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Ethylene Receptor Antagonists: Strained Alkenes Are Necessary but Not Sufficient

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SUMMARY

Plants use ethylene as a hormone to control many physiological processes. Ethylene perception involves its binding to an unusual copper-containing, membrane-bound receptor. Inhibitors of ethylene action are valuable to study signaling and may have practical use in horticulture. Past investigation of alkene ligands for this receptor has identified strain as the key factor in antagonism of ethylene binding and action, consistent with known trends in metal-alkene complex stability. However, in this work, this principle could not be extended to other alkenes, prompting development of the proposal that a ring-opening reaction accounts for the unusual potency of cyclopropene ethylene antagonists. Another factor augmenting the affinity of alkenes for the copper binding site is pyramidalization, as in *trans*-cycloalkenes. The enantiomeric selectivity in the binding of one such alkene to the ethylene receptor demonstrates its protein-composed asymmetric environment.

INTRODUCTION

Although ethylene has been known to exhibit hormonal properties in plants since the early 20th century, only sketchy details of how it functions have been available. Burg and Burg (1965) suggested, based on a correlation of biological activity with the ability of 1-alkenes to bind to silver ion (Winstein and Lucas, 1938; Muhs and Weiss, 1962), that plants possess a metal-containing ethylene binding site. Advances in molecular biology have provided information concerning the ethylene receptor protein ETR1 from *Arabidopsis* (Chang et al., 1993). Bleecker showed that its ethylene binding site is assembled from a copper ion bound in a transmembrane helix of this kinase (Rodríguez et al., 1999). A model for receptor function based on this binding site has been proposed (Pirrung, 1999).

Antagonists and agonists for the ethylene receptor are known, but theories that explain the action of antagonists vis-à-vis agonists are still needed (Pirrung, 2000, 2001). Ethylene is the strongest agonist, but other agonists include 1-alkenes and compounds capable of coordination to a metal (isonitriles, CO) (Sisler, 1991). Antagonists of ethylene action are desired owing to its effects in promoting senescence, abscission, and wilting. Silver ion is an ethylene antagonist used classically in plant physiology and commercially in floriculture (Beyer, 1979; Le Masson and Nowak, 1981; Veen, 1983). Silver was likely investigated originally as an ethylene inhibitor because, inspired by Burg and Burg, it was envisioned to sequester ethylene. However, the basis of the effects of silver ion on ethylene perception was unknown until Bleecker showed that silver ion can replace copper in the receptor and support ethylene binding yet prevent activation of the receptor by ethylene (Rodríguez et al., 1999). Antagonists of the receptor also include strained alkenes such as norbornadiene and *trans*-cyclooctene (Sisler et al., 1990; Hirayama et al., 1999), which have greater affinity than ethylene for metal ion π-complexation. These compounds were presumably also investigated owing to their strong binding to silver ion. *trans*-Cyclooctene is unique among known ethylene antagonists in being chiral. Molecular interactions (and therefore the potency) of chiral compounds with biological macromolecules that exist in single enantiomer forms are expected to be dependent on absolute configuration. Compounds whose chiralities are based on even very subtle differences can still show chiral discrimination in their activity. For example, the inhalation anesthetics halothane and enflurane, whose enantiomers differ only by pairwise exchange of halogen atoms, show a dependence of both potency and metabolism on stereochemistry (Meinwald et al., 1991; Franks and Lieb, 1991; Sedensky et al., 1994). *trans*-Cyclooctene should therefore exhibit configuration-dependent activity if it acts at a site on the receptor protein.

The most prominent of currently known ethylene antagonists are 1-alkyl-cyclopropenes (Sisler et al., 1996, 2003; Serek et al., 1995a, 1995b; Sisler and Serek, 1997), and one such compound, 1-methylcyclopropene (MCP) (Sisler et al., 1999; Mueller et al., 1998; Serek et al., 1995a, 1995b, 1998), is marketed as the antisenescence agent EthylBloc (Sisler and Serek, 2003; Serek

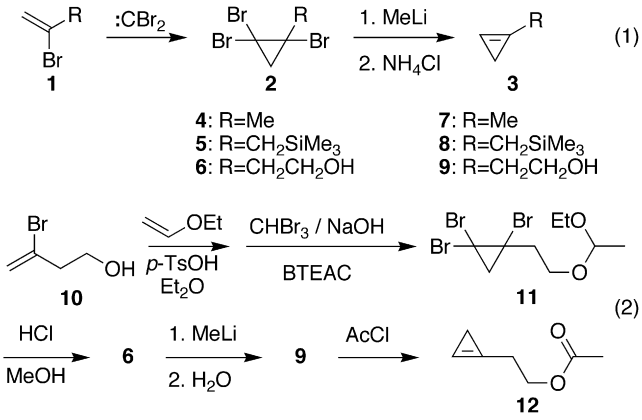

Figure 1. Synthetic Routes to 1-Substituted Cyclopropenes

et al., 2006). 1-Substituted cyclopropenes with higher alkyl groups are also antagonists, and their action can persist for days following treatment (Sisler et al., 2003). Cyclopropenes are highly strained, with a strain energy of about 55 kcal/mol (Bach and Dmitrenko, 2004), and consequently they are quite reactive. Neat 1-methylcyclopropene itself dimerizes via an ene reaction within minutes at room temperature (Fisher and Applequist, 1965), and cyclopropenes are light (Sastry and Lakshminarayana, 1969) and acid sensitive (Kircher, 1964; Saracoglu et al., 1995). Foreshadowing implications of this study, strain may not be the only determinant of ethylene antagonist activity: cyclopropene itself and 3,3-dimethyl-cyclopropene are less potent ethylene antagonists than MCP.

