mL of this chloroform solution was extracted during 5 min with an equal volume of aqueous 0.2 M NaBr (pH 7.0). The pH of the aqueous phase was readjusted to 7.0. The flask was shaken again for 5 more min. At the end of the back-extraction, the nucleotide concentration in chloroform was measured again by UV spectroscopy to calculate the extraction yield. UV absorption of the nucleotide bases in chloroform: ATP λ_{max} 260 nm (ϵ 1.3 $\times 10^4$ L mol^{-1} cm^{-1}); CTP λ_{max} 275 nm (ϵ 6.6 $\times 10^3$ L mol^{-1} cm^{-1}); ddTTP and AZTTP λ_{max} 268 nm (ϵ 8.4 $\times 10^3$ L mol^{-1} cm^{-1}). The extraction yields in Table II are averages of triplicate runs.

Acknowledgment. We thank the National Institutes of Health (Grant GC-R-123558) for the support of this work.

Trichodiene Synthase. Synergistic Inhibition by Inorganic Pyrophosphate and Aza Analogs of the Bisabolyl Cation

David E. Cane* and Guohan Yang

Department of Chemistry, Box H, Brown University, Providence, Rhode Island 02912

Robert M. Coates* and Hyung-Jung Pyun

School of Chemical Sciences, 1209 West California Street, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Thomas M. Hohn

Mycotoxin Research Unit, USDA/ARS, National Center for Agricultural Utilization Research, Peoria, Illinois 61604

Received January 13, 1992

A series of aza analogs of the bisabolyl and α-terpinyl cations were tested as inhibitors of the sesquiterpene cyclase, trichodiene synthase. Both (*R*)- and (*S*)-**16** and (*R*)- and (*S*)-**13** as well as trimethylamine were only weak inhibitors when incubated alone. In the presence of inorganic pyrophosphate, itself a known competitive inhibitor of trichodiene synthase, all five amines showed strong cooperative competitive inhibition with an enhancement factor estimated to be 10–40. The apparent induced inhibition constant αK_J decreased in going from trimethylamine to the monoterpene analogs **13** and was strongest for the sesquiterpene analogs **16**, indicating that both electrostatic and hydrophobic interactions are important in the binding of each intermediate analog. The cyclase showed little discrimination, however, between the individual enantiomers of each inhibitor.

Trichodiene synthase catalyzes the cyclization of farnesyl diphosphate (**1**) (FPP) to trichodiene (**4**),[1] the sesquiterpene hydrocarbon precursor of the trichothecenes, a large family of antibiotic metabolites produced by several genera of phytopathogenic fungi as well as the higher plant genus *Baccharis*.[2] The presence in contaminated grain of trichothecenes, which are potent inhibitors of protein synthesis, has been associated with serious incidents of mycotoxicoses. Trichodiene synthase has been isolated from a variety of fungal sources including *Trichothecium roseum*,[1,3] *Gibberella pulicaris*,[4] and *Fusarium sporotrichioides*.[5] The *F. sporotrichioides* cyclase has been purified to homogeneity and shown to be a homodimer, M_r 90 000. Cyclization of the acyclic substrate *trans,trans*-FPP requires no cofactors other than the divalent metal cation Mg^{2+}, in common with the majority of known terpene cyclases. The calculated k_{cat} of 0.15 s^{-1}, while modest, is typical of such cyclases, and well in excess of the competing Mg^{2+}-catalyzed solvolysis of the allylic substrate. The trichodiene synthase genes of both *F. sporotrichioides* and *G. pulicaris* have been cloned and sequenced, showing 89% homology at the nucleic acid level.[6,7] After deletion of a 60-nt intron, the trichodiene synthase gene has been expressed in *Escherichia coli* and the recombinant cyclase appears to be identical to the native enzyme in all respects.[8]

Extensive mechanistic and stereochemical studies have supported a cyclization mechanism, illustrated in Scheme I, in which *trans,trans*-FPP is initially isomerized to the corresponding tertiary allylic isomer, nerolidyl diphosphate (**2**) (NPP), which by rotation about the newly generated 2,3-single bond can adopt a conformation capable of cyclization to the bisabolyl cation (**3**).[1,9] Further cyclization, followed by a succession of well-documented hydride and methyl migrations, can then yield, after deprotonation, trichodiene. Although the isomerization and cyclization components of this mechanism are clearly distinct reactions, these transformations are believed to take place by

(1) Cane, D. E.; Ha, H.; Pargellis, C.; Waldmeier, F.; Swanson, S.; Murthy, P. P. N. *Bioorganic Chemistry* **1985**, *13*, 246. Cane, D. E.; Swanson, S.; Murthy, P. P. N. *J. Am. Chem. Soc.* **1981**, *103*, 2136.

(2) Grove, J. F. *Nat. Prod. Rep.* **1988**, *5*, 187. Tamm, C.; Breitenstein, W. In *The Biosynthesis of Mycotoxins*; Steyn, P. S., Ed.; Academic Press: New York, 1980; pp 69–104.

(3) Evans, R.; Holton, A. M.; Hanson, J. R. *J. Chem. Soc., Chem. Commun.* **1973**, 465.

(4) Hohn, T. M.; Beremand, M. N. *Appl. Environ. Microbiol.* **1989**, *55*, 1500.

(5) Hohn, T. M.; VanMiddlesworth, F. *Arch. Biochem. Biophys.* **1986**, *251*, 756.

(6) Hohn, T. M.; Beremand, P. D. *Gene* **1989**, *79*, 131.

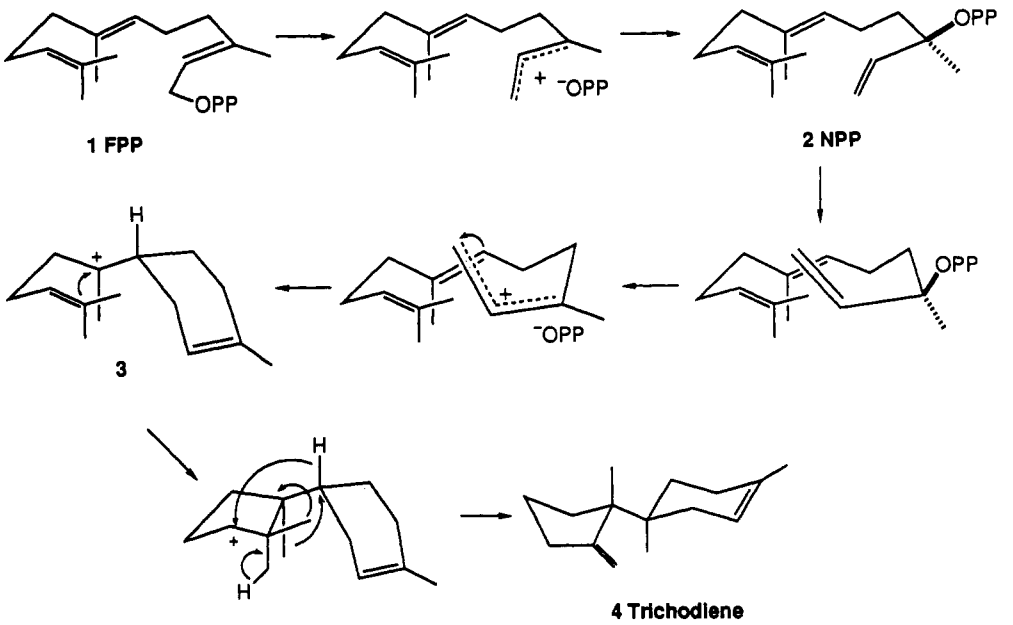
(7) Hohn, T. M.; Desjardins, A. E. *Mol. Plant-Microbe Interact.* In press.

(8) Hohn, T. M.; Plattner, R. D. *Arch. Biochem. Biophys.* **1989**, *275*, 92.

(9) Cane, D. E.; Ha, H. *J. Am. Chem. Soc.* **1986**, *108*, 3097. Cane, D. E.; Ha, H. *J. Am. Chem. Soc.* **1988**, *110*, 6865.

0022-3263/92/1957-3454$03.00/0

Scheme I



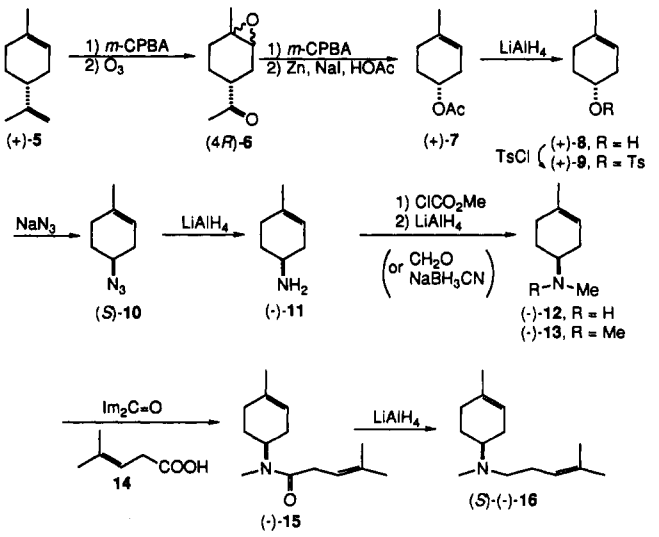
microscopically similar mechanisms, each involving the ionization of the relevant allylic diphosphate ester to the corresponding transoid or cisoid allylic cation–pyrophosphate anion pair. Competition experiments have established that (3*R*)-NPP is normally a tightly enzyme-bound intermediate of the overall conversion of FPP to trichodiene[9] and further insight into the otherwise cryptic isomerization step was obtained by analysis of the mixture of acyclic olefins and alcohols obtained upon incubation of the anomalous substrate 6,7-dihydrofarnesyl diphosphate with trichodiene synthase.[10]

The assertion that the proximal product of NPP cyclization is the bisabolyl cation rests on analogy to numerous chemical model reactions. For example, solvolysis of (+)-(3*R*)-nerolidol under a variety of conditions leads to formation of an enantiomeric excess of a mixture of α- and β-bisabolene isomers.[11] Moreover, the analogous solvolysis of linalyl esters results in enantiospecific formation of α-terpineol or its derivatives by way of an *anti-endo* transition state.[12] On the other hand, neither bisabolol nor any related derivatives are directly observable as free intermediates of the cyclization of NPP to trichodiene.[13] The problem, therefore, is to devise methods for the direct observation of the succession of cationic intermediates in the cyclization cascade or, failing that, to adduce supporting evidence for the existence of these chemically reasonable species. On a more general level, there is a need for strategies to elucidate the factors which govern the interaction between the active site of any cyclase and both the charged and lipophilic portions of the substrate and derived intermediates. As one approach to this problem, we have examined the role of ammonium ion analogs of the bisabolyl cation as inhibitors of trichodiene synthase.

