,3′R)-[1-^{3}H]-1-OH

(1S,1′R,3′R)-[1-^{3}H]-1-OPyl (1S,1′R,3′R)-[1-^{3}H]-1-OH (1′R,3′R)-[1-^{3}H]-10

pro-R position at C(1) of (1′*R*,3′*R*)-[1-^{3}H]-**1**-OH by oxidation of the C(1) diastereomers to (1′*R*,3′*R*)-[1-^{3}H]-**10**, followed by reduction of the aldehyde with horse liver alcohol dehydrogenase. A control experiment showed that C(1) of the aldehyde did not epimerize under the conditions of the reaction, and the labeled aldehyde was analyzed by radio-TLC to insure that no alcohol remained after the oxidation step. In repeated oxidation–reduction cycles the horse liver enzyme only exchanges the *pro-R* hydrogen of primary alcohols and has been used repeatedly to synthesize *S*-hydroxymethyl groups from isotopically labeled aldehydes.[3a,9] We employed a catalytic amount of NADH and regenerated the expensive coenzyme by a coupled oxidation of ethanol. The radioactive pyridinium salt of (1*S*,1′*R*,3′*R*)-[1-^{3}H]-**1**-OH was prepared and solvolyzed in aqueous bicarbonate at 25 °C as previously described.[6,7d] Analysis of the mixture by GLC showed two major products which coeluted with yomogi alcohol and artemisia alcohol on a 500 ft open tubular column.

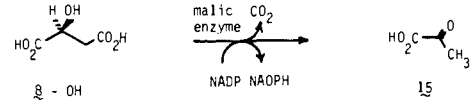
C. Isolation, Degradation, Resolution, and Stereochemical Analysis of [5-^{3}H]-2,7-Dimethylocta-2,6-dien-4-ol. The isolation of radiolabeled 7-OH from the mixture of solvolysis products was aided by the addition of cold carrier, prepared as shown below. A preliminary isolation was performed by



11 12 13 7-OH 14

GLC before the hydroxyl group was protected as the acetate, and the double bonds were cleaved with ozone. An oxidative workup with hydrogen peroxide, followed by a hydrolysis step, yielded radioactive malic acid of undetermined configuration at C(2) and C(3).

The radiolabeled malic acid was resolved by addition of a large excess of *S*-malic acid and crystallization of the mixture to constant specific activity. The efficiency of the resolution was checked enzymatically by incubating a portion of the resolved material with malic enzyme and NADP. The enzyme catalyzes the oxidation–decarboxylation shown below and is specific for *S*-malic acid.[10] After the reaction had gone to



8-OH NADP NAOPH 15

completion, the mixture was separated by TLC. Radioactivity comigrated with authentic samples of pyruvic and malic acid. From the distribution of radioactivity in the two peaks, we conservatively estimate that our sample contained 85% *S*-malate.

The radiochemical yields of the synthesis, degradation, and resolution steps are summarized in Table I. The synthetic steps up to (1*S*,1′*R*,3′*R*)-[1-^{3}H]-**1**-OH proceeded in good yield as expected. Acetate, [5-^{3}H]-7-OAc, isolated after solvolysis of chrysanthemyloxypyridinium iodide gave a much larger radiochemical yield than expected. In several experiments with cold material, we never saw more than 0.02% of 7-OH.[7d] Thus, it is likely that [5-^{3}H]-7-OAc contained radioactive contaminants, which may explain the unexpectedly low yield of the ozonolysis step. Crude malic acid was crystallized three times

Table I. Radiochemical Yields

Compound	^3H μCi	Yield, %
(1′*R*,3′*R*)-[1-^{3}H]-**1**-OH	40.0×10^3	100
(1′*R*,3′*R*)-[1-^{3}H]-**10**	15.5×10^3	78
(1*S*,1′*R*,3′*R*)-[1-^{3}H]-**1**-OH	14.3×10^3	92
[5-^{3}H]-7-OAc	16.5	0.115
2*S*-[3-^{3}H]-**8**-OH (Crude)	0.548	3.3
(1st crystallization)	0.132	24
(2nd crystallization)	0.120	91
(3rd crystallization)	0.117	98

Table II. Assay of (2*S*,3*S*)-[3-^{3}H]Malic Acid

Run	Incubation time, min	dpm	% ^{3}H retained[a]
Blank		4441 ± 107	
Blank		4453 ± 133	
1[b]	21	3761 ± 102	85 ± 3
2[b]	37	3793 ± 102	85 ± 3
3[b]	60	3912 ± 106	88 ± 3

[a] Average value for blanks of 4447 was used. [b] Three units of fumarase.

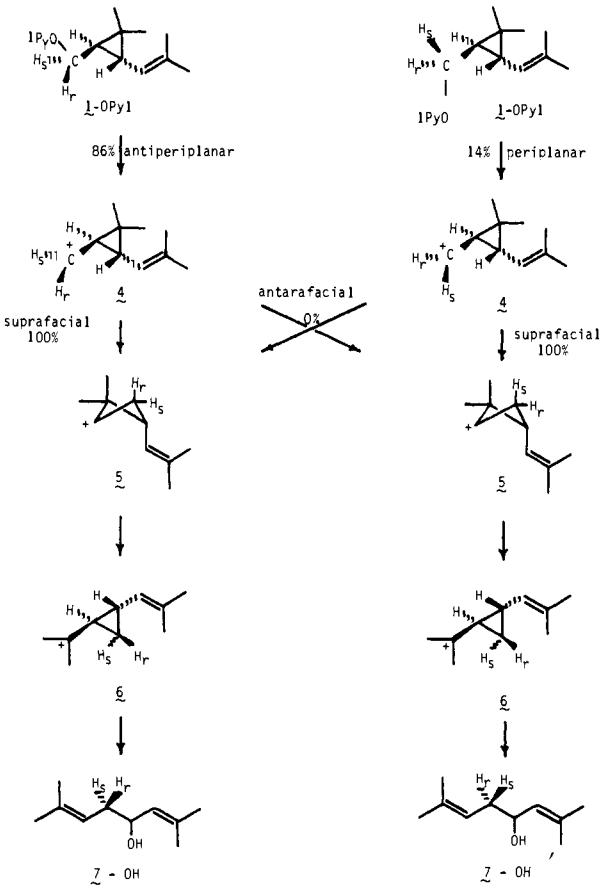
with an overall recovery of 21% of the original activity. Previous work indicates that 4*R*-[5-^{3}H]-7-OAc should be the predominant C(4) stereoisomer from hydrolysis of the 1′*R*,3′*R*-chrysanthemyl system[5b,c] (see below). Therefore, the overall yield of 0.0008% of 2*S*-[3-^{3}H]malic acid from (1*S*,1′*R*,3′*R*)-[1-^{3}H]-**1**-OH represents a good (ca. 10–15%) recovery of the maximum expected radioactivity.