Beyond the intrinsic activity of ethylene antagonists at the receptor, their mode of application is a practical concern. The classical antagonist silver ion is typically applied as silver thiosulfate in water, but the volatility of MCP makes it hard to handle and to formulate. EthylBloc is a cyclodextrin inclusion complex of MCP that slowly releases the active antagonist. Here, aerial diffusion of the antagonist to the receptor must occur. Therefore, the physiological potency of an antagonist as an antisenescence agent could relate to its boiling point. A novel approach to delivering strained alkene ethylene antagonists might be based on systemic application of a compound that is water soluble and moves in the vasculature of the plant to the ethylene receptor site, like silver. Such compounds would be far more convenient to handle and administer.

We were initially interested in targeting complex, heteroatom-containing cyclopropenes that could offer superior properties of handling outside of conventional laboratory settings and perhaps exert superior action at the ethylene binding site. Preparation of cyclopropenes can be challenging. 1-Methylcyclopropene was originally prepared from methallyl chloride and sodamide (Fisher and Applequist, 1965), and a preparation using phenyllithium as base gives a 54%–73% yield (Magid et al., 1971; Koester et al., 1973). This reaction has even been performed on a large scale (Daly and Kourelis, 2000), but this route is not widely applicable. After mixed results using several other known strategies to access 1-alkylcyclopropenes (Zhou, 2002), we applied the general synthetic approach described here and examined the antagonist properties of some of the resulting cyclopropenes. Further, we reasoned that another class of strained alkenes, cyclobutenes (with strain energies of about 28 kcal/mol; Bach and Dmitrenko, 2004), would be more easily accessed than cyclopropenes. We also examined the enantiomeric selectivity of *trans*-cyclooctene (with a strain energy of 16.4 kcal/mol; Wiberg, 1986). A potency increase over the racemate of up to 2-fold might be observed, and the observation of differential activity of the enantiomers would establish the intrinsic asymmetry of a protein-derived ethylene binding site.

Table 1. Preparations of Cyclopropenes by the Routes in Figure 1

R	Yield of **2** (%)	Yield of **3** (%)	Boiling Point (°C)
Me	61	90[a]	8
CH_2CH_2OH	55[b]	80	138
CH_2SiMe_3	84	80	121

[a] Calculated from NMR integration.
[b] Calculated over three steps.

RESULTS

Synthesis

We were attracted by the reports of Baird on the production of 1-octylcyclopropene by metalation-protonation of 1-octyl-2-bromo-cyclopropene, itself available by reductive debromination of the tribromocyclopropane (Al Dulayymi et al., 1996; Baird et al., 1986; Dent et al., 1986). We envisioned a general route to 1-alkylcyclopropene ethylene antagonists (Figure 1, Equation 1) based on treatment of alkenes **1** with dibromocarbene to give tribromocyclopropanes **2** followed by exhaustive debromination to give the 1-substituted cyclopropene **3**. On reaction of **2** with an organolithium, the vicinal dibromide is eliminated to the bromocyclopropene, which is converted to the lithiocyclopropene by metal-halogen exchange. Quenching of this organometallic gives the cyclopropene. This route is ideal for the preparation of volatile and difficult-to-handle cyclopropenes, in particular MCP, because the byproducts (bromomethane, methane, lithium bromide) are volatile or salts and the solvent (ether) is low boiling.

This route was applied to three commercial 2-bromo-1-alkenes, the first two as written in Equation 1. Product **8** was targeted because its higher boiling point would make it easier to handle than **7** and it could be more potent owing to the benefit of the allyl silane on transition metal binding (Stoebenau and Jordan, 2006). It would also be potentially convertible to **7** by protiodesilylation, a unique mechanism of activation for a ''prodrug.'' Product **9** was targeted because it would be more water soluble and higher boiling. It also has the potential for increased potency, in this case owing to an additional donor ligand for the copper ion that forms the ethylene binding site (Rodríguez et al., 1999). Using this synthetic route, the targeted cyclopropenes are obtained efficiently (Table 1).

This route could not be applied directly to obtain alcohol **9** because alkene **10** does not undergo dibromocyclopropanation; a protecting group for the alcohol was required (Figure 1, Equation 2). An ethoxyethyl group provides transient protection, permitting cyclopropanation to give **6** following acid hydrolysis of acetal **11**. The free alcohol does not interfere in the elimination-metalation sequence, and cyclopropene-alcohol **9** can be obtained from **6** in high yield (Table 1). To prepare a prodrug form of an ethylene antagonist that is more traditional than **8**

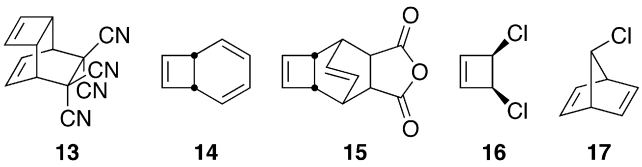

Figure 2. Other Strained Alkenes Examined in This Work

(Beaumont et al., 2003), acetylation of **9** under standard conditions gave acetate **12**.

Because one goal of this work was to create MCP derivatives that are more stable than the parent **7**, the stabilities of **8** and **9** were evaluated by NMR monitoring of their solutions in d_6-acetone. Decomposition was indicated by several new signals in the NMR spectra and, after longer times, visible precipitates. These compounds exhibit no sensitivity to light and, as expected, are more stable at lower temperature ($-20°C > 4°C > > 25°C$). They lose only about 20% of their cyclopropene signals over 9 d at 4°C. They decompose much faster in $CDCl_3$ solution, presumably as a result of trace amounts of acid. Both compounds are quite unstable when neat. They are colorless, free-flowing liquids when freshly prepared, but the neat compounds become red, very viscous oils over several hours at room temperature. These materials exhibit complex NMR spectra with only small cyclopropene signals.

Cyclobutenes are (like cyclopropenes) highly strained. Formation of cyclobutene-metal complexes should consequently be highly favorable, making cyclobutenes good candidates for ethylene receptor antagonists. Cyclobutenes are also more accessible than cyclopropenes, with many available synthetic routes (Figure 2). Cyclobutene **13** can be prepared in a single step from cyclooctatetraene and tetracyanoethylene (Paquette et al., 1974; Scheiner and Vaughan, 1961). Cyclooctatetraene is in equilibrium with its bicyclo[4.2.0]octa-2,4,7-triene (**14**) isomer, which undergoes Diels-Alder reaction with many dienophiles. When the dienophile is maleic anhydride, adduct **15** (stereochemistry unassigned) results (Avram et al., 1960; Cookson et al., 1961). *cis*-3,4-Dichlorocyclobutene (**16**) is well known (Ullah and Kabir, 1983; Pettit and Henery, 1970) and commercially available, as is 7-chloronorbornadiene (**17**), an analog of the weak antagonist norbornadiene.

