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Molecular Modeling of the (S)-ABA Binding Site of the Receptor Involved in the Induction of Freezing Tolerance: A Hypothetical Receptor Model*

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Abstract. Molecular modeling was used to compare abscisic acid (ABA) analogs to rationalize reported structure–activity relationships based on the induction of freezing tolerance in bromegrass (*Bromus inermis* Leyss.) cell culture. A modified version of the active analog approach was employed with (S)-ABA used as the standard to which other compounds were superimposed. Common conformations that present similar three-dimensional steric and electronic patterns were identified through conformational searches. From this analysis, a hypothetical model for the putative (S)-ABA receptor was constructed with the following features. The ring is in the pseudochair conformation with an axial side chain. The C-1′ has the same absolute stereochemistry as (S)-ABA. The model suggests that discrimination between (S)-ABA, phaseic acid, and (R)-ABA is due to the presence of an ether bridge or a methyl group below the C-2′ of (S)-ABA, in a region proposed to be occupied by the receptor. The side chain is *syn* with the ring and positioned approximately above the 2′-carbon of the ring. The model serves as a working hypothesis for testing receptor requirements and can be used to direct future analog studies.

The molecular requirements of the active sites of the phytohormone abscisic acid (ABA) are poorly characterized. Only three putative models of ABA active sites have been proposed (Milborrow 1985, 1987, Milborrow and Rubery 1985), despite considerable data relating ABA analog structure to biological activity (Milborrow 1978; Walton 1983). Hypothetical receptor models are valuable tools for designing novel analogs (Hilbert et al. 1988). For example, Milborrow's (1985) model spurred the synthesis and biological testing of novel allenic ABA analogs (Abrams and Milborrow 1991). Furthermore, knowledge of the molecular requirements for the processes governed by ABA would provide fundamental insight to the number of receptors and their evolutionary relationship, and, on a practical level, compounds could be rationally designed for specific effects.

Ideally, these requirements could be obtained from the three-dimensional structure of the receptor, but this information is not available. In fact, unlike other phytohormones (Napier and Venis 1990, Venis and Napier 1992), the single report of an ABA receptor on the plasma membrane of guard cells (Hornberg and Weiler 1984) has not been repeated to date. Therefore, the active site requirements only can be deduced indirectly by studying the activity of analogs on physiological processes.

Although the analog approach has several drawbacks (e.g., uptake, compartmentation and metabolism; see Walton 1983), approaches developed in the field of medicinal chemistry address the problem of rationalizing structure–activity data at the

* The following convention will be used throughout this paper: the ring face from which the side chain extends will be referred to as the upper face. This will simplify later discussion of analogs with multiple stereocenters that interact with a common active site. From this it follows that the upper face of (S)-ABA is the α-face, and in (R)-ABA it is the β-face (nomenclature of Rose et al., 1980).

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molecular level when the receptor is only postulated, or its structure is unknown (Marshall 1987, Marshall and Cramer 1988). Similarities and differences between active and inactive analogs are uncovered with approaches such as quantitative structure–activity relationship (QSAR) analysis, which relates physiochemical parameters such as charge, hydrophobicity, and molecular volumes to biological activity (Fujita 1990, Topliss 1983), and molecular modeling, which allows structural comparisons such as shape and conformation (Cohen 1985, Cohen et al. 1990, Hilbert et al. 1988, Langridge and Klein 1990, Marshall 1987, Marshall and Motoc 1986, Seiler et al. 1989). Conformational analysis, particularly the active analog approach (Marshall et al. 1979, Motoc et al. 1986), in which conformations of analogs are compared to elucidate common steric and electronic features in three dimensions, has been used extensively to construct hypothetical receptors models, from which drugs can be designed rationally (Charifson et al. 1989, Cohen 1985, Hilbert et al. 1988, Hopfinger 1985, Marshall 1987, Seiler et al. 1989). An integral facet of these approaches has been the recent accessibility of powerful computer-assisted molecular modeling software and hardware (Cohen et al. 1990, Langridge and Klein 1990).

