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Monoclonal Antibody Recognition of Abscisic Acid Analogs

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ABSTRACT

Specificities of three monoclonal antibodies (15-I-C5, DBPA 1, and MAC 62) raised against the plant hormone (S)-(+)-abscisic acid (ABA) have been compared. Immunological cross-reactivities against fifteen biologically active analogs of ABA were measured. The ABA analogs were altered at one or more of four positions: the double bonds in the ring, at C-2 C-3 and at C-4 C-5, and in the oxidation level at C-1. Several analogs were optically active with chiral centers at C-1′ and C-2′. For cross-reactivity, all three monoclonal antibodies required the carboxylic acid group, and the *cis* configuration of the double bond at C-2 C-3 of the ABA molecule. Monoclonals 15-I-C5 and DBPA 1 required the entire ABA sidechain from the C-1 to C-1′, but these monoclonals did cross-react with analogs with the ring double bond reduced and the C-2′ methyl *cis* to the sidechain. Only MAC 62 recognized analogs containing an acetylene at C-4 C-5. MAC 62 had more strict requirements for the ring double bond, but gave some cross-reactivity with acetylenic analogs having a saturated ring. All three monoclonals had higher specificity for analogs having the same absolute configuration at C-1′ as (S)-(+)-ABA. This work provides new information about the spatial regions of the ABA molecule that elicit immunological recognition, and serves as a basis for future investigations of the ABA receptor using ABA analogs and anti-idiotypic antibodies.

Even though the plant hormone ABA is critical for environmental stress responses and embryo maturation (23), little is known about the ABA hormone receptor. A recent review on receptors for plant growth regulators did not include a section on the ABA receptor because there has been little progress in ABA receptor identification (10). Understanding of the processes regulated by ABA-responsive genes depends on the characterization of the ABA receptor (16). There are two promising strategies to investigate the ABA hormone receptor and the signal transduction pathway. These are: (a) systematic modification of the ABA molecule (21) to identify the spatial and electronic regions that elicit recognition and (b) use of anti-idiotypic antibodies as receptor probes. As a first step in using these strategies to examine the ABA receptor, we have assessed the effects of systematically modifying the ABA molecule on immunological recognition. Specificities of three different ABA MAbs[1] to each ABA analog were compared.

The ABA molecule **1** has a single chiral center (C-1′) and the natural product has the (S)-(+) configuration (Fig. 1). The preferred conformation of the molecule is that with the sidechain axial, and the cyclohexenone ring in a pseudo-chair (Fig. 1) (9). Different MAbs including 16-I-C5 (8) and 15-I-C5 (5), MAC 62 (13, 14), and DBPA 1 (18) have been produced against ABA antigens made by linking the ABA molecule to a protein through hydrazones at C-4′. Similar cross-reactivities for the three monoclonal antibodies with ABA analogs and metabolites have been reported. Each of the monoclonals has less than 0.1% cross-reactivity with (R)-(−)-ABA, 2-*trans*-(S)-(+)-ABA, (S)-(+)-ABA esters (methyl and glucose), phaseic acid, dihydrophaseic acid, and α- and β-ionone. Quarrie *et al.* (14) found some cross-reactivity of MAC 62 and 16-I-C5 with 1′-deoxyabscisic acid, and MAC 62 gave high response to *cis*-α-ionylideneacetic acid.

Anti-idiotypic antibodies have been successfully used to examine animal hormone receptors (1, 3). With similar techniques monoclonal antibodies to ABA could possibly be used to produce anti-idiotypic antibodies that could interact with the ABA cellular receptor. Such anti-idiotypic antibodies could block the binding of ABA or mimic ABA activity (3). More knowledge of the specificities of the available ABA monoclonal antibodies would be useful in the selection of the most appropriate monoclonals for anti-idiotype production.

To compare the specificities of three ABA monoclonal antibodies (15-I-C5, DBPA 1, and MAC 62), we have synthesized a systematic series of ABA analogs. The ABA molecule has been altered at four positions: the ring double bond, the C-2 C-3 double bond, the C-4 C-5 double bond, and the oxidation levels at C-1. This set of compounds has been used to probe the regions of the ABA molecule recognized by each antibody. The ABA analogs identified in this study that have a three-dimensional structure so similar to ABA that monoclonal antibody recognition is, elicited, will be of interest in the future design of receptor probes and in screening of biological activity.

