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ARTICLE

Molecular Mechanism of Enantioselective Inhibition of Acetolactate Synthase by Imazethapyr Enantiomers

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Chiral compounds usually behave enantioselectively in phyto-biochemical processes. Imidazolinones are a class of chiral herbicides that are widely used. They inhibit branched-chain amino acid biosynthesis in plants by targeting acetolactate synthase (ALS). It has been reported that the imidazolinone enantiomers show different inhibiting activities to maize (*Zea mays* L.) seedlings and ALS. However, to date, the mechanism of enantioselective inhibition of imazethapyr (IM) on ALS activity has not been well studied. In this study, pure enantiomers of IM were used for characterizing their differences in activity to ALS. Computational molecular docking was performed to discover the molecular interaction between IM enantiomers and ALS at the first time. Results showed that the IM enantiomers enantioselectively suppressed the in vitro and in vivo ALS activity of maize leaves. *R*-(−)-IM was more active than *S*-(+)-IM. The in vivo ALS activity study showed only a 2-fold difference between *R*-(−)-IM and *S*-(+)-IM. Quite different from the in vivo study, the in vitro study showed that the difference in inhibition between the enantiomers fell sharply as concentration increased. At the lowest concentration of 40 μg L^{-1}, *R*-(−)-IM appeared 25 times more active than *S*-(+)-IM, but only 7 times at 200 μg L^{-1}. At the highest concentration of 25 mg L^{-1}, in vitro ALS activity was almost completely inhibited by *S*-(+)-, *R*-(−)-IM and (±)-IM, there was only 1.1 times differences between *S*-(+)- and *R*-(−)-IM. Molecular modeling results provide the rational structural basis to understand the mechanism of enantioselective inhibition of IM on ALS activity.

KEYWORDS: Imidazolinones; acetolactate synthase (ALS); enantioselectivity; chiral; molecular mechanism; molecular docking

INTRODUCTION

Chirality is an important element of biology, chemistry and physics, which exists extensively in nature. The enantiomers of a chiral compound have identical physical and chemical properties in an achiral environment (e.g., air−water exchange, sorption, abiotic transformation), but they interact with biological systems enantioselectively and may behave as drastically different compounds (*1*, *2*). Often, one enantiomer is solely active or it is more active than the other enantiomer, which is inactive or less active and simply adds an extra chemical load to the environment (*1*, *3*). Application of the pure active enantiomer reduces the dosage and possible unwanted effects of the racemates.

Because of the vital importance of the chirality, the enantioselective ecological fate and effects of chiral herbicides have gradually received much attention. The present studies show enantioselectivities in the toxicity and environmental fate of chiral herbicides (*4*−*12*). The behavior of the active enantiomer, instead of just the racemate, may have more relevance to the herbicidal effects and ecological safety. This is why some herbicides can legally be sold and used only as their active enantiomers; e.g., some aryloxyphenoxypropanoic, acetanilide herbicides and so on (*13*, *14*).

Imidazolinones are a class of chiral herbicides targeting acetolactate synthase (ALS),which is a key enzyme in the synthesis of the branched-chain amino acids valine, leucine and isoleucine. They are widely used because of their high weed control efficacy, the large range of crops to which they can be applied, their low use rates and their low mammalian toxicity (*15*). All the imidazolinones are chiral herbicides typically consisting of two enantiomers. However, they are usually produced commercially as racemates. It has been reported that the imidazolinone enantiomers have different herbicidal activities, with the *R*-enantiomer being 8 to 10 times more inhibitory to ALS than the *S*-enantiomer (*16*, *17*). In our previous studies, we also found that the enantiomers of imazethapyr (IM), one of the imidazolinone herbicides, selectively inhibited the plant growth of maize by damaging root morphostructure and ultrastructure. The *R*-(−)-IM affected the root growth of maize seedlings almost two times

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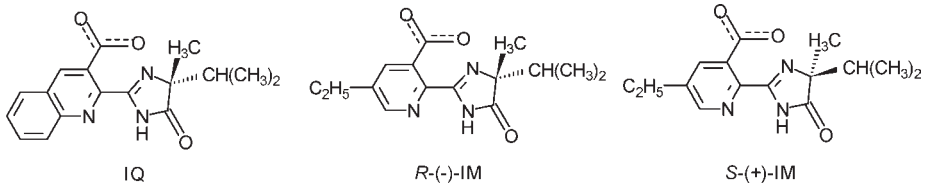