Molecular modeling has been used in the plant sciences for selecting auxin transport inhibitors (Bures et al. 1991), and QSARs were used both to construct a receptor map for cytokinins (Iwamura et al. 1985) and to rationalize the activity of diverse dormancy breaking compounds (Cohn et al. 1989). However, there are only two reports of the use of these techniques for analyzing ABA analog activity (Dimoglo et al. 1990, Schubert et al. 1991).

This study was initiated with the intent of developing a hypothetical receptor model for the putative ABA receptor involved in the induction of freezing tolerance in bromegrass (*Bromus inermis* Leyss.) cell cultures. To this end, a modified version of the active analog approach (Marshall et al. 1979) was employed to rationalize the biological activities of ABA analogs (Churchill et al. 1992, Gusta et al. 1990, Robertson et al. 1994) and metabolites (Reaney 1989, Robertson et al. 1994). From this analysis it was apparent that seemingly unrelated and complex structure–activity trends could be explained by simple geometric, stereochemical, and conformational considerations, which are summarized in a hypothetical receptor model. Although the validity of this model will remain equivocal until ABA receptors are isolated and studied, it is nevertheless useful as a summary of our current understanding of the molecular basis of the structure–activity relationships and as a predictive tool for directing future synthetic and screening efforts (see Hilbert et al. 1988).

Materials and Methods

The biological activity of the ABA analogs compared in this study, in regard to the induction of freezing tolerance in Bromegrass (*Bromus inermis* Leyss.) cell-suspension cultures, was obtained from published data (Churchill et al. 1992, Gusta et al. 1990, Reaney 1989, Robertson et al. 1990, 1993).

All molecular modeling was performed with Chem3D Plus (Cambridge Scientific Computing Inc., Cambridge, MA). A modified active analog approach (Marshall et al. 1979, Motoc et al. 1986) was used with all compounds compared by superimposition to (S)-ABA, which was taken as the pharmacophore (the required three-dimensional functional group pattern). Compounds were minimized with the MM2 force-field (Allinger 1977) routine provided with Chem3D. Minimization was initiated with molecules in a chair or a pseudochair conformation with an axial side chain. The conformation of the ABA-like molecule when bound to the receptor was deduced from systematic comparisons of conformations that presented a similar pharmacophore. Specific details of the analyses are provided in the figure captions where necessary.

Results and Discussion

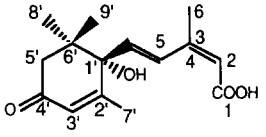
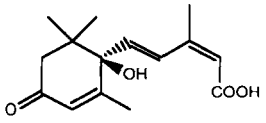
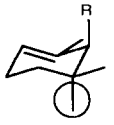
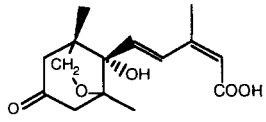
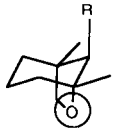
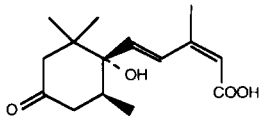
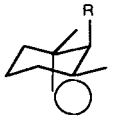
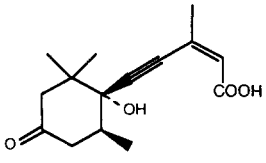
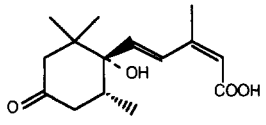
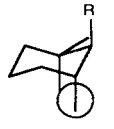
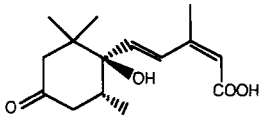
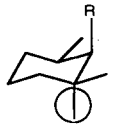
Side Chain Extends off the Alpha Face of the Ring

In contrast to other "slow responses" mediated by ABA in which (R)- and (S)-ABA are approximately equally active (Milborrow 1980, 1987), (S)-ABA (1 in Table 1) induces a much higher degree of freezing tolerance than (R)-ABA (2 in Table 1; Gusta et al. 1990). In (S)-ABA, and in the pure, optical isomers of other analogs that are active (4 and 5 in Table 1; Gusta et al. 1990, Churchill et al. 1992), the side chain extends off the α- or upper face* of the ring (Table 1). Therefore, this absolute configuration at C-1′ is required for activity.

