Research Article

Effects of Quinizarin and Five Synthesized Derivatives on Fifth Larval Instar Midgut Ecdysone 20-Monooxygenase Activity of the Tobacco Hornworm *Manduca sexta*

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The plant allelochemical, quinizarin (1,4-dihydroxy-9,10-anthraquinone), and five anthraquinones that were synthesized from quinizarin, namely, 1,4-anthraquinone; 2-hydroxy-1,4-anthraquinone; 2-methoxy-1,4-anthraquinone; 9-hydroxy-1,4-anthraquinone; and 9-methoxy-1,4-anthraquinone, were assessed as to their effects on the essential, P450-dependent ecdysone 20-monooxygenase system of the insect model *Manduca sexta* (tobacco hornworm). This steroid hydroxylase converts the arthropod molting hormone, ecdysone, to the physiologically required 20-hydroxyecdysone form. *M. sexta* fifth larval instar midgut homogenates were incubated with increasing concentrations (10^{-8} to 10^{-3} M) of each of the six anthraquinones followed by ecdysone 20-monooxygenase assessments using a radioenzymological assay. Four of the five anthraquinones exhibited I_{50} 's of about 4×10^{-6} to 6×10^{-2} M. The most effective inhibitors were 2-methoxy-1,4-anthraquinone and 1,4-anthraquinone followed by 9-hydroxy-1,4-anthraquinone and 9-methoxy-1,4-anthraquinone. At lower concentrations the latter anthraquinone stimulated E20M activity. Quinizarin was less inhibitory and 2-hydroxy-1,4-anthraquinone was essentially without effect. Significantly, these studies make evident for the first time that anthraquinones can affect insect E20M activity, and thus insect endocrine regulation and development, and that a relationship between anthraquinone structure and effectiveness is apparent. These studies represent the first demonstrations of anthraquinones affecting any steroid hydroxylase system.

"This paper is dedicated with admiration to the legacy of Dr. Stan L. Smith, without whom this work and studies like it would not have been initiated."

1. Introduction

Ecdysone 20-monooxygenase (E.C. 1.14.99.22, E20M) is the insect cytochrome P450-dependent steroid hydroxylase responsible for the conversion of the arthropod molting hormone ecdysone (E) to its more active metabolite, namely, 20-hydroxyecdysone (20E) [1, 2]. The nature, regulation, and molecular biology of E20M were elucidated predominantly

employing the tobacco hornworm, *Manduca sexta*, as the model [3–8]. Indeed, the developmental impact of E20M was made evident in midgut tissue of the *M. sexta* fifth larval instar where E20M activity increases 50-fold between days four and five of the stadium and this increase is inextricably tied to the onset of wandering stage behavior [1, 8, 9]. Moreover, the timing conferred by pulses of 20E was found to be critical to all stages of insect development [1, 10].

The E20M system, therefore, represents a crucial target in terms of potential remediation and regulation of pestilent insects.

A number of plant allelochemicals, considered to have evolved to aid plants in their resistance to insect phytophagy, can affect insect steroid hydroxylase function [11–14]. Amongst these allelochemicals are limonoids, labdane diterpenoids, and polyketides that include flavonoids and some naphthoquinones [12–17].

Anthraquinones are compounds that are noted in plants, fungi, lichens, and insects and these compounds, or derivatives, are commonly used commercially in dyestuffs and for antineoplastic drug preparations [18–20]. The anthraquinone, quinizarin, is the usual starting material for the syntheses of other anthraquinones given its availability, affordability, and adaptability to a variety of chemical procedures, in addition to the fact that these products are readily purified chromatographically [19–21]. Based on the relationship of quinizarin to other plant allelochemicals and, more importantly, the fact that it lends itself readily to chemical manipulations allowing for substituted anthraquinone syntheses, the potential for quinizarin and synthetic derivatives to serve as endocrine/developmental effectors in insects by affecting the E20M system was apparent. Aside from the fact that the syntheses of anthraquinones permitted, for the first time, a study of chemical structure/function relationships as related to changes in E20M activity, they also permitted a first evaluation of the effects of these compounds on any steroid hydroxylase. Accordingly, an evaluation was pursued of the degrees to which quinizarin and five anthraquinone derivatives synthesized from quinizarin affect E20M activity in midgut tissues of the insect model, *M. sexta*.

2. Materials and Methods

2.1. Animal Maintenance. Animals used in these studies were day five, gate II, nondiapausing fifth instar larvae of the tobacco hornworm, *M. sexta*. They were reared and staged as described previously [22].