Results

Both the *S* and *R* enantiomers of the aza analogs (16) of the bisabolyl cation were prepared from the corresponding primary amines, (*S*)-(−)-11 and its antipodal form, by an alternating sequence of acylations and hydride reductions (overall yield, 52–55%)[14] as shown in Scheme II. The related aza analogs of the α-terpinyl cation, (*S*)- and (*R*)-13, were obtained in one step by reductive methylation of (−)- and (+)-11 ($NaBH_3CN$, CH_2O, CH_3CN, 55–57%).[15,16] The primary amines were secured by S_N2 displacements of the enantiomerically pure, crystalline tosylates (*R*)-(+)- and (*S*)-(−)-9 with azide ion (NaN_3, DMF, 50 °C) followed by $LiAlH_4$ reduction (73–75% from 9).

Scheme II



The previously unknown enantiomers of 4-methyl-3-cyclohexenol (8) were synthesized by 5-step degradations of (*R*)-(+)- and (*S*)-(−)-limonene (5) (overall yield,

(10) Cane, D. E.; Pawlak, J. L.; Horak, R. M.; Hohn, T. M. *Biochemistry* **1990**, *29*, 5476.
(11) Andersen, N. H.; Syrdal, D. D. *Tetrahedron Lett.* **1972**, 2455.
(12) Stephan, K. *J. Prakt. Chem.* **1898**, *58*, 109. Gotfredsen, S.; Obrecht, J. P.; Arigoni, D. *Chimia* **1977**, *31*(2), 62.
(13) Forrester, J. M.; Money, T. *Can. J. Chem.* **1972**, *50*, 3310.
(14) Attempts to introduce the 4-methyl-3-pentenyl side chain first were unsuccessful. Although acylation of 11 occurred smoothly by the same procedure used for 12, $LiAlH_4$ reduction of the resulting secondary amide was very slow and inefficient, presumably for solubility reasons. Attempts to effect N-methylation of the secondary amide were similarly unproductive.
(15) McGeady, P.; Pyun, H.-J.; Coates, R. M.; Croteau, R. Manuscript in preparation.
(16) Borch, R. F.; Hassid, A. I. *J. Org. Chem.* **1972**, *37*, 1673.

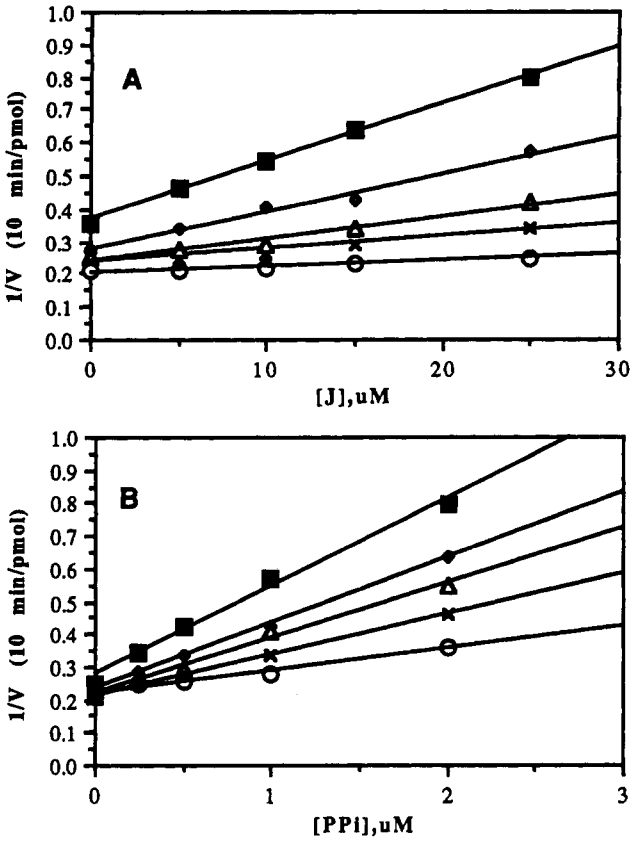


Figure 1. Cooperative inhibition of trichodiene synthase by PP_i and (*R*)-**16**. Incubations were carried out for 10 min at 30 °C with constant amounts of trichodiene synthase as described in the Experimental Section in the presence of 0.525 μM FPP and varying amounts of PP_i and (*R*)-**16**, as indicated. (A) Plot of $1/v$ against concentration of (*R*)-**16** [J] at variable [PP_i]: (○) [PP_i] = 0 μM; (X) [PP_i] = 0.25 μM; (△) [PP_i] = 0.50 μM; (◇) [PP_i] = 1.0 μM; (■) [PP_i] = 2.0 μM. (B) Plot of $1/v$ against [PP_i] at variable [J]: (○) [J] = 0 μM; (X) [J] = 5.0 μM; (△) [J] = 10.0 μM; (◇) [J] = 15.0 μM; (■) [J] = 25.0 μM.

36–38%), largely based on the scheme reported by Delay and Ohloff.[17] The enantiomeric purities of (*R*)-**8** (99 ± 2% ee) and (*S*)-**8** (86 ± 2% ee) were determined by NMR analysis of their respective (*S*)-camphanate esters in the presence of a chiral shift reagent. The lower enantiomeric purity of (*S*)-**8** is consistent with the lower optical purity of the (*S*)-limonene from which it was derived. Consequently five recrystallizations of the (*S*)-tosylate were needed to obtain optical purity corresponding to that of the (*R*)-tosylate.

The stereochemistry of the crucial substitution reaction was established by S_N2 displacement of (*R*)-(+)-**9** with acetate ion (n-Bu_4NOAc, DMF, 70 °C, 7 h) to optically pure (*S*)-(−)-acetate (**7**). This demonstrates that nucleophilic substitution can be effected with clean inversion, despite the significant possibility of competing double bond participation which might have caused partial, or complete, retention of configuration. Therefore it seems safe to assume that the displacement reactions of (+)- and (−)-**9** with the highly nucleophilic azide ion[18] under similar conditions occurred with complete inversion. These 4-methyl-3-cyclohexenyl derivatives as well as the related thiols,[15] now readily accessible in enantiomerically pure form of either absolute configuration, are potentially useful substrates for asymmetric synthesis.

(17) Delay, F.; Ohloff, G. *Helv. Chim. Acta* **1979**, *62*, 2168.
(18) (a) Pearson, R. G.; Sobel, H.; Songstad, J. *J. Am. Chem. Soc.* **1968**, *90*, 319. (b) Parker, A. J. *Chem. Rev.* **1969**, *69*, 1.

Table I. Cooperative Inhibition of Trichodiene Synthase by Inorganic Pyrophosphate (PP_i) and Ammonium Analogs (*R*)- and (*S*)-16**, (*R*)- and (*S*)-**13**, and Trimethylamine (TMA)**

inhibitor	PP_i, K_I (μM)	αK_J (μM)
(*R*)-**16**	0.51 ± 0.07	2.6 ± 0.2
(*S*)-**16**	0.47 ± 0.07	2.9 ± 0.2
(*R*)-**13**	0.48 ± 0.09	10.9 ± 1.2
(*S*)-**13**	0.53 ± 0.09	18.6 ± 2.8
TMA	0.68 ± 0.22	172 ± 24

Each of the four aza analogs was next tested as an inhibitor of trichodiene synthase. At pH 7.2, the tertiary amines should be essentially completely protonated, thereby mimicking the corresponding bisabolyl and α-terpinyl cations. The first analog examined, (*R*)-(+)-**16**, had the same absolute configuration as that for the proposed bisabolyl cation intermediate (**3**). Suprisingly, when trichodiene synthase was incubated with FPP (525 nM, K_m 75 nM) in the presence of (*R*)-(+)-**16** at concentrations up to 25 μM, the added ammonium ion analog had only a minor effect on the observed rate of formation of trichodiene, resulting in a maximum of 15% inhibition. This is illustrated in Figure 1A, in which a plot of $1/v$ against ammonium ion concentration [J] has a slope of near zero. (Figure 1A, [I] = [PP_i] = 0). This behavior could be contrasted with that of the known competitive inhibitor, inorganic pyrophosphate (PP_i),[10] itself a product of the cyclization reaction. As illustrated in Figure 1B, the plot of $1/v$ versus PP_i concentration [I] from 0 to 2.0 μM gave the expected straight line indicating a decrease in the rate of trichodiene formation with increasing inhibitor concentration. More interestingly, in the presence of inorganic pyrophosphate, (*R*)-(+)-**16** became a strong competitive inhibitor, with the potency of inhibition increasing in proportion to the PP_i concentration (Figure 1A). In like manner, increasing concentrations of (*R*)-(+)-**16** enhanced the apparent inhibitory effect of PP_i (Figure 1B). This type of cooperative competitive inhibition is well-known and can be described by the general kinetic expression, eq 1, where v is the observed rate of formation of trichodiene,

