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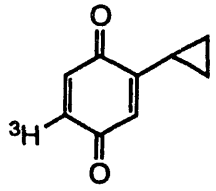
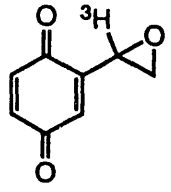
As suggested by the reviewers of our grant application, most of our efforts have focused on the first set of our primary objectives. We have pursued inhibitor development and found that the inhibitors (both the cyclopropyl- and oxiranequinones) are intolerant of other substituents on the quinone nucleus and are only marginally tolerant to substitution on the three-membered rings. Therefore the production of labelled compounds have been restricted to ^{3}H and ^{14}C isotopes. The tritiated cyclopropane and oxirane benzoquinones were prepared as labeled inhibitors.



[^{3}H] 2-cyclopropyl-5-tritio-p-benzoquinone
(22 mCi/mol)



[^{3}H] 2-oxirane-1'-tritio-p-benzoquinone
(330 mCi/mmol)

NAD(P)H - 2,6-DMBQ oxidoreductase. Striga plants were regenerated from callus cultures and maintained under quarantine conditions. Culture conditions were optimized for root growth; stem and root tissues were removed and fractionated by the procedures of Larsson (1). The material from each step of the fractionation procedure were evaluated for NAD(P)H - 2,6-DMBQ oxidoreductase activity that was inhibited by the cyclopropyl and oxirane compounds. A single activity was identified; one that fractionated not with the plasma membranes (the upper phase of the two-phase system but with the other membrane fraction (lower phase) as determined by marker enzymes. It required several hours of incubation to inhibit the activity with the cyclopropylquinone, similar to the time dependence for the *in vivo* inhibition (2,3), suggesting a rather high turnover number before the inhibitor leads to enzyme inactivation. Proceeding on that assumption, the product distribution of the [^{3}H] cyclopropyl compound following incubation with the lower phase membrane fraction was monitored with HPLC. A proposal to explain the products that were found is given in Scheme 1. The dominant reaction is the reversible addition and removal of one electron from the quinone. The subsequent mechanisms shown are only one possibility and are postulated to explain the observed products.