Incubation of recrystallized [3-^{3}H]-**8**-OH with fumarase gave the results shown in Table II. The reaction was followed spectrophotometrically, and equilibrium between malate and fumarate was achieved in 3 min in runs 1–3. An average of 86% of the radiolabel was retained in the substrate. If one assumes that 15% of the counts in 2*S*-[3-^{3}H]-**8**-OH is due to a contaminant (perhaps the 2*R* diastereomers), a correction of 668 dpm for the blanks and runs 1–3 gives 83 ± 3%[11] retention of radiolabel after equilibration. Therefore, the ^{3}H-containing malic acid is an 83/17 mixture of 2*S*,3*S* and 2*S*,3*R* diastereomers, respectively. The degradation of **7**-OH to malic acid does not alter the configuration of the methylene group, and the resolution of **8**-OH by crystallization should not fractionate the 2*S*,3*S* and 2*S*,3*R* diastereomers. Thus, we conclude that the *R*/*S* ratio at C(5) in [5-^{3}H]-2,7-dimethyl-2,6-octadien-4-ol is also 83/17.

D. Stereochemistry of the Head-to-Head Rearrangement in a C_{10} Model System. The data presented above clearly demonstrate that the rearrangement of **1**-OPyI to **7**-OH involves predominant inversion of configuration at C(1), with a ratio of inversion to retention of 83/17. The pyridinium iodide contained approximately 2% of the 1*S*,1′*S*,3′*S* diastereomer[12] and correcting for "mislabeling" in the starting material[6] changes the ratio slightly to 84/16. This value is the same, within experimental error, as that for the ratio of ionization from the antiperiplanar (86%) and periplanar (14%) conformations. We conclude that the cyclopropylcarbinyl–cyclopropylcarbinyl rearrangement of **4** to **6** is initiated by a stereospecific suprafacial migration of the C(1′)–C(3′) cyclopropane bond (see Scheme IV). Recently, several examples of cyclopropylcarbinyl–cyclopropylcarbinyl rearrangements that require suprafacial bond migrations have been reported in simple systems.[13]

In relating the model work to the enzymatic reactions, it is

Scheme IV

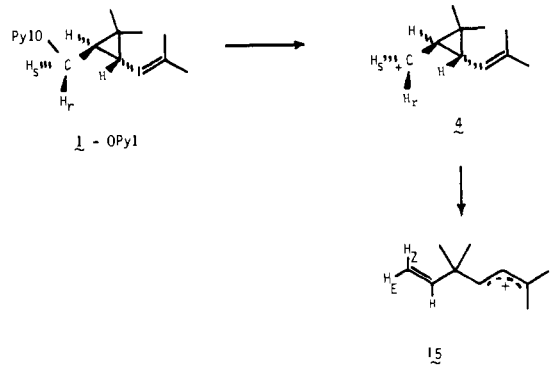


instructive to consider the ionization and bond migration steps separately (see Scheme IV). The difference in activation energies for ionization from the two conformers is only 1.08 kcal/mol at 25 °C,[6] and the stereospecificity required in this step of the enzymatic reaction is undoubtedly due to orientation of the substrate in the active site prior to ionization. However, a suprafacial bond migration like that found for rearrangement of **4** to **5** appears to be a general phenomenon for cyclopropylcarbinyl cations.[13,14] The difference in the activation energies for suprafacial and antarafacial rearrangements in cyclopropylcarbinyl systems is not known, but the antarafacial mode requires decoupling of the carbinyl carbon with the rest of the system and should be highly endergonic with respect to a suprafacial migration.[15] We conclude that the antiperiplanar–suprafacial path is preferred in the solvolytic and enzymatic head-to-head rearrangements.

C(3′) of presqualene pyrophosphate is also inverted during biosynthesis of squalene.[3a] In this case, the configuration of the center is not altered until the reaction of tertiary cation **6** (R = $C_{11}H_{19}$) with NADPH. In a related model study, we showed that head-to-head alcohol **7**-OH obtained by trapping **6** (R = CH_3) with water is inverted at C(5).[5b] Thus, the stereochemistry of the individual steps that we proposed for the head-to-head rearrangement is duplicated in our model studies.

E. Chrysanthemyl Cation. During solvolysis, the chrysanthemyl cation isomerizes to allylic cation **15** by rupture of the C(1′)–C(3′) cyclopropane bond.[7d] This is the most facile reaction that **4** undergoes, with more than 97% of the products from hydrolysis of **1**-OPyI coming from **15**. The configuration about the C(1)–C(1′) bond of **1**-OPyI during ionization was determined by replacing H_s with deuterium and measuring the ratio of protium to deuterium in the H_Z and H_E positions in products derived from **15**. Although we showed that **4** isomerized to **15**, it was still possible that **1**-OPyI ionized directly

to **4** and **15** by two competing paths. One could argue that a direct fragmentation of **1**-OPyI to **15** is stereospecific and occurs from the antiperiplanar conformation while the 14% of ionization from the periplanar conformation must occur via **1**-OPyI → **4**. That leaves the contribution of the antiperiplanar orientation to **1**-OPyI → **4** undetermined. However, direct



ionization of **1**-OPyI to **15** must be negligible since the stereoselectivity at C(1) for the head-to-head rearrangement (84/16) is the same as that for ionization (86/14). The congruent stereoselectivities also support our assertion that isomerization of **4** to **15** is irreversible.[7d] The loss of chirality at C(1′) and C(3′) which accompanies the isomerization effectively randomizes C(1) because reclosure gives (1′*R*,3′*R*)-**4** (shown above) and its enantiomer. Further isomerization of the reclosed enantiomers would randomize C(5) in head-to-head **7**-OH, and this was not observed. Our data also suggest that **4** is a discrete intermediate in the head-to-head rearrangement and that migration of the C(1′)–C(3′) cyclopropane bond in **1**-OPyI is not concerted with ionization for that fraction which rearranges to alcohol **7**-OH.

Experimental Section

General. Melting points were obtained on a Fisher-Johns melting point stage or in open capillaries in a Thomas-Hoover melting point apparatus and are uncorrected. Carbon–hydrogen microanalyses were performed by Chemalytics, Inc., Tempe, Ariz.

Nuclear magnetic resonance (NMR) spectra were obtained on Varian Associates A-60, EM-360, EM-390, and XL-100 spectrometers and are reported in parts per million (δ, ppm) downfield from tetramethylsilane (Me_4Si) internal standard. Unless otherwise indicated, NMR spectra were obtained in carbon tetrachloride (Mallinckrodt, SpectrAR grade) containing 1 vol % tetramethylsilane (Diaprep, Inc.) and chloroform (Mallinckrodt, SpectrAr grade) as internal standards. NMR solvent giving equally intense Me_4Si and chloroform resonances of amplitude suitable for use with 10% solutions was prepared by adding 0.5 ml (0.32 g, 3.68 mmol, 44.2 mmol of protons) of Me_4Si and 5.30 g (44.2 mmol) of chloroform to 50 ml of carbon tetrachloride. Spectra were corrected when necessary for nonlinearity using the 436 Hz (at 60 MHz) singlet resonance of chloroform. Infrared (IR) spectra were obtained on a Beckman IR-5 spectrophotometer.