Figure 1. Chemical structures of IQ, *R*-(−)-IM and *S*-(+)-IM.

more severely than the *S*-(+)-IM. The inhibition abilities of the racemate was between *S*-(+)- and *R*-(−)-IM (*18*). However, the specific mechanisms of IM enantioselectively inhibiting the growth of maize are still unclear. According to the three-point model proposed by Easson and Stedman in 1993 (*19*) and four-location model developed by Mesecar in 2000 (*20*), the interaction between compounds and enzyme are stereospecific. Therefore, the enantioselective activity of the herbicide to plant may be due to the highly stereospecific interaction between the enantiomer and the enzyme. As the enantiomers are mirror images of each other, they differ in their three-dimensional configurations, and the enzyme is also chiral with a specific steric structure (*21*). Therefore, there are good chances with chiral herbicides that one enantiomer could combine more tightly than the other, consequently causing differences in enzyme activity. With the developments of structural biology and computer-aided drug design methods, molecular modeling has emerged as a powerful tool to investigate stereoselective protein−ligand interactions (*22*, *23*).

To understand the mechanisms of enantioselective inhibition on ALS by IM, molecular docking was performed to explore the structural explanation for the experimentally observed enantioselective results. Individual enantiomers of IM were used for characterizing their differences in toxicity to ALS. Computational molecular docking was performed, for the first time, to reveal the interaction modes between IM enantiomers and ALS.

MATERIALS AND METHODS

Chemicals. Analytical standards of racemic imazethapyr (98%) (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid; trade names include Contour, Hammer, Overtop, Passport, Pivot, Pursuit, Pursuit Plus, and Resolve) were kindly donated by the Shenyang Research Institute of Chemical Industry (Shenyang, China). Solvents and other chemicals used in this study were of analytical or HPLC grade.

Preparation of IM Enantiomers. Preparation of IM enantiomers was described in a previous study (*18*). Enantiomers were separated using the method developed in a previous study (*24*). Briefly, a Jasco LC-2000 series HPLC system (Jasco, Tokyo, Japan) was used with a chiral OJ column and a hexane/ethanol/acetic acid solution (75/25/0.5 by volume) as the mobile phase with a flow rate of 1 mL min^{-1}. A volume of 20 μL was injected for analysis. The circular dichroism (CD) detector was operated at 250 nm for detection. Chromatographic data were acquired and processed with the ChromPass software (Jasco, Tokyo, Japan). The octant rule was used to establish the absolute configuration of IM enantiomers. Therefore, the eluting sequence was *S*-(+)-IM, followed by the *R*-(−)-IM. The resolved enantiomers were manually collected into separate glass vials at the HPLC outlet. The fractions were dried under a stream of nitrogen and redissolved in ethanol. The purity and concentration of the recovered enantiomers were verified by HPLC with the same conditions used for separation. IM enantiomers were stable, and there were no signs of enantiomer conversion or degradation occurring during the experiment.

Plant Materials. Seeds of maize (*Zea mays* L.) were bought from the Hangzhou Seed Station, Hangzhou, China. They were surface-sterilized by 10% sodium hypochlorite (NaOCl) for 10 min and rinsed thoroughly with distilled water. After soaking in distilled water for 12 h at room temperature, seeds were then placed on moist gauze for germination. Maize seedlings of all experiments were grown in a growth chamber under controlled environmental conditions with a 12 h light period (light intensity of 10,000 lx), a 25 °C/20 °C light/dark temperature regime and 60% relative humidity (*18*). In the in vitro enzyme assays, uppermost-expanded leaves of plants were collected after growing for five days. The herbicide treatment was applied during the enzyme assay process. In the in vivo enzyme assays, when the first leaf of the plant grew out, the seedlings were treated with an herbicide solution (*S*-(+)-, *R*-(−)- or (±)-IM dissolved in distilled water) with various concentrations (0, 100, 200, 400, and 800 μg L^{-1}). The uppermost-expanded leaves of IM treated seedlings were harvested 24 h after the treatment for enzyme assays.

