Chapter Seven

TERPENOID ANTI–HERBIVORE CHEMISTRY OF ENCELIA SPECIES
(ASTERACEAE)

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Introduction.......................................................... 249
Terpenoid Chemistry of Encelia.................................. 250
Bioactivity of Encelia Terpenoids to Insects.................. 254
Bioactivity of Foliar Extracts of Encelia Species.......... 255
Sources of Bioactivity in Inhibitory Species............... 257
Conclusion.......................................................... 261

INTRODUCTION

The role of terpenoid natural products in plant defense against herbivory by insects has been well established.1-2 This phenomenon extends to arid and semi-arid plants, even though taxa from these latter habitats have been less frequently examined.3 In this chapter, we compare the terpenoid chemistry of desert sunflowers of the genus Encelia, with the performance of a generalist insect feeding on extracts of these plants, in an attempt to define the role of the terpenoid allelochemicals as protective agents against herbivory.
The genus *Encelia* Adans. includes approximately twenty taxa of shrubby perennials that inhabit the Sonoran and Mojave deserts of the southwestern United States and adjacent areas of Mexico, particularly Baja California; however, there are two disjunct species that occur in South America. Several of the species are widespread and are frequently dominant elements of the desert floras. There is scant information on natural enemies of *Encelia*, but the common brittle bush, *E. farinosa* A. Gray, is known to be attacked by a specialist leaf beetle, *Trirhabda geminata* (Chrysomelidae), and a generalist grasshopper, *Cibolacris parviceps* (Acrididae) (R.F. Chapman, personal communication).

The terpenoid chemistry of *Encelia* has been the subject of considerable investigation, with respect to both chemotaxonomy and quantitative variation. The insecticidal and antifeedant actions of one class of *Encelia* terpenoids, the benzopyrans/benzofurans, have been well documented against several types of insects. However, these previous reports have been based solely on laboratory bioassays utilizing isolated compounds, without establishing the exact role of these natural products as defensive agents in the plants themselves.

**TERPENOID CHEMISTRY OF ENCELIA**

Most species of *Encelia* are characterized by two distinct classes of terpenoids, sesquiterpene lactones and benzopyrans/benzofurans. The latter group are biogenically related, both arising from prenylation of a phenolic ring by a C₅ unit from the mevalonic acid pathway. The phenolic ring including the C-acetyl substituent has recently been shown to be derived from the shikimic acid pathway, *via cinnamic acid*. Formation of the heterocyclic ring can take place in two ways, resulting in 2,2-dimethylchromenes (benzopyrans, 2-4) (Fig. 1), or 2-isopropylbenzofurans (5-6) (Fig. 1).

The quantities of benzopyrans and benzofurans accumulated vary widely both between and within species of *Encelia*. Qualitative variation occurs interspecifically, but the overall pattern of these compounds intraspecifically is relatively constant, which makes them useful as chemotaxonomic markers. This approach has allowed a subgeneric division of the genus into three groups (Table 1); these groupings based on benzopyran/furan patterns are well supported by morphological characters.
ANTI-HERBIVORE CHEMISTRY OF *ENCELIA*

![Chemical Structures]

**Key Structures:**

1. Farinosin (1)
2. Demethoxyencecalin (2)
3. Demethylencecalin (3)
4. Encecalin (4)
5. Euparin (5)
6. Methyleuparin (6)

**Figure 1.** Structures of the major terpenoid (benzopyrans/benzofurans) constituents of *Encelia* species.

As part of our investigation, we quantified the major benzopyrans/furans from the foliage of fourteen taxa of *Encelia* (Table 1) by reverse-phase HPLC. Our system included a 3.9 mm x 15 cm NovaPak™ C18 column (4 μ), a linear gradient from 10-60% aqueous acetonitrile in 20 minutes at a flow rate of 1.5 ml/min, and UV detection at 254 nm. Crude methanolic extracts of air-dried foliage were prepared for bioassay (see section on bioactivity of foliar extracts); a small aliquot of each sample was saved for analysis. With the exception of samples of *E. palmeri* and *E. farinosa*, plant material was harvested from plants growing in an experimental garden at Pomona, California.
Table 1. List of Encelia taxa investigated.