Many methods have been reported to prepare enantiomerically enriched *trans*-cyclooctene, but none affords *both* antipodes in enantiomerically pure form (Bach et al., 2003; Adam and Stegmann, 2002). It was therefore necessary to balance access to both enantiomers with their enantiomeric purity. We thought it essential to study both enantiomers rather than the racemate and a single enantiomer. We chose a method for *trans*-cyclooctene preparation developed by Inoue based on the photosensitized geometrical isomerization of *cis*-cyclooctene using (*R*)- and (*S*)-hexakis(1-methylheptyl) benzenehexacarboxylates as chiral sensitizers (Inoue et al., 1992, 1998). Using this method, both (*R*)- and (*S*)-*trans*-cyclooctene are obtained in 50% enantiomeric excess.

Biological Evaluation

Arabidopsis seedlings have proven to be ideal model organisms for the study of ethylene receptor signaling and action. Prior studies of etiolated wild-type seedlings revealed two phases of ethylene-induced growth inhibition for hypocotyls (Binder et al., 2004a, 2004b, 2007; Potuschak et al., 2006). Phase I inhibition is transient, begins approximately 10 min after ethylene is added, and reaches a new, lower steady-state plateau in growth rate after an additional 15 min. This plateau in growth rate is maintained for approximately 20 min, after which the phase II inhibition starts reaching a lower steady-state growth rate 20 min later. This second phase growth inhibition lasts as long as ethylene is present at saturating concentrations (Binder et al., 2004a, 2007) (Figure 3A).

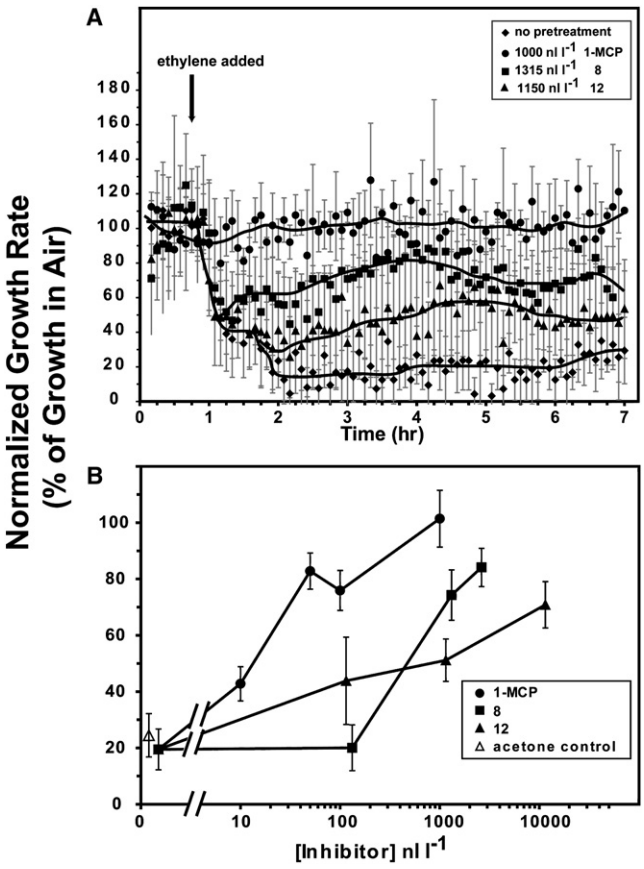

Figure 3. The Effects of MCP and MCP Analogs on Growth Responses to Ethylene

Seedlings were pretreated for 17 hr with the indicated concentrations of each compound. For comparison, measurements from untreated or acetone-treated seedlings are shown.

(A) Rapid ethylene response kinetics were made by taking images every 5 min. Measurements were made in air for 45 min before the introduction of 10 μl l^{-1} ethylene (arrow). Growth rate was normalized to the average growth rate in air for each condition. Lines were drawn by hand.

(B) Dose responses were determined for several of the MCP analogs and compared to MCP and to an acetone solvent control. Growth rates were determined by averaging growth rates from data collected 3–6 hr after the addition of 10 μl l^{-1} ethylene. For both panels, data represent the average ± SD for at least three seedlings total from at least three separate experiments.

It is known that MCP is an effective antagonist of ethylene responses in etiolated *Arabidopsis* seedlings (Hall et al., 2000; Binder et al., 2004b). We compared the effects of MCP on ethylene growth inhibition kinetics with two analogs (Figure 3A).

As reported previously (Binder et al., 2004b), a 17 hr pretreatment with 1000 nl l^{-1} MCP completely eliminated both phases of the ethylene growth response. In contrast, pretreating seedlings with either 1350 nl l^{-1} **8** or 1150 nl l^{-1} **12** only partially affected responses to ethylene. When pretreated with **8** at this concentration, seedlings had an attenuated phase I response and failed to enter the initial plateau in growth rate. Instead, the growth rate increased to an intermediate level. Seedlings pretreated with **12** showed ethylene responses similar to wild-type for the first 55 min after ethylene was added. Afterward, the responses diverged, with seedlings pretreated with **12** having a slow increase in growth to an intermediate growth rate (Figure 3A).