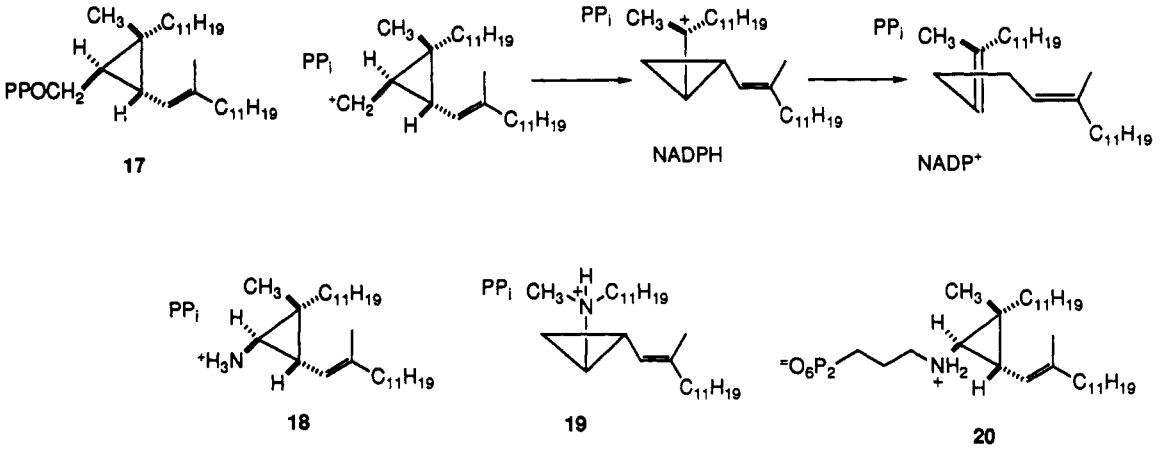
$$v = \frac{V \cdot S}{K_m\left\{1 + \frac{I}{K_I} + \frac{J}{K_J} + \frac{I \cdot J}{\alpha K_I K_J}\right\} + S} \quad (1)$$

V is v_{max}, S is the substrate [FPP] concentration, I and J are the concentrations of PP_i and amine, respectively, K_I and K_J are the corresponding inherent inhibition constants, and α is a cooperativity factor.[19] For positive cooperativity, α is <1. The values for K_I and αK_J were obtained by nonlinear regression fitting of the experimental data summarized in Figure 1 to eq 1. Since at the concentrations of (*R*)-(+)-**16** examined, the aza analog alone was ineffective as a competitive inhibitor, K_J must be ≫ [J] and the J/K_J term could be ignored.[20] In this case, the

(19) Cleland, W. W. *Steady State Kinetics*; In *Enzymes*, 3rd ed.; Boyer, P. D.; Academic: New York, 1970; Vol. 2, pp 1–65.
(20) Attempts to fit the experimental data directly to the full eq 1 led to large uncertainties in the calculated values of K_J and the interaction coefficient α. This problem arose because the concentrations of amine [J] which were tested were small compared to the inherent inhibition constant K_J, which probably lies between 30 and 100 μM. The J/K_J term is therefore ≤1 and is small compared to the overall expression i, which is dominated by the I/K_I and $(I \cdot J)/(\alpha K_I K_J)$ terms for all non-zero values of [PP_i] (I).

$$1 + \frac{I}{K_I} + \frac{J}{K_J} + \frac{I \cdot J}{\alpha K_I K_J} \quad (i)$$

Scheme III



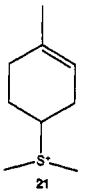
value αK_J can be considered to be an induced inhibition constant for the ammonium ion analog. The calculated K_I for PP_i, 0.50 ± 0.08 μM, was in close agreement with that previously obtained for PP_i alone[10] and the induced inhibition constant for (*R*)-(+)-**16**, αK_J, was 2.5 ± 0.2 μM (Table I).

Unexpectedly, the enantiomeric bisabolyl cation analog (*S*)-(−)-**16** showed identical behavior to (*R*)-(+)-**16** when tested as an inhibitor of trichodiene synthase. While (*S*)-(−)-**16** alone had little effect on the normal rate of conversion of FPP to trichodiene, in the presence of PP_i the ammonium ion analog acted as a strong competitive inhibitor. More surprising yet, the calculated induced inhibition constant for (*S*)-(−)-**16**, αK_J = 2.8 ± 0.2 μM, was experimentally indistinguishable from that for the (*R*)-(+)-enantiomer.

To explore further the effect of amine structure, we also tested the enantiomeric monoterpene analogs (*R*)- and (*S*)-**13** as inhibitors of trichodiene synthase (Table I). In fact, both (*R*)- and (*S*)-**13** exhibited behavior qualitatively similar to that of the bisabolyl cation analogs, but with induced inhibition constants that were 5- to 8-fold higher than those for (*R*)-(+)- and (*S*)-(−)-**16** (Table I). Finally, to evaluate the effect of the ammonium moiety alone, we also examined trimethylamine as a potential inhibitor. When incubated with trichodiene synthase, trimethylamine exhibited the same absolute requirement for PP_i as did the isoprenoid aza analogs, albeit with an induced inhibition constant some 70–80 times larger than that observed for the $C_{14}N$ analogs (+)- and (−)-**16** (Table I).

Discussion

It has long been recognized that molecules which mimic the structural and electronic features of metastable intermediates or transition states can act as potent inhibitors of enzyme-catalyzed reactions.[21] Ammonium ion analogs of carbocation intermediates have previously been used to inhibit a variety of terpenoid metabolizing enzymes.[22] For example, 2,3-iminosqualene is a potent inhibitor of oxidosqualene-lanosterol cyclase;[23] similarly, 2-aza-2,3-dihydrosqualene and several derivatives are effective inhibitors of 2,3-oxidosqualene-β-amyrin and -cycloartenol cyclases.[24] A variety of nitrogen-containing triterpene analogs have been shown to act as effective inhibitors of steroid biosynthesis by mimicking various carbonium ion species postulated to be involved in lanosterol or cycloartenol formation or side-chain alkylation.[25] Poulter has reported an important study of the inhibitory properties of **18** and **19**, ammonium analogs of the postulated primary and tertiary cyclopropylcarbinyl cation intermediates in the formation of squalene from presqualene diphosphate (**17**)[26] (Scheme III). Interestingly, while neither **18** nor **19** alone had any measurable effect on squalene synthase activity, addition of PP_i to the incubation mixtures led to significant synergistic inhibition of squalene synthesis. Moreover, the phosphonophosphate **20**, a bisubstrate analog containing both the ammonium ion and diphosphate moieties, was itself a potent inhibitor of squalene synthase. In similar fashion, Croteau and Oehlschlager have shown that racemic **21**, the sulfonium



ion analog of the α-terpinyl cation, is an effective inhibitor of various monoterpene synthases and that this effect is potentiated by added inorganic pyrophosphate.[27] More recently, Croteau and Coates have found that there is little discrimination by either pinene cyclase I or II between the individual enantiomers of the ammonium ion analog **13**.[15] In the absence of PP_i, the observed inhibition patterns were apparently uncompetitive. Addition of PP_i enhanced the effective inhibition by a factor of 2–3 and changed the inhibition mode to noncompetitive or competitive.

(21) Jencks, W. P. *Adv. Enzymol.* **1975**, *43*, 219. Wolfenden, R. *Annu. Rev. Biophys.* **1976**, *5*, 271.
(22) Rahier, A.; Taton, M.; Benveniste, P. *Biochem. Soc. Trans.* **1990**, *18*, 48.
(23) Corey, E. J.; Ortiz de Montellano, P. R.; Lin, K.; Dean, P. D. G. *J. Am. Chem. Soc.* **1967**, *89*, 2797.
(24) Delprino, L.; Balliano, G.; Cattel, L.; Benveniste, P.; Bouvier, P. *J. Chem. Soc., Chem. Commun.* **1983**, 381. Duriatti, A.; Bouvier-Navé, P.; Benveniste, P.; Schuber, F.; Delprino, L.; Balliano, G.; Cattel, L. *Biochem. Pharm.* **1985**, *34*, 2765.
(25) Avruch, L.; Fischer, S.; Pierce, H. J.; Oehlschlager, A. C. *Can. J. Biochem.* **1976**, *54*, 657. Oehlschlager, A. C.; Angus, R. H.; Pierce, A. M.; Pierce, H. D.; Srinivasan, R. *Biochemistry* **1984**, *23*, 3582. Oehlschlager, A. C.; Pierce, H. D.; Pierce, A. M.; Angus, R. H.; Quantin-Martenot, E.; Unrau, A. M.; Srinivasan, R. In *Biogenesis and Function of Plant Lipids*; Mazliak, P., Benveniste, P., Costes, C., Douce, R., Eds; Elsevier/North Holland Biomedical Press: Amsterdam, 1980; pp 395–403. Narula, A. S.; Rahier, A.; Benveniste, P.; Schuber, F. *J. Am. Chem. Soc.* **1981**, *103*, 2408. Rahier, A.; Genot, J. C.; Schuber, F.; Benveniste, P.; Narula, A. S. *J. Biol. Chem.* **1984**, *259*, 15215.
(26) Poulter, C. D.; Capson, T. L.; Thompson, M. D.; Bard, R. S. *J. Am. Chem. Soc.* **1989**, *111*, 3734. Sandifer, R. M.; Thompson, M. D.; Gaughan, R. G.; Poulter, C. D. *J. Am. Chem. Soc.* **1982**, *104*, 7376.
(27) Croteau, R.; Wheeler, C. J.; Aksela, R.; Oehlschlager, A. C. *J. Biol. Chem.* **1986**, *261*, 7257.