<table>
<thead>
<tr>
<th>Group</th>
<th>Code</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CAL*</td>
<td>E. californica Nutt.</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>E. conserpse Benth</td>
</tr>
<tr>
<td></td>
<td>DEN</td>
<td>E. densifolia Clark &amp; Kyhos</td>
</tr>
<tr>
<td></td>
<td>FAR</td>
<td>E. farinosa A. Gray</td>
</tr>
<tr>
<td></td>
<td>PHE</td>
<td>E. farniosa var. phenicodonta Blake</td>
</tr>
<tr>
<td></td>
<td>HAL</td>
<td>E. halimfolia Cav.</td>
</tr>
<tr>
<td></td>
<td>LAC</td>
<td>E. X laciniata Vasey &amp; Rose</td>
</tr>
<tr>
<td></td>
<td>PAL</td>
<td>E. palmeri Vasey &amp; Rose</td>
</tr>
<tr>
<td></td>
<td>RAD</td>
<td>E. radians Brandegee</td>
</tr>
<tr>
<td></td>
<td>VEN</td>
<td>E. ventorum Brandegee</td>
</tr>
<tr>
<td>II</td>
<td>ACT</td>
<td>E. actoni Elmer</td>
</tr>
<tr>
<td></td>
<td>RES</td>
<td>E. frutescens A. Gray var. resinosa M. E. Jones</td>
</tr>
<tr>
<td>III</td>
<td>ASP</td>
<td>E. asperifolia Clark &amp; Kyhos</td>
</tr>
<tr>
<td></td>
<td>FRU</td>
<td>E. frutescens var. frutescens A. Gray</td>
</tr>
</tbody>
</table>

Taxa grouped according to Proksch and Clark.  

*species codes used in Figures 2 and 3.

Material of E. palmeri was collected from plants in Baja California. Material of E. farinosa was a composite of several samples collected in Arizona.

Five of the fourteen taxa analyzed contained relatively large quantities of the dominant benzopyran, encecalin (4) (Fig. 2). For E. palmeri, E. densifolia (also see Fig. 4) and E. conspersa, encecalin constitutes greater than 90% of the total benzopyrans/furans in foliar extracts.

E. californica has almost equally large amounts of encecalin and methyleuparin (6) (also see Fig. 4). E. halimifolia has little encecalin, but considerable quantities of demethylencecalin (3) and methyleuparin (6). E. frutescens var. resinosa was notable in that no benzopyrans/furans were detected in the sample analyzed (Fig. 2). All of the other taxa had minor amounts of these compounds. It is noteworthy that the sample of E. farinosa analyzed contained little encecalin, but an appreciable quantity of demethylencecalin. This
Fig. 2. Concentration of benzopyrans and benzofurans in foliar extracts of Encelia species, based on HPLC analysis of methanol extracts. DMX = demethoxy; DME = demethyl. Numbers in parentheses refer to structures in Figure 1, codes for species refer to Table 1 (dwt = dry weight).

species has been documented to be extremely variable with respect to benzopyran/furan content,\textsuperscript{6,7} existing as two races or chemotypes based on encecalin content. Populations of \textit{E. farinosa} from the Mojave desert of California generally have concentrations of encecalin ten-fold greater than those of populations from the Sonoran desert of Arizona.\textsuperscript{6} An earlier analysis of a sample from the Mojave desert indicated that it contained approximately 20 \(\mu\text{mol/g dwt}\) (unpublished data).

Four structurally related sesquiterpene lactones of the eudesmanolide skeletal class have been isolated from species of \textit{Encelia}.\textsuperscript{13} These arise from an eremophilane C15 precursor. The predominant compound of this group, farinosin (1) (Fig. 1) has been quantified, along with encecalin (4) and euparin (5) in several populations of \textit{E. farinosa}.\textsuperscript{7} Values given for the latter two compounds in the report appear erroneously high (foliar encecalin concentrations averaging 20\% dwt!), but the salient observations are that, on average, there is about one-eighth as much farinosin as encecalin, but slightly more farinosin than euparin.\textsuperscript{8} These authors report that for Sonoran
populations, farinosin is the predominant terpenoid compound in foliage of *E. farinosa*.

