Table 1. Incorporation of 14β-cholest-5-en-3β-ol-[15β-^{3}H] and cholesterol-[4-^{14}C] (1.16×10^8 dpm of ^{14}C, ^{3}H:^{14}C ratio = 1.36) into cardenolides in *Digitalis lanata*

Products	Specific activity (dpm of ^{14}C/mM)	^{3}H:^{14}C ratio
Digitoxigenin (1)	1.086×10^6	0.055
Gitoxigenin (2)	6.404×10^5	0.099
Digoxigenin (3)	1.026×10^5	0.050

diluted with cold material, purified and crystallized to constant specific activity. The results are summarized in Table 1.

The ^{3}H:^{14}C ratio of the labelled cardenolides undoubtedly shows that 14β-cholest-5-en-3β-ol-[15β-^{3}H] is not incorporated by *Digitalis lanata*, whereas in the same experiment cholesterol-[4-^{14}C] is transformed in the usual yields.

This strongly suggests that the epimerization at C-14, provided that this process is on the main biosynthetic pathway, does not occur with cholesterol itself, but with a more advanced precursor such as pregnenolone or progesterone.

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REFERENCES

1. Jacobsohn, G. M. (1972) *Recent Advances in Phytochemistry*, Vol. 3, p. 229. Appleton-Century-Crofts, Educational Division/Meredith Corporation, New York.
2. Canonica, L., Ronchetti, F. and Russo, G. (1970) *J.C.S. Chem. Comm.* 1675.
3. Aberhart, D. J., Lloyd-Jones, J. G. and Caspi, E. (1973) *Phytochemistry* **12**, 1065.
4. Caspi, E. and Lewis, D. O. (1968) *Phytochemistry* **7**, 683.
5. Tschesche, R., Hulpke, H. and Scholten, H. (1967) *Z. Naturforsch* (B) **22b**, 677.
6. Tschesche, R., Hombach, R., Scholten, H. and Peters, M. (1970) *Phytochemistry* **9**, 1505.
7. Gorodetsky, M., Kogan, D. and Mazur, Y. (1970) *J. Am. Chem. Soc.* 1094.
8. Anastasia, M., Scala, A. and Galli, G. (1976) *J. Org. Chem.* 1064.

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INHIBITION OF LIMONOID BIOSYNTHESIS IN LEAVES OF *CITRUS LIMON* BY TRIETHYLAMINE DERIVATIVES

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Key Word Index—*Citrus limon*; limonoids; triethylamine derivatives; inhibition; biosynthesis; metabolism.

Abstract—Both 2-(4-ethylphenoxy)triethylamine and 2-(3,4-dimethylphenoxy)triethylamine markedly inhibited the biosynthesis of limonoids in lemon leaves. However, neither significantly affected the biodegradation of limonoids.

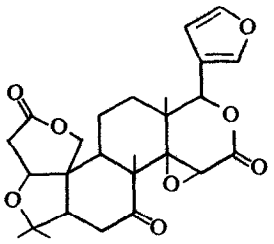
INTRODUCTION

Limonin (1) is an intensely bitter, tetracyclic, triterpenoid dilactone present in citrus seeds [1, 2]. Fruit and leaf tissues do not contain 1 but contain a precursor, limonoate A-ring lactone (2), which is gradually converted to 1 after juice extraction [3, 4]. The limonin bitterness in certain citrus juices and other processed products continues to be an important economic problem in citrus industry.

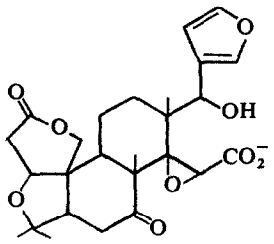
Limonoids have been shown to be synthesized in citrus leaves and translocated to the fruit [5]. Therefore, citrus leaves should be especially suitable for the study of limonoid biochemistry.

Derivatives of triethylamine, such as 2-(4-chlorophenylthio)triethylamine chloride and many others, have been shown to inhibit the cyclase(s) in carotenogenesis in citrus [6, 7] and microorganisms [8, 9]. Since limonoids are cyclic terpenoids, we believe that cyclase(s) must be involved in the biogenesis of limonoids. If so, triethylamine derivatives should inhibit the formation of limonoids in citrus.

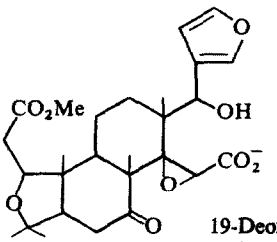
We report the effects of two such derivatives,



Limonin
(1)



Limonoate A-ring lactone
(2)



19-Deoxylimonoate
3-methyl ester
(3)

2-(4-ethylphenoxy)triethylamine and 2-(3,4-dimethylphenoxy)triethylamine on the biosynthesis and biodegradation of limonoids in lemon leaves.

