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Terpenoid cyclases: design and function of electrophilic catalysts

David E. Cane

Department of Chemistry, Brown University, Providence, RI 02912, USA

Abstract. Terpenoid cyclases catalyse the cyclization of the universal acyclic precursors geranyl and farnesyl diphosphate to monoterpenes and sesquiterpenes, respectively. All such cyclases investigated to date are operationally soluble, moderately lipophilic proteins of relative molecular weight 40 000–100 000, requiring no cofactors other than a divalent metal, usually Mg^{2+} and occasionally Mn^{2+}. The focus of most work has been on the mechanisms of the cyclization reactions themselves. It is currently proposed that the cyclase binds the acyclic substrate in a suitable conformation and initiates the cyclization by ionization of the labile allylic diphosphate moiety. The use of stereospecifically labelled substrates and analysis of the sites of labelling in the derived cyclization products has allowed the proposal of detailed cyclization mechanisms. Further insight into the architecture and function of the cyclase active site has come from the study of substrate and intermediate analogues designed to act as potential inhibitors or anomalous substrates of the normal cyclization reaction. Progress has also been made on the cloning of the relevant structural genes for sesquiterpene cyclases. This has led to new insights into the basic requirements for cyclase catalysis and specificity.

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As pointed out by J. W. Cornforth nearly 25 years ago in a review entitled 'Olefin alkylation in biosynthesis', while the majority of biologically formed carbon–carbon bonds are generated by condensations of the aldol and Claisen type, the biosynthesis of iosprenoids is distinguished by its nearly exclusive reliance on electrophilic reactions involving carbon–carbon double bonds (Cornforth 1968). The characteristic reactions of polyisoprenoid chain assembly and cyclization are initiated by one of two general reactions: (1) protonation or alkylation of a double bond or epoxide; or (2) ionization of an allylic diphosphate ester. Attack of the resulting electrophilic species on an olefinic bond, in an intermolecular or intramolecular fashion, generates one or more carbocationic intermediates, with positive charge ultimately being quenched by loss of a proton or capture of an external nucleophile such as water.

Although the biosynthesis of prenylated proteins, acyclic polyisoprenoids and cyclized terpenoids has been the subject of intensive study, the fascinating family of proteins which mediate the formation of this functionally diverse and structurally rich family of metabolites is still poorly understood from the point of view of structure and catalytic mechanism. Our own group has been particularly interested in the terpenoid cyclases, a family of enzymes which catalyse the cyclization of farnesyl diphosphate to any of 200 different sesquiterpene hydrocarbons or alcohols, as well as the related monoterpene cyclases mediating the cyclization of geranyl diphosphate (Cane 1990, Croteau 1987). Only a relative handful of such enzymes have so far been isolated and characterized, yet certain trends have already begun to emerge. All cyclases investigated to date are operationally soluble, moderately lipophilic, monomeric or homodimeric proteins of subunit relative molecular weight 40 000–65 000, requiring no cofactors other than a divalent metal, usually Mg^{2+} and occasionally Mn^{2+}. Experiments with inhibitors are consistent with the notion that all the events in a multistep cyclization take place at a single active site; there is no evidence supporting the intervention of any covalently bound intermediates. On the other hand, the few cyclases which have been cloned and sequenced show no homology at either the DNA or protein level to any other known protein, nor is there any substantive information about the nature and role of the amino acid residues which make up the cyclase active sites.

How, one might reasonably ask, can one claim to know something about the 'design and function of electrophilic catalysts' when so little is known about the structure and mode of action of the catalysts themselves? Fortunately, we now know enough about terpenoid cyclization reactions that we can at least begin to formulate appropriate questions and even to discern the shape, if not the details, of the answers we shall eventually obtain. The current state of affairs can best be put in perspective by considering what is perhaps the most thoroughly studied sesquiterpene cyclase, trichodiene synthase.

Trichodiene synthase has been isolated from a variety of *Fusarium* species (a group of plant pathogenic fungi which produce a number of potent metabolites toxic to livestock and humans). The synthase catalyses the cyclization of *trans,trans*-farnesyl diphosphate (FPP) (**1**) to trichodiene (**2**), the parent hydrocarbon of the trichothecene family of mycotoxins (see Fig. 1) (Hohn & VanMiddlesworth 1986, Hohn & Beremand 1989a). Extensive experiments with labelled substrates have led us to propose the cyclization mechanism illustrated in Fig. 1 in which the primary allylic substrate is initially rearranged to its tertiary allylic isomer, nerolidyl diphosphate (NPP) (**3**), which then undergoes ionization and cyclization, by way of a series of rearranged intermediates, with ultimate formation of trichodiene (Cane & Ha 1988, Cane et al 1985, 1990a). Although the isomerization and cyclization components of the reaction obviously differ in their gross consequences, they are believed to be very similar in