**BIOACTIVITY OF ENCELIA TERPENOIDS TO INSECTS**

Pure benzopyrans isolated from *Encelia* have contact insecticidal action against several types of insects. Encecalin (4) is toxic to neonate larvae of the variegated cutworm (*Peridroma saucia*, Noctuidae) via residue contact on glass surfaces (LD$_{50}$ = 1.4 µg/cm$^2$) or when incorporated into an artificial diet (LD$_{50}$ = 2.4 µmol/g fwt). This latter value is equivalent to 12 µmol/g dwt, suggesting that at least five species of *Encelia* should be more than adequately defended chemically against this insect based on their encecalin content alone (see Fig. 2).

In both tyes of bioassay (residue-contact and dietary incorporation), demethylencecalin (3) is significantly less toxic than encecalin. This differential toxicity is also apparent for both the milkweed bug (*Oncopeltus fasciatus*, Lygaeidae) and the mosquito (*Culex pipiens*, Culicidae). In the case of the migratory grasshopper (*Melanoplus sanguinipes*, Acrididae), demethylencecalin is somewhat more toxic than either encecalin or demethoxyencecalin when the compounds are applied topically to neonate nymphs. In contrast, the benzofurans euparin and methyleuparin lack toxicity in all bioassays used for the above mentioned insects. However, these compounds have been shown to have antimicrobial properties and thus they may serve in a defensive capacity against plant pathogens. It is noteworthy that the benzofurans antagonize the toxicity of encecalin in bioassays using the cutworm, grasshopper and milkweed bug.

The antifeedant action of the *Encelia* benzopyrans and euparin was recently assessed, using a leaf disc choice test with 5th instar variegated cutworms (Table 2). All three of the benzopyrans tested have considerable antifeedant activity; the EC$_{50}$ (concentration reducing feeding by 50%) for encecalin corresponds to an approximate value of 12 µmol/g dwt (cf. Fig. 2). As the sensitivity of this insect to allelochemicals is known to diminish with age, it would be expected that smaller larvae would be deterred by lesser foliar concentrations. In contrast, euparin has only weak antifeedant activity, being about seven times less active than encecalin in this bioassay (Table 2).
Table 2. Antifeedant action of Encelia benzopyrans/furans to 5th instar larvae of Peridroma saucia.

<table>
<thead>
<tr>
<th>Compound*</th>
<th>EC50</th>
<th>nmol/cm²</th>
</tr>
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<tbody>
<tr>
<td>encecalin (4)</td>
<td>17.1</td>
<td>73.9</td>
</tr>
<tr>
<td>demethylenecalcalin (3)</td>
<td>17.0</td>
<td>78.1</td>
</tr>
<tr>
<td>demethoxyencecalin (2)</td>
<td>13.1</td>
<td>68.8</td>
</tr>
<tr>
<td>euparin (5)</td>
<td>114.8</td>
<td>531.3</td>
</tr>
</tbody>
</table>

Based on a cabbage leaf disc choice test.

*numbers in parentheses refer to structures in Figure 1.

In the only report of bioactivity of farinosin against insects, this sesquiterpene lactone did not significantly deter feeding or growth of the corn earworm (Heliothis zea, Noctuidae) when added to an artificial diet at 5.3 μmol/g fwt.17 We have tested a related eudesmanolide sesquiterpene lactone, helenin (a mixture of alantolactone and its isomer isoalantolactone) against the variegated cutworm, and found that this mixture inhibited growth and reduced survivorship of neonate larvae in a dose-dependent manner with an EC50 and LD50 of approximately 2.5 and 3.0 μmol/g fwt respectively (unpublished data). Helenin is therefore similar in bioactivity to a series of pseudogualanolide sesquiterpene lactones previously tested.18-20