Enzyme Isolation. The procedure used was a modification of one used by Shaner (*25*). Leaves of the maize seedlings were homogenized in an extraction buffer (50 mM K-phosphate buffer (pH 7.0), containing 1 mM pyruvate, 5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), 10 μM flavin adenine dinucleotide (FAD)) (w/v 1/2). The homogenate was centrifuged at 12000*g* for 20 min. The supernatant fraction was brought to 50% saturation with respect to $(NH_4)_2SO_4$ and allowed to stand for 2 h on ice. Then the mixture was centrifuged as described above and the supernatant was discarded. The proteins were resuspended in 50 mM K-phosphate buffer (pH 7.0), containing 20 mM pyruvate, 0.5 mM $MgCl_2$, and used for the assay procedure. All operations were carried out at 4 °C.

Enzyme Assays. The assay solution contained 30 mM pyruvate, 1 mM $MgCl_2$, 1 mM TPP, 20 μM FAD, 50 mM K-phosphate buffer (pH 7, 1 mL), and 0.1 mL of enzyme solution. The inhibitors dissolved in acetone were placed in test tubes, and the acetone was evaporated; the series of concentrations were 0, 0.04, 0.2, 1, 5, 25 mg L^{-1}. This mixture was incubated at 37 °C for 1 h, then stopped with 0.2 mL of 3 M H_2SO_4. The reaction tubes were assayed for acetolactate by decarboxylation at 60 °C for 15 min and subsequent measurement of the acetoin formed by the method of Westerfield (*26*), as follows: 1 mL each of 0.083% (w/v) creatine and 0.83% (w/v) a-naphthol in 4 N NaOH was added to the tubes and incubated at 60 °C for 15 min. Absorbance at 520 nm was measured. Color formation was linear with incubation time at 37 °C for at least 60 min. Furthermore, protein was then measured by the method of Coomassie blue (*27*).

Molecular Docking. Because the IM and imazaquin (IQ) belong to the same category of herbicides with similar parent structure, and the amino acid sequences of *Arabidopsis thaliana* and maize are similar (identities 60−70%), especially the nearly identical residues in the ALS active pocket of both plants, the crystal structure of IQ in complexes with ALS of *Arabidopsis thaliana* (PDB ID: 1Z8N) (*28*) was selected to be the receptor. The configurations of enantiomer IM were modified based on IQ and then optimized (**Figure 1**). During the docking process, receptor and enantiomer IM were prepared following the original publication protocols Audock 3.05 (*29*), and the active site was defined by a three-dimensional grid (60 × 60 × 60 points) at the center of quinoxaline's mass. Autodock was used with the Lamarckian genetic algorithm (LGA) as conformation search method; and the number of generations, energy evaluations and docking run were set to 27,000, 1,000,000 and 100, respectively. Default values of the other parameters were used. The cluster rmsd 1.5 Å is acceptable, and the best docking conformation was selected according to the criteria of binding energy combined with geometrical matching quality.

Data Analysis. All data processing and graphical work were carried out by Origin software (OriginLab Corporation, MA, and Version 8.0). The analysis of variance was conducted according to the experimental designs employed, and the treatment means were compared by Fisher's protected least significant difference (LSD) at the 5% level of probability.

RESULTS

Enantioselective Effects of IM on in Vitro ALS Activity. The primary mode of action of the herbicide IM is interfering with the

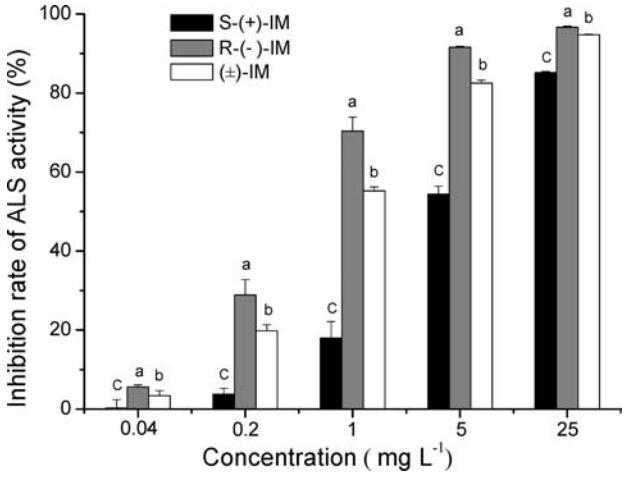

Figure 2. Inhibition of in vitro ALS activity of maize (*Zea mays*) by IM enantiomers and the racemate. Data points and error bars represent means ± SD of three replicates. Different letters indicate significant differences ($P < 0.05$) among the *R*-(−)-, *S*-(+)- and (±)-IM.