To further delineate the effectiveness of **8** and **12**, we tested the ability of various concentrations of these compounds to block the phase II, long-term ethylene growth response (Figure 3B). When we treated seedlings with 10 μl l^{-1} ethylene without pretreatment with MCP or analogs, growth during phase II diminished to approximately 20% of the growth rate seen in air. MCP caused a measurable effect on long-term resistance to ethylene at all concentrations tested (10–1000 nl l^{-1}), as evidenced by a higher growth rate in the presence of ethylene. By contrast, **8** and **12** were required at higher concentrations to measurably block ethylene growth responses. Dose-response curves show that approximately 15 nl l^{-1} MCP led to partial inhibition of the ethylene growth response so that growth was occurring at 50% of the rate seen in air (Figure 2B). In contrast, much higher concentrations of **12** (1000 nl l^{-1}) and **8** (500 nl l^{-1}) were needed for similar inhibition of the ethylene growth response. It is unclear whether saturating concentrations of **8** and **12** were reached in the current study to cause maximal inhibition of the ethylene growth response. Therefore, it is unknown whether very high concentrations of these compounds completely block ethylene responses or whether they are only capable of partially blocking responses to ethylene. Application of acetone (used as a solvent for the MCP analogs) as a vehicle control was without effect on long-term growth inhibition caused by exposure to ethylene (Figure 3B). Thus, **8** and **12** do block responses to ethylene, but are less effective than MCP under these conditions.

The transgenic expression of the full-length ethylene receptors and individual receptor domains has proven to be a powerful tool to evaluate ethylene binding and other enzymatic activities associated with the receptors (Rodríguez et al., 1999; Hall et al., 2000; Gamble et al., 1998; Moussatche and Klee, 2004; O'Malley et al., 2005; Schaller and Bleecker, 1995; Schaller et al., 1995; Zhang et al., 2004; Wang et al., 2006). Further studies of the action of ethylene antagonist candidates exploited one of these expression systems in radioligand binding assays. The cyclobutenes **13**, **15**, and **16** were examined along with norbornadiene analog **17**. Compounds **13** and **15** were chosen as models for systemic rather than aerial administration because they are nonvolatile. All were applied as DMSO solutions. Two radioligand binding assays were used, one on membranes derived from yeast expressing the full-length *Arabidopsis* ETR1 protein, and one on intact yeast cells expressing ETR1. In the former (Figure 4A), compounds **13** and **15** compete with ethylene in a dose-dependent manner, but **16** and **17** have a much smaller effect. In the assay on intact yeast cells (Figure 4B), all four (**13**, **15**, **16**, **17**) exhibit

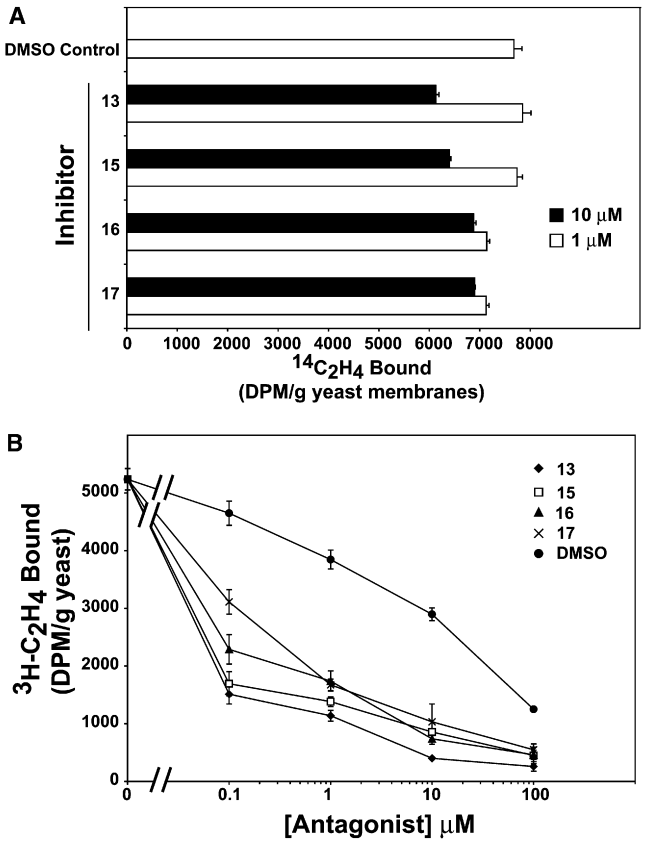

Figure 4. The Effect of Cyclobutenes on Ethylene Binding to the ETR1 Receptor

(A) Membranes from yeast expressing full-length *Arabidopsis* ETR1 were assayed for ethylene binding using [^{14}C]ethylene after being pretreated with the indicated concentrations of compounds **13**, **15**, **16**, or **17**. Each compound was dissolved in DMSO and diluted with medium to the stated concentration. For this and the following figure, saturable ethylene binding activity is indicated as disintegrations per min (DPM) and was calculated by subtracting the amount of radioactivity bound in the presence of excess unlabeled ethylene from that bound in the absence of added nonradioactive ethylene. DMSO solvent controls (0.5% solution of DMSO in medium lacking inhibitors) are shown in both panels.

(B) Ethylene binding was assayed in yeast cells expressing full-length ETR1 receptor using [^{3}H]ethylene (0.1 ppm). Cells were preincubated for 30 min at room temperature with the indicated concentration of each compound at the indicated concentration. The cells were then filtered and the filters were tested for ethylene-binding activity. Results are the average DPM ± SD in triplicate samples. Background ethylene binding in the presence of 100 ppm cold ethylene was 535 ± 28 DPM/100 mg yeast.

similar action, significantly greater than DMSO controls. Of the four compounds tested, ethylene binding was most sensitive to **13** and **15**, which caused half-maximal inhibition of ethylene binding at concentrations below 0.1 μM. Cyclobutene **16** caused half-maximal inhibition of ethylene binding at approximately 0.1 μM, whereas higher concentrations of **17** were needed to achieve half-maximal inhibition. All compounds tested significantly inhibited ethylene binding at 100 μM, but **17** was somewhat less effective than the others.