Several studies have examined the role of the pyrophosphate moiety in allylic diphosphate metabolism. Cane and Croteau observed that conversion of geranyl diphosphate to either (+)- or (−)-bornyl diphosphate, by way of the tertiary allylic isomers (3*R*)- and (3*S*)-linalyl diphosphate, respectively, took place without detectable scrambling of the original ester oxygen atom of either allylic diphosphate substrate.[28] These results indicated a substantial restriction on the internal motion of the inorganic pyrophosphate moiety which is generated during the course of the enzymatic cyclization. These findings contrasted with those of an earlier study of the isomerization of farnesyl to nerolidyl diphosphate, in which the oxygen atoms attached to the proximal phosphorus atom were shown to undergo positional isotope exchange, an observation which was used to argue in support of an ion-pair mechanism for the allylic rearrangement.[29] Further insight into the role of the pyrophosphate moiety in terpenoid cyclizations has come from the finding that whereas the enzymatic conversion of FPP to the sesquiterpene hydrocarbon pentalenene was not inhibited by either 10 μM pentalenene or inorganic pyrophosphate alone, the combination of the two products together at 10 μM each increased the apparent K_m for FPP by a factor of 7.[30]

In the inhibition of trichodiene synthase by the ammonium ion derivatives of (*R*)- and (*S*)-**16** and (*R*)- and (*S*)-**13** as well as by trimethylamine the following three trends were apparent.

1. All five tertiary amines required inorganic pyrophosphate (PP_i) in order to act as effective competitive inhibitors. Together each amine and PP_i showed typical cooperative kinetic behavior. Although only the induced inhibition constant for each amine (αK_J) was determined, the enhancement factor in each case was probably between 10 and 40, corresponding to an α value of 0.025–0.1. These results presumably reflect the fact that during the course of the normal cyclization, the inorganic pyrophosphate moiety generated by ionization of the intermediate (3*R*)-NPP remains tightly bound to the active site of the cyclase. Indeed, it is tempting to speculate that the pyrophosphate group is not simply a passive spectator to subsequent catalytic events but may play an active role in the stabilization of the various cationic intermediates lying between **2** and trichodiene.

2. The effectiveness of each amine increased by a factor of ca. 10 in going from Me_3N to the α-terpinyl cation analogs, while the corresponding bisabolyl cation analogs were yet again 4–8 times more effective than the C_9N compounds. The affinity of each inhibitor for the active site of the cyclase would therefore appear to depend not only on the net positive charge of the tertiary alkyl ammonium moiety but on the degree to which the hydrocarbon residue mimics the structure of the actual intermediate. At the very least, these results indicate a substantial hydrophobic component to the binding of the various inhibitors.

3. For a given enantiomeric pair of aza analogs, there was little discrimination by trichodiene synthase. The small experimental differences between the observed values of αK_J for the α-terpinyl cation analogs, although slightly greater than the calculated statistical deviation, was probably not significant, while the individual bisabolyl cation analogs were indistinguishable in their effect on the rate of FPP cyclization. From simple steady-state kinetic measurements, it is of course impossible to determine whether the various ammonium ion analogs are all bound in the same fashion or, indeed, whether any individual inhibitor is bound in a single orientation, provided that each binding event results in an identical mode of inhibition.[31] The answer to such questions must await crystallographic studies on recombinant enzyme carrying bound inhibitors. In the meantime, it is still useful to consider the implications of the apparent lack of enantiomeric discrimination.

The tetrahedral tertiary ammonium ions are inherently imperfect geometric analogs of the planar carbocations they are intended to mimic. Despite the fact that the protonated forms of **16** are necessarily diastereomeric, these two species are of course rapidly interconvertible. Although the corresponding sulfonium ion analogs would avoid the dependence of net charge on pH characteristic of the aza analogs, they would still suffer from the complication of having an additional stable stereogenic center at the tetrahedral sulfur atom. Assuming for the moment that the enantiomeric $C_{14}N$ analogs both bind in a comparable fashion, the conformation of bound inhibitor would be unlikely to mimic precisely that of the corresponding bisabolyl cation intermediate (**3**), which, at least according to the favored stereochemical model of trichodiene formation, is expected to be generated initially in a boat-like conformation from the *anti-endo* form of NPP (**2**). At the moment, there is no way of knowing whether the initially generated bisabolyl intermediate relaxes to a half-chair conformation immediately prior to further cyclization or at some subsequent stage in the course of the various hydride and methyl migrations which result in an additional ring flip of the cyclohexene moiety. What the cyclization mechanism does suggest, however, is that the formation of trichodiene requires considerable rearrangement of the original substrate and that the requisite internal reorganizations involving substantial changes in hybridization, bonding, and configuration at the majority of the carbon atoms of the original substrate must somehow be accommodated by the cyclase active site. The ability of trichodiene synthase to bind both enantiomers of **16** with equal affinity is consistent with the notion that the cyclase active site is *permissive* rather than *restrictive*, thereby allowing a family of related structures derivable from the initial chiral folding of the FPP substrate without being rigidly complementary to any single member of this family of carbocationic intermediates. This idea of a

(28) Cane, D. E.; Saito, A.; Croteau, R.; Shaskus, J.; Felton, M. *J. Am. Chem. Soc.* **1982**, *104*, 5831. Croteau, R. B.; Shaskus, J. J.; Renstrom, B.; Felton, N. M.; Cane, D. E.; Saito, A.; Chang, C. *Biochemistry* **1985**, *24*, 7077.

(29) Cane, D. E.; Iyengar, R. *J. Am. Chem. Soc.* **1979**, *101*, 3385.

(30) Cane, D. E.; Pargellis, C. *Arch. Biochem. Biophys.* **1987**, *254*, 421.

(31) The ammonium ion analog (*R*)-**13** can exist in a variety of low energy conformations, illustrated in Figure 3, which have been calculated to lie within 1.8 kcal of one another by using the program MacroModel and a modified MM2 force field.[32] The two gauche equatorial conformers **13a** and **13b** have the trans (anti) relationship of the C-6 methine proton and the C-7 methyl group (GPP/FPP numbering) which is presumably characteristic of the initially generated α-terpinyl or bisabolyl cation intermediates, whereas conformers **13c** and **13d** correspond to the lowest energy equatorial and axial conformers, respectively, of the ammonium ion analog. (An analogous set of low energy half-chair conformers differing somewhat in relative energy but having approximately the same range of energies has previously been calculated for p-menthene, a monoterpene in which the ammonium moiety of **13** has been replaced by an isopropyl group: Singh, R. D.; Keiderling, T. A. *J. Am. Chem. Soc.* **1981**, *103*, 2387.) In principle, any or several of the conformers **13a–d** might be the principle species bound by trichodiene synthase.

(32) (a) MacroModel V3.0: Still, W. C.; Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Lipton, M.; Liskamp, R.; Chang, G.; Hendricksoon, T.; DeGunst, F.; Hasel, Department of Chemistry, Columbia University, New York, 10027. (b) Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440. (c) Allinger, N. L. *J. Am. Chem. Soc.* **1977**, *99*, 8127; force field modified 6/87. (d) Colucci, W. J.; Gandour, R. D.; Mooberry, E. A. *J. Am. Chem. Soc.* **1986**, *108*, 7141.

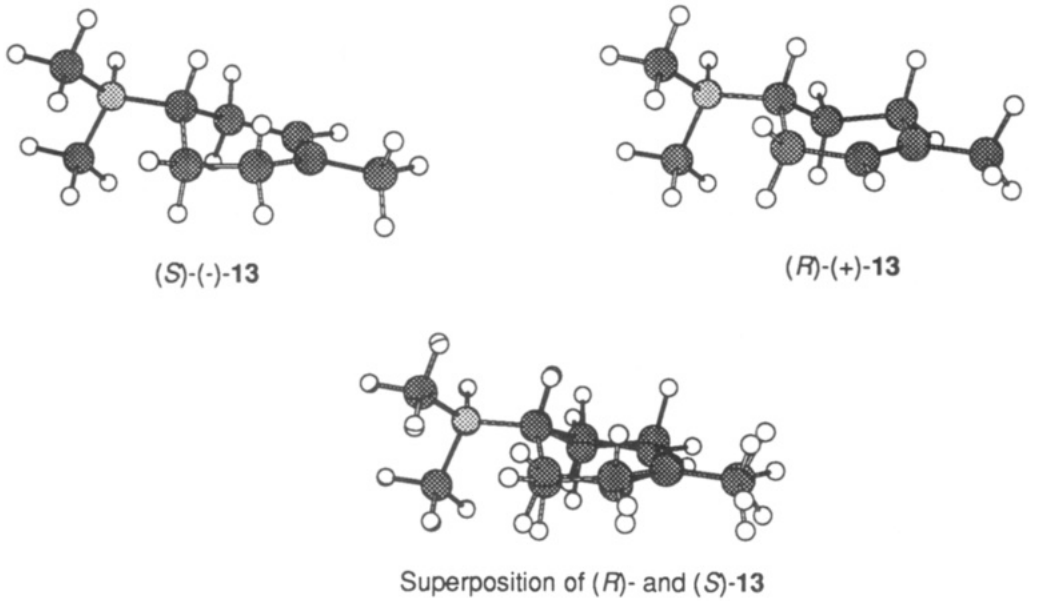


Figure 2. Comparison of gauche equatorial conformations of (*S*)- and (*R*)-**13** showing superposition of both enantiomers.