RESULTS AND DISCUSSION

Both 2-(4-ethylphenoxy)triethylamine (**4**) and 2-(3,4-dimethylphenoxy) triethylamine (**5**) markedly inhibited the biosynthesis of **2** in lemon leaves. Table 1 shows the large differences in **2** content between the **4**-treated and control leaves. Eight days from treatment, control leaves contained 3 or 12.7 times as much **2** as leaves sprayed with 300 or 500 ppm of **4**, respectively.

Leaves, particularly young ones, appeared yellow when sprayed with 500 ppm of **4** and slightly yellow when sprayed with 300 ppm of **4**, a few days after treatments. Leaves treated with 300 ppm of **5**, however, appeared to be healthy and normal.

Compound **5** was less effective than **4** but also decreased **2** content significantly. After 6 and 10 days of treatment, control leaves contained about 2.5 times as much **2** as leaves sprayed with 300 ppm of **5** (Table 2). Detailed quantitative analyses of nomilinic acid, which is the other major limonoid in lemon, were not carried out. However, we estimated that **4** and **5** inhibited the synthesis of nomilinic acid to about the same extents that they inhibited the synthesis of **2**.

These results suggested the involvement of a cyclase(s), which is inhibited by the derivatives of triethylamine, in the biosynthesis of limonoids in lemon leaves.

The significant differences between the treated and control leaves with respect to **2** and nomilinic acid contents, together with the fact that young leaves contain much more **2** than old leaves [5], strongly suggest that limonoids turn over continuously in leaves.

We tested the effect of the inhibitors on the biodegradation of limonoid in leaves. Experience has shown that labelled 19-deoxylimonoate 3-methyl-^{14}C ester (**3**), an analogue of **2**, is an excellent substrate for limonoid metabolizing enzymes, and moreover, it is easily prepared and stable [5, 10, 12]. About 20000 cpm of **3** were fed via the stem to detached control leaves and leaves that had been treated with 300 ppm of **4** or **5**, 3 days before being fed the labelled compound. The leaves, which were supplied water through the stem, were then placed in a room illuminated 14 hr per day by fluorescent light. After 3 days, the leaves were extracted, and radiochromatograms were obtained for each extract. Both the 4-treated and control produced very similar metabolites. Compound **5** gave practically the same pattern of metabolites. These results showed that both **4** and **5** had no significant effect on the biodegradation of limonoids in lemon leaves.

Previous findings [10, 11, 12] support the above conclusions. Limonoids are metabolized through at least two pathways: one via 17-dehydrolimonoids, and the other via deoxylimonoids. Neither pathway appears to involve cyclase activity, at least at the early stages.

Table 1. Effect of 2-(4-ethylphenoxy)triethylamine on the biosynthesis of limonoate A-ring lactone in lemon leaves

	Wt. of leaves (mg)	Limonoate A-ring lactone (μg)	(ppm)
Exp. 1*			
Control	755	260	344
Treated	724	20	27
Exp. 2†			
Control	652	255	391
Treated	671	86	128

* Exp. 1 A branch of the lemon tree was sprayed with 500 ppm of 2-(4-ethylphenoxy)triethylamine. After 8 days, young leaves were analyzed for limonoate A-ring lactone. Exp. 2 Treatments were similar to those of Exp. 1, except that a branch was sprayed with 300 ppm.

Table 2. Effect of 2-(3,4-dimethylphenoxy)triethylamine on the biosynthesis of limonoate A-ring lactone in lemon leaves*

	Wt. of leaves (mg)	Limonoate A-ring lactone (μg)	(ppm)
Exp. 1			
Control	412	200	485
Treated	424	80	189
Exp. 2			
Control	760	1120	1474
Treated	786	477	606
Exp. 3			
Control	412	676	1640
Treated	425	260	612

* Branches of Meyer (Exp. 1) and of an unidentified (Exp. 2 and 3) lemon tree were sprayed with 300 ppm of 2-(3,4-dimethylphenoxy) triethylamine. After 6 days (Exp. 1 and 2) and 10 days (Exp. 3), leaves were analyzed for limoniate A-ring lactone.

EXPERIMENTAL*

Materials. Sil gel G plates were used for quantitative and radiochromatographic analyses. The following solvent systems were used: (1) C_6H_6–EtOH–H_2O–HOAc (200:47:15:1, upper

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

layer) and (2) cyclohexane–EtOAc (3:7). Radioactively labelled 19-deoxylimonoate 3-methyl-^{14}C ester was obtained from R. D. Bennett of our laboratory. Compound **4** was synthesized by the method of Poling *et al.* [7], and **5** was synthesized from 2-diethylaminoethylchloride and 3,4-dimethylphenol according to Schuetz and Baldwin [13]. X-77 Spreader, Ortho, was purchased from Chevron Chemical Company, San Francisco, California. A Meyer tree and an unidentified lemon tree were used. The latter was grown from a seedling.