The selectivity of the receptor for the *trans*-cyclooctene enantiomers was determined by the radioligand binding assay

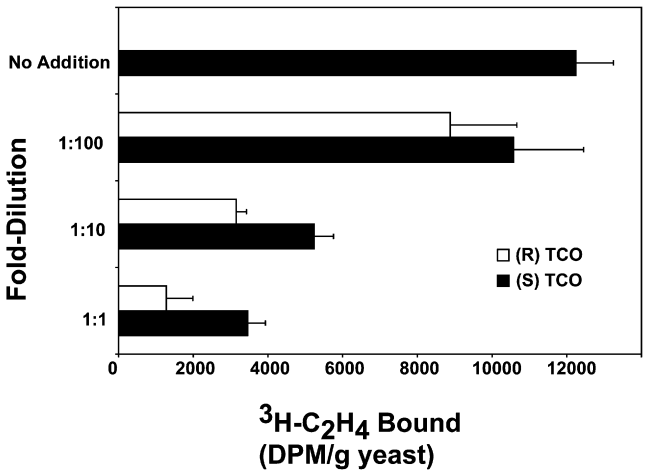

Figure 5. The Effect of *trans*-Cyclooctene Enantiomers on the Binding of Ethylene to the ETR1 Receptor

Yeast cells expressing the full-length ETR1 were incubated with the indicated dilution of either the (*S*)- or (*R*)-enriched *trans*-cyclooctene (abbreviated TCO, 50% enantiomeric excess) for 30 min at room temperature. Saturable ethylene binding activity was assayed as described in the Experimental Procedures and is indicated as disintegrations per min (DPM). Results are the average DPM ± SD in triplicate samples.

on intact yeast cells expressing the ETR1 protein. Selectivity is modest but consistent with dose, with the (*R*)-enriched isomer being more potent (Figure 5). At the highest treatment level, it is about twice as effective as the (*S*)-enriched isomer. Extrapolating these data to enantiomeric excesses of 100% suggests about a 5:1 enantiomer discrimination.

DISCUSSION

The ethylene antagonists described and tested here, in both the cyclopropene and cyclobutene families, do not rival MCP in potency but offer greater convenience in handling and administration. However, if applied aerially, this may decrease their penetration to the ethylene receptor. Some are not at all volatile and *must* reach the ethylene binding site systemically, whereas some could diffuse to the site. The boiling points of the more volatile antagonist candidates were used to represent their diffusive ability. The bp of **16** is 74°C–76°C, the bp of **17** is calculated to be 179°C, and the boiling points of the cyclopropene antagonists **8** and **9** were determined to be 120°C–140°C (see Supplemental Data available with this article online). The boiling point of **12** should be very similar to that of **9**. Unlike MCP, these cyclopropenes offer little risk of adventitious loss during preparation. There is no evidence from physiological data that **8** is protiodesilylated to MCP during the assay, because its potency is weaker than MCP itself. The theory that its vinyl silane structure would make it a more potent metal binder than MCP was not supported. Likewise, the reduced potency of **12** compared to MCP does not support its conversion to the free alcohol or its increased potency via chelation.

Cyclobutenes represent a novel class of ethylene receptor ligands. In the binding assays described here, their effects were modest and comparable to the weak ethylene antagonist norbornadiene. There is no question about the ability of these specific compounds to serve as excellent ligands for metal ions, as an Ni(II) complex (Froehlich and Hoberg, 1981) of dichlorocyclobutene (**16**) and a dimeric Rh(I) complex (Abel et al., 1959) of **15** are known, inter alia. Although the structures of **13**, **15**, and **17** suggest their potential for chelation, with both alkenes binding to one metal ion, we disfavor this view. When norbornadiene binds to copper(I), it forms a complex with one metal at each alkene (Zou, 2006; Abel et al., 1959) (similar to the Rh_2Cl_2[**15**]$_2$ complex) rather than forming a chelating complex.

The modest enantiomeric selectivity in the binding of *trans*-cyclooctene to the ethylene receptor was somewhat surprising. In many ligand-macromolecule interactions, enantioselectivity is 100%. Typically, such interactions are based on multiple parts of a small organic molecule interacting with several different atoms in a protein backbone, whereas the key interactions we believe are responsible for ethylene antagonism involve only the copper ion and the two alkene carbons, constrained here in such a way that (1) makes the alkene a much better ligand and (2) makes it chiral. Prior to the cloning of ETR1, there was a significant question as to whether a receptor for ethylene even existed, or whether ethylene's actions represented a general, macroscopic effect on cell membranes. This situation parallels the inhalation anesthetics, where similar questions were asked. Investigations of the influence of chirality on the biological actions of halothane and enflurane provided a useful guide in this study. The observation of enantiomeric selectivity in the binding of *trans*-cyclooctene to the ethylene binding site, though modest, is strong indication that the binding site is composed from a chiral molecule, the ETR1 protein.

The actions of cyclopropenes and *trans*-cyclooctene at the ethylene binding site have in the past been strongly linked to their strain. Yet, theoretical studies of the binding of transition metals to strained alkenes have come to the remarkable conclusion that the binding of cyclopropene and cyclobutene is far weaker than would be expected on the basis of their very high strain energy (Cedeño and Sniatynsky, 2005). This behavior is ascribed to the significant structural reorganization required of an alkene upon metal binding, primarily involving pyramidalization, which requires strong back bonding. This thinking runs counter to a significant literature on ethylene action that says the strong back-bonding power of ethylene enables it to *promote* signaling at its receptor (Sisler and Yang, 1984). If cyclopropenes also promote back bonding when binding to metal ions, they should be agonists of the ethylene receptor, not antagonists. One explanation for this conflict may simply be that the theoretical studies were performed on Group 6 metals, not Group 11 to which copper belongs. Another may be a different view of the action of cyclopropenes, 1-alkylcyclopropenes in particular.