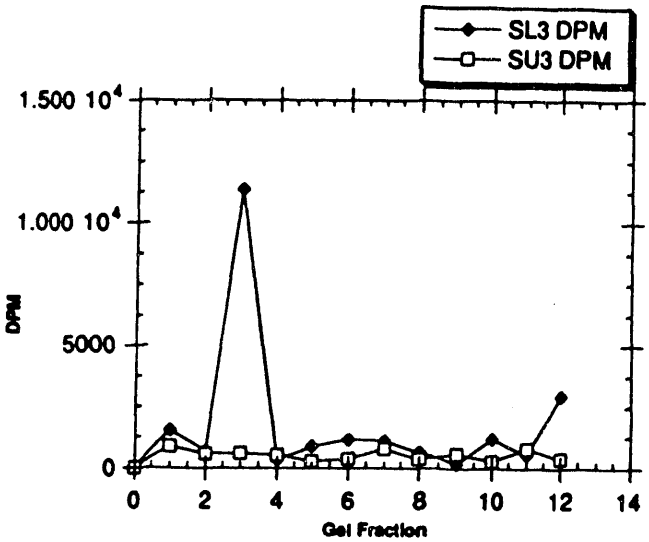
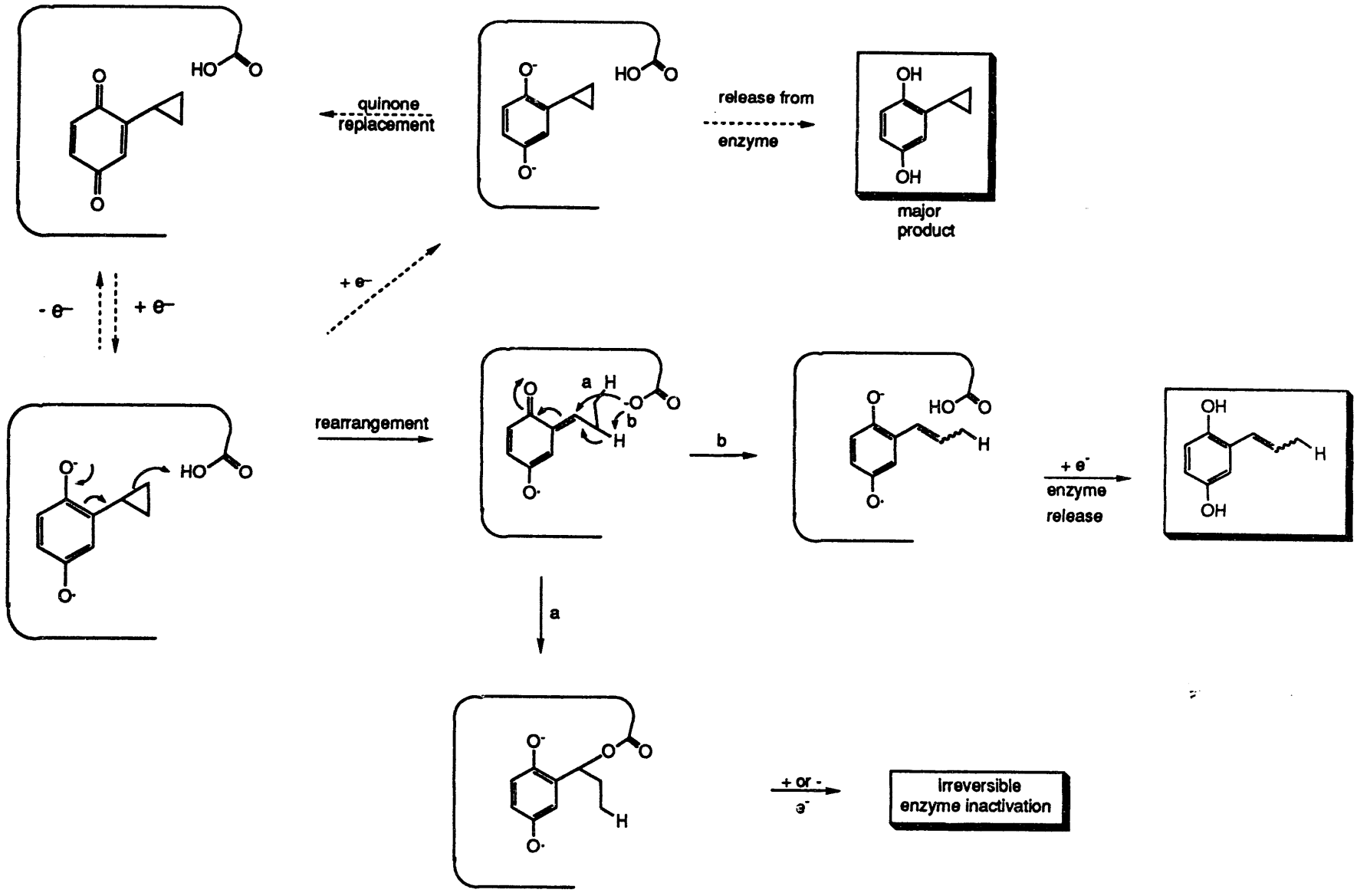


Figure 1. The radioactivity associated with specific regions of the SDS-PAGE gel of the lower (SL_3) and upper (SU_3) cell fractions following exposure to NADH and [H]oxirane-p-benzoquinone for 4 hrs.