2.2. Tissue Preparation and Homogenization. Midgut tissues from day five, gate II, fifth instar larvae of *M. sexta* were dissected and rinsed in Lepidopteran Ringer's solution at 4°C [14]. Following dissection, tissues were blotted to surface dryness on bibulous paper, weighed, and homogenized, at 20 mg tissue/mL, in 50 mM sodium phosphate buffer (pH 7.5) containing 250 mM sucrose, using a tissue grinder with a motor driven Teflon pestle (275 rpm, 10 strokes) at 0°C.

2.3. E20M Assay. E20M activity was quantified using an *in vitro* radioenzymological assay [3, 9]. For these assessments, 0.05 mL aliquots of insect midgut homogenate (containing 1 mg tissue equivalent) were added to 0.05 mL of homogenization buffer, without sucrose, containing 3.3×10^{-7} M [23,24- $^3\text{H}_2$]-E; 1.6×10^{-3} M NADPH; and one of the six anthraquinones evaluated in concentrations from 1×10^{-8} to 1×10^{-3} M in the assay (assuming full anthraquinone solubility

in the assay medium and no selective partitioning into the membrane environment).

Anthraquinones were suspended in 100% ethanol so that the concentrations in the assays were as listed. Ethanol was evaporated prior to assessments as detailed for ecdysteroid additions to the assay [3]. Contents were mixed by vortexing the assay tubes and incubated at 35°C for 30 min with constant agitation. All assays were run in duplicate with zero-time controls and the incubations were terminated with the addition of 1.5 mL of 100% ethanol. Following termination, the assay tubes were centrifuged at $8,000 \times g$ for 10 min to collect precipitated protein. Subsequently, 0.15 mL aliquots of the assay supernatant (containing 10,000 dpm) plus 10 μL of ethanol containing 200 μg of non-radiolabeled E and 20E standards were evaporated to dryness.

The residues were dissolved in methanol and streaked onto analytical thin layer chromatography plates (0.25 mm silica gel 60, F-254, E. Merck, Darmstadt, Germany). The plates were developed in a solvent system of chloroform and 95% ethanol (4:1, v/v). After chromatogram development the E and 20E bands were visualized under short wavelength UV light. The visualized ecdysteroid bands were scraped into individual scintillation vials, suspended in 10 mL of scintillation fluid, and counted using a Beckman model 3800 scintillation counter (^3H counting efficiency, 60%).

Control E20M activity was expressed as pg 20E formed/min/mg tissue equivalent. The effects of anthraquinones on E20M activity were expressed as a percent of controls ($\pm$ SEM). Prior studies, using both normal and reverse phase chromatography, confirmed 20E as the product of this *in vitro* assay [3, 23–25].

2.4. Spectrophotometric Assessments of Potential Background Oxidation or Reduction of NADP(H) by the Anthraquinones. Potential oxidation of NADPH was evaluated spectrophotometrically employing a 1 mL reading volume containing 0.50 mL of 5 mM sodium phosphate (pH 7.5) and 1.6×10^{-4} M NADPH. To this was added 0.48 mL buffer without NADPH followed by 0.02 mL of the six individual anthraquinones in 100% ethanol to contain the concentrations, that is, 1×10^{-8} to 1×10^{-3} M, evaluated. Absorbance at 340 nm was measured over a 10 min period employing a Shimadzu UV-1700 spectrophotometer.

To assess potential background reduction of NADP⁺, acetylpyridine NADP⁺ (AcPyADP⁺) was used as the acceptor. The 1 mL reading volume contained 0.50 mL buffer containing 5 mM sodium phosphate (pH 7.5) and 1.6×10^{-4} M AcPyADP⁺. To this was added 0.48 mL buffer without AcPyADP⁺ followed by 0.02 mL of the six individual anthraquinones in 100% ethanol to contain the concentrations, that is, 1×10^{-3} to 1×10^{-8} M, evaluated. Absorbance at 375 nm was measured over 10 min employing a Shimadzu UV-1700 spectrophotometer.

2.5. Anthraquinone Synthesis. 1,4-Anthraquinone was prepared by quinizarin reduction according to established methods [20].