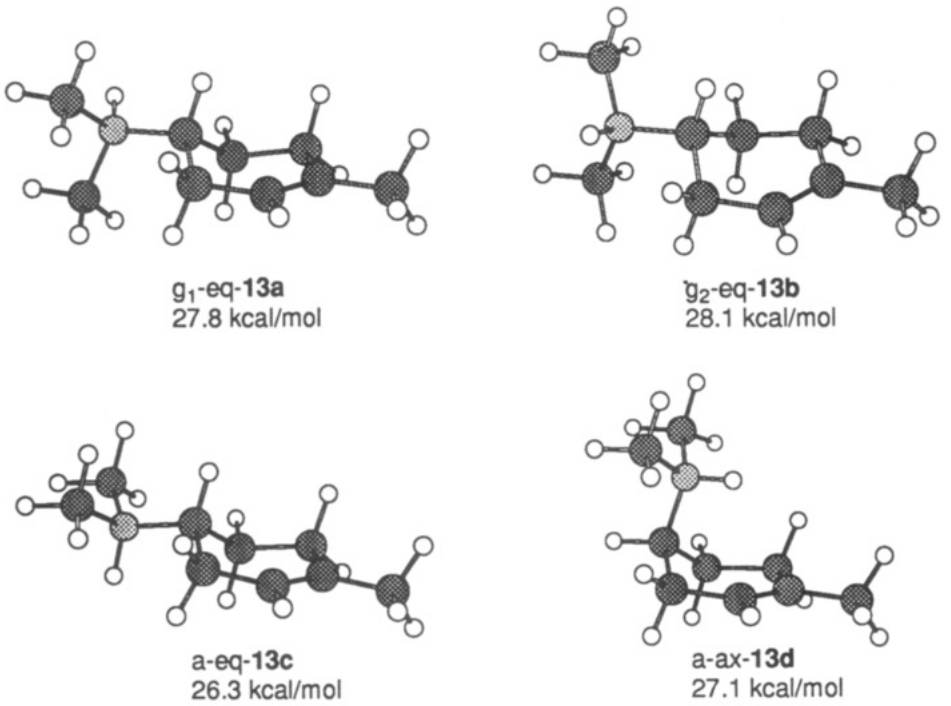


Figure 3. Lowest energy conformers of **13** calculated by molecular mechanics. The (+)-equatorial and (−)-axial conformers are illustrated for convenience of comparison.

permissive active site topology is reinforced by several earlier observations which established the apparent inability of several monoterpene cyclases to discriminate cleanly between the enantiomers of the intermediate LPP, in spite of the fact that the same cyclases normally produce exclusively one enantiomer of their natural product from the achiral precursor GPP.[33]

In the case of (*R*)- and (*S*)-**16**, the enantiomeric ammonium analogs can also be considered as simple double bond positional isomers. The lack of discrimination by trichodiene synthase may simply reflect the failure of the active site to recognize the difference between a vinylic methine and an allylic methylene carbon and thus an inability to distinguish between the most stable conformers of each double bond isomer. The close geometrical similarity between the monoterpene analogs (*S*)- and (*R*)-**13** is illustrated in Figure 2. Superposition of one of the low energy gauche equatorial half-chair conformers of (*S*)- and (*R*)-**13**, as calculated using a modified MM2 force field and the program MacroModel,[31,32] results in only a 0.144 Å/atom root mean square deviation for the cyclohexenyl carbon atoms and the attached methyl carbon and nitrogen atoms. The same close geometric relationship would also be expected for the individual antipodes of **16**. It is evident that for each pair of enantiomeric ammonium ions the two structures can be readily superimposed with little distortion at any position. The differences between the individual enantiomers are apparently small in comparison to the much larger changes in intermediate structure which must be accommodated by the trichodiene synthase active

(33) Croteau, R.; Satterwhite, D. M.; Cane, D. E.; Chang, C. C. *J. Biol. Chem.* **1986**, *261*, 13438. Croteau, R.; Satterwhite, D. M.; Cane, D. E.; Chang, C. C. *J. Biol. Chem.* **1988**, *263*, 10063.

site in the course of the normal cyclization.

In summary, we have found that ammonium ion analogs of the intermediate bisabolyl cation act in synergy with PP_i to become effective competitive inhibitors of trichodiene synthase, supporting the intermediacy of the proposed bisabolyl cation and reinforcing the idea that the inorganic pyrophosphate plays an active role in the normal cyclization. Moreover, the fact that both enantiomers of the inhibitor are equally effective is consistent with a permissive model of cyclase active site structure, according to which an active site must accommodate a variety of rearranged intermediates of varying shape and charge distribution without being rigidly complementary to a single intermediate or transition state species.

Experimental Section

General Procedures. Melting points were determined in open-ended capillary tubes and are uncorrected. The IR, ^1H NMR, and ^{13}C NMR spectra of enantiomers prepared by the same procedure were identical to those of their mirror image forms.

Materials and Methods. Preparation of buffers and substrates and general methods of biochemical and radiochemical analysis were as previously described.[9,10] All buffers were prepared with doubly deionized water. Trichodiene synthase was isolated from *Fusarium sporotrichioides* and purified as previously described.[5] The homogeneous enzyme was stored frozen, either at −75 °C or liquid N_2 temperature, in 10 mM Hepes buffer (pH 7.5) that contained 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol. The enzyme preparation consisted primarily of the homodimer of the M_r 45 000 polypeptide (≥90%) and had a concentration of 0.2 mg of protein/mL. The enzyme was thawed in an ice bath and diluted to the appropriate amount with the same buffer for kinetic studies immediately prior to use. [1-^3H]Farnesyl pyrophosphate (682 mCi/mmol) was prepared as previously described.[34]

Kinetic Studies of Trichodiene Synthase Inhibition. Studies of the inhibition of trichodiene synthase by inorganic pyrophosphate and the aza analogs (+)- and (−)-16, (+)- and (−)-13, and trimethylamine were carried out using the previously described assay system[10] with a constant amount of purified trichodiene synthase in a total volume of 400 μL of 25 mM Hepes buffer, pH 7.2, containing 3 mM $MgCl_2$ and 1 mM DTT. For each inhibition series the enzyme was diluted with enough ice-cold storage buffer to give less than 10% conversion of substrate during a 10-min assay and was used within 10 min of dilution. Each assay was initiated by adding trichodiene synthase to the incubation mixture containing 0.525 μM FPP and varying amounts of PP_i and/or aza analog. The aqueous layer was overlaid with 0.5 mL of hexane to prevent evaporative loss of product. After incubation for 10 min at 30 °C, the enzymatic reaction was quenched by addition of 150 μL of ethanol followed by 1 mL of hexane. The contents of the reaction vial were vortexed for 20 s and the extracts were passed through a silica gel pipet column (0.5 × 3 cm) directly into scintillation vials containing 5 mL of Optifluor. The column was washed with an additional 0.5 mL of hexane and the combined eluents were analyzed by liquid scintillation. Five different values of both PP_i and aza analog concentration were used, and all combinations were run in duplicate.

Data Analysis. The data from each experiment were initially analyzed qualitatively by Dixon plots,[35] by computer fitting the values of $1/v$ vs inhibitor concentration to straight lines for each series of experiments (see Figure 1 and supplementary material). The steady-state kinetic parameters were obtained by computer fitting of the data to eq 1 using the nonlinear least-squares regression program KINFIT written by Professor Vernon Anderson of Brown University. Best fits were obtained by ignoring the J/K_J term and fitting the values of K_I, the inherent inhibition constant for PP_i, and αK_J, the apparent induced inhibition constant for each aza analog in the presence of PP_i.

(1*R*,2*S*,4*R*)- and (1*S*,2*R*,4*R*)-1-methyl-4-(1-methylethenyl)cyclohexene 1,2-epoxide ((4*R*)-limonene oxide) was prepared by a literature procedure for α-pinene epoxide preparation.[36] A slurry of 20.5 g (150 mmol) of (+)-(*R*)-limonene ((+)-(*R*)-5, Aldrich Chemical Company, $[\alpha]^{20}_D$ = +123° (neat)) and 25.2 g (300 mmol) of $NaHCO_3$ in 150 mL of CH_2Cl_2 was stirred at 0–5 °C as a solution of 47.0 g (150 mmol) of 55% *m*-CPBA in 250 mL of CH_2Cl_2 was added over 50 min. The mixture was stirred at 0 °C for 30 min and a solution of 10 g of Na_2SO_3 in 100 mL of water was added. The mixture was stirred for 30 min at rt and diluted with 100 mL of water before separating the organic fraction. The aqueous layer was extracted with CH_2Cl_2 (100 mL) and the combined organic fractions were dried ($MgSO_4$) and concentrated. Distillation at 76–81 °C (9.8 mm) afforded 14.42 g (63%) of (4*R*)-limonene oxide which was a 60:40 mixture of two isomers. TLC and GC behavior of the product were consistent with a commercially available authentic sample except minor impurities (~5%) were present as judged by TLC and GC analysis. ^1H NMR analysis indicated the presence of diepoxide. The product mixture was used for ozonolysis without further purification.

(1*S*,2*R*,4*S*)- and (1*R*,2*S*,4*S*)-1-methyl-4-(1-methylethenyl)cyclohexene 1,2-oxide ((4*S*)-limonene oxide) was prepared from (−)-(*S*)-limonene ((−)-(*S*)-5, Aldrich, $[\alpha]^{19}_D$ = −94° (*c* = 10, EtOH)) as described previously, yield 15.03 g (66%); bp 75–81 °C (9.7 mm). GC analysis indicated that the product was a 60:40 mixture of the two isomers (96% purity).