Analytical gas–liquid chromatography was performed on a Varian-Aerograph Model 1200 instrument equipped with a flame ionization detector and an Autolab Model 6300 digital integrator. Repetitive injections showed that the system's reproducibility averaged ±0.5% and never exceeded ±2%. Analytical separations were achieved using 500 ft × 0.03 in. (i.d.) stainless steel open tubular columns utilizing the stationary phases Carbowax 20M (recrystallized from ethanol), 95:5 OV-101/Igepal CO-880 (both obtained from Analabs, Inc.), or SF-96 (viscosity 50 cSt at 25 °C; Analabs, Inc.) as indicated and operated at the specified temperature at a nitrogen flow rate of 10 ml/min. Preparative gas–liquid chromatographic separations were performed on a Varian-Aerograph Model A90-P3 thermal conductivity instrument, using 12 ft × ¼ in. (o.d.) glass columns packed with 10% Carbowax 20M on 60/80 mesh Anakrom ABS diatomaceous earth support, or on a 3 ft × ¼ in. (o.d.) stainless steel column packed with 95:5 OV-101/Igepal CO-880 on 50/60 mesh Anakrom ABS.

Thin-layer chromatography was carried out on 75 mm × 25 mm "Bakerflex" precoated silica gel 1B-F sheets (silica gel G with fluorescer; J. T. Baker Co.) in vapor-saturated tanks, visualizing the developed spots first with 254 nm light and then with iodine staining.

Liquid scintillation counting was performed on a Packard Tri-Carb scintillation spectrometer for periods such that the 99% probable error limit was no greater than 3% of the counting rate (cpm).[16] Over a 2-week period, background counts averaged 15 cpm in polyethylene vials and 20 cpm in glass vials. Toluene-soluble materials were counted in 10.0 ml of a cocktail prepared by dissolving 15 g of "Omnifluor" premixed scintillator (New England Nuclear) in 8 pints of reagent-grade toluene. Counting efficiencies (determined by adding a known activity of tritium-labeled toluene as internal standard and recounting) averaged 0.39 over a period of 8 months for this system, varying only between 0.38 and 0.40. Counting errors and statistical uncertainties in the counting rates obtained were determined using standard tables,[16] based on observed background, total counts and the counting period. The reported uncertainties are the 99% probable values. Aqueous samples not exceeding 25 μl were rendered soluble in the above cocktail by adding 0.6–0.8 ml of absolute ethanol and not more than 0.1 ml of formic acid. Larger amounts of ethanol lowered the observed counting efficiencies to 0.17–0.19, and larger amounts of formic acid caused phase separation. Best results for counting water-soluble samples were obtained by taking up the sample to be counted in 0.9 ml of deionized distilled water, transferring to a glass counting vial, and adding 10.0 ml of a commercial Triton X-100 (New England Nuclear)–Omnifluor–toluene cocktail (500 ml: 8.5 g: 1000 ml). The resulting emulsion was clarified by addition of 50 μl aliquots of water (three usually required) until a clear, stable solution was obtained. An additional 25 μl of water insured stability of the solution at the lower temperature (5 °C) of the scintillation counter. Larger amounts of water in this counting system produced unsatisfactory gels. The Triton cocktails had counting efficiencies averaging 0.2 for alcohol-free samples.

Thin-layer radiochromatograms were analyzed by locating radioactive bands using a Packard Model 7200 radiochromatogram scanner equipped with a Packard Model 385 recording ratemeter, and spots were counted, if necessary, by scraping bands of adsorbent from glass plates into counting vials or by cutting bands from precoated sheets and placing each section in a counting vial. Compounds were eluted from the adsorbent with the appropriate solvent (0.1 ml) before adding counting solution. TLC visualizing agents such as iodine or indicator spray produced variable quenching so that internal standardization of each vial was necessary for quantitative work.

(1′*R*,3′*R*)-Chrysanthemal (10). Optically active aldehyde was prepared by stirring 2.0 g of (1′*R*,3′*R*)-chrysanthemol (98% enantiomeric excess)[7d] over 25 g of active manganese dioxide in pentane for 24 h at room temperature. Filtration and careful evaporation of solvent at reduced pressure afforded 1.45 g (72%) of a fragrant yellow oil which was distilled, bp 54–55 °C (0.8 mm). Analytical samples were collected by GLC, taking care to insure acid-free conditions and minimum operating temperatures: $[\alpha]^{30}D$ −44.97° (*c* 2.2, $CHCl_3$); NMR (CCl_4) 1.10 and 1.20 (6, pr of s, methyls at $C_{2'}$), 1.56 (1, d of d, $J_{1',3'}$ = 6.0 Hz, H at $C_{1'}$), 1.67 (6, d, *J* = 1.7 Hz, allylic methyls), 2.16 (1, d of d, $J_{3',1''}$ = 7.5 Hz, H at $C_{3'}$), 4.80 (1, d of septets, olefinic H), and 9.34 ppm (1, d, $J_{1,1'}$ = 4.5 Hz, H at C_1); IR (neat) 2960, 2875, 2825, 2685, 1705, 1450, 1380, 1345, 1260, 1180, and 1105 cm^{-1}.

Anal. $C_{10}H_{16}O$: C, H.

(1′*R*,3′*R*)-[1-^{3}H]Chrysanthemol ([1-^{3}H]-1-OH). Freshly distilled (1′*R*,3′*R*)-chrysanthemal (100 mg, 0.658 mmol) was dissolved in 0.5 ml of anhydrous methanol (freshly distilled from magnesium turnings) in a 12-ml ignition (thick-wall) tube clamped above a stainless steel tray. Sodium borotriteride (NaB^3H_4, 100 mCi, Amersham/Searle) was dissolved in 1 ml of methanol in the original ampule, similarly clamped. A small portion was removed for other experiments, and one-half of the remaining solution was carefully transferred to the reaction tube. Vigorous bubbling was immediately evident. The reaction tube was gently swirled, tightly stoppered, and allowed to stand at ambient temperature for 16 h.

Any remaining aldehyde was reduced by adding a methanolic solution of sodium borohydride with thorough manual vortexing until no further reaction was evident. Excess borohydride was decomposed by addition of 1 ml of aqueous acetic acid. The organic product was extracted into three 2-ml portions of Skellysolve, and the combined extracts were washed with two 1-ml portions of water and stored at 5 °C over anhydrous granular sodium sulfate in a tightly stoppered ignition tube.

The solution was brought to 5.0 ml total volume with distilled hexane. A 1-μl sample was diluted 1000-fold and 1-μl of the diluted solution was counted (6919 cpm, 1.78×10^4 dpm) to give an approximate radiochemical yield (40 mCi, 400 μCi/mg, specific activity 61.5 mCi/mmol). A 1-μl sample was chromatographically homogeneous (R_f 0.49) on silica gel G developed with 15% 2-propanol/heptane after 4 months of storage at 5 °C.

