Biological and Chemical Properties of the Epidioxide Isomer of Abscisic Acid and its Rearrangement Products

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Abstract. The growth inhibitory activity of the epidioxide (II), a precursor in the synthesis of abscisic acid (ABA), has been confirmed with additional assay systems. Under physiological conditions the epidioxide is rearranged to give ABA and an isomer of ABA which has probably the structure V. This major product has very low, if any, biological activity. The biological activity of the epidioxide is explained by its partial conversion (about 20%) to ABA. The reaction rate was enhanced by heavy metal ions and decreased by EDTA. At pH 12.5, the decomposition of the epidioxide is slower than it is near neutrality and ABA is the predominant product. In the biological systems studied the activity of the epidioxide can be accounted for by nonenzymatic conversion to ABA.

Abscisic acid (ABA) (I) (1) is a recently discovered hormone which is detected at low concentration in many species of higher plants (8). In addition to rather broad-spectrum growth inhibitory properties, ABA promotes abscission in young cotton explants (10) induces dormancy in leaf buds (17) and may have a regulatory role in senescence (2) and seed germination (12, 13). Growth inhibitions produced by exogenously applied ABA are characterized by a general lack of injurious side reactions and can be reversed with other plant growth hormones. Only limited information is available on the relation between biological properties and structure of compounds related to ABA. Yet this information would be useful for the solution of many problems. For example, while one can often elicit a response to exogenously applied ABA this in no way establishes that the compound has a regulatory role under normal circumstances. But if a biogenetic precursor were available that could elicit the typical response only after enzymatic conversion to ABA, then the effect would be detected only in those plants that have the necessary biochemical machinery to convert the precursor to ABA. Routine screening for ABA effects on different plant materials with such a compound would, therefore, be a much more selective procedure. In an attempt to find such a compound, we have studied the chemical and biological behavior of the 1,4-epidioxide isomer (II) of abscisic acid. We chose this substance because it is the direct precursor of ABA in the chemical synthesis devised by Cornforth et al. (3) and because its inhibitory activity on the growth of excised wheat embryos is quite high—one sixth that of the naturally occurring hormone and more than 16 times that of all trans (RS)-ABA (3). According to Cornforth et al. (3) the relatively high biological activity of II in comparison to ABA is a possible indication of the biosynthetic route.
Experimental

Preparation of 3-Methyl-5-(1',4'-epidioxy-2',6',6'-trimethyl-2'-cyclohexenyl)-cis, trans-2,4-pentadienoic acid (II) (3). Two hundred and 40 mg 3-methyl-5-(2',6',6'-trimethyl-1',3'-cyclohexadienyl)-cis, trans-2,4-pentadienoic acid (II) (11) and 4.7 mg eosin yellowish were dissolved in a mixture of 47 ml benzene and 47 ml anhydrous ethanol. The solution was kept at 3 to 10°C and photolyzed with a 500 watt photospot lamp in an oxygen atmosphere. The rate of oxygenation was monitored by following the loss of the 340 m\textmu absorption peak. After about 80% of the compound had reacted the oxygenation was stopped. The solvent was removed by evaporation below 30°C, the residue taken up in benzene and extracted with water to remove most of the eosin. The benzene solution was concentrated below 30°C, dried over magnesium sulfate and the product isolated by addition of hexane, yield 94 mg; m.p. 161 to 163°C. The \textit{trans, trans} epidioxide (III) was prepared from 3 - methyl - 5 - (2',6',6' - trimethyl - 1',3' - cyclohexadienyl)-\textit{trans,trans}-2,4-pentadienoic acid by the same procedure.

\begin{equation}
\text{(III)}
\end{equation}

Separation and Quantitative Determination of the Epidioxide and Its Reaction Products. Thin layer chromatography (TLC) on silica-gel GF₂₅₄ permitted separation of II and its major reaction products with benzene acetic acid:water (8:3:5-v/v-upper phase) as the developing solvents. The compounds could be detected with a short wavelength UV light. II has an \textit{Rₚ} value of 0.29 on a 0.5 mm plate and gives a cherry red color with \textit{p}-dimethylaminobenzaldehyde (6). When II is allowed to decompose in aqueous solution near neutrality 2 products are detected neither of which gives a positive test for an epidioxide. The major product has an \textit{Rₚ} value of 0.15 and is designated compound A, and the minor compound which has an \textit{Rₚ} value of 0.04 was shown to be ABA. Reaction of II at pH 12.5 gives only 1 major product, asbiscic acid, \textit{Rₚ} = 0.04. The \textit{trans, trans} epidioxide (III), \textit{Rₚ} = 0.37, behaves similarly to the \textit{cis, trans}-isomer, yielding a compound with \textit{Rₚ} = 0.27 as the major product under neutral conditions and a compound with \textit{Rₚ} = 0.07, \textit{trans, trans}-asbiscic acid as the minor product.