There is good reason to question the existence of any complex between the copper at the ethylene binding site and methylcyclopropene. Very few stable transition metal-cyclopropene complexes are known, limited mainly to Group 10 metals (Visser et al., 1971, 1973; De Boer and Bright, 1975), and they are specifically unknown with copper. We made extensive efforts to form complexes between copper(I) (bearing a wide variety of ligands) and several different cyclopropenes, including MCP (Zou, 2006). Without exception, the only products observed were the precipitated copper-ligand complexes and organic

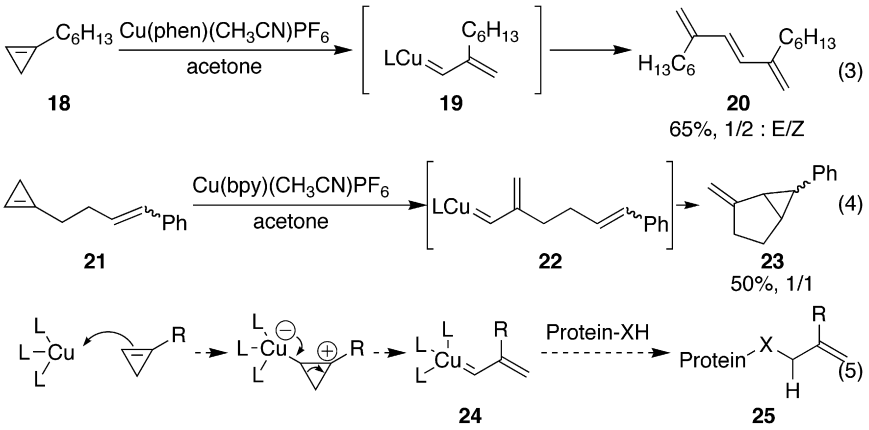

Figure 6. Reaction of Copper(I) Complexes with Cyclopropenes and an Alternative Mechanism for the Action of Cyclopropene Ethylene Receptor Antagonists

products in the supernatant that turned out to be hexatrienes. This observation held regardless of the steric environment of the cyclopropene. A specific example of a reaction using a copper complex in catalytic quantities is given in Figure 6, Equation 3. The observed product **20** may be formed by dimerization of a putative copper carbenoid **19** derived from ring opening of 1-hexylcyclopropene (**18**), or perhaps by reaction of the carbenoid with another cyclopropene, forming a bicyclobutane that undergoes ring opening. Similar observations were made with cyclopropenes **8** and **9** (Zou, 2006; Supplemental Data). Evidence in favor of the carbenoid intermediate was obtained by trapping it with an alkene in an intramolecular reaction (Figure 6, Equation 4; Zou, 2006; Supplemental Data).

These observations prompt our speculation (Figure 6, Equation 5) that the copper ion at the ethylene receptor's binding site could convert a 1-alkylcyclopropene to a carbenoid such as **24**. This intermediate could be protected from dimerizing like **19** by being sequestered deep within the protein. Rather, it could react covalently with neighboring protein residues and lead to inactive receptor **25**. Supporting a very strong, perhaps covalent, interaction between the receptor and some 1-alkylcyclopropenes is a long period of insensitivity of plant tissue to ethylene following treatment (Sisler and Serek, 2003; Serek et al., 2006). This observation could be attributed to irreparable damage of the receptor that requires it to be replaced. Return of sensitivity of that tissue to ethylene would presumably require degradation of inactive protein and the biosynthesis of new receptors. Ethylene receptor proteolysis is known (Chen et al., 2007; Kevany et al., 2007), but specific information on synthesis of new receptors is unavailable. In other organisms, cell-surface receptors are often internalized, degraded, and replaced via protein synthesis on relatively long time scales.

Returning to theoretical considerations of the strength of binding of alkenes to transition metals, *trans*-cyclooctene is the strained alkene that best fulfills its metal-binding potential based on its strain energy. This conclusion was attributed by Cedeño and Sniatynsky (2005) to the significant alkene pyramidalization in its unbound state. *trans*-Cyclooctene can be seen as preorganized to bind most effectively to the copper in the ethylene receptor. In addition to strain, pyramidalization may be a key trait to consider in understanding the potency of alkene ethylene receptor ligands. Pyramidalized alkenes have been the subjects of significant chemical investigation (Borden, 1989). Many of them are also highly strained and are therefore good candidates for novel ethylene receptor antagonists.

SIGNIFICANCE

This study further defines the molecular properties of high-affinity alkene ligands (generally, antagonists) for the copper-containing ethylene receptor in higher plants. It shows that alkene strain is not the sole requirement for high potency, and that alkene pyramidalization or reactivity with the copper ion may be important. Based on chemical models, it offers a novel mechanism of action for known antagonists such as 1-methylcyclopropene involving covalent modification of the receptor. Using the enantiomeric *trans*-cyclooctenes, it reveals the enantioselectivity that is expected for a protein-based receptor. Structure-activity relationships among ethylene receptor ligands and the ability to predict the actions of new ligands are important because of the role of ethylene antagonists in postharvest plant physiology and in practical horticultural applications.

EXPERIMENTAL PROCEDURES

General

Compounds **13**, **15** (isomer II), **16**, and **17** were obtained from commercial suppliers.

Trimethyl-(1,2,2-Tribromo-Cyclopropylmethyl)-Silane (5)

NaOH solution (50%, 1 g NaOH in 1 ml H_2O, 25 mmol) was added to a rapidly stirring solution of (2-bromoallyl)-trimethylsilane (0.48 g, 2.5 mmol) and benzyltriethylammonium chloride (0.057 g, 0.25 mmol) in bromoform (2.2 ml, 25 mmol) cooled in an ice-water bath. The mixture was vigorously stirred for 24 hr at room temperature, diluted with CH_2Cl_2, and poured into cold water. The mixture was filtered through celite and the organic phase was separated. The solvent was removed in vacuo and the crude product was purified by flash chromatography (hexanes) to give **5** as a colorless oil (0.764 g, 84%). ^{1}H NMR ($CDCl_3$): δ 2.00 (d, J = 9.3 Hz, 1H), 1.63–1.79 (m, 3H). ^{13}C NMR ($CDCl_3$): δ 43.85, 39.76, 36.07, 31.48, −0.14. IR (film): 3060, 2960, 2880, 1380, 1220, 800 cm^{-1}. Analytical: calculated for $C_7H_{13}Br_3Si$: C, 23.04; H, 3.59; found: C, 22.78; H, 3.44.