[1] Abbreviations: MAb, monoclonal antibody; RIA, radioimmunoassay; TBS, Tris-buffered saline.

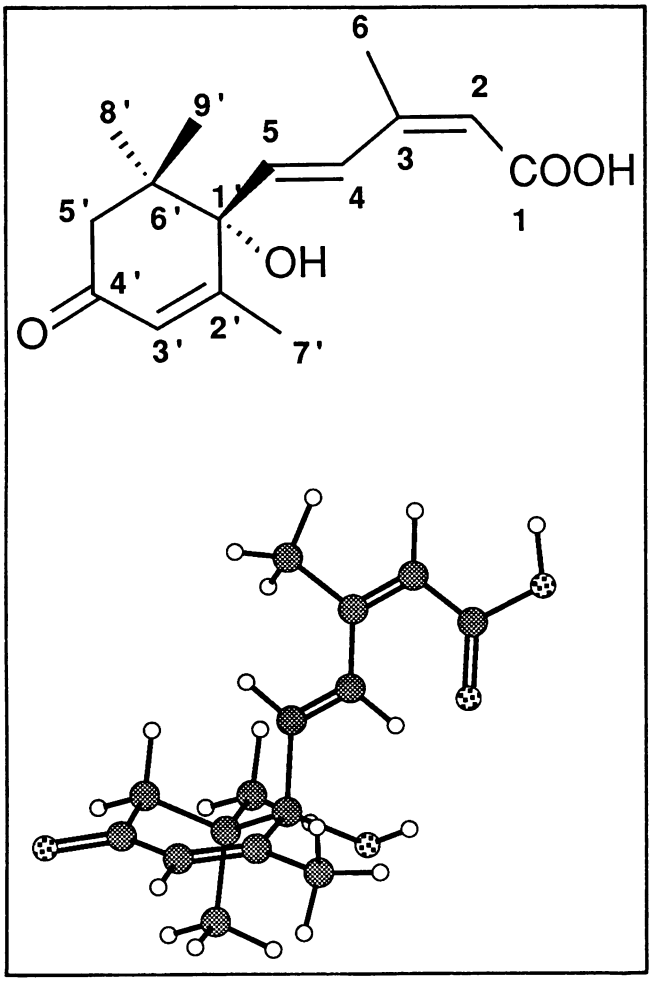

Figure 1. Structural formula of (S)-(+)-ABA showing conventional numbering system (upper), and preferred conformation of the molecule (lower).

MATERIALS AND METHODS

Sources of Chemicals

Racemic 2-*cis*-4-*trans*-ABA and natural (S)-(+)-ABA were purchased from Sigma Chemical Co.[2] ABA-related compounds (Fig. 2) were synthesized as described. Abscisyl alcohol **2** and aldehyde **3** were prepared by the procedure of Mayer *et al.* (7). Methyl abscisate **4** was obtained by methylation of ABA with diazomethane. 2-*Trans* abscisyl aldehyde **5** and 2-*trans*-ABA **6** were prepared by the procedure of Mayer *et al.* (7) except that *trans*-3-methylpent-2-en-4-yn-1-ol was used in place of the *cis* isomer. The acetylenic aldehyde **9** was synthesized by oxidation of the known alcohol with manganese dioxide (7) and the acid **10** was obtained after oxidation of the aldehyde with manganese dioxide, sodium cyanide and acetic acid in methanol employing the procedure of Corey *et al.* (2), and subsequent saponification of the ester. Epoxy-β-ionylideneacetic acid **7** and methyl ester **8** were prepared according to Tamura and Nagao (17). The 2′,3′-dihydroabscisic acids were synthesized according to Lamb and Abrams (6). The two 2′,3′-dihydroacetylenic ABA analogs **15** and **16** were prepared by oxidation of acetylenic alcohols (12) by the procedures analogous to those used by Lamb and Abrams (6).