Information on the rates of reaction and the proportions of the 2 products was obtained by chromatography on 0.5 mm TLC plates, development with the above solvent system, extraction of the separated substances with ethanol and determination of the UV absorption in 95% ethanol containing 0.002 n hydrochloric acid. Since the molar extinction coefficient (\textepsilon) for each compound is known, the amount of each substance formed can be calculated.

Isolation of Compound A From Reaction of II. A solution of 15 mg II in 150 ml water was gently shaken at 26°C for 6 days, the solvent evaporated under reduced pressure below 30°C and the residue dissolved in a small amount of acetone. The acetone solution was streaked across 5 TLC plates coated 1 mm thick with silica gel GF₂₅₄ and developed with the above solvent system. The zone containing the desired material was scraped off the plates and removed from the silica gel by repeated washings with acetone. Evaporation of the solvent yielded a slightly colored oil which crystallized, m.p. 158 to 161°C, from concentrated benzene solution on addition of hexane. The long reaction time, while probably leading to a decreased yield, aided in the isolation procedure since interference due to remaining II had been eliminated. The compound III on similar treatment yielded crystals with m.p. 165 to 169°C.

The mass spectrum of compound A was obtained with a Hitachi mass spectrometer Model RMU 6E by the direct inlet method at 70 electron volts and 240°C to 250°C. A low but distinct parent peak was found at m/e 264. Since this is also the parent peak of ABA and II these 3 compounds are isomeric. The fragmentation patterns of II and compound A show distinct similarities while that for ABA is different. The major peaks above m/e 35 in the mass spectrum for compound A were as follows (m/e; relative intensity in percent of the base peak at m/e 56 in brackets): 264 (<1), 220 (45), 209 (20), 190 (20), 125 (41), 11 (27), 57 (90), 56 (100), 55 (57), 44 (57), 43 (45), 42 (46), 41 (28), 39 (59).

Compound A has a UV absorption peak at 264 m\textmu in 0.002 n hydrochloric acid in 95% ethanol \textepsilon₂₅₄ m\textmu = 17,000. While this is also the UV maximum for ABA, the shoulder exhibited by ABA at 245 m\textmu is absent in the spectrum of compound A. The UV absorption characteristics of II are similar to those of compound A.

Results and Discussion

Chemical Stability of II. The reported inhibitory activity of II could be readily confirmed with additional test systems involving excised bean axes (16), wheat coleoptile sections (9) and decreased alpha-amylase synthesis with gibberellic acid-treated barley half seeds (15). In all cases II was less inhibitory than ABA by a factor of 5 to 10. This difference carries over into the all-\textit{trans} series also. These results can be explained either by assuming that the unchanged epidioxides have a somewhat lower biological activity than the corresponding asbiscic acids.
or that they are relatively inert but are partially converted to abscisic acids or other active substances. If the latter explanation is correct it becomes important to ascertain whether the conversion is enzymatic or chemical.

Chromatography of recrystallized II, spotted from acetone solution showed that the material survives chromatography essentially unchanged. Approximately 90% of the applied material is recovered as 1 spot, \( R_F = 0.29 \). This spot gives a positive peroxide test with \( p \)-dimethylaminoaniline and has the same UV spectral characteristics after elution as the material prior to chromatography. If, however, II is first stored in 0.01 M phosphate buffer pH 6.0, at 26° for 2 hr complete conversion is observed after chromatography. Two reaction products are detected; compound A accounts for approximately 80% of the starting material and a second compound with \( R_F = 0.04 \) accounts for the rest. Kinetic studies showed that the decomposition of the epidioxide follows a first order rate equation, but that the rate is highly variable and difficult to reproduce. For example, the reaction is faster in phosphate and tris buffers than in citrate buffer even though the pH and molarity of the solutions were the same. The lack of stability of II under the conditions frequently used for bioassays is, therefore, of obvious significance in the interpretation of the biological responses.

In attempts to account for the variability in the rates of decomposition of II it was observed that the reaction rate is highly susceptible to metal ion catalysis; iron, copper and tin salts enhanced the rate. Furthermore, ethylenediaminetetraacetic acid (EDTA) had a protective action. Typical data are summarized in table I. The enhanced reactivity of compound urith metal ions probably favored the decomposition.