Table 1. Inhibition of IM Treatments on in Vivo ALS Activity (OD 520/mg protein/h) in Maize[a]

concn (μg L^{-1})	*S*-(+)-IM	*R*-(−)-IM	(±)-IM
0 (control)		8.93 ± 0.18 a	
100	7.96 ± 0.18 b	7.14 ± 0.39 c	7.60 ± 0.33 bc
200	6.34 ± 0.19 b	3.39 ± 0.04 d	4.41 ± 0.26 c
400	3.18 ± 0.39 b	1.56 ± 0.34 d	2.26 ± 0.18 c
800	2.06 ± 0.05 b	0.97 ± 0.06 c	1.90 ± 0.11 b

[a] Each value represents the mean of three replicates of each treatment. Different letters in the same row indicate significant differences ($P < 0.05$) among the *S*-(+)-, *R*-(−)- and (±)-IM.

activity of ALS enzyme. In the present study (**Figure 2**), we found that IM inhibited the in vitro ALS activity at very low concentrations, and the injury generally increased with increasing dose. Furthermore, the inhibition by IM is enantioselective. *R*-(−)-IM was more effective than *S*-(+)-IM. However, the enantioselective differences between the enantiomers were complex and variable. At the lowest concentration of 40 μg L^{-1}, significant differences in in vitro ALS activity could be observed among *S*-(+)-, *R*-(−)-IM and the racemate. *R*-(−)-IM appeared to be 25 times more active than *S*-(+)-IM. The differences in inhibition between the enantiomers fell sharply as concentration increased. At 200 μg L^{-1}, *R*-(−)-enantiomer was only 7 times more inhibitory than the *S*-(+)-enantiomer. The inhibition of in vitro ALS activity exposed to *R*-(−)-IM was 28.9%, while the *S*-(+)- and (±)-IM inhibitions were 3.8% and 19.8%, respectively. At the highest concentration of 25 mg L^{-1}, in vitro ALS activity was almost completely inhibited by *S*-(+)-, *R*-(−)- and (±)-IM. It is only 1.1 times the difference between *S*-(+)- and *R*-(−)-IM.

Enantioselective Effects of IM on in Vivo ALS Activity. In the hydroponics experiment, the in vivo ALS activity was enantioselectively inhibited by IM enantiomers relative to the control (**Table 1**). *R*-(−)-IM again caused the most effective inhibition in comparison to the *S*-(+)-IM and racemate mixtures at equal concentrations. At low concentration of 100 μg L^{-1}, there were significant differences between *S*-(+)-and *R*-(−)-enantiomer, but no significant differences between *S*-(+)-and (±)-IM. With increased concentrations, the amount of the *R*-(−)-enantiomer in (±)-IM also increased, and the (±)-IM became more inhibitory. Therefore, a significant difference was observed between *S*-(+)-and (±)-IM at 400 and 800 μg L^{-1} (**Table 1**). At the concentration of 800 μg L^{-1}, the difference between *R*-(+)-and

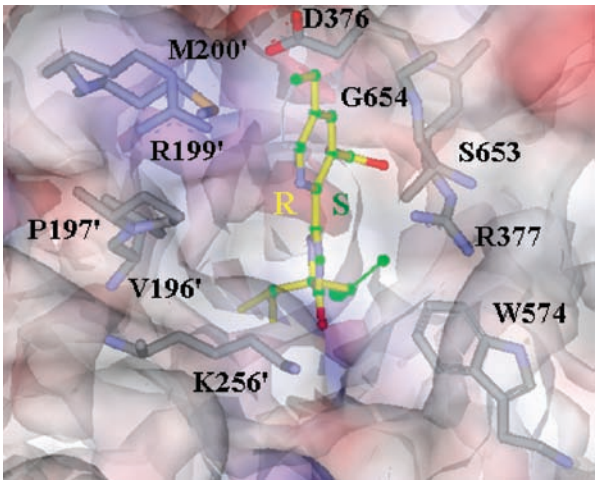

Figure 3. The superposion of *R*-(−)-IM (carbon atoms shown in yellow color), *S*-(+)-IM (carbon atoms displayed in green color) and IQ in the binding pocket of ALS.