(1′*R*,3′*R*)-[1-^{3}H]Chrysanthemal ([1-^{3}H]-10). The solution of radiolabeled chrysanthemol was carefully transferred to a 25-ml recovery flask equipped with a Teflon stopper and a magnetic stirrer. The residual drying agent was washed with eight 1-ml portions of distilled pentane, and the total volume was brought to 20 ml with pentane before 1.5 g (15 weight equiv) of active manganese dioxide (recently assayed for activity) was added. The brown slurry was allowed to stir for 12 h at ambient temperature, at which time radio-TLC showed no radioactive alcohol remaining. The reaction was filtered through a medium-porosity glass Buchner funnel, washing the reaction vessel and then the filter cake with each of two 5-ml portions of pentane and two 5-ml portions of distilled ether. All but 5 ml of solvent was removed from the combined filtrates by distillation through a short-path apparatus at atmospheric pressure, heating the distillation flask with a warm water bath so that the head temperature never exceeded 31 °C. The remainder of the solvent was removed with a stream of dry nitrogen just before use in the next step. Counting of duplicate aliquots during the next step established recovery of 15.5 mCi from the oxidation (radiochemical yield 78% from [1-^{3}H]-chrysanthemol).

(1*S*,1′*R*,3′*R*)-[1-^{3}H]Chrysanthemol ([1-^{3}H]-1-OH). The pentane solution (5 ml) of [^{3}H]aldehyde was concentrated with a stream of dry nitrogen until the flask was no longer cold, and 0.25 ml of absolute ethanol was added. A solution of 25.1 mg of crystalline horse liver alcohol dehydrogenase (liver alcohol: NAD oxidoreductase, E.C. 1.1.1.1., Worthington Biochemical Corp.; assayed at 2 units/mg) and 5.8 mg (8.1 μmol) of reduced nicotinamide adenine dinucleotide (NADH, Sigma Chemical Co.) in 25 ml of 0.01 M potassium phosphate buffer, pH 7.0, was added, and the homogeneous solution was swirled gently. Radio-TLC analysis (silica gel G, 15% 2-propanol/heptane; aldehyde R_f 0.63, alcohol R_f 0.49) after 2 h showed reduction to be 70% complete. Another 2 mg each of enzyme and cofactor were added along with 0.25 ml of ethanol, and the solution was allowed to stand with occasional swirling for an additional 6 h.

The reaction mixture was divided equally between two 50-ml centrifuge tubes. Each tube was saturated with sodium chloride and extracted with three 4-ml portions of Skellysolve. The aqueous layers could not be deproteinated with trichloroacetic acid due to the acid sensitivity of the product alcohol, and the presence of protein caused extensive emulsification after each extraction, which was cleared by centrifugation for 10 min at 3000 rpm in a Model HN-S centrifuge (International Equipment Co.) equipped with a swinging bucket rotor. The combined organic layers were washed in two portions with 2 ml of water before drying over anhydrous granular sodium sulfate. Counting of duplicate aliquots established recovery of 14.3 mCi of radioactivity (radiochemical yield 92%), and the product was homogeneous by TLC (same system used for following the reaction), migrating with authentic (1′*R*,3′*R*)-chrysanthemol, co-spotted as standard. Cold (1′*R*,3′*R*)-chrysanthemol (200 mg) was added, and solvent was evaporated under nitrogen just before the next step.

4-[(1*S*,1′*R*,3′*R*)-[1-^{3}H]Chrysanthemyloxy]pyridine ([1-^{3}H]-1-OPyI). Sodium dimsylate was prepared from 81 mg (3.38 mmol) of sodium hydride (Alfa-Ventron; obtained by washing 162 mg of 50% oil dispersion with dry hexane) and 10 ml of dry dimethyl sulfoxide. The mixture was heated to 65 °C with stirring until a homogeneous dark green solution was obtained (2.5 h). A solution of (1*S*,1′*R*,3′*R*)-[1-^{3}H]chrysanthemol (270 mg, 1.75 mmol; 14.3 mCi) in 5 ml of dry tetrahydrofuran (along with 5 ml of Me_2SO used to rinse the flask) was added over 10 min. The reaction mixture (now dark brown) was stirred at 65 °C for 2 h before 305 mg (2.69 mmol, 50% excess) of freshly prepared 4-chloropyridine in 5 ml of dry Me_2SO was added over 5 min. The reaction mixture was then stirred at 65 °C under nitrogen for 8 h.

Radio-TLC (silica gel G, ethyl acetate) of a 1-μl sample washed into water and then extracted into pentane showed 97% of the radioactivity to be associated with the alkoxypyridine as shown by comparison with authentic alkoxypyridine (R_f 0.52) co-spotted as standard and only 1.3% with authentic (1′*R*,3′*R*)-chrysanthemol (R_f 0.85). Furthermore, no unreacted alcohol was visible on a separate chromatogram visualized with iodine vapor. The reaction mixture was allowed to cool to room temperature and then divided equally between two 50-ml test tubes, each containing 10 ml of distilled water. The yellow aqueous emulsions were extracted with 5-ml portions of pentane until the extracts were colorless (four extractions were required), and the combined organic extracts were dried (sodium sulfate) and evaporated under nitrogen to give a clear golden oil which was used immediately in the next step.

A parallel reaction with 250 mg of radioinactive material was run under identical conditions (using 4 equiv of sodium hydride) as a control and afforded a 90% yield of a golden oil whose NMR and IR spectra were identical with those previously reported.[7d]

***N*-Methyl-4-[(1*S*,1′*R*,3′*R*)-[1-^{3}H]chrysanthemyloxy]pyridinium Iodide ([1-^{3}H]-1-OPyI).** The radiolabeled alkoxypyridine was treated with 1.12 g (7.9 mmol, 4.5 equiv) of iodomethane (MCB, reagent) in a stoppered test tube. The rapid exothermic reaction was moderated by the use of a water bath. The mixture began darkening within 30 s and was a familiar dark green after 5 min. The reaction mixture was allowed to stand at ambient temperature for 24 h and was then placed in an ice bath for an additional 12 h, during which crystallization occurred.