**Table I. Stability of Epidioxide II in 0.01 M Phosphate Buffer at pH 6 and 26°**

<table>
<thead>
<tr>
<th>Concentration of compounds μg/ml</th>
<th>% Epidioxide after 2 hr 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidioxide (II) EDTA CuCl₂·2H₂O</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>300</td>
<td>20</td>
</tr>
<tr>
<td>300</td>
<td>20</td>
</tr>
<tr>
<td>300</td>
<td>38</td>
</tr>
</tbody>
</table>

1 Determined by quantitative chromatography on TLC plates and UV absorption spectra.

II in the presence of cupric or ferric salts in phosphate buffer indicates that only small quantities of metal ions are required for observable effects since the solubility of these salts is extremely low. Additional examples of metal ion-catalyzed reactions involving epidioxides (5) hydroperoxides (4) and the protective effects of EDTA (4) have been reported.

When the pH of the reaction medium is above pH 10.0 the nature of the reaction changes; the rate is decreased and the proportion of ABA increases, but the solubility of the metal ions is very low and a base-catalyzed path is probably favored.

The possibility that the 2 reaction products obtained from the epidioxide near neutrality arise by sequential reactions could be eliminated. The ratio of the 2 products does not change with time. Also, isolation of the products and storage at pH 7 and 26° gives no evidence for interconversions or resynthesis of II. Therefore, both compounds are formed from II in an irreversible reaction.

**Characterization of the Major Products Obtained From II Under Neutral Reaction Conditions.** The products obtained from II have slightly lower \( R_F \) values than those of the substances obtained from III. This difference in \( R_F \) values is also found in the epidioxides themselves and therefore indicates that no isomerization of the double bonds of the side chain are involved in the rearrangement. Although no attempt was made to crystallize the reaction product with \( R_F \) 0.04, sufficient evidence was obtained to show that this material is abscisic acid. The \( R_F \) value of the substance is the same as that of the sole product detected at pH 12.5. Under the latter conditions one would expect to obtain ABA, since base is used in the synthesis of ABA from the epidioxide (3). In 3 different systems, ABA and the reaction product with \( R_F \) 0.04 showed identical behavior: benzene:propionic acid:water (8:3:5 v/v) TLC plates coated with 0.5 mm silica gel GF₂₅₄, \( R_F \) for ABA = 0.23, \( R_F \) for the isolate 0.22; isopropanol:acetic acid (95:5 v/v) TLC plates coated with 0.5 mm silica gel GF₂₅₄, \( R_F \) for ABA = 0.15, \( R_F \) for the isolate 0.16; n-butanol:ammonia:water (24:1:7 v/v) Whatman number 3 paper \( R_F \) for ABA = 0.53, \( R_F \) for the isolate = 0.54. The UV spectrum of the eluted material is the same as that of crystalline ABA and shows the characteristic shoulder at 245 mµ attributable to the presence of a

**Table II. Decomposition of Epidioxide (II) at Different pH Values**

<table>
<thead>
<tr>
<th>pH</th>
<th>% Compound remaining 1</th>
</tr>
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<tbody>
<tr>
<td>6.0</td>
<td>0-5 75 20</td>
</tr>
<tr>
<td>10.0</td>
<td>0-5 75 20</td>
</tr>
<tr>
<td>12.5</td>
<td>70 5 25</td>
</tr>
<tr>
<td>2</td>
<td>12 Hr reaction time</td>
</tr>
<tr>
<td>6.0</td>
<td>0 85 15</td>
</tr>
<tr>
<td>10.0</td>
<td>0 80 20</td>
</tr>
<tr>
<td>12.5</td>
<td>0-5 0 95</td>
</tr>
</tbody>
</table>

1 Substances were separated on 0.5 mm thick silica gel GF₂₅₄ TLC plates. Compounds were eluted and quantities present determined spectrophotometrically. Prior to spotting the coated plates were washed 3 times with chloroform and twice with ethanol. Actual recoveries averaged 85% and are given in the table on a 100% recovery basis.
conjugated ketone. In its growth inhibitory activity on bean axes the material with R\textsubscript{f} 0.04 is also as active as crystalline ABA while II and the other reaction product are much less effective.

