# Isolation and structural elucidation of compounds from Australian populations of *Portulaca oleracea*

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# Declaration of Originality

I, Jack Keiran Micklewright, declare that the work presented in this thesis has not previously been submitted for a degree, nor has it been submitted as part of requirements for a degree to any university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and in the preparation of the thesis itself have been appropriately acknowledged.

# Acknowledgements

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Finally, I would like to thank Macquarie University Department of Molecular Sciences for the opportunity to follow my passion and do something I love.

# List of Abbreviations

| | |
|---|---|
| 1D | One-dimensional |
| 2D | Two-dimensional |
| ABTS | 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) |
| br | Broad |
| COSY | Correlation spectroscopy ($^{1}$H-$^{1}$H) |
| CSU | Charles Sturt University |
| d | Doublet |
| DAD | Diode array detection |
| DI | Deionised |
| DMSO | Dimethyl sulfoxide |
| DPPH | 2,2-Diphenyl-1-picrylhydrazyl |
| DQF | Double quantum filter |
| ECD | Electronic circular dichroism |
| ESI | Electrospray ionisation |
| FRAP | Ferric reducing antioxidant power |
| HMBC | Heteronuclear multiple bond correlation |
| HPLC | High performance liquid chromatography |
| HSQC | Heteronuclear single quantum correlation |
| LC | Liquid chromatography |
| NOE | Nuclear Overhauser effect |
| NOESY | Nuclear Overhauser effect spectroscopy |
| NMR | Nuclear magnetic resonance |
| m | Multiplet |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| MRes | Master of Research |
| *m/z* | Mass to charge ratio |
| ROE | Rotating frame Overhauser |
| ROESY | Rotating frame Overhauser effect spectroscopy |
| s | Singlet |
| RP | Reversed phase |
| TIC | Total ion current |
| TLC | Thin layer chromatography |
| UV | Ultraviolet |
| UV-vis | Ultraviolet-visible |

# Abstract

*Portulaca oleracea* is a globally significant medicinal herb and food source. It has been used to treat diabetes, infection, pain and numerous other ailments. Many of *P. oleracea*'s medicinal uses have been verified by scientific studies. Populations of *P. oleracea* found in Australia are morphologically distinct from those found in other countries. There is also record of this plant's use for food and medicine by multiple Australian Aboriginal communities. Despite this, Australian populations of *P. oleracea* have received little scientific attention. This study looked at *P. oleracea* collected in Wagga Wagga, Australia, believed to be a globally prevalent variety, and compared it with samples bred from two Australian populations, one from Lake Eyre and the other from the Kimberley. Previous LC-MS work conducted by collaborators at Charles Sturt University had shown the two Australian populations to contain potentially novel betacyanins and oleraceins. Both classes of chemicals are known to be antioxidants and have been shown to have other medicinal properties. This study covers the successful extraction of betacyanins and oleraceins from each of the three populations and confirmation by structural elucidation of the presence of oleraceins C and D in two of the three populations investigated.

# Chapter 1: Introduction

*Portulaca oleracea*, known as common purslane or pigweed, is a succulent herb with a long history of use both as a customary medicine and food source[1]. *P. oleracea* has a wide distribution, but most commonly grows in tropic and sub-tropic regions and it is very often found in urban and rural environments[1]. *P. oleracea's* growth is usually prostrate though some varieties form small erect shrubs. Leaves are small, ovate and opposite, green or reddish in colour, with red stems (Fig. 1).

*P. oleracea* is one of the most common plants in the world and its ability to survive in many varied environments has contributed to its widespread growth and subsequent regional diversification[2, 3]. Determining where it is native and where a population has become naturalised is difficult, and regional divergence has led to a vast array of *P. oleracea* varieties, each with distinct morphology and phytochemistry.

Figure 1: *Portulaca oleracea* omega red, an Australian population of this species, originally from Lake Eyre, South Australia. Picture taken by Colette Geier 2018.

The earliest recorded use of *P. oleracea* as a medicinal herb dates from almost 2000 years ago in Roman Greece where it was described as a panacea[4]. There is also evidence of its use in ancient Rome, Persia and China[4-6]. Its seeds have been found associated with settlements around the Mediterranean dating back to prehistory[5]. Its use as a customary medicine and food source in North America dates back hundreds of years, and likely predates European arrival[7]. Records of medicinal use of the plant by Australian Aboriginal people as a 'blood cleanser' and to treat scurvy (antiscorbutic) date back over 100 years, and the use itself likely predates European arrival on the continent[8, 9]. In many areas of Australia, *P. oleracea* also appears to have been an important part of the diet of Australian Aboriginal people[8]. The broad range of medicinal uses of *P. oleracea* has persisted, and in many areas of the world it is still in use as a customary medicine[5]. Modern

ethnobotanical research has led to descriptions of its use for many medical conditions, some of the most common being as an antidiabetic, analgesic and antiseptic[10-12]. However, due to its global distribution and the varied needs of the people who use *P. oleracea*, it has many other uses, some of them unique to a culture and some more widespread.

*P. oleracea's* spread and diversity, coupled with its widespread use as a food and medicine, has resulted in it being a plant of continued scientific interest. This has led to an accumulation of a vast wealth of knowledge of this plant, both from an ethnobotanical and chemical perspective[6]. Even with this considerable scientific investigation, little chemical research has been conducted into Australian varieties of *P. oleracea*. This is despite *P. oleracea's* use as both a food source and customary medicine by Australian Aboriginal communities, and it being found in every Australian state and territory with many diverse populations[13, 14].

### 1.1 Customary medicines

Customary medicines are medicines based on traditional and contemporary knowledge of Indigenous peoples. For many Indigenous peoples their main form of healthcare[15]. The largest group of these medicines is customary plant medicines, which have long recorded histories for treatment of a wide range of diseases[15].

Cultures around the world have, over thousands of years, developed plant-based customary medicines. These practices have been informed by experimentation with the plant materials available to each culture, and accumulation of knowledge over generations. This has led to the development of extensive botanical pharmacies. For many Indigenous people, knowledge of customary medicinal plants is an important part of their culture, and maintaining this knowledge is an important way to perpetuate their culture. These cultures and their customary medicines have received sustained interest from the scientific community, which has found evidence of efficacy in the use of many of these medicines[16]. These medicines are also the source of a vast array of novel bioactive chemicals, many of which have gone on to become drugs, either in their original forms or as semisynthetic derivatives[17].

### 1.2 *Portulaca oleracea*: evidence of efficacy of customary uses

*P. oleracea* is globally significant for its customary uses as a medicinal plant[6, 16]. Four of the most widespread medicinal uses of *P. oleracea* are in the treatment of pain and inflammation, infections, diabetes and mental disorders (Table 1). These uses are broad in range and even when treating the

same condition, in some cases a different part of the plant or method of preparation has been employed (Table 1).

Table 1: Common medicinal uses of *P. oleracea*

| **Customary medicinal use** | **Countries/regions where this use is practiced** | **Plant parts/preparation used** |
|---|---|---|
| Analgesic | India, Nepal, Pakistan | Aerial plant material chewed, poultice and tea[11, 12] |
| Antidiabetic | China, Iran, Turkey | Leaves, stems and seeds all eaten or used in tea[10, 18, 19] |
| Anti-inflammatory | China, India, Pakistan | Tea of aerial plant material is drunk. Juice from leaves and stems applied to inflammation[12, 20] |
| Antiparasitic | Dominica, Iran, Pakistan | Seeds and whole plant are eaten. A tea is made of the leaves[21-23] |
| Antiscorbutic | Australia, India | Aerial plant material is eaten[6, 9] |
| Antiseptic | China, Iran, Italy, Pakistan, India, Mexico, Turkey | Aerial plant material is eaten[12, 18, 24-26] |
| Blood cleanser | Arabian Peninsula, Australia | Aerial plant material is eaten[8, 27] |
| Cardiovascular | Cyprus | Aerial plant material is eaten[28] |
| Diuretic | India, Pakistan | Seeds are eaten. Whole plant material, leaves or seeds are eaten[26, 27] |
| Kidney diseases | India, Pakistan | Whole plant material, leaves or seeds are eaten[22, 26] |
| Lung diseases | India, Iran | Tea of whole plant material[26, 29] |
| Neurological disorders | Cyprus, Iran | Aerial plant material is eaten[28, 30] |

### 1.2.1 Analgesic and anti-inflammatory activity

One of the most common customary uses of *P. oleracea* is for relief of pain, most often toothache, earache and headache, as well as pain associated with fever and inflammation[16]. Aerial plant material or seed material is primarily applied to the area of the pain as a poultice, or in the case of toothaches, chewed[16]. In some cases it is taken orally either as a tea or the aerial plant material is chewed to treat pain associated with fever and inflammation[16]. Both intraperitoneal injection of 10% ethanolic extracts of aerial plant material and oral administration of water extracts of aerial plant material have been found to be effective in reducing pain responses of mice[31, 32]. Another study looking at petroleum ether extracts of aerial plant material reported oral analgesic activity[33]. These studies suggest there are both polar and non-polar analgesic compounds in *P. oleracea*[32, 33].

Many analgesic drugs owe part of their action to anti-inflammatory effects. Aqueous ethanolic extracts of the aerial parts of *P. oleracea* at 200 $mg.kg^{-1}$ and 400 $mg.kg^{-1}$ have been shown to significantly reduce inflammation in mice[31].

*P. oleracea* is used to treat inflammatory lung diseases such as asthma in some countries (Table 1)**.** Oral doses of a 70% ethanolic extract of leaves caused a significant dose dependant reduction in the inflammatory markers interleukin-1β, interleukin-6, tumour necrosis factor α, prostaglandin-E2 and transforming growth factor β1[34] in rats with lipopolysaccharide-induced lung damage. This suggested a potential partial mechanism for the anti-inflammatory effects.

Another study looked for potential chemical causative agents for *P. oleracea*'s anti-inflammatory effects. This led to the discovery of four novel compounds in the aqueous extract of the aerial parts: oleracimine, oleracimine A, oleracone A and oleracone B (Fig. 2)[35]. Oleracimine was found to show good anti-inflammatory activity in macrophages stimulated with lipopolysaccharides to induce inflammatory cytokines[35]. This treatment caused a significant reduction in the secretion of the cytokines interleukin-6 and tumour necrosis factor α, and other species associated with inflammation responses *e.g.* nitric oxide and prostaglandin-E2[35]. Interleukin-6, tumour necrosis factor α and nitric oxide were also found to be reduced in a mouse study investigating lung inflammation, providing evidence that oleracimine was responsible for some of the anti-inflammatory effects of this plant[34, 35]. Additionally, mRNA expression of the cyclooxygenase 2 (COX2) gene was significantly reduced[35]. COX2 is a key protein involved in inflammation and a common target of anti-inflammatory drugs.

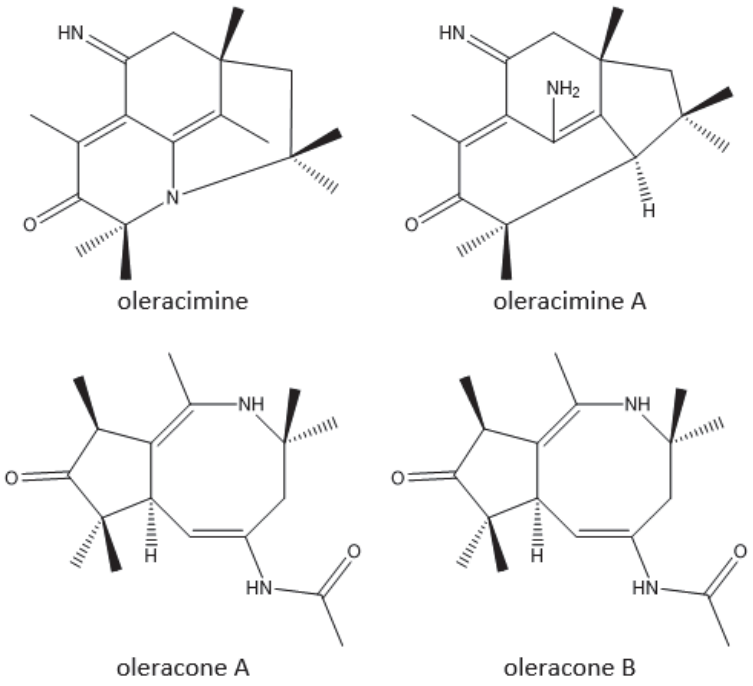



Figure 2: Four novel compounds found in aqueous extracts of *P. oleracea*

### 1.2.2 Antiseptic and antiparasitic activity

*P. oleracea* has been used for treatment of infections in multiple countries (Table 1). This includes its use in the treatment of mouth, throat, skin and urogenital infections as both an oral and topical medicine[6]. It is also used to treat bacillary dysentery, applied to burns, and used as a broad range antiparasitic[6, 16]. An 85% aqueous ethanolic extract of aerial parts of *P. oleracea* has been shown to be bactericidal, most notably against *Staphylococcus aureus* [36]. The ethanolic extract of the aerial parts of *P. oleracea* has been found to inhibit the *in vitro* growth of *Leishmania major*[37], which is one of the pathogens responsible for leishmaniasis, a significant parasitic infection spread by sand flies and still prevalent in many tropical regions. The extract had an $IC_{50}$ of 360 µg.mL$^{-1}$ and of 680 µg.mL$^{-1}$, respectively, [37] against a standard laboratory and a clinical strain of *L. major* [37].

A study on the 70% aqueous ethanolic extract of aerial parts of *P. oleracea* led to the isolation of four novel compounds, portulacerebroside B, portulacerebroside C, portulacerebroside D and portulaceramide A, all with strong antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri* and *Salmonella typhi* (Fig. 3)[38].

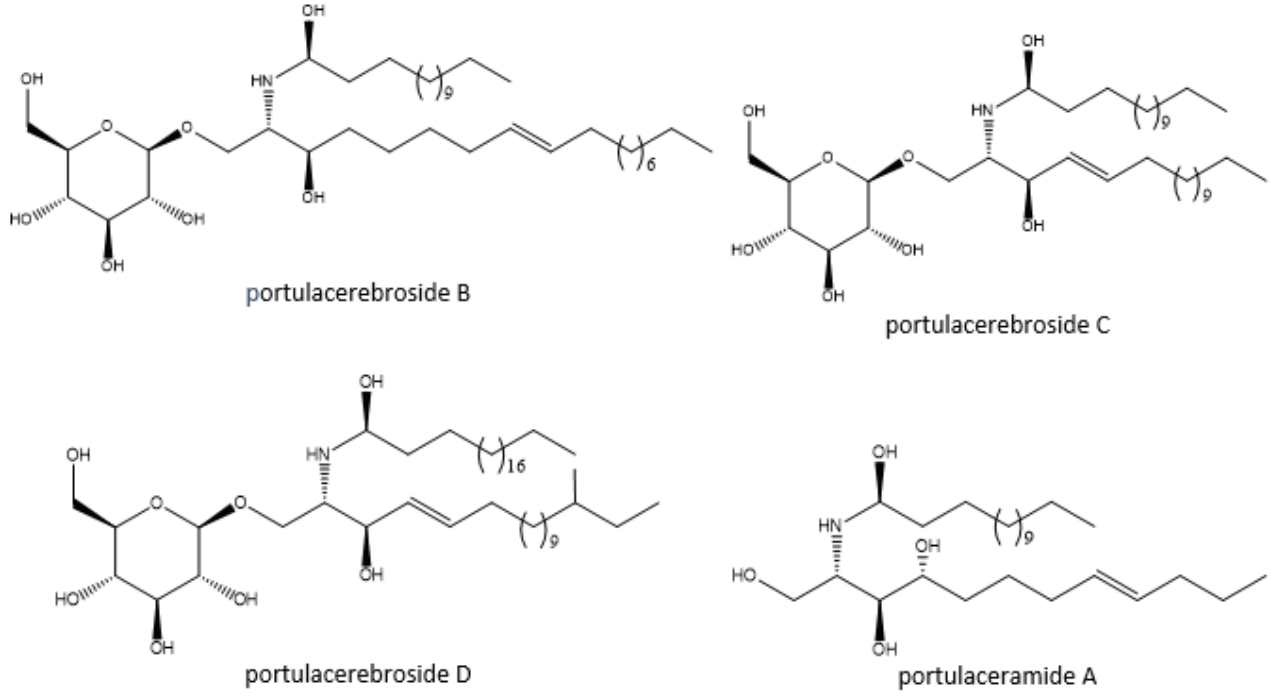



Figure 3: Novel antibacterial compounds found in 70% ethanolic extracts of *P. oleracea*

### 1.2.3 Antidiabetic activity

*P. oleracea* has been used customarily for the treatment of diabetes in several Mediterranean and Middle Eastern countries as well as China[10, 18, 19]. As diabetes is a disease with increasing global

prevalence, research into the antidiabetic properties of customary medicines is a field of research experiencing a lot of activity[39].

One study comparing customary medicines from Iran used to treat diabetes looked at a range of extracts of *P. oleracea* aerial plant material made using solvents of varying polarity (*n*-hexane, dichloromethane, chloroform, ethyl acetate and methanol)[40]. This study found that the extract of *P. oleracea* made using methanol was most effective at inhibiting the activity of α-glucosidase[40]. A subsequent study looked at the *in vivo* effects of *P. oleracea* on diabetes in rats induced by alloxan injections[41]. Alloxan is a drug specifically toxic to β-cells, which are the pancreatic cells responsible for insulin production[42]. Some of the rats were fed 250 mg of an aqueous extract of *P. oleracea* per kilogram of body mass daily for 4 weeks prior to injections of alloxan[41]. The rats being treated with *P. oleracea* exhibited better outcomes than the rats exposed only to alloxan[41]. This showed that *P. oleracea* could protect β-cells from alloxan or aid in their recovery. As maintaining pancreatic β-cells is an important part of treating diabetes, this did show another possible route for *P. oleracea's* potential antidiabetic effects.

A study of women with type 2 diabetes found that daily *P. oleracea* seed consumption was more effective than just exercise at managing many of the biomarkers of type 2 diabetes including blood glucose levels[43]. This study also linked consumption of the seeds to reduced biomarkers for inflammation, increased biomarkers for β-cell activity and reduced blood cholesterol levels. These were all indicators of positive therapeutic effects on the diabetic state[43].

Each of the above studies showed the efficacy of *P. oleracea* in treating diabetes and the importance of assessing the chemical mechanisms by which *P. oleracea* elicits these effects[40, 41, 43]. *P. oleracea* is known to be a rich source of α-linolenic acid and is also known to contain β-sitosterol (Fig. 4)[43, 44]. Both of these chemicals have been linked to various antidiabetic effects[45, 46]. More comprehensive work on the phytochemicals of *P. oleracea* is necessary to better understand its antidiabetic effects[47, 48].

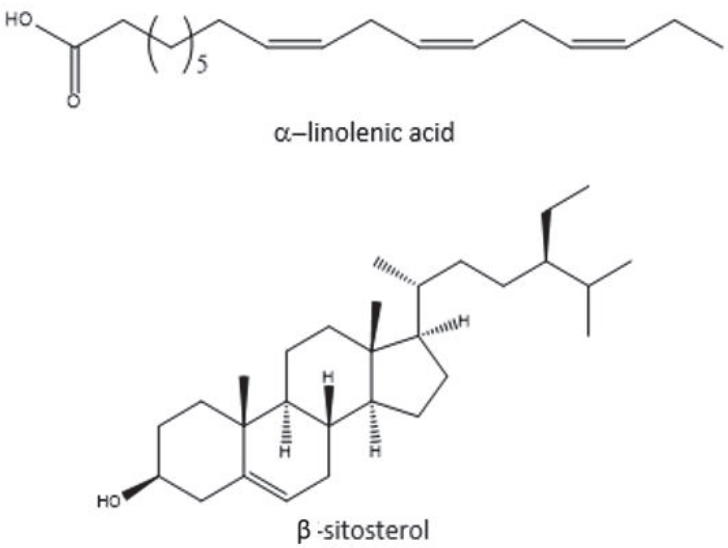



Figure 4: Two well-known antidiabetic compounds found in *P. oleracea*

1.2.4 Neuroprotective activity

*P. oleracea* is used by some cultures to treat melancholia, madness and mental disorders in general. Experiments looking at 10% aqueous ethanolic extracts of aerial portions of *P. oleracea* as an analgesic found reduced movement in rats and mice after injection into the bloodstream, suggesting a neurological component to the plant's activity[31, 32]. Additionally, when the mice injected with the 10% ethanolic extract were also treated with naloxone, the analgesic activity was blocked[31]. As naloxone blocks other drugs from interacting with opioid receptors, this is evidence of neurological action of this plant specifically associated with opioid receptors.

Another study looked at 10% ethanolic extracts of aerial portions of *P.* oleracea and its effect on chemically induced convulsions in mice[49]. The study used injection of pentylenetetrazole for inducing convulsions and found that the onset of convulsions was delayed and the convulsions were less severe if the mice were pre-treated with intraperitoneal injections of *P. oleracea* extract[49]. However, the rate of deaths from convulsions was the same in the control and the extract-treated groups[49]. This study also found that injection with the extract caused a reduction in grip strength and in skeletal muscle contraction from an external electrical impulse[49]. All these effects were seen to follow a dose dependant pattern[49].

A later study looked at *P. oleracea* for its possible neuroprotective effects. This study used microinjection of 6-hydroxydopamine into the striatum of rat brains as a model for Parkinson's disease[50]. Following the injections, groups of rats received daily oral doses of either an aqueous or ethanolic extract of *P. oleracea* aerial plant material for 15 days[50]. This study found that 15 days after 6-hydroxydopamine treatment the rats that had the subsequent *P. oleracea* treatment were behaving more like the negative control group, using standard metrics of their movements[50].

Though these tests are subjective, they showed a dose dependant effect. The aqueous extract groups performed better than the ethanolic extract groups. At 28 days the rats were euthanased and their brains dissected. The rats that had received the extracts had higher density of tyrosine hydroxylase-positive neurons in the striatum, with similar dose dependant effects to the activity tests[50]. Tyrosine hydroxylase is used as a biomarker for catecholaminergic neurons. This was considered evidence that the *P. oleracea* extracts had protected these neurons. The 400 $mg.kg^{-1}$ aqueous extract group had 80% of the tyrosine hydroxylase-positive neurons when compared to the negative control group and the positive control group had < 40%[50]. The authors of this paper suggested that the neuroprotective activity of the extracts may have been due to *P. oleracea*'s antioxidant activity[50].

Another study had previously found that oral doses of an 80% ethanolic extract of *P. oleracea* aerial material protected mice from hypoxia in normobaric and chemical hypoxia models[51]. For both models, the mice given the extracts had significantly longer survival times in a dose dependant manner[51]. This study also found that the mice given the extracts had higher levels of ATP and glycolysis molecules than the control group[51]. The mice treated with the extracts did not show increased sedation or decreased movement[51]. This suggested that the sedative effects of some *P. oleracea* extracts were not responsible for the longer survival times[51].

### 1.3 Betalains and oleraceins

Many medicinal effects including the antidiabetic effects of *P. oleracea* have been partially attributed to the high antioxidant activity of this plant[34, 47, 50]. Oxidative stress is strongly linked with many diseases; the development of type 2 diabetes being an important example[52]. Common antioxidant supplements such as ascorbic acid (vitamin C) and vitamin E have been shown to have a positive impact in managing diabetes in human trials[53]. Another class of antioxidants of which *P. oleracea* is a rich source, the betalains, has shown promise in managing diabetes[54].

#### 1.3.1 Betalains

The betalains are a large class of alkaloids that are known for their antioxidant activity and for their use as natural red and yellow food dyes[55]. They were first discovered in beetroots (*Beta vulgaris*). Since then they have also been detected in many other organisms throughout the order Caryophyllales, including the genus bougainvillea and many cacti, and several genera of higher fungi and bacteria[56]. The betalains consist of betalamic acid, a derivative of tyrosine bonded to other small molecules (Fig. 5). When betalamic acid is bound to an (*S*)-cyclodopa group, the compounds are

known as betacyanins and produce a red to purple colour (Fig. 5)[57]. Betacyanins are the molecules that give *P. oleracea* its distinctive red hues. When betalamic acid is linked to other small molecules, usually amino acids or catecholamines, the betalains produced are known as betaxanthins and have yellow to orange colours (Fig. 5)[57]. Betalains, in particular betacyanins, are also often glycosylated and can contain many other additional moieties[55, 58].