FIGURE 1: (a) and (c), the structures of quinizarin (QZ) and 1,4-anthraquinone (AQ); (b) and (d), the effects QZ and AQ, expressed as percent of controls, on E20M activity of day 5 midgut homogenates of gate II fifth instar *Manduca sexta*. Each value including controls is the mean $\pm$ SEM of three determinations done in duplicate. Control E20M activity for QZ was 403 ± 2.3 pg 20E formed/min/mg tissue, while control E20M activity for AQ was 485 ± 4.9 pg 20E formed/min/mg tissue. Asterisks indicate significant differences compared to controls: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Purification by flash chromatography using chloroform as the eluent afforded pure 1,4-anthraquinone (2.8 g, 81%). The identity and purity of this compound were confirmed by melting point determination, UV-Vis spectroscopy, infrared spectroscopy, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, and mass spectroscopy.

The 9-hydroxy and 9-methoxy derivatives of quinizarin were prepared as previously described [26]. The 2-hydroxy and 2-methoxy derivatives were prepared by known methods [27–29].

2.6. Statistical Analyses. All statistical differences between E20M activities of anthraquinone treated and control preparations were determined using ANOVA analysis followed by Tukey's Post Hoc comparison of the means of the group. Differences with a P value ≤ 0.05 were considered significant (JMP11, SAS).

2.7. Materials. Radiolabeled $[23,24\text{-}^3\text{H}_2]\text{-E}$ (stock of 55 ci/mmol, radiopurity > 99%) used in the E20M assays

was purchased from New England Nuclear, Boston, MA. The standards for the assay, that is, E and 20E, were purchased from Fluka Chemical, Ronkonkoma, NY. NADPH, AcPyADP⁺, and quinizarin (96% pure) were from Sigma-Aldrich, St. Louis, MO. Quinizarin was purified further (99%) by flash chromatography on silica gel using chloroform as the solvent. Salts and organic solvents were purchased from Fisher Scientific, Cleveland, OH. Scintillation fluid (Ultima Gold) was obtained from PerkinElmer, Waltham, MA.

3. Results

The abbreviations used for the anthraquinones evaluated are as follows: the parent compound quinizarin, QZ; 1,4-anthraquinone, AQ; 2-hydroxy-1,4-anthraquinone, 2OH; 2-methoxy-1,4-anthraquinone, 2MeO; 9-hydroxy-1,4-anthraquinone, 9OH; 9-methoxy-1,4-anthraquinone, 9MeO.

FIGURE 2: (a) and (c), the structures of 2-hydroxy-1,4-anthraquinone (2OH) and 2-methoxy-1,4-anthraquinone (2MeO); (b) and (d), the effects of 2OH and 2MeO, expressed as percent of controls, on E20M activity of day 5 midgut homogenates of gate II fifth instar *Manduca sexta*. Each value including controls is the mean $\pm$ SEM of three determinations done in duplicate. Control E20M activity for 2OH was 516 ± 4.5 pg 20E formed/min/mg tissue, while control E20M activity for 2MeO was 644 ± 5.4 pg of 20E formed/min/mg tissue. Asterisks indicate significant differences compared to controls: * $P < 0.05$; *** $P < 0.001$.

In the absence of enzyme, none of the anthraquinones evaluated acted to either reduce NADP^+ or oxidize NADPH nor did these compounds elicit conversion of E to 20E.

The parent compound, QZ (Figure 1(a)), significantly inhibited *M. sexta* midgut E20M activity in the concentration range of 10^{-6} to 10^{-3} M. Although at 10^{-4} M inhibition was suggested, it was not statistically significant (Figure 1(b)). Whereas the degrees of inhibition at these concentrations significantly differed from controls, with the greatest inhibition being 61% of controls at 10^{-3} M, and a decrease in inhibition was suggested with decreasing QZ content, the degrees of inhibition in the 10^{-6} to 10^{-3} M range did not differ significantly from one another. At the lower concentrations of QZ, that is, 10^{-8} and 10^{-7} M, the changes in E20M activity were not statistically significantly different from controls (Figure 1(b)).

With the chemical placement of the quinone moiety at a terminal position, accompanied by the removal of hydroxyl

groups, that is, synthesis of AQ (Figure 1(c)), significant inhibition of midgut E20M activity was noted. At concentrations of 10^{-4} and 10^{-3} M, E20M activity was significantly diminished to 34% and 25%, respectively, as compared to controls (Figure 1(d)). Whereas degrees of stimulation at 10^{-8} and 10^{-7} M followed by inhibition at 10^{-6} and 10^{-5} M were suggested with AQ, these latter differences in E20M activity did not differ statistically from controls. At 10^{-4} and 10^{-3} M, the inhibitions of enzyme activity significantly differed from controls as well as the activities noted at 10^{-8} and 10^{-7} M. AQ treatment at 10^{-3} M was significantly more inhibitory than at 10^{-5} M and both treatments significantly differed from treatment with 10^{-8} M AQ (Figure 1(d)).