The Side Chain Is Axial

The equatorial or axial positioning of the side chain, when the molecule is bound to the receptor, can be deduced from the dihydro isomers and their activities. Only isomers in which the 7′-methyl group and side chain are *cis* are active (Churchill et al. 1992). Activity is lost when these groups are *trans*, even if the configuration at C-1′ is correct for activity (Churchill et al. 1992). Considering the position of

Table 1. Correlation between optical isomerism at C-1′ and/or C-2′ and biological activity with respect to the space below the C-2′ of (S)-ABA.

Designation	Compound	Freezing Tolerance Assay Activity [a]	3D Structure
(S)-ABA 1		High (< -40)[bcd]	
(R)-ABA 2		Slight (-19)[c]	
Phaseic Acid 3		Inactive (-9)[bd]	
4		High (-35)[c]	
5		High (-35)[e]	
6		Inactive (-7)[e]	
7		Inactive (-10)[c]	

Note. In conformations where the side chain is axial, activity is always correlated with occupancy of the region below the C-2′ of (S)-ABA (circled area). This indicates that this region is not accessible to the agonist and may be due to occupancy by the receptor.

[a] Freezing tolerance is expressed as LT_{50} (temperature at which 50% of the cells are killed).

[b] Reaney 1989.

[c] Gusta et al. 1990: Gusta unpublished data.

[d] Robertson et al. 1994.

[e] Churchill et al. 1992.

the 7′-methyl group in the dihydro analogs relative to that in ABA further illustrates the importance of these conformational differences. In ABA the 7′-methyl group is locked in an equatorial position by the sp^2hybridized 2′-carbon. Therefore, it is likely that the 7′-methyl group must be equatorial in active dihydro analogs. When the side chain and 7′-methyl group are *cis*, as in the active analogs 4 and

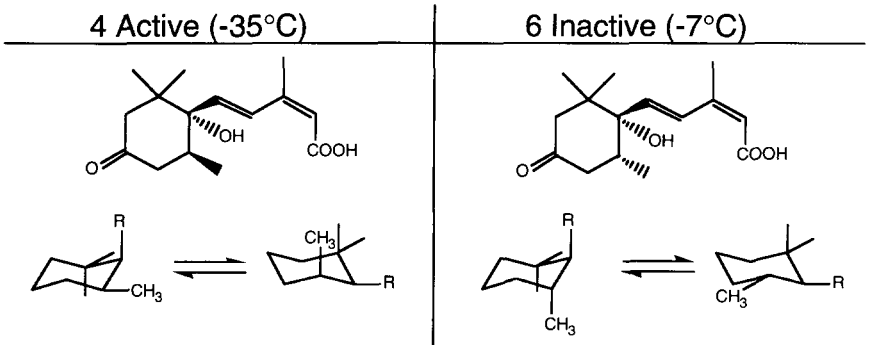


Fig. 1. Effect of ring conformation on the axial/equatorial positioning of the side chain (R) and methyl groups. The active isomer has its 7′-methyl group in an equatorial position, similar to its position in (S)-ABA, only when the side chain is axial. In contrast, the inactive isomer has its 7′-methyl group equatorial only when the side chain is equatorial. Therefore, the conformation with the axial side chain is likely the active conformation. The conformations with the 7′-methyl group equatorial are also energetically more favorable, as the other conformation has deleterious 1,3-diaxial interactions between the methyl groups.

5, the side chain must be axial for the 7′-methyl to be equatorial (Fig. 1). In this position it can occupy a similar, although not identical, region of space as in (S)-ABA. Conversely, when the side chain and 7′-methyl group are *trans*, as in the inactive analog 6, the side chain must be equatorial for the 7′-methyl to be equatorial (Fig. 1).

