

From Small Rings to Big Things: Fruit Ripening, Floral Display and Cyclopropenes*

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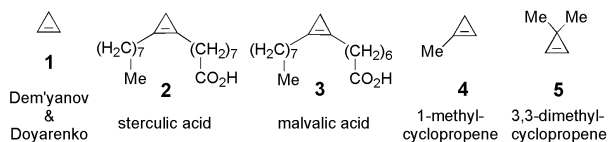
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*Dedicated to Bede Squire FRCS who, fortunately, left the NZ native bushes and trees until after his retirement.

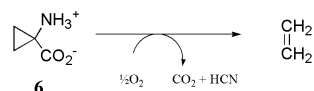
Introduction

Cyclopropene chemistry first graced the chemical literature by way of assumptions for the presence of symmetrical derivatives in works by Wolff and Merling as referred to by Franz Feist¹ when he reported the isolation of the 3-methyl-1,2-dicarboxylic acid in 1893. The paper, on cumulene ring decomposition, includes a 1,3-dehydrobromination that provides the diacid in direct analogy to the preparation of cyclopropane some ten years earlier.¹ Another twenty years passed before the parent, gaseous C₃H₄ hydrocarbon **1** was recognised, initially in a Russian publication,² and, more widely, one year later in *Berichte*.³ Following these reports and the synthesis of various derivatives over the ensuing thirty years, the naturally occurring sterculic and malvalic acids were confirmed as **2** and **3** (Chart 1) in the early to mid-1950s.⁴ The latter half of the last century saw the essential chemistry of the class of compounds explored, but it is only in the last fifteen years that the commercial importance of 1-methylcyclopropene⁵ (**4**) and other alkyl homologues, e.g. **5**, has been recognized and implemented.

Chart 1



The occurrence and biological activity of cyclopropane derivatives in nature is well known^{4,6} with all green plant matter containing 1-aminocyclopropane carboxylic acid (**6**, known also as ACC). It is converted by ACC oxidase to ethylene,⁷ an important plant hormone. Environmental and endogenous signals regulate the biosynthesis of ethylene primarily through differential expression of ACC synthase genes whose activity controls the rate of production; its regulation is the key to controlling ethylene biosynthesis, the final step of which involves a radical process (Scheme 1),⁸



Scheme 1

Recognition that ethylene causes premature senescence (ageing) and defoliation of plants dates to the nineteenth century when several reports of leaks of illuminating gas in greenhouses and near trees appeared.⁹ Neljubov, a Russian plant physiologist, recorded etiolated pea seedlings (ones with small yellow leaves) growing horizontally in his laboratory but upright in outside air. He showed that the abnormal growth habit was caused by contaminating

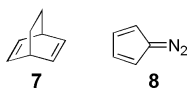
illuminating gas and went on to prove, in 1901, that the active principle in the gas was ethylene. Chemical proof that plants produce ethylene dates back some 75 years,⁹ since which time the regulation of a multitude of plant processes by ethylene has been recognised. Sometimes ethylene is called the *death hormone* because it promotes the ripening and aging of many fruits and flowers.

As a fruit approaches maturity a *climacteric* (critical) event takes place and, during the build-up to this, ethylene production increases within the fruit and cell respiration rises. The production of the ethylene is associated with the expression of hundreds of genes that influence various ripening related changes, e.g. in fruit colour and sugar release; it marks the end of fruit maturation and the beginning of fruit senescence. For fruits that are food, the climacteric event equates to the peak of edible ripeness when the best texture and taste are present. After the event, fruit is more susceptible to fungal infection and begins to degrade. The climacteric fruits include apples, apricots, bananas, melons and tomatoes while citrus fruit, grapes and strawberries are non-climacteric as they ripen without ethylene and the associated respiration bursts. Many fruits are picked prior to full ripening because ripened fruits do not ship well. Thus, bananas are picked when green, shipped, and may be subsequently artificially ripened by treatment with ethylene to mimic the natural process. Left alone, bananas actually produce enough ethylene to accelerate ripening in other fruits when placed next to them.

Cut flowers such as the carnation and geranium, as well as a range of plants, can become stressed during transport and suffer as a result of the ethylene they produce – it reduces significantly the floral display and the consequent shelf-life. Thus, ethylene production can cause economic loss in the market place for growers, suppliers, and florists alike and for this reason such flowers are cooled prior to packing and kept between 1 and 4 °C during shipment. It is not surprising, therefore, that there has been a search for compounds that delay the onset of fruit ripening and extend the shelf-life of cut flowers.