(1*R*,2*S*,4*R*)- and (1*S*,2*R*,4*R*)-1-methyl-4-(1-oxoethyl)-cyclohexane 1,2-oxide ((4*R*)-6) was prepared according to the procedure of Delay and Ohloff with modifications.[17] A solution of 14.42 g (95 mmol) of (4*R*)-limonene oxide and 20.5 mL (506 mmol) of methanol in 500 mL of CH_2Cl_2 was magnetically stirred at −65 to −70 °C as a stream of ozone in oxygen was bubbled through it until a blue color appeared. The pale blue color of ozone disappeared when 150 mL of dimethyl sulfide was added at −78 °C. The solution was warmed, stirred for 4 h, and washed with water (2 × 500 mL). The organic fraction was dried ($MgSO_4$) and concentrated. Distillation at 72–83 °C (1.7 mm) gave 12.92 g (89%) of a cis and trans mixture of (4*R*)-6.

The two isomers were separated from the mixture resulting from a small scale run by flash chromatography, and the ^1H NMR spectra of the two isomers were in agreement with the literature data.[17]

(1*S*,2*R*,4*S*)- and (1*R*,2*S*,4*S*)-1-methyl-4-(1-oxoethyl)-cyclohexane 1,2-oxide ((4*S*)-6): yield 13.11 g (86%); bp 73–79 °C (1.7–1.8 mm).

(1*R*,2*S*,4*R*)- and (1*S*,2*R*,4*R*)-1-methyl-4-acetoxycyclohexane 1,2-oxide was prepared according to the procedure of Delay and Ohloff with modifications.[17] A solution of (4*R*)-6 (12.9 g, 84 mmol) in 500 mL of CH_2Cl_2 was stirred at −10 °C as 52.6 g (168 mmol) of *m*-CPBA (55% pure) was added. After stirring for 60 h, the reaction mixture was washed with ice-cold saturated Na_2SO_3 (2 × 500 mL), ice-cold 2 N NaOH (500 mL), water (2 × 500 mL), and saturated NaCl (500 mL). Some insoluble materials produced during washing were removed by filtration. After the organic fraction was dried ($MgSO_4$) and concentrated, the residue was purified by chromatography on 400 g of silica gel by gravity using ethyl acetate–hexane (1:8) as eluent. Distillation of the product at 65–67 °C (1.1 mm) gave 11.60 g (81%) of (4*R*)-1-methyl-4-acetoxycyclohexane 1,2-oxide as a 57:43 mixture of cis and trans isomers.

The two isomers were also prepared separately from the purified cis and trans isomers of (4*R*)-3. Their ^1H NMR spectral data were in agreement with the literature data.[17]

(1*S*,2*R*,4*S*)- and (1*R*,2*S*,4*S*)-1-methyl-4-acetoxycyclohexane 1,2-oxide: yield 11.89 g (82%); ratio of two isomers, 57.43.

(+)-(1*R*)-4-Methyl-1-acetoxy-3-cyclohexene ((+)-7). Method A: Reduction of Epoxide. Reduction of (4*R*)-1-methyl-4-acetoxycyclohexane 1,2-oxide was performed by a modified procedure of Cornforth et al.[17,37] A slurry of zinc powder (12.2 g, 187 mmol), sodium acetate (27.9 g, 340 mmol), NaI (81.7 g, 545

(34) Cane, D. E.; Iyengar, R.; Shiao, M. *J. Am. Chem. Soc.* **1981**, *103*, 914. Davisson, V. J.; Zabriskie, T. M.; Poulter, C. D. *Bioorg. Chem.* **1986**, *14*, 46.

(35) Dixon, M. *Biochem. J.* **1953**, *55*, 170.

(36) Coxon, J. M.; Dansted, E.; Hartshorn, M. P. *Organic Syntheses*; Wiley: New York, 1988; Collect. Vol. VI, p 949.

(37) Cornforth, J. W.; Cornforth, R. H.; Mathew, K. K. *J. Chem. Soc.* **1959**, 112.

mmol), and acetic acid (70 mL, 1.22 mol) in 250 mL of CH_2Cl_2 was magnetically stirred at 0 °C as a solution of 11.48 g (67.4 mmol) of (4*R*)-1-methyl-4-acetoxycyclohexane 1,2-oxide in 25 mL of CH_2Cl_2 was added over 5 min. A rinse with 25 mL of CH_2Cl_2 was added to the reaction mixture. After being stirred at rt for 4 h, the mixture was partitioned between 200 mL of CH_2Cl_2 and 500 mL of water. The two-phase mixture was filtered over Celite to remove excess zinc dust, and the separated organic fraction from the filtrate was washed with water (500 mL), saturated $NaHCO_3$ (500 mL), and saturated NaCl (500 mL). The organic layer was dried ($MgSO_4$) and concentrated. Purification of the residue by chromatography on 150 g of silica gel by gravity using ether–pentane (1:10) as eluent and subsequent distillation at 78.5–79.5 °C (9.5 mm) afforded 9.31 g (90%) of (+)-7: $[\alpha]^{25}_D$ +40.0° (*c* 1.00, $CHCl_3$); IR (thin film) ν_{max} 2926 (CH), 2851 (CH), 1738 (C=O), 1444, 1370, 1246, 1036 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.66 (s, 3 H, C*H*$_3$), 1.73–1.95 (m, 2 H, H at C6), 2.05 (s, 3 H, C*H*$_3$CO), 2.05–2.13 (m, 3 H, H at C2 and C5), 2.32 (br m, 1 H, H at C5), 4.98 (m, 1 H, C*H*OAc), 5.27 (br s, 1 H, —C*H*=); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 21.5, 23.3, 27.5, 27.9, 30.7, 69.8 (*C*HOAc), 117.7 (—*C*H=), 133.9 (=*C*Me), 170.8 (*C*=O). Anal. Calcd for $C_9H_{14}O_2$: C, 70.10; H, 9.15. Found: C, 70.15; H, 9.17.

(−)-(1*S*)-4-Methyl-1-acetoxy-3-cyclohexene ((−)-7): yield 9.37 g (87%); bp 79 °C (10 mm); $[\alpha]^{25}_D$ −35.9° (*c* 1.00, $CHCl_3$). Anal. Calcd for $C_9H_{14}O_2$: C, 70.10; H, 9.15. Found: C, 70.11; H, 9.11.

Method B: Displacement of (+)-9. A slurry of 266 mg (1.0 mmol) of (+)-**9** and 1.5 g (5.0 mmol) of tetrabutylammonium acetate in 2 mL of DMF was stirred in a thick-walled tube and heated at 70 °C for 7 h. The mixture was dissolved with water (10 mL) and extracted with hexane (2 × 10 mL, 2 × 5 mL). The combined extracts were washed with water (20 mL) and dried ($MgSO_4$). The yield before purification was estimated to be 69% by quantitative GC analysis. The solution was concentrated and the residue was purified by flash chromatography using ether–pentane (1:10) as eluent. Kugelrohr distillation at 75–85 °C (9 mm) gave 43 mg (28%) of (−)-7: $[\alpha]^{24}_D$ −40.0° (*c* 1.00, $CHCl_3$). ^1H NMR spectral data were in agreement with those of an authentic sample. GC analysis indicated that the product was >99% pure.

(+)-(1*R*)-4-Methyl-3-cyclohexen-1-ol ((+)-8). A slurry of 5.54 g (139 mmol) of $LiAlH_4$ was stirred at 0 °C as 9.09 g (59 mmol) of (+)-7 in 25 mL of ether was added. After being stirred at rt for 2 h, the reaction mixture was cooled at 0 °C and quenched by successive addition of water (5.5 mL), 15% NaOH (5.5 mL), and water (16.5 mL).[38] The mixture was stirred at 0 °C for 1 h and the precipitate was removed by filtration. The ethereal filtrate was washed with water (2 × 400 mL), dried ($MgSO_4$), and concentrated. Distillation of the residue at 78–79 °C (9 mm) provided 6.20 g (94%) of (+)-8: $[\alpha]^{25}_D$ +70.3° (*c* 1.00, $CHCl_3$); ^1H NMR, ^{13}C NMR, and IR spectral data were in agreement with the literature values for the racemic compounds.[27,39]

The enantiomeric purity was determined on the camphanate ester. A solution of 17 mg (0.15 mmol) of (+)-8 and 101 mg (0.47 mmol) of (−)-(1*S*)-camphanic chloride in 0.5 mL of pyridine was stirred for 24 h at rt. The mixture was diluted with 10 mL of ether and washed with 3 N HCl (2 × 10 mL), saturated $NaHCO_3$ (2 × 10 mL), and saturated NaCl (10 mL). The organic fraction was dried ($MgSO_4$) and concentrated. Purification of the residue by chromatography using ether–pentane (1:3) as eluent provided 41 mg (93%) of the camphanate ester. A ^1H NMR spectrum on a solution of 5.26 mg of the camphanate ester and 10.81 mg of tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato)europium(III) ($Eu(fod)_3$) in 0.65 mL of C_6D_6 showed the presence of a very small amount (~1%) of diastereomeric impurity ≥99 ± 2% ee).

(−)-(1*S*)-4-Methyl-3-cyclohexen-1-ol ((−)-8): yield 5.78 g (88%); bp 77–78 °C (10 mm); $[\alpha]^{25}_D$ −63.0° (*c* 1.00, $CHCl_3$). The enantiomeric purity was 86 ± 2% ee).