The biologically active conformation of the dihydro analogs coincides with the preferred conformation of ABA in solution, in which the side chain is axial (Milborrow 1984). Affinity for molecules with an axial side chain makes this active site distinctly different from the ones proposed by Milborrow (1978) for slow responses, and the active site of the uptake carrier (Milborrow and Rubery 1985), both of which bind ABA with an equatorial side chain (Milborrow 1987). Although molecules with axial side chains were depicted in the original carrier model (Milborrow and Rubery 1985), molecules likely are bound with an equatorial side chain since β-ionone is an inhibitor (Astle and Rubery 1987) with a fixed pseudoequatorial side chain due to a C-1′, C-2′ double bond.

Recognition of an axial side chain makes this active site similar to those that recognize phaseic acid (PA), which are involved in responses such as stomatal closure in certain species (Sharkey and Raschke 1980) and inhibition of GA stimulated α-amylase production in barley aleurone cells (Nolan and Ho 1988). These receptors must recognize ABA and ABA-like molecules with an axial side chain since PA has its side chain fixed in the axial position (see below). Furthermore, stomata of *Cyperus dispersa* and *Commelina communis* were unresponsive to allenic analogs, in which the side chain is fixed in a pseudoequatorial position by two contiguous double bonds at the C-4, C-5 and C-5, C-1′ positions (Abrams and Milborrow 1991).

Occupancy of the Region Below the C-2′ of (S)-ABA May Be Responsible for Enantiomeric Discrimination

Conformational analysis of the enantiomers of ABA and optical isomers of the dihydro analogs, in which the *cis/trans* relationship between the side chain and the 7′-methyl group is varied, can be used to define the axial/equatorial positioning of the methyl groups when the molecule is bound to the receptor. For this analysis, an approach similar to that of Milborrow (1978, 1985, 1987) is employed in which the different methyl groups of (R)- and (S)-ABA can be accommodated in the same receptor site.

In Milborrow's model the side chain is equatorial, which enables the 9′- and 7′-methyl groups of (R)-ABA to be accommodated in positions normally occupied by the 7′- and 8′-methyl groups of (S)-ABA, respectively (Milborrow 1987). In our proposal, the side chain is axial, and the 7′- and 8′-methyl groups of (R)-ABA can occupy positions normally occupied by the 9′- and 7′-methyl groups of (S)-ABA, respectively (Table 1). In contrast to Milborrow's model, which explains the equal activity of (R)- and (S)-ABA in slow responses (Milborrow 1985), our model must account for enantiomeric discrimination because only (S)-ABA is highly active (Gusta et al. 1990).

Given these reasons, (R)-ABA can achieve a similar conformation to that of (S)-ABA, resulting in