Solvolysis of *N*-Methyl-4-[(1*S*,1′*R*,3′*R*)-[1-^{3}H]chrysanthemyloxy]pyridinium Iodide ([1-^{3}H]-1-OPyI). The partially crystallized green-brown oil containing the pyridinium iodide was dissolved in 50 ml of distilled water containing 3.27 g (38.9 mmol, 22 equiv) of sodium bicarbonate (Mallinckrodt USP) by washing the reaction tube with the solution in several portions. The tan solution was sealed in a 50-ml round-bottom flask with a standard taper stopper and Parafilm, and the flask was clamped in a 25.0 °C water bath for 32.5 h (7.1 half-lives,[7d] 99.2% reaction). The resulting solution was divided equally among three 50-ml test tubes. Sodium chloride was added until saturation, and each portion was extracted with 5-ml portions of pentane until the extracts were colorless (four extractions). The combined extracts were dried (sodium sulfate) and concentrated to 10 ml volume with a stream of dry nitrogen. A 1-μl sample was diluted 100-fold with carbon disulfide (Mallinckrodt SpectrAR), and 0.3 μl (0.75 μg of products, approximately 3700 cpm) was analyzed by GLC (500 ft Carbowax 20M, 150 °C). The major products were identified by coinjection with authentic material.[7d]

2-(2′-Methylpropen-1′-yl)-1,3-dithiane (12).[17] A solution of 22.52 g (0.268 mol) of freshly distilled 3-methyl-2-butenal in 15 ml of anhydrous ether was cooled to 0 °C. 1,3-Propanedithiol (30.40 g, 0.282 mol, 5% excess) was added, and the resulting cloudy mixture was stirred for 10 min before boron trifluoride-diethyl ether complex (19.66 g, 0.139 mol, 0.5 equiv) was added dropwise over 10 min, beginning carefully with 5-drop portions which initially caused bubbling and self-reflux. The ice bath was removed and the cloudy yellow reaction mixture allowed to stir for 30 min at ambient temperature, during which time the initially formed water droplets disappeared. The pale-yellow reaction mixture was poured into 100 ml of 10% aqueous sodium hydroxide and extracted with three 100-ml portions of chloroform. The combined organic layers were washed with 100 ml each of water and brine, dried over anhydrous magnesium sulfate, and solvent was removed at reduced pressure to give 45.05 g (97% crude) of a slightly cloudy, pale-yellow, offensive-smelling liquid. Short-path distillation afforded 42.05 g (90%) of clear, light-yellow liquid, bp 84–86 °C (0.2 mm), which NMR spectroscopy indicated was greater than 96% pure. The sample crystallized after being allowed to stand for 4 weeks at −15 °C, mp 41–43 °C (open capillary). The crystals were removed by filtration with suction, dried on the fritted glass filter, and used in the next step of the synthetic sequence. NMR of the crystalline material was identical with that of the liquid except for the improved purity of the former; NMR (CCl_4) 1.72 (6, d, J = 1.5 Hz, allylic methyls), 1.82–2.15 (2, m, J = 4 Hz, H at C_5), 2.65–2.98 (4, m, H at C_4 and C_6), 4.67 (1, d, J = 10 Hz, H at C_2), and 5.02 ppm (1, d of m, J = 10 Hz, J = 1.5 Hz, olefinic H at $C_{1'}$); IR (neat) 2950 sh, 1665 sh, 1445, 1425, 1378, 1278 sh, 1170 brd, 1110, 1032, 908, 855, 812, and 757 s cm^{-1}.

Standardization of *n*-Butyllithium Solutions. All manipulations of open containers of *n*-butyllithium were performed in a phosphorus pentoxide-charged drybox under an atmosphere of dry nitrogen. *n*-Butyllithium in hydrocarbon (100 g, 90% in hydrocarbon, Alfa Inorganics) was diluted by pouring approximately one-half of the thick slurry into each of two oven-dried pint bottles half-filled with dry hexane (Mallinckrodt AR, dried over sodium wire) and then completely filling each container with dry hexane. Each solution was

standardized using the procedure of Watson and Eastham,[18] as follows: a 5.00-ml aliquot of *n*-butyllithium solution was carefully pipetted into a 125-ml Erlenmeyer flask containing 20 ml of benzene (Mallinckrodt AR, dried over 3A molecular sieves) and approximately 10 mg of 1,10-phenanthroline (G. F. Smith Chemical Co.; monohydrate dried in vacuo over phosphorus pentoxide). A bright rust-orange color formed instantly and the flask was then sealed with Parafilm and removed from the drybox. The flask was quickly unsealed and restoppered with a two-hole rubber stopper fitted with a 10-ml buret and a dry nitrogen inlet. The magnetically stirred solution was then titrated with a 1.00 M solution of 2-butanol in xylene (both dried overnight over 3A molecular sieves), the end point being the *sudden* fading of the orange color to a pale yellow. Titration of several aliquots of each sample, using both dehydrated indicator and commercial material without drying showed that: (1) the analytical method is reproducible to better than ±4%; and (2) the 0.67% of lithium alkyl hydrolyzed by water of hydration in the indicator is insignificant compared with the other sources of experimental error.

2,7-Dimethylocta-2,6-dien-4-one Propylene Dithioacetal (13). Glassware and stir bar were dried overnight at 110 °C, assembled hot with foil spacers, and allowed to cool under dry nitrogen. Freshly distilled 2-(2′-methylpropen-1′-yl)-1,3-dithiane (20.1 g, 0.115 mol) was dissolved in 100 ml of dry tetrahydrofuran and cooled to −40 °C (acetone–ice–dry ice). *n*-Butyllithium in hydrocarbon (66 ml of 1.8 M solution, 0.118 mol, 3% excess) was added over 20 min, accompanied by formation of a bright-yellow solution.

The reaction mixture was then allowed to stir for 1 h at −25 °C before sampling for NMR analysis, as follows. A 1-ml aliquot was withdrawn by syringe and injected into 3 ml of deuterium oxide (Stohler Isotopic Chemicals, 99.8% D). The two-phase mixture was shaken well before extracting with two 5-ml portions of pentane, and the combined organic layers were filtered through anhydrous granular sodium sulfate and evaporated at reduced pressure to give 70 mg of a clear colorless oil. NMR analysis indicated that only 5% of the proton at C(2) had not been replaced by deuterium, representing 95% formation of the lithio anion. Accordingly, the doublet of septets for the olefinic proton at C(1′) was reduced to a single multiplet at 5.25 ppm. Sampling, workup, and NMR analysis again after 2 h at −25 °C revealed complete anion formation.

The reaction mixture was then cooled to −78 °C, and 17.12 g (0.115 mol, 1 equiv) of 1-bromo-3-methyl-2-butene was added by syringe over 5 min, causing the highly colored solution to fade to a pale yellow. The reaction mixture was allowed to stir at −78 °C and checked by TLC at 1-h intervals, as follows: A 0.1-ml aliquot was withdrawn by syringe, injected into 0.5 ml of water, and extracted with 0.5 ml of pentane. The upper layer was drawn off and 2 μl thereof spotted on silica gel sheets which were developed with 1:1 chloroform/heptane and visualized by 254 nm light and by iodine staining. TLC showed essentially complete reaction from 2 h with no change thereafter (R_f's: product, 0.45; starting dithiane, 0.54). The reaction vessel was stoppered and placed (still in the −78 °C bath) in the freezer (−15 °C) for 14 hr. The pale-yellow reaction mixture was then poured into 500 ml of distilled water and the two-phase mixture extracted with three 200-ml portions of pentane. The combined organic layers were washed in succession with 200-ml portions of water, 7% aqueous potassium hydroxide (twice), and brine before drying over magnesium sulfate. Solvent was removed at reduced pressure to give 24.89 g (89% crude) of a pale-yellow oil. Short-path distillation afforded 22.82 g (82%) of material, bp 94–96 °C (0.1 mm), which was 98% pure by NMR. Analytical samples were collected by GLC (3 ft × 0.25 in. stainless steel, 10% [95% OV-101/5% Igepal CO-880] on 60/80 mesh Anakrom ABS, 180 °C) for spectra and microanalysis: NMR (CCl_4) 1.70 (2, d, C_5 methylene of dithiane ring), 1.73 (6, d, J = 1.4 Hz, allylic methyls at C_7), 1.97 (6, d, J = 1.2 Hz, allylic methyls at C_2), 2.73 (6, m, protons at C_4 and C_6 of dithiane ring and at C_5), 5.13 (1, t of septets, ^3J = 6.60 Hz, ^4J = 1.4 Hz, olefinic H at C_6), and 5.27 ppm (1, septet, ^4J = 1.2 Hz, olefinic H at C_3); IR (neat) 2950, 1650, 1445 s, 1380 s, 1275 sh, 1105, 905, 868, 834, and 810 cm^{-1}.