Affinity Labelling. The discovery of the styrene derivative shown in Scheme 1 was consistent with the model we had proposed for the cyclopropyl benzoquinone inhibition. The specific activity of the tritiated cyclopropyl derivative was not high enough to use for affinity labeling studies and the oxirane derivative was employed. Even this material was not hot enough for autoradiography and higher specific activity material is currently being prepared. Nevertheless, scintillation counting of sections of the

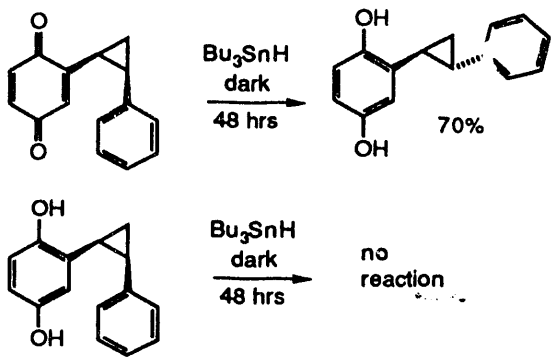


Scheme 1. Proposed pathways to explain the products observed in the in vitro reaction of the lower phase fraction with the cyclopropyl-quinone. Dotted arrows ⇢ indicate the major pathway, solid → is more minor.

SDS-PAGE gel run on the lower phase fraction (SL_3) that had been incubated with the oxirane for four hours did show a single region (75 to 90 kDa) of incorporated radioactivity (Figure 1, The labeling (i) showed the same time dependence of incorporation as the time dependence for the inhibition, (ii) required the addition of NADH and an active protein fraction, (iii) was partially protected with added 2,6-DMBQ (the natural ligand), and (iv) was completely protected by cyclopropylquinone.

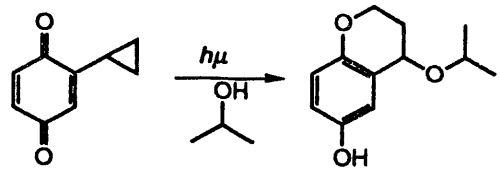
Model Reactions of the Inhibitors. There is currently no direct proof that the identified cell free fraction is required for haustorial induction. However, our interest in this fraction has been increased by further work on the chemistry of these inhibitors. There were other NADH-quinone oxidoreductases that were identified during the fractionation. These other enzymatic activities were not inhibited by the cyclopropyl derivative nor did they appear to induce the ring-opening reactions observed here. Moreover, under a series of reaction conditions where the quinone was reduced, the cyclopropyl ring could not be induced to open.

Earlier studies (4) had established that delocalized radical density would not give cyclopropyl ring opening whereas a primary radical localized α to a cyclopropane opened at a rate of 10^8 sec^{-1} (5). With the cyclopropyl quinones the absence of observed ring opened products is apparently a thermodynamic result. The ring-opening reaction can be monitored by the loss of stereochemistry of the *cis*-disubstituted cyclopropanes. For example, **1** racemizes to **2** under the reduction conditions (6) whereas **3** under the same conditions does not react.



Therefore we can propose that the *Striga* oxidoreductase changes the equilibrium position enabling ring opening and inactivation. Scheme 1 suggests that the structural feature causing this difference is the proximity of a carboxylic acid residue to the quinone binding site. Therefore, these inhibitors are very specific and may uniquely inactivate the enzymes of haustorial induction. Further characterization of the enzyme will allow this proposal to be tested.

The Photochemical Reaction. A second new reaction discovered in the course of these studies is the ring-opening on the photochemical surface. Photochemical activation of the cyclopropylquinone leads to the unprecedented reaction with nucleophilic solvents as shown below.



The photolysis of the cyclopropylquinone in the proposed binding site shown in Scheme 1 could lead to the same product as that of reductive alkylation or could react with other proximal nucleophiles. The discovery of this reaction makes it possible to exploit a photoaffinity labeling strategy as well. In fact, the development of this chemistry may make it possible to covalently label any general quinone binding protein. This approach could then be extended to other parasitic plants or even other plants and animals generally.

