References

1. Carpenter, J.H. *Limnol. Oceanogr.* **1965**, *10*, 141-143.

2. Murphy, J.; Riley, J.P. *Anal. Chim. Acta* **1962**, *27*, 31-36.

3. Parsons, T.R.; Maitra, Y.; Lalli, C.M. *A Manual of Chemical and Bio-* *logical Methods for Seawater Analysis* Pergamon: New York, 1984.

4. *Levin*, M.A.; *Fischer*, J.R.; *Cabelli*, V.J. *Appl. Microbiol.* **1975**, *30, 66*–71.

5. Lewis, E. L. *Marine Geodesy* **1982**, *5*, 350-357.

6. Hickey, C.W.; Norton, N.; Broekhuizen, N. *Proposed dissolved reactive phosphorus guidelines for the Ruamahanga River,* NIWA: Wellington, 2004.

7. ANZEE & ARMCANZ *Australian and New Zealand guidelines for fresh and marine water quality,* Canberra, 2000.

8. *Recreational Water Quality Annual Monitoring Summary 2008/09,* Otago Regional Council, 2009.

Article

Hot Chemistry from Horopito

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Introduction: The New Zealand Pepper Tree

Many will be familiar with horopito, *Pseudowintera colorata*. This shrub is often found in disturbed bush and along track margins. It is readily recognised by its red blotched leaves (see front cover and dark spots in Fig. 1), and by using one of our chemical senses, namely taste. A cautious nibble on a horopito leaf shows why it is called pepper tree, as the pungent, burning sensation develops on one's tongue. Past and present research on this unique NZ plant is discussed and brings together its hot and colourful chemistry.

Fig. 1. Blotched leaves of horopito, *Pseudowintera colorata* (courtesy of Luke Youard, Botany Dept., Otago University); see front cover for colour illustration.

Horopito has traditionally been used by Māori and European settlers for a variety of ailments, including toothache (by chewing) and skin diseases (by external application).[1] The first chemical studies were reported in 1958 by Corbett and Grant on a steam distilled oil from horopito leaves.[2] A range of mono- and sesquiterpenes were identified, together with the phenylpropanoid, eugenol. Eugenol, from clove oil, has been used as a local antiseptic and anaesthetic, but is not present in horopito in sufficiently high levels leaves to explain the medicinal activities. Corbett and his colleagues also found (and named) the sesquiterpene cyclocolorenone in their steam distillate, and correctly established its unusual structure.[3] This was achieved by chemical transformations without access to the NMR spectroscopy or X-ray crystallography that now we rely upon so heavily for structure determination.
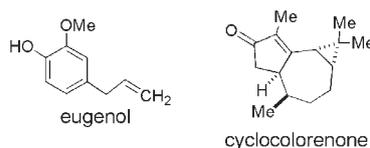
Pungent Polygodial

An Anti-Candida Agent

Screening of NZ plants for antibiotic activity by Canterbury University researchers showed that a horopito leaf extract stood out for its activity against *Candida albicans*, a yeast that can cause thrush infections.[4] Bioactivity-directed isolation work gave the main active compound identified as the sesquiterpene dialdehyde polygodial (**1**, Chart 1).[5] US researchers later found that the anti-*Candida* activity of polygodial was strongly increased by the presence of anethole,[6] a phenyl propanoid natural product that gives the characteristic flavour of anise. An NZ company, Forest Herbs Research in Golden Bay, is growing horopito and marketing a range of medicinal products based on these results. Forest Herbs and IRL have patented a process for extracting **1** from horopito using supercritical carbon dioxide.[7]

Polygodial (**1**) was known before it was found in horopito. It was first reported from, and named after, the water pepper *Polygonum hydropiper* by Australian chemists.[8] Water pepper, now classified as *Persicaria hydropiper*, is used in some Japanese dishes because of the pungency of polygodial.[9] Polygodial was also reported at the same time from the Australian mountain pepper, *Tasmannia* (earlier *Drimys*) *lanceolata*.[10] It is the most widely occurring sesquiterpene dialdehyde, having been found in flowering plants, ferns, liverworts, fungi, and marine molluscs from all around the world.[11] The drimane skeleton of **1**, and many of the other dialdehydes, is named after the plant genus *Drimys*, also in the family Winteraceae, the source of medicinal winter's bark from the South American *Drimys winteri*. The presence of **1** in horopito leaves is high as simple CDCl$_3$ extraction shows the two obvious aldehydic protons in the ^{1}H NMR spectrum of **1**, allowing quantitative analyses, with levels up to 40 mg/g of dry leaf.[12] The earlier work on steam-distilled leaf oil probably missed polygodial because of its low volatility and high reactivity.