2-(1,2,2-Tribromo-Cyclopropyl)-Ethanol (6)

3-bromo-3-buten-1-ol (**10**, 0.30 g, 2.0 mmol) and *p*-toluenesulfonic acid monohydrate (0.019 g, 0.10 mmol) were cooled in Et_2O at 0°C and ethyl vinyl ether (0.57 ml, 6.0 mmol) was added dropwise. After 1 hr, the reaction mixture was quenched by saturated $NaHCO_3$ solution. The aqueous phase was extracted with Et_2O and the combined organic phase was dried with anhydrous Na_2SO_4. The solvent was removed in vacuo to give the crude 2-bromo-4-(1-ethoxy-ethoxy)-but-1-ene, which was used directly in the next step. NaOH solution (50%, 1 g NaOH in 1 ml H_2O, 25 mmol) was added to a rapidly stirring solution of crude 2-bromo-4-(1-ethoxy-ethoxy)-but-1-ene and benzyltriethylammonium chloride (0.057 g, 0.25 mmol) in bromoform (2.2 ml, 25 mmol) cooled in an ice-water bath. The mixture was vigorously stirred at room temperature for 60 hr, diluted with CH_2Cl_2, and poured into cold water. The mixture was filtered through celite. The organic phase was separated and the solvent

was removed in vacuo to give crude 1,1,2-tribromo-2-(2-[1-ethoxy-ethoxy]-ethyl)-cyclopropane **11** as a dark oil, which was used directly in the next step. To a slurry of crude **11** in MeOH (7 ml) and H_2O (2 ml) was added *p*-toluenesulfonic acid monohydrate (9 mg) and 6 M HCl (7 ml). The mixture was stirred for 1 hr. MeOH was removed by rotary evaporator and the aqueous phase was extracted with CH_2Cl_2. The organic layer was concentrated to give the crude product, which was purified by flash chromatography (hexanes: EtOAc, 2:1) to give **6** as a light yellow oil (0.358 g, 55% for three steps). ^{1}H NMR ($CDCl_3$): δ 4.04 (td, *J* = 6.4, 2.2 Hz, 2H), 2.35 (t, *J* = 6.6 Hz, 2H), 1.98 (s, 2H), 1.73 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ 61.64, 43.67, 42.70, 38.06, 32.53. IR (film): 3320, 3060, 2960, 2880, 1380, 1040 cm^{-1}. HRMS (EI): calculated for $C_5H_5Br_3$ [M − H_2O]$^+$: 301.7941; found: 301.7944.

Cycloprop-1-Enylmethyl-Trimethyl-Silane (8)

MeLi (1.6 M in Et_2O, 3.3 ml, 5.28 mmol) was added to a solution of **5** (0.642 g, 1.76 mmol) in Et_2O (4 ml) stirring at −78°C. The solution was allowed to warm to 0°C after 10 min, stirred at 0°C for another 10 min, and saturated NH_4Cl solution was added. The aqueous phase was extracted with Et_2O and the combined organic phase was washed with water, then brine, and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo to give **8** as a light yellow oil (0.178 g, 80%). ^{1}H NMR (acetone-d_6): δ 6.40 (m, 1H), 1.99 (s, 2H), 0.83 (d, *J* = 1.8 Hz, 2H), 0.27 (s, 9H). ^{13}C NMR (acetone-d_6): δ 118.31, 94.89, 16.94, 6.04, −1.84. IR (film): 2956, 2873, 1767, 1478, 1400, 1249, 1142, 1108, 1026, 998, 934, 838, 763, 697 cm^{-1}. HRMS (ESI): calculated for $C_7H_{14}Si$ [M + H]$^+$: 126.0865; found: 126.0859.

2-Cycloprop-1-Enyl-Ethanol (9)

MeLi (1.6 M in Et_2O, 0.75 ml, 1.2 mmol) was added to a solution of **6** (0.097 g, 0.30 mmol) in Et_2O (2.5 ml) stirring at −78°C. The solution was allowed to warm to 0°C after 10 min, stirred at 0°C for another 10 min, and saturated NH_4Cl solution was added. The aqueous phase was extracted with Et_2O and the combined organic phase was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo to give **9** as a light yellow oil (0.020 g, 80%). ^{1}H NMR ($CDCl_3$): δ 6.60 (m, 1H), 3.85 (t, *J* = 6.3 Hz, 2H), 2.75 (td, *J* = 6.3, 1.5 Hz, 2H), 2.27 (brs, 1H), 0.91 (d, *J* = 1.8 Hz, 2H). ^{13}C NMR (acetone-d_6): δ 118.19, 98.97, 59.55, 30.62, 4.29. IR (film): 3345, 2963, 2879, 1778, 1631, 1418, 1378, 1045, 1025, 944, 893, 849, 689 cm^{-1}. HRMS (ESI): calculated for C_5H_8O [M]$^+$: 84.0575; found: 84.0570.

7,10-Dimethylene-Hexadec-8-Ene (20)

1-hexyl-cyclopropene (0.5 mmol) in acetone (2 ml) was added to copper phenanthroline acetonitrile hexafluorophosphate (0.05 mmol) in acetone (3 ml) and the mixture was stirred at room temperature for 10 min. The solvent was removed in vacuo and Et_2O was added. The mixture was filtered through a pad of celite and concentrated to give a crude product that was purified by flash chromatography to give the title compound in 69% yield as a 2:1 mixture of (*Z*)- and (*E*)-alkenes. ^{1}H NMR ($CDCl_3$): δ 6.27 (s, 0.72H), 5.86 (s, 1.28H), 4.90–5.04 (m, 4H), 2.12–2.26 (m, 4H), 1.22–1.54 (m, 16H), 0.84–0.92 (m, 6H). ^{13}C NMR ($CDCl_3$): δ 146.81, 146.75, 131.27, 130.61, 115.61, 114.40, 36.19, 32.26, 32.01, 29.51, 29.34, 28.64, 28.54, 22.86, 14.31. IR (film): 2956, 2926, 2857, 1723, 1466, 1378, 1174, 1022, 909, 732 cm^{-1}. GC-MS: 248 (M$^+$, two peaks on GC, one at 10.6 min, one at 11.5 min, both with the same molecular weight). HRMS (FAB): calculated for $C_{18}H_{31}$ [M − H]$^+$: 247.2426; found: 247.2423. The stereochemistry was assigned based on the chemical shift of the olefinic proton of the internal double bond. In (*E*)-hexatrienes, this is in the range of 6.0–6.5, whereas in (*Z*)-hexatrienes it is in the range of 5.6–6.2 (Vroegop et al., 1973). As the integration of the 6.27 and 5.86 ppm signals in this compound is 1:2, the Z isomer is major.