[2] Mention of a specific product name by the U.S. Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.

Sources of Monoclonal Antibodies

Three MAbs were compared in this study. MAb 15-I-C5 was purchased from Idetek, Inc., 1057 Sneath Lane San Bruno, CA 94066. We prepared lyophilized ascites fluid of MAC 62 (13, 14) and DBPA 1 (18). MAb 15-I-C5 was prepared to ABA conjugated to human serum albumin via a tyrosyl hydrazone bridge. DBPA 1 and MAC 62 were prepared to ABA conjugated to keyhole limpet hemocyanine via an aminobenzoyl hydrazone bridge.

ELISA Assay

Monoclonal cross-reactivities were compared utilizing an indirect ELISA as previously described (19). The ABA-bovine serum albumin-C-4′ conjugate used in the ELISA was prepared according to Weiler (22) as modified by Norman *et al.*

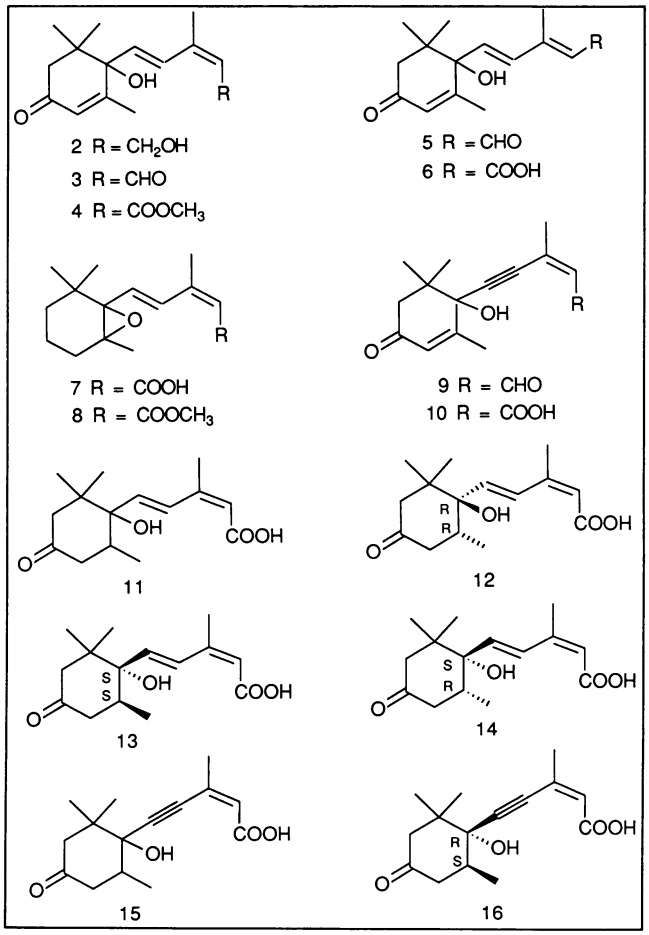

Figure 2. Structures of ABA analogs employed in this study to determine cross-reactivities of monoclonal antibodies.

Table I. *Comparison of MAb ELISA Characteristics*

Monoclonal Antibody	Optimum MAb concentration for ELISA	ABA Assay Range
	μg/mL	*pg/well*
15-I-C5	3.75	5 to 250
DPBA 1	7.50	50 to 2000
MAC 62	37.5	500 to 6000

(11). Monoclonal antibodies (lyophilized powder) were diluted into TBS: 6.05 g Tris-HCl, 0.2 g $MgCl_2$, 8.8 g NaCl/L (pH 7.8), containing 0.1% (w/v) bovine serum albumin (ELISA grade, Sigma Chemical Co., St. Louis, MO) for immunoassay. Dilution media for MAC 62 also contained soluble PVP (PVP-40, Sigma) at a concentration of 4.5 mg/mL as previously described (14).