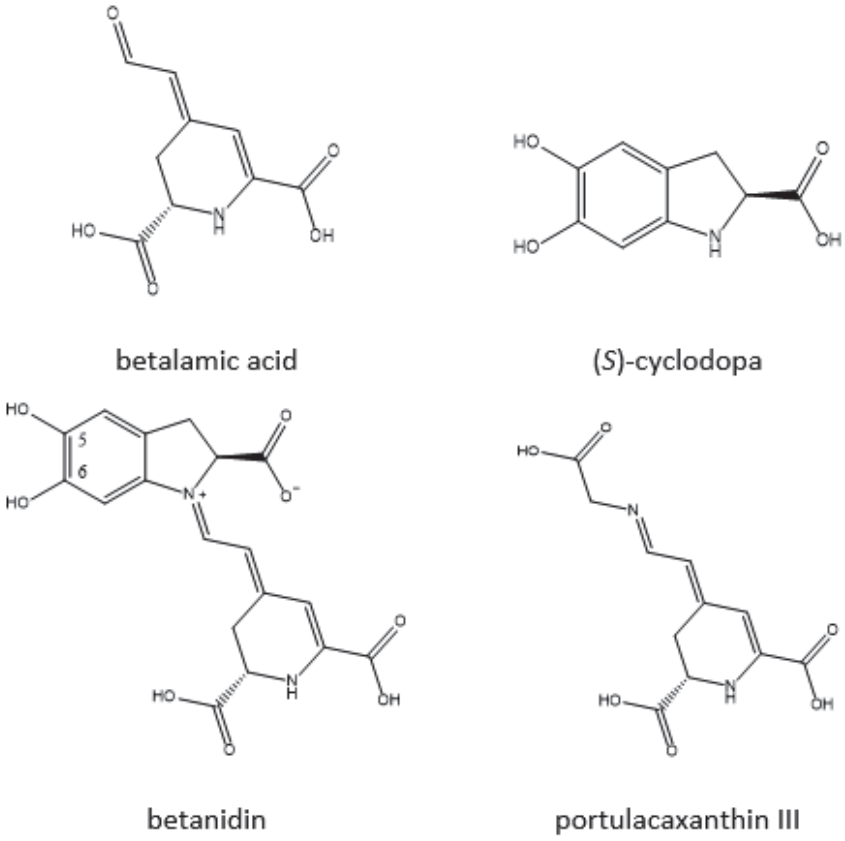



Figure 5: Betalain structure: betalamic acid, cyclodopa, betanidin the simplest betacyanin, and portulacazanthin III, a simple betaxanthin

Betalains have been shown to have numerous potential medicinal properties, many of which are linked to their antioxidant activity. Several betalains have been shown to prevent lipid peroxidation and DNA oxidation, two forms of oxidative damage that can have significant negative impacts on cell viability[59]. The betalains from *Opuntia ficus-indica* (prickly pear) have shown an anti-inflammatory effect in rats[60]. The betalains from beetroots have also shown potential in relieving osteoarthritic pain in a human trial, which was thought to be in part due to their anti-inflammatory action[61]. The betalains from *Hylocereus ocamponis* (dragon fruit) showed antidiabetic effects in mice, reducing blood sugar levels[62]. These studies all found effects of betalains that coincide with customary medicinal uses of *P. oleracea*. However, the betalains of *P. oleracea* have not been studied in detail.

### 1.3.2 Oleraceins

The oleraceins are another group of molecules found in *P. oleracea* that are known to be responsible for a substantial part of its antioxidant activity and have been connected to other medicinal properties[63, 64]. The oleraceins, like the betacyanins, have a cyclodopa group; however, with no betalamic acid group attached. The cyclodopa group instead typically has a cinnamoyl derivative attached by an amide bond to the nitrogen. To date this cinnamoyl derivative has always been either a *p*-coumaroyl or feruloyl group**.** There is also a glucose attached to the oxygen at the C-6 position. Additional groups, most notably glucosyl, feruloyl or caffeoyl are often attached to the glucose, and an additional glucosyl group to the central cinnamoyl derivative. (Fig. 6 and Supplementary 1).

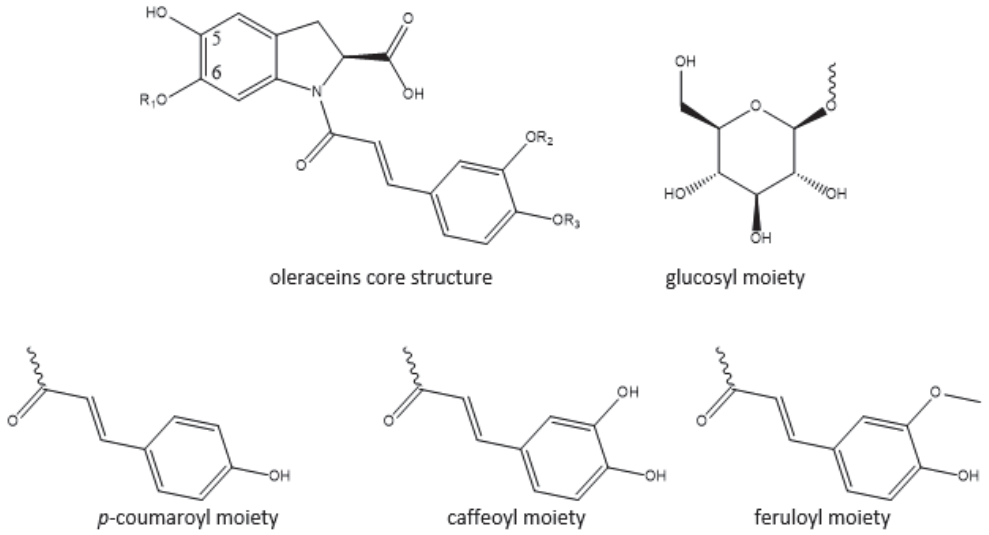



Figure 6: Structural components of oleraceins

The oleraceins were first identified in 2005, when oleraceins A, B, C, D and E were extracted from a Chinese cultivar of a common globally distributed variety, *P. oleracea* Linn[65]. In 2011 two more oleraceins were structurally elucidated using the same variety of *P. oleracea*[66]. These oleraceins, F and G, were methyl esters of oleraceins A and B (Fig. 7). As methanol was used for the extraction and purification processes, it is possible that these methyl esters were an artefact of the experimental procedures rather than naturally occurring oleraceins[66, 67].

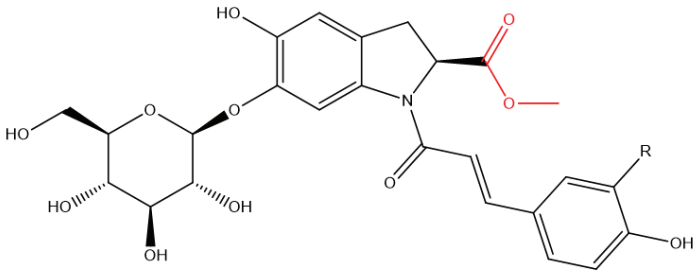
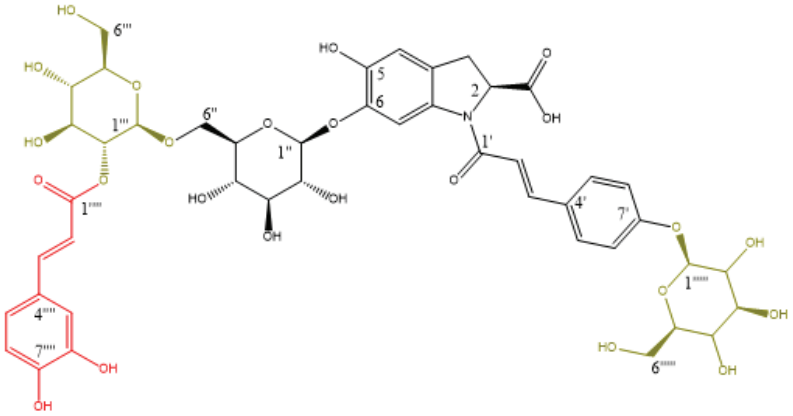
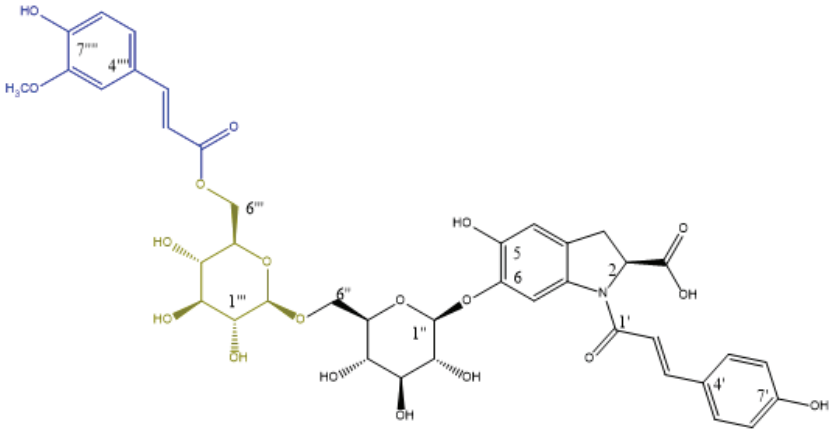


Figure 7: Oleracein F (R = $OCH_3$) and oleracein G (R = H), with the methyl ester group in red.

In 2015 ten new oleraceins were isolated from *P. oleracea*, oleraceins H, I, K, L, N, O, P, Q, R and S (Supplementary 1). None of these oleraceins contained methyl ester moieties[64]. They contained the same backbone as oleraceins A and B with the addition of a glucosyl moiety to the C-6'' position of the first glucosyl moiety[64]. Six of these new oleraceins (N, O, K, L, R and S) also contained an additional caffeoyl or feruloyl moiety attached to this second glucosyl moiety at O-2 or O-6 (Supplementary 1). Three oleraceins (P, Q and R) contained an additional glucosyl moiety attached to C-7' of the core cinnamoyl derivative (Fig. 8)[64].



Oleracein R



Oleracein S

Figure 8: Oleraceins structurally elucidated in 2015, with additional moieties; glucosyl in green, caffeoyl in red and feruloyl in blue, all around oleracein A core in black

In the 2011 and 2015 studies, the antioxidant activity of each oleracein was assessed *via* a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay[64, 66]. This work showed that each of the oleraceins had some antioxidant activity and that the activity varied based on which moieties were attached[64]. Comparison of the results revealed patterns of structural modification that correlated with the antioxidant activity. The most significant increase in antioxidant activity appeared to be linked to addition of cinnamoyl moieties, with feruloyl moieties being more potent than *p*-coumaroyl moieties and caffeoyl moieties being more potent than feruloyl[64].

To date these are all the oleraceins that have been reported as natural products and structurally elucidate. No work has been published on the variation of oleracein content between *P. oleracea* varieties or whether different oleraceins are present in other populations. Oleracein E, which is structurally dissimilar to the other oleraceins has received the most attention due to its neuroprotective effects and has since been found in the roots of *Aconitum carmichaelii* (Chinese wolfsbane), another medicinal herb, and in the larvae of *Tenebrio molitor* (mealworm) (Fig. 9)[68, 69].

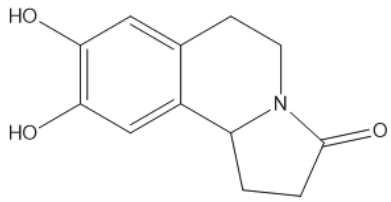



Figure 9: Oleracein E

1.4 Background to this study

The betalains have been of considerable interest to scientists, for their antioxidant properties and their use as food colourants and preservatives[70]. No substantial effort has been made to identify which betalains are present in *P. oleracea* and what role they play in antioxidant activity. Additionally, the secondary metabolites of Australian varieties of *P. oleracea* have not received the same attention as those of many other populations in other countries[16]. This is despite the well-known use of *P. oleracea* as a plant medicine and food source by Indigenous Australians[8, 9]. Due to the geographic isolation and extreme environments that Australian populations have been subjected to, it is likely that the oleracein profile of Australian populations is distinct.

Unpublished work recently completed by collaborators at Charles Sturt University (CSU), Colette Geier, Associate Professor Paul Prenzler and Dr Russell Barrow, looked at two Australian populations of *P. oleracea* and a cosmopolitan population gathered in Wagga Wagga, Australia. The Australian populations were omega gold and omega red, bred from samples gathered in the Kimberley and around Lake Eyre, respectively. This work focused on identifying novel phytochemicals or phytochemicals not previously detected in *P. oleracea* that may be of medicinal value.

The research was further focused on the antidiabetic activity of this plant and consequently targeted betalains, oleraceins and other antioxidant molecules. To target these molecules, 50% aqueous ethanol extracts of plant materials (stems, seeds, leaves and roots) of the two Australian and the cosmopolitan populations were washed with *n*-hexane to remove lipids and then analysed[71]. The extracts were subjected to reversed phase high performance liquid chromatography (RP-HPLC) with an online 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) antioxidant assay to detect

antioxidant activity of eluting peaks[71]. ABTS is a well-established assay for assessing antioxidant activity and has been widely and successfully used in natural products chemistry[72]. The online HPLC ABTS assays identified several promising antioxidant molecules, many of which appeared to be betalains and oleraceins. The structures of these molecules were tentatively identified *via* electrospray ionisation tandem mass spectrometry (ESI-MS/MS) of all three population extracts[71].

All the previously known oleraceins except for oleracein R and the methyl esters oleraceins F and G were tentatively detected in this study based on reported MS profiles[71]. However, there was substantial variation in quantities of each of the oleraceins between the three populations and between plant parts. Omega red was the only population shown to tentatively contain oleraceins K, L, O, P and Q, which were detected in the stems, leaves and buds only[71].

Additionally, a potentially novel oleracein with an *m/z* of 680 (ESI negative ion mode) was also detected in each of the populations studied (Fig. 10)[71]. There were substantial amounts of this compound in all three populations studied, with the greatest quantity detected in omega gold. The ESI-MS/MS fragmentation pattern (ESI negative ion mode) of this *m/z* of 680 ion showed fragment ions of *m/z* of 518 and 356, which were in agreement with the tentative assignment as an oleracein (Fig. 10)[65]. The fragment ion at *m/z* 518 corresponds to the loss of a single glucose and *m/z* 356 to a loss of both glucoses. The fragmentation data also suggested that the cinnamoyl derived moiety attached to the cyclodopa core *via* the amide bond is a caffeoyl group; this is because this ion's *m/z* was 16 amu greater than its *p*-coumaroyl equivalent. Caffeoyl groups, being *ortho*-diphenols, are known to be stronger antioxidants than most other simple phenols, in part due to resonance stabilisation and intramolecular hydrogen bonding[73]. The strong signal shown for this molecule in the online ABTS experiments also supported the assertion that it contained a caffeoyl moiety[71]

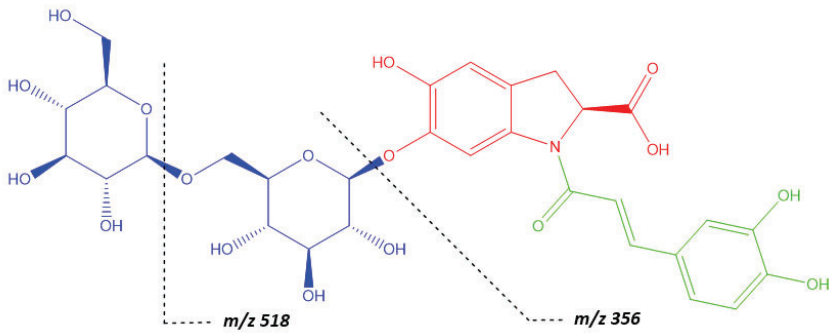



Figure 10: Oleracein X tentative structure, showing structure and two fragment ions

To date, no oleraceins with a caffeoyl-cyclodopa core have been structurally elucidated by NMR, though one (oleracein W) was reported in an MS/MS study[74]. The oleraceins K and L are the only oleraceins known to have caffeoyl moieties, but these are attached *via* a disaccharide (Fig. 11). These oleraceins were shown *via* the DPPH radical scavenging assay to be the most potent

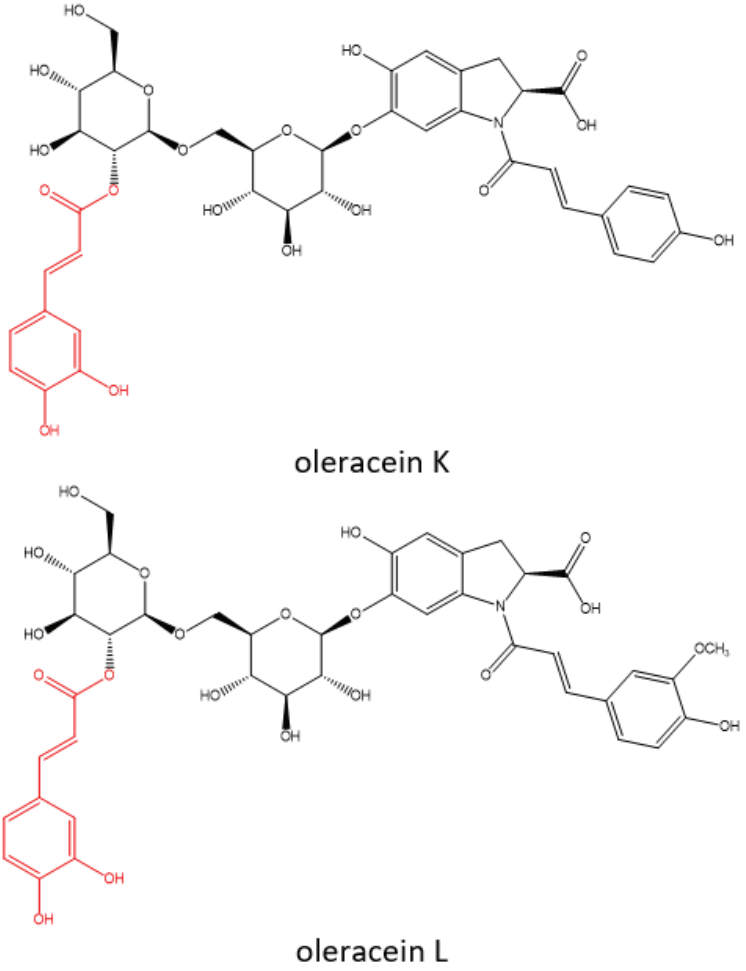



Figure 11: Oleraceins with caffeoyl moieties in red

antioxidants of the currently known oleraceins[71].

In the work at CSU, eight betacyanins were detected, with substantial differences between the three populations of *P. oleracea* tested. Four of these molecules had fragmentation data matching published data, *i.e.* for 15*S*-betanidin 6-*O*(6'-*O*-*E*-caffeoyl)-*β*-sophoroside, 15*S*-isobetanidin 6-*O*(6'-*O*-*E*-caffeoyl)-*β*-sophoroside, 6'-*O*-*E*-sinapoyl-2'-*O*-glucosyl-betanin and 6'-*O*-*E*-sinapoyl-2'-*O*-glucosyl-isobetanin[58]**.** However, standard samples were not available, so further structural elucidation was deemed necessary to confirm their identities. The other four betacyanins had different fragmentation data to those that had been previously published, suggesting they could be novel betacyanins. They still had a characteristic betanidin peak at *m/z* 389 (in ESI positive ion mode), providing strong evidence that they were in fact betacyanins (Fig. 12)[71]. This peak is

produced when betacyanins lose alkyl and glycosyl groups attached to the oxygens at C-5 and C-6. The variation in fragmentation data may mean they are isomers of previously detected betacyanins.

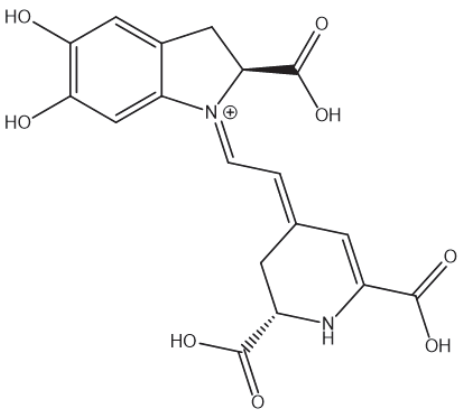



Figure 12: Characteristic betanidin peak at *m/z* 389 $[M+H]^+$

## 1.5 Objectives of this study

The objectives of this study were to isolate and structurally elucidate the oleraceins and betacyanins tentatively identified in the three Australian populations of *P. oleracea* that had been studied at CSU. The focus of this work was to be on the oleraceins and betacyanins due to the known medicinal properties of these classes of molecules and the lack of knowledge relating to these molecules in Australian populations of *P. oleracea.* An additional component of this study was to gain a greater understanding of the variation in phytochemistry of these three Australian populations.

# Chapter 2: Results and Discussion

Multiple studies have looked at the oleraceins as a novel class of chemicals with potential in treating a range of conditions[63]. The betacyanins have also received much attention on their various biological properties[75]. As both classes of chemicals are known to be contained in *P. oleracea*, their isolation and structural elucidation is an important step in understanding the medicinal properties of this plant. Furthermore, work conducted by collaborators at Charles Sturt University (CSU) showed that Australian populations of *P. oleracea* appear to be rich in these compounds. This Master of Research (MRes) study was primarily focused on formally identifying, through isolation and structural elucidation, the betacyanins and oleraceins that the CSU collaborators had tentatively identified in three Australian populations of *P. oleracea*.

Both betacyanins and oleraceins are polar and therefore water soluble. This makes their presence in traditional plant remedies such as teas, poultices and external washes of *P. oleracea* very probable. Many of the medicinal effects that have been observed in studies of *P. oleracea* have also been produced by polar extracts of this plant[32, 41, 50]. Therefore, these chemicals are potentially responsible for some component of *P. oleracea's* medicinal properties and were selected as the main target molecules for this work. Structural elucidation of these molecules would be an important step in understanding the mechanisms of action for the various customary preparations of *P. oleracea*.

Previous studies that have successfully isolated betacyanins and oleraceins from *B. vulgaris*, *O. ficus-indica* and *P. oleracea*, have extracted the plant materials with acetonitrile, ethanol, methanol and/or water, and used repeated size exclusion, reversed phase and/or ion-exchange chromatographies[64]. These have often involved large quantities of the plant material and ended up with 1-100 mg quantities of the betacyanins and oleraceins. At least in the case of the betacyanins, and most likely also for the oleraceins, yields of the compounds have been limited due to their instability. For this MRes study, only small quantities (~30 g) of dried material of each of the three populations of *P. oleracea* being studied were available. Therefore, it was essential to design an experimental protocol for their extraction and isolation to minimise degradation or loss of target molecules. To assist with this design, a detailed analysis of previous literature relating to the stability (or instability), extraction and isolation of these classes of compounds was conducted, and the extraction and purifications were attempted on a small-scale (~1 g plant material) before moving to a larger scale (~30 g).

## 2.1 Experimental design

### 2.1.1 Target molecule stability

A key issue in the extraction and analysis of betacyanins is their instability in various solvents, including water and alcohols, at temperatures above 20 °C, as well as at pH below 5 or above 7, and when exposed to oxygen or ultraviolet (UV) light[76]. At temperatures as low as 5 °C, many betacyanins experience instability when in solvent, even when protected from UV light[76]. Vulnerability to different forms of degradation, in particular those relating to pH and temperature, vary substantially between betacyanins.

The most common decomposition route for the betacyanins is *via* decarboxylation at C-2, C-15 and/or C-17 (Fig. 13)[77, 78]. Each of these carboxyl groups is easily lost *via* thermal- and photo-degradation, and also in basic or acidic conditions[79, 80]. The C-2 and C-17 positions are the most reactive sites as both have adjacent nitrogens and conjugated systems that help to stabilise intermediates of the decarboxylation reactions (Supplementary 2-7) [77, 80].

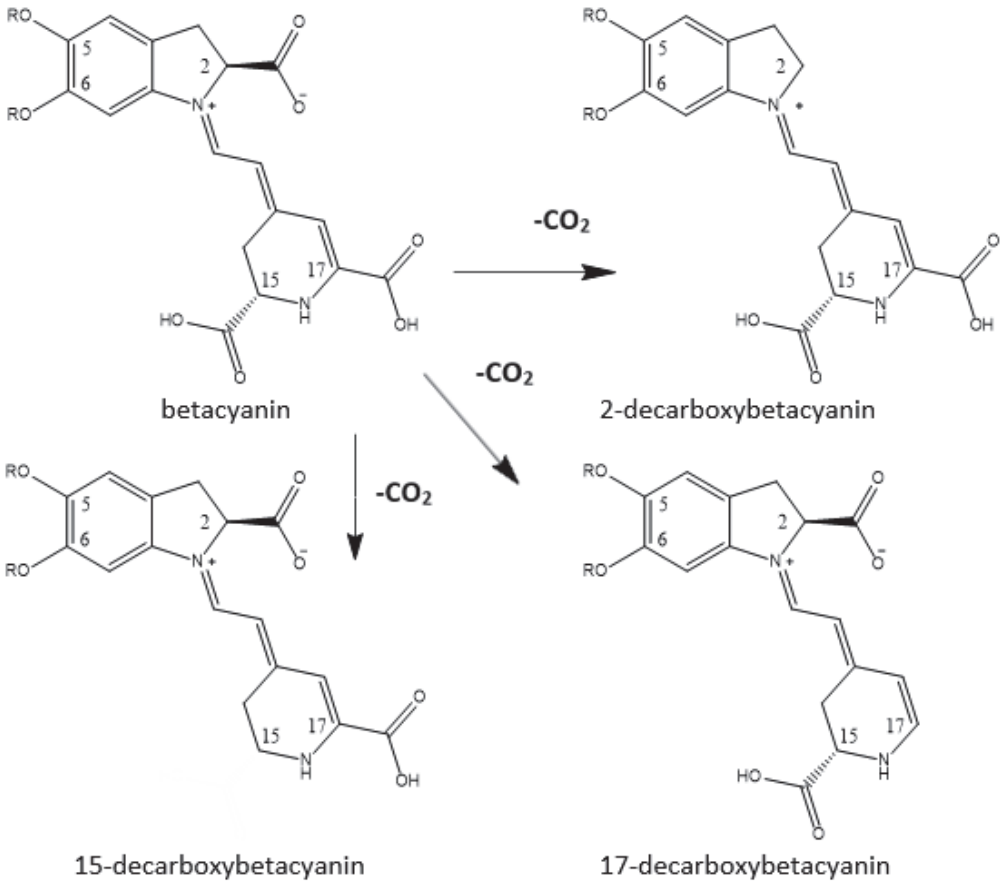



Figure 13: Decarboxylation of core betacyanin structure

Another common point of instability is at the aldimine bond attaching cyclodopa to betalamic acid[81]. This bond can be hydrolysed, especially in alkaline conditions, but also in acidic conditions (pH < 3) (Fig. 14)[81]. Although this hydrolysis is partially reversible, substantial quantities of betacyanin are

typically lost when the pH rises above 7 or falls below 3[80]. The aldimine bond can also be cleaved in the presence of methanol and ethanol *via* nucleophilic attack, an effect that is exacerbated by UV light[78, 82]. Aldimine bond alcoholysis of betacyanins has been observed to occur quickly in the presence of alcohols; at 50 °C in the presence of ethanol the half-life of betacyanins can be less than an hour, and even when at 4 °C in solutions containing 50% methanol or ethanol, some betacyanins have half-lives of a period of only days[82, 83].