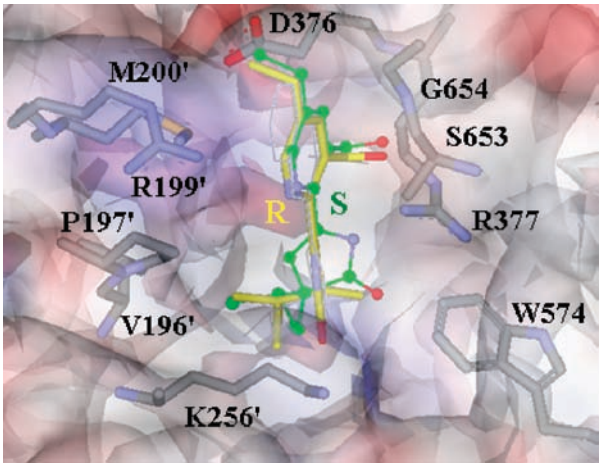

Figure 4. Comparative binding modes of *R*-(−)-(carbon atoms shown in yellow color) and *S*-(+)- IM (carbon atoms displayed in green color) with ALS.

(±)-IM became smaller. However, quite different from the results of the in vitro ALS activity study, the in vivo ALS activity study showed only a 2-fold difference between *R*-(−)-IM and *S*-(+)-IM.

Stereospecific Interaction between IM Enantiomers and ALS. McCourt et al. have elucidated the structural basis for how *R*-IQ (*R*-imazaquin) acted with the ALS (*28*). Similarly, if the IM enantiomers are positioned in the active site of IQ-ALS complex (**Figure 3**), besides the overlapped sites including the dihydroimidazolone ring and quinoline ring, the remarkable difference between them is that the isopropyl substituent of *R*-(−) and S-(+)- IM point to the opposite directions; the former is nearly identical to the corresponding part of IQ, while the latter forms repulsive forces with the side chain of R377 and W574, which may be used as a crude explanation to elucidate the stereospecificity. And also the numerous contacts between ALS and the isopropyl and methyl groups are important for anchoring the herbicide to the protein (*16*, *30*).

Additionally, the docked conformations of enantiomers present the detailed and precise interaction mode of IM with ALS. As shown in **Figure 4**, the conformation of *R*-(−)-IM is sandwiched in a hydrophobic core consisting of side chains of M200, P197 and V196 at one side and G654, S653 and W574 at another side.

Furthermore, the salt bridge is found between the carboxylate group and the side chain of R377, which is crucial for herbicide to bind with ALS (*28*). However, *S*-(+)-IM with the distorted dihydroimidazolone ring, although located in the same binding pocket, cannot form orderly interactions with ALS as *R*-(−)-IM does. One hypothesis is that the dihydroimidazolone ring has to rotate to avoid the steric hindrance between isopropyl and W574. Therefore *R*-(−)-IM could bind to ALS in its preferred orientation, thereby explaining the greater binding potential of *R*-(−)-IM relative to *S*-(+)-IM.

DISCUSSION

Acetolactate synthase, the first enzyme catalyzing two reactions in the branched-chain amino acid biosynthesis pathway, is the target enzyme of imidazolinones. The primary mode of action of the imidazolinones that interfere with the activity of ALS enzyme has been well studied (*31*). Acetolactate synthase inhibition can be achieved at a very low herbicide concentration. For example, ALS enzyme extracted from maize cell suspension culture was half maximally inhibited in vitro by imidazolinones at the concentration of 3 μM (*32*). However, most of the previous studies reporting the inhibition of IM on ALS activity were merely based on the racemate of IM. The enantioselectivity of ALS inhibition has not been studied. In the present study, we found IM inhibited the in vitro ALS activity at very low concentration, and the injury generally increased with increasing dose, which was in agreement with the previous studies. Furthermore, the inhibition of the IM enantiomer is enantioselective. It has been reported that the *R*-enantiomer was almost 8−10 times more inhibitory against the enzyme ALS than the *S*-enantiomer (*16*, *17*). Our results showed a similar trend, i.e. *R*-(−)-IM was more effective than *S*-(+)-IM. However, in this study we showed for the first time that the enantioselective differences between the enantiomers were complex and variable. The biggest differences of in vitro ALS activity inhibition could be observed among *S*-(+)-, *R*-(−)-IM and the racemate at the lowest concentration of 40 μg L^{-1}. The difference in inhibition between the enantiomers fell sharply as concentration increased.