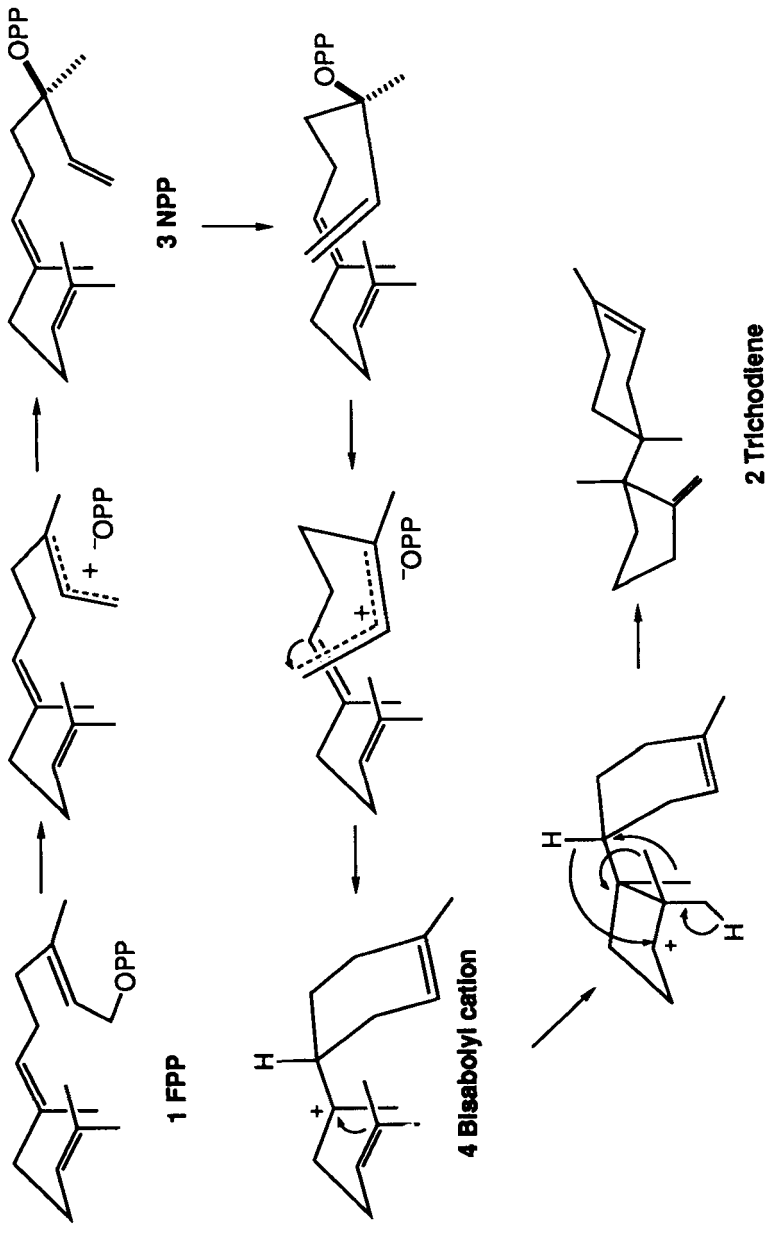

FIG. 1. Cyclization of farnesyl diphosphate (FPP) (1) to trichodiene (2).

microscopic mechanism, involving the ionization of an allylic diphosphate ester to an allylic cation–pyrophosphate anion pair, followed in each case by either recapture of the pyrophosphate anion or electrophilic attack on the neighbouring central double bond, respectively. The mechanism itself can be considered a prototype for all such cyclization reactions, in that the initial folding of the acyclic substrate, FPP, as well as that of the derived intermediate NPP, is believed to be a major determinant of the structure and stereochemistry of the eventually formed cyclic product (Cane 1985).

Allylic diphosphate ionization

Physical organic studies have suggested the special advantages of allylic diphosphate esters from the point of view of both acid lability and cation stability (Tidd 1971, Cane 1980). Moreover, studies of prenyl transferases, whose mode of action bears many mechanistic and catalytic similarities to those of the terpenoid cyclases, have established that the actual ionization substrate is the allylic diphosphate–divalent magnesium ion complex, the role of the metal presumably being to hold the diphosphate moiety in a defined conformation and to assist in its ionization by neutralization of the negative charge of the phosphoryl oxygen atoms (Poulter & Rilling 1981). How then is this ionization initiated at the cyclase active site? To date, none of the amino acids present at the active site of trichodiene synthase, nor any other sesquiterpene cyclase, has been identified, although the closely related monoterpene cyclases can be inhibited by sulphydryl-directed reagents. On the other hand, photoaffinity labelling of avian liver FPP synthase, which catalyses the mechanistically related intermolecular coupling of an allylic diphosphate substrate with the olefinic acceptor isopentenyl diphosphate, has implicated an arginine residue found within an active site peptide, EERYK, as possibly being involved in binding of the pyrophosphate moiety (Brems et al 1981). Indeed, closely related sequences have been found in the rat and human fetal liver FPP synthases, although the corresponding yeast gene shows only a weakly homologous sequence, with a basic lysine residue in place of the arginine (Cane 1992). Interestingly, cloning of the trichodiene synthase genes of both *Fusarium sporotrichioides* and *Gibberella pulicaris* has revealed the presence of similar basic amino acid-rich sequences (DRRYR in *F. sporotrichioides* and DHRYR in *G. pulicaris*) (Hohn & Beremand 1989b, Hohn & Desjardins 1991). The functional significance of these regions in trichodiene synthase remains to be demonstrated, although Hohn has found that replacement of the conserved arginine with lysine reduces the observed cyclase activity by 98%, while substitution by glutamate abolishes all but 0.1% of the trichodiene synthase activity (T. M. Hohn, personal communication 1991). In spite of these suggestive observations, a comparable basic amino acid-rich domain is absent from aristolochene synthase (T. M. Hohn, personal communication 1991)

and preliminary sequence data on a third sesquiterpene cyclase, pentalenene synthase, have so far failed to reveal any such homologous sequences (D. E. Cane & C. R. Lamberson, unpublished results).

Edwards has noted the presence of two aspartate-rich domains in yeast hexaprenyl diphosphate synthase and has pointed out that the same (I,L, or V) XDDXXD consensus sequence is present in several FPP synthases as well (Ashby & Edwards 1990). This has led him to propose that the aspartate residues may somehow be involved in substrate binding, perhaps by chelation of the divalent metal. It is therefore of some interest that the *F. sporotrichioides* trichodiene synthase contains the sequence VLDDSKD beginning at amino acid 98, while the corresponding trichodiene synthase gene from *G. pulicaris* (*Tox5*) has the inferred peptide sequence VLDDSSD at the same site. Similarly, sequencing of aristolochene synthase has established the presence of a related heptapeptide, LIDDVLE, starting at amino acid 113 (T. M. Hohn, personal communication 1991). Homologous sequences are also present in pentalenene synthase, on the basis of preliminary sequence data (D. E. Cane & C. R. Lamberson, unpublished results). On the other hand, a scan of the PIR™ Protein Sequence database using the consensus sequence (I,L,V,H)X(D,N)DXX(D,E) provides some 1200 matches out of 27 000 sequences surveyed, close to the statistically expected frequency of one out of every 20 proteins, thereby raising interesting questions about the functional significance of this acidic peptide sequence. The identity of the active site residues involved in binding and ionization of the diphosphate moiety of allylic diphosphate esters therefore remains to be firmly established.