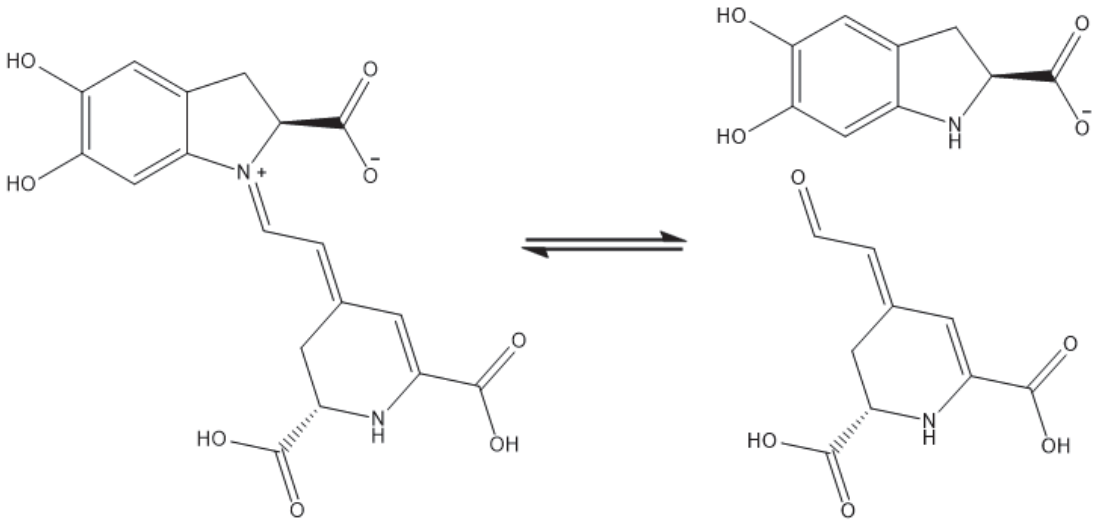


Figure 14: Equilibrium between betanidin (left) and cyclodopa + betalamic acid (right)

Betacyanins are also readily oxidised, to produce neobetacyanins[79]. This irreversibly produces neobetacyanins, which have a far paler orange colour (Fig. 15)[79]. This oxidation is favoured in the presence of oxygen and in acidic conditions, especially below a pH of 4[79]. However, recently some plants have been shown to naturally contain neobetacyanins[84-86].

The C-15 carboxyl readily epimerises in solution to produce isobetacyanins (Fig. 15). The isobetacyanins are usually detected along with their betacyanin counterparts in plants, though the betacyanin is always in a higher concentration[87].

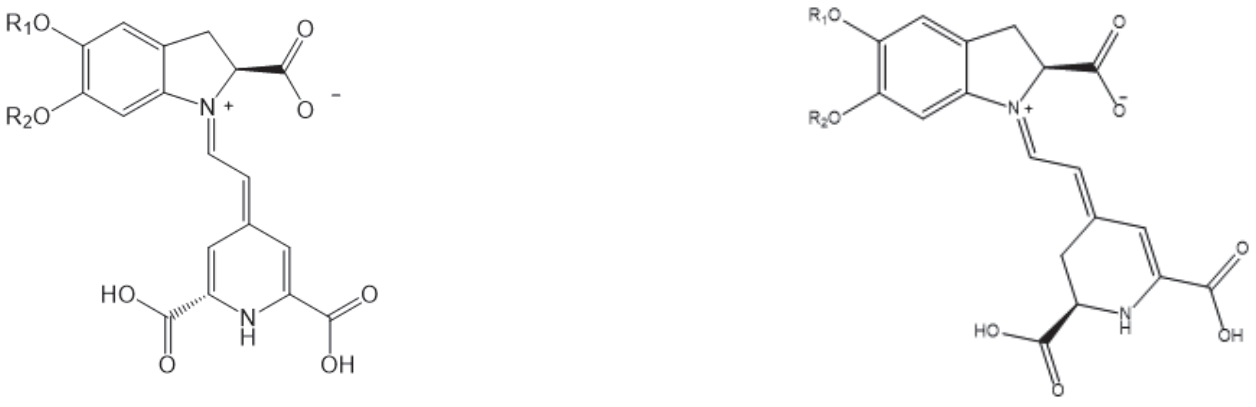


Figure 15: Core structure of neobetacyanins (left) and core structure of the isobetacyanins (right)

There is far less published work on the stability of the oleraceins due to their relatively recent discovery. They have been shown to be stable over a wider range of pH and at higher temperature when compared to the betacyanins[64]. In contrast to the aldimine bond of the betacyanins, the oleraceins have a more stable amide bond. Like the betacyanins, the oleraceins are likely to be vulnerable to degradation, most notably at the C-2 carboxyl group and glycosidic linkages (Fig 16). There is also a substantial potential for oxidation of the oleraceins containing the *ortho*-diphenol caffeoyl moiety to *o*-quinones[88].

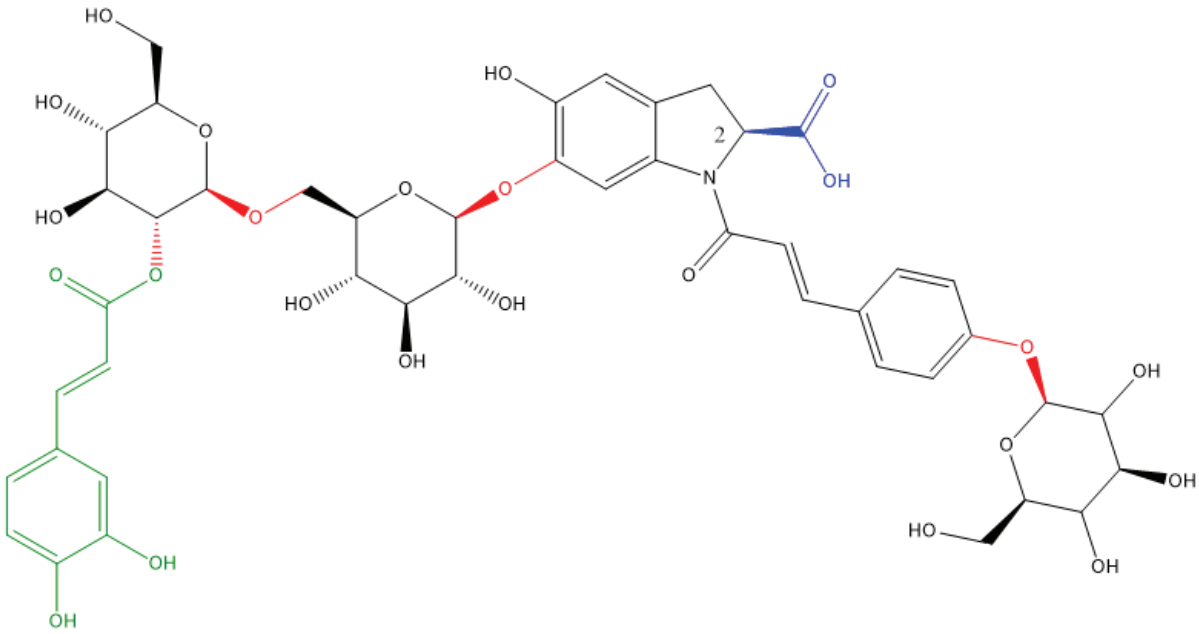



Figure 16: Oleracein R with 4 glycosidic linkages (red), C-2 carboxyl group (blue) and *ortho*-diphenol caffeoyl moiety (green)

### 2.1.2 Extraction

A review of the literature showed that both the betacyanins and oleraceins are very soluble in water, which is expected due to their highly polar functional groups. This is particularly true of betacyanins, which have a positively charged quaternised nitrogen (of the aldimine) and are insoluble in ethanol, methanol and dimethyl sulfoxide (DMSO)[89]. Water was initially considered for extraction; however, previous work had shown that water was substantially less selective for extraction of betacyanins than an alcoholic water mixture. When water had been used as the sole extraction solvent a far greater quantity and range of unwanted molecules were also extracted. Additionally, despite betacyanins being insoluble in ethanol they are highly soluble in water ethanol (alcohol) mixtures [76]. Use of alcoholic-water mixtures for extraction have also been the main published methods for

oleracein extraction and was also successfully employed by the CSU collaborators[64, 71]. A review of the literature showed that a methanol:water (20:80) mixture gave good results for betacyanin extraction at low temperatures (10 °C) and over short periods of time (< 20 min)[76]. However, use of methanol for natural product extraction has the disadvantage that it is unclear to discern whether the presence of methyl ethers or esters are natural or artefacts[67]. Thus, ethanol is often used instead of methanol for natural product extractions as ethoxy groups, unlike methoxy groups, are rare in natural products, and any artefacts produced by using ethanol would be apparent. Ethanol concentrations of 20-50% in water have been shown to be almost as effective at extracting betacyanins as the analogous aqueous methanol mixtures[76]. Aqueous ethanol mixtures, like aqueous methanol mixtures, show greater selectivity for betacyanins over other polar compounds such as proteins and polysaccharides[76, 87]. Ethanol water mixtures of 60-70% ethanol are also the dominant method in the literature for initial extraction of oleraceins[64]. This is also consistent with the method used by collaborators at CSU, who extracted *P. oleracea* plant material with 50% aqueous ethanol[71].

### 2.1.3 Separation and purification

Considerable research has been conducted in recent years to optimise betacyanin separation and purification from complex plant extracts[76, 90]. This is in part due to the increasing demand for natural food colourants and antioxidants, as well as the potential medicinal value of betacyanins[55]. A 2012 study investigated optimisation of the separation of betanin from aqueous beetroot extracts[90]. Betanin is one of the simplest betacyanins, consisting of the betacyanin core with a glucose attached to the oxygen at C-5 (Fig. 17). This red pigment is found in high levels in beetroot, making beetroot betanin a viable red food colourant and antioxidant[90]. This study compared seven methods for chromatographic separation of the beetroot extract. The purity of betanin obtained from each method was determined *via* reversed phase (C18) high performance liquid chromatography (RP-HPLC) measuring absorbance at 536 nm[90].

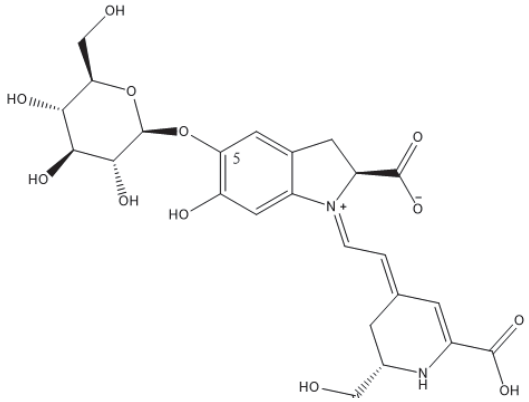



Figure 17: Betanin structure

Anionic ion-exchange chromatography using Q Sepharose high performance resin, a cross-linked agarose with quaternary ammonium as the exchanger group, gave the most efficient separation of betanin. However, a further purification step would be required (typically size exclusion chromatography) to remove salts[90]. RP-HPLC, eluting with water, also gave good separation, with the betanin almost as pure as that obtained by ion-exchange chromatography[90]. Size exclusion chromatography, using either Sephadex LH-20 or G-25, eluting with water, was almost as effective as RP-HPLC in separating betanin[90]. Sephadex LH-20 size exclusion chromatography has also been shown to be an effective method for removing colourless phenolics from betacyanin fractions, whereas RP-HPLC was not as effective[84, 90].

A review of the limited literature on purification of oleraceins showed that in each case size exclusion chromatography had been employed[64-66]. In the three papers describing oleracein purification from *P. oleracea*, Sephadex LH-20 was always used as a stationary phase for size exclusion, and aqueous ethanol or aqueous methanol as the mobile phase[64-66]. Thus, size exclusion chromatography was deemed as an appropriate choice for this MRes study for the initial separation of betacyanins and oleraceins from *P. oleracea* extracts. However, further purification would be needed as size exclusion has only been successful in partial purification of oleraceins and betacyanins[64, 90].

### 2.1.4 Further separation and isolation

On reviewing the literature, C18 RP chromatography appeared to be the logical choice for further separation and potential isolation of the betacyanins and oleraceins. Preparative RP-HPLC with a methanol mobile phase has been effective in cleaning up size exclusion fractions containing oleraceins[64]. However, as mentioned above, there is a risk of methanol artefacts. Combinations of water and acetonitrile or ethanol have also been employed in the C18 RP purification of oleraceins[64, 65]. The literature showed that although the solubility of betacyanins in acetonitrile is low, their stability in 50% aqueous acetonitrile is greater than 50% aqueous methanol or 50% aqueous ethanol[78]. As a result of this high stability and relatively low solubility, an aqueous acetonitrile system was considered ideal for separation of betacyanins by C18 RP chromatography. Oleraceins have previously been shown to be stable in acetonitrile, and aqueous acetonitrile has been employed to successfully separate oleraceins from semi-pure mixtures[64].

### 2.1.5 Experimental design based on literature

Guided by the above literature review, the following experimental design was proposed (Fig. 18): The whole plant material (leaves, stems, roots, seeds and buds) of *P. oleracea* would be extracted using 50% aqueous ethanol, and the extract washed with *n*-hexane to remove lipids, chlorophyll and other non-polar molecules. Aerial parts, especially leaves and seeds, of *P. oleracea* are known to contain large quantities of lipids[71, 91]. The remaining aqueous-ethanolic extracts would be concentrated by rotary evaporation at 20 °C to remove the ethanol, and then freeze-dried to remove the residual water. This follows the protocol designed by the CSU collaborators and would allow direct comparison of the extract profile obtained to their previous studies. Aqueous ethanol was favoured over aqueous methanol for the extraction to enable ester and ether artefacts to be readily identified[67].

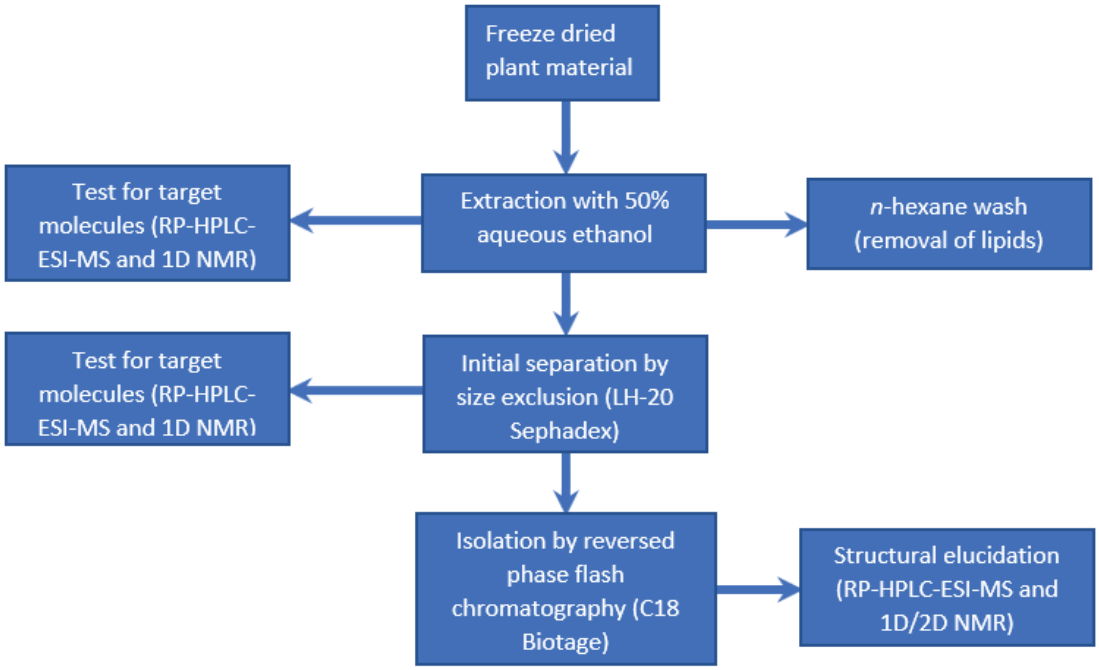



Figure 18: Workflow overview diagram

Chromatographic purification was proposed to be conducted using LH-20 size exclusion chromatography eluting with water for partial fractionation of the freeze-dried extracts, followed by C18 RP flash chromatography[64, 92]. While RP-HPLC is most commonly used, there was limited access to RP-HPLC, and flash chromatography with a Biotage Isolera Four system was readily available. In recent years flash chromatography had been employed successfully in the purification of a wide range of natural products including betacyanins[92]. Use of the Biotage system would allow for the separation of large samples with better reproducibility than free standing columns and with

a greater sample capacity than the available C18 HPLC columns and semi-preparative HPLC facility would allow.

Due to difficulties in obtaining plant materials, 30 g was the most that could be obtained of each population of *P. oleracea*. Although this would make structural elucidation challenging, work at CSU had suggested that these populations were rich in the target molecules. Structural elucidation was therefore regarded as feasible if the focus was made on efficiency of extraction and target molecule stability. Hence all extraction steps would be conducted at ~20 °C and for the extraction and purification stages, glassware/flasks would be wrapped in alfoil to minimise thermal- and photo-degradation[77, 78].

$^{1}$H NMR experiments, RP-HPLC-ESI-MS and TLC would be run on all extracts and fractions to track the presence of the betacyanins and oleraceins and to monitor their stability. Both betacyanins and oleraceins have characteristic $^{1}$H NMR signals for their cyclodopa core, as do the cinnamoyl derived moieties of the oleraceins and the betalamic moiety of the betacyanins, making their tracking relatively straightforward. Structural elucidation of semi-pure and pure compounds would employ extensive 1D and 2D NMR and ESI-MS.

## 2.2 Extraction of *P. oleracea* whole plant

Whole plant material (leaves, stems, roots, seeds and buds) was obtained from three populations of *P. oleracea*, *i.e.* omega red, omega gold and common purslane grown at Eurongilly, NSW. The material was collected and freeze-dried by collaborators at CSU. This plant material was stored at -80 °C before being sent overnight *via* courier (unrefrigerated). Upon arrival the plant material was stored in a freezer at -20 °C.

The three populations were selected to assess the betacyanins and oleraceins in *P. oleracea* from a range of climates and lineages. The omega red population was bred from seeds originally sourced from the Lake Eyre region of central South Australia, and as the name suggests, had a far more distinct red colour than that of the other two populations (Fig. 19). This as an extremely arid region with substantial annual fluctuation in temperature and saline soils. The omega gold population was bred from seeds originally sourced in the Kimberley Ranges region of northern Western Australia. This area is in the tropics and as such has a more consistent annual temperature, but rainfall varies greatly with the vast majority falling in the wet season and comparatively little in the wet season. Both these populations of *P. oleracea* have more intense red pigmentation than that of the common purslane (Fig. 19). This pigmentation was likely due to betacyanins, which in many plants are known to be produced as part of a stress response in coping with salinity and temperature extremes[93]. Additionally, it is possible that these populations would have higher levels of oleraceins, as

antioxidants more generally are known to be important in plant adaptation to extreme environments such as those of Australian deserts[94]. Common purslane was selected as a population representative of *P. oleracea* found in agricultural and urban areas globally - common purslane near the east coast of Australia is thought to largely have descended from imported cosmopolitan populations.

Figure 19: The three populations of *P. oleracea*, from left to right: omega red, omega gold and common purslane. Pictures taken by Colette Geier 2018.

Each of the three populations of *P. oleracea* underwent the extraction procedure described in 2.1.5, *i.e.* extraction with 50% aqueous ethanol at 20 °C, and washing with *n*-hexane to remove lipids and other non-polar material. The procedure was first performed on a small-scale (~1.0 g) so that ease of target molecule extraction could be tested before full-scale extraction was attempted. This employed 3 x 10 mL extractions with 50% aqueous ethanol. The resultant extracts, labelled as 'polar extracts', had substantially different colours, reflecting the colours of the dried plant materials and the plants themselves at maturity (Fig. 19, 20, 21). Three washes with *n*-hexane (3 x 15 mL) was performed for the omega red and omega gold extracts. The procedure had to be modified for the common purslane, which required five *n*-hexane washes (5 x 15 mL) before the *n*-hexane came off colourless rather than green. This was likely due to higher concentrations of chlorophyll in common purslane, which was consistent with the common purslane having much darker green leaves and stems than the other populations (Fig. 19). The pooled *n*-hexane washes also differed in colour, though not as much as the polar fractions (Fig. 21). These were the first observations other than the colour of the dried plant material that these populations had substantial differences in phytochemistry.

Figure 20: polar extracts 10 mg.mL$^{-1}$ in 50% aqueous ethanol, left to right: omega red, omega gold and common purslane

Figure 21: Concentrated *n*-hexane washes, left to right: omega red, omega gold and common purslane

The small-scale polar extracts were analysed by RP-HPLC-ESI-MS and $^{1}$H NMR. The extracts were first assessed *via* RP-HPLC utilising an online diode array detection (DAD). Based on work by collaborators at CSU, the wavelengths of 280 nm (phenolic compounds), 320 nm (cinnamic acids) and 540 nm (betacyanins) were used for detection. Additionally, the MS data presented in this study was produced using a low-resolution system, unlike the work at CSU. The RP-HPLC-ESI-MS work was conducted at Macquarie University using an Agilent 1260 liquid chromatography system connected to a 6130 Series Quadrupole LC/MS System. The CSU research employed Agilent 1290 Infinity connected to a 6540 UHD accurate mass spectrometer. As a result, the peaks produced in this study were not as well resolved as those produced at CSU[71]. In order to get HPLC peaks of a somewhat similar intensity and resolution to that seen for the CSU studies, sample concentration had to be increased from 0.5 µg.mL$^{-1}$ to 5.0 µg.mL$^{-1}$, the flow rate had to be slowed from the 0.4 mL.min$^{-1}$ used to 0.3 mL.min$^{-1}$. With these modifications, the results were broadly analogous to those of the CSU collaborators at CSU (Fig. 22), but the data obtained for the MRes study could not reproduce the resolution quality of the CSU.

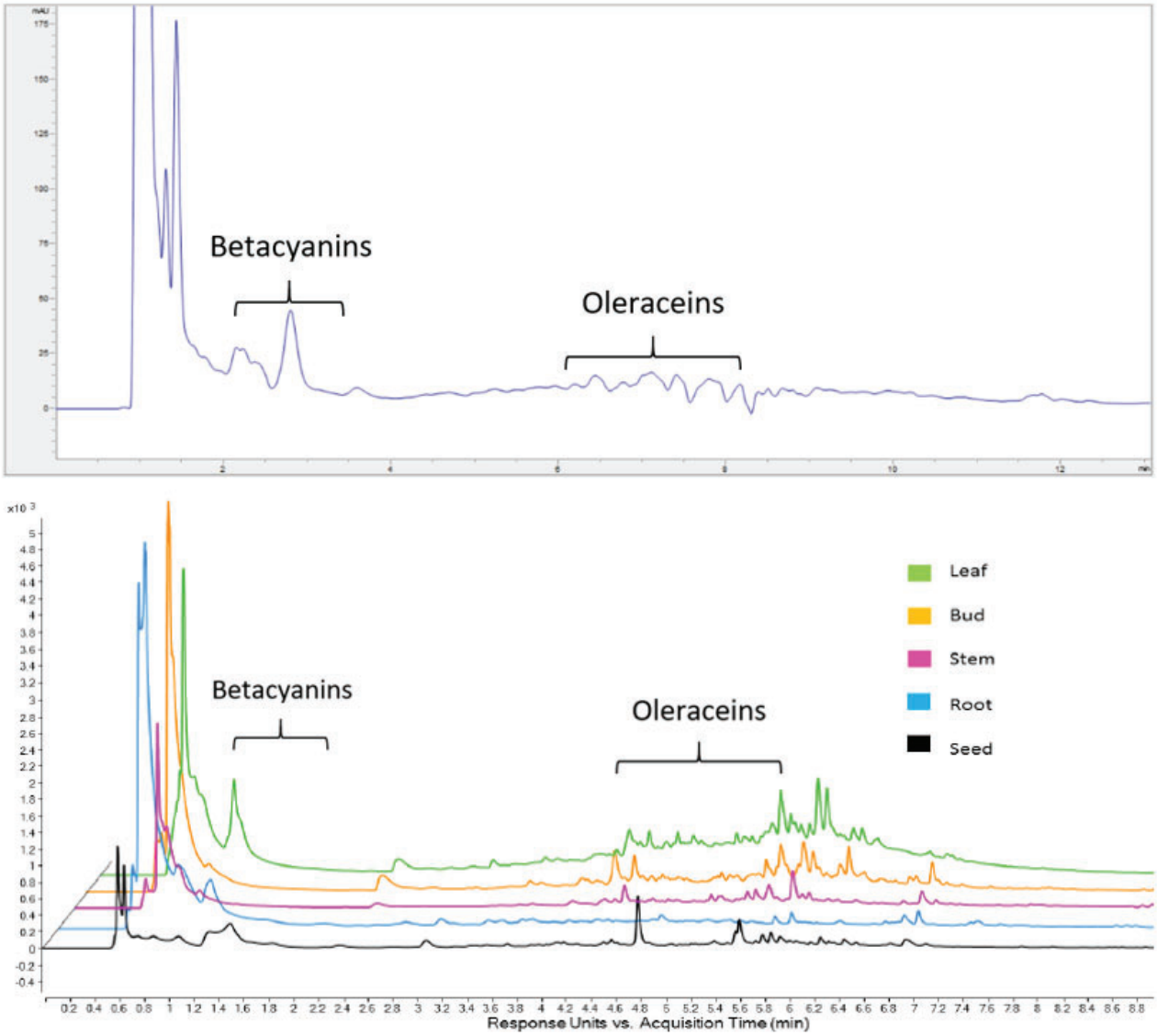



Figure 22: RP-HPLC chromatogram of omega red at 280 nm, top MRes work and bottom work of Colette Geier (CSU collaborator)

Analysis of the HPLC peaks by ESI-MS in both positive and negative ion modes identified ions characteristic of the pseudo molecular ions of betacyanins and oleraceins, including those detected by the CSU collaborators (*m/z* 502, 644, 680, 875) (Table 2)[71] . Several peaks (*m/z* 191, 313) consistent with other compounds previously reported in *P. oleracea*, were also observed (Table 2). This data is summarised in Table 2.