Molecular Genetic Strategies. Even though Striga samples are now available through tissue culture, the identification of these proteins will require molecular genetics approaches. For that reason and because we are additionally interested in the events following quinone reduction, we have begun investigating gene expression following 2,6-DMBQ exposure to Striga seedlings. The inducible proteins were identified by in vivo protein labeling. At the selected times following 2,6-DMBQ treatment, Striga seedlings were incubated with ^{35}S-Met for 30 min and the labelled proteins were analyzed by 12.5 % PAGE and fluorography. As shown in Fig 2, we identified the most strongly induced protein at 25kDa. The induction of this protein was very rapid and directly related to the presence of the active quinone. The 25kDa protein was induced after only a 1hr exposure of 2,6-DMBQ and disappeared within 2hr after replacing 2,6-DMBQ with water. In order to identify the 2,6-DMBQ inducible

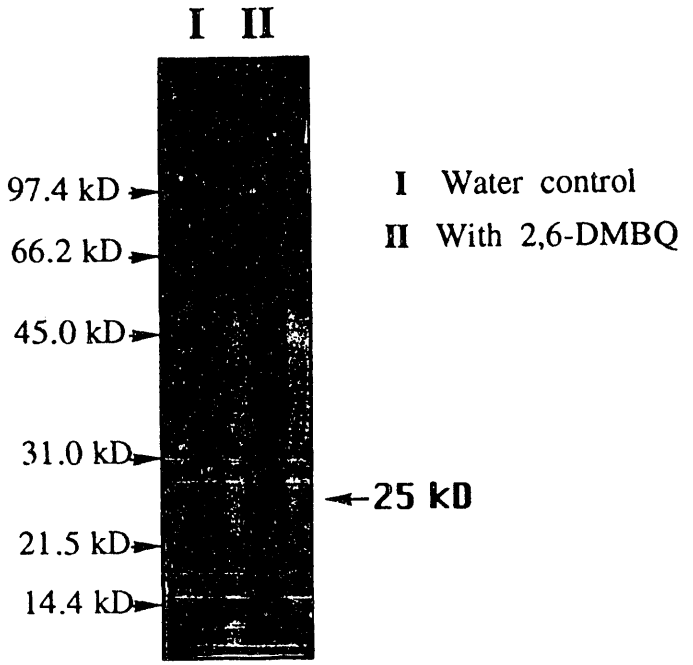


Figure 2. Fluorograph of 12.5% PAGE separation of *in vivo* labeled proteins of *Striga* seedlings

gene, mRNA was prepared by Micro-FastTrack™ (Invitrogen) using germinated seedlings (10mg) with (+) and without (-) a 4hr 2,6-DMBQ exposure. The two different cDNA libraries (+/-) were c structed using Uni-AmpTM Plus kit (Clontech). D ble stranded cDNA primed by oligo-dT wa gated with oligonucleotide adaptor and am d by PCR (average cDNA length was 1.2kb). Th nplified cDNA was ligated to the PUC19 vector and nsformed into the E.coli. About 50,000 transformants from (+)cDNA library were screened with ^{32}P labelled (+/-) total cDNA probes and four individual clones (1.5kb, 0.5kb, 0.3kb, 0.2kb) which strongly hybridized to the (+) cDNA and weakly to the (-) cDNA were selected after the third screening. By Southern and Northern blots, we have confirmed that these clones were different from one another, mRNAs were transcriptionally induced more than two-fold and the mRNA sizes were 5kb,4kb, 3kb, and 4kb . To investigate the function of these cloned genes, we have sequenced the four clones and are now attempting to clone the full-length cDNAs using a random-primed cDNA library and the PCR-RACE technique (7). These cDNAs, which will be first cloned genes from Striga, will provide insight into the signal transduction events in Striga haustorium development and the regulation of the quinone induced gene expression.

Conclusion. The outstanding question that remains is whether the *in vitro* enzymatic activity that we have observed is the activity that is required for and inhibited by these inhibitors *in vivo*. While the specificity of the inhibitors are certainly promising, we are now attempting labeling on the intact seedlings to test whether the same proteins are labeled *in vivo*. We are excited that the molecular biology experiments are proceeding smoothly. The development of this technology with *Striga* will ultimately be essential to the identification of the receptor.

References:

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