Anal. $C_{13}H_{22}S_2$: C, H.

2,7-Dimethylocta-2,6-dien-4-one (14). A 19.8-g sample (81.8 mmol) of the propylene dithioacetal was dissolved in 400 ml of 95% ethanol and warmed to 40 °C. A 5% excess of silver nitrate (29.19 g, 172 mmol; Eastman) in 100 ml of deionized water was added over 10 min, causing the formation of a heavy yellow precipitate within 5 min. The reaction mixture was allowed to stir for 2 h at 40 °C, filtered, and the filtrate poured into 1 l. of saturated aqueous sodium chloride solution. The resulting suspension was extracted with three 250-ml portions of pentane, and the combined organic layers were washed with three 200-ml portions of saturated brine before drying over anhydrous magnesium sulfate. Solvent was removed at reduced pressure to give 5.35 g (43% crude) of a fragrant yellow liquid. Short-path distillation afforded 3.78 g (30%) of a colorless liquid, bp 42–43 °C (0.15 mm), which GLC (500 ft Carbowax 20M, 150 °C) showed to be 94% pure. Analytical samples were collected by GLC (Carbowax 20M, 125 °C) to give material of 95% purity (500 ft Carbowax 20M, 140 °C): NMR (CCl_4) 1.61 and 1.72 (6, pr of d, $J_{1,3}$ = 1.2 Hz, methyls at C_7), 1.82 and 2.07 (6, pr of d, J = 1.6 Hz, methyls at C_2), 2.93 (2, brd d, J = 7.2 Hz, H at C_5), 5.18 (1, t of septets, J = 7.2 Hz, J = 1.2 Hz, H at C_6), and 5.85 ppm (1 septet, J = 1.6 Hz, H at C_3); IR (neat) 2950, 1690s, 1625 s, 1445 s, 1380, 1300 w, 1208 w, 1110, 1013 w, 842 w, 827, and 709 cm^{-1}.

2,7-Dimethylocta-2,6-dien-4-ol (7-OH). A suspension of 0.320 g (8.42 mmol, 33.7 mequiv) of lithium aluminum hydride (Alfa-Ventron) in 25 ml of anhydrous ether was cooled to −10 °C in an ice–methanol bath before 3.27 g (21.5 mmol, 50% excess hydride) of 2,7-dimethylocta-2,6-dien-4-one in 40 ml of dry ether was added over 30 min. The reduction was followed by TLC (silica gel G, 15% ethyl acetate–benzene) to guard against reduction of the conjugated double bond. Reduction was about 60% complete after 2 h at −10 °C, and at that time an additional 0.103 g (for a total of 11.1 mmol, 2 equiv) of lithium aluminum hydride was added along with 10 ml of dry ether, and stirring was allowed to continue for 1 h at −10 °C. TLC analysis then revealed only a trace of starting material. The bath was removed for 10 min before excess hydride was quenched, and the salts hydrolyzed by dropwise addition of 2.90 ml of saturated aqueous sodium chloride. The mixture was allowed to stir vigorously until the inorganic salts precipitated cleanly. The mixture was filtered by suction, the filter cake washed with ether, and the filtrate was dried (magnesium sulfate) and evaporated at reduced pressure to afford 3.31 g (100%) of a nearly colorless liquid which GLC (500 ft Carbowax 20M, 150 °C) showed to be 95% pure, containing less than 1% of unreduced ketone. NMR and IR spectra were identical with those previously reported.[5b]

Isolation of [5-^{3}H]-2,7-Dimethylocta-2,6-dien-4-ol ([5-^{3}H]-7-OH). Unlabeled 2,7-dimethylocta-2,6-dien-4-ol (75 μl, 68 mg) was added to the products, from solvolysis of [1-^{3}H]-**1**-OPyl, and the pentane solution was concentrated to ca. 1 ml. The three major components were collected by preparative GLC (Carbowax 20M, 139 °C) giving radiolabeled yomogi, artemisia, and 7-OH alcohols (78.0, 7.7, and 18.3 mg, respectively; 30% recovery).

[5-^{3}H]-2,7-Dimethylocta-2,6-dien-4-yl Acetate ([5-^{3}H]-7-OAc). Additional carrier (18 mg) was added to the Halovka collector containing the GLC purified sample of [5-^{3}H]-7-OH, and the contents were transferred by syringe (with several 50 μl rinses of dry pyridine) to a 5-ml pear-shaped flask. Two equivalents of acetic anhydride (48.9 mg, 475 μmol; MCB, 99%) in 0.5 ml of dry pyridine were added, and the colorless solution was allowed to stir at ambient temperature for 24 h. Control experiments had shown the reaction to be essentially complete in 12 h under these conditions.

The reaction mixture was diluted with 1 g of ice and 0.5 ml of water, acidified to pH 2 with 3 N hydrochloric acid, transferred to a 12-ml test tube, and extracted with four 2-ml portions of ether. The combined extracts were similarly washed with 2-ml portions of 1 N hydrochloric acid, saturated aqueous sodium bicarbonate (twice), and water before drying over anhydrous granular sodium sulfate. Duplicate aliquots taken for counting assayed the total radioactivity as 16.5 μCi (0.115% radiochemical yield from (1*S*,1′*R*,3′*R*)-[1-^{3}H]chrysanthemol). GLC analysis (500 ft Carbowax 20M, 145 °C) of a fivefold-diluted sample showed the acetate to be 94% pure, containing only 2% unreacted alcohol. Aliquots of the two aqueous solutions were counted to determine that more than 99% of the radioactivity was recovered in the ether extracts.

A sample for characterization was prepared from unlabeled alcohol using the same procedure: NMR (CCl_4) 1.62 and 1.68 (12, pr of brd s, allylic methyls), 1.93 (3, s, acetoxy methyl), 2.15 (2, m, H at C_5), 4.67–5.20 (2, m, olefinic H), and 5.33 ppm (1, d of d, $J_{3,4}$ = 9.0 Hz, $J_{4,5}$ = 6.5 Hz, H at C_4); IR (neat) 3000, 2950, 1735s, 1660w, 1445, 1385, 1240s, 1128, 1105, 1048, 1018, 954, and 840 cm^{-1}.