Table 2: Peaks of suspected target oleraceins in the three populations of *P. oleracea* analysed

| Putative compound | ion *m/z* | Elution time (min) | Omega red | Omega gold | Common purslane |
|---|---|---|---|---|---|
| Oleracein A $(M-H)^-$ | 502 | 6.6 | + | + | + |
| Oleracein B $(M-H)^-$ | 532 | 6.9 | | | + |
| Oleracein C +/or H $(M-H)^-$ | 664 | 7.1 | + | + | + |
| Oleracein D +/or I $(M-H)^-$ | 694 | 7.3 | + | + | + |
| Oleracein N $(M-H)^-$ | 840 | 8.6 | + | | |
| Putative oleracein X $(M-H)^-$ | 680 | 7.2 | + | + | + |
| Oleracein K $(M-H)^-$ | 826 | 8.5 | + | | |
| Oleracein L $(M-H)^-$ | 856 | 8.5 | + | | |
| Quinic acid $(M-H)^-$ | 191 | 3.2 | + | + | + |
| Oleracimine A $(M+H)^+$ | 301 | 4.2 | | + | + |
| 15*S*-betanidin 6-*O*(6'-*O*-*E*-caffeoyl)-$\beta$-sophoroside $(M+H)^+$ | 875 | 2.9 | + | + | + |
| 15*S*-isobetanidin 6-*O*(6'-*O*-*E*-caffeoyl)-$\beta$-sophoroside, 6'-*O*-*E*-sinapoyl-2'-*O*-glucosyl-betanin $(M+H)^+$ | 899 | 2.8 | + | + | + |
| 6'-*O*-*E*-sinapoyl-2'-*O*-glucosyl-isobetanin $(M+H)^{+-}$ | 919 | 3.1 | + | + | + |
| Portulacanone A $(M-H)^-$ | 313 | 7.1 | + | + | + |

The mass peaks matching those of the target oleraceins exhibited absorbance maxima at 320 nm, which was consistent with them being cinnamoyl derivatives. The MS data also suggested that some of the oleraceins had multiple isomers. A peak with a pseudo molecular ion of *m/z* 680 eluted in all three samples, with the most intense peak being in the omega gold population and the least intense in the omega red population. This was thought to be the main target oleracein, oleracein X. It eluted at ~4.1 minutes, surrounded by several other suspected oleraceins, also detected at 320 nm, for each of the three polar extracts. Two of these peaks had pseudo molecular ions of *m/z* 664, another two with *m/z* of 694, and another with an *m/z* of 502. These ions were in agreement with the published masses of oleraceins C and H (665 amu), D and I (695 amu) and A (503 amu), respectively in positive ion mode $(M+H)^+$. The ESI-MS data were also in agreement with that found by the CSU collaborators. This work at CSU found the pseudo molecular ion tentatively assigned as oleracein X partially coeluted with another suspected oleracein with *m/z* of $[664-H]^-$. Experiments employing MS/MS suggested this had been oleracein H, due to fragmentation suggesting two glycosyl moieties were linked[71].

The omega red extract also showed a second smaller peak with an *m/z* of 680 $[M-H]^-$ eluting shortly after what was believed to be oleracein X. This suggested that there may be an isomer of oleracein

X, possibly with a rearrangement of the glucosyl moieties. It is known that many of the oleraceins have isomers with differing placement of glucosyl moieties.

Each of the *P. oleracea* populations appeared to have large quantities of oleracein C and/or H, both with an *m/z* of 664 $[M-H]^-$. Without fragmentation data, it was impossible to tell which was which. What was clear was that in each of the populations, the putative oleracein X eluted between these two isomers and in far smaller quantities.

The mass peaks in ESI positive ion mode matching those of betacyanins exhibited absorbance maxima at ~540 nm, which was consistent with this class of compounds[95]. These pseudo molecular ions detected included *m/z* 701, 889 and 919. In comparison to other MS peaks, the peaks in the 540 nm chromatograms were quite weak for all three populations. This suggested that the betacyanin concentrations were low. The intensity of the red colour seen is not surprising given the reported high extinction coefficients of the betacyanins, for example, betanin has an extinction coefficient of $6.5 \times 10^4$ $l.mol^{-1}.cm^{-1}$ at 535 nm[96].

$^1H$ NMR experiments were also performed on each of the polar and *n*-hexane extracts. The polar extracts appeared to have large quantities of sugars as determined by signals between δ 3.0 - 4.0 ppm, which suggested the possibility of oxymethines and oxymethylenes of sugars, and possibly methoxy signals (Fig. 23)[97]. Crucially, there were substantial aromatic components in the polar extracts, and their peaks were in the right regions (between δ 6.5 and 8.5 ppm) to be cyclodopa and cinnamoyl derived moieties (Fig. 23)[64]. Many of these peaks also had splitting patterns characteristic of hydrogens attached to the rings of *p*-coumaroyl, caffeoyl and feruloyl moieties of oleraceins and betacyanins, though these samples were far too complex to accurately assign any moieties with a high level of confidence (Fig. 23)[64, 98]. Additionally, there were no discernible *trans* doublets ($J$ ~ 16 Hz), characteristic of the double bond of cinnamoyl derived moieties at ~6.2-7.7 ppm[64, 98]. This was likely an indication of the low concentration of the oleraceins and betacyanins in the complex mixtures of these polar extracts.

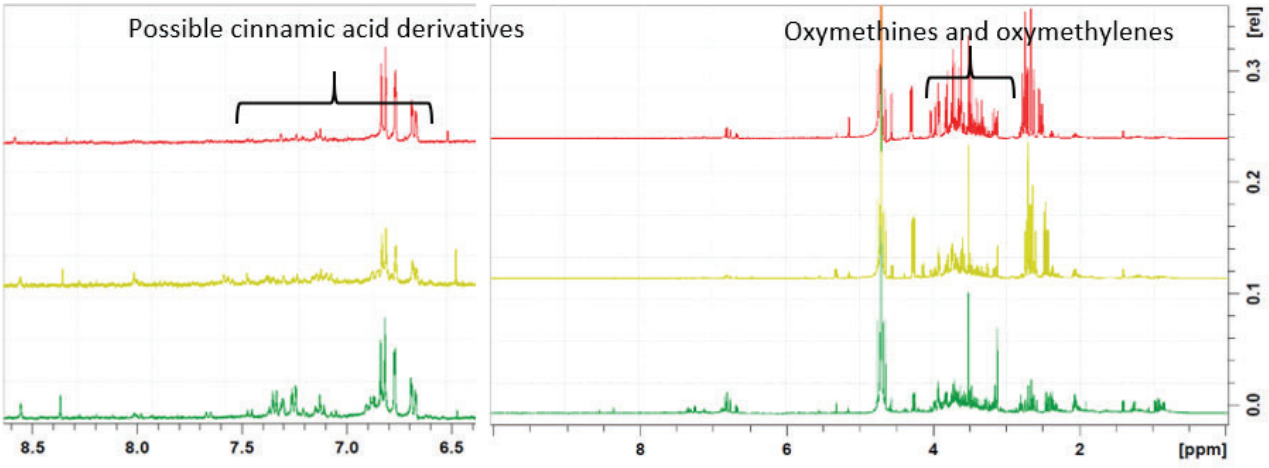



Figure 23: $^{1}$H NMR spectra of polar extracts in deuterated water at concentrations of 40 mg.mL$^{-1}$ Top to bottom: omega red, omega gold and common purslane. Right: all visible peaks, left: the aromatic region from δ 6.5 ppm and δ 8.5 ppm.

The $^{1}$H NMR results for the *n*-hexane wash samples revealed strong similarities between the three populations; however, omega red had several peaks not shared by the other two populations in the range from δ 0.0 ppm to δ 2.5 ppm and more broadly the *n*-hexane washes of the omega gold and common purslane extracts were more similar in terms of relative integration of shared peaks (Fig. 24). The *n*-hexane washes appeared to have collected large quantities of long carbon chains, which were likely *cis* unsaturated fatty acids based upon the characteristic signals between δ 0.5 ppm and δ 4.5 ppm (Fig. 24)[99]. This was in agreement with the literature in which *P. oleracea* has been found to be an extremely rich source of these acids, in particular α-linolenic acid[91]. Omega red and omega gold also showed a greater variety and quantity of aromatic signals than that of the common purslane. However, these aromatic signals were a small part of the overall NMR spectra for the *n*-hexane washes and were likely coming from non-polar aromatics. Indeed many of these aromatic peaks were in agreement with a mixture of chlorophyll A and chlorophyll's degradation product methyl pheophorbide A[100]. Due to the very low level of aromatic signals from the *n*-hexane washes and the lack of any red hue in these fractions or characteristic peak for the betacyanin or oleracein molecules, these molecules appear to have been retained in the polar extract.

The colour of the *n*-hexane washed polar extracts, combined with the RP-HPLC-ESI-MS and $^{1}$H NMR data, suggested that the target molecules had been successfully extracted and not lost during the washing step and that the *n*-hexane washes had successfully removed unwanted compounds from the aqueous ethanol extracts.

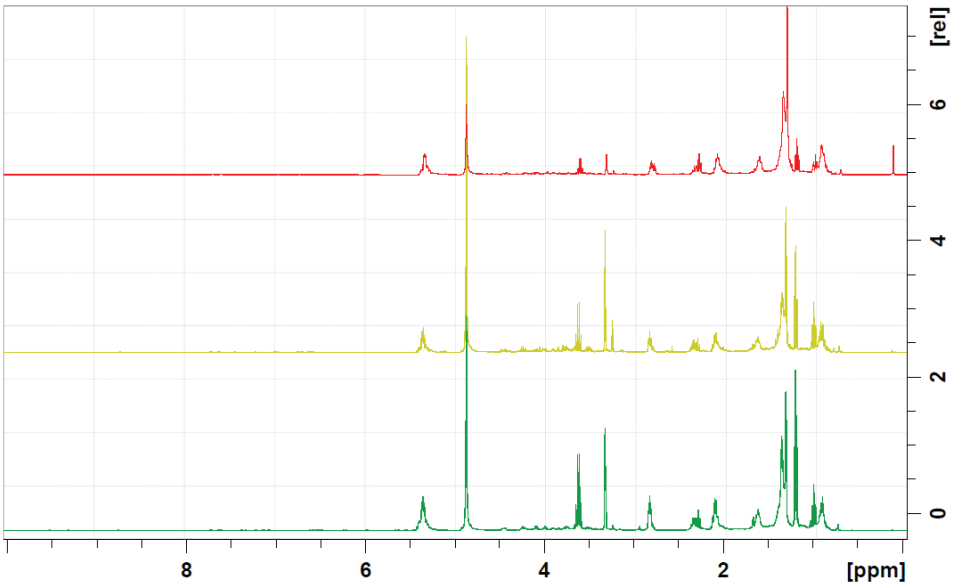



Figure 24: $^{1}$H NMR spectra of n-hexane extracts in deuterated methanol, top to bottom: omega red, omega gold and common purslane (30 mg.mL$^{-1}$).

Following on from what appeared to be successful extraction of the target molecules in the small-scale studies, the full-scale extraction was performed on each of the three populations. Like the small-scale extraction, two additional *n*-hexane washes were required for the common purslane. Once completed, the full-scale and small-scale extracts of each population were pooled, revealing that there was also a substantial difference in the quantity of material being extracted by the procedure in each population (Table 3). The most substantial differences between populations were in the quantity of material obtained in the *n*-hexane extract (Table 2).

Table 3: Total extraction yields of *P. oleracea*

| | Omega red | Omega gold | Common purslane |
|---|---|---|---|
| Dried plant material | 28.1 g | 28.4 g | 30.1 g |
| Polar extract | 6.7 g (23.8%) | 7.5 g (26.4%) | 7.6 g (25.2%) |
| *n*-Hexane extract | 1.0 g (3.6%) | 0. 8 g (2.8%) | 1.4 g (4.7%) |
| Waste material | 20.4 (72.6%) | 20.1 g (70.6%) | 21.1 (70.1%) |

## 2.3 Separation and purification

### 2.3.1 Initial small-scale size exclusion

Initial size exclusion chromatography was conducted on the omega gold extract obtained from the 1 g plant material extraction using LH-20 Sephadex. LH-20 Sephadex is a hydroxypropylated dextran resin with both polar and non-polar properties that separates molecules based upon their masses and largely regardless of polarity. It has been used successfully in the separation with good recovery of many complex mixtures of natural products, including separation of many small glycosylated molecules including the two classes of target molecules[64, 90, 101].

The omega gold polar extract, ~35 mg, was applied to the column (400 x 10 mm), and eluted with water, collecting 50 fractions (~5 mL each). Mass recovery was ~90%. A small amount of green material remained at the top of the column, which eluted when the column was flushed with ethanol. By visual inspection, five distinct bands were seen in the column: the first two bands being fuchsia and the third orange, the fourth orange-red, and the fifth orange.

The electron withdrawing glucosyl and alkyl groups attached to the 5- and 6-positions of betacyanins are known to cause a bathochromic shift from $\lambda_{max}$ ~535 nm to between ~540-550 nm in absorbance spectra due to lowering the energy between the highest occupied molecular orbital and the lowest unoccupied molecular orbital (Fig. 25)[95, 102]. Due to this bathochromic shift, the first two eluting bands were thought to be betacyanins with additional moieties attached to the cyclodopa core, likely sugars or small organic acids, and the third band betanidin (the unmodified betacyanin core)[95, 102]. This is consistent with what had been the expected order of elution *via s*ize exclusion chromatography, the larger compounds eluting first, the order essentially unaffected by other features such as polarity. Due to the orange-yellow colour, the third and fourth bands were thought to contain oleraceins[64].

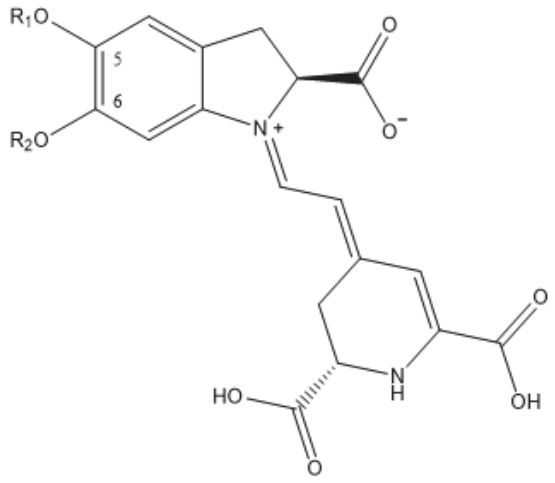



Figure 25: Core betacyanin structure showing 5 and 6 positions where all known additional moieties are connected

RP-HPLC-ESI-MS supported that the first two bands contained what appeared to be target betacyanins and the fourth band (orange) contained the target oleraceins. The first band showed a peak in ESI positive mode with an *m/z* of 919 and the second darker and wider band showed peaks with ions with *m/z* of 889 and 875. These masses agreed with what was observed by the CSU collaborators who tentatively identified them as betacyanins *via* MS/MS[71]. Though these betacyanins shared their pseudo molecular masses with previously describe betacyanins, their

different fragment ions from those in the literature suggested that they were novel compounds[71]. The second band also contained in its tail a substance with a high absorbance at 540 nm and an *m/z* of 701. Target molecules were not detected in the third and fourth bands. The fifth band showed ions in ESI negative mode corresponding to the deprotonated pseudo molecular ions of oleraceins; the largest peaks corresponding to known oleraceins with *m/z* 664 $[M-H]^-$ and 694 $[M-H]^-$[65]. This band also exhibited an ion with *m/z* 680, corresponding to the potential novel oleracein X detected by the CSU collaborators[71]. The colours of the bands seen and the presence of potential target betacyanins in red and oleraceins in yellow/orange were in agreement with the published colour of these target classes of molecules and the DAD profiles of eluting peaks[55, 64]. Given the success of the small-scale size exclusion chromatography of omega gold, the same procedure was applied to the polar extracts of common purslane and omega red.

Common purslane showed an almost identical elution profile to omega gold, though as was to be expected from the colour of the plant, the red bands were far less obvious. It exhibited the same ions as seen for omega gold in the ESI-MS, but the putative betacyanins were less intense in the total ion current (TIC) than that seen for the omega gold size exclusion chromatography fractions. The mass recovery was ~85% from the water elution, likely due to a larger amount of green material remaining on the top of the column that was eluted with ethanol. In the tail of the fifth band, common purslane showed ions corresponding to the pseudo molecular ions of two additional oleraceins, at *m/z* 502 (oleracein A) and 532 (oleracein B)[65]. This was consistent with the findings of the CSU collaborators, which indicated common purslane had higher concentrations of these oleraceins than omega gold[71]. Omega red showed similar bands as the other two *P. oleracea* polar extracts, but the red bands were darker (Fig. 26). The fourth and fifth bands of omega red were harder to distinguish than for the other polar extracts, and tailed for longer. Mass recovery from the water elution was ~90%, but the green material left on top of the column was fainter than that of the other two populations. The omega red polar extract showed the same ions from the same bands as common purslane, including what was consistent with oleracein A and B.

Figure 26: Sephadex LH-20 size exclusion, omega red (left) and omega gold (right)

### 2.3.2 Full-scale size exclusion

Once the small-scale size exclusion chromatography had been completed for each of the polar extracts, larger portions (~1 g) of each of the polar extracts were subjected to LH-20 Sephadex (column 580 x 40 mm) size exclusion chromatography, eluting with water, with fractions of 10 mL each. The three populations showed similar profiles to that seen with the small-scale studies, and all showed ~70% of the mass coming out between fractions 20 and 50 (Fig. 27). Both omega red and omega gold showed ~95% mass recovery and common purslane showed ~85% mass recovery. Due to the complexity of the mixtures clearly defined peaks were not produced; however, each of the samples did show semi-defined peaks (Fig. 27). The omega red separation showed a broader separation of the bulk of the mass and what appeared to be more peaks than the other two samples (Fig. 27). As in the small-scale experiments each of the fractionations left a small amount of green material at the top of the column which was likely the main reason for a loss of mass on the column. Analysis by RP-HPLC-ESI-MS confirmed that many of the fractions contained betacyanins or oleraceins (Fig. 27). This analysis also suggested that the oleraceins made up between 1-5% of the polar extract's mass.

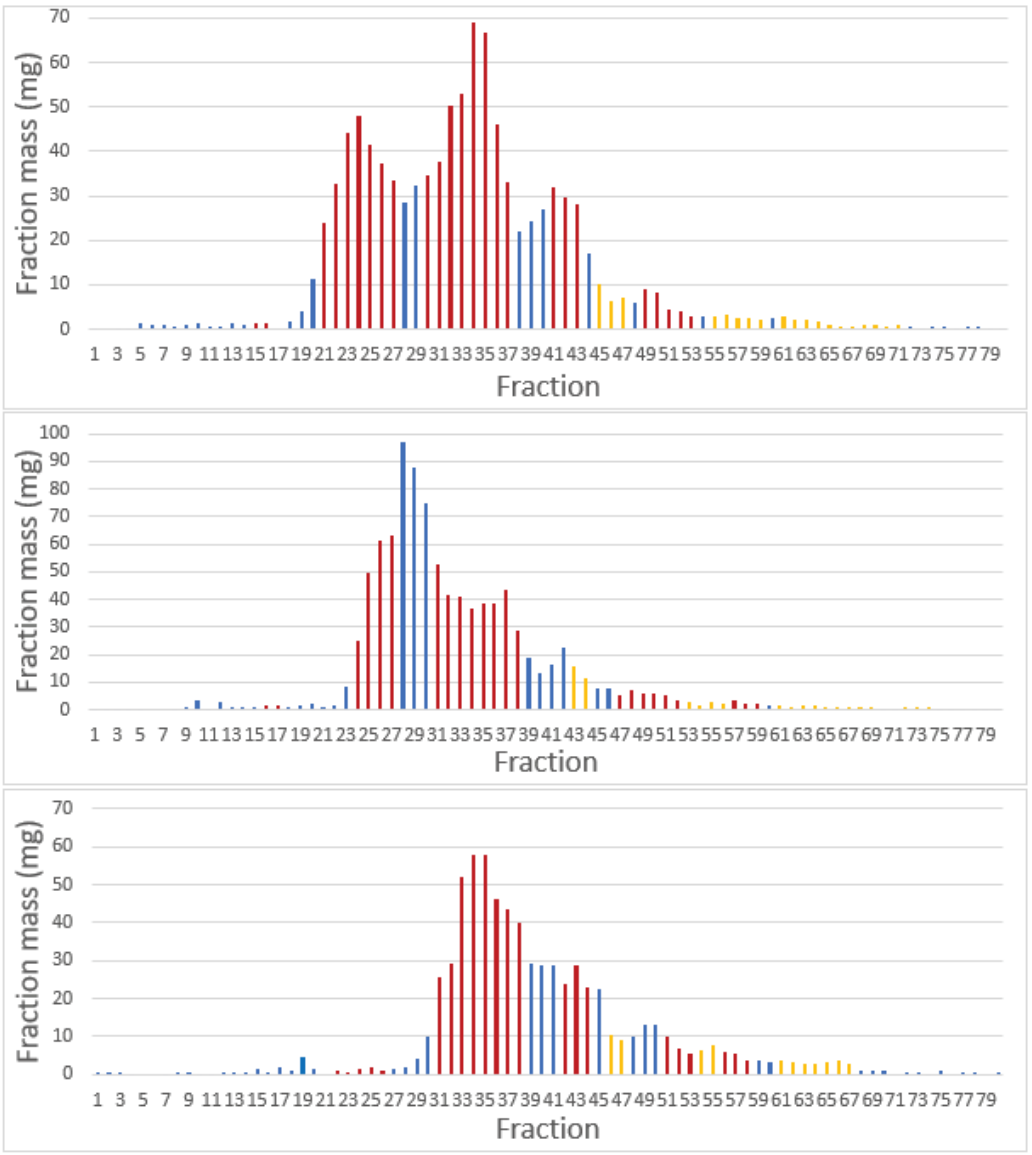



Figure 27: Elution profiles of full-scale size exclusion, from top to bottom: omega red, omega gold and common purslane. Red fractions rich in betacyanins and orange fractions rich in oleraceins.

### 2.3.3 Further purification of betacyanins

Due to the ease with which they could be visually detected, the betacyanins were the first class of target molecules selected for further purification. Visual inspection of the polar extracts and size exclusion fractions, combined with the initial chromatograms, MS traces and $^{1}$H NMR of the polar extracts, suggested that the omega red population had the greatest concentrations of betacyanins. Thus, omega red was selected for the first attempt to purify betacyanins from a polar extract.

There were six fractions (fractions R32-R37, total mass 200 mg) obtained from the second band of the omega red size exclusion chromatography experiment that had a dark pink/red hue. Each of these showed peaks in the RP-HPLC with a $\lambda_{max}$ at ~540 nm, consistent with betacyanins. The $^{1}$H

NMR of these six fractions suggested that they were still very complex mixtures (Supplementary 8). However, in each fraction peaks were observed at *m/z* in the ESI-MS (positive mode) of 875 and 889, which were consistent with the pseudo molecular ions of the betacyanins 15*S*-betanidin 6-*O*(6'-*O*-*E*-caffeoyl)-$\beta$-sophoroside and 6'-*O*-*E*-sinapoyl-2'-*O*-glucosyl-betanin, respectively, or novel isomers of these two molecules. The HPLC elution and MS profiles were consistent with the findings of the CSU collabrators[71]. MS/MS was not conducted in this MRes study and thus no further data could be gleamed *via* RP-HPLC on whether these were the target betacyanins or other molecules sharing a similar mass. Previous study at CSU had found the ions at *m/z* 875 and 889 fragmented to *m/z* 551 and 389 in the positive ESI mode. In the context of betacyanins, the ion at *m/z* 389 is characteristic of the cyclodopa-betalamic acid core (betanidin) and the ion at *m/z* 551 characteristic of that same core with the addition of a glucosyl moiety (betanin).