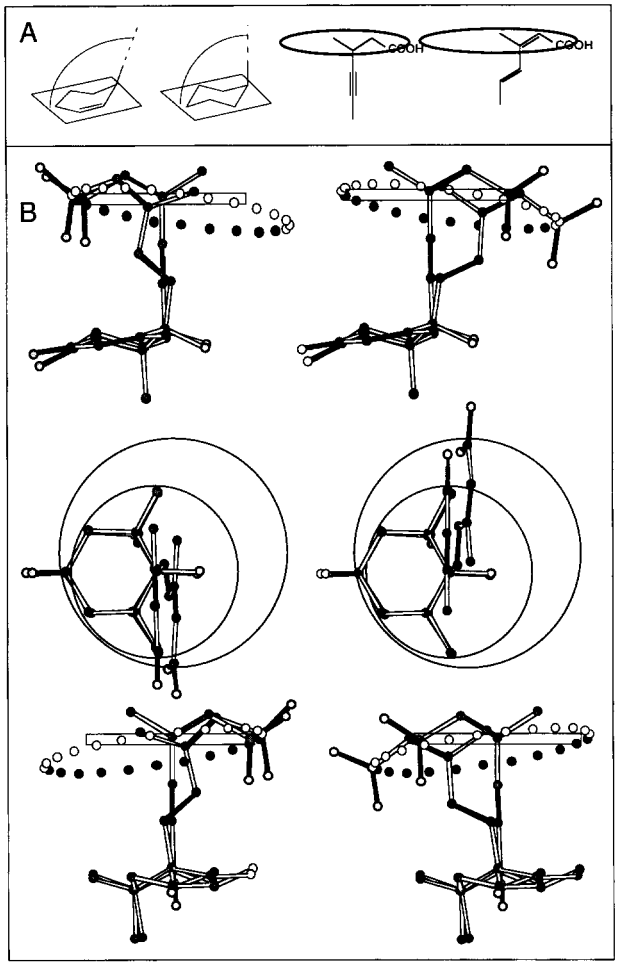


Fig. 2. Conformational analysis of the effect of ring saturation and side chain type on the positioning of the carboxyl group relative to the plane of the rings. (A) Schematic representation of the effects of ring saturation on the angle formed between the plane of the ring and the rotational axis of the side chain and the effects of bond order at C-4, C-5 on the rotational radius of the carboxyl group. Note that the angle in A is greater when the ring is unsaturated and that the radius of rotation is greater when a double bond is present at C-4, C-5 than when a triple bond is present. (B) Conformational sweep graphs illustrate the space available to the carboxyl and the region of overlap between the two most active combinations of ring saturation and side chain type (S)-ABA and active analog **4**. Three views were chosen to depict the closest approach of the two carboxyl carbons. The upper pair of molecules are shown from the plane of the ring with C-4′ on the left and C1′ on the right. The center pair of molecules are shown from above the plane of the ring with C-4′ on the left and C1′ on the right. The lower pair of molecules are depicted from the plane of the ring with C-4′ at the center front position and C-1′ behind, and slightly above C-4′. Following MM2 minimization, the molecules were superimposed, and the side chains were rotated about the C-1′, C-5 bond. Note that the closest contact between the two carboxyl carbons occurs in a region where they are rotated toward the ring and approximately above the 7′-carbon (image on the right in each pair). Superimposition was performed by minimizing the root mean square distance between the carbons 9′, 6′, 1′, 2′, 7′, and 4′. The C-1′, C-5 bond was set to the vertical axis. The two side chains were then rotated in 15° increments. The path of the C-1 carbon is depicted by marking each position for (S)-ABA (open circles extending toward the viewer and shaded away), and as a solid rectangle for active analog **4**, for the side views perpendicular to the vertical axis. For the view normal to the vertical axis, the paths of the carbonyl carbons are depicted by lines. Only selected views are shown, which most clearly illustrate the carbon paths and permit identification of the individual molecules. Molecular modeling was performed with Chem3D Plus software (Cambridge Scientific Computing Inc., Cambridge, MA).

only a slight deviation in the three-dimensional positioning of the equatorial methyl groups. Therefore, discrimination likely stems from the difference in the positioning of the geminal methyl group that is axial on the lower face of the ring, i.e., the 8′-methyl on the β-face of (S)-ABA and the 9′-methyl on the α-face of (R)-ABA (Table 1). Inactivity of (R)-ABA could then be attributed to steric hindrance caused by the 9′-methyl of (R)-ABA in a region below (β-face) what would be the C-2′ of (S)-ABA (Table 1).

The importance of the region below the C-2′ of (S)-ABA is also consistent with the correlation between dihydro analog activity and the presence of a methyl group in this region, when the side chain is axial (Table 1). Furthermore, PA is inactive (Gusta et al. 1990, Reaney 1989, Robertson et al. 1993) even though the ring is in a chair conformation with the side chain locked in an axial position (correct for biological activity), due to an ether bridge on the lower face of the ring. However, the oxygen in the second ring occurs in the region below the C-2′ of (S)-ABA (Table 1).