Substrate folding and stabilization of positive charge

In order to mediate the conversion of FPP to a single cyclic sesquiterpene, trichodiene synthase must not only control the folding of the substrate as well as that of several derived intermediates; it must also stabilize the various carbocationic species which have been generated at the active site, all the while avoiding accidental annihilation by these highly reactive electrophiles. Little if anything is known about the manner in which an enzyme can enforce a particular conformation on the lipophilic portion of a substrate such as FPP. Indeed, the problem is further complicated by the recognition that in the course of the formation of the eventual cyclized product, FPP must undergo substantial changes in bonding, hybridization and configuration. To illustrate the dimensions of the problem, Fig. 2 shows the presumed shapes of several key intermediates of the trichodiene synthase reaction, no two of which would be expected to be bound by an active site rigidly complementary to either one or the other. The single active site of trichodiene synthase must therefore somehow accommodate and control the reactivity of all of them. The necessity of allowing considerable freedom of movement in related squalene cyclizations was recognized long ago by Cornforth, leading him to postulate so-called X-group

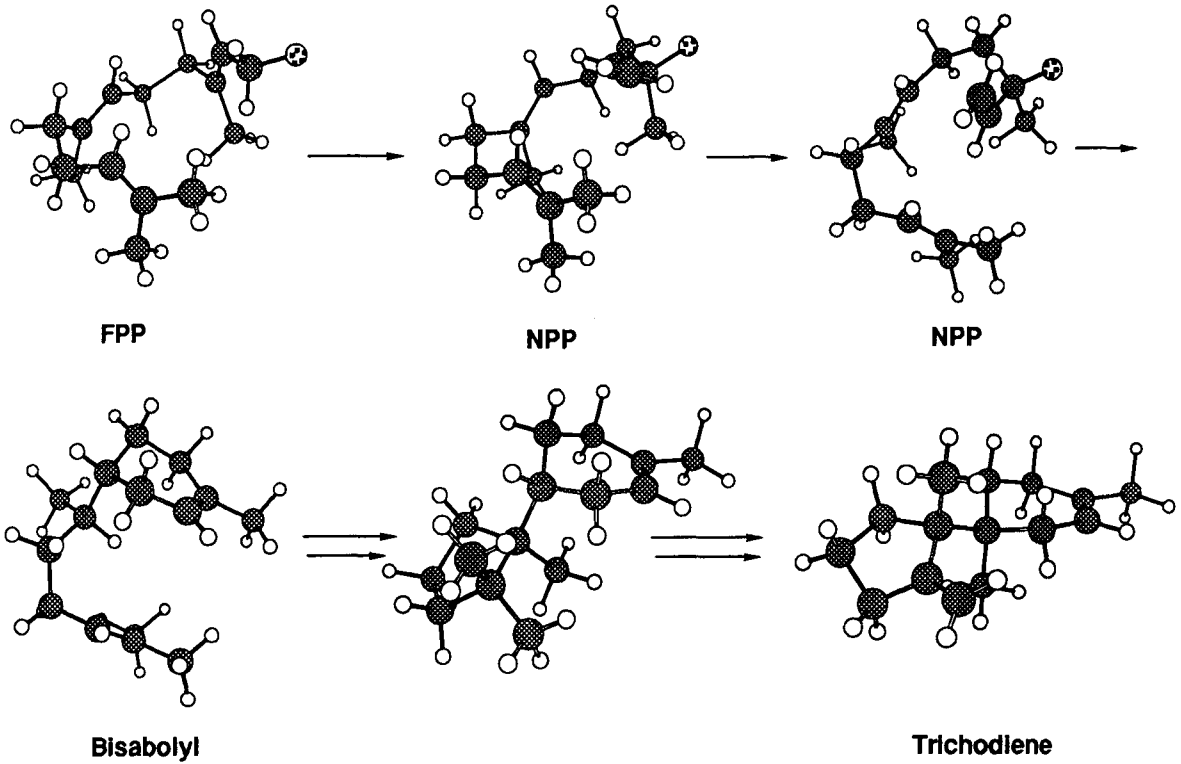

FIG. 2. Likely conformations of substrate FPP and intermediates in the formation of trichodiene. For simplicity, only the ester oxygen of the diphosphate moiety is shown. Carbocationic centres which undergo rearrangement are shown as tetrahedral centres carrying an extra hydrogen atom.

mechanisms to account for stereospecific rearrangements of carbonium ions at a single active site (Cornforth 1968). Unfortunately, there is still no firm evidence which explicitly supports the intervention of X-groups in terpenoid cyclizations. The problem of accounting for the ability of a cyclase active site to accommodate a variety of intermediates of varying shape and charge distribution remains unresolved.

In collaboration with Professor Robert Coates of the University of Illinois, we have probed the manner in which trichodiene synthase interacts with its various charged intermediates by testing a family of ammonium ion analogues of the proposed bisabolyl cation intermediate (**4**) (Fig. 1) for their relative ability to inhibit the normal cyclization reaction (Cane et al 1992). It was initially observed that the aza analogue (*R*)-**5** alone was at best a weak inhibitor of trichodiene synthase (Fig. 3). Interestingly, the protonated amine became a strong competitive inhibitor of the cyclization when incubated with inorganic pyrophosphate, itself a known competitive inhibitor of trichodiene synthase. Unexpectedly, the enantiomeric analogue, (*S*)-**5**, showed identical behaviour, indicating that the cyclase could not distinguish between the individual enantiomers of this inhibitor. Inhibition by the corresponding α-terpinyl cation analogues, (*R*)- and (*S*)-**6**, showed the same requirement for pyrophosphate ion but with a 4–8-fold weaker

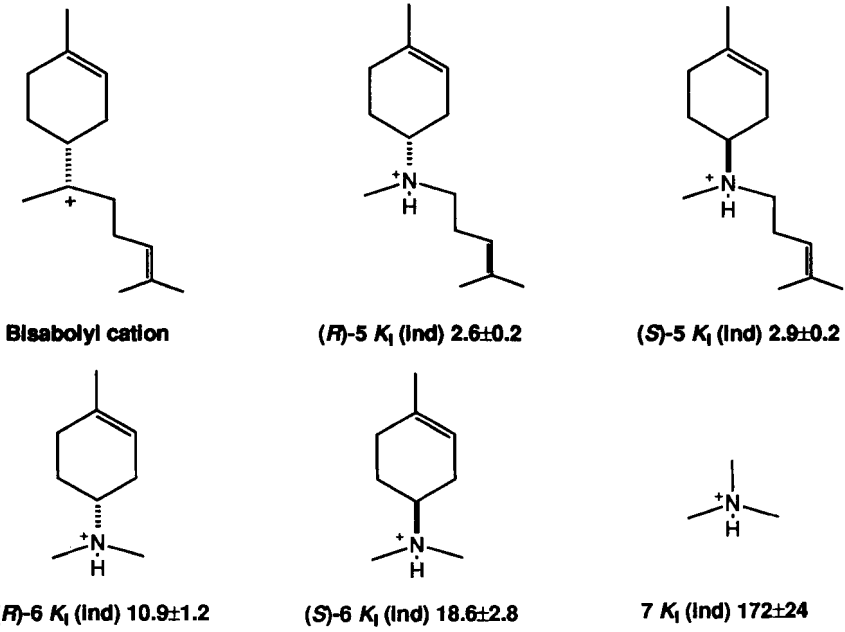

FIG. 3. Ammonium ion analogues of the bisabolyl cation as inhibitors of trichodiene synthase. Values for K_I (ind) are competitive inhibition constants in the presence of added inorganic pyrophosphate.

