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Compounds controlling the ethylene receptor

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Abstract. Many organic compounds interact with the ethylene receptor and diffuse free after different periods of time. Some are agonists and mimic ethylene, and some are antagonists, which prevent ethylene action by blocking the receptor. Some of the antagonists have proven useful in scientific studies, and some promise to be commercially important in protecting against ethylene. The times that different compounds remain bound may be important clues to how ethylene transmits its signal to the signal transduction pathway. Ethylene diffuses from the site with a $t_{1/2}$ of 2–10 minutes and is an active compound. For 2,5-Norbornadiene (2,5-NBD) and some other strained olefins that block ethylene action, the half diffusion time is 3–6 h. For other more strained compounds (cyclopropenes) the half diffusion time is estimated to be 7–12 days; they block ethylene action during this time. The time of diffusion from the receptor appears to be the major difference between compounds that block the receptor and those that are active. It is suggested that this time constant may be the controlling factor in ethylene action.

Keywords: 1-methylcyclopropene, 1-MCP; 2,5-norbornadiene, 2,5-NBD; 3,3-dimethylcyclopropene, 3,3-DMCP; Cyclopropene; Diazocyclopentadiene, DACP; Ethylene antagonist; Ethylene receptor; Methylenecyclopropane; *trans*-Cyclooctene.

Abbreviations: 1-MCP, 1-methylcyclopropene; **2,5-NBD,** 2,5-norbornadiene; **3,3-DMCP,** 3,3-dimethylcyclopropene; **DACP,** diazocyclopentadiene.

Contents

Introduction

In recent years some very effective agents for blocking the ethylene receptor have been discovered by Sisler and coworkers. These block ethylene action, and hold the promise of being a new way of controlling ripening, senescence, and other ethylene responses. Since the ethylene receptor is ubiquitous in plants, these compounds should control all ethylene responses in plants. Rather than being a totally new discovery, they are the consequence of efforts to develop a better understanding of the ethylene receptor. This paper is not intended to cover all of the many papers in which these compounds have been used, but instead to cover the important findings and uses of the compounds. We will initially address what has been

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learned from inhibitor studies in plants, and then address how these relate to the recent identification of a putative ethylene receptor from *Arabidopsis*. Some aspects of receptor blocking agents have recently been reviewed (Sisler and Serek, 1997).

Although ethylene has been recognized as a plant hormone since 1901 (Neljubov, 1901) and many important discoveries of ethylene action have been made in the intervening years, the paper by Burg and Burg (1967), which proposed that the ethylene receptor acted *via* a metal, was the beginning of work at the receptor level. Burg and Burg (1967) reported that compounds such as ethylene, propylene, butene, and vinyl methyl ether bind to silver ion in the same order as their ability to induce ethylene responses in plants. They postulated that a metal was involved in ethylene action. To this date, no one has proved that the ethylene receptor contains a metal, but the evidence strongly supports that view.

Sisler and Pian (1973) reported that some cyclic olefins appeared to block ethylene responses rather than to induce a response. Of these, 2,5-norbornadiene (2,5-NBD) blocked at the lowest concentration, but all of these compounds require continuous exposure to be effective. Sisler and Yang (1984) showed that the relative blocking ability of these compounds also paralleled their ability to bind to silver ion and proposed ring strain as an important factor. It has never been definitively shown exactly how these compounds work although much more effective compounds have become available since that time.

A number of other compounds such as *cis*-butene, cyclopentene (Sisler and Yang, 1984), and some heterocycle compounds were subsequently shown to inhibit ethylene action. In 1990 it was shown that the highly strained *trans*-cyclooctenes, but not the less strained *cis*-cyclooctenes, were much more effective, in terms of concentration, than 2,5-NBD (Sisler et al., 1990). These also required continuous exposure to the tissues to be effective in blocking ethylene responses.

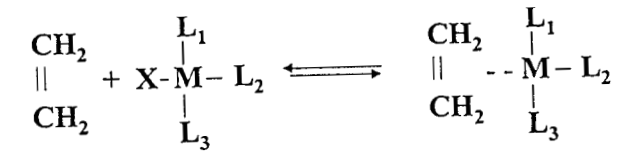
While searching for a photoaffinity label for the receptor (Sisler and Blankenship, 1993a,b), the irradiation product of diazocyclopentadiene (DACP) was shown to block the ethylene receptor for many days. The product appears to be a gas at room temperature; however, it is very unstable and has not been identified. The product of photolysis was shown to block ripening and senescence in banana (*Musa sapientum*), tomatoes (*Lycopersicon esculentum*), kiwi (*Actinidia chinensis*), persimmons (*Diospyros virginiana)*, and carnation (*Dianthus caryophyllus*) and prevent ethylene inhibition of pea (*Pisum sativum*) growth (Sisler and Blankenship, 1993a,b; Sisler and Lallu, 1994; Serek et al., 1994). Using ^{14}C-ethylene, it has been shown to block the receptor in mung bean (*Vigna radiata*) sprouts and in tobacco (*Nicotiana tabacum*) leaves.