These correlations may be due to a receptor that discriminates between (S)-ABA and PA by the presence or absence of a group below the C-2′ of (S)-ABA, which is then, coincidentally, manifested as discrimination between the naturally occurring and synthetic enantiomer. From this evidence, it can be deduced that the lower face of the ring of (S)-ABA is recognized, and that it requires close contact with the receptor for binding. It has been suggested that certain compounds that contain the correct pharmacophore for activity but are inactive (such as (R)-ABA and PA), extend into a novel volume occupied by the receptor (Marshall 1987, Marshall and Cramer 1988, Marshall et al. 1979, Seiler et al. 1989). The size of the substituent at this position is also important because a methyl group or oxygen atom in this region causes loss of activity, whereas a hydrogen atom does not. This can be deduced from the high activity of several dihydro analogs,

which have an axial hydrogen atom below the ring (Churchill et al. 1992).

The Side Chain Is Rotated so that the Carboxyl Group Is over the 7'-Carbon

Two lines of evidence suggest that the carboxyl group is important for binding to the receptor: 1) analogs have to be at the acid oxidation state to be active, and 2) geometric isomerism of the C-2, C-3 double bond from *cis* to *trans* invariably eliminates analog activity (Churchill et al. 1992). The latter is also true for all other ABA bioassay systems (Milborrow 1978, Walton 1983) and could indicate that positioning of the carboxyl group may be of critical importance for binding (Walton 1983). Furthermore, molecular superimposition of ABA and acetylenic ABA (both were the "R-like" enantiomer) led Schubert et al. (1991) to suggest that acetylenic analogs were both active because they retained the same geometry between the ring and the C-1 functional group. Similar acetylenic analogs, with protected aldehyde and ketone groups, increased freezing tolerance in both whole plants and tissue culture of wheat (Flores et al. 1988).

The preferred position of the carboxyl group is difficult to determine because of conformational flexibility arising from a low energy barrier of rotation around these single bonds (C-1', C-5 and C-3, C-4). However, a statistical interaction between side chain type and ring saturation enables determination of the probable position of the carboxyl group. Specifically, the decrease in activity of cyclohexenone analogs, and the increase in activity of cyclohexanone analogs, when the *trans* double bond is replaced by a triple bond (Churchill et al. 1992) can be explained by geometrical arguments.

Ring saturation, in addition to the steric and conformational effects just discussed, alters the geometry between the plane of the ring and the carboxyl group. In cyclohexenone analogs, the side chain axis and the plane of the ring form a greater angle than in cyclohexanone analogs (Fig. 2A). A triple bond in the side chain restricts the rotational space potentially occupied by the carboxyl group, and positions the carboxyl group closer to the axis of rotation compared with the *trans* double bond (Fig. 2A). If the carboxyl group is *anti* to the ring, an acetylenic bond would compound the shortening effect of the cyclohexanone ring, making such compounds even less active. However, if the carboxyl group is *syn* with the ring, the chain and ring effects would compensate for one another and the carboxyl group could remain almost stationary, and thus account for the statistical interaction.

To test this idea with modeling, the two most biologically active combinations of ring saturation and side chain type where superimposed, and the side chains were rotated to explore regions were the two molecules could exhibit the same geometry between the ring and the carboxyl carbon (Fig. 2B). The area of potential overlap occurs in a region where the carboxyl carbons are rotated toward the ring (Fig. 2B: views on left side of each pair of views show the closer proximity of the carboxyl carbons). Further positional refinement is possible by taking into account the steric interactions between the axial hydrogen atom(s) on the upper face of the ring and the hydrogen atom bonded to C-5 of the diene-based side chain (Fig. 3: upper and center pairs of views). In (S)-ABA close contacts occur between the side chain hydrogen atom and the hydrogen atom bonded to the C-5' of the ring when the side chain is rotated over the 5'-carbon, making this conformation unfavorable (Fig. 3: upper view pair). A second axial hydrogen atom on C-3' places even greater conformational restriction on dihydro analogs, limiting free rotation of the side chain toward the ring to a region over the 2'-carbon (Fig. 3: center view pair). However, no steric restriction exists in analogs with an acetylenic side chain because the electron cloud surrounding the triple bond is compact and symmetrical (Fig. 3: lower view pair).