Quantitative analyses of limonoids. Compound **2** was extracted from leaves in the form of **1**, and its quantity was estimated by the procedure described previously [5].

Spray treatments. The aq. solns of **4** and **5** containing 0.1% of the X-77 spreader were sprayed on branches from the top to about 50 cm down, where phloem tissues were cut to prevent translocation of the chemicals to other branches.

Analyses of labelled metabolites. Labelled metabolites were extracted from leaves as described previously [5]. A portion of the extract, which contained about 2500 cpm, was spotted on a thin layer plate and developed with solvent (2). The chromatogram was analyzed with a radiochromatogram scanner.

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REFERENCES

1. Arigoni, D., Barton, D. H. R., Corey, E. J., Jeger, O., Caglioti, L., Dev, S., Ferrini, P. G., Glazier, E. R., Melera, A., Pradhan, S. K., Schaffner, K., Sternhell, S., Templeton, J. F. and Tobinaga, S. (1960) *Experientia* **16**, 41.
2. Barton, D. H. R., Pradham, S. K., Sternhell, S. and Templeton, J. F. (1961) *J. Chem. Soc.* 255.
3. Maier, V. P. and Beverly, G. D. (1968) *J. Fd Science* **33**, 488.
4. Maier, V. P. and Margileth, D. A. (1969) *Phytochemistry* **8**, 243.
5. Hasegawa, S. and Hoagland, J. E. (1977) *Phytochemistry* **16**, 469.
6. Coggins, Jr., C. W., Henning, G. L. and Yokoyama, H. (1970) *Science* **168**, 1589.
7. Poling, S. M., Hsu, W. J. and Yokoyama, H. (1975) *Phytochemistry* **14**, 1933.
8. Hsu, W. J., Yokoyama, H. and Coggins, Jr., C. W. (1972) *Phytochemistry* **11**, 2985.
9. Batra, P. P., Gleason, Jr., R. M. and Louda, J. W. (1973) *Phytochemistry* **12**, 1309.
10. Hasegawa, S., Bennett, R. D., Maier, V. P. and King, Jr., A. D. (1972) *J. Agr. Fd Chem.* **20**, 1031.
11. Hasegawa, S., Maier, V. P., Border, S. N. and Bennett, R. D. (1974) *J. Agr. Fd Chem.* **22**, 1093.
12. Hasegawa, S., Maier, V. P. and Bennett, R. D. (1974) *Phytochemistry* **13**, 103.
13. Schuetz, R. D. and Baldwin, R. A. (1958) *J. Am. Chem. Soc.* **80**, 162.

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ANTIMICROBIAL COMPOUNDS OF THE MARINE RED ALGA *MARGINISPORUM ABERRANS*

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Key Word Index—*Marginisporum aberrans*; Corallinaceae; red alga; *p*-hydroxybenzaldehyde; dichloroacetamide; 3,5-dinitroguaiacol; antimicrobial activity.

The fresh alga (15 kg) was washed with water, air-dried and extracted with MeOH. The extract showed marked antimicrobial activity against *Bacillus subtilis*. After removing the solvent, the residue was separated into *n*-hexane- and Et_2O-soluble neutral, EtOAc-soluble acidic and Et_2O-soluble basic fractions. Si gel chromatography of the Et_2O-soluble neutral fraction (*n*-hexane–EtOAc, 3:1) gave an active compound (8 mg), mp 113–114°, which was found to be identical with *p*-hydroxybenzaldehyde by comparison (mmp, IR, NMR, MS) with authentic sample. Recently, Fenical and McConnell [1] also isolated *p*-hydroxybenzaldehyde as an antimicrobial component of the red alga *Dasya pedicellata* var. *stanfordiana*.

Continued elution gave dichloroacetamide (15 mg), mp 98–99°, which was identical in all respects (IR, NMR, MS) to an authentic sample. In 1967, Khaskin and coworkers [2] synthesized a variety of amides and measured their antimicrobial activities, and they found dichloroacetamide showing moderate activity against *Botorytis cinerea* and *Alternaria radicina*.

Si gel chromatography of the EtOAc-soluble acidic fraction using $CHCl_3$–EtOAc (5:1) and crystallization from *n*-hexane–Et_2O afforded 3,5-dinitroguaiacol (10 mg), mp 124–125°; $\nu_{max}^{CCl_4}$ cm^{-1}: 3500, 1625, 1570, 1550 and 1355; λ_{max}^{MeOH} nm (log ε): 213(4.33), 266(3.98), 332(3.81) and 410(3.04); $\delta_{TMS}^{CDCl_3}$ (100 MHz): 4.06(3H, s), 8.02(1H, d J = 3 Hz), 8.74(1H, d, J = 3 Hz) and 11.22(1H, s).