ABA Standards and Cross-Reactivity Tests

Standard solutions of (S)-ABA and (R,S)-ABA were prepared by dissolving the compounds in TBS. A series of ABA standards (usually eight concentrations) appropriate for the linear range of each MAb was assayed for each microtiter plate. For each MAb the following ABA standard concentration ranges were utilized: 15-I-C5, 5 to 250 pg/100 μL; DBPA-1, 50 to 2000 pg/100 μL; MAC 62, 500 to 6000 pg/100 μL. Absorbance of ABA standards was plotted against the log of the ABA concentrations. Data were calculated at 50% saturation of the immunotitration curves. The value for (S)-(+)-ABA was taken as 100% cross-reactivity. Percentage of cross-reactivity for each ABA analog was calculated on a molar basis.

Preparation of ABA Analogs

Solutions of compounds tested in Table II were prepared by dissolving samples of known weights in 100 μL methanol and then immediately diluting 100-fold in water. For assay the stock solutions were further diluted in TBS. Compounds tested in Figure 3 were treated similarly except that the compounds were dissolved in 100 μL of 1% (w/v) sodium bicarbonate solution, followed by immediate 100-fold dilution into TBS.

Computer Graphics

Stereochemical representations of ABA and analogs showing calculated preferred conformations of the molecules were obtained on a Macintosh IIcx using Chem 3D plus Molecular Modelling program version 2.01 (Cambridge Scientific Computing, Inc., Cambridge, MA).

RESULTS AND DISCUSSION

Comparison of Three Monoclonal Antibodies in an Indirect ELISA Assay

Two antibodies, MAC 62 and DBPA 1, previously used in RIAs, were used successfully in an indirect ELISA, that had been developed with the Idetek MAb (5). A comparison of ELISA characteristics is presented in Table I. A higher final concentration of MAC 62 monoclonal antibody was required for the ELISA compared to the two other MAbs. Larger quantities of MAC 62 were also required for RIA analysis compared to other MAbs (14). The reliable range of ABA measurement varies with each MAb (Table I).

Cross-Reactivity with ABA C-1 Oxidation Levels and C-2 C-3 Double Bond Isomers

ABA analogs (Fig. 1) altered at the C-1 oxidation level and at the C-2 C-3 double bond were synthesized as described in "Materials and Methods." Cross-reactivities of the MAbs for these ABA analogs were measured by indirect ELISA (Table II). All three monoclonals are specific for the (+)-antipode of ABA. The MAbs did not cross-react with racemic ABA molecules having alcohol **2**, aldehyde **3**, or methyl ester groups **4** at C-1. The 2-*cis* double bond is required for recognition and only very slight cross-reactivity was observed for 2-*trans*-ABA **6** and the corresponding aldehyde **5**, possibly due to traces of the *cis* isomer contaminating the *trans* form.

Cross-Reactivity with Epoxy-β-Ionylideneacetate Analogs

Racemic methyl-epoxy-β-ionylideneacetate ***8*** and the corresponding acid *7* were synthesized and tested against the MAbs (Table II). As expected from the ABA series, the ester did not cross-react with any of the MAbs. However, the acid *7* showed significant cross-reactivity with MAC 62 even though *7* lacks the α,β unsaturated ketone group of ABA as well as having the C-1′ oxygen bound in a three membered ring to C-2′. These results show that MAC 62 antibody recognizes the sidechain, which is common to both *7* and (+)-ABA, but has less stringent requirements for structural changes at C-1′ and C-2′ as in the epoxide containing compounds.

Table II. *Specificity of the Monoclonal Antibodies for S-(+)-ABA Compared with an Equal Molar Amount of ABA Analogs Determined by Indirect ELISA*

Data were calculated at 50% saturation of the immunotitration curves. Each data point represents the mean of at least five separate dilutions.