induced binding affinity. Once again, however, both enantiomers were essentially equally effective as inhibitors. Finally, the simplest analogue, trimethylammonium ion (7), while weaker by another factor of 10, also exhibited the same requirement for inorganic pyrophosphate. These results indicated that both electrostatic and hydrophobic interactions are important for the binding of each intermediate analogue. Moreover, the observation that inorganic pyrophosphate potentiates—indeed, that it is required for—inhibition suggests that during the normal course of the cyclization the pyrophosphate moiety is not simply a passive product of the initial ionization of the FPP substrate but may well play a role in the stabilization of the cascade of cationic intermediates which link FPP and trichodiene. Similar synergistic effects of inorganic pyrophosphate on terpenoid cyclase or chain elongation enzymes have previously been reported. For example, although neither pentalenene nor inorganic pyrophosphate alone at concentrations of 10 μM significantly inhibited the cyclization of FPP to the tricyclic sesquiterpene pentalenene, the combination of the two together at the same concentration increased the apparent K_m for FPP by a factor of seven (Cane & Pargellis 1987). Similarly, various cyclopropyl ammonium ion analogues of proposed intermediates in the conversion of presqualene diphosphate to squalene become potent inhibitors only in the presence of added inorganic pyrophosphate (Poulter et al 1989). The apparent inability of trichodiene synthase to discriminate between the enantiomeric ammonium ion analogues of the bisabolyl cation is itself reminiscent of our earlier observation that several monoterpene cyclases were unable to discriminate cleanly between the individual enantiomers of their natural tertiary allylic intermediate, linalyl diphosphate (Croteau et al 1986, 1988).

Permissive model of cyclase active sites

Taken together, these results are all supportive of what may be termed a *permissive* model of terpenoid cyclase active site structure. Rather than being rigidly complementary to some or all of the various cyclization intermediates, the cyclase appears to be considerably looser fitting, thereby accommodating a family of related structures derived from the initial folding and ionization of the precursor FPP. According to this model, the structure and stereochemistry of each intermediate is largely the consequence of the geometry of its immediate precursor. The cyclase need not be able to discriminate among all *possible* geometries of any given intermediate, since these substances are normally not encountered free in solution, but need only provide sufficient constraints so as to limit the conformations available to an intermediate which has been generated at the active site itself. In this sense, one can think of the active site of a cyclase such as trichodiene synthase as more closely resembling a mitten than a glove in its interaction with its natural substrates and intermediates. Whether this model is at all accurate will have

to be determined by detailed structural analysis of the interaction of this and other cyclases with a range of substrate and intermediate analogues.

Cyclization of anomalous substrates

Further evidence for the ability of cyclase active sites to accommodate a range of anomalous substrate geometries has come from work carried out recently in our laboratory aimed at elucidating the intermediates of the reaction catalysed by the fungal cyclase, aristolochene synthase, isolated from *Aspergillus terreus*. On the basis of the cyclization of various labelled precursors, we have proposed that the formation of aristolochene (**8**) from FPP involves an initial cyclization to germacrene A (**9**) which in turn undergoes protonation of the 6,7-double bond and cyclization to aristolochene through the intermediacy of a eudesmane-type cation (Fig. 4) (Cane et al 1989, 1990b, Hohn & Plattner 1989b). In the course of the normal cyclization reaction, the postulated germacrene A intermediate never leaves the cyclase active site. Unfortunately, the thermal lability of germacrene A renders impractical any attempt to study the enzymic cyclization of synthetically prepared **9**. We therefore chose the alternative of incubating aristolochene synthase with (7*R*)-6,7-dihydroFPP (**10**). It was expected that cyclization of **10** would result in the formation of 6,7-dihydrogermacrene A (**11**). Since the latter intermediate lacks the double bond which would normally be the target of further protonation, **11** would be expected to be released from the active site, allowing expression of the otherwise cryptic initial cyclization step. Furthermore, since **11** is not subject to the facile Cope rearrangement characteristic of germacrene A, the abortive cyclization product would be able to accumulate in the incubation medium. In the event, **10** proved to be an effective competitive inhibitor of aristolochene synthase, with K_I 0.15 μM, compare to K_m 3.0 μM for FPP. Even more interestingly, aristolochene synthase converted **10** to a hydrocarbon of molecular mass 206 Da which proved to be identical by gas chromatography/mass spectrometry (GC/MS) comparison with one of two synthetically prepared epimers of dihydrogermacrene **11**, differing from one another only in the configuration of the isopropenyl side chain. Work is in progress to establish the full stereochemistry of the anomalous cyclization product (D. E. Cane & Y. S. Tsantrizos, unpublished).

In the meantime, we have also examined the role of **10** as a competitive inhibitor and anomalous substrate of *epi*-aristolochene synthase, a cyclase isolated and purified from tobacco (*Nicotiana tabacum*) tissue cultures by Professor Joseph Chappell of the University of Kentucky, which converts FPP to *epi*-aristolochene (**12**) (Vögeli et al 1990). The mechanism of the latter cyclization is believed to be closely related to the aristolochene synthase reaction, the major difference being the precise stereochemistry and folding of the FPP substrate as well as that of the derived germacrene A intermediate (Fig. 5).