BIOACTIVITY OF FOLIAR EXTRACTS OF ENCELIA SPECIES

To assess the role of benzopyrans in the chemistry of defense against insect herbivory in Encelia species, HPLC analysis of crude foliar extracts as described previously was combined with parallel bioassay of the extracts against neonate larvae of the variegated cutworm, a generalist herbivore. This insect, which feeds on an extremely wide range of plants including many economically important vegetable and fruit crops, ornamental plants and even seedling conifers, has been used as a test insect for many other plant extracts and purified natural products.19-22 Dry, powdered foliar material of Encelia species was extracted once with hot methanol, and
Fig. 3. Larval weight of *Peridroma saucia* larvae feeding on artificial diets spiked with foliar extracts of *Encelia* species at 25% of natural concentration. Encecalin concentrations in respective species are shown for comparison. Codes for species refer to Table 1.

Following concentration of the extracts, they were added to an artificial medium in the standard manner such that the final concentration in the diet represented 25% of the natural concentration (i.e., extract from 1 g dwt foliage added to 4 g dwt of diet ingredients). Preliminary bioassays indicated that this concentration would permit some larval growth but might indicate differences between species with respect to their ability to support larval growth. Phytochemical analyses (results shown in Fig. 2) were conducted on an aliquot of the same extracts which were used for bioassay, allowing for direct comparison. For each plant species, forty neonate larvae were reared (two per cup) on diet containing extract for 7 days at 27°C, after which time all larvae were weighed. Our rationale for using crude foliar extracts (as opposed to adding leaf powder to the diet) is that this method permits us to evaluate the effect of the major extractable substances without interference from physical characteristics of the plants and without altering the nutritional value of the diet.

Results of the experiment are shown in Figure 3. Diets spiked with foliar extracts of the different *Encelia* taxa differ markedly in their ability to support larval growth, with mean larval growth for the treatments ranging from 92% of con-
trols (*E. farinosa* var. *phenicodonta*) to 26% of controls (*E. actoni*). At this extract concentration, there was no appreciable mortality in any of the treatments. However, when an extract of *E. palmeri* was bioassayed against neonate larvae at 20, 40, 60 and 80% of natural concentration, all larvae died at the highest concentration and only 65% survived at 60% nat. conc. These observations collectively suggest that foliage of at least half of the species investigated would not be expected to support larval growth, based on extractable substances.

What is the relationship between encecalin (and/or benzopyrans/furans) and larval growth on diets containing crude foliar extracts? The data plotted in Figure 3 indicate unambiguously that encecalin does not play a major role in the relative suitability of *Encelia* extracts to the variegated cutworm. This observation is paradoxical in light of the considerable toxicity and deterrency of pure encecalin to this insect in several laboratory bioassays. The best current explanation of our data is that other substances, such as the benzofurans (but probably other compounds) antagonize or 'mask' the toxicity/deterrency of encecalin in this type of bioassay. One exception may be *E. palmeri*, which is relatively inhibitory to the cutworm (though not the most) and possesses the highest concentration of encecalin amongst the taxa examined.

**SOURCES OF BIOACTIVITY IN INHIBITORY SPECIES**

The most inhibitory foliar extracts in our bioassay were those of *E. actoni*, *E. X laciniata*, *E. asperifolia* and *E. ventorum* (Fig. 3). Of these, only *E. X laciniata* has appreciable quantites of benzopyrans/furans (Figs. 2, 3). That this species possesses considerable concentrations of encecalin is not surprising as it consists of hybrids between *E. palmeri* (which has the highest concentration of encecalin in the collection) and *E. ventorum*. Does chromatographic analysis provide any clues as to the substances in these inhibitory species that may be responsible for their bioactivity?