While the MS data and colour of the six fractions suggested that they contained betacyanins, $^{1}$H NMR experiments suggested that the betacyanins were in very low concentrations. The $^{1}$H NMR spectra of the six fractions showed only very weak signals in the aromatic region (δ 6.0 to 8.7 ppm), and had very strong signals between δ 2.0 to 4 ppm that were thought to be due to sugars[97]. There was a doublet at δ ~8.5 ppm, which is often characteristic of H-11 of the cyclodopa-betalamic acid core in betacyanins[89, 103].

The darkest pink fraction, R32 (50.2 mg), was subjected to C18 RP flash chromatography, eluting with a water acetonitrile mobile phase. After freeze-drying of the eluted fractions, a large quantity of white material was seen in the first 7 fractions (of 25) and two fractions (13 and 14) containing what looked like a red wax (1.9 mg). None of the fractions contained any yellow colour, which is characteristic of most betacyanin degradation. However, the fractions containing the red material showed little UV response at 254 or 280 nm in the flash chromatography trace. This suggested that despite the strong visible colour, any betacyanins present were still quite impure, which given this class of molecules' high extinction coefficient was not an unreasonable notion[96]. $^{1}$H NMR of the pooled two red fractions showed peaks consistent with betacyanins at between δ 7.0 ppm and δ 8.7 ppm; however, they were still a very small component of the mixture (Supplementary 9) [103].

C18 RP flash chromatography was also attempted on the first pink band of the omega red polar extract, with very similar results to above. The population showing the second highest level of betacyanins, in the work by collaborators at CSU was omega gold. Thus, the same process was attempted with the brightest pink fractions from omega gold. This showed even weaker peaks in the $^{1}$H NMR diagnostic of betacyanins (Supplementary 10-11).

At this point due to the poor yield of suspected betacyanins and time allocated for this MRes study the decision was made to move onto the oleraceins, which from the RP-HPLC and ESI-MS experiments appeared to be in far higher concentrations.

### 2.3.4 Further purification and isolation of oleraceins

Analysis of the polar extract of omega gold had shown it to likely possess the most significant amount of oleracein X. Based upon the results of the small-scale size exclusion chromatography experiment, fractions from the second yellow/orange band (Q58-Q71) were analysed by RP-HPLC-ESI-MS (negative ESI-MS) searching for an MS peak at *m/z* 680. Four fractions showed the presence of this pseudo molecular ion. These four fractions also showed in negative ESI-MS two other peaks corresponding to potential oleraceins at *m/z* 664 (oleracein C or H) and *m/z* 694 (oleracein D or I). At CSU all four of these oleraceins had been tentatively identified in omega gold *via* MS/MS. The peak intensity at *m/z* 664 was larger than the other two by TIC and HPLC trace. Each of these suspected oleraceins showed absorbance at 320 nm, consistent with the presence of a cinnamoyl moiety. The $^{1}$H NMR spectra also showed evidence for the presence of oleraceins in these four samples, with peaks characteristic of the cyclodopa and cinnamoyl moieties in the aromatic region including peaks between δ 8.0 and δ 8.4 ppm corresponding to C-7 of the cyclodopa, and doublets between δ 6.6 ppm and δ 7.6 ppm with coupling constants of 6.8 Hz and 9.0 Hz corresponding to aromatic protons of the *p*-coumaroyl, caffeoyl and feruloyl moieties[64, 98]. Any glucosyl moieties were obscured by signals from what appeared to be other sugars. Only one of the two characteristic *trans* doublets coming from C-2' and C-3' of the dominant oleracein's cinnamoyl derivative was visible[64]. There were also a variety of other small unidentified impurities and what appeared to be grease contamination (δ 0.5 ppm to δ 1.5 ppm).

Further review of the literature revealed that cinnamoyl derivatives can have one of their *trans* peaks concealed by a doublet produced by the aromatic part of the residue[98]. This problem had been observed in 1D experiments utilising deuterated water, where the two doublets overlapped at δ ~7.5 ppm and may have explained the missing *trans* signal encounter in earlier 1D NMR analysis of these fractions[98]. There was an asymmetrical triplet at 7.55 ppm, which could have been two overlapping doublets with different *J* values. To overcome this potential problem the $^{1}$H NMR experiment utilised deuterated DMSO, which in the literature did not cause the concealment of this *trans* signal in any known oleracein[64]. This successfully revealed the second *trans* splitting signal from what was now suspected to be a *p*-coumaroyl moiety at δ 7.57 ppm (Fig. 29). The shift of this

*trans* doublet was in agreement with the tentative assignment of the main oleracein in this set of fractions being either oleracein C or H based on the MS peak of *m/z* 664.

Importantly, there was also a small *trans* doublet at δ 6.34 ppm. This was too far upfield to be the result of a *p*-coumaroyl or feruloyl moiety, but was in the correct range to be the C-2 doublet of a caffeoyl moiety in deuterated DMSO(Fig. 28)[64, 98]. More promising still, this doublet did not match that produced by either of the oleraceins already known to contain caffeoyl moieties (attached to disaccharides)[64].

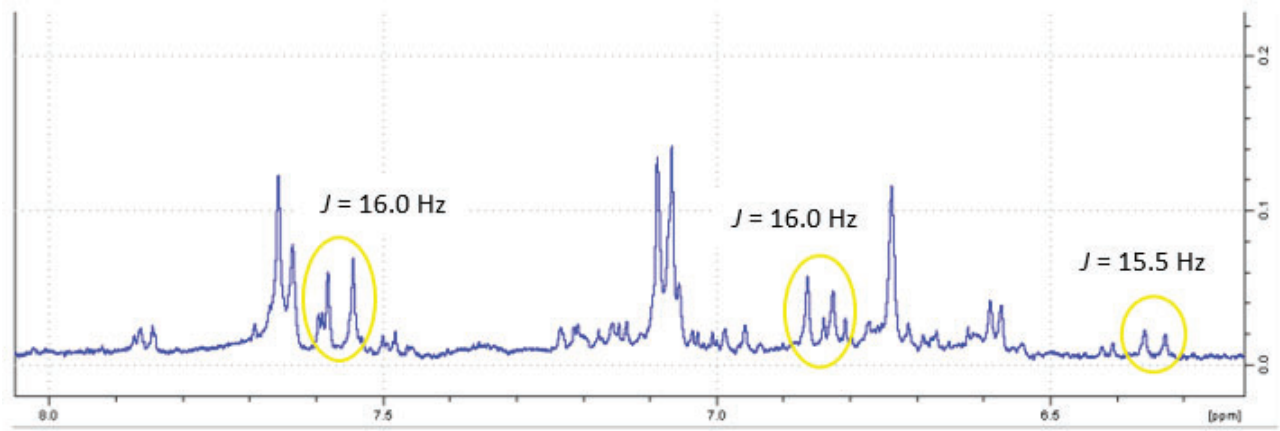



Figure 28: $^{1}$H NMR of omega gold fraction Q64 in deuterated DMSO, aromatic region (δ 6.2 to 8.1 ppm), showing key *trans* doublets at δ 7.57, δ 6.85 and 6.34 ppm

The aromatic region in the $^{1}$H NMR and the three mass peaks suggested these fractions were likely a mixture dominated by an oleracein with a mass of 665 amu and several other oleraceins in much lower concentrations, one of which was the main target, oleraceins T. C18 RP thin layer chromatography (TLC) experiments with mobile phases ranging from 100% DI water to 100% acetonitrile were run at 5% intervals on each of the four fractions containing the substance with *m/z* 680. These showed that between 5% acetonitrile and 15% acetonitrile what was otherwise a single yellow spot (likely oleraceins) in each of the fractions could be resolved into two, one large and a smaller trailing band shortly behind [64]. Visualisation with UV light showed a third spot, which based on its position was far more polar than the two yellow/orange dots and was likely the suspected sugar (s). As the TLC, RP-HPLC-ESI-MS and the $^{1}$H NMR had shown four fractions (Q63-Q66) to be similar they were pooled, and small portion of the pooled sample was analysed *via* $^{1}$H NMR and RP-HPLC-ESI-MS. Both these analyses showing similar results to the original four unpooled fractions.

The pooled samples were subjected to repeated C18 RP flash chromatography in an acetonitrile water system, with a gradient from 100% water to 80% water. At each stage of this purification samples were subjected to further analysis by $^{1}$H NMR and RP-HPLC-ESI-MS. The NMR data showed

a progression from a mixture of oleraceins and large quantities of contaminants to one putative oleracein (compound 1) purified to a point where structural elucidation could be achieved (Fig. 29). Another putative oleracein was also approaching the point of structural elucidation (compound 2).

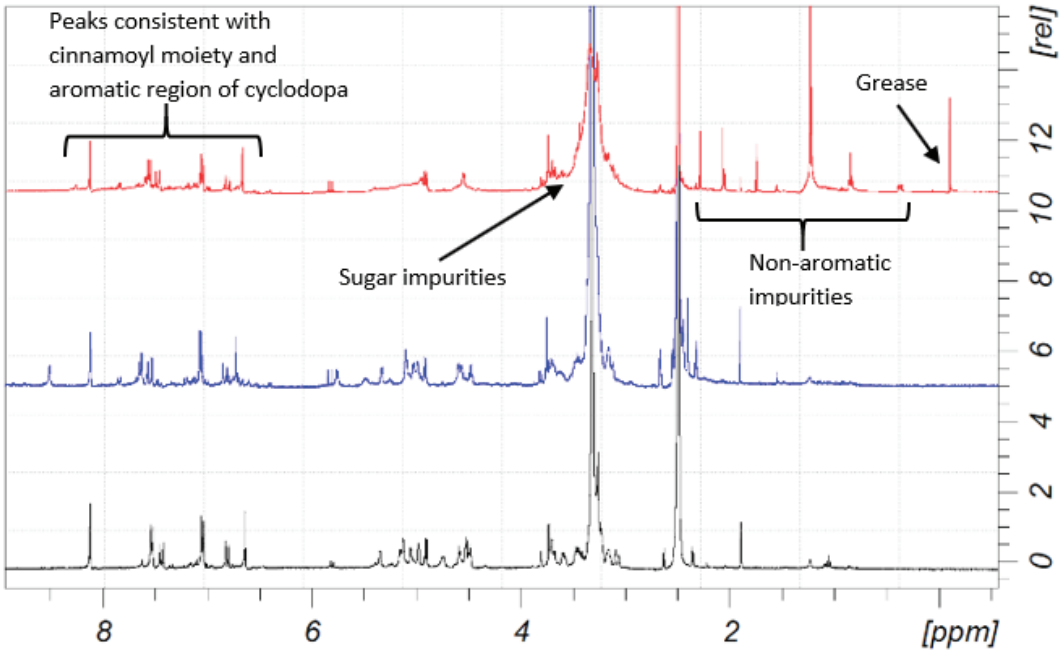



Figure 29: $^{1}$H NMR of compound 1 from omega gold, initial complex mixture (top), partially purified by RP Biotage (middle) and pure compound (bottom)

The above procedure was also used in an attempt to purify oleraceins from common purslane, which had also shown evidence of oleracains includign the possible novel oleracein X. As in the case of omega gold, the oleracein in highest quantity was of *m/z* 664 and was the only one recovered in large enough quantities for structural elucidation. Unlike, omega gold the oleracein with *m/z* of 994 was a much smaller fraction of the total oleraceins in the fractions selected for purification. As such no data other that the pseudo molecular mass were collected on this compound. 2.4 Structural elucidation

### 2.4.1 Structural elucidation of compound 1 (tentatively identified as oleracein C):

Compound 1 (4.6 mg) was obtained as an off-white solid. ESI-MS in negative mode showed a pseudo molecular ion at *m/z* 664, suggesting that this was either oleracein C or oleracein H, each having a molecular mass of 665.2 amu (Fig. 30)[64, 65]. No fragmentation was seen to assist in structural elucidation; however, the studies from CSU showed fragmentations that suggested that both of these molecules were present in each of the three populations. The elution profile of compound 1 was very similar to those of oleraceins C and H in the studies conducted at CSU, having similar elution times and the impure sample being flanked by the same pseudo molecular ions. In each of the *P. oleracea* populations collaborators at CSU saw two pseudo molecular ions at m/z 664, one

which fragmented losing a disaccharide (oleracein H) and the other which lost one glucosyl at a time (oleracein C).

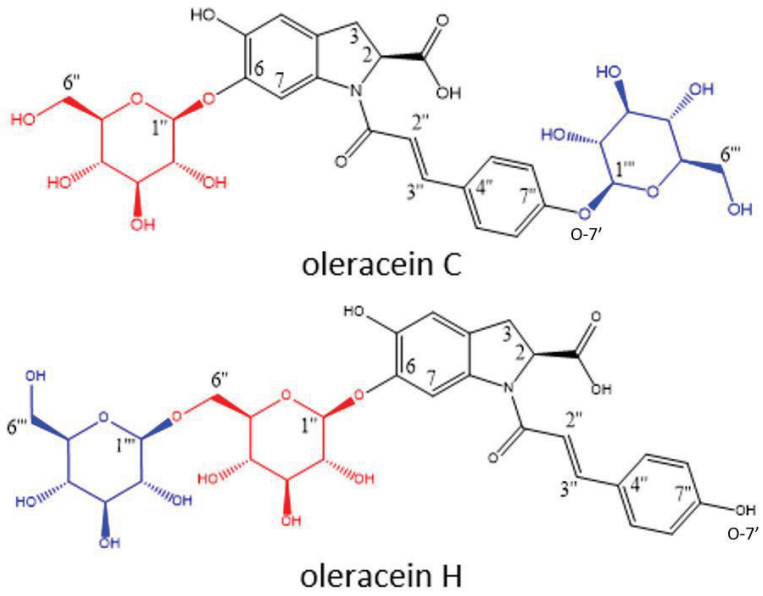



Figure 30: The two oleraceins with pseudo molecular ions of m/z 664 $[M-H]^-$, glucosyl 1 (red) and glucosyl 2 (blue)

Compound 1 was further analysed by one-dimensional (1D) and two-dimensional (2D) NMR experiments [correlation spectroscopy ($^1$H-$^1$H) (COSY), heteronuclear single bond correlation (HSQC), heteronuclear multiple bond quantum correlation (HMBC) and rotating frame Overhauser effect spectroscopy (ROESY)], using a 500 MHz spectrometer (unlike the tracking at 400 MHz) to enhance resolution (Supplementary 12-17) . As during the isolation process, it had been shown that deuterated water as an NMR solvent caused overlap of the diagnostic C-3' *trans* doublet signals for *p*-coumaroyl moieties. Thus, deuterated DMSO was used for all these NMR experiments. A summary of the results is presented in Supplementary 18.

Oleracein C and H have both been studied in detail using NMR spectroscopy[64, 65]. As shown in Fig. 30, oleracein C has one glucosyl group attached to the cyclodopa moiety at *O*-6 and the other to the *p*-coumaroyl moiety at *O*-7', while oleracein H differs in that both the glucosyl moieties are connected as a disaccharide and to cyclodopa at *O*-6 (Fig. 30). The most striking differences in the NMR spectra for these compounds have been reported to be a more downfield shift of the anomeric proton attached to *O*-7 in oleracein C compared to that of the second glucosyl in the disaccharide of oleracein H[64, 65]. The H-6'' and H-6''' protons likewise have substantially different environments for both oleraceins, and consequently different chemical shifts.

Analysis of the literature indicated that compound 1 closely matched oleracein C[65]. Compound 1 showed all the diagnostic features. *Trans* coupled doublets at δ 6.81 ppm and 7.48 ppm (*J* = 15.5 Hz) were consistent with the *p*-coumaroyl moiety (Fig. 31)[64, 65]. HMBC revealed that these protons were also both coupled with an aromatic carbon with a shift of δ 128.4 ppm (C-4'). The proton at δ 7.48 ppm had far stronger coupling to δ 128.4 ppm, suggesting it was H-3'. The signal at δ 128.4 ppm was in turn coupled to an AA'BB' *para*-disubstituted benzene ring with shifts of δ 7.56 ppm (d, *J* = 8.7 Hz, C-5'/C-9') and 7.05 ppm (d, *J* = 8.7 Hz, C-6'/C-9'). These values were in close agreement with the *p*-coumaroyl group of oleracein C. Oleracein H has no glucosyl moiety attached to its *p*-coumaroyl moiety, consequently its C-6'/C-8' doublet is shifted upfield to δ 6.74 ppm. The C-6'/C-8' doublet produced by compound 1 was at δ 7.05 ppm, and thus could not be oleracein H (Supplementary 18)[64, 65]. There was also a C-7' at δ 157.8 ppm. Though this was too close to the values of the C-7's of both oleracein C and H to be differentiated, its HMBC interaction with an anomeric hydrogen (H-1''') at δ 4.91 ppm was the strongest indication that this was oleracein C. Consequently compound 1 could not be oleracein H. This is because oleracein H has no $^{1}$H NMR peaks in this range and no glycosyl bound to the *p*-coumaroyl[65]. This interaction between the *p*-coumaroyl moiety and the downfield glucosyl was further evidenced by ROESY interactions between the two (Fig. 31). Though this ROESY correlation was not listed in the literature for either oleracein C or H, it was expected for oleracein C. Additionally, oleracein H showed HMBC correlations between the two glucosyl groups in its disaccharide moiety, which were not observed in the HMBC spectra of compound 1[65].

The above data were all in close agreement with compound 1 being oleracein C. There were, however, inconsistencies in the comparison between the NMR spectra of compound 1 and the literature values for oleracein C. The first was the signal produced by H-2 in the cyclodopa moiety. The literature for oleracein C showed this to be a doublet at δ 5.39 ppm (*J* = 9.0 Hz), whereas compound 1 had a broad singlet at δ 4.99 ppm[65]. All COSY and HMBC correlations were in agreement with this assignment. The second difference was the doublet splitting for H-1''. While having the same chemical shift, the literature had a coupling constant of *J* = 4.5 Hz, but this study found *J* = 7.1 Hz. As the glucosyl groups in oleracein C are in the β–conformation, a value of 7.1 Hz is within the expected range[65, 97]. There was no difference in solvent used in this study when compared to the literature[65].

The compound purified from common purslane was subjected to the same suite of NMR experiments and revealed near identical spectra, suggesting that this was also oleracein C. TLC and LC-MS data were also consistent.

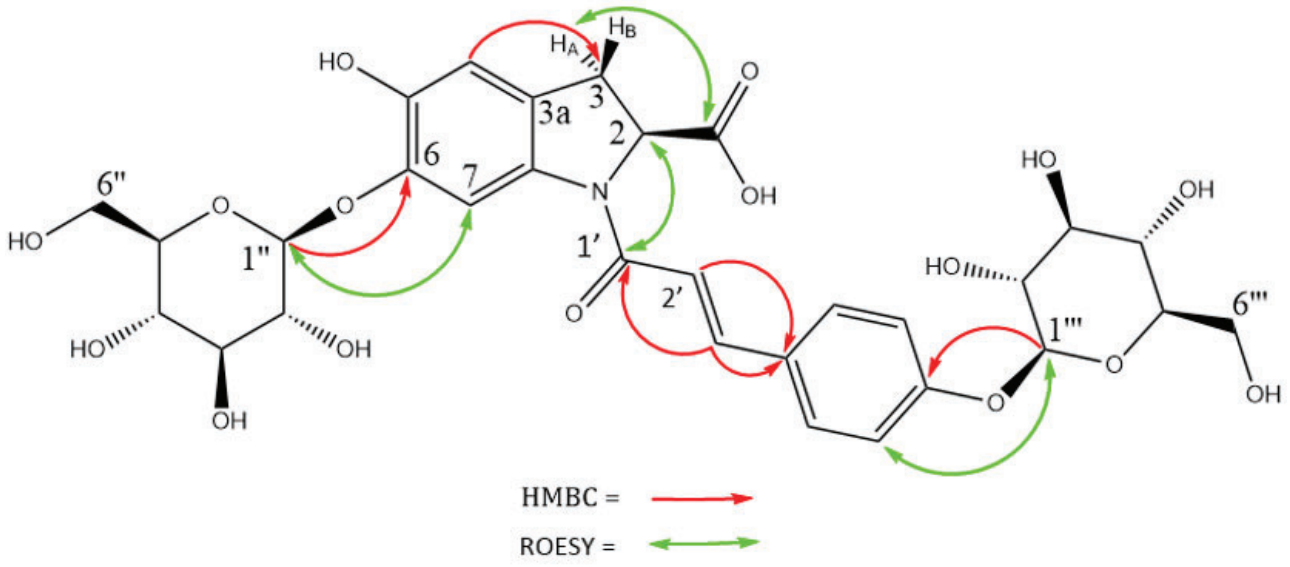



Figure 31: Structure of oleracein C, showing key positions and correlations

2.4.2 Structural elucidation of compound 2 (tentatively identified as oleracein D):

In the purification of compound 1 from omega gold, a second oleracein was partially purified. Although it was still clearly a mixture with compound 1, ESI-MS in negative mode showed a pseudo molecular ion at *m/z* 694, consistent with the molecular mass (695.2 amu) of oleracein D and oleracein I (Fig. 32)[64, 65]. As was the case with compound 1, there was no fragmentation data available for compound 2 from the MRes studies; however, MS/MS data from CSU suggested both oleracein D and oleracein I were in all three *P. oleracea* populations[71]. This was based on fragmentation data that showed two pseudo molecular ions with *m/z* of 994 in each population sample, one of which fragmented losing a disaccharide (oleracein I) and the other which lost its glucosyl groups one at a time (oleracein D)[71].

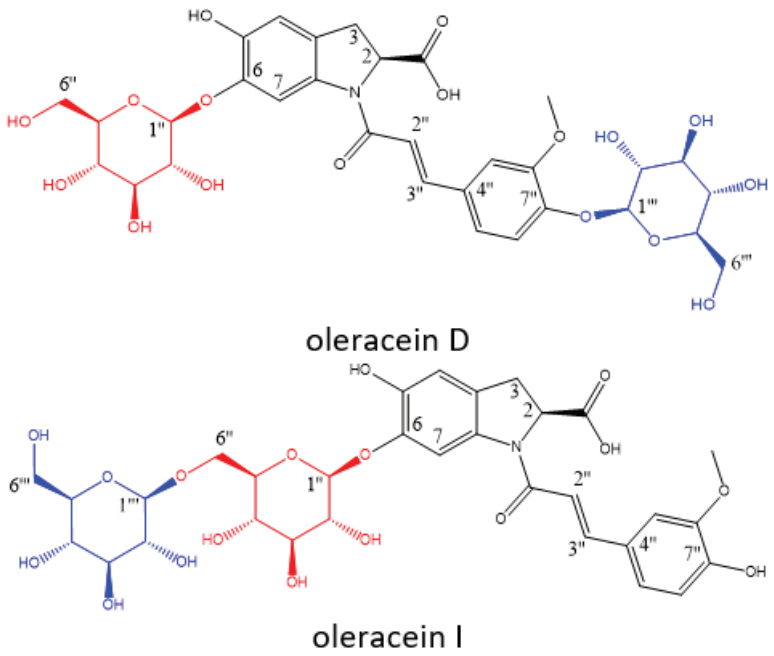



Figure 32: The two oleraceins with pseudo molecular ions of *m/z* 694 $[M-H]^-$, glucosyl 1 (red) and glucosyl 2 (blue)

Compound 2 was further analysed by 1D and 2D NMR experiments, using a 500 MHZ spectrometer and with deuterated DMSO as solvent. Along with signals for compound 1, there were several signals consistent with oleracein D and oleracein I, which both contain a feruloyl moiety[65]. Both of these oleraceins have detailed published NMR data, and comparison of the spectra of compound 2 with these data indicated that compound 2 closely matched oleracein D[64, 65]. However, due to overlapping signals with compound 1, not all diagnostic features could be observed (Fig. 33). What initially suggested that there was an oleracein with a feruloyl moiety in this sample was the presence of a methoxy singlet at δ 3.74 ppm in the $^1$H NMR experiment that showed coupling to an aromatic carbon in the HMBC experiment at δ 148.1 ppm (Supplementary 19). This closely matched the literature value of C-6' in oleracein D (δ 149.7 ppm) and oleracein I (δ 148.2 ppm)[64, 65]. This carbon was shown to also have an HMBC correlation with the anomeric proton of a glycosyl group, ruling out oleracein I as a possibility (Fig. 34)[64]. Additionally, the data for H-8' (d, δ 7.05 ppm (*J* = 7.4 Hz)) was in close agreement with literature values for oleracein D H-8' (d, δ 7.08 ppm (*J* = 7.5 Hz)), but was substantially different to the published values for oleracein I H-8' (d, δ 6.79 ppm (*J* = 7.2 Hz))[64, 65]. Each of these signals (C-6', C-7' and C-8') correlated in the HMBC experiment with H-9' (d, δ 7.19 ppm (7.4 Hz)), which also closely matched the published data for oleracein D (d, δ 7.15 ppm (7.5). H-5', H-8' and H-9' all correlated with another aromatic carbon in the HMBC experiment at δ 128.0

pm, which was assigned as C-4’ due to these correlations and its closeness to the literature value for C-4’ of oleracein D, δ 128.0 ppm.