Efforts to understand the effects that ethylene has on plant materials began by storing fruits under controlled atmospheres and assessing the changes that took place.¹⁰ They then moved on to examine the nature of the ethylene binding site. Many compounds interact with the receptor, some reversibly, e.g. 2,5-norbornadiene (**7**), and some irreversibly, e.g. diazocyclopentadiene (**8**).^{10,11} An assumption that the site involves coordination of ethylene by copper(I) has been confirmed, and studies have found that of several transition metals, only silver(I) mimicks the effect of copper, nicely consistent with the similarities of the two ions. Silver

thus proved capable of replacing copper and interacting with ethylene, but not in transducing the signal to downstream effectors.^{9,12} Hence, the possible use of silver ion on an industrial scale to prolong flower life emerged and was taken seriously once silver thiosulfate became available.¹⁰ However, usage of a heavy metal, coupled with the associated contamination concerns led researchers, notably Sisler and his colleagues, to seek organic analogues of ethylene that would do the same job.¹⁰⁻¹⁴



Cyclopropenes

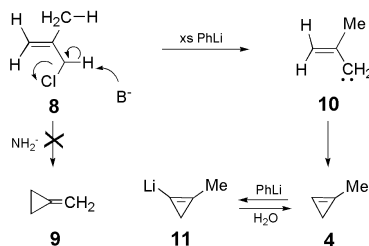
There are two approaches by which the effects of ethylene can be minimised. The first seeks to prevent (or minimise) ethylene synthesis while the second aims to block the ethylene binding site completely. Inhibiting ethylene synthesis is the least effective option because ethylene from other sources is still able to bind and impact. In comparison, a blocking of the binding site prevents all ethylene from binding irrespective of its source such that fruits, plants and flowers remain in a state of limbo until the block is removed and ethylene production recommences - and this is independent of whether the ethylene source were endogenous or exogenous.

Work from the 1960's had shown that not only CO₂, but also CO and the lower members of the alkene family are ethylene antagonists and, of the last group, the longer chain members are inactive.¹⁵ This led to the belief that activity was inversely related to molecular size and the idea that likely inhibitors of ethylene perception would be analogues of ethylene with comparable, but by no means identical spatial requirements. Thus cyclopropene and its simple alkyl derivatives received early attention^{10,11,14,15} and the discoveries made stem in large measure from the laboratories of Sisler and Blakenship at North Carolina State University.

What was found in the mid-1990's was that cyclopropenes **1**, **4**, and the 3,3-dimethyl derivative **5** (Chart 1) are especially effective blockers of the ethylene binding site and prevent the physiological action of ethylene for extended periods of time.¹³ After a 24 hour exposure to a concentration of **4** as low as 0.5 nL/L (1 mL in 2,000,000 L), carnation flowers (*Dianthus caryophyllus*) are protected for several days against the effects of ethylene, and 0.7 nL/L of **1** or **4** will prevent the ripening of banana (*Musa sapientum*) for 12 days at 24 °C after which they then ripen normally. By comparison the dimethylated analogue **5** has higher spatial demand; it requires a concentration 1000 times greater and then only provides a 7 day protection. Methylcyclopropene **4** is more easily handled than parent **1** and, as it appeared to be non-toxic at the concentrations that are active, its future use in regulating the ripening of fruits and preventing the deleterious effects of ethylene in plants and flowers seemed assured.¹² Cyclopropene **4** is now used in a number of horticultural products that are registered in more than 26 countries.

The first report of 1-methylcyclopropene (**4**) came from Fisher and Applequist⁵ in 1965. These authors found that attempts to produce methylenecyclopropane **9** by 1,3-dehy-

drochlorination of 2-chloromethylpropene **8** with sodamide gave instead the endocyclic cyclopropene **4** (Scheme 2). The cyclization proceeds by way of α -elimination to vinylcarbene **10**, which then self inserts. The gaseous product was condensed in a dry ice trap and formally characterized. Improvements were made to the procedure in 1971 from use of phenyllithium as base^{16,17} and exclusion of lithium bromide and non-PhLi bases, e.g. lithium alkoxide. Yields of **4** were in the 60-80% range when crystalline phenyllithium in ether was used. However, with excess of PhLi the cyclopropene is transformed into its 1-lithio salt **11** and this is stable for months in ether in the freezer.¹⁷ With water **4** is rapidly regenerated and Sisler has provided a convenient laboratory procedure for this.¹³



