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Metabolism of Thymol and *trans*-Anethole in Larvae of *Spodoptera litura* and *Trichoplusia ni* (Lepidoptera: Noctuidae)

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Metabolism of the monoterpenoid thymol and the phenylpropanoid *trans*-anethole, both constituents of essential oils, was investigated following topical and oral administration of the compounds to the tobacco cutworm (*Spodoptera litura*) and the cabbage looper (*Trichoplusia ni*). In both species and irrespective of route, administration of thymol resulted in the excretion of its 3-*O*-β-glucoside, whereas *trans*-anethole was hydroxylated on the side chain methyl group. Both metabolites were isolated and their structures elucidated by interpretation of their mass and NMR spectra. Previous experiments indicated that *trans*-anethole synergized the toxicity of thymol in *S. litura*, but analyses of feces indicated that metabolism of thymol was not significantly suppressed when the two compounds were orally coadministered to *T. ni* larvae.

KEYWORDS: Metabolism; *Spodoptera litura*; *Trichoplusia ni*; thymol; *trans*-anethole; synergistic effects

INTRODUCTION

The characteristic smell of plants is mainly due to the presence of compounds volatile at room temperature. Such compounds can be separated from the plant by steam distillation or suet extraction ("en fleurage"), often used in the perfume industry. The resulting crude mixture of many volatile compounds is referred to as the essential oil of a plant. Essential oils often consist of a complex mixture of lipophilic compounds with relatively low molecular weights (e.g., mono- or sesquiterpenes) and/or biogenically different phenylpropanes. Essential oils are usually stored in special compartments in plants (e.g., oil cells, oil streams, or glandular trichomes).

Many essential oils have antiseptic activities and some have spasmolytic activities and therefore can be used to treat different kinds of gastrointestinal and respiratory disorders. However, essential oils also likely serve a defensive role for the plants that produce them. Many of the oils and their major constituents are acutely toxic to insects, and/or repellent or deterrent (*1*). Metabolism of these substances by insects can therefore represent a system of detoxification, making it possible for the insect to tolerate these toxins in their diet. Miyazawa and colleagues investigated metabolism of (+)- and (−)-limonene (*2*), (+)- and (−)-menthol (*3*), and β-myrcene (*4*) in *Spodoptera litura*. They found that these monoterpenes were oxidized through diol formation (via epoxidation), aliphatic hydroxylation, and glycol formation, respectively, all of which would render the substrates more hydrophilic and thus readily excretable by the insect. Metabolism of 1,8-cineole (=eucalyptol) has been investigated in a number of insect species, the majority of which produce metabolites formed through monohydroxylation of the carbon ring system (*5*−*7*).

In a recent paper, the toxicity of thymol (**1**), the main constituent of the essential oil of thyme, *Thymus vulgaris* (Lamiaceae) against late instar larvae of *Spodoptera litura* was reported (*8*). Additionally, it was shown that simultaneous administration of the phenylpropanoid *trans*-anethole (**2**), from the essential oil of anise, *Pimpinella anisum* (Lamiaceae), had a synergistic effect on the toxicity of thymol to the larvae (*8*).

Because such effects can either result from different modes-of-action of two structurally different compounds or by the impact of one of the synergists on the metabolism (detoxification) of the other, we investigated the metabolism of **1** and **2** after oral and topical administration in last instar larvae of two agricultural pests, the tobacco cutworm, *Spodoptera litura*, and the cabbage looper, *Trichoplusia ni*.

MATERIALS AND METHODS

Test Compounds. Thymol (5-methyl-2-(1-methylethyl) phenol, CAS number 89−83−8) and *trans*-anethole (1-methoxy-4-(1-propenyl)-benzene, CAS number 4180−23−8) were purchased from Sigma-Aldrich. Purity (>99%) was confirmed by GC and HPLC.

NMR. NMR was performed on a Bruker ARX 500 and AM 500.

GC-MS. EI (70 eV) HP MSD 5972 with GC 5890 plus (HP); Optima-1 (MN), 25-m × 0.25-mm; 150 °C (3 min) to 280 °C at 10°

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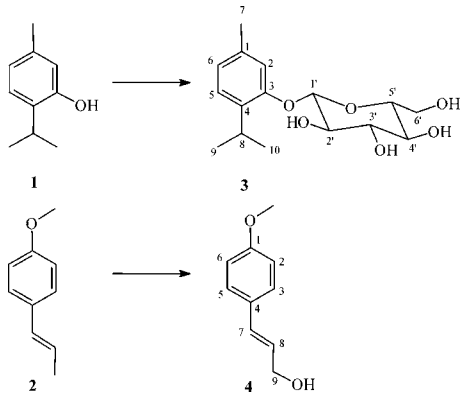
min^{-1}. R_t-value: **1**, 8.44 min; **3**, 20.86 min. MS: 252 (2), 235 (2), 150 [M-glucosyl]$^+$ (30), 135 [150-CH_3]$^+$ (100), 115 (20), 107 (10), 91 (35), 65 (10), 44 (15), 41 (10).