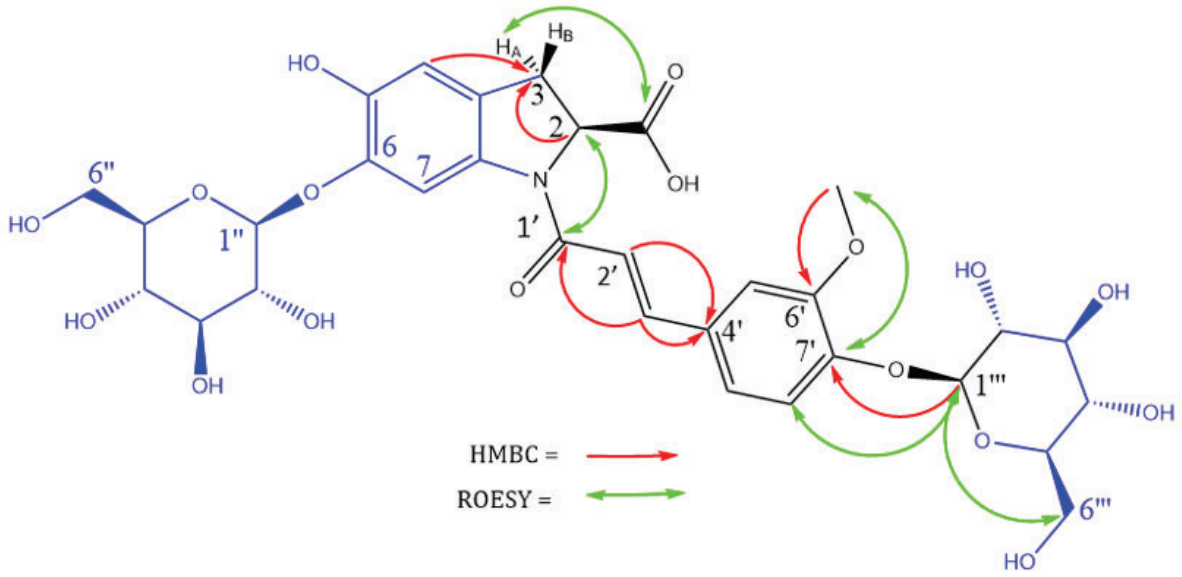



Figure 33: Structure of oleracein D, showing key positions and correlations, section in blue shows area not structurally elucidated by NMR

In purifying compound 1 from common purslane, a semi-pure compound with a pseudo molecular ion of *m/z* of 694 was found. This was subjected to the same suite of NMR experiments and revealed near identical spectra. This suggested that this was also oleracein D, though as it was for the compound from omega gold isolation and further structural elucidation would be required for this to be confirmed.

# Chapter 3: Experimental

## 3.1 Plant material

Omega gold and omega red *Portulaca oleracea* were grown in Eurongilly (-34.943724, 147.795722) over February-March 2019, New South Wales (NSW), Australia, by Colette Geier from samples purchased from Kapitany Enterprises, Melbourne. Common purslane was gathered in Eurongilly (-34.943724, 147.795722), NSW, Australia by Colette Geier. Whole plant material (leaves, stems, roots, seeds and buds) was collected at maturity, freeze-dried (Christ® Alpha 2-4 LDplus Freeze Drier) for 5 days at -76 °C and 0.0010 mbar, and stored in a freezer at -20 °C. Plant materials were shipped overnight and unrefrigerated from CSU, Wagga Wagga, to Macquarie University, Macquarie Park, NSW. On arrival they were immediately placed in a freezer at -20 °C.

## 3.2 Reagents and equipment

All solvents used for extraction, size exclusion chromatography, flash chromatography and HPLC-MS were HPLC grade and were used without further purification. Ethanol was supplied by Chem-Supply, Australia. 2-Propanol, ethyl acetate and *n*-hexane were manufactured by Sigma-Aldrich, USA. Acetonitrile, acetone, chloroform and dichloromethane were supplied by Merck, Germany. Water used was purified by a Direct-Q 5UV water purification system manufactured by Millipore, USA. Deuterated chloroform, deuterated dimethyl sulfoxide (DMSO), deuterated water and deuterated methanol were manufactured by Cambridge Isotope Laboratories, USA. Reversed phase thin layer chromatography plates (fluorescent, silica gel 60 RP-18 $F_{254}$s) were manufactured by Merck, Germany, and the plates were visualised using UV light of 254 nm. Size exclusion chromatography was performed using Sephadex LH-20 packing material manufactured by Sigma-Aldrich, USA. Reversed phase flash chromatography was performed using a Biotage Isolera Four system manufactured by Biotage, Sweden. 10 g Biotage cartridges and 1 g Biotage samplets (loading columns) were packed with LiChroprep® RP-18 (40-63 µm) manufactured by Merck, Germany. The freeze-dryer system was manufactured by Labconco, USA. Plant material was ground using a Blend-X Pro 1400 W blender Kenwood, Thailand. Plant extracts were filtered using grade 1 filter paper manufactured by Advantec, USA. Organic solvents were removed using a Buchi rotary evaporator.

$^{1}$H NMR spectra were recorded on a 400‘54 Ascend NMR spectrometer (Bruker, USA) operating at 400.2 MHz or a 500‘54 Ascend NMR spectrometer (Bruker, USA) operating at 500.2 MHz, using standard Bruker pulse sequences. $^{13}$C and 2D NMR spectra were recorded on the 500‘54 Ascend NMR spectrometer operating at 125.7 MHz for $^{13}$C and using standard Bruker pulse sequences. Chemical shifts were assigned relative to $H_2O$ ($^{1}$H δ 4.79 ppm), DMSO ($^{1}$H δ 2.50 ppm and $^{13}$C δ 39.52

ppm), methanol ($^{1}$H δ 3.31 ppm and C$^{13}$ δ 49.00 ppm) and chloroform ($^{1}$H δ 7.26 ppm and $^{13}$C δ 77.16 ppm).

RP-HPLC-ESI-MS analysis was conducted on a 1260 liquid chromatography system connected to a 6130 Series Quadrupole LC/MS System, Agilent, USA. The column used for RP-HPLC-ESI-MS analysis was an InfinityLab Poroshell 120 SB-C18 2.7 µm 2.1 x 100 mm, Agilent, USA.

### 3.3 RP-HPLC-ESI-MS

For RP-HPLC-ESI-MS experiments crude plant extracts were diluted to 5.0 mg.mL$^{-1}$ and filtered with nylon syringe filters (0.22 µm).

HPLC was run on a gradient from 10% solvent B to 100% solvent B over 20 min, with a further 8 minutes for equilibration per run. Solvent A was DI water with 0.1% (v/v) formic acid, solvent B was 100% acetonitrile with 0.1% (v/v) formic acid. The flow rate was 0.3 mL.min$^{-1}$, with an injection volume of 10 µl. The needle wash was 3 seconds with 2-propanol. The temperature was set to 20 °C. UV-visible chromatograms were recorded at 210, 254, 280, 320, 380, 480 and 540 nm *via* DAD with a spectral range was 190 to 600 nm with a step of 2 nm**.** ESI-MS was run with a fragmentor voltage of 70 V and nozzle voltage of 800 V. The system was run in both positive and negative modes, scanning from *m/z* of 100 to *m/z* of 1200.

### 3.4 Data acquisition and analysis

ChemStation (1.3.4) software (Agilent, USA) was to run all RP-HPLC-ESI-MS experiments and analysis of all DAD and MS data.

Topspin (4.0.7) software (Bruker, USA) was used for analysis of all NMR data.

### 3.5 TLC experiments

Reversed phase TLC plates were used to analyse extracts, fractions and semi-pure compounds. Experiments were conducted on 100 mm long plates. Water and acetonitrile were used as the mobile phase, and the experiments were conducted in 5% increments from 100% water to 100% acetonitrile, each solvent system being trialled with fresh sample on fresh plates. This method determined the ideal solvent ratio for RP separation and analysis of each extract, fraction and semi-pure compound. Plates were visualised under UV light at 254 nm.

### 3.6 Extraction

#### 3.6.1 Omega red

28.1 g of freeze-dried omega red whole plant material (leaves, stems, roots, seeds and buds) was ground in a blender (~1200 rpm for 60 s) to a fine pink powder. Next it was suspended in 290 mL of

a 50% aqueous ethanol solution. The solution was agitated regularly by swirling and after 15 minutes it was vacuum filtered. The filtrate was collected, and the same extraction procedure was repeated on the plant material another two times, pooling the filtrate each time. The filtrate was then placed in a separatory funnel and washed with *n*-hexane (3 x 300 mL) over ~15min. The filtrate (polar extract) was frozen in liquid nitrogen and freeze-dried at -50 °C and 0.15 mbar protected from UV light with aluminium foil. This yielded 6.7 g (*w/w* 23.8%) of a purple red semi-crystalline solid with a small amount of what appeared to be a white salt sitting at the base of the container. This was stored under nitrogen in a freezer at -20 °C. The *n*-hexane washes were pooled, concentrated on a rotary evaporator at 25 °C and dried under high vacuum at ~0.3 mbar to yield 1.0 g (*w/w* 3.6%) of a dark green sticky oil, which was then stored in a freezer at -20 °C where it became waxy.

### 3.6.2 Omega gold

The above procedure was repeated on 28.4 g of freeze-dried omega gold whole plant material (leaves, stems, roots, seeds and buds) with no modifications. This yielded 7.5 g (*w/w* 26.4%) of the polar extract and 0.8 g (*w/w* 2.8%) of the *n*-hexane extract. The polar extract was a semi-crystalline solid; red with orange hues. The *n*-hexane extract was a medium yellow/green sticky oil, which became waxy upon freezing.

### 3.6.3 Common purslane

The above procedure was repeated on 30.1 g of freeze-dried common purslane whole plant material (leaves, stems, roots, seeds and buds). The modifications were that 300 mL of *n*-hexane was used for each wash and 2 additional washes were carried out. This yielded 7.6 g (*w/w* 25.2%) of the polar extract and 1.4 g (*w/w* 4.7%) of the *n*-hexane extract. The polar extract was a semi-crystalline red solid. The *n*-hexane extract was a medium green sticky oil, which became waxy upon freezing.

## 3.7 Size exclusion chromatography

### 3.7.1 Column preparation

A 400 x 10 mm LH-20 Sephadex column was used to separate the polar extracts for the ‘small-scale’ work. The column was prepared by soaking Sephadex LH-20 in excess DI water. The column was then loaded, and 500 mL of DI water was run through the column prior to addition of the plant material.

A 580 x 40 mm LH-20 Sephadex column was used to separate the polar extracts for the ‘large scale’ work. The column was prepared by soaking Sephadex LH-20 in excess DI water. The column was then loaded, and 5 L of DI water was run through the column prior to addition of the plant material.

### 3.7.2 Omega red

35 mg of the polar extract of *P. oleracea* omega red was loaded onto the small-scale column. Fractions were collected as follows, for the first 30 minutes all eluate was collected in a single fraction. 5 mL fractions were then collected until a total of 50 fractions had been collected. After collection of each fraction, they were frozen in liquid nitrogen and stored in a freezer at -20 °C. Once all fractions from a single run had been collected, they were placed on a freeze-dryer at -50 °C and 0.15 mbar.

1.10 g of the polar extract of *P. oleracea* omega red was loaded onto the column. Fractions were collected as follows, for the first 45 minutes all eluate was collected in a single fraction of ~170 mL. 10 mL fractions were then collected until a total of 80 fractions had been collected. A further fraction of ~200 mL was then collected. After collection of each fraction, they were frozen in liquid nitrogen and stored in a freezer at -20 °C. Once all fractions from a single run had been collected, they were placed on a freeze-dryer at -50 °C and 0.15 mbar.

Fractions from the size exclusion chromatography were labelled R0 (the initial 170 mL fraction), R1-R80 (the 10 mL fractions) and R81 (the final 200 mL fraction).

Fractions R19-R40 were pink, fractions R41-R51 were orange, fractions R52-R60 were red and fractions R61-R74 were yellow/tan.

### 3.7.3 Omega gold

The procedure above was repeated for the omega gold extract, with the only modification being that 1.02 g of extract was used for the full-scale separation.

Fractions from the size exclusion chromatography were labelled Q0 (the initial 170 mL fraction), Q1-Q80 (the 10 mL fractions) and Q81 (the final 200 mL fraction).

Fractions Q21-Q27 and Q31-Q38 were pink, fractions Q41-Q51 were orange/red, fractions Q52 to Q57 were red and fractions Q59-Q77 were orange/tan.

### 3.7.4 Common purslane

This procedure was repeated as above for the common purslane extract, with the only modification being that 0.81 g of extract was used for the full scale process.

Fractions from the size exclusion chromatography were labelled P0 (the initial 170 mL fraction), P1-P80 (the 10 mL fractions), P81 (the final 200 mL fraction).

Fractions P23-P28 and P32-P37 were pink, fractions P40-P45 were orange, fractions P50-P57 were red and fractions P60-P74 were orange/tan.

## 3.8 Further fractionation and isolation

Each fraction from the size exclusion chromatographies were frozen in liquid nitrogen and water was removed by freeze-drying at -50 °C and ~0.15 mbar. Finally, fractions were stored in the freezer under a nitrogen atmosphere at -20 °C.

$^{1}$H NMR experiments were also used to track the presence initially of aromatics and phenolics, and once samples were semi-pure to track potential target molecules. These tracking experiments were performed on a Bruker 400‘54 Ascend, at 400MHz, 64 scans, spinning with small quantities of sample dissolved in $D_2O$. A mixture of TLC, $^{1}$H NMR and RP-HPLC-ESI-MS (see Section 3.7) was used to analyse the fractions.

Flash chromatography samplets were packed with ~1 g of RP C18 (40-63 µm) packing material. Samples were dissolved in minimal DI water and were then forced onto the samplet packing material by pressurised nitrogen. The loaded samplets were then frozen at -20 °C, freeze-dried at -50 °C and ~0.15 mbar before being loaded onto the flash chromatography column.

### 3.8.1 Omega red

Fraction R24 (48.1 mg) was fractionated *via* flash chromatography running a gradient from 100% water to 70% water and 30% acetonitrile. 5 mL fractions were collected and labelled R24F1-R24F25. Fractions R24F4 and R24F5 contained of a white substance (29.5 mg). Fractions R24F7-R24F10 contained an impure unknown compound with characteristic betacyanin features, and so was labelled as betacyanin X (2.0 mg).

Fractions R31-R38 were the darkest pink fractions, with fraction R32 (50.2 mg) being the darkest of these. A portion (50 mg) of fraction R32 was fractionated *via* flash chromatography running a gradient from 100% water to 70% water and 30% acetonitrile. 5 mL fractions were collected and labelled R32F1-R32F25. Fractions R24F13-R24F14 contained an impure unknown compound with characteristic betacyanin features, and so was labelled as betacyanin Y (1.9 mg) (Compound 3).

### 3.8.2 Omega gold

Fractions Q63-Q66 were combined and fractionated *via* flash chromatography running a gradient from 100% acetonitrile to 80% acetonitrile and 20% water. 10 mL fractions were collected and labelled QOF1-QOF20. Fractions QO1F2 and QOF3 were a bright yellow/orange. These fractions were combined and fractionated *via* flash chromatography running a gradient from 100% water to 80% water and 20% acetonitrile. 5 mL fractions were collected and labelled QO1F2/3F1-45. Fractions QO1F2/3F10-12 contained compound 1 (4.6 mg).

### 3.8.3 Common purslane

Fraction P36-P43 were bright pink. Fractions P41-P42 were pooled (81.5 mg). Fractions P64-P68 were combined (13.6 mg) and fractionated *via* reversed phase flash chromatography running a gradient from 100% water to 80% water and 20% acetonitrile. 10 mL fraction were collected and labelled POF1-POF20. Fractions PO1F2 and POF3 contained a yellow/orange oily substance. These fractions were combined and further fractionated *via* reversed phase flash chromatography running a gradient from 100% water to 80% water and 20% acetonitrile. 5 mL fractions were collected and labelled PO1F2/3F1-45. Fractions PO1F2/3F11-12 contained compound 1 (2.3 mg).

## 3.9 Structural elucidation

**Compound 1** (tentatively identified as oleracein C): Yellow/pale orange solid, *m/z* 664.2 [ESI-MS, M-H]$^-$, $^1$H NMR (500.2 MHz, D6-DMSO) δ 8.12 (1H, *br s*, H-7), δ 7.56 (2H, *d*, *J* 8.7 Hz, H-5' and H-9'), δ 7.48 (1H, *d*, *J* 16.0 Hz, H-3'), δ 7.05 (2H, *d*, *J* 8.7 Hz, H-6' and H-8'), δ 6.81 (1H, *d*, *J* 16.0 Hz, H-2'), δ 6.67 (1H, *s*, H-4), δ 4.98 (1H, *br s*, H-2), δ 4.91 (1H, *d*, *J* 7.5 Hz, H-1'''), δ 4.56 (1H, *d, J* 6.7, H-1''), δ 3.71 (2H, *m*, H-6'' and H-6'''), δ 3.2-3.5 (8H, *m*, H-2'', H-3'', H-4'', H-5'', H-2''', H-3''', H-4''' and H-5'''). $^{13}$C NMR (125.7 MHz, DMSO) δ 173.0 (COOH), δ 163.1 (C-1'), δ 157.8 (C-7'), δ 143.2 (C-6), δ 142.6 (C-5), δ 139.4 (C-3'), δ 135.7 (C-7a), δ 129.0 (C-5' and C-9'), δ 128.4 (C-4'), δ 125.8 (C-3a), δ 118.8 (C-2'), δ 116.2 (C-6' and C-8'), δ 111.5 (C-4), δ 108.1 (C-7), δ 103.8 (C-1''), δ 99.8 (C-1'''), δ 77.1 (C-3''), δ 76.9 (C-3'''), δ 76.8 (C-2''), δ 76.6 (C-5'''), δ 75.6 (C-5''), δ 73.3 (C-2'''), δ 70.0 (C-4''), δ 69.0 (C-4'''), δ 60.4 (C-6''), δ 60.3 (C-6''').

**Compound 2** (tentatively identified as oleracein D): Yellow/pale orange solid, *m/z* 694.6 [M-H]$^-$, $^1$H NMR (500.2 MHz, DMSO) δ 7.46 (1H, *d*, *J* 16.0 Hz, H-3'), δ 7.31 (2H, *br s*, *J* 8.7 Hz, H-5'), δ 7.19 (1H, *d*, *J* 7.4 Hz, H-9'), δ 7.05 (1H, *d*, *J* 7.4 Hz, H-8'), δ 6.81 (1H, *d*, *J* 16.0 Hz, H-2'), δ 6.78 (1H, *d*, *J* 16.0 Hz, H-2'), δ 4.98 (1H, *br s*, H-2), δ 4.91 (1H, *d*, *J* 7.5 Hz, H-1'''), δ 3.79 (2H H-6'''), δ 3.74 (3H $OCH_3$), δ 3.2-3.5 (4H, *m*, H-2''', H-3''', H-4''' and H-5'''). $^{13}$C NMR (125.7 MHz, DMSO) δ 176.1 (COOH), δ 165.8 (C-1'), δ 148.1 (C-6'), δ 147.5 (C-7'), δ 139.5 (C-3'), δ 128.0 (C-4'), δ 123.5 (C-9'), δ 118.7 (C-2'), δ 114.1 (C-8'), δ 112.3 (C-5'), δ 99.8 (C-1'''), δ 76.9 (C-3'''), δ 76.6 (C-5'''), δ 73.3 (C-2'''), δ 60.3 (C-6'''), δ 55.3 ($OCH_3$).

# Chapter 4: Conclusions and future directions

*P. oleracea* is a plant of globally significance both as a customary medicine and as a food source. Interest in this plant has been rapidly increasing and it is becoming increasingly valuable as a crop. Concordantly, a better understanding of this plant, its chemistry and its value as a food, medicine and nutritional supplement is important.

This work confirms the presence of oleraceins C and D in two of the three Australian populations of *P. oleracea* investigated. There are other compounds in these populations which merit further investigation to identify and understand the diversity between populations.

In continuing this work, the next step is to complete the structural elucidation of the oleraceins. Measurement of optical rotation through a polarimeter and/or circular dichroism will be used both to compare with published data and to determine stereochemistry of these molecules. For the current and future isolated compounds, MS/MS data will be produced to assist in the structural elucidation, using the facilities at CSU that carried out the original work to ensure consistency. Enzymatic degradation will also be considered to confirm the glucosyl unit linkages, including if they are mono or disaccharides and their alpha- or beta-anomeric linkages. Derivatisation of the sugar moieties by methylation, followed by cleavage and GCMS comparison with standards, will also assist in the complete structural elucidations[104].

Assays into the medicinal properties of these molecules will also be performed, primarily to examine antioxidant activity, but also $\alpha$-/$\beta$-glucosidase and $\alpha$-amylase inhibition assays, and antibacterial assays, following on from the customary uses and biological activities seen for some *P. oleracea* populations[16].

Further work is also needed to investigate the Australian populations of *P. oleracea* in more depth. Of interest would be the investigation of oleracein and betacyanin concentration and composition at different stages of *P. oleracea*'s life cycle and in different growing conditions, as well as looking at the variability between populations, harvest age and growing conditions, and examining further Australian populations.

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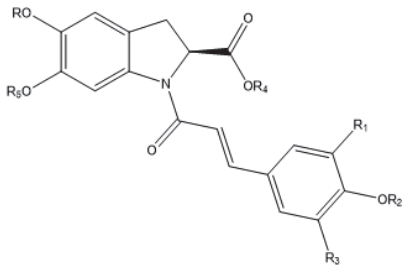
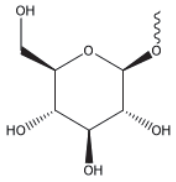
# Supplementary Material

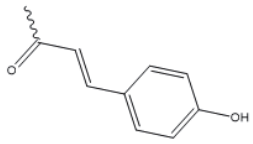
## Supplementary 1:



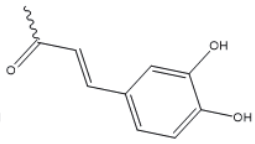
oleraceins core structure



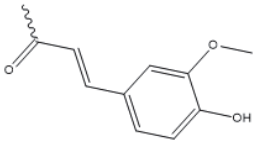
glucosyl moiety



*p*-coumaroyl moiety

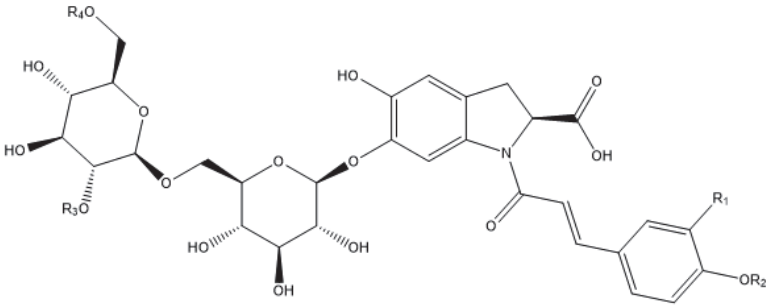


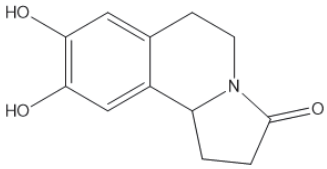
caffeoyl moiety



feruloyl moiety

| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| Oleracein A | H | H | H | glucosyl | H |
| Oleracein B | $OCH_3$ | H | H | glucosyl | H |
| Oleracein C | H | glucosyl | H | glucosyl | H |
| Oleracein D | $OCH_3$ | glucosyl | H | glucosyl | H |
| Oleracein F | $OCH_3$ | H | H | glucosyl | $CH_3$ |
| Oleracein G | H | H | H | glucosyl | $CH_3$ |
| Oleracein T* | H | H | H | *p*-coumaroyl | H |
| Oleracein U* | H | H | H | H | H |
| Oleracein V* | OH | H | OH | glucosyl | H |
| Oleracein W* | OH | H | H | H | H |



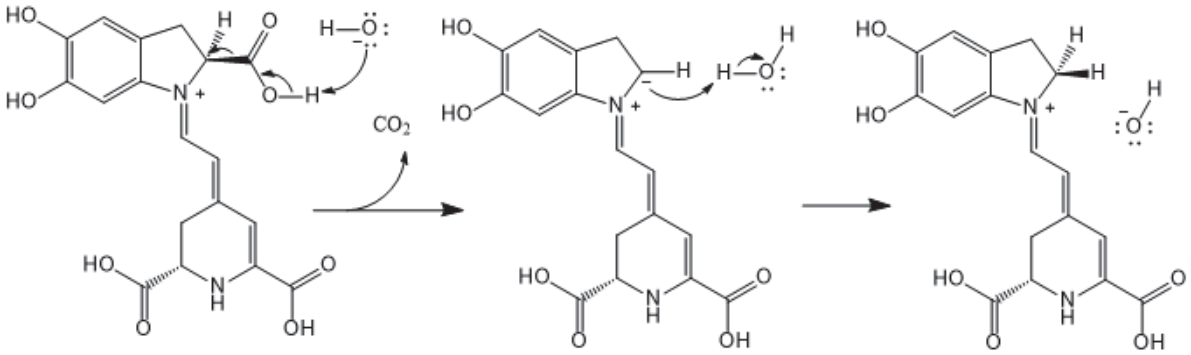


oleracein E

| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Oleracein H | H | H | H | H |
| Oleracein I | $OCH_3$ | H | H | H |
| Oleracein K | H | H | caffeoyl | H |
| Oleracein L | $OCH_3$ | H | caffeoyl | H |
| Oleracein N | H | H | feruloyl | H |
| Oleracein O | $OCH_3$ | H | feruloyl | H |
| Oleracein p | H | glucosyl | H | H |
| Oleracein Q | $OCH_3$ | glucosyl | H | H |
| Oleracein R | H | H | feruloyl | H |
| Oleracein S | H | glucosyl | H | feruloyl |
| Tentative oleracein X* | OH | H | H | H |