Subsequently, it was discovered that some cyclopropenes counteract the effects of ethylene for 10–12 days in tissues given a single exposure at very low concentrations (Sisler et al., 1996a,b; Serek et al., 1995; Dupille and Sisler, 1995). Some cyclopropenes block for shorter times and others appear to be inactive. These compounds appear not only to be of practical value as ethylene antagonists, but also may give valuable clues as to how ethylene is able to enter into signal transduction (Figure 1).

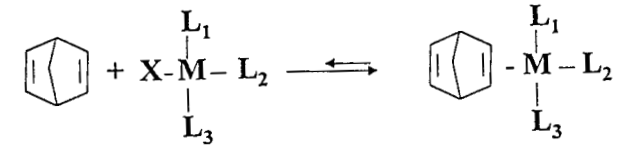
Uses of Commercial or Scientific Value

There are four compounds which are or have been used extensively in scientific investigations: 2,5-NBD, *trans*-cyclooctene, DACP and 1-methylcyclopropene (1-MCP).

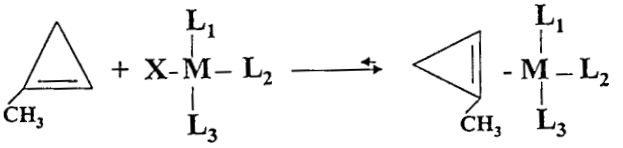
1) 2,5-NBD (2,5 bicyclohepta-2,5-diene) is a liquid (bp 89 C) at room temperature and is sufficiently volatile to be applied in the vapor state either by allowing it to evaporate in the treating chamber or inserting it with a stream of air. Since it was the best of the first group of receptor blockers discovered, it has been used in numerous scientific studies to block the ethylene receptor. It has a very disagreeable odor but has the advantage of being commercially available. It is still being used in some studies. It must be continually applied to be effective, and at high



Ethylene



2,5-Norbornadiene



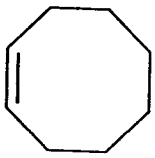
1-Methylcyclopropene

Figure 1. Types of interaction with the ethylene receptor. After binding, a rearrangement of the ligands in some unknown manner is thought to occur in each case. (Top) Ethylene and many other pi acceptor compounds bind to the receptor and induce an ethylene response. Ethylene, acetylene, carbon monoxide, and isocyanides are examples. (Middle) Strained olefin compounds which bind to the receptor and prevent ethylene responses but require continuous exposure. High levels of ethylene will overcome the effect by competition. 2,5 NBD and *trans*-cyclopropene are examples of these compounds. (Bottom) Strained olefins compounds which bind to the receptor and prevent ethylene responses for up to 12 days by a single exposure, during which time ethylene does not overcome the response. 1-MCP, cyclopropene and 3,3-DMCP are examples of these compounds.

concentrations stimulate ethylene production which may overcome its effect if the ethylene is not vented away. The noxious odor limits its use to scientific investigations.

2) *trans*-cyclooctene is also a liquid (bp 75 C, 100 torr) but is sufficiently volatile to be applied as a vapor and is a good inhibitor of ethylene responses. It is usually effective at concentrations 50–100 times lower than 2,5-NBD. This compound should not be confused with *cis*-cyclooctene, which is much less active and requires concentrations more than 600 times higher (Figure 2). Despite the superior properties of *trans*-cyclooctene, it has seen only limited use since it is not commercially available and must be synthesized (Hiyama and Nozaki, 1973) for use. It too must be continuously supplied to be effective and has an unpleasant odor.