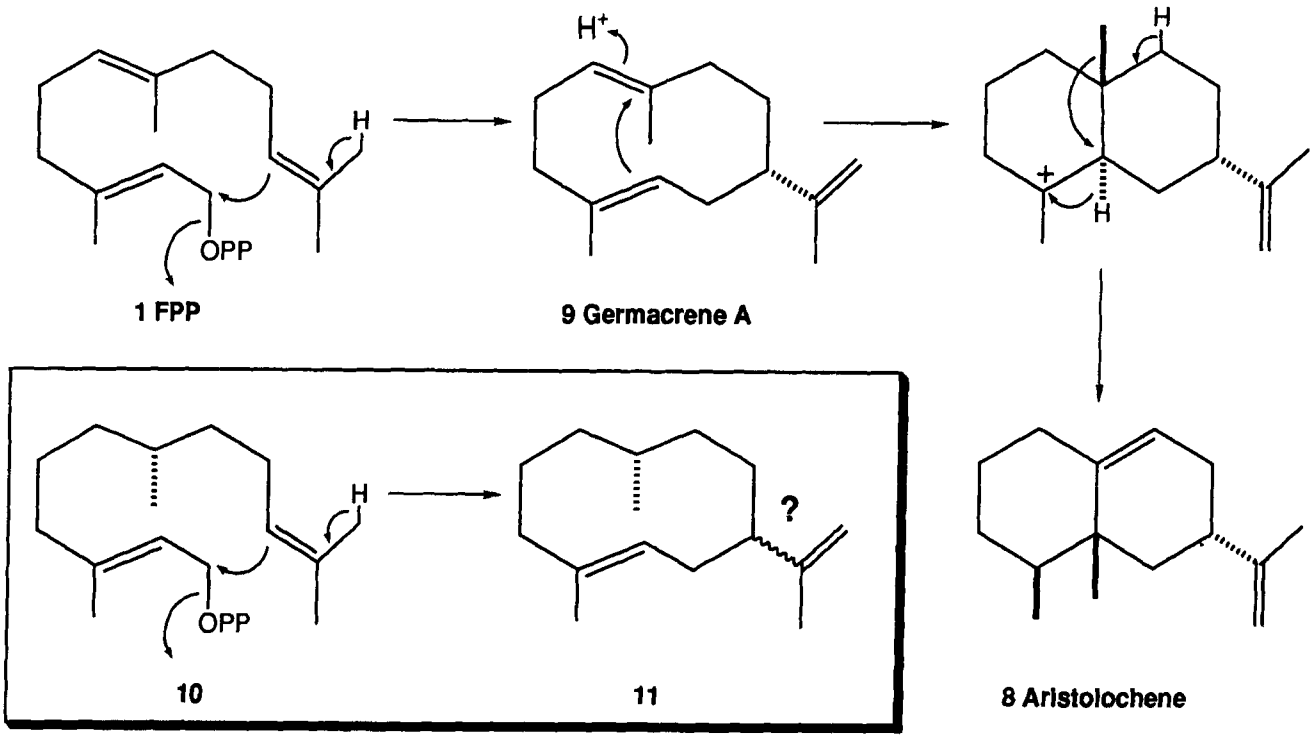

FIG. 4. Cyclization of FPP to aristolochene (**8**) through the intermediacy of germacrene A, and the cyclization of dihydroFPP (**10**) to dihydrogermacrene (**11**) by aristolochene synthase.

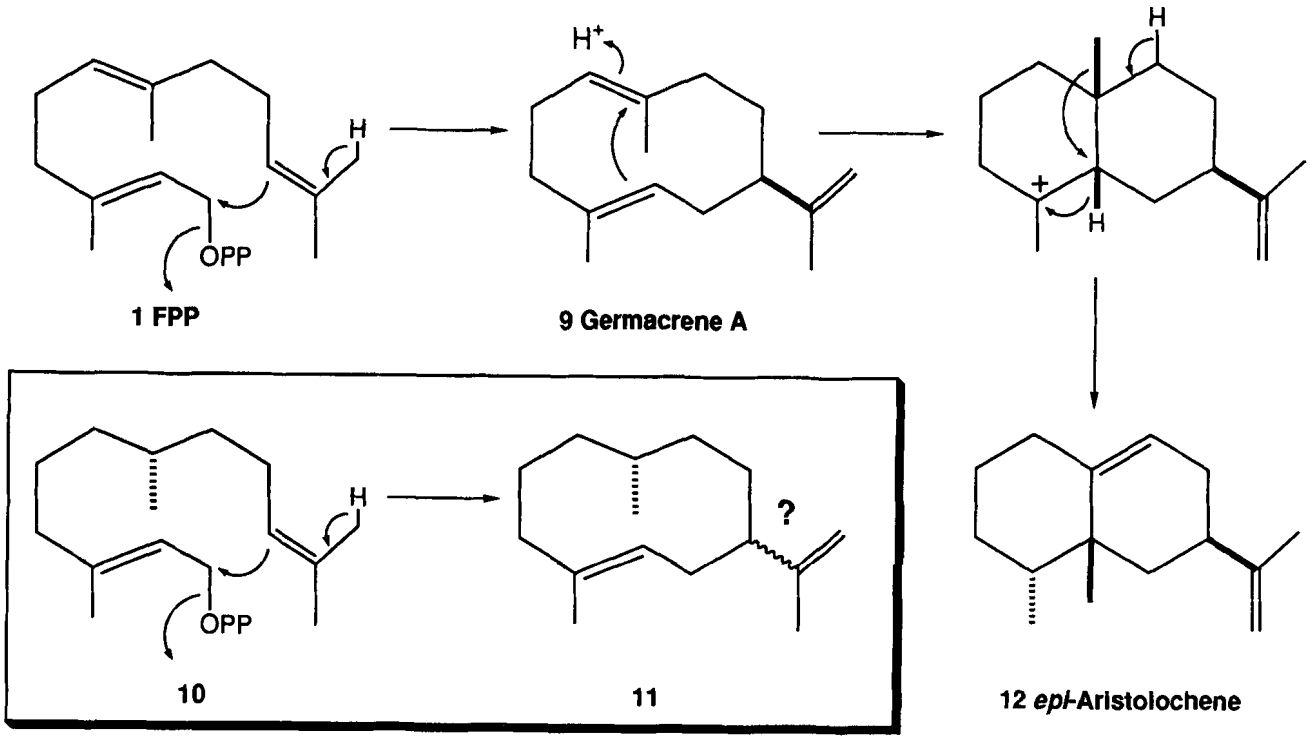

FIG. 5. Cyclization of FPP to *epi*-aristolochene (**12**) through the intermediacy of germacrene A and cyclization of dihydroFPP (**10**) to dihydrogermacrene (**11**) by *epi*-aristolochene synthase.