The in vivo ALS activity was also enantioselectively inhibited by IM enantiomers. *R*-(−)-IM also caused the most effective inhibition in comparison to the *S*-(+)-IM and racemate mixtures at equal concentrations. However, quite different from the results of the in vitro ALS activity study, the in vivo ALS activity study showed only a 2-fold difference between *R*-(−)-IM and *S*-(+)-IM. In our previous studies (*18*), we also found a 2-fold difference between *R*-(−)-IM and *S*-(+)-IM in retarding plant growth of maize. The enantioselectivity of the in vivo ALS activity inhibition is in agreement with the enantioselective inhibition of plant growth, which means there are some other elements involved in the enantioselective inhibition of plant growth, not only the target enzyme ALS. In vivo ALS activity rather than in vitro ALS activity has a much closer relationship to the herbicidal inhibition of plant growth. The difference between in vivo and in vitro ALS activity requires further investigation into the mechanisms whether the difference is caused by the enantioselective absorption or enantioselective degradation in plants.

According to the theory of three-point model and four-location model, we proposed a hypothesis that the enantioselective difference of ALS inhibition between *R*-(−)-IM and *S*-(+)-IM is due to the highly stereospecific interaction between the enantiomers and the enzyme. Consequently, studies on the interaction mechanism between IM enantiomers and ALS were explored by molecular docking.

Wang et al. found that the organophosphate insecticide fenamiphos enantioselectively interacted with acetylcholin-esterase by molecular docking (*23*). The structure of the binding site of the stereoselective anti-D-amino acid antibody 67.36 was modeled by Ranieri et al. They found that the incorporation of side-chain flexibility within the binding site resulted in a protein structure that stereoselectivity binds to the D-enantiomer of the model ligand; the L-enantiomer of the model ligand cannot access the binding site due to steric hindrance (*33*). In our study, the combination of IM with ALS also shows chiral discrimination. *R*-(−)-IM could bind to ALS in its preferred orientation more than *S*-(+)-IM could. Different interaction modes of *R*-(−)- and *S*-(+)-IM with ALS obtained from molecular docking provide structural explanation for the more potential activity of *R*-(−)-IM in contrast to *S*-(+)-IM.

Molecular docking provides further evidence for the link between inhibition of this enzyme and the phytotoxicity of imidazolinone herbicides. This study also provides important evidence for developing novel and further selective and effective herbicides, that is, we can choose the proper optically pure enantiomer which has a stronger combination ability with the target enzyme to yield higher herbicidal effects.

In conclusion, the IM enantiomers enantioselectively suppress the in vitro ALS activity in maize leaves. The *R*-(−)-IM was more active than the *S*-(+)-IM. The enantioselective differences between the enantiomers were complex and variable. The difference in the inhibition rate between the enantiomers fell sharply with concentration increases. The in vivo ALS activity study showed only a 2-fold difference between the *R*-(−)-IM and the *S*-(+)-IM, which is accordance with the previous established difference between the *R*-(−)-IM and the *S*-(+)-IM in retarding the plant growth of maize. Compared to the in vitro ALS activity, the in vivo ALS activity has a much closer relationships with plant growth. In the study of molecular docking, the combination of the IM with ALS shows chiral discrimination. *R*-(−)-IM could bind to ALS in its preferred orientation. The different interaction modes of the *R*-(−)- and the *S*-(+)-IM with ALS obtained from molecular docking provide a structural explanation for the more potent activity of the *R*-(−)-IM in contrast to the *S*-(+)-IM.

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