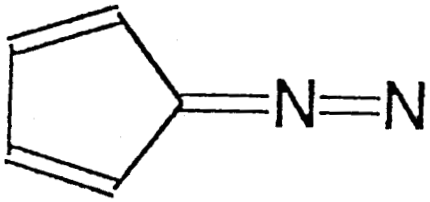
3) DACP (bp 52–53, 50 torr) (Regitz and Leidhegener, 1967; Sisler and Blankenship 1993a,b) binds to the receptor but is not very effective in blocking ethylene action. In fluorescent light, it photodecomposes to form a very effective inhibitor of ethylene responses (Figure 3). The photolysis product has not been identified, and the use of DACP has been limited because it is not commercially available. It is potentially explosive when concentrated, and should be kept in a solution of pentane or other organic compound. Anyone using this compound should be aware of its explosive nature and avoid ground glass joints (which promote the decomposition of diazo compounds) and avoid shocking it. It can be crystallized from alcohol or pentane at -70°C.



cis–cyclooctene

trans–cyclooctene

Figure 2. Structure of *cis*-cyclooctene and *trans*-cyclooctene. Both of these compounds block the ethylene receptor, but the highly strained *trans*-cyclooctene is concentration-wise 650 times more active than the *cis* form.



diazocyclopentadiene

Figure 3. Photolysis of DACP. Fluorescent light causes the photodisintegration of DACP. DACP interacts with the receptor and blocks it, but requires continuous exposure. The photolytic product blocks up to 12 days, but requires continuous exposure. After light activation the unidentified light product is concentration-wise 5000 times more active and blocks the receptor up to 12 days.

4) 1-MCP represents the best example of a group of active cyclopropene compounds based on concentration and stability considerations. This compound is being commercially developed by Biotechnologies for Horticulture Inc., 751 Thunderbolt Dr. Waltersboro, SC 29488, USA. It can be synthesized easily by those familiar with air sensitive reagents (Magid et al., 1970). This compound will probably be the ethylene inhibitor of choice for the immediate future and holds considerable commercial potential since at its active concentration of 0.5 $nl.l^{-1}$ on carnation, it has no detectable odor and has not been reported to have toxic properties.

Effects of Concentration

A relatively high concentration of 2,5-NBD is required to block the receptor. Table 1 shows that 55,000 $nl.l^{-1}$ are required to protect bananas. Like ethylene binding, 2,5-NBD binding is an equilibrium reaction, and if the 2,5-NBD is vented away, the plant material becomes sensitive to ethylene in a short time.

It requires 780 $nl.l^{-1}$ of *trans*-cyclooctene and continuous exposure to protect ethylene-induced ripening of bananas. 512,000 $nl.l^{-1}$ of *cis*-Cyclooctene is required for protection. Carnations exposed to DACP in the dark re-

Table 1. Concentration of compound needed to protect plants against ethylene.

Compound	Plant	Concentration ($nl.l^{-1}$)
2-5-NBD	Banana	55,000
trans-Cyclooctene	Banana	780
cis-Cyclooctene	Banana	512,000
DACP (dark)	Carnation	700,000
DACP (light)	Carnation	140
1-MCP	Banana	0.7
1-MCP	Carnation	0.5
1-MCP	Pea growth	40
3,3-DMCP	Banana	700

quire 700,000 $nl.l^{-1}$ for protection against 10 $\mu l.l^{-1}$ of ethylene, but after the DACP is pre-irradiated with fluorescent light, 140 $nl.l^{-1}$ of DACP is required. This shows a considerable activation of effect. Some of this effect may be due to the conversion to an unidentified product which binds more rapidly than DACP, but much of it probably is due to the fact that the unidentified product remains bound for many days while most of the unconverted DACP comes off the receptor within 60 minutes (Sisler et al., 1993).

To prevent ethylene induced ripening of bananas requires 2 $nl.l^{-1}$ 1-MCP for a 6 h exposure or 0.7 $nl.l^{-1}$ for a 24 h exposure. It is not known if this is an equilibrium reaction or not, but since the inhibition lasts for 12 days, in a practical sense it probably can be considered a non-equilibrium reaction. It is possible that at least part of the regained sensitivity to ethylene is due to the synthesis of new receptors. Although these values are for bananas, they can be considered approximate for most fruits and flowers.

Competition for the Receptor

Ethylene is the natural hormone for the receptor; however, other active compounds such as acetylene, carbon monoxide, isocyanides and other olefins compete for it (Burg and Burg, 1967; Sisler, 1977). Likewise, compounds that block the receptor compete with ethylene for it. 2,5-NBD (Sisler et al., 1985), *trans*-cyclooctene (Sisler et al., 1990) and many other cyclic olefins have competitive kinetics for the receptor. The photolytic product of DACP and 1-MCP show competitive kinetics with ethylene before the compound is bound. After these "permanent" blocking compounds have bound, competition has not been demonstrated because the receptor has been inactivated for too long. In ripening fruit even large amounts of ethylene (e.g. 1,000 $\mu l.l^{-1}l$) do not give a response after these compounds have bound. The difference in the two groups seems to be that the "permanent" blocking agents dissociate too slowly to demonstrate competition. However plant material does become sensitive to ethylene after 12 days at 25°C, and some dissociation is thought to take place. It is possible that new receptors are made, but tissues treated with 3,3-dimethylcyclopropene (3,3-DMCP) become sensitive to ethylene within 7 days after treatment, suggesting that the receptor becomes free. It would thus appear that the difference between compounds that must be used continuously and those that are "permanent" is a matter of the dissociation time from the receptor (Figure 1).