(*S*)-Malic Acid ([3-^{3}H]-8-OH). Optically active carrier material was purified by recrystallization of the commercial product (Aldrich, 99%) from acetone and from acetone–chloroform to constant melting point (103–104 °C); $[\alpha]^{20}D$ −1.53° (*c* 9.68, H_2O), (lit. mp 100.5 °C, $[\alpha]^{20}D$

−1.63 (H_2O), R_f 0.50 (silica gel G, 7:1 moist ether/formic acid) and 0.55 (cellulose, 7:2:1 ether/formic acid/water).

(*S*)-Malic Acetate (8-OAc). An authentic sample for characterization and use as a standard was prepared from recrystallized (*S*)-malic acid by the literature procedure[19] and recrystallized from acetone/chloroform: mp 136–137 °C (lit.[19] 134–135 °C); R_f 0.75 (silica gel G, 7:1 moist ether/formic acid); NMR ($CDCl_3/Me_2SO$-d_6) 2.11 (3, s, acetoxy methyl), 2.88 (2, d, $J_{2,3}$ = 6 Hz, H at C_3), 5.44 (1, t, H at C_2), and 11.19 ppm (2, brd, carboxyl H).

[3-^{3}H]Malic Acetate ([3-^{3}H]-8-OAc). The dried ether solution of dienyl acetate, (see preparation of [5-^{3}H]-7-OAc) was transferred to a graduated 12-ml centrifuge tube and concentrated slowly with a stream of dry nitrogen. Three 2-ml pentane washes of the drying agent were sequentially added, and the total volume was slowly reduced to 0.5 ml before 7.5 ml of ethyl chloride (Eastman Kodak; prechilled in an ice bath to allow handling) was added. The solution was cooled to −78 °C (dry ice–2-propanol), and a stream of 2% ozone in oxygen (generated with a Welsbach T-23 ozonizer operating at 110 V and 6 psi of dry oxygen) was bubbled into the solution until the deep-blue color of excess ozone appeared (20 s). The solution was quickly swirled to insure the persistence of the color and then subjected to ozone for another 10 s. The deep-blue solution was allowed to stand at −78 °C for 5 min before excess ozone was removed at that temperature by bubbling a stream of dry nitrogen through the solution until the blue color had disappeared. The solution was allowed to warm to room temperature to insure complete rearrangement to final ozonide, and ethyl chloride was evaporated under nitrogen. The white residue was dissolved in 0.35 ml each of glacial acetic acid and 30% aqueous hydrogen peroxide and allowed to stand for 10 min. The tube equipped with a magnetic stirrer and a one-hole stopper was connected to a U-tube bubbler (to allow escape of evolved gases) and then heated to 70 °C in a hot water bath. Hydrogen peroxide additions of 0.2 ml were made after 1 h and 4 h of stirring at 70 °C.

The oxidative workup was followed by TLC (silica gel sheets developed with 7:1 moist ether/formic acid), counting bands located by visualizing a lane containing malic acid (R_f 0.50) and malic acetate (R_f 0.75) standards with bromocresol green spray. Stirring at 70 °C was continued until the percentage of radioactivity associated with malic acetate standard reached a constant value (35% of total counts after 6 h) for a total of 7.5 h. Excess peroxide was destroyed by addition with vigorous stirring of 10-mg portions of manganese dioxide (Mallinckrodt, reagent) until effervescence ceased and the gray powder remained unconsumed (required five additions and 30 min). The aqueous solution and associated solids were transferred with three 1-ml water washes to a 10-ml recovery flask, acidified to pH 4 with 0.5 N sulfuric acid, and evaporated to dryness at reduced pressure. The tan residue was dissolved in 4 ml of water to give a brown suspension which was again taken to dryness. The tan solid was dissolved in 4 ml of water and extracted continuously with 25 ml of ether for 28 h. The ether extract was found to contain 4.59 μCi of radioactivity (28% of the activity in the starting acetate) with 0.39 μCi (2.4% of the total) retained by the aqueous solution. The extract was dried over anhydrous granular sodium sulfate and concentrated with a dry nitrogen stream just before use in the next step.

(2*S*)-[3-^{3}H]Malic Acid ([3-^{3}H]-8-OH). The dried ether extract, along with three 5-ml ether washings of the drying agent, was concentrated to 0.5 ml in a 12-ml conical tube equipped with magnetic stirring. Two ml of ethanolic ammonium hydroxide (pH 9) was added, the tube was stoppered, and the clear solution was allowed to stir at ambient temperature for 24 h. The solution was acidified, the solvent was evaporated with a gentle stream of dry nitrogen directed into the tube overnight, and the residue was gently heated in 5 ml of ethyl acetate (Mallinckrodt AR) to give a cloudy solution which was clarified by drying (sodium sulfate). The solution was transferred with two 2-ml washes to a weighed 12-ml conical tube containing 11.18 mg of (*S*)-malic acid as carrier. The reaction tube with drying agent was vortexed with 1.0 ml of acetone and duplicate 10-μl aliquots were counted, finding 1.5 × 10^5 dpm remaining or 1.5% of the total radioactivity recovered from the ozonolysis. The crude carrier-diluted malic acid contained 0.548 μCi of radioactivity (3.3% from [5-^{3}H]-7-OAc).

The ethyl acetate solution was gently heated to clarity in a hot water bath, triturated to cloudiness with pentane, reclarified by careful heating, and placed in ice. Feathery white crystals appeared on the walls of the tube within 30 min and were allowed to grow at 5 °C overnight. The mother liquor was removed with a disposable pipet to a weighed tube and evaporated to dryness. Both tubes were evacuated at 200 μ for 30 min on a lyophilizer, weighed, and the contents of each dissolved in 2.0 ml of acetone (Mallinckrodt AR). Duplicate aliquots of each were counted in the ethanol/formic acid cocktail to establish initial specific activity (Table I) and first radiochemical yield (23%, from the crude hydrolysate). Crystallization from ethyl acetate–pentane was carried out twice more in the same manner, recovering 91 and 98% of the initial specific activity. The radiochemically pure malic acid was identified by comparison with authentic material on two TLC systems (silica gel G, 7:1 moist ether/formic acid, R_f 0.50; cellulose, 7:2:1 ether/formic acid/water, R_f 0.55). The crystalline material finally obtained represented 61% of the activity found in the first crystals, 14% of the ^{3}H recovered from the ammonolysis reaction, and 0.47% of the radioactivity in [5-^{3}H]-7-OAc.

Equilibration of (2*S*)-[3-^{3}H]Malic Acid ([3-^{3}H]-8-OH) with Fumarase. Enzymatic assays were performed and incubations were followed spectrophotometrically. L-Malate hydrolyase (E.C. 4.2.1.2, fumarase) was obtained from Sigma Chemical Co. as a crystalline suspension in 3.2 M ammonium sulfate solution. Stock solutions of enzyme were prepared by pipetting aliquots of the suspension into 2.0-ml portions of 0.1 M potassium phosphate buffer, pH 7.4, and were assayed by the literature procedure[8] (modified for maximum sensitivity as recommended by the supplier). Stock solutions containing 1.5, 5, 15, and 40 units of enzyme per milliliter were prepared and stored at 5 °C for up to 72 h.