Compound	Cross-Reactivity		
	15-I-C5	DPBA 1	MAC 62
		%	
(S)-(+)-ABA	100	100	100
(±)-ABA	49.4	52.3	51.0
2	0	0	0.02
3	0.02	0.02	0.02
4	0.1	0	0.06
5	0.09	0.13	0.24
6	0.98	1.13	1.39
7	0.03	0.082	20.9
8	0	0	0.42
9	0	0	0
10	0.43	0.64	36.4

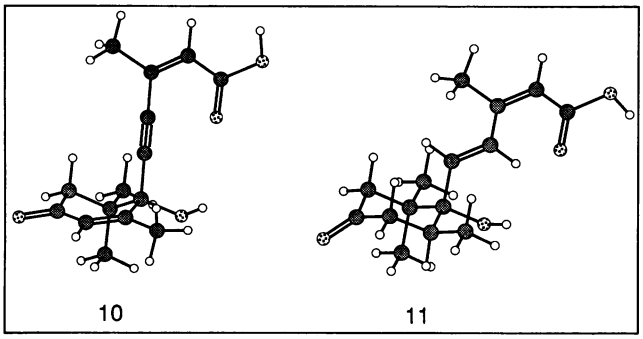

Figure 3. Preferred conformations of ABA analogs *10* and *11*.

Cross-Reactivity with C-4 C-5 Acetylenic Analogs

The acetylenic aldehyde **9** was synthesized and assayed along with its corresponding acid **10**. The acid **9** differs from (±)-ABA in that a triple bond replaces the *trans* double bond of the sidechain. The acetylene alters the shape of the sidechain because C-1′, C-3, C-4, and C-5 are colinear, and the sidechain is perpendicular to the ring (Fig. 3). All three MAbs had no cross-reactivity (Table II) with the acetylenic aldehyde **9**, and two (15-I-C5, DBPA 1) did not cross-react with the acid. MAC 62, however, gave 36% cross-reactivity with racemic **10**, compared to 50% for (±)-ABA, which shows that this MAb has tolerance for the altered sidechain. Coupled with the observed binding of MAC 62 to 1′-deoxyabscisic acid (14), and *7*, this result shows considerable insensitivity of MAC 62 to the hydroxyl at the junction of the ring and the sidechain. The two other MAbs have strict requirements for the ABA structure at the *trans* double bond at C-4 C-5.

Cross-Reactivity with 2′,3′-Dihydroabscisic Acid Analogs

Cross-reactivities of the three MAbs were compared with a series of ABA analogs, compounds **11–16**, synthesized with the ring double bond reduced (6). 2′,3′-Dihydroabscisic acid **11** resembles racemic ABA in the sidechain, but has a saturated six-membered ring, with the methyl and the sidechain on the same face of the ring. In this case the change to the ABA structure is close to the C-4′ carbon and greater cross-reactivity with MAbs generated from C-4′-linked ABA might be expected. As shown in Figure 4, significant cross-reactivity was obtained with 15-I-C5 (23%) and DBPA 1 (36%), but not for MAC 62 (<0.01%). Compound **11** can adopt a conformation very similar to that preferred for ABA (Fig. 3). The methyl groups at C-2′ of ABA and **11** are both nearly in the plane of the ring. The main differences from the ABA structure in **11** are the axial protons at C-2′ and C-3′. These may be impeding binding with MAC 62.

Because 2′,3′-dihydroabscisic acid, compound **11**, elicits immunological cross-reactivity, it would be expected that this ABA analog would have biological activity. This ABA analog has been found to increase freezing tolerance in *Bromus inermis* cell cultures (15) as well as to partially inhibit germination (12).

Two optical isomers of **11** were compared for cross-reactivity in the MAb assays. Compound **12**, which has the same absolute configuration at C-1′ as (R)-ABA, did not cross-react with any of the MAbs (Fig. 4). This is analogous to the specificity observed for (S)- *versus* (R)-ABA. However, the mirror image of **12**, acid **13**, which has the same absolute configuration at C-1′ as the natural hormone, had high cross-reactivity to 15-I-C5 (72%) and DPBA 1 (81%). The values obtained are over twice as high as for the racemic mixture, **11**. These MAbs recognize the saturated C-2′ carbon and its methyl group as nearly equivalent to the C-2′ vinyl carbon and methyl group of ABA.

Compound **14** is also a 2′,3′-dihydroabscisic acid, epimeric with **11**, and has the same configuration at C-1′ as (S)-ABA, but an axial methyl group at C-2′. Again, MAbs 15-I-C5 and DBPA 1 behaved in a similar manner, displaying no recognition of the molecule, while MAC 62 gave a slight cross-reactivity (4.6%). We conclude that an axial methyl group at C-2′ severely restricts recognition by all three MAbs.