Hypocotyl Growth Rate Measurements

Kinetic analyses of hypocotyl elongation were performed using etiolated *Arabidopsis thaliana* seedlings of the Columbia ecotype as described previously. Seeds were surface sterilized by treatment with 70% ethanol for approximately 30 s, placed on sterile filter paper to dry, and plated on half-strength Murashige and Skoog medium (pH 5.7) containing 0.8% agar and B5 vitamins, consisting of inositol (100 mg ml^{-1}), nicotinic acid (1 mg ml^{-1}), pyridoxin HCl (1 mg ml^{-1}), thiamine HCl (10 mg ml^{-1}), and 5 μM L-α-(2-aminoethoxyvinyl)-glycine (Rohm Haas, Philadelphia, PA, USA), with no added sugar. Seeds were stratified for 2–4 days at 4°C and exposed to white light for 2–8 hr prior to being grown vertically in the dark for 2 days at 22°C. Once seedlings reached a height of 2–4 mm, hypocotyl growth rates were measured by a modification of methods used previously (Binder et al., 2004a). Briefly, following a 45 min treatment with air to establish a basal elongation rate, ethylene was introduced at a flow rate of 10 ml min^{-1} giving a final concentration of 10 μl l^{-1}. Gas flow was maintained at 100 ml min^{-1} using Side-Trak mass flow meters and controller (Sierra Instruments, Bolsuen, The Netherlands). Hypocotyl growth was measured from digital images that were captured every 5 min for 8 hr with an EDC-1000N CCD camera (Electrim, Princeton, NJ, USA) and light was provided by an infrared light-emitting diode. Growth rates were calculated using custom software generated by Spalding in LabVIEW 5.0 (National Instruments, Austin, TX, USA) as previously described (Folta and Spalding, 2001; Parks and Spalding, 1999). Data were normalized to the average growth rate in air prior to treatment with ethylene. All data presented represent the average of at least three seedlings from a minimum of three separate experiments.

To assess inhibition of ethylene responses, seedlings were pretreated with MCP or MCP analogs for 17 hr prior to growth rate measurements. Agar plates with etiolated seedlings were placed in a sealed chamber containing the indicated concentration of MCP or analog. MCP was supplied by Rohm Haas in solid form as EthylBloc and was released by the addition of hot water through a septum in the sealed chamber. The analogs of MCP were dissolved in acetone and injected into the sealed chamber through a septum. Acetone controls were performed by injecting acetone without MCP analogs. After 17 hr of pretreatment, growth rate analysis was carried out as described above. The effects of each compound were determined for long-term responses to ethylene by averaging growth rates between 3 and 6 hr after addition of 10 μl l^{-1} ethylene.

Ethylene Binding Assays on Intact Yeast Cells and Isolated Yeast Membranes

Cultures of *Saccharomyces cerevisiae* (strain LRB 520) expressing full-length ETR1 protein were grown to mid-log phase at 30°C and harvested by centrifugation as previously described (O'Malley et al., 2005). Yeast cells were washed with water and collected on glass microfilters by vacuum filtration to give 1 g yeast per filter. For some experiments, instead of concentrating on filters, the yeast cells were disrupted and membranes were isolated by centrifugation as previously described (Rodríguez et al., 1999). Membranes were rapidly frozen in liquid nitrogen and stored at −80°C until used.

Saturable ethylene binding on intact yeast or isolated yeast membranes expressing full-length ETR1 protein was determined using the methods of Sisler (1979) as modified by others (Schaller and Bleecker, 1995; Schaller et al., 1995; Rodríguez et al., 1999). Either [^{3}H]ethylene or [^{14}C]ethylene at a concentration of 0.1 μl l^{-1} was used to measure ethylene binding. Data represent saturable ethylene binding calculated by subtracting the amount of radioactive ethylene bound in the presence of 100-fold excess $^{12}C_2H_4$ from the amount of radioactive ethylene bound in the absence of nonradioactive ethylene. As found in earlier studies (Schaller and Bleecker, 1995; Schaller et al., 1995; Rodríguez et al., 1999), no saturable binding of ethylene was detected in yeast or yeast membranes not expressing ETR1 (data not shown).

To test the effects of scalemic mixtures of *trans*-cyclooctene, yeast cells were preincubated for 30 min at room temperature with the indicated dilution of a mixture enriched in either the (*S*)- or (*R*)-enantiomer. Cells were washed and filtered onto glass microfilters and assayed for ethylene binding as described.

The effect of compounds **13**, **15**, **16**, and **17** on saturable ethylene binding was examined. Each compound was dissolved in DMSO. Intact yeast cells were mixed with a compound at the indicated concentration and treated for 30 min at room temperature. The cells were then filtered and assayed for ethylene binding as described above. Isolated yeast membranes were also preincubated with these compounds under these conditions, then centrifuged, and the resulting membrane pellet was resuspended and assayed for ethylene binding. Control experiments were carried out using DMSO without additions.

SUPPLEMENTAL DATA

Supplemental Data include procedures for the preparation of **4**, **7**, **12**, **21**, **23**, and *trans*-cyclooctene enantiomers; copper-promoted ring opening of

cyclopropenes; boiling point determination by gas chromatography; and ^{1}H NMR spectra of **6**, **8**, and **9**, and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/4/313/DC1/.

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