A stock solution of labeled malic acid was prepared by adding 1.0 ml of water to the crystalline acid after evaporation of acetone. Duplicate 25 μl aliquots were taken for counting, with one placed directly into a counting vial and the other in 1.0 ml of 0.1 M potassium phosphate buffer in a 12-ml ignition tube, to serve as a control for the enzymic incubations. The control sample was lyophilized, redissolved in 0.5 ml of deionized water and relyophilized (both cycles at 200 μ pressure). The freeze-dried residue was transferred to a counting vial with three 0.3-ml washes of deionized water and counted in the Triton cocktail. Both samples were counted for three 10-min periods and found to contain 4441 and 4453 dpm, respectively (ratio 0.997). The mean value of 4447 dpm was utilized for subsequent calculations.

The results of multiple incubations of the (2*S*)-[3-^{3}H]malate are listed in Table II. Each sample consisted of 25 μl of substrate solution in 1.0 ml of phosphate buffer with 0.2 ml of the appropriate enzyme stock solution (3 units of enzyme) added at time zero. The first sample of each run was equilibrated in the spectrophotometer to allow determination of the equilibrium point (indicated by constant A_{240}). The remaining samples were clamped in the same bath used to thermostat the cuvette holder. The samples were lyophilized after the indicated times in exactly the same way as the blank sample (2 cycles at 200 μ) and counted in the aqueous Triton cocktail.

Decarboxylation of (2*S*,3*S*)-[3-^{3}H]Malic Acid ([3-^{3}H]-8-OH). The optical purity at C(2) of the radioactive malate was checked by conversion to pyruvate with malic enzyme (L-malate: NADP oxidoreductase (decarboxylating), E.C. 1.1.1.40; obtained from Sigma Chemical Co.). Each run consisted of 25 μl of malate stock solution (the same used for the fumarase experiments; 1.35 μmol), 60 μmol of NADP (Sigma), and 30 μl of enzyme solution (0.03 unit) in a measured amount of 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM mercaptoethanol and 4 mM magnesium ion. A concentrated sample was prepared by adding the above components (except enzyme) to 1 ml of buffer in a quartz cuvette and equilibrating the solution to 26 °C. The reaction was started by adding the enzyme. After 15 min the absorbance at 340 nm (NADPH) had increased from 0.32 to a stabilized value at 2.65. A dilute sample was run in the same way except in 3 ml of buffer solution. After 15 min the A_{340} of this sample had increased from 0.24 to 1.85. A blank sample was prepared identically with the first run except enzyme was omitted.

Each sample was then freeze-dried, acidified with 3 drops of concentrated hydrochloric acid, and washed with three 1-ml portions of acetone. The acetone washes were combined and evaporated, and the residue was washed with two 1-ml portions of acetone. The final washes were evaporated, and the residue taken up in 25 μl of acetone and spotted on a cellulose thin-layer plate. After development with 7:2:1 ether/formic acid/water, the region between the origin and the solvent front was divided into ten equal zones and each was scraped into a counting vial containing 0.7 ml of ethanol and 0.07 ml of formic acid. Cold pyruvate (R_f 0.86) and malate (R_f 0.62) were chromatographed under similar conditions and visualized with bromocresol green. Radioactivity peaked between R_f 0.60–0.70 in the blank and

between R_f 0.80–0.90 in the concentrated and dilute runs with enzyme. Although recovery of total counts after scraping was only ca. 25%, starting material and product were well separated on the TLC plate and at least 85% of the radioactivity migrated with pyruvic acid.

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References and Notes

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Communications to the Editor

Activation through Impregnation. Permanganate-Coated Solid Supports[1]

Sir:

Although potassium permanganate is a powerful oxidizing agent, its utility in organic synthesis has been severely limited by solubility problems.[2] One recent development that has significantly expanded the scope of this reagent was the demonstration that $KMnO_4$ forms a stable complex with dicyclohexyl-18-crown-6 which is soluble in benzene and reactive toward a variety of organic substrates.[3] In the absence of the crown ether, potassium permanganate has no detectable solubility in benzene and is unreactive.

We have discovered that potassium permanganate can be activated for certain oxidations in benzene by the very simple process of impregnation onto inorganic supports such as Linde Molecular Sieves, silica gel, and certain clays and now wish to report preliminary results obtained for alcohol oxidations. The following procedure has been used to prepare sieve/$KMnO_4$ reagents. A 2-L round-bottomed flask was charged with 500 mL of 0.06 M aqueous potassium permanganate and 20 g of Linde 13X Molecular Sieves ($\frac{1}{16}$-in. pellets)[4] was added to it in one portion.[5] The flask was then transferred to a rotary evaporator and water was removed under reduced pressure.[6] The coated pellets were separated physically from nonadsorbed potassium permanganate by screening (20 mesh). Analysis of the reagent revealed a loading of 0.27 mmol of $KMnO_4$/g of reagent.[7]

When 15.0 g of sieve/$KMnO_4$ was suspended in 20 mL of freshly distilled benzene containing 0.250 g (1.36 mmol) of cyclododecanol and the resulting mixture heated to 70 °C for 1.5 h, analysis of the liquid phase by GLC indicated complete conversion to cyclododecanone. The ketone was isolated by filtering the product mixture through Celite, washing the pellets with 70 mL of benzene, and removing the solvent from the combined filtrate under reduced pressure yielding 0.226 g (90%) of cyclododecanone as a colorless solid which melted at 56–59 °C.[8] The infrared and NMR spectra were identical with those of an authentic sample. Control experiments carried out in which sieve/$KMnO_4$ was replaced by either reagent grade potassium permanganate[9] or an aqueous potassium permanganate solution (organic–aqueous phase oxidation) showed no loss of alcohol in the organic phase after heating for 50 h at 70 °C. We have also found that potassium permanganate can be activated by impregnation onto various other supports; our results are summarized in Table I.[10]

Most of our work has been done with molecular sieves in pelleted form in order that nonadsorbed permanganate could be removed and thereby make analysis of loading and reactivity possible. Comparison of loading with oxidizing capacity for reagents **1–6** indicates reasonably efficient utilization of impregnated permanganate.[11] On the basis of loading, oxidizing capacity, and reactivity, Linde 13X Molecular Sieves ($\frac{1}{16}$-in. pellets) appear optimal as a support system. Other useful reagents which were derived from inorganic support material but which could not be analyzed for adsorbed permanganate are listed in Table I (reagents **7–11**).

Further examples of oxidations by a sieve/$KMnO_4$ are illustrated in Table II. In general, high yields of ketones and modest yields of aldehydes can be obtained.[12] Although we are still exploring the scope of sieve/$KMnO_4$-based oxidations, our results thus far indicate the above procedure to be com-