How Long do Compounds Remain Bound to the Receptor?

In tobacco leaves half of the bound ethylene diffuses out in 10 minutes. In tomato leaves, half appears to diffuse out in less than 2 minutes. In some cases, ethylene diffuses from the binding site(s) in two or three phases, depending on the plant material (Sisler, 1991; Sanders et al., 1991). In mung bean sprouts, there is a rapid phase with a half-life of from 8–10 minutes. Another lasts about 1.5–2 h, and another from 10–24 h. Growth experiments (Warner and Leopold, 1971) in pea suggest the short time site is a receptor.

Diffusion of ethylene blocking compounds from mung bean sprouts has been measured in a few cases (Table 2). 2,5-NBD diffuses away with a half-life of 3 h, and *trans*-cyclooctene diffuses away with a half life of 6 h. Both are compounds that require continuous exposure. The time constants of compounds omit which bind for long periods of time have not been measured, because the tissue usually has changed during that time interval. Some tissues require 12 days to become sensitive to ethylene again. When tissues are treated with other compounds they may require less time to become sensitive again; 7 days in the case of 3,3-DMCP and 5 days for others (Sisler et al., unpublished). These widely divergent times to regain activity suggest that the same receptor is becoming active again rather than that new receptors are being produced, although both may be partly responsible. It probably can be assumed that these periods represent, roughly, the time required for half of the receptor to be free.

Why do Olefins Bind to Metals?

Burg and Burg (1967) reported that the concentration needed for the biological activity of olefins in an ethylene response was of the same order as those compounds that bind to silver (Muhs and Weiss, 1962). Binding to silver is reported to be of the same order as ring strain (Traynham and Olechowski, 1959). All double bonds have inherent

Table 2. Time required for the receptor to become free. Time is for 1/2 of the receptor to become free after being exposed to the compound. In the case of 3,3-DMCP and 1-MCP, time refers to time for bananas become sensitive after a single exposure to ethylene.

Compound	Plant	Time (Minutes)
Ethylene	Mung bean sprout	10
Ethylene	Tobacco leaf	10
Ethylene	Tomato leaf	2
2,5-NBD	Mung bean sprout	180
Trans-c*yclooc*tene	Mung bean sprout	360
3,3-DMCP	Banana	25,200
1-MCP	Banana	43,200

strain (Wiberg, 1987). Even ethylene may be looked at as having considerable inherent strain, which can be relieved by accepting electrons. Some other olefins have considerably more strain and accept electrons to relieve that strain. The greater the strain, the tighter the binding and presumably the longer the bonds would last, unless some other factor such as steric effects came into play.

How do these Compounds Prevent Ethylene Action?

When ethylene binds to the receptor, it must do something which the blocking compounds do not do, and these compounds must also prevent ethylene from doing it. Molecular size could be involved, but 1-MCP and methylenecyclopropane have the same empirical formula and differ only in the position of the double bond (Figure 4). 1-MCP is an ethylene antagonist while methylenecyclopropane is an agonist. They should be similar in size. Chatt et al. (1955) have proposed that binding of compounds such as ethylene to metals makes the position *trans* to the bound ethylene more susceptible to substitution by other ligands by withdrawing electrons from the metal and giving the metal more affinity for electrons in the opposite position. The process probably has at least two steps: In Step 1, both those compounds which induce a response and those that block the receptor should bind to the supposed metal and withdraw electrons from the metal. This would likely result in a rearrangement or change in ligands on the metal. In Step 2, ethylene likely dissociates from the metal (or is pushed off) in a few minutes while those that block receptors appear to take at least several hours to leave, and some many days (Table 2). The leaving time from the metal seems to be the thing that is different. If high levels of compound are used, binding time is rapid (a few minutes), which rules it out as the controlling factor. It would thus appear that ethylene leaving the complex causes the complex to become active probably by further ligand rearrangement since those that do not leave rapidly do not form an active complex.