In fact, we have found that *epi*-aristolochene synthase converts **10** to a dihydrogermacrene A (**11**) isomer, but, unexpectedly, this isomer is *identical* by GC/MS comparison with the epimer of **11** generated by aristolochene synthase. Thus abortive cyclization of the anomalous substrate dihydroFPP (**10**) supports the proposed intermediacy of germacrene A in the normal cyclization catalysed by aristolochene and *epi*-aristolochene synthases. One of these two cyclases, however, is obviously converting dihydroFPP to the dihydrogermacrene product with the 2-propenyl moiety in an unnatural configuration. Presumably, the natural permissiveness of the cyclase active site is being reflected in binding of the anomalous substrate analogue in an unnatural conformation.

Future directions

Clearly, the understanding of terpenoid cyclase active site topology and catalytic mechanism is still at a rudimentary stage. Nonetheless, recent progress in the cloning and expression of terpenoid cyclase genes has opened up the possibility of further advances on a number of fronts:

1. The identification of substrate and intermediate analogues which act as effective competitive cyclization inhibitors, combined with the cloning and expression of cyclase genes (Hohn & Plattner 1989a), suggest that it may soon be possible to obtain X-ray crystallographic data on the precise geometry of binding of these inhibitors to the cyclase itself.
2. Experiments are in progress to identify effective analogues acting as irreversible inhibitors which can be used to identify active site residues by covalent modification.
3. Once sequences of additional cyclases become available, it should be possible to look for homologies at the DNA and amino acid sequence level, in order to identify conserved features of functional importance.
4. The availability of larger quantities of recombinant cyclases could allow detection of cyclization intermediates through the application of rapid quench and related kinetic techniques.

When these advances are combined with parallel advances in the understanding of terpenoid chain assembly (prenyl transferases, squalene synthase) and cyclization (oxidosqualene:lanosterol cyclase) enzymes, the way now seems open to an understanding of the way in which this important family of electrophilic catalysts carry out their complex and fascinating reactions.

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DISCUSSION

Chater: Can one imagine a situation in which the substrate goes in through one 'door'and the product comes out through another, and everything possible happens inside, but the only thing that can get out is the product you want?

Cane: The experiments with the anomalous substrates indicate that abortive products can come out *earlier*. These cyclases show product inhibition and one can prevent the binding of the substrate with inorganic pyrophosphate. In fact, binding is potentiated for pentalenene synthase by the presence of pentalenene, so pentalenene plus inorganic pyrophosphate constitutes a more effective inhibitor than either alone.

There is a limitation to steady-state kinetics, but what those kinetics mean is that there is a single active site, so the inhibitors and substrate both compete for the same place. Steady-state kinetics do not tell you how you reach the active site or whether, when something leaves the enzyme, it flies off in the same or a different direction as when it approaches the enzyme. But there's no evidence that the various catalytic events happen in different places.

There are monoterpene cyclases which make product mixtures. In experiments in collaboration with Rod Croteau at Washington State University, we diverted the formation of the mixed products formed by pinene cyclase (Croteau et al

1987). The monoterpene cyclases tend to be more 'sloppy'; of course in any single turnover you form one product, but in a collection of turnovers, you tend to form different products, derived from quenching the common carbocations at different stages.

Orgel: What are the obstacles to obtaining enough of the enzyme to crystallize it and then proceed to determine the structure in the normal way?

Cane: As with many enzymes involved in this type of metabolism, where the products accumulate over a relatively long period, and are not themselves essential to the organism, there are very low titres of the enzyme. For example, from 20–30 litres of growth culture of *Streptomyces* we could isolate sufficient purified pentalenene synthase for sequencing, namely 100–200 pmol. In principle, there is no natural impediment to the over-expression of the cyclase gene.

Davies: What organisms are you using to produce the enzyme?

Cane: We are expressing trichodiene synthase in *E. coli*. Dr Hohn has shown that not only is the enzyme expressed, but it seems to appropriate endogenous farnesyl diphosphate, because the *E. coli* will produce trichodiene. This cyclase, as well as aristolochene synthase, does not seem to express well in the traditional vectors. This probably has nothing to do with it being lethal, because you can express the cyclase and the cells function perfectly well. It is probably more to do with secondary structure and codon usage. But I think it can be done. [Note added in proof: With trichodiene synthase, we have recently achieved substantial levels of over-expression, 15–25% of total protein (D. E. Cane & Z. Wu, unpublished).]

Rinehart: I gather that aristolochene synthases are different from *Penicillium roqueforti* and from *Aspergillus terreus*. Is that based on sequencing?

Cane: There are three pieces of evidence. One is the gross molecular size: the *Penicillium* cyclase is just under 40 kDa, whereas the *Aspergillus* enzyme is close to 60 kDa. Secondly, antibodies to the aristolochene synthase from *Penicillium roqueforti* do not cross-react with the *Aspergillus terreus* enzyme. And, thirdly, the cloned *P. roqueforti* structural gene for aristolochene synthase, when used as a probe against *A. terreus* DNA, shows no stringent hybridization; so there appear to be sequence differences.

Trichodiene synthase is present in a large number of *Fusarium* species that are closely related. You can use DNA from *F. sporotrichioides* as a probe for *Gibberella pulicaris* DNA. But *Myrrothecium verrucaria* also produces other trichothecanes and there's no hybridization to *M. verrucaria* DNA. So there is already a strong divergence, even within closely related fungal species.

Rinehart: Do different *Penicillium* species give the same aristolochene synthase, and do different *Aspergillus* species all give the same synthase?

Cane: I can't say, because the aristolochene synthase activity has been detected only in the two fungi. In *P. roqueforti*, aristolochene is the hydrocarbon precursor of a toxin, called PR toxin; this is presumably present in roquefort cheese! There is a family of structurally related compounds, such as the

eremofortins, which occur as mycotoxins in various other *Aspergillus* species. Some of these are also plant pathogens. Nobody knows the oxidation product of aristolochene in *Aspergillus terreus*. We found aristolochene completely by accident; we were looking for a cyclase that would make the precursor of quadrone. We still have no idea what the quadrone cyclase is, but we found three other sesquiterpene hydrocarbons, and the cyclase for one of them.