Compound A was purified sufficiently to yield crystalline material. A parent peak of m/e = 264 in its mass spectrum shows it to be isomeric with ABA and II. Absence of reaction with p-dimethylaminoniline shows the loss of the epidioxide group. The UV absorption spectrum has no shoulder at 245 m\textmu, and therefore indicates the absence of a conjugated ketone. This is confirmed by the infrared spectrum which has a narrow band at 5.9 \mu, but no doublet at 6.1 \mu as shown by ABA. Unlike ABA the infrared spectrum of compound A does not have a sharp band at 3.1 \mu. Together with the chromatographic evidence this shows the absence of an alcohol group in compound A.

The above data, previous work with ascaridole (7) and the epidioxide of levopimaric acid (5) suggest 2 possible structures (IV) or (V) for compound A. If II reacts in a manner similar to

\[
\text{CO}_2\text{H}
\]

(IV)

\[
\text{CO}_2\text{H}
\]

(V)

ascaridole and the levopimaric acid derivative than V is preferred. However, attempts to prove the presence of an epoxide ring in compound A either from the mass spectral data, the IR absorption or by chemical means have thus far been unsuccessful. Therefore, the assignment of structure V to compound A, while consistent with our data, is nevertheless not to be considered firmly established.

**Physiological Activity of II and Its Reaction Products.** Quantitative information on the effectiveness of II and its reaction products was obtained with 2 methods. Increase in the length of wheat coleoptile sections after 24 hr incubation is a very sensitive procedure, while fresh weight increase of bean axes can be used as a method when short exposure time is desirable. A 50% inhibition of coleoptile elongation was obtained with 0.06 \mu g/ml (RS)-ABA and 0.5 \mu g/ml epidioxide; 50% inhibition required, 0.01 \mu g/ml (RS)-ABA and 0.065 \mu g/ml epidioxide. Under these conditions compound A was completely ineffective; 0% inhibition was obtained in a concentration range of 0.003 to 0.3 \mu g/ml.

In the bean axes assay many compounds related to ABA show a temporary growth inhibition which is fairly strong 12 to 14 hr after start of water imbibition but is much weaker after 20 hr (14). In this assay, ABA is again the most effective compound, the epidioxide has one-third to one-fourth the activity and compound A is least active, table III. That the activity of II is not due primarily to the presence of an epidioxide moiety is shown by comparison with ascaridole, an epidioxide that has significantly lower activity than II, table III.

Since EDTA increases the chemical stability of II enzymatic conversion of II to (S)-ABA should be reflected in the growth responses. If there are no enzymes that catalyze the conversion of II to ABA then the presence of EDTA would decrease the growth inhibition. On the other hand, if enzymatic conversion can occur and if exogenously added EDTA does not interfere with the enzyme, then the effectiveness of II would be enhanced. This follows because one would expect a 50% yield of (S)-ABA. Bean axes were used as assay material since their response is fast and they will tolerate fairly high EDTA concentrations without interference with growth. As seen from table III, EDTA has no effect on the activity of ABA, either at 12 or 20 hr. However, there is a significant decrease in the effectiveness of II in the presence of 100 \mu g/ml EDTA.

The above results lead to the following conclusions: 1) The effectiveness of II is due primarily to the presence of ABA which is formed in about 20% yield in a rapid nonenzymatic metal ion catalyzed reaction near neutrality. 2) The major product formed from the epidioxide near pH 7 and the epidioxide itself are either ineffective or have a lower order of activity than ABA. 3) No evidence for enzymatic conversion of the epidioxide to ABA is as yet available. 4) Substitution of II for ABA does not lead to a more selective bioassay procedure.

**Acknowledgment**

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Table III. Activity of Epidioxide (II) and Related Compounds in the Inhibition of Growth of Bean Axes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration necessary for 50% inhibition¹</th>
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<tbody>
<tr>
<td></td>
<td>12 hr</td>
</tr>
<tr>
<td></td>
<td>No EDTA 100 µg/ml EDTA 10 µg/ml No EDTA 100 µg/ml EDTA 10 µg/ml</td>
</tr>
<tr>
<td>Epidoxide (II)</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>(RS)-ABA</td>
<td>3 µg/ml</td>
</tr>
<tr>
<td>Compound A</td>
<td>8 µg/ml</td>
</tr>
<tr>
<td>Ascaridole²</td>
<td>10 µg/ml</td>
</tr>
</tbody>
</table>

¹ The assay was performed in triplicate with bean axes using the procedure of Walton (16). The beans were shaken at 26° in 0.01 M potassium phosphate buffer containing 20 µg/ml chloramphenicol. The concentration required for 50% inhibition was obtained graphically.

² This value, which represents a minimum, was obtained by extrapolation above the 30% inhibition, the highest experimentally available point.


Literature Cited