Chromatographic profiles of *E. actoni* and *E. asperifolia*, two of the most inhibitory species, have in common a dominant peak occurring at just under 5 minutes in our HPLC system (Fig. 5). We have isolated this peak using preparative HPLC, and have tentatively identified this substance as the sesquiterpene...
lactone farinosin, based on $^1$H-NMR and GC-MS analyses (unpublished data). To determine if this compound is the source of bioactivity in the foliar extracts of these two plants, we fractionated each extract using preparative HPLC, and bioassayed the fractions as before.

In the case of the *E. asperifolia* extract, the fraction corresponding to 2.5-5.0 minutes (Fig. 5), which contains the peak tentatively identified as farinosin, has considerable larval growth inhibiting activity, however, the following fraction (5.0-7.5 minutes), containing the only other UV-visible substances in the extract, is even more biologically active, though not extremely so. Additionally, we analyzed and bioassayed a second collection of *E. asperifolia* (from a different locale). The extract of this latter sample contained approximately 60% as much 'farinosin' as the first sample, did not have the second major peak, but most important, did not significantly inhibit larval growth. In the case of *E. actoni*, the fraction containing the 'farinosin' peak had only modest activity (though statistically significant, $p < 0.05$), whereas again the fraction representing 5.0-7.5 minutes was the most active. However, in this species, the following two fractions (7.5-10 and 10.0-12.5 minutes) were also quite active. However, in this species, the following two fractions (7.5-10 and 10.0-12.5 minutes) were also quite active, though less so than the 5.0-7.5 minute fraction. Collectively these observations suggest that although the peak corresponding to farinosin contributes to the bioactivity of the most inhibitory extracts, it cannot account for most of the activity observed.

The extract of *E. ventorum* contains a series of moderate sized peaks in the 5.0-7.5 minute range (Fig. 5), which provides further circumstantial evidence that the important bioactive substances of *Encelia* species reside in this area of the chromatogram. *E. X laciniata* is a complete anomaly. This species has no apparent UV-visible (232/254 nm) substances which elute in less than 10 minutes (Fig. 4), yet this is one of the most inhibitory taxa (Fig. 3). The only feasible explanation for the pronounced bioactivity of this species (and, to a lesser extent, the aforementioned species) is that

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Fig. 4. HPLC chromatograms of foliar extracts of *E. densifolia* (top), *E. californica* (middle), and *E. laciniata* (bottom), monitored at 254 nm. Numbered peaks refer to structures in Figure 1.
the substance(s) accounting for the bioactivity are UV-invisible or irreversibly bound to the chromatographic column.

CONCLUSION

The present investigation serves to underline the undesirable, but well documented, discrepancy between the bioactivity of isolated natural products in laboratory bioassays and the bioactivity of these substances in planta.24

In Encelia, the lack of a relationship between encecalin content and bioactivity in foliar extracts is especially surprising given the degree of biological activity of this compound against different types of insects. It should be noted, however, that amongst noctuid species, _P. saucia_ is known to be relatively less sensitive to many allelochemicals than other frequently used bio assay species,20 and therefore it is probably a very conservative model for this type of study. In spite of the results presented here, it may well be that the benzopyrans/furans serve in a defensive capacity for _Encelia_ species against other insect herbivores. For example, a mixture of terpenoids (farinosin, encecalin and euparin) representative of the compounds in _E. farinosa_ (of which encecalin made up 85%) significantly reduced larval growth and survival of the beetle _Trihabda geminanta_, even though this insect feeds exclusively on this host plant.4 Nonetheless, the key unanswered questions from our study (i.e. the nature of the bioactive constituents) indicate that there remains considerable scope for phytochemical investigation in the genus Encelia.

ACKNOWLEDGMENTS

We wish to thank Anna Luczynski and Nancy Brard for their expert technical assistance and unflagging enthusiasm. Opender Koul conducted the antifeedant bioassay, the results of which are presented in Table 2. We also thank Don Champagne for

Fig. 5. HPLC chromatograms of foliar extracts of _E. asperifolia_ (top), _E. actoni_ (middle), and _E. ventorum_ (bottom), monitored at 254 nm. The numbered peak (1) has been tentatively identified as farinosin (see Fig. 1 for structure).
assistance with spectroscopic analyses, and for preparation of
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