Davies: Have you ever used specific antibodies to look at the presence of cyclases in other strains? In general, does the anti-cyclase or anti-synthase antibody pick up this kind of activity?

Cane: We are doing experiments using polyclonal antibodies that people have given us; some experiments are being done in other labs. An antibody raised by Tom Hohn of the US Department of Agriculture against aristolochene synthase from *Penicillium roqueforti* does not cross-react with the *epi*-aristolochene synthase from the tobacco plant (T.M. Hohn, personal communication); antibodies to trichodiene synthase do not cross-react with any other cyclases. The idea of an essentially universal DNA probe with which to look for cyclases in a range of organisms doesn't seem to be on.

Brückner: Do you know whether there are different enzymes for the first steps in steroid biosynthesis, for the construction of membranes, and for secondary metabolite synthesis?

Cane: This is not known. We have not looked at the prenyl transferases of the fungal systems; in the streptomycetes, as far as one knows, there are no steroids; so the cyclic sesquiterpene pathway is in that sense unusual. The *Streptomyces* which produces pentalenene is not the only streptomycete with the ability to make cyclic terpenes (Gerber 1971). In the fungi we don't know whether there is any specialization in terms of the source of acyclic prenyl units.

Brückner: In yeast there are two different HMG-CoA reductases, but in higher fungi, I don't know.

Haslam: It's my experience in the area of plant phenolic chemistry that far from things being totally promiscuous, they are only partially promiscuous, in the sense that of the number of pathways of phenol oxidation which are possible, only a certain number of these are found. I wonder whether you agree, Dr Cane, with the proposition that once a certain chemical type of intermediate has been set up, part of the whole process has already been determined, and then follows a purely chemically determined pathway.

Jeremy Knowles has worked on the cyclization of a seven-carbon sugar (DAHP) to give 3-dehydro-quinate, at the beginning of the shikimate pathway (Knowles 1989, Bender et al 1989a,b). The enzyme 3-dehydroquinate synthase catalyses in principle four distinct chemical steps: an oxidation, an elimination, a reduction, and then a cyclization. The thrust of his work is that only the oxidation and reduction are enzymically controlled. The elimination and cyclization reactions are determined purely by the intrinsic chemistry of the system, and do not need enzyme catalysis. I wonder whether, once you have

set up a particular type of carbocation intermediate, the rest of the reaction then follows as a result of the inherent chemistry of the intermediates.

Cane: I would think so. The notion is that even though the chemistry is not mediated by the enzyme, it is still taking place within the cavity of the enzyme, and therefore one is preventing side reactions. For the cyclases, one is preventing interception of intermediates by water. The idea that one would have to make a carbocationic rearrangement go faster doesn't work out, from what is known about the rates, in a super acid solution or gas phase. One is looking for control, basically, and this has to come from (among other things) holding groups in proximity to one another, or holding them in the right stereo-electronic orientation, in the same way, for example, that many pyridoxal-dependent processes depend on the conformation of the amino acid relative to the pyridoxine ring. The chemistry needs little catalysis, but the product-forming event needs channelling, to prevent all possible reactions from happening. By retarding the processes that you don't want, you are not necessarily making the reactions that you do want go substantially faster, because the turnover rates are very small compared to what are probably the inherent chemical rates. Isn't that what you think about IPNS, Dr Baldwin?

Baldwin: In reactions that proceed through potentially very reactive intermediates, which can be intercepted or diverted, it must be a critical role of the enzyme protein to channel the pathway.

It seems to me that in all these terpene cation cyclizations, once you have established the first carbonium ion, the process of the reaction is the translation in space of a positive charge, through (in the case of the squalene cyclization) an enormous distance of about 12Å. Control of the pathway may be better achieved, not by conformational interactions with the surrounding environment at the active site, but by some means of stabilizing the motion of a positive charge, because the electrostatic effect is a much more potent effect than non-bonding interactions. This would fit in with what you seem to find, namely a very sloppy active site that can adjust quite readily even to different enantiomers.

Cane: The squalene case is a good comparison. When you are separating positive charge, after a separation of about 3 Å it doesn't cost much to get it further, because the attraction is falling off as the square of the distance. But with squalene, the charge goes out to one end of the molecule, after which there is a series of rearrangements by which the charge comes back quite close to where it started. With the squalene oxide to lanosterol cyclization, the whole molecule turns inside out, so there is a gross change in shape. The eventually formed sterol is not going to look anything like the initial folding of the substrate.

Bu'Lock: To what extent is this due not so much to the conformation of the cyclizing molecule, but to the location of the ultimate acceptor site, which in many cases will be a potential proton acceptor? In the alternative cyclizations of squalene oxide in mammalian systems versus plant systems, in the plant

systems, cycloartenol, a sterol with a cyclopropane ring, is generated, where the animal systems generate lanosterol. This seems a *priori* to depend on whether you have the proton acceptor on the α or β side of the system.

Cane: The position of counter bases may be critical. The only piece of relevant information that I have, because no one knows anything about what's present at the active sites, is in the formation of pentalenene. We have shown that a proton is removed in an initial cyclization, but that this proton is re-added to the adjacent carbon without any 'cross-talk' with the medium (Cane et al 1984). There is presumably a single base mediating deprotonation–reprotonation. We have looked at the stereochemistry of the final deprotonation which quenches this system (Cane et al 1991). We can't prove that this deprotonation involves the same base, but we know that the proton which is removed is very close to where the original base would be present. It is conceivable that a single base does triple duty: it pulls off a proton; it puts the proton back on an adjacent carbon; and then, after a further cyclization and rearrangement, it removes a proton from a site immediately adjacent to the one where it removed a proton initially. In principle, the reaction could have stopped after the first deprotonation, but that is not sufficient, because it is capable of going on further. We want to find out what is present around the substrate; this will address some of these questions. My own view is that once one knows the full structure of any enzyme, all understanding of how it works immediately disappears; that is either a psychological or a conceptual principle!

Leadlay: Are you saying you don't *need* many of these counter groups on the enzyme? You seem to be saying that there is quite a sloppy interaction; is it that you just have one base, or very few, and the intermediates can actually rotate around, and in this way you can get deprotonation and quench at different points?