An Insect Antifeedant

The attention of the Plant & Food Research group at Otago was drawn to horopito because tests run at AgResearch in Hamilton on leaf extracts showed strong antifeedant activity against insects. Bioactivity-directed isolation work showed that the main antifeedant compounds were **1** and **2**, the closely related 9-deoxymuzigadial (**2**, Chart 1).[13] Dial **2**, with its rare rearranged drimane skeleton, had only been reported once before this discovery, from a Caribbean plant *Cannela winterana* (family Canellaceae).[14] It was not reported in the Canterbury work,[5] and the Plant & Food Research group found out why when they analyzed different horopito collections from around the South Island, using HPLC and by quantitative 1H NMR spectroscopy. The ratio of dialdehydes **1** to **2** varied regionally with West Coast and Nelson horopito leaves dominated by **1**, whereas Otago and Canterbury leaves contained almost equal levels of **1** and **2**.[12]

Dialdehydes **1** and **2** had similar insect antifeedant activity against the two pests tested by AgResearch, namely caterpillars of the webbing clothes moth and larvae of the Australian carpet beetle.[13] However, storage trials on wool treated with dialdehyde showed that the protective antifeedant activity was lost after a few weeks and so no commercial development was possible.

A Chemical Sensor of Electrophiles

Polygodial was also found to have other biological activities by the Plant & Food Research group. Thus, anthelmintic activity (the expulsion of parasitic worms) against the sheep parasite *Trichostrongylus colubriformis* was found in collaboration with AgResearch,[15] and anti-inflammatory activity in a mouse model of gout by working with the Malaghan Institute and NIWA.[16] Others have found cytotoxicity, plant growth inhibition, and piscicidal and molluscicidal activities for **1** and other unsaturated dialdehydes.[11]

The broad, non-selective range of biological activities of **1** is ascribed to its electrophilic reactivity[11] that binds it to

Chart 1. Structures of sesquiterpene dialdehydes from *Pseudowintera* and other organisms.

3, paxidal
R^1 = cinnamate

4, R^1 = cinnamate

5, R^1 = cinnamate
6, R^1 = coumarate

7
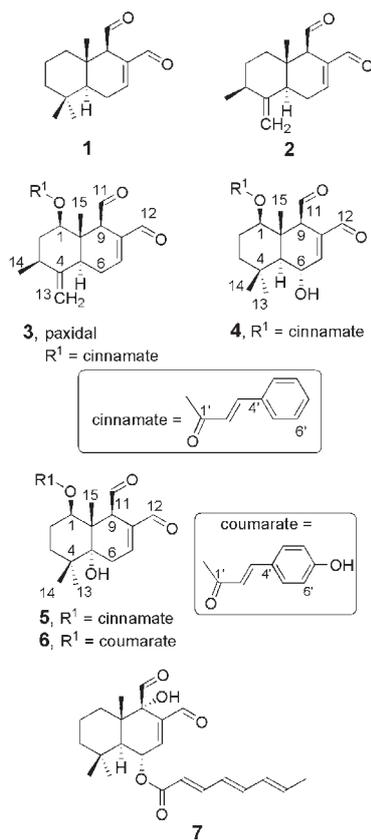
thiols and amines in proteins. Italian researchers showed that the reactivity of 1,4-dialdehydes with methylamine (under conditions mimicking cellular systems) correlated with their hot taste, and they found that stable pyrrole derivatives were formed.[17] More specifically, polygodial has been shown to activate an ion channel receptor TRPA1 (transient receptor potential A1) involved in inflammatory pain signalling.[18] This receptor is present in vertebrates and invertebrates, and acts as a chemical sensor for toxic electrophiles.[19] Other pungent compounds such as allyl isothiocyanate (responsible for the *hit* of wasabi) and cinnamaldehyde are active at this receptor, which is unusual; receptors generally respond to particular molecular shapes rather than chemical reactivity. The cysteines within TRPA1 are covalently modified by electrophiles, causing channel activation and rapidly signalling potential tissue damage through the pain pathway.[20]

Red Hot

The other chemical characteristic of horopito that we can study directly with our senses is the red marking of its leaves (front cover), which gives the specific name *colorata*. This coloration is a response to damage, which can be induced if you find horopito on a favourite bush walk – simply select a leaf close to the growing tip at the end of a branch, mark your initials (or those of some significant other) on this with a finger nail. Within a few days the initials will be highlighted in red.