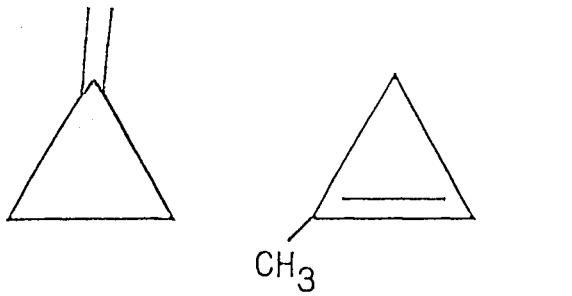


Figure 4. Structure of 1-MCP and methylenecyclopropane. 1-MCP is a very effective antagonist of ethylene action, and methylenecyclopropene is an ethylene agonist.

What are the Ligands?

Exactly what ligands are is unknown, but in proteins groups such as histidine, methionine, thiol groups, tyrosine groups, and, in a hydrophobic environment such as a membrane, perhaps the double bonds in unsaturated fatty acids could all be ligands. The ligands could be on a single protein or perhaps form a cross link between two proteins. A small molecule could also supply a ligand. It has been shown (Schaller and Bleecker, 1995) that replacing a cysteine or histidine (Schaller et al., 1997) eliminates ethylene responses in *Arabidopsis* and eliminates ethylene binding in yeast. This suggests that cysteine and histidine are ligands although the function of cysteine could be to put a metal in a low oxidation state , causing it to bind ethylene in the hydrophobic environment. The receptor complex probably needs to be isolated from the plant tissue and the binding site located by radioactive label to determine with certainty what the metal is and to what it is bound. Even this approach may be difficult if the metal in the receptor is labile.

How does the Active Complex Work?

At this time there is no information on how the active complex works except that ethylene responses are manifest. We know that compounds which induce responses also accept electrons, and those that block are those acceptors that are strained. This does not tell us how a ligand substitution process turns on the signal transduction pathway. This problem needs to be addressed in future research.

Where is the Site of Ethylene Action?

Recent molecular biology studies indicate the gene product of ETR1 as the site of ethylene action (Schaller and Bleecker, 1995). When ETR1 is cloned in yeast, it binds ethylene. Ethylene diffuses from this site with a $t_{1/2}$ of 12.5 h. The major binding component in *Arabidopsis* diffuses from the binding site rapidly (Sanders et al., 1991) unless the plants are incubated with ^{14}C-ethylene many hours when another binding site appears from which diffusion is much slower. Plants have a number of ethylene binding sites. Mung bean seeds bind large amounts of ethylene, far in excess of vegetative tissue, and most of it dissociates over a period of many hours. Inorganic Cu^{+} binds ethylene in aqueous suspension, and it has a very definite dissociation pressure. It must be realized that the discrepancy in dissociation times for ethylene in yeast and in *Arabidopsis* is a serious discrepancy. The growth evidence of Warner and Leopold (1971) in pea strongly suggest a short time association-dissociation for ethylene in controlling growth. Plants containing a mutant ETR1 gene bind less ethylene than normal plants, and this is the component that seems to disappear in mutant *Arabidopsis* plants (Bleecker et al., 1988). Histidine and cysteine are essential for ethylene binding in this cloned ETR1 protein, and binding is thought to be Cu^{+}. Many

metals in proteins are bound through histidine, and cysteine in a hydrophobic environment would likely reduce Cu^{++} to Cu^{+}. This should bind ethylene. Binding alone by this over expressed protein does not mean that the ethylene binding has anything to do with physiological activity. The discrepancy in dissociating time does not rule out this binding being the site of the physiological ethylene response. The discrepancy may mean the Cu^{+} is not properly oriented or some other component is missing, and it could mean the metal is different from copper. It should be mentioned that when mung bean sprouts are blended, the short time-binding component disappears and does not appear to bind ethylene *in vitro* (Sisler, 1991). This could be due to the loss of the metal, or possibly the change of valence by the metal when it is exposed to high oxygen levels or exposed to water thus releasing bound ethylene. It will be necessary to show that the ethylene binding in ETR1 has physiological activity before it can be said on that basis that ETR1 is the receptor, although the evidence does favor ETR1 as being at least part of the receptor.

Future Work

Despite the vast amount of work done in the past, some problems remain. Perhaps the biggest are identifying the supposed metal in the receptor, finding to what ligands is it bound and where they are located. Other questions are: Is there more than one type of receptor? How does ethylene turn the ethylene response pathway on, and how is it regulated? Still other challenges include better control of these responses and extending the shelf life of fruits and vegetables using these materials.

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