Upon chemical addition of an adjacent hydroxyl function to the terminal quinone of AQ, resulting in the synthesis of 2OH (Figure 2(a)), the anthraquinone was essentially ineffective in altering E20M activity. Although a degree of stimulation at 10^{-4} M and inhibition at 10^{-3} M, respectively,

FIGURE 3: (a) and (c), the structures of 9-hydroxy-1,4-anthraquinone (9OH) and 9-methoxy-1,4-anthraquinone (9MeO); (b) and (d) the effects of 9OH and 9MeO, expressed as per cent of controls, on E20M activity of day 5 midgut homogenates of gate II fifth instar *Manduca sexta*. Each value including controls is the mean $\pm$ SEM of three determinations done in duplicate. Control E20M activity for 9OH was 377 ± 1.9 pg 20E formed/min/mg tissue, while control E20M activity for 9MeO was 237 ± 1.4 pg of 20E formed/min/mg tissue. Asterisks indicate significant differences compared to controls: * $P < 0.05$; *** $P < 0.001$.

were suggested, no significant differences were noted between experimental and control assessments throughout the concentration range of 2OH evaluated (Figure 2(b)).

Addition of a methoxy group, in lieu of a hydroxyl group, to the terminal quinone of AQ resulted in an inhibitory anthraquinone, namely, 2MeO (Figure 2(c)). At the three highest concentrations examined, that is, 10^{-5} to 10^{-3} M, midgut E20M activities were decreased significantly, 30%, 19%, and 27%, respectively, as compared to controls. At the concentration range of 10^{-8} to 10^{-6} M, lesser degrees of inhibition were suggested, although the 10^{-7} M treatment proved significant at 73% of control activity (Figure 2(d)).

With the placement of a hydroxyl function at carbon 9 of AQ, that is, the synthesis of 9OH (Figure 3(a)), a molecule capable of inhibiting E20M activity was synthesized (Figure 3(b)). At 10^{-5} to 10^{-3} M, 9OH exerted significant inhibitions, namely, 63% to 40% of controls. At the lower concentrations of 9OH examined, that is, 10^{-8} to 10^{-6} M, no significant changes in E20M were noted, although a suggested

increase in activity at 10^{-7} M 9OH was apparent. However, the levels of E20M activity observed at 10^{-5} and 10^{-4} M 9OH were significantly lower than that observed for 10^{-7} M (Figure 3(b)).

With chemical substitution of a methoxy function for the 9 hydroxyl group of 9OH, that is, the synthesis of 9MeO (Figure 3(c)), a molecule that displayed concentration dependent stimulatory and inhibitory effects on E20M was made (Figure 3(d)). In the concentration range of 10^{-8} to 10^{-6} M, stimulation was apparent with statistically significant increases noted at 10^{-7} and 10^{-6} M, that is, 136% and 130% of controls, respectively. Conversely, in the concentration range from 10^{-5} to 10^{-3} M, 9MeO inhibition was apparent with statistically significant effects noted at 10^{-4} and 10^{-3} M, that is, 59% and 46%, respectively (Figure 3(d)).

The efficacy of QZ and the synthesized anthraquinones in terms of E20M inhibition is reflected in the I_{50} values listed in Table 1. The most effective diminution of E20M

TABLE 1: I_{50} Values for the anthraquinones tested.

Anthraquinone	I_{50} value
2-Methoxy-1,4-anthraquinone	4×10^{-5} M
1,4-Anthraquinone	3×10^{-4} M
9-Hydroxy-1,4-anthraquinone	1×10^{-3} M
9-Methoxy-1,4-anthraquinone	2×10^{-3} M
Quinizarin	6×10^{-2} M
2-Hydroxy-1,4-anthraquinone	2×10^4 M

activity was noted with 2MeO. The I_{50} for AQ was essentially an order of magnitude greater and both 9OH and 9MeO, respectively, displayed values reflecting a lesser potency than AQ. The parent compound, QZ, was less effective than the above mentioned derivatives. In turn, the anthraquinone least capable of exerting an effect on *M. sexta* E20M was 2OH (Table 1).