DCI. Kratos Compact HQ MS 80, DCI+, NH_3 (rel. Int.). **4**: 182 (10) [M + NH_4]$^+$, 164 (29), 147 (100), 121(40), 108 (21), 91 (8), 79 (12).

LC-MS. Esquire LC 85, ESI, 87.4 V, infusion. **3**: 335 [M + Na]$^+$ (100), 249 (10), 236 (28), 180 (5), 107 (5).

HPLC. HP 1050, DAD. 215 and 260 nm, Hibar RP 18 LiChrosorb (7-μm, 25.0- × 7-mm), flow 3.0 mL min^{-1}. CH_3CN-H_2O (27:75). R_t-value: **3**, 7.1 min; **4**, 0.3.

TLC. Silica gel 60 F254, toluene/EtOAc (4:1). R_f-value: **3**: 0.19, **4**: 0.3.



Metabolism Experiments. Larvae of *Spodoptera litura* and *Trichoplusia ni* were obtained from laboratory colonies maintained for at least 10 generations on artificial diet. A total of 80 last instar larvae (4 cohorts of 20 larvae each) of *S. litura* or *T. ni*, respectively, were placed on artificial diet (*8*) containing the test compounds **1** or **2**, respectively, at a concentration of 0.1% fwt. A group of 20 larvae fed on regular diet served as the control. After 3 days of feeding, the feces from each group was collected and immediately extracted with CH_2Cl_2. After evaporation of the solvent, 28.7 mg (thymol feeding) and 81.2 mg (anethole feeding) of extract were obtained. Spots representing putative metabolites in TLC were detected by comparison of the feces extracts from the treatment groups with that from the control. The respective metabolites were then isolated by preparative TLC after dissolution in CH_2Cl_2. We obtained 3.2 mg of **3** and 5.8 mg of **4**.

In a second experiment, 30 last instar larvae of *T. ni* were each treated topically on the abdominal terga with 100 μg of **1** in 1 μl MeOH. Feces of these caterpillars, fed on the regular diet, was collected and extracted as described above. The thymol metabolite **3** was detected in the extract by direct comparison in TLC and HPLC and LC-MS, and its identity was unambiguously proven. We did not attempt to quantify rates of metabolism or excretion in the first or second experiment as our goal was strictly to identify putative metabolites.

In a third experiment, last instar larvae of *T. ni* (n = 4 replicates with 20 larvae each) were fed diets containing 0.1% fwt of **1** and concentrations of **2** varying from 0 to 0.1% fwt. Feces from each cohort on each treatment was collected and extracted with CH_2Cl_2. After evaporation of the solvent, all resulting extracts were dissolved in MeOH to give 5% solutions. An aliquot (10μl) of each was analyzed by HPLC for **1** and **3** with four replicates. A solution of thymol (0.1%) was used as an external standard.

RESULTS AND DISCUSSION

The incorporation of thymol (**1**) (0.1% fwt in the regular artificial diet) resulted in the isolation and identification of **3** from the collected feces of late instar larvae of *Spodoptera litura*. No other metabolite was found, but unchanged thymol (**1**), was also excreted. The molecular ion and the fragmentation in the ESI LC-MS spectrum indicated that thymol was combined with a hexose type sugar. The fragmentation in the additionally recorded EI mass spectrum showed that the thymol moiety was unchanged. The structure of **3** was then unambiguously identified as thymol 3-*O*-β-glucoside by its ^{1}H and ^{13}C NMR spectra (**Table 1**). Assignment of the signals was made through interpretation of additionally recorded 2D-COSY, HMQC, and HMBC experiments.

Table 1. NMR Data of **3** and **4** (125 and 500 MHz, respectively; DMSO-*d*6)

	3			4	
	^{13}C	^{1}H		^{13}C	^{1}H
1	135.68		1	129.46	
2	115.83	6.88 s (br.)	2	113.96	7.34 d, J = 8.4 Hz
3	154.63		3	127.27	6.87 d, J = 8.6 Hz
4	134.12		4	158.53	
5	125.43	7.04 d, J = 7.7 Hz	5	127.27	6.87 d, J = 8.6 Hz
6	122.43	6.75 d (br.), J = 7.45 Hz	6	113.96	7.34 d, J = 8.4 Hz
7	20.94	2.22 s	7	128.26	6.46 d, J = 16.0 Hz
8	25.62	3.30 m	8	128.28	6.20 m
9	22.99	1.12 d, J = 6.9 Hz	9	61.57	4.07 s (br.)
10	22.72	1.10 d, J = 6.95 Hz	OCH_3	55.02	3.74 s
1‘	101.29	4.74 d, J = 7.25 Hz			
2‘	73.50	3.24 dd, J = 3.15 u. 7.55 Hz			
3‘	76.89	3.26 m			
4‘	69.88	3.16 m			
5‘	77.05	3.25 m			
6‘	60.81	3.69 m			
		3.44 m			

Compounds **1** and **3** were also isolated from the feces of *T. ni*, confirming the same metabolic pathway in this insect.