The red pigments were identified as anthocyanins by IRL's Ken Markham when he worked on this wounding response with plant physiologists at Auckland University.[21] They used real-time imaging of hydrogen perox-

ide to show that mechanical injury elicited an oxidative burst from chloroplasts. H_2O_2 accumulated in green leaf portions for 10 minutes, and then decreased slowly. By contrast, red regions recovered rapidly and maintained consistently low levels of H_2O_2. The red regions were enriched in anthocyanins, flavonols, dihydroflavonols, and hydroxycinnamic acids, all of which are antioxidants, but only the anthocyanins were suitably located to account for enhanced rates of H_2O_2 scavenging. The data support the hypothesis that red cells have elevated antioxidant capabilities *in vivo*, protecting the leaves from further damage from reactive oxygen species.

Red Alert?

The combination of the results on insect antifeedant activity and on inducible red coloration led Gould and Perry to speculate that the two observations might be linked. Youard, in his Otago PhD work, found that that the redder horopito leaves contained greater polygodial levels.[22] This led to the question of whether horopito was an example of a plant using colour to warn approaching herbivores that it is chemically well-defended.

Aposematic (warning) colouration is a well-known phenomenon in animals, but has only recently been considered in plants. Nevertheless, there are plants from all terrestrial biomes whose leaves are pigmented red rather than green, and the possibility that their bright colours are involved in defence has begun to attract keen theoretical support. The hues of autumn foliage, for example, are postulated to serve as an honest signal of defensive commitment against colonising insect pests. The implications of this hypothesis are profound. However, foliar aposematism lacks solid experimental proof.

A Marsden funded project is now underway in our laboratories to test for aposematism directly using horopito.[23] Our multidisciplinary team combines expertise in native plant biochemistry, anthocyanin function, plant-insect interactions and community ecology, based at Victoria and Plant & Food Research at Otago universities. There is also collaboration with Martin Schaefer (University of Freiburg) who has expertise on the evolutionary ecology of visual communication in distinct signalling systems. Combining these skills to study the ecological biochemistry of horopito and its predators will engender a significant contribution to the understanding of the role of plant colouration in ecosystems. There are also implications for agriculture with proof of aposematism presenting the possibility of using leaf colour as a novel tool to mitigate crop damage, thereby reducing the increasingly evident ecotoxicological problems associated with pesticide use.

Cooler, Greener Plant Protection

There is another plant protection opportunity offered by another *Pseudowintera* species. The shrub know as *P. axillaris* is also called horopito. It grows throughout the NZ North Island but only at the top of the South Island, whereas *P. colorata* is found throughout most of NZ including Stewart Island. The two species can readily be distinguished by appearance and taste since *P. axillaris* has larger leaves with no red blotching and *axillaris* is

much less pungent than *colorata*. The two species also showed up quite differently in various screening assays as *P. axillaris* extracts show much less biological activity than *P. colorata* extracts. However, screening in an agrochemical discovery collaboration with Dow AgroSciences showed good activity for an extract of *P. axillaris* against *Phytophthora infestans*,[24] the plant pathogen that caused the Irish potato famine.

Bioactivity-directed fractionation of an extract of *P. axillaris* provided the main active compound with a ^{1}H NMR spectrum with aldehydic signals akin to those of **1** and **2** and signals consistent with a cinnamate group. 2D NMR spectra indicated that the structure was **3** (Chart 1), a new structure named paxidal.[24] This was available at quite high levels from leaves, allowing preparation of a series of derivatives for further antifungal testing. Structure-activity studies showed that both the dialdehyde and the bulky cinnamate groupings were important for the fungicidal activity.[24] However, none of the derivatives met the stability requirements for commercial development

The switch in biological activity provided by the cinnamate substituent is interesting. Cinnamate **3** was not active against *Candida albicans*, in contrast to the strong activity of **1** and **2**, but neither **1** nor **2** showed any activity against the plant pathogens tested. The lower pungency of *P. axillaris* suggests a lowered electrophilic reactivity of the unsaturated dialdehyde moiety owing to the bulky cinnamate substituent, at least towards TRPA1.