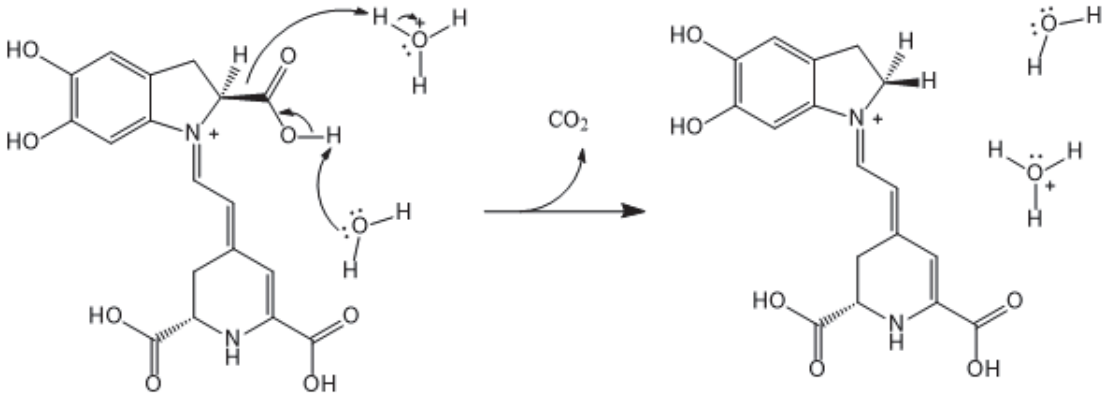
Structurally elucidated oleraceins and the tentative structure of oleracein X (* denotes structures tentatively identified from MS/MS data)[64-66, 71, 74]

Supplementary 2:



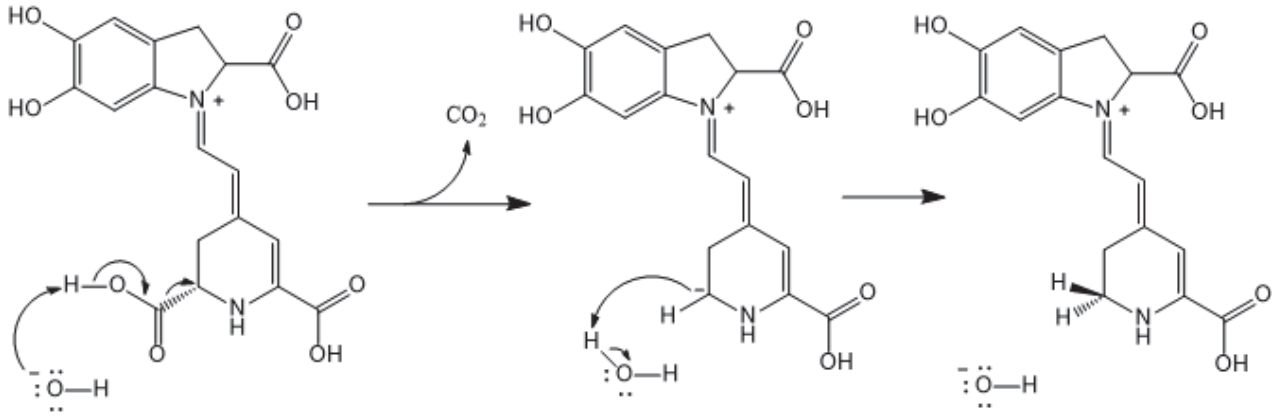
Decarboxylation of betanidin yielding 2-decarboxybetanidin in basic conditions

Supplementary 3:



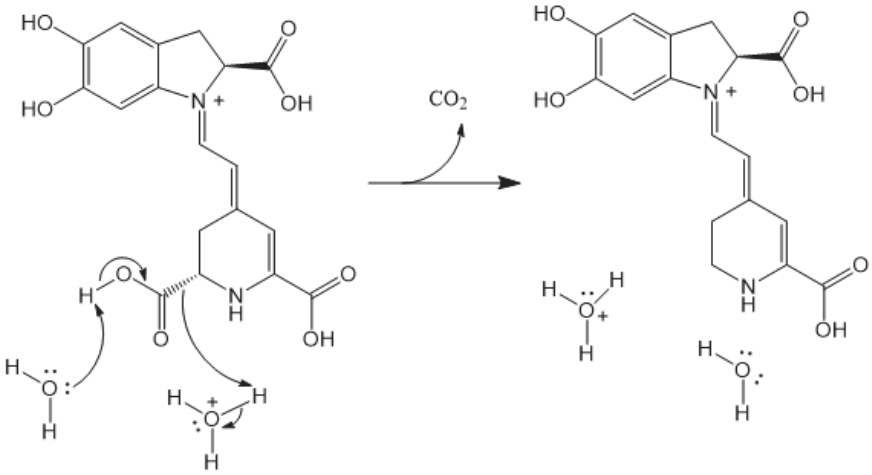
Decarboxylation of betanidin yielding 2-decarboxybetanidin in acidic conditions

Supplementary 4:



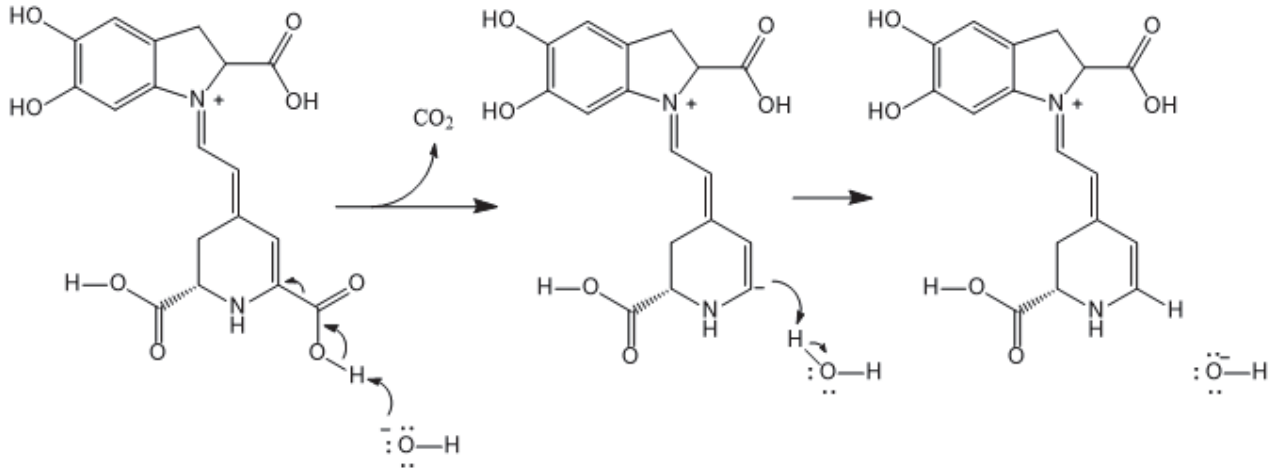
Decarboxylation of betanidin yielding 15-decarboxybetanidin in basic conditions

Supplementary 5:



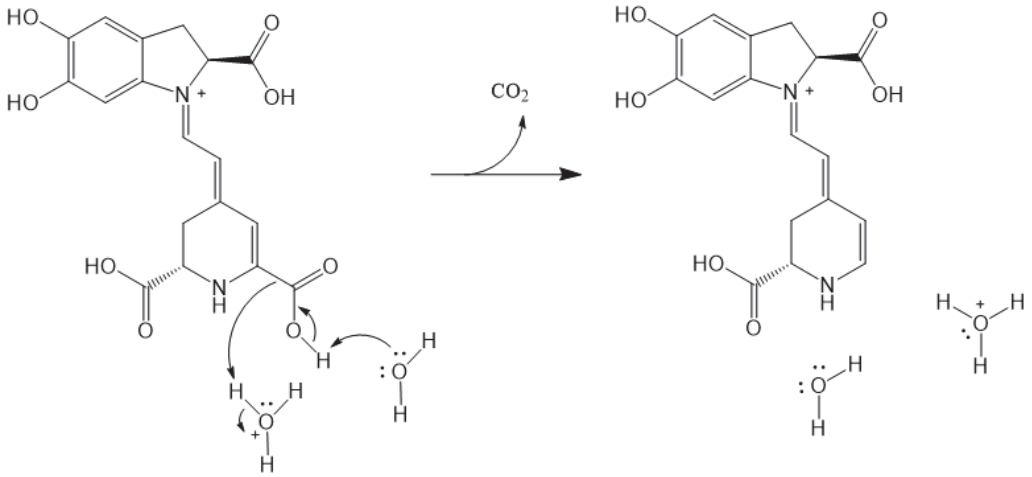

Decarboxylation of betanidin yielding 15-decarboxybetanidin in acidic conditions

Supplementary 6:



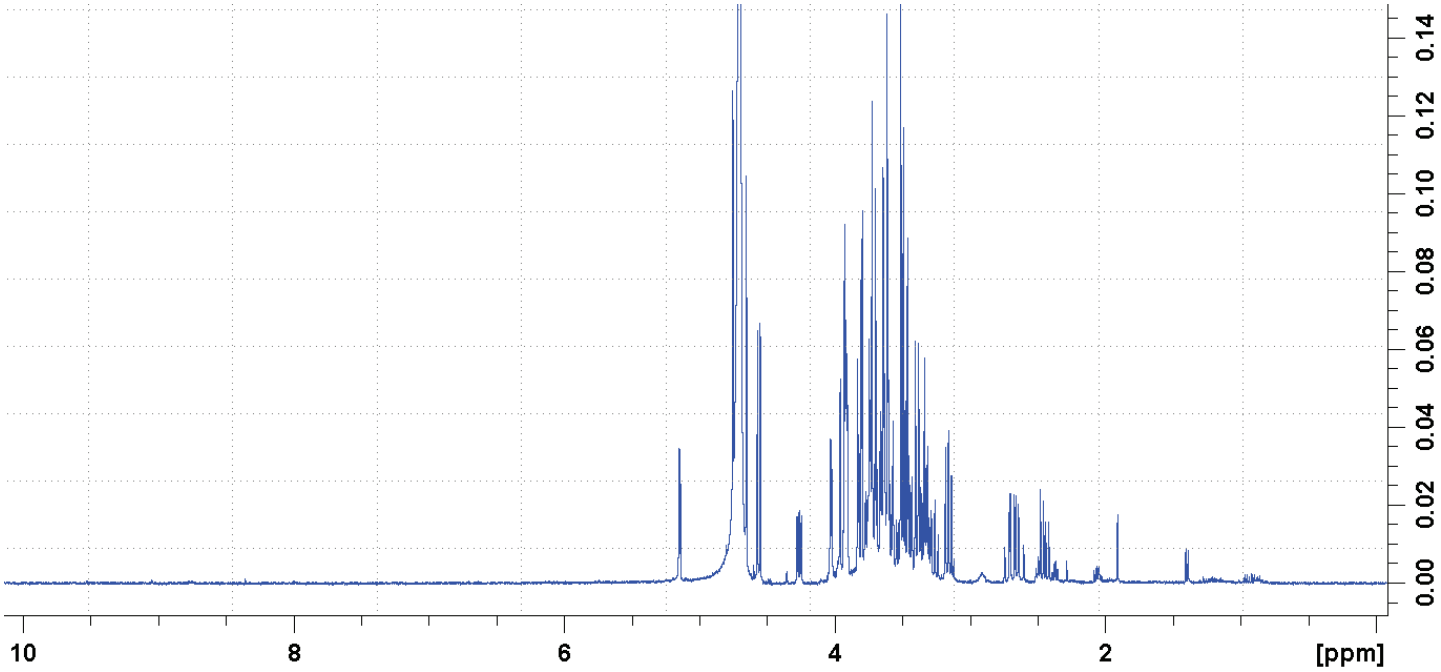

Decarboxylation of betanidin yielding 17-decarboxybetanidin in basic conditions

Supplementary 7:



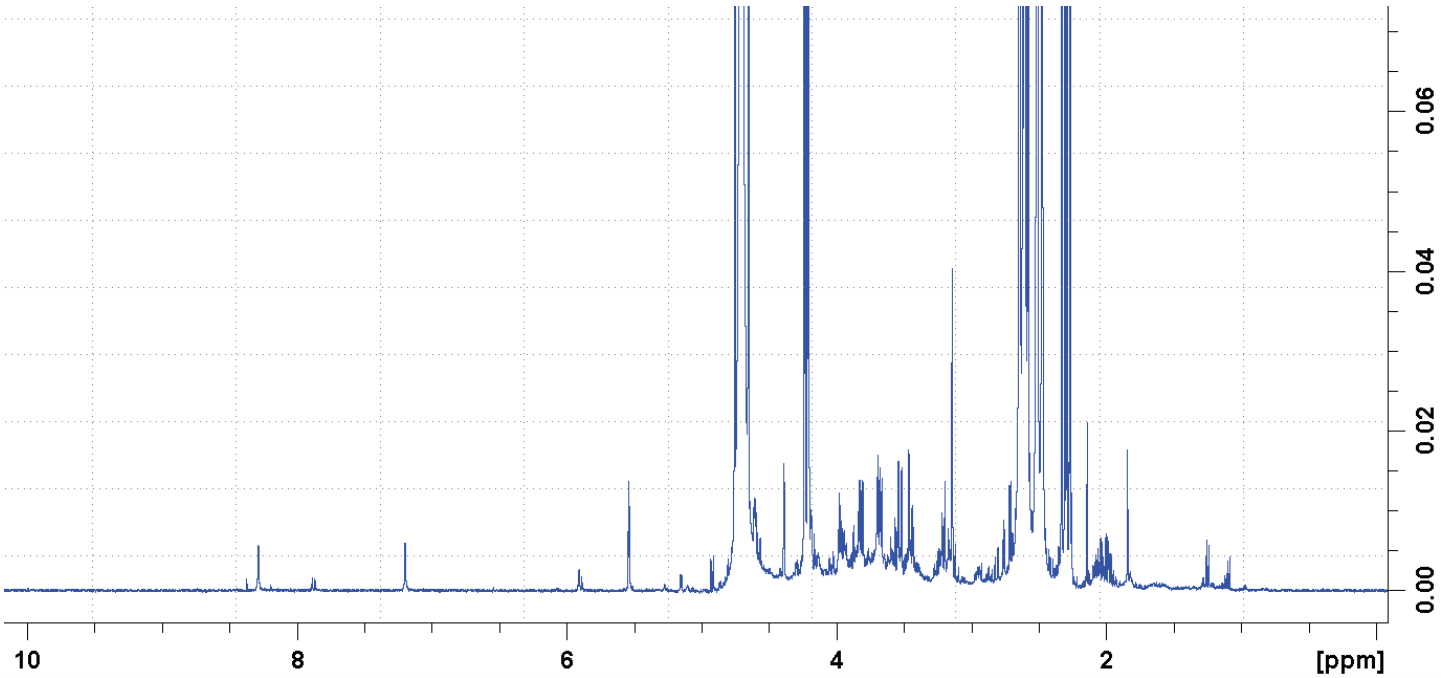

Decarboxylation of betanidin yielding 17-decarboxybetanidin in acidic conditions

Supplementary 8:



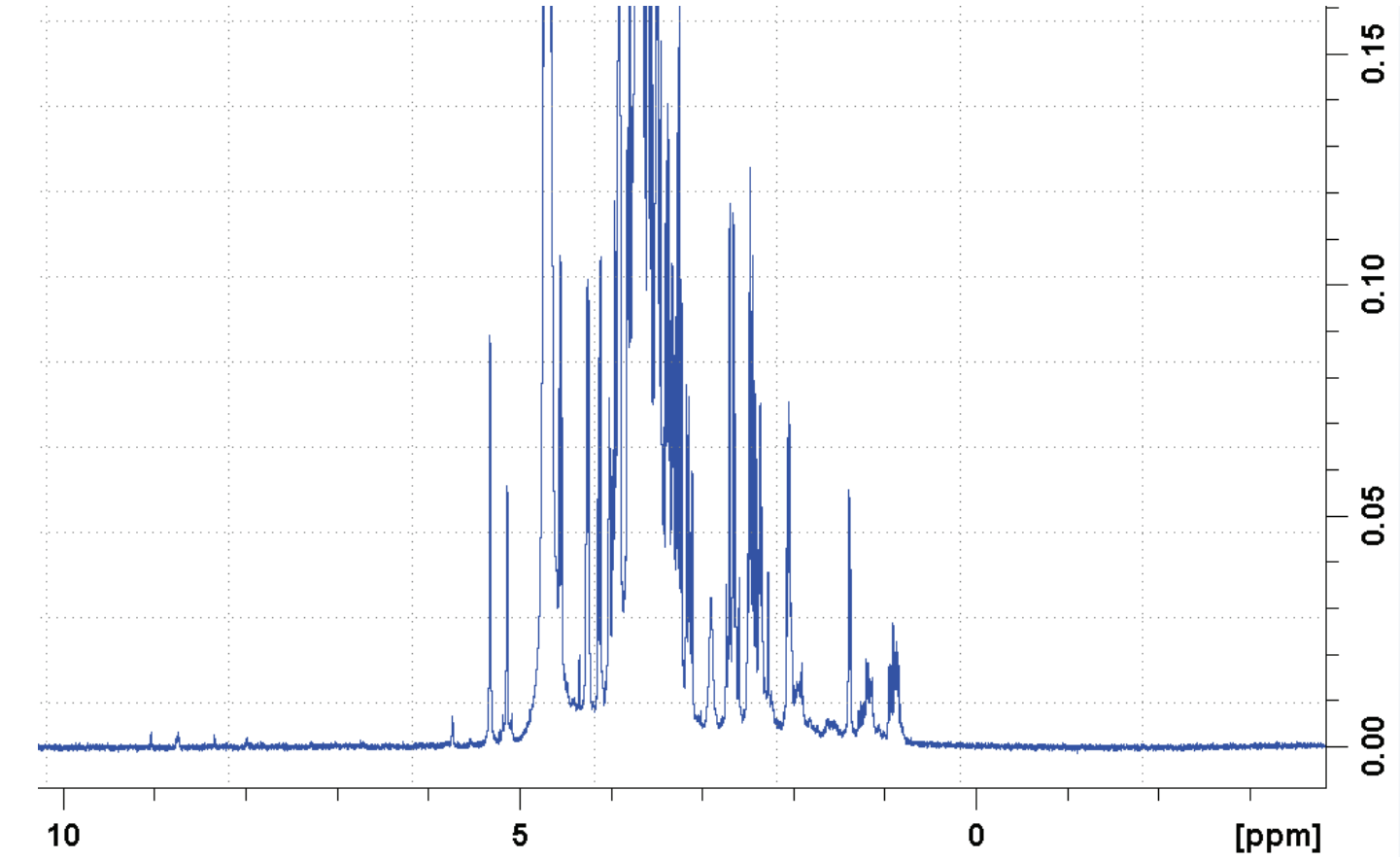

$^{1}$H NMR of fraction R32 in deuterated water produced on Bruker 400‘54 Ascend NMR spectrometer

Supplementary 9:



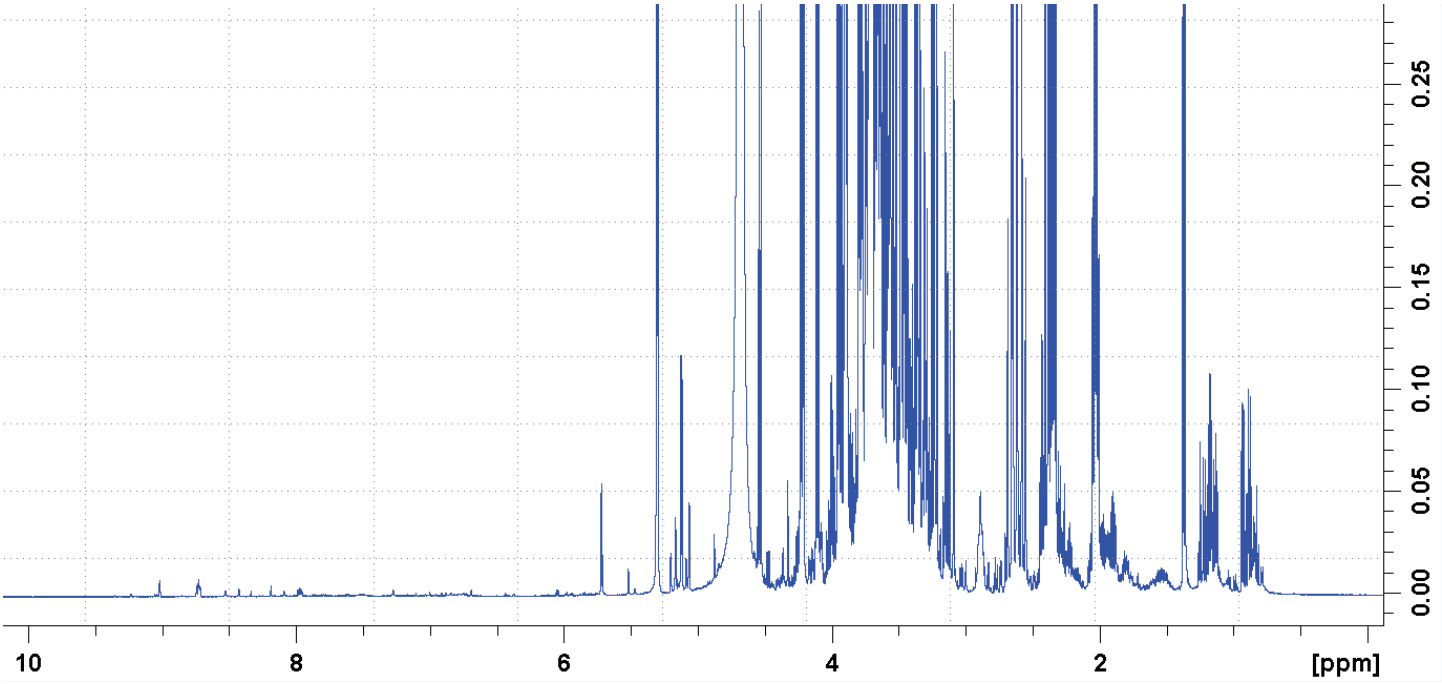

$^{1}$H NMR of fraction R32 fraction 13 and 14 in deuterated water produced on Bruker 400‘54 Ascend NMR spectrometer

Supplementary 10:



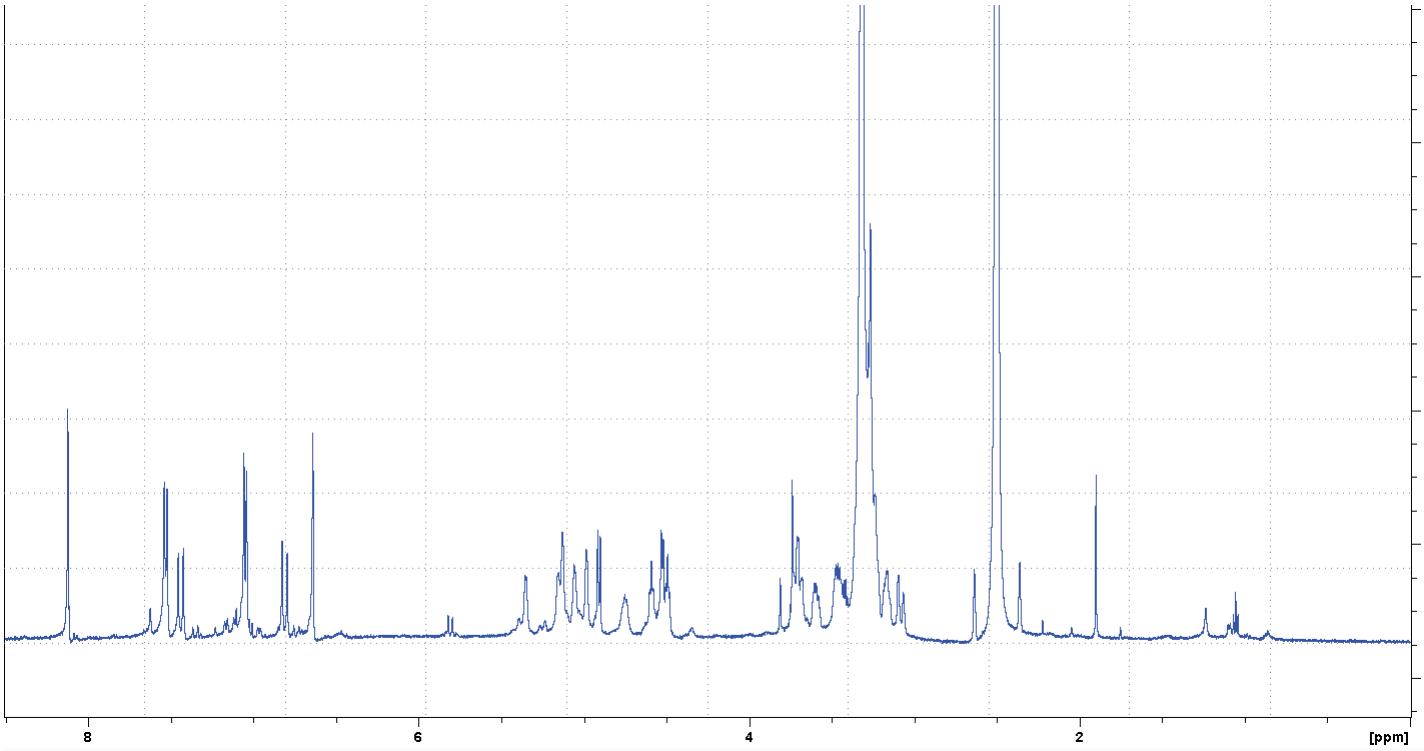

$^{1}$H NMR of fraction O34 in deuterated water produced on Bruker 400‘54 Ascend NMR spectrometer

