The Synthesis and Biological Evaluation of Potential ABA-Like Analogues: Prospective Substrates to Control Berry Ripening of Wine Grapes.

A thesis presented in fulfilment of the
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Doctor of Philosophy

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Abstract

The control of ripening of grape berries has not yet been clearly defined and a better understanding of the role of carotenoids and the plant hormone abscisic acid (ABA) may offer a useful means of manipulating berry composition and flavour profiles and the timing and synchronicity of ripening itself. ABA is an important hormone in a range of higher plant processes such as stress response, seed maturation and dormancy and fruit ripening. This thesis details the development and early biological evaluation of a library of potential ABA-like substrates as potential inhibitors of ABA biosynthesis / catabolism that are similar structurally to the backbone of ABA itself.

This thesis begins with an overview of grape berry ripening and details the reasons why it may be of importance to develop substrates that can be utilised to manipulate the ripening period of grapes grown in Australia and elsewhere in the world. As ABA is a key substrate that has been extensively linked to plant development and maturation, details into numerous previous studies including the current understanding of the biosynthesis of ABA within plants are also summarised. In particular, previous studies have reported on the synthesis of ABA-like analogues and their evaluation as ABA biosynthesis inhibitors or suicide substrates to prevent catabolism which is the term given to the natural breakdown of ABA within a plant. Consequently, these studies are summarised and aided in the selection of a new library of substrates to be synthesised herein and biologically evaluated.

Chapter two details the successful synthesis of eleven target ABA-like compounds with two of them existing as mixtures of related compounds. Confirmation of their structures required extensive 2D NMR evaluation, in particular for elucidating the (*E/Z*) stereochemistry of the polyene backbones of the majority of these substrates. In addition, ^{13}C NMR analysis was paramount and the most decisive tool for confirmation of the carbon-carbon double bond stereochemistries. Whilst the proposed biological tests only required small quantities of pure chemical material for evaluation, it was important to consider that if any of the target compounds displayed excellent biological activity, then the chemical processes for their synthesis should also be able to be scaled up to ensure ample quantities for biological / field evaluation. Pleasingly, all chemical transformations carried out herein were almost exclusively performed within the 0.5 grams to multi-gram scale.

Whilst there were a few proposed substrates that were not able to be synthesised, the reasons for which are discussed, there was one particular newly synthesised analogue (novel substituted 2,2-dimethyl-6-methylidenecyclohexylidenes (**60** and **61**)) that showed reasonable biological activity. There are no published reports on the use of these type of substrates being evaluated for ABA-like activity and consequently they represented a new class of substrates to be further explored along with those originally proposed.

Chapter three details the use of the *Lactuca* (Lettuce) seed germination assay to explore the ABA-like activity of these new bioactive chemicals along with a discussion of the current understanding of the modes of ABA inhibitors and where they are likely to act in the biosynthetic pathway. ABA acts as a dormancy inhibitory hormone and in simple terms prevents germination until levels are reduced. Investigating dormancy of seeds by supplying them with ABA or ABA-like substrates (our synthesised targets) allows for the opportunity to study certain physiological relevant phenomenon that may provide information not only on the regulation of seed dormancy but also on the molecular mechanism of ABA action in plants. It was found that five of the test compounds displayed no ABA-like activity and included the simple polyene ethyl esters (**30**) and (**31**), the mixture of related esters (**52**)/(**53**) and the endoperoxides (**37**) and (**67**), *Figure 3.10*. Three substrates displayed good to excellent ABA-like activity ((**33**), (**68**) and (**60**)/(**61**)) at the highest level tested of 1 mM but quickly become ineffective at the lower concentrations evaluated. Finally, three substrates displayed excellent ABA-like activity ((**28**), (**29**) and (**34**)) both at 1 mM and also at 100 μM, *Figure 3.10*. Given that many of these new bioactive molecules are structurally similar to each other and also to the structure of ABA it was possible to elucidate some early structure-activity relationships (SAR's).

Given that the seed germination assays provided some insight into whether these compounds behave in an ABA-like manner, and indeed some appeared to, we next turned our attention to screening the compounds in a bean dehydration assay, which is the subject of this Chapter four. ABA is known to close leaf stomates and therefore reduce water loss resulting upon dehydration. Such effects were able to be seen visually and quantified based on the assay developed. Unfortunately, evaluation of all eleven new substrates revealed that none of them appeared to prevent dehydration thus it can be concluded that they do not appear to be able to act like ABA itself. However, the delay they caused in lettuce seed

germination and the lack of protection of bean shoots response to an imposed dehydration event suggested that these molecules delayed the breakdown of ABA i.e. they act to decrease the rate of ABA catabolism by either blocking the 8'-hydroxylase enzyme or by acting as suicide substrates that are also catabolised / oxidised and deactivated in a similar manner to ABA itself by the 8'-hydroxylase enzyme. Importantly, a range of new ABA-like substrates have been synthesised and biologically evaluated with some of them clearly displaying reasonable activity on the ABA biosynthetic pathway in plant tissues and as such provide new lead analogues to be further studied in the future.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Abbreviations

ABA	Abscisic acid
ABA-GE	ABA glucosyl ester
Å	Angstroms
app. d	Apparent doublet
Ar	Aromatic
Bn	Benzyl
br	Broad
COSY	Correlation spectroscopy
cm	Centimetres
d	Doublet
DCM	Dichloromethane
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
DPA	Dihydrophaseic acid
Et	Ethyl
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
g	Grams
GA	Gibberellic acid
GC	Gas chromatography
GCMS	Gas chromatography mass spectrometry
h	Hours
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
Hz	Hertz
hν	Light/irradiation
J	Coupling constant
L	Litre
Lit.	Literature
m	Multiplet
M	Molar (moles/litre)

MB	Methylene blue
min.	Minutes
m/z	Mass to charge ratio
mg	Milligrams
$MgSO_4$	Magnesium sulphate
MHz	Megahertz
mL	Millilitre
mmol	Millimoles
mol	Moles
M.pt.	Melting point
MS	Mass spectrometry
NCED	9-*cis*-Epoxycarotenoid dioxygenase
nm	Nanometres
NMR	Nuclear magnetic resonance
PA	Phaseic acid
Ph	Phenyl
ppm	Parts per million
psi	Pounds per square inch
q	Quartet
R_f	Retension factor
rt	Room temperature
s	Singlet
SAR	Structure activity relationship
SEM	Standard error means
t	Triplet
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV	Ultra-violet
d	Chemical shift
m	Micro

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学而不思则罔，思而不学则殆 孔子 (前 551-前 479, 哲学家)

Learning without thinking leads to confusion, thinking without learning ends in danger.

Confucius (551B.C-479 B.C, Chinese Philosopher)

Genius only means hard-working all one's life.

Mendeleyer (1834-1907, Russian Chemist)