Assays on two final compounds, **15** and **16**, again show the similarity in specificity between the 15-1-C5 and DPBA 1 MAb, and their dissimilarity with MAC 62. In this case, the molecules resemble **11** in the six-membered ring, but contain an acetylenic bond at C-4 C-5. Acetylenic acid **15** is the racemic mixture, and **16** is one optical form, having the same relative absolute configuration at C-1′ as (S)-ABA. (The designation of the chiral center at C-1′ is R as the order of precedence changes on substitution of the triple for double bond in the sidechain.) Only MAC 62 shows cross-reactivity and the optically active compound gives a higher value than the racemic mixture (**15**, 29%; **16**, 44%). MAC 62 cross-reacts with acetylenic acids either with or without a double bond in the ring. This finding is interesting, as MAC 62 does not cross-react with the 2′,3′-dihydro ABA analog **13**, which has the ABA sidechain.

Our results demonstrate that monoclonal antibodies pre-

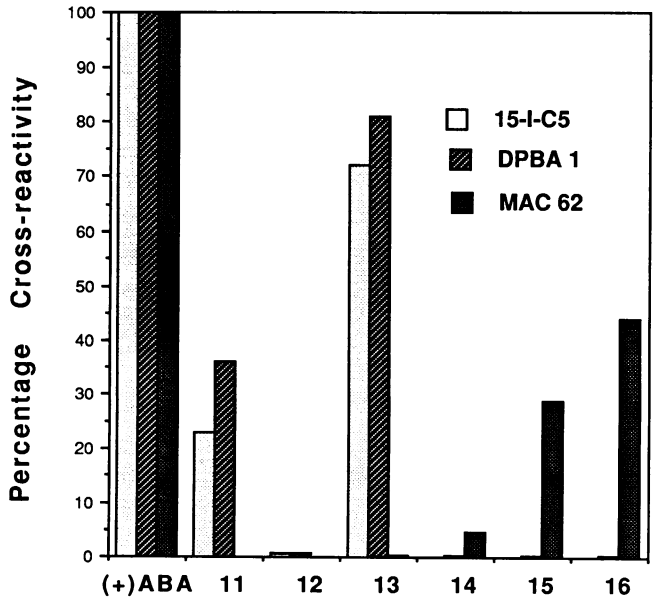

Figure 4. Cross-reactivities of MAbs 15-I-C5, DPBA 1, and MAC 62 with (S)-(+)-ABA and analogs *11–16*.

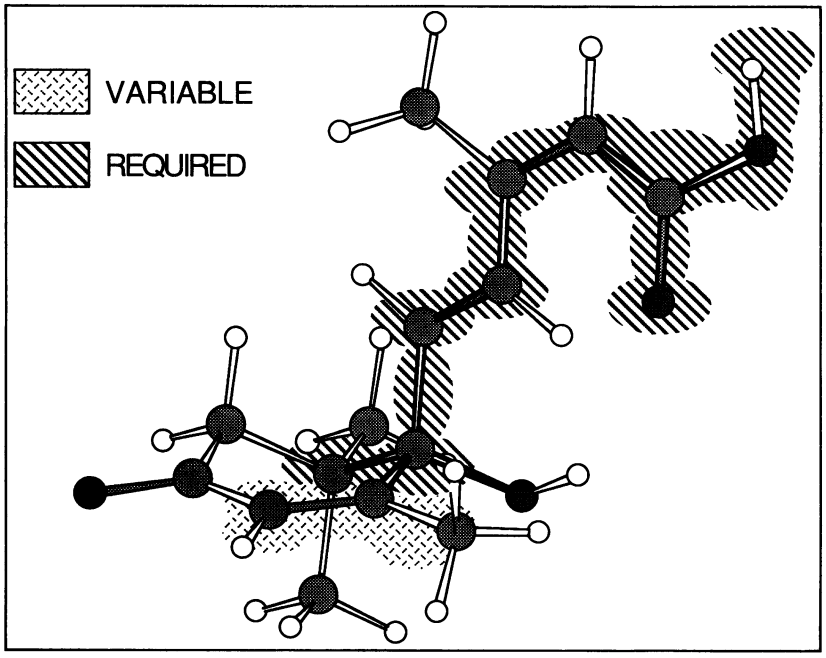

Figure 5. ABA molecule, showing structural requirements for cross-reactivity with MAbs 15-I-C5 and DPBA 1.