Cane: I think you need a few counter groups, because of the way the charge moves; if you track it in any given cyclization, it moves from one side of the active site to the other. Some people have speculated that a major tactic for controlling the charge is that the enzyme uses the pyrophosphate moiety to steer things. Our own inhibition studies, and also some nice work by Dale Poulter on squalene synthase, have shown that the pyrophosphate group is critical to the effectiveness of certain ammonium ion analogues (Poulter et al 1989). He has also made bi-substrate analogues of prenyl transferase and shown that these can be cyclized. His argument is that the pyrophosphate is acting as a base.

Williams: On the question of the tolerances that the enzymes will allow, it was striking that in your ammonium ion analogue of the cation intermediate, you could remove very large hydrocarbon side chains; the compound was then just a weaker inhibitor, by a factor of four to eight. If in fact removal of the hydrocarbon side chain left a cavity, you would expect an enormously greater effect. For example, in selecting a given amino acid in protein synthesis, you select very nicely between the subtle differences of glycine and alanine. Here,

we are removing a very large side chain and only getting a small effect. This would seem, from the evidence, to preclude a cavity being left when that large side chain is removed; the enzyme may somehow rearrange itself to accommodate the structural change so that a cavity does not remain. Does that seem reasonable?

Cane: It's reasonable, but I can't say whether it's true or not! You certainly would not fill up the cavity with water, or you would have a big problem of maintaining free carbocations without quenching. The trouble with the steady-state experiment is that I can't even claim, for example, that any two of these inhibitors bind in the same way, or that any single one binds in a unique way. Obtaining insight into what else is happening is impossible from a steady-state experiment. All one can say is that there is some hydrophobic component. How the enzyme compensates for the absence of a component which would normally be bound, I don't know. One can conceive of ways of binding the ammonium ion inhibitors in which the inhibitor goes into the active site completely backwards, and all the enzyme is really seeing is the ammonium ion analogue and some extra pieces of hydrocarbon.

Williams: I agree that you wouldn't expect the channel to be filled by water, but it's conceivable that hydrophobic side chains that were originally apart move in to fill the cavity.

Cane: Yes, phenylalanine rings, say, might just move closer; that is perfectly conceivable.

Cavalier-Smith: I would like to ask about the evolutionary implications of the enzyme not speeding-up reactions, but selecting from various potential pathways. When the enzyme initially evolved, did it just reduce the number of possibilities, and was therefore rather sloppy, yielding not one specific product, but a mixture? Then, later in evolution, it would be improved, further reducing the number of products. Or do you think that initially it would be rather precise, perhaps having evolved from some other enzyme that was already precise, and therefore be capable of selecting just one of the products, right from the start?

Cane: I would imagine that sloppiness would precede precision, because in the natural cyclization, the first thing it has had to learn is to isomerize the 2,3 double bond. Once it has evolved to do that, controlling the conformation of the rest of the cyclizing molecule, as well as the points of deprotonation, in principle follows. I don't know whether there is any selective advantage for an enzyme in remaining sloppy; that is, if an organism at some point derives an advantage from having not one but five different monoterpenes, then there would be no more pressure to get better. But although sloppiness might precede precision, it's not clear to me, at this stage, that one is inherently more primitive than the other.

Cavalier-Smith: There are different chlorophylls in different groups of algae, and there is some evolutionary indication that their chloroplasts were 'experimenting' with diversity, to start with, and then, as they diverged, stabilized

in one direction or another. Perhaps the sort of side chain modifications that are seen in the chlorophylls are analogous to what you are discussing? The enzymes could initially have been sloppy as to what they were adding, and then became more precise.

Cane: I don't know. You can propose that this is an adaptation to particular types of membrane interactions, with multiple ways of solving the same problem; I would tend to favour that view.

Orgel: In relation to the metal ion dependence, where the enzyme works better with manganese than with magnesium, is the suggestion that manganese is the naturally occurring metal?

Cane: It was a *quantitative* effect, in that one can measure the rate of reaction as a function of ion concentration, and, as with many kinases, manganese is often more effective than magnesium at low concentrations; but in many cases manganese becomes inhibitory at higher concentration, presumably because of non-specific interactions with thiols. There are a small number of cyclase enzymes for which manganese is simply superior. I don't think anyone has a way of sorting out which particular complex of geranyl diphosphate is the physiological substrate.

Orgel: Is it clear that the function of the metal ion is to complex the pyrophosphate group?

Cane: Yes; studies of prenyl transferases have shown that the metal ion is essential; the stoichiometry of binding has also been worked out. It has also been shown by Rod Croteau that monoterpene cyclases can be inhibited by sulphydryl-directed reagents. In at least one case, you cannot protect the active site with geranyl diphosphate alone, but you can protect it against inactivation with geranyl diphosphate–magnesium complex (R. B. Croteau, personal communication).

Baldwin: On the question of channelling, and how this could have evolved towards more precision, it seems to me that there is, underlying this discussion, the idea that these kinds of enzymes may be acting not as classical Pauling catalysts but as devices that restrict reaction pathways. The implication is that those pathways existed before the enzymes did, and the proteins came later, to provide selectivity.

Orgel: Particularly if they have something to do with eliminating water as a potential competitor, rather than accelerating a reaction.

Baldwin: That would be another version of the same idea of restricting the selection of the pathway from all of those reactions that are open to the system, once the reaction began to proceed.

Cavalier-Smith: Did Dr Cane say that the reaction is actually a lot slower than if it didn't have the enzyme there?

Cane: No; without the enzyme, there is a magnesium ion-catalysed solvolysis, but in a typical enzymic incubation, the cyclization rates are usually a factor of a hundred or a thousand more than the background solvolysis catalysed solely

by magnesium. I was saying that the rate of the allowed reaction must be substantially faster than the rate of the competing reactions which would give off other possible products. So, what has been catalysed, essentially, has been the ionization of the substrate. Upon ionization, the formation of one or another product should follow quite rapidly. You now have to channel things so that only one or a few of the many conceivable products are generated. But without ionization there is no generation of the product.

Cavalier-Smith: So there is catalysis—an effective speeding up of the reaction—and selection, and the two are equally important.

Leadlay: Are these enzymes under thermodynamic control, or kinetic control? That is to say, is the final quenching of the cation essentially irreversible?

Cane: It is irreversible; there's no case known in which one can take a product and convert it back to the starting material.

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