(+)-(1*R*)-4-Methyl-3-cyclohexen-1-yl 4-Methylbenzenesulfonate ((+)-9). A solution of 5.87 g (52.4 mmol) of (+)-8 in 18.6 mL (230 mmol) of pyridine was stirred at 0 °C as 19.97 g (105 mmol) of *p*-toluenesulfonyl chloride was added. The reaction mixture was stirred at 0 °C for 1.5 h and at rt for 6 h. The solution was cooled to 0 °C before the remaining *p*-toluenesulfonyl chloride was destroyed by adding 2 mL of water. A 10-mL portion of CH_2Cl_2 was added after 0.5 h for efficient stirring. After 2 h at rt, the slurry was dissolved with 200 mL of CH_2Cl_2. The solution was washed with ice-cold 1.5 N HCl (2 × 250 mL), 1 N NaOH (250 mL), water (250 mL), and saturated NaCl (250 mL). After the organic solution was dried ($MgSO_4$) and concentrated, the residue was purified by chromatography on 100 g of silica gel by gravity using ether–hexane (1:5) as eluent. Recrystallization of the collected product (13.70 g, 98%) from hexane afforded 10.90 g (78%) of optically pure (+)-9: mp 52–52.5 °C; $[\alpha]^{25}_D$ +9.0° (*c* 1.00, $CHCl_3$); IR (KBr) ν_{max} 2940 (CH), 2913 (CH), 1345 (S=O), 1167, 912 (S—O), 818 cm^{-1}; ^1H NMR ($CDCl_3$), 300 MHz) δ 1.62 (s, 3 H, C*H*$_3$), 1.80–1.87 (m, 2 H, CH(OTs)C*H*$_2$CH$_2$), 1.92–2.25 (m, 4 H, C*H*$_2$CH=CMeC*H*$_2$), 2.45 (s, 3 H, C*H*$_3$Ph) 4.70 (overlapping tt, 1 H, *J* = 5.6, 6.7 Hz, C*H*OTs), 5.28 (s, 1 H, C*H*=CCH$_3$), 7.33 (d, 2 H, *J* = 8.2 Hz, PhH), 7.80 (d, 2 H, *J* = 8.2 Hz, PhH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 21.6, 23.1, 27.7, 28.2, 31.3, 78.7 (*C*HOTs), 116.8 (*C*H=CMe), 127.6 (*o*-Ph*C*), 129.7 (*m*-Ph*C*), 134.0 (CH=*C*Me), 134.5 (*p*-Ph*C*), 144.4 (*C*SO$_3$). Anal. Calcd for $C_{14}H_{18}O_3S$: C, 63.13; H, 6.81; S, 12.04. Found: C, 63.35; H, 6.87; S, 11.86.

(−)-(1*S*)-4-Methyl-3-cyclohexen-1-yl 4-methylbenzenesulfonate ((−)-9) was prepared as described above. The tosylate purified by chromatography was crystallized to yield 11.43 g (88%) of white crystals; mp 49.5–51 °C; $[\alpha]^{24}_D$ −8.1°. After five recrystallizations, the mp and $[\alpha]_D$ values were comparable to those of the enantiomer. The yield was 6.41 g (49%) of (−)-9: mp 52–52.5 °C; $[\alpha]^{25}_D$ −9.0° (*c* 1.00, $CHCl_3$). Anal. Calcd for $C_{14}H_{18}O_3S$: C, 63.13; H, 6.81; S, 12.04. Found: C, 63.37; H, 6.90; S, 11.84.

(−)-(1*S*)-4-Methyl-3-cyclohexenamine ((−)-11). A slurry of 2.66 g (10 mmol) of (+)-9 and 3.25 g (50 mmol) of NaN_3 in 10 mL of DMF was stirred at 50 °C for 18 h. The reaction mixture was dissolved in 50 mL of water and the product was extracted with pentane (50 mL, 2 × 10 mL). The combined pentane extracts were washed with water (50 mL), dried ($MgSO_4$), and concentrated. Purification of the residue by chromatography using ether–pentane (1:10) as eluent afforded (1*S*)-1-azido-4-methyl-3-cyclohexene ((*S*)-**10**) containing some residual ether: IR (KBr) ν_{max} 2967 (CH), 2930 (CH), 2842 (CH), 2099 (N_3), 1443, 1258 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 1.66 (s, 3 H, C*H*$_3$), 1.69–1.77 (m, 1 H, H at C6), 1.88–1.97 (m, 1 H, H at C6) 2.00–2.09 (m, 3 H, H at C2 and C5), 2.31 (br m, 1 H, H at C5), 3.62 (m, 1 H, C*H*N$_3$), 5.30 (br m, 1 H, —C*H*=).

The azide (*S*)-**10** in 10 mL of ether was stirred at 0 °C as 15 mL of 0.67 M $LiAlH_4$ in ether was added.[40] The mixture was stirred for 3 h at 0 °C and at rt for 0.5 h. The mixture was cooled at 0 °C and quenched as described in the (+)-8 preparation.[38] After the inorganic precipitate was removed by filtration, the filtrate was concentrated. Kugelrohr distillation of the residue at 80 °C (50 mm) yielded 831 mg (75%) of (−)-11: $[\alpha]^{24}_D$ −107.0° (*c* 1.00, $CHCl_3$); IR (neat) ν_{max} 3353 (NH), 3281 (NH), 2963 (CH), 2913 (CH), 2853 (CH), 1593, 1439, 849 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.3–1.5 (m, 3 H, H at C6 and N*H*$_2$), 1.65 (s, 3 H, C*H*$_3$), 1.70–1.85 (m, 2 H, H at C5 and C6), 2.01 (br, 2 H, H at C2), 2.19–2.27 (m, 1 H, H at C5), 2.90–2.97 (m, 1 H, C*H*NH$_2$), 5.29 (m, 1 H, —C*H*=); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.3, 29.1, 32.6, 35.3, 46.5, 119.1 (—CH=), 133.6 (—*C*Me=). Anal. Calcd for $C_7H_{13}N$: C, 75.62; H, 11.79; N, 12.60. Found: C, 73.40; H, 11.89; N, 12.23.

(+)-(1*R*)-4-Methyl-3-cyclohexenamine ((+)-11): yield 814 mg (73%); $[\alpha]^{24}_D$ +104.7° (*c* 1.00, $CHCl_3$). Anal. Calcd for $C_7H_{13}N$: C, 75.62; H, 11.79; N, 12.60. Found: C, 73.15; H, 11.69; N, 12.28.

(−)-(1*S*)-1-Methyl (4-Methyl-3-cyclohexenyl)carbamate. A solution of 334 mg (3.0 mmol) of (−)-11 and 0.3 mL (3.7 mmol) of pyridine in 20 mL of CH_2Cl_2 was stirred at 0 °C as 0.28 mL (3.6 mmol) of methyl chloroformate was added. The progress of the reaction was followed by TLC. After 0.5 h at 0 °C and 8 h at rt, the solution was washed with ice-cold 1 N HCl (25 mL), 5% NaOH (2 × 25 mL), and saturated NaCl (25 mL). After the organic fraction was dried ($MgSO_4$) and concentrated, Kugelrohr

(38) Micovic, V. M.; Mihailovic, M. L. *J. Org. Chem.* **1953**, *18*, 1190.
(39) Danheiser, R. L.; Martinez-Davila, C.; Sard, H. *Tetrahedron* **1981**, *37*, 3943.
(40) Freiberg, L. A. *J. Org. Chem.* **1965**, *30*, 2476.

distillation of the residue at 115 °C (0.7 mm) yielded 374 mg (73%) of (-)-(1*S*)-1-methyl (4-methyl-3-cyclohexenyl)carbamate: mp 50–50.5 °C; $[\alpha]^{20}_D$ -14.3° (*c* 1.00, $CHCl_3$); IR (KBr) ν_{max} 3281 (NH), 2965 (CH), 2911 (CH), 2838 (CH), 1686 (C=O), 1551, 1272, 1237, 1057 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.54–1.63 (m, 1 H, H at C6), 1.65 (s, 3 H, CH_3), 1.83–1.89 (m, 2 H, H at C5 and C6), 1.93–2.11 (m, 2 H, H at C2), 2.34 (br m, 1 H, H at C5), 3.66 (s, 3 H, CH_3O), 3.80 (br, 1 H, CHN), 4.65 (br, 0.2 H, NH), 4.80 (br, 0.8, NH), 5.29 (m, 1 H, —CH=); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.3, 28.1, 28.4, 28.5, 31.9, 45.9, 51.8, 118.2 (—CH=), 118.3 (—CH), 134.0 (—CMe=), 156.3 (CO), 156.4 (CO). Anal. Calcd for $C_9H_{15}NO_2$: C, 63.88; H, 8.93; N, 8.28. Found: C, 63.98; H, 8.99; N, 8.17.

(+)-(1*R*)-1-Methyl (4-methyl-3-cyclohexenyl)carbamate: yield 357 mg (70%); mp 50–50.5 °C; $[\alpha]^{27}_D$ +13.3° (*c* 1.00, $CHCl_3$). Anal. Calcd for $C_9H_{15}NO_2$: C, 63.88; H, 8.93; N, 8.28. Found: C, 63.94; H, 8.92; N, 8.34.

(-)-(1*S*)-*N*,4-Dimethyl-3-cyclohexenamine ((-)-12). A solution of 364 mg (2.2 mmol) of (-)-(1*S*)-1-methyl (4-methyl-3-cyclohexenyl)carbamate in 13 mL of ether was stirred at 0 °C as 7 mL (7 mmol) of 1.0 M $LiAlH_4$ was added. The mixture was refluxed for 4 h and cooled at 0 °C before it was quenched as described above for (+)-8.[38] After concentration of the ethereal filtrate, Kugelrohr distillation of the residue at 80 °C (20 mm) yielded 232 mg (86%) of (-)-12: $[\alpha]^{27}_D$ -84.6° (*c* 1.00, $CHCl_3$); IR (KBr) ν_{max} 3289 (NH), 2963 (CH), 2917 (CH), 2853 (CH), 1445, 1138, 799 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.1 (br, 1 H, NH), 1.39–1.48 (m, 1 H, H at C6), 1.65 (s, 3 H, CH_3), 1.75–1.92 (m, 2 H, H at C5 and C6), 2.01 (m, 2 H, H at C2), 2.25 (br, 1 H, H at C5), 2.45 (s, 3 H, CH_3N), 2.60 (m, 1 H, CHN), 5.31 (m, 1 H, —CH=); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.4, 28.9, 29.0, 32.2, 33.8, 54.6, 119.1 (—CH=), 133.9 (—CMe=). Anal. Calcd for $C_8H_{15}N$: C, 76.74; H, 12.07; N, 11.19. Found: C, 75.82; H, 12.03; N, 11.02.