pared to the same plant hormone can have variable specificity. The results show the limitations of these MAbs for measuring free ABA in the presence of structurally similar compounds. A summary of the specificity results for antibodies 15-I-C5 and DPBA 1 is shown in Figure 5. Both these MAbs have strict requirements for the structure of the sidechain from carboxyl at C-1 to hydroxyl group at C-1′, while there is some flexibility at C-3′ through C-2′ methyl group. The required and variable regions of the ABA molecule recognized by MAC 62 are shown in Figure 6. MAC 62 recognizes analogs with the C-1 carboxyl and C-2 C-3 *cis* double bond, but does not require the *trans* double bond at C-4 C-5. In summary, we have identified ABA analogs with a similar enough three-dimensional structure to ABA to elicit immunological recognition, but which differ markedly in their affinities for three MAbs raised to C-4′ conjugates of ABA. Although the use of ABA conjugated via the ring as the immunizing antigen would make parts of the ABA ring inaccessible to these MAbs, the availability of the free keto-group at C-4′ may not be essential to allow interaction of ABA with specific receptors to elicit a physiological effect. Hornberg and Weiler (4) have shown that the C-4′-tyrosine hydrazone of ABA has 30% of the activity

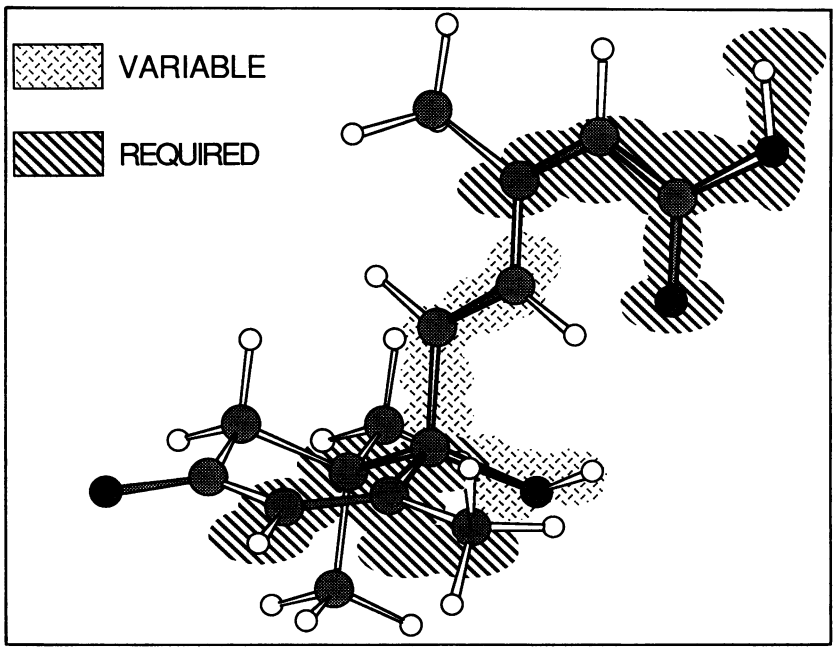

Figure 6. ABA molecule, showing structural requirements for cross-reactivity with MAb MAC 62.

of the free acid in eliciting stomatal closure, whereas the methyl ester of ABA was completely inactive in this bioassay. It is therefore possible that these MAbs would be suitable for the production of anti-idiotypic antibodies or a means of providing information about different ABA receptors. We have preliminary results already (20) demonstrating differences in the biological activity of some of these ABA analogs at both the physiological level (wheat embryo germination) and the molecular level (gene expression). Our results presented here will also aid the design of photoaffinity probes for the ABA receptor(s).

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