Scheme 2

Sisler and Blakenship^{14,18} acquired the first patent for commercial applications of **4** in the USA but had Floralife Inc. in South Carolina commercialize for the floral market with later development for fruits and vegetables planned.¹⁹ A preparation approved by the US Environmental Protection Agency in 1999 for plants and flowers is sold as Ethyl-Bloc™. Rohm and Haas Company recognized the opportunity to purchase a product with a very favourable safety profile, formed subsidiary AgroFresh Inc. to develop **4** for food production, and purchased the rights for development in fruits and vegetables from Floralife in 1999. The development led to the subsidiary trading SmartFreshSM,²⁰ principally for the apple industry, following EPA approval in 2002. Although FloraLife was acquired by Rohm and Haas in 2006, it was sold to Smithers-Oasis Co. (a manufacturer of floral foam and related products) a year later. Since then, AgroFresh have provided a new liquid formulation of **4** under the Invinsa™ label²¹ for use in stress protection of crops in the field. Their March 2008 press release says that they plan to have the technology commercial within two years and direct it to major field crops including canola, corn, cotton, rice, soybean, sunflower and wheat. Although set to trade independently, the Rohm and Haas company was taken over by The Dow Chemical Company in July 2008.

Because **4** is a gas under normal conditions commercial development demanded a simpler, easier, and more convenient synthesis, and then the means to get the product to market. Here, the advances in supramolecular chemistry dominate. Encapsulation of **4** can be brought about by hosts such as the cyclodextrins, crown ethers, zeolites, etc., and patents for these have appeared.^{22,23} Use of α -cyclodextrin gives rise to a powder (from which subsequent tablet formulation is presumed) that is ideal for shipping and handling. On addition to water under normal conditions, the host-guest complex releases gaseous **4** within 20-30 min.¹¹ The original US patent²³ involved a batch synthesis and encapsulation of **4**. However, patent rights were granted to Rohm and Haas for a continuous process in 2005 (applica-

tion 2002).²² In this the α -elimination from chloropropene **8** is brought about using a slurry of sodium amide in mineral oil with added hexamethyldisilazane ($\text{Me}_3\text{Si-NH-SiMe}_3$) which when mixed with **8** generates gaseous cyclopropene **4**. After purification, the cyclopropene is bubbled into a solution of cyclodextrin to precipitate the inclusion complex which is filtered off.²²

The marketed formulation, *SmartFresh*SM, is applied to apples soon after harvest in a cone-like device within storage rooms. The treatment takes one day and leaves no detectable residue. As a side benefit, this treatment often replaces the need for diphenylamine (PhNH_2) to control the postharvest disorder, scald; the toxicity of **4** is less than that of the amine. *SmartFresh*SM was approved by the European Union in 2005 and is used in some 26 countries including NZ. Rohm and Haas gained ERMA approval for importation of **4** within the *SmartFresh*SM technology in late 2003. The much more recent *Invinsa*TM is a sprayable liquid formulation of **4** and the first product to specifically protect crop yield during extended periods of high temperature, mild-to-moderate drought, and other crop stresses. Although still under development, it is approved in Chile and Argentina and US regulatory approval was expected during 2008; it is to be marketed in a joint venture between AgroFresh and Syngenta AG.

*SmartFresh*SM has reaped a number of awards for providing fresher fruit and it is now used on apples, apricots, avocados, bananas, cantaloupe melons, kiwifruit, mangoes, nectarines, papayas, peaches, pears, plums, persimmons and tomatoes. Of course critics are plentiful. The use of **4** came under scrutiny in 2005 when it was revealed that the ripening of apples was being delayed by up to a year so that consumers purchased year-old produce without being aware of it; that similar storage lengths had been standard industry practice for many years was not mentioned. However, *SmartFresh*SM was given a *balanced* (author emphasis) account in the *Dining and Wine* section of the *New York Times* by David Karp²⁴ under the headline *Puff the Magic Preservative: Lasting Crunch, but Less Scent*. Despite some concerns about the effects of very low levels impurities,²⁵ the safety, toxicity, and environmental profiles of **4** are exceptionally favourable, and no death or clinical signs of systemic toxicology were seen.²⁶ The impact of **4** on the nutritional content of fruits is still under investigation although fruits appear to maintain antioxidant and vitamin levels.

The effective concentration of **4** needed varies widely with the commodity concerned,¹¹ and the temperature and the method of application, but the uses to which it continues to be put have placed 1-methylcyclopropene **4** among the significant players in the agrochemicals market. At the present time, something in excess of 60% of the apples sold on the North American market have been treated with *SmartFresh*SM, while cantaloupes imported into the US from Central America can be harvested riper and stay firm and free from decay after treatment with *SmartFresh*SM. The use of **4** is widespread. It is applied to substantial quantities of Asian pears in Korea, on South African and Chilean avocados and Chilean kiwis sent to Europe, and on small lots of plums in France and Chile.