4. Discussion

The effects of plant allelochemicals on insect E20M activity have been noted not only with respect to the model, *M. sexta*, but with other insect systems as well, for example, *Aedes aegypti* (mosquito), *Drosophila melanogaster* (fruit fly), and *Spodoptera frugiperda* (fall army worm) [12–17, 30, 31]. However, until the present study, no data relating to the effects of anthraquinone allelochemicals on insect E20M were available. This is of particular significance inasmuch as the anthraquinone allelochemical, QZ, readily lends itself to chemical derivatization, thereby allowing for studies of the relationship of chemical structure to the effects of the resulting synthetic compounds. The data presented in our study constitute the first demonstrations of anthraquinones effectively inhibiting (and to a lesser degree stimulating) *M. sexta* E20M activity in a dose-dependent fashion. Moreover, the present study represents the first demonstration that anthraquinones affect a P-450 dependent steroid hydroxylase in any invertebrate or vertebrate system.

Considered within the context of chemical reactivity, some characteristics of the examined anthraquinones could impact potential interactions with the *M. sexta* E20M system [32]. For example, terminal placement of the quinone moiety and removal of the hydroxyl groups from QZ allow for an increased potential for nucleophilic activity on the part of the resulting AQ. Furthermore, the placement of a hydroxyl moiety at the number 2 carbon of AQ allows for a tautomeric shift and orthoquinone formation of the resulting 2OH compound. In turn, such tautomerization could reduce potential interactions with the E20M system. Placement of a hydroxyl group at the number 9 carbon position of AQ potentiates hydrogen bonding between hydroxyl and carbonyl functions of 9OH while addition of a methoxy group to the number 2 carbon of AQ allows the electron-donating ability of the methoxy group to render the adjacent position of the 2MeO quinone more susceptible to cationic interaction.

The present study made evident that the effects of the anthraquinones examined on *M. sexta* E20M activity were

not attributable to any nonenzymatic reactions affecting either the NADP(H) content in the assays or the conversion of E to E20. Clearly, with the exception of 2OH, syntheses performed using the QZ parent resulted in compounds displaying enhanced inhibition of *M. sexta* day five, fifth larval stage E20M activity. In terms of comparative inhibitory efficacy, the most active synthetic product was 2MeO followed by AQ, the latter being about an order of magnitude less inhibitory. Although treatment of *M. sexta* enzyme preparations with 2OH proved essentially ineffective and QZ was the least effective of the compounds displaying significant inhibition, it is noteworthy that both the 9OH and 9MeO derivatives were about an order of magnitude more inhibitory than QZ as reflected in I_{50} values.

The impact of the chemical characteristics of the anthraquinones and the mechanism(s) of anthraquinone inhibition of *M. sexta* E20M certainly merit further study; our data make apparent marked differences in inhibition that are dependent upon the positional alkyl and hydroxyl substitutions of QZ-derived AQ. An additional underpinning for further studies is noted in our findings that lower concentrations of 9MeO significantly stimulated E20M activity while higher concentrations were significantly inhibitory. Prior evaluations of plant allelochemicals on insect E20M activity demonstrated both inhibitory and stimulatory effects [12–17]. Yu [30, 31] noted allelochemical stimulation of the juvenile hormone III oxidase (a monooxygenase) as well as E20M of the fall army worm, *S. frugiperda*. Indeed, more recently, several plant flavonoids were found to be inhibitory/stimulatory with respect to other insect enzymes, namely, the mitochondrial, membrane associated NADPH $\rightarrow$ NAD⁺ transhydrogenase, NADH oxidase, and succinate dehydrogenase systems of *M. sexta* fifth larval instar midgut and fat body [33]. Thus, additional potential targets for the study of anthraquinone effects also merit consideration.

In light of their effects on E20M, and therefore insect endocrine regulated development and potential insect control, further studies of the anthraquinones evaluated as well as other anthraquinone derivatives are warranted. In addition, the relationship between anthraquinone molecular structure and effects on E20M was made apparent for the first time. As the data presented are the first indications of anthraquinones affecting a steroid hydroxylase system, comparative evaluations of the impact of anthraquinones on steroid hydroxylases generally also will be most informative.

5. Conclusions

This study shows for the first time that QZ and anthraquinones synthesized from the QZ parent compound warrant evaluation as effectors of a crucial enzyme mediating arthropod molting and metamorphosis. This study revealed that strategic placement of functional groups on the anthraquinone nucleus has significant effects on the inhibitory/stimulatory activity of these compounds. In light of their effects on insect development, via affecting E20M activity, and potential effects on steroid hydroxylases in general, further studies are clearly warranted.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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