(+)-(1*R*)-*N*,4-Dimethyl-3-cyclohexenamine ((+)-12): yield 228 mg (90%); bp 80 °C (20 mm); $[\alpha]^{27}_D$ + 83.9° (*c* 1.00, $CHCl_3$). Anal. Calcd for $C_8H_{15}N$: C, 76.74; H, 12.07; N, 11.19. Found: C, 72.58; H, 11.87; N, 10.48.

4-Chloro-2-methyl-2-butene:[41] yield 13.44 g (51%); bp 63–66 °C (150 mm). ^1H NMR analysis indicated the presence of 6% of 3-chloro-3-methyl-1-butene.

4-Methyl-3-pentenenitrile was prepared according to a literature procedure for homogeranic acid preparation with some modifications.[42] A slurry of 7.40 g (151 mmol) of NaCN in 65 mL of DMF was magnetically stirred at 0 °C as 13.40 g (128 mmol) of 4-chloro-2-methyl-2-butene in 5 mL of DMF was added. The cooling bath was removed after 10 min, and the temperature spontaneously rose to 50 °C and remained so for 1 h. After 2 h, the reaction mixture was diluted with water (350 mL) and the aqueous solution was extracted with pentane (1 × 100 mL, 2 × 50 mL). The combined organic extracts were washed with water (300 mL) and saturated NaCl (200 mL), dried ($MgSO_4$), and concentrated. Purification of the residue by chromatography using ether–pentane (1:15 to 1:10) as eluent and subsequent distillation at 64–65 °C (18 mm) gave 7.78 g (64%)[43] of 4-methyl-3-pentenenitrile. ^1H NMR and IR spectral data were in agreement with the literature values.[44]

4-Methyl-3-pentenoic Acid (14). A solution of 7.52 g (79 mmol) of 4-methyl-3-pentenenitrile in 150 mL of 2 N NaOH in 50% aqueous methanol was refluxed for 5 h. After concentration to 60 mL, the solution was diluted with water (250 mL) and washed with ether (2 × 150 mL). The aqueous fraction was acidified by adding 26 mL of concd HCl, and the solution was extracted with ether (2 × 150 mL). The combined organic extracts were washed with saturated NaCl (200 mL), dried ($MgSO_4$), and concentrated. Distillation of the crude product at 62–63 °C (0.4 mm) gave 5.43 g (60%) of 14 which was 95% pure as judged by ^1H NMR and GC analysis: IR (KBr) ν_{max} 2973 (CH), 2919 (CH), 2728 (COOH), 1711 (C=O), 1416, 1300, 1225, 939, 831 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.65 (s, 3 H, CH_3), 1.76 (s, 3 H, CH_3), 3.09 (d, 2 H, *J* = 7.1 Hz, CH_2), 5.30 (br t, 1 H, *J* = 7.1 Hz, =CH—). Further distillation of the remaining pot residue at 58 °C (0.2 mm) provided another 1.81 g (20%) of 90% pure 14.

(-)-(1*S*)-*N*,4-Dimethyl-*N*-(4-methyl-3-cyclohexenyl)-3-pentenamide ((-)-15). A slurry of 368 mg (2.3 mmol) of *N*,*N*′-carbonyldiimidazole in 3 mL of THF was stirred at rt as 285 mg (2.5 mmol) of 14 in 3 mL of THF was added. After 1 h at rt, 220 mg (1.8 mmol) of (-)-12 in 3 mL of THF was added, the solution was stirred for 10 h, the solvent was evaporated, and the residue was dissolved in ether (25 mL). The ethereal solution was washed with water (2 × 25 mL), saturated $NaHCO_3$ (25 mL), and saturated NaCl (25 mL) before it was dried ($MgSO_4$) and concentrated. Purification of the residue by chromatography using ether–hexane (1:1) as eluent and Kugelrohr distillation at 150 °C (0.7 mm) gave 348 mg (89%) of (-)-15: $[\alpha]^{27}_D$ -11.2° (*c* 1.00, $CHCl_3$); IR (KBr) ν_{max} 2965 (CH), 2919 (CH), 1642 (C=O), 1443, 1402, 1375, 1154, 1100 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.65 (s, 3 H, CH_3), 1.67 (s, 3 H, CH_3), 1.74 (s, 3 H, CH_3), 1.65–1.88 (m, 1 H, H at C6), 1.97–2.28 (m, 5 H, H at C2, C5, and C6), 2.79 (s, 1.5 H, CH_3N), 2.81 (s, 1.5 H, CHN), 3.06 (d, 1 H, *J* = 6.8 Hz, CH_2CO), 3.10 (d, 1 H, *J* = 7.0 Hz, CH_2CO), 3.82 (m, 0.5 H, CHN), 4.69 (m, 0.5 H, CHN), 5.27–5.34 (m, 2 H, —CH=). Anal. Calcd for $C_{14}H_{23}NO$: C, 75.97; H, 10.47; N, 6.33. Found: C, 75.94; H, 10.47; N, 6.31.

(+)-(1*R*)-*N*,4-Dimethyl-*N*-(4-methyl-3-cyclohexenyl)-3-pentenamide ((+)-15): yield 348 mg (89%); $[\alpha]^{25}_D$ +11.1° (*c* 1.00, $CHCl_3$). Anal. Calcd for $C_{14}H_{23}NO$: C, 75.97; H, 10.47; N, 6.33. Found: C, 76.04; H, 10.56; N, 6.29.

(-)-(1*S*)-*N*,4-Dimethyl-*N*-(4-methyl-3-pentenyl)-3-cyclohexenamine ((-)-16). A solution of 333 mg (1.5 mmol) of (-)-15 in 13 mL of ether was stirred at 0 °C as 7 mL (7 mmol) of 1.0 M $LiAlH_4$ in ether was added. The mixture was refluxed for 6 h. After cooling at 0 °C, the excess hydride was destroyed as described previously for (+)-8.[38] After concentration of the ethereal filtrate, Kugelrohr distillation of the residue at 125 °C (0.7 mm) gave 308 mg (99%) of (-)-(1*S*)-16: $[\alpha]^{27}_D$ -62.3° (*c* 1.00, $CHCl_3$); IR (KBr) ν_{max} 2965, 2924, 2843, 1449, 1375, 1123, 1103, 1051 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.47 (m, 1 H, H at C6), 1.62 (s, 3 H, CH_3), 1.64 (s, 3 H, CH_3), 1.69 (s, 3 H, CH_3), 1.84–1.91 (m, 1 H, H at C6), 1.95–2.07 (br, 4 H, H at C2 and C5), 2.15 (q, 2 H, *J* = 7.7 Hz, CH_2CH_2CH=), 2.29 (s, 3 H, CH_3N), 2.42–2.47 (m, 2 H, CH_2N), 2.61–2.70 (m, 1 H, CHN), 5.10 (br t, 1 H, *J* = 7.7 Hz, —CH=), 5.33 (br, 1 H, —CH=); ^{13}C NMR (75 MHz, $CDCl_3$) δ 17.8, 23.2, 25.6, 25.7, 26.6, 27.3, 30.8, 37.9, 53.5, 58.9, 120.0 (—CMe=), 122.1 (—CMe=), 132.6 (—CH=), 133.9 (—CH=). Anal. Calcd for $C_{14}H_{25}N$: C, 81.09; H, 12.15; N, 6.75. Found: C, 81.07; H, 12.17; N, 6.75.

(+)-(1*R*)-*N*,4-Dimethyl-*N*-(4-methyl-3-pentenyl)-3-cyclohexenamine ((+)-16) was prepared as described above. GC analysis of the product indicated the presence of 1% of amide (+)-15. The product was dissolved in ether and the solution was extracted with 1 N HCl (1 × 10 mL, 2 × 5 mL) to remove this impurity. The combined aqueous extracts were basified by adding 1.5 g of NaOH, and the solution was extracted with ether (1 × 10 mL). The combined ether extracts were dried ($MgSO_4$) and concentrated. Kugelrohr distillation of the residue at 125 °C (0.7 mm) gave 259 mg (93%) of (+)-(1*R*)-16: $[\alpha]^{27}_D$ +62.6° (*c* 1.00, $CHCl_3$). Anal. Calcd for $C_{14}H_{25}N$: C, 81.09; H, 12.15; N, 6.75. Found: C, 81.07; H, 12.14; N, 6.78.

Acknowledgment. This work was supported by NIH grants GM30301 to D.E.C. and GM13956 to R.B.C. We would like to thank Professor Vernon Anderson for providing a copy of the program KINFIT and for helpful discussions.

Supplementary Material Available: Plots of inhibition kinetics (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(41) Naruta, Y.; Nishigaichi, Y.; Maruyama, K. Unchecked procedure submitted to *Org. Synth.*; see Vol. *70*, 1991, 279, procedure No. 2551R*.

(42) (a) Mori, K.; Funaki, Y. *Tetrahedron* **1985**, *41*, 2369. (b) Barnard, D.; Bateman, L. *J. Chem. Soc.* **1950**, 926.

(43) About 10% of the solution was lost during workup.

(44) Kanai, T.; Kanagawa, Y.; Ishii, Y. *J. Org. Chem.* **1990**, *55*, 3274.