In 2003 the series of 1-alkyl substituted cyclopropenes from methyl to decyl were prepared and assessed as ethylene antagonists for banana.²⁷ Each member of the series showed activity but, quite unexpectedly, from the 1-butyl derivative onwards *the effective minimum concentration fell below that of 1-methylcyclopropene*. The 12 day protection period of **4** was attained by 1-ethyl-, 1-propyl-, and 1-butylcyclopropene at ambient temperature (22–23 °C), but only from use of higher concentrations (4–6 nL/L vs 0.7 nL/L). However, 1-pentyl- was effective for 14 days, 1-hexyl- for 20 days, 1-heptyl- 21 days, 1-octyl- 25 days, 1-nonyl- 35 days, and 1-decylcyclopropene for 36 days. These results were achieved using concentrations of 0.3 – 0.5 nL/L and *lower* than the 0.7 nL needed for **4**; 1-decylcyclopropene gave the longest protection from ripening at the lowest concentrations (0.3 nL/L).²⁷ Some 20 cyclopropenes were assessed in a 2006 study reported in the Russian literature²⁸ and some of these compounds extended the exhibition life of cut mini-carnation flowers as well as delaying senescence of bean leaves.

During 2008 a further study employing a variety of 1-alkyl substituted cyclopropenes was reported.²⁹ This encompassed testing their potency to inhibit ethylene-induced plant processes on a variety of climacteric fruit like avocado and tomato, on ethylene-induced growth modification in etiolated pea seedlings, and on abscission in citrus leaf explants. Fruits responded differently than other plant organs to the same inhibitor, indicating possible differences in characteristics and availability of the ethylene receptors in the various tissues. The potency of the inhibitors was markedly dependent on their molecular structure and size, but each inhibitor gave the highest potency when treatment was prior to the onset of ethylene action. All of the fruits resumed normal ripening after recovery from the inhibition, a crucial factor when considering putative inhibitors for practical use.²⁹ It would seem from these studies that the cyclopropene moiety of the 1-alkyl derivatives blocks the binding site and that the long alkyl chain fits into the available volume and exerts little to no effect. Whether any of these more complex cyclopropenes will make it to the market remains to be seen, but any development will demand a simple synthesis and delivery mode as well as retaining the minimal toxicological impact of 1-methylcyclopropene (**4**).

There has been much recent activity in the Asian scientific communities with a number of reviews on the uses and applications of **4** appearing.³⁰ Thus, treatment of varieties of pears delayed fruit ripening and prolonged storage periods whilst keeping fruit quality and palatability.³¹ It also inhibited the occurrence of melanoderma and black heart of pear.³² The number of patent applications for formulations and uses of **4** in these countries has also increased over the last four years.

The strawberry typifies non-climacteric fruits but its ripening is not yet well understood and the role of ethylene is unclear. Recent studies have included use of **4** in the four different ripening stages (green, white, pink and fully ripe) of the fruit. As **4** specifically blocks the ethylene signal transduction pathway, its use enables the indirect identification of genes activated by ethylene.³³

Those who prize organic and/or seasonal produce may well find that the premium prices increase in parallel to the use of 4. However, the complete loss of 4 after removal of the fruit from containment is apparently encouraging AgroFresh to seek US National Organic Standards Board to allow fruits treated with SmartFreshSM to be labeled as *organic*.

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Patent Proze

But what does it actually do?

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The first Court decision from the United Kingdom to consider the validity of a gene sequence patent, *Eli Lilly vs. Human Genome Sciences*,¹ has accentuated the need for an invention to have a practical purpose.

The Background of the Decision

Patents are granted for inventions which are considered inventive and to be useful to society. Patents are not granted for mere discoveries. So, whilst the synthesis of a new compound may be the result of a significant investment, the compound itself cannot be patented without knowl-

edge of a potential use, or more specifically, a commercial application. Similarly, while it is often possible to patent a gene sequence - even if identical to a gene found in its natural environment - the function or some commercial use for the gene sequence must also be disclosed.

In 2005, Human Genome Sciences (HGS) was granted a European patent covering the Neutrokin- α protein, the DNA sequence encoding it, antibodies binding to it, and corresponding pharmaceutical and diagnostic compositions. Neutrokin- α is a member of the TNF (Tumour