New Species, New Chemistry

The genus *Pseudowintera* is endemic to NZ, with *P. axillaris*, *P. colorata* and the Nelson species *P. traversii* long known. Recently, Conservation Department and Landcare botanists proposed a new species, *P. insperata*.[25] This is a threatened species known only from 46 mature individuals confined to the volcanic plugs above the Whangarei Harbour. In view of results on the other species, it seemed likely that *P. insperata* would also contain sesquiterpene dialdehydes and possibly new ones.

DoC provided samples of *P. insperata* leaves from cultivated plants. These were found to be much less pungent than leaves of *P. colorata*, implying low levels of **1**. Crude extracts from the leaves showed the characteristic ^{1}H NMR signals of unsaturated 1,4-dialdehydes, and two were isolated. The minor component, dialdehyde **5** (Chart 1), showed ^{1}H NMR signals characteristic of a polygodial derivative with a cinnamate moiety at C-1, similar to cinnamate **4** that is found in fruits of *P. colorata*.[26] Mass spectrometry on **5** suggested a molecular formula isomeric with **4**, but the 2D NMR data placed an α-OH group at C-5. This led to the proposed structure of **5**, namely the previously unknown (*E*)-1β-cinnamoyl-5α-hydroxypolygodial. However, prior to the *P. insperata* results being written up, the structure of **5** was published as occurring in *Zygozynum* spp. (also in the family Winteraceae) from New Caledonia.[27]

The major new dialdehyde was identified as **6** and differed from **5** only by having ^{1}H NMR signals characteristic of a coumarate moiety at C-1. The structure was assigned as

(*E*)-1β-*p*-coumaroyl-5α-hydroxypolygodial (insperadial, **6**, Chart 1). This structure remained unreported until this year when the *P. insperata* results were published.[28] Thus, the new species did yield new chemistry.

We extended this study by analysing multiple samples of *P. axillaris*, *P. colorata* and *P. insperata* collected from around the North Island by DoC staff. HPLC analyses showed that the main dialdehydes in the leaves conformed to the species boundaries. Thus, *P. insperata* individuals all had high levels (3.0–6.9% of leaf dry wt.) of coumarate **6**, *P. axillaris* had high levels (2.2–6.9%) of paxidal (**3**), and *P. colorata* from different regions of NZ contained varying levels of polygodial (**1**) (1.4–2.9%) and 9-deoxymuzigadial (0–2.9%) (**2**),[28] as previously found for South Island collections.[12] Therefore, the sesquiterpene dialdehydes are good species markers in *Pseudowintera*, but potential hybrids with different chemical composition were also found.[28]

Hot Questions

We have shown how the natural products chemistry and bioactivity of horopito and polygodial (**1**) has interested, and brought together, researchers from many of NZ's universities and research institutes. However, questions remain to be answered, most particularly the wide-spread occurrence, both geographical and biological, of **1**.

The results described herein have shown the chemotaxonomic links between the two vascular plant families Canellaceae and Winteraceae, which both consistently contain sesquiterpene dialdehydes, from plants growing in the Caribbean, South America, Africa, Australia, New Caledonia and New Zealand. However, **1** has also been found in other, unrelated organisms from all around the world – ferns from NZ and South America;[29,30] liverworts from NZ, Europe and Japan;[29,31] and in marine molluscs from the Mediterranean and Pacific.[32] At the microbial level, polygodial derivative **7** (Chart 1) has been reported from a fungal mold *Aspergillus* species, from a soil sample cultured in Japan.[33]

The diverse and potent biological activities of the sesquiterpene dialdehydes described herein presumably offer evolutionary advantages to all of these organisms. What is not known is how the same secondary metabolites, with quite complex structures, arise in so many different life forms. Could this be from parallel evolution, production by a symbiotic microorganism, or even horizontal gene transfer? More research and increased levels of funding are needed, as we scientists like to tell anyone who will listen.

References

1. Riley, M. *Māori Healing and Herbal*; Viking Sevenseas N.Z. Ltd: Paraparaumu, 1994.

2. Corbett, R. E.; Grant, P. K. *J. Sci. Food Agric.* **1958**, *9*, 733-738.

3. Corbett, R. E.; Speden, R. N. *J. Chem. Soc.* **1958**, 3710-3715.

4. Calder, V. L.; Cole, A. L. J.; Walker, J. R. L. *J. Royal Soc. NZ* **1986**, *16*, 169-181.

5. McCallion, R. F.; Cole, A. L. J.; Walker, J. R. L.; Blunt, J. W.; Munro, M. H. G. *Planta Med.* **1982**, *44*, 134-138.