After feeding on a diet containing *trans*-anethole (**2**) (0.1% fwt), the feces from a group of *T. ni* was extracted, and one main metabolite (**4**) was isolated. We did not observe any parent compound (**2**) in the feces. Interpretation of the NMR spectra (**Table 1**) clearly showed that **2** was hydroxylated by the insects on the side chain. In comparison to the NMR spectra of **2**, the signals for the methyl group of the side chain (δ 17.7 (^{13}C) and 1.80 ppm (3H,^{1}H)) are replaced by signals at δ 61.6 (^{13}C) and 4.07 ppm (2H,^{1}H), which clearly indicated the presence of a hydroxy group at this position. The signals for the other carbons and protons of the side chain were subsequently shifted and found at the appropriate shift values (**Table 1**) expected for an unsaturated three-carbon side chain containing a terminal hydroxy function.

As previously found for other monoterpenes (*2*−*7*), both metabolic pathways led to more hydrophilic products in a single step. The introduction of a single hydroxy-group to monoterpenes in *S. litura* was already shown for menthol (*3*), and monohydroxylation appears to be the major metabolic process for 1,8-cineole in three species of chrysomelid beetles (*5*), a pyrgomorphid grasshopper (*6*) and a pergid sawfly (*7*). In the case of limonene metabolism in *S. litura*, a diol is formed, presumably through epoxidation of a double bond. However, for *trans*-anethole, such reactions are hindered by conjugation of the double bond in the side chain with the aromatic system. The terminal methyl group of the propane side chain in **2** is therefore the only possible target for such metabolic hydroxylation reactions.

In contrast to this, **1** is combined with glucose without change of the monoterpenoid structure. This can be explained by the presence of the phenolic hydroxy group, which may be responsible for the neurotoxic activity of thymol. The combination with glucose offers a quick detoxification by masking the phenolic OH, and the resulting glucoside can easily be eliminated. This metabolic pathway was also found following

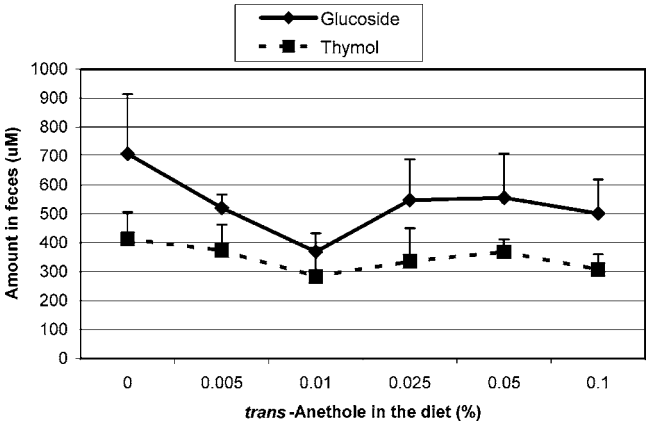


Figure 1. Relative quantities of parent thymol and its glucoside recovered from feces of *T. ni* larvae fed on artificial diets containing 0.1% (fwt) thymol and varying concentations of *trans*-anethole. Each point represents the mean of four replicates, error bars represent standard error of the mean.

topical administration of **1**. This indicates that thymol penetrates the insect integument and is excreted by the Malpighian tubules into the hindgut. The site of glucosylation thus remains unclear, although we can state that the transformation does not appear exclusive to the midgut.

Simultaneous treatment of *S. litura* larvae with **1** and **2** resulted in an enhancement of the toxicity of **1** (*8*)**.** As **2** was also previously shown to synergize toxicity of the insecticide parathion in insects (*9*), we hypothesized that **2** might synergize **1** by preventing its detoxicative metabolism to the glucoside **3**. To test this hypothesis, *T. ni* larvae were reared on diets containing 0.1% (fwt) **1** and concentrations of **2** varying from 0 to 0.1% (fwt). Compound **2** did not significantly suppress formation of the glucoside **3** (**Figure 1**). In the material excreted, the ratio of glucoside to parent was approximately 63:37 (on a molar basis) in the absence of **2**. Across treatment groups including **2**, the percentage of glucoside excreted ranged from 56.5 to 62.1%, with no dose-dependent trend nor significant differences between treatments. Our data therefore do not support the hypothesis that **2** suppresses metabolism of **1** in this insect.

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