Supplementary 11:



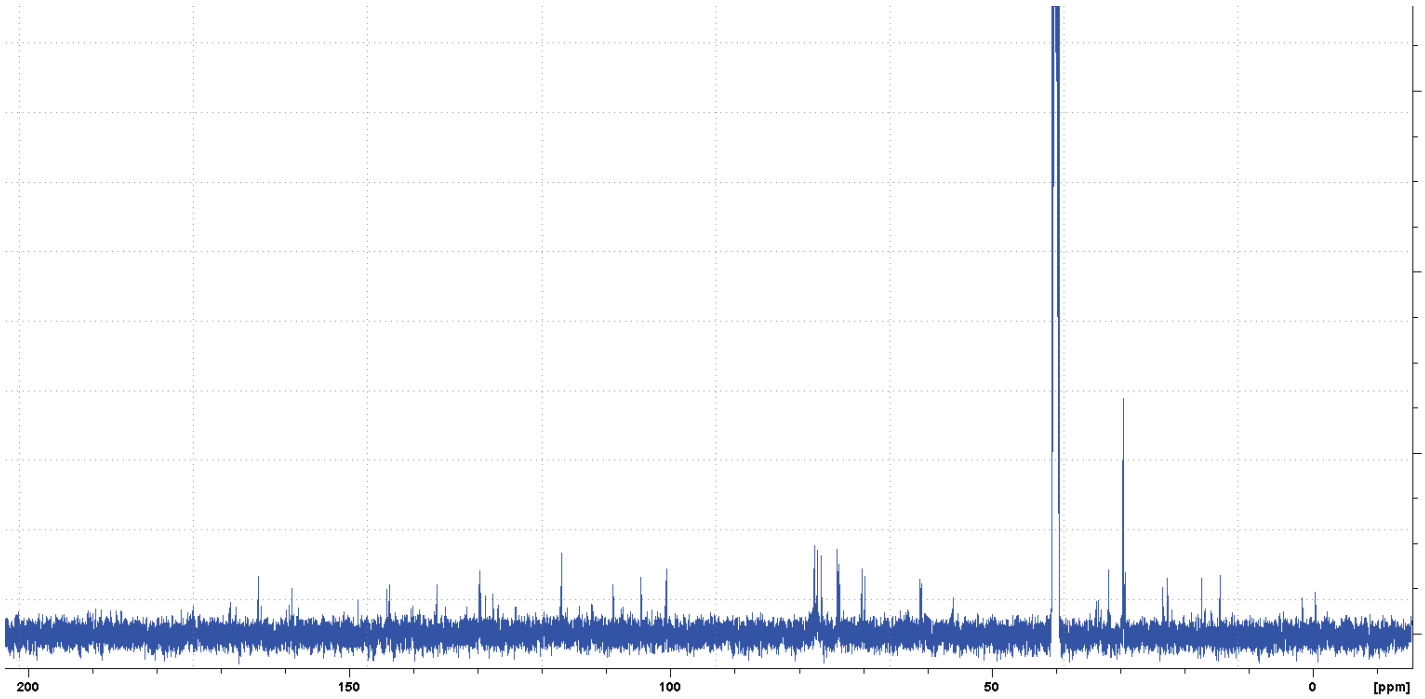

$^{1}$H NMR of fraction O33 fraction 13 in deuterated water produced on Bruker 400‘54 Ascend NMR spectrometer

Supplementary 12:



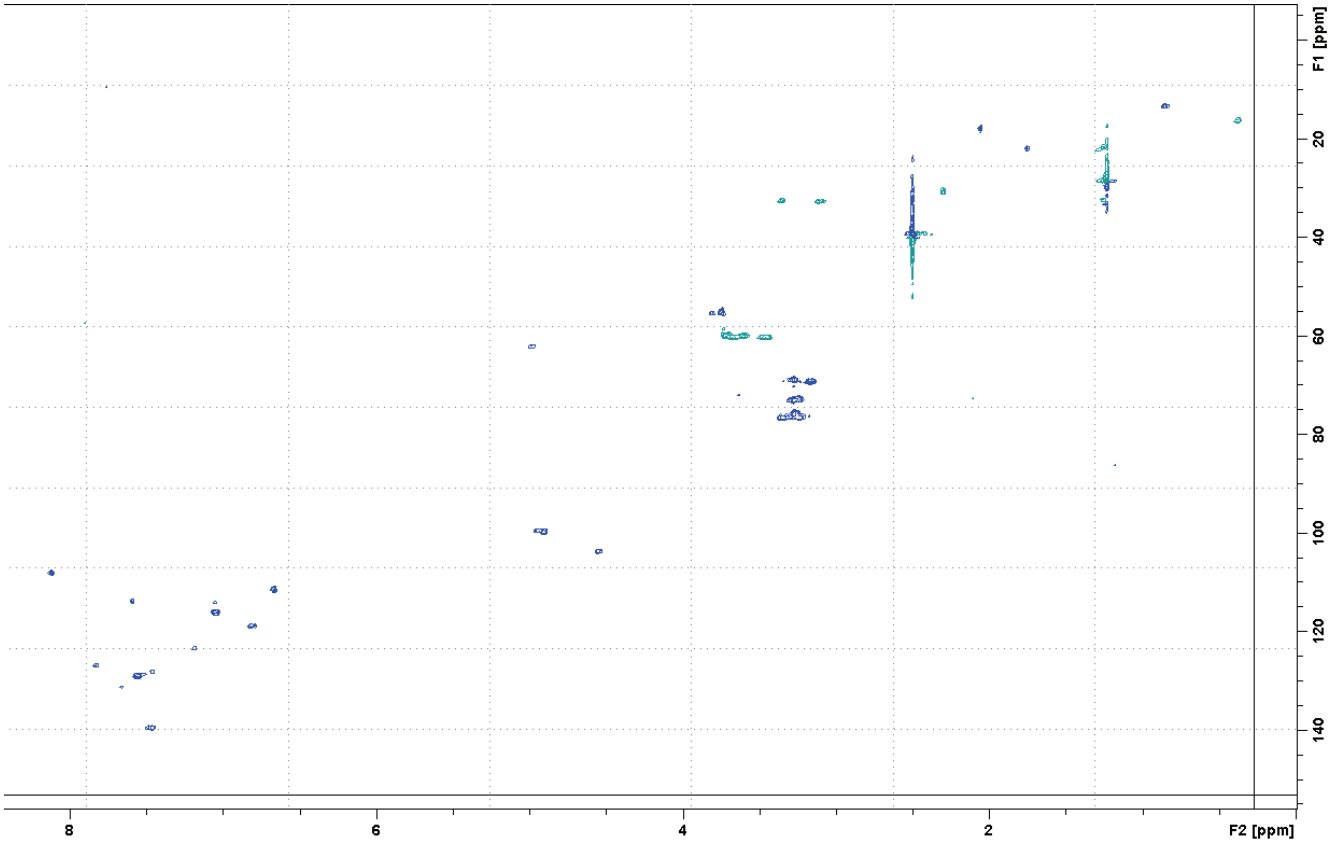

$^{1}$H NMR of compound 1 in deuterated DMSO produced on Bruker 500‘54 Ascend NMR spectrometer

Supplementary 13:



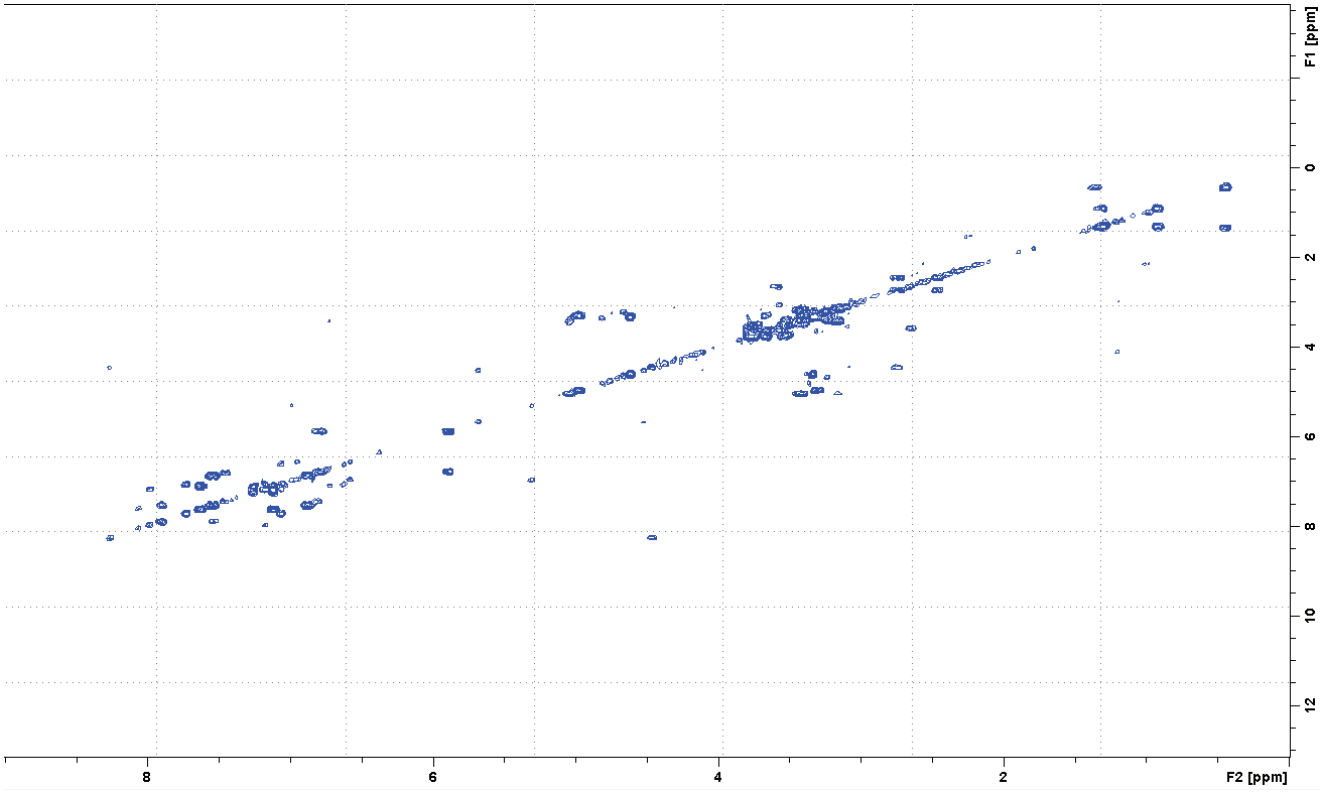

$^{13}$C NMR of compound 1 in deuterated DMSO produced on Bruker 500‘54 Ascend NMR spectrometer

Supplementary 14:



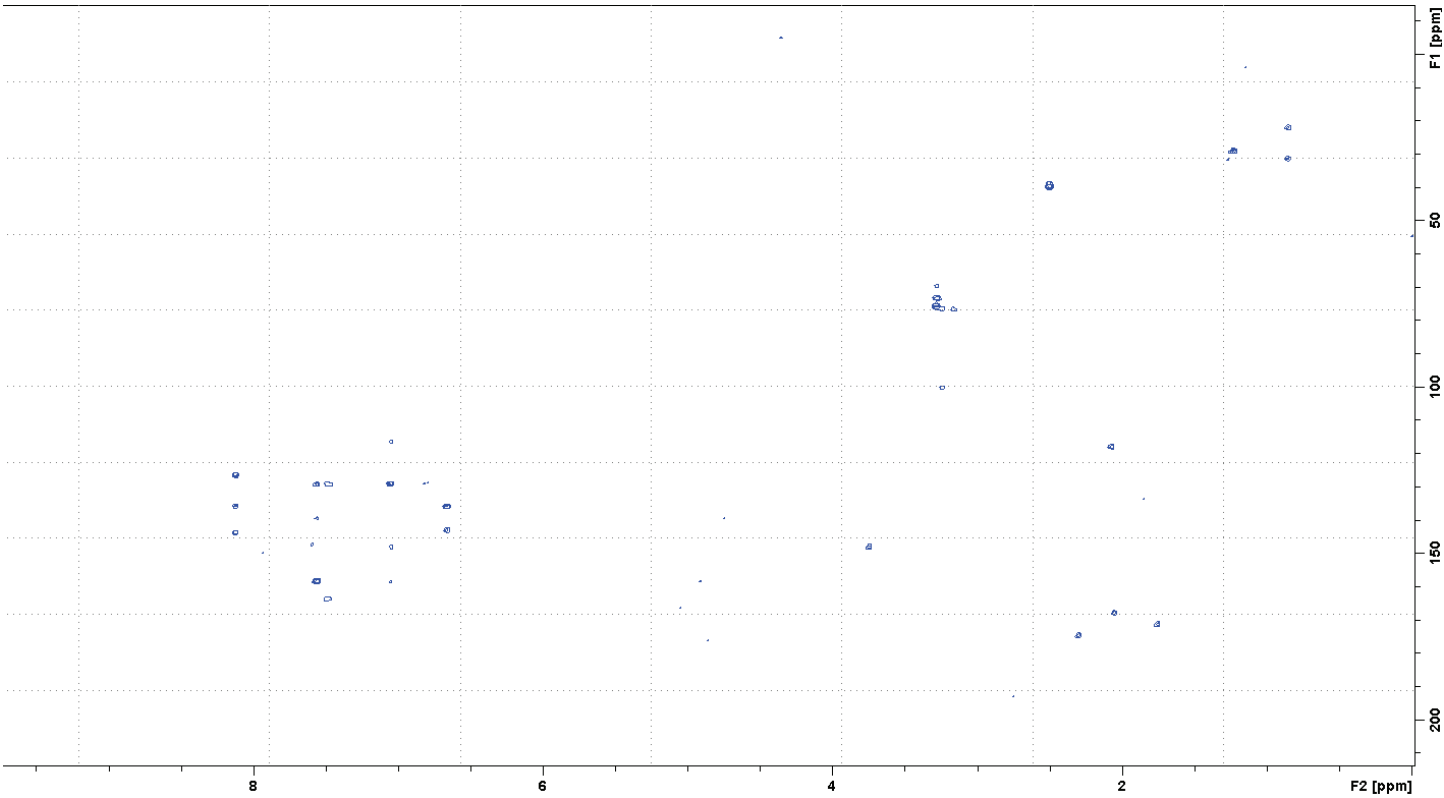

HSQC of compound 1 in deuterated DMSO produced on Bruker 500‘54 Ascend NMR spectrometer

Supplementary 15:



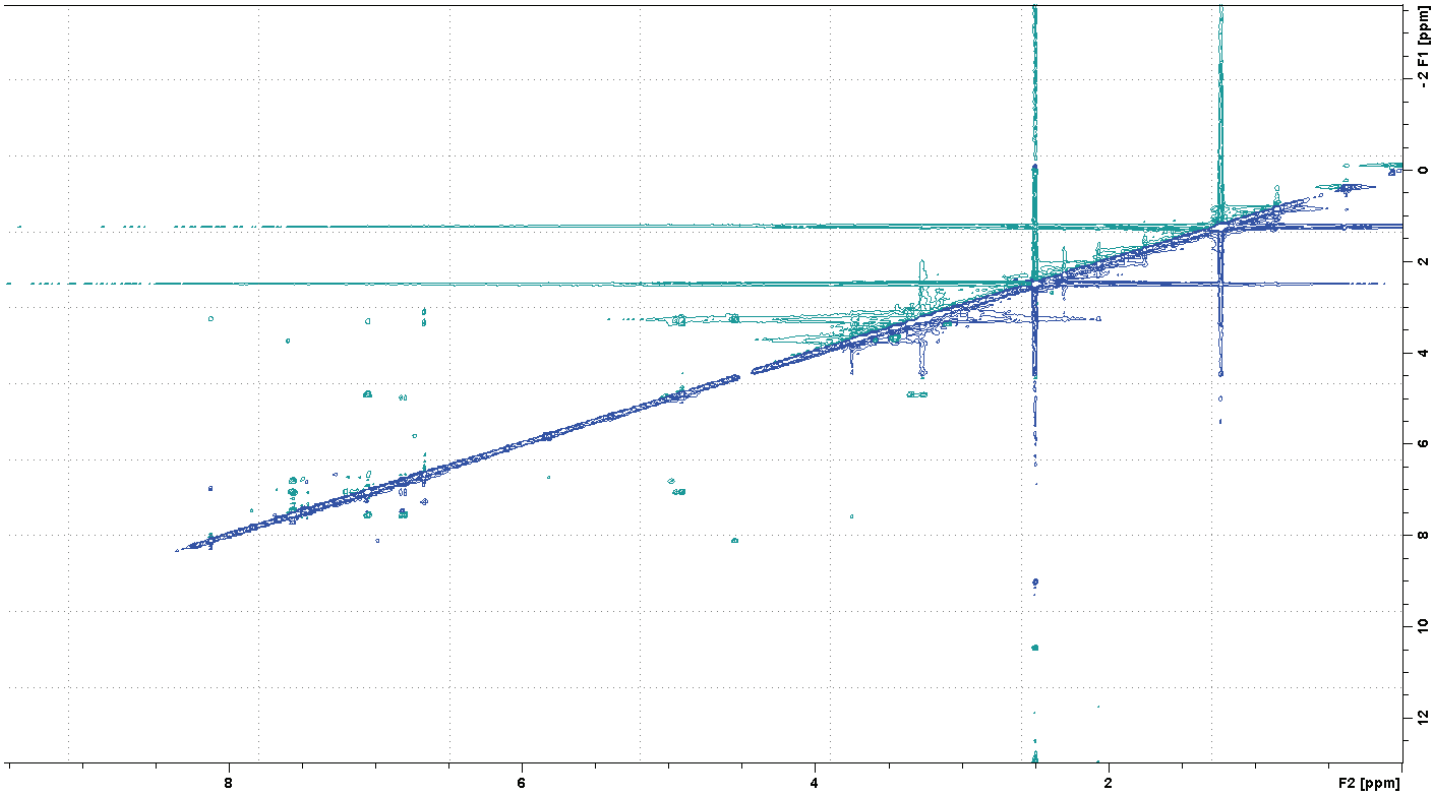

COSY ($^{1}$H-$^{1}$H) of compound 1 in deuterated DMSO produced on Bruker 500‘54 Ascend NMR spectrometer

Supplementary 16:




HMBC of compound 1 in deuterated DMSO produced on Bruker 500‘54 Ascend NMR spectrometer

Supplementary 17:




ROESY of compound 1 in deuterated DMSO produced on Bruker 500‘54 Ascend NMR spectrometer

Supplementary 18:

Table S1: NMR results of compound 1 and literature values of oleracein C all in deuterated DMSO[65]

| Position | δ C (ppm) | Literature δ C (ppm) | δ H (ppm) (*J* in Hz) | Literature δ H (ppm) (*J* in Hz) | Multiplicity | HMBC | COSY |
|---|---|---|---|---|---|---|---|
| 2 | 62.2 | 61.5 | 4.99 br s | 5.39 d (9.0) | 1 | C-3 | H-3 |
| 2-COOH | 173.0 | 174.4 | | | | | |
| 3A | 33.2 | 33.4 | 3.34 m | 3.45 m | 1 | COOH | H-2 |
| 3B | 33.2 | 33.4 | 3.08 d (16.5) | 3.12 d (16.5) | 1 | | |
| 4 | 111.5 | 112.6 | 6.67 s | 6.70s | 1 | C-3, C-5, C-6, C-7a | |
| 5 | 142.6 | 144.2 | | | | | |
| 6 | 143.2 | 144.6 | | | | | |
| 7 | 108.1 | 108.7 | 8.12 br s | 8.12s | 1 | C-3a, C-6, C-7a | |
| 3a | 126.5 | 125.7 | | | | | |
| 7a | 135.7 | 136.1 | | | | | |
| 1’ | 163.1 | 164.3 | | | | | |
| 2’ | 118.8 | 118.7 | 6.81 d (15.5) | 6.83 d (15.0) | 1 | C-4’ | H-3’ |
| 3’ | 139.4 | 141.5 | 7.48 d (15.5) | 7.53 d (15.0) | 1 | C-4’ | H-2’ |
| 4’ | 128.4 | 129.4 | | | | | |
| 5’ 9’ | 129.0 | 130.2 | 7.56 d (8.7) | 7.62 d (8.0) | 2 | C-3’, C-7’ | H-6’, H-8’ |
| 6’ 8’ | 116.2 | 117.2 | 7.05 d (8.7) | 7.06 d (8.0) | 2 | C-7’ | H-5’, H-9’ |
| 7’ | 157.8 | 159.3 | | | | | |
| 1’’ | 103.8 | 104.4 | 4.56 d (7.1) | 4.60 d (4.5) | 1 | C-6 | |
| 2’’ | 76.8 | 74.1 | 3.2-3.5 m | 3.2-3.8 m | 1 | | |
| 3’’ | 77.1 | 77.8 | 3.2-3.5 m | 3.2-3.8 m | 1 | | |
| 4’’ | 70.0 | 70.3 | 3.2-3.5 m | 3.2-3.8 m | 1 | | |
| 5’’ | 75.6 | 76.7 | 3.2-3.5 m | 3.2-3.8 m | 1 | | |
| 6’’ | 60.4 | 61.3 | 3.71 m | 3.82 m | 2 | | |
| 1’’’ | 99.8 | 100.8 | 4.91 d (7.4) | 4.92 d (7.5) | 1 | C-7’ | |
| 2’’’ | 73.3 | 73.8 | 3.2-3.5 m | 3.2-3.8 m | 1 | C-1’’’ | |
| 3’’’ | 76.9 | 77.7 | 3.2-3.5 m | 3.2-3.8 m | 1 | | |
| 4’’’ | 69.0 | 69.9 | 3.2-3.5 m | 3.2-3.8 m | 1 | | |
| 5’’’ | 76.6 | 77.3 | 3.2-3.5 m | 3.2-3.8 m | 1 | | |
| 6’’’ | 60.3 | 61.0 | 3.71 m | 3.82 m | 2 | | |

Supplementary 19:

Table S2: NMR results of compound 2 and literature values of oleracein D all in deuterated DMSO[65]

| Position | δ C (ppm) | Literature δ C (ppm) | δ H (ppm) (*J* in Hz) | Literature δ H (ppm) (*J* in Hz) | Multiplicity | HMBC | COSY |
|---|---|---|---|---|---|---|---|
| 2 | 63.6 | 63.2 | 5.05 | 5.05 | 1 | C-3 | H-3 |
| 2-COOH | 176.1 | 175.0 | | | | C-3 | |
| 3A | 33.2 | 33.9 | 3.37 | 3.45 m | 1 | COOH | |
| 3B | 33.2 | 33.9 | 3.11 | 3.15 d (16.0) | 1 | | |
| 1’ | 165.8 | 164.3 | | | | | |
| 2’ | 118.7 | 119.9 | 6.78 d (16.0) | 6.84 d (15.0) | 1 | C-4’ | |
| 3’ | 139.5 | 140.7 | 7.46 d (16.0) | 7.46 d (15.0) | 1 | C-4’, C-1’ | |
| 4’ | 128.0 | 129.9 | | | | C-3’, C-5’, C-8’, C-9’ | |
| 5’ | 112.3 | 112.4 | 7.31 br s | 7.26 br s | 1 | C-4’ | H-6’, H-8’ |
| 6’ | 148.1 | 149.7 | | | | | H-5’, H-9’ |
| $OCH_3$ | 55.3 | 56.5 | 3.74 s | 3.84 s | 3 | C-6’ | |
| 7’ | 147.5 | 148.5 | | | | | |
| 8’ | 114.1 | 115.9 | 7.05 d (7.4) | 7.08 d (7.5) | 1 | C-4’, C-6’, C-7’, C-9’ | |
| 9’ | 123.5 | 121.9 | 7.19 d (7.4) | 7.15 d (7.5) | 1 | C-7’ C-4’ | |
| 1’’’ | 99.5 | 100.6 | 4.95 d (7.5) | 4.94 d (6.0) | 1 | C-7’ | |
| 2’’’ | 73.3 | 73.8 | 3.2-3.5 m | 3.2–3.8 m | 1 | | |
| 3’’’ | 76.9 | 77.7 | 3.2-3.5 m | 3.2–3.8 m | 1 | | |
| 4’’’ | 69.0 | 70.0 | 3.2-3.5 m | 3.2–3.8 m | 1 | | |
| 5’’’ | 76.6 | 77.5 | 3.2-3.5 m | 3.2–3.8 m | 1 | | |
| 6’’’ | 60.03 | 61.1 | 3.79 m | 3.82 m | 2 | | |