6. Himejima, M.; Kubo, I. *J. Agric. Food Chem.* **1993**, *41*, 1776-1779.

7. Catchpole, O. J. *Recovery of polygodial from Horopito plant by extraction with supercritical carbon dioxide*: NZ Patent Applic. NZ 2002-520178, 20020715; *CAN 142:394427*, **2005**.

8. Barnes, C. S.; Loder, J. W. *Aust. J. Chem.* **1962**, *15*, 322-327.

9. See Gernot Katzer 's spice page: http://www.uni-graz.at/~katzer/engl/Poly_hyd.html (accessed 30 July 2010).

10. Loder, J. W. *Aust. J. Chem.* **1962**, *15*, 389-390.

11. Jansen, B. J. M.; de Groot, A. *Nat. Prod. Rep.* **2004**, *21*, 449-477.

12. Perry, N. B.; Foster, L. M.; Lorimer, S. D. *Phytochem.* **1996**, *43*, 1201-1203.

13. Gerard, P. J.; Perry, N. B.; Ruf, L. D.; Foster, L. M. *Bull. Entomol. Res.* **1993**, *83*, 547-552.

14. Al-Said, M. S.; El-Khawaja, S. M.; El-Feraly, F. S.; Hufford, C. D. *Phytochem.* **1990**, *29*, 975-977.

15. Lorimer, S. D.; Perry, N. B.; Foster, L. M.; Burgess, E. J., *et al. J. Agric. Food Chem.* **1996**, *44*, 2842-2845.

16. Martin, W. J.; Herst, P. M.; Chia, E. W.; Harper, J. L. *Free Rad. Biol. Med.* **2009**, *47*, 616-621.

17. Cimino, G.; Sodano, G.; Spinella, A. *Tetrahedron* **1987**, *43*, 5401-5410.

18. Escalera, J.; von Hehn, C. A.; Bessac, B. F.; Sivula, M.; Jordt, S.-E. *J. Biol. Chem.* **2008**, *283*, 24136-24144.

19. Kang, K.; Pulver, S. R.; Panzano, V. C.; Chang, E. C., *et al. Nature* **2010,** *464*, 597-600.

20. Macpherson, L. J.; Dubin, A. E.; Evans, M. J.; Marr, F., *et al. Nature* **2007**, *445*, 541-545.

21. Gould, K. S.; McKelvie, J.; Markham, K. R. *Plant Cell Env.* **2002**, *25*, 1261-1269.

22. Youard, L.; Lord, J.; Gould, K. S.; Perry, N. B. *Chemistry and the Biosphere Conference, (Dunedin)*, Dec 2008,. unpublished results.

23. Menzies, I. J.; van Klink, J. W.; Perry, N. B.; Schaefer, H. M., *et al. Combio 2009 Conference, Christchurch* Dec 2009, poster presentation; results unpublished.

24. Brennan, N. J.; Larsen, L.; Lorimer, S. D.; Perry, N., *et al. J. Ag. Food Chem.* **2006**, *54*, 468-473.

25. Heenan, P. B.; de Lange, P. J. *NZ J. Bot.* **2006**, *44*, 89-98.

26. Larsen, L.; Lorimer, S. D.; Perry, N. B. *Biochem. Syst. Ecol.* **2007**, *35*, 286-292.

27. Allouche, N.; Apel, C.; Martin, M.-T.; Dumontet, V. *et al. Phytochem.* **2009**, *70*, 546-553.

28. Wayman, K. A.; de Lange, P. J.; Larsen, L.; Sansom, C. E.; Perry, N. B. *Phytochem.* **2010**, *71*, 766-772.

29. Asakawa, Y.; Toyota, M.; Oiso, Y.; Braggins, J. E. *Chem. Pharm. Bull.* **2001**, *49*, 1380-1381.

30. Socolsky, C.; Muruaga, N.; Bardón, A. *Chem. Biodiversity* **2005**, *2*, 1105-1108.

31. Cullmann, F.; Becker, H. *Z. Naturforsch.* **1999**, *54c*, 151-155.

32. Avila, C.; Cimino, G.; Crispino, A.; Spinella, A. *Experientia* **1991**, *47*, 306-310.

33. Uosaki, Y.; Yoshida, M.; Ogawa, T.; Saito, Y. *J. Antibiotics* **1996**, *49*, 6-12.