An axial side chain with the carboxyl group rotated over the ring may also be the conformation of (S)-ABA bound by the MAC 62 monoclonal antibody because its recognition depends on the same reciprocal relationship between ring saturation and side chain type (Walker-Simmons et al. 1991). This affords a unique opportunity to test the inferences on which the model is based. Specifically, through collaboration, we are using X-ray crystallography to directly determine the conformation of (S)-ABA bound to MAC 62.

Hypothetical Receptor Model

The characteristics of the active site for the receptor linked to the induction of freezing tolerance are summarized in the hypothetical model shown in Fig. 4. The similarities and differences between the requirements of this putative receptor and those reported for other ABA-mediated responses (Milborrow 1980, 1985, 1987, Sharkey and Raschke 1980, Walton 1983) may relate to the degree of homology at the level of DNA. For example, the early pharmacologic separation of members of the adrenergic receptor family was later shown to correspond to differences in the DNA sequences of the receptors (Liggett and Raymond 1993). Similarity to the re-

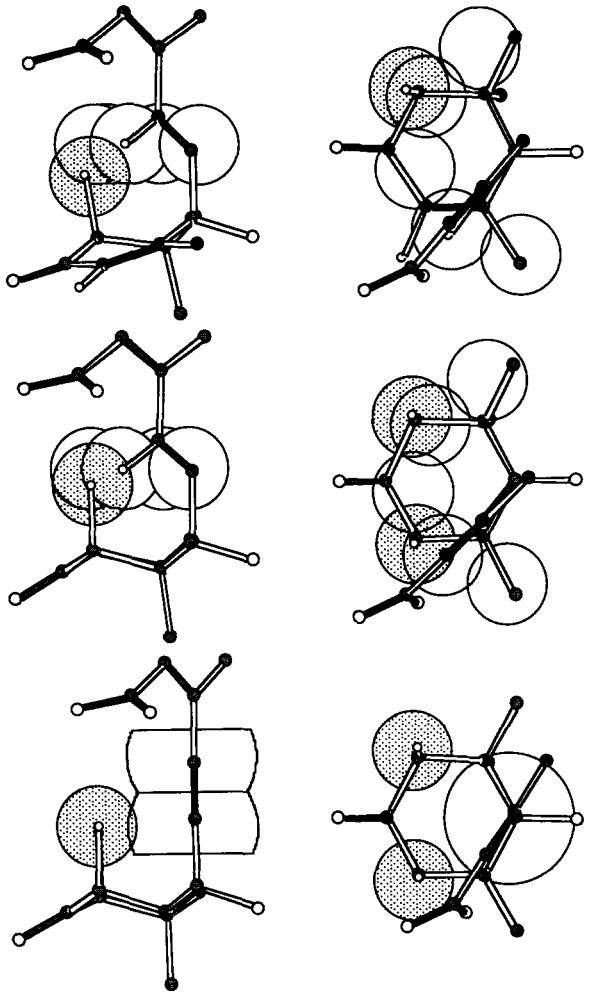


Fig. 3. Comparison of the effect of steric interactions between the axial ring hydrogen atom(s) and the hydrogen atom bonded to the carbon at position 5 in the side chain, on the rotational freedom of the side chain in three analogs with unsaturated or saturated rings and a double or triple bond in the side chain shown in two views: (S)-ABA (upper pair), 4 (middle pair), and 5 (lower pair). In all cases the right view of each pair is in the plane of the ring with C-1′ on the right, and the left view is taken from above the ring with C-1′ on the right. Note that reducing the ring double bond restricts the rotational freedom of the *cis, trans* side chain more than in (S)-ABA, but that oxidation of the C-4, C-5 bond in the side chain to a triple bond releases this restriction. In fact, an acetylenic side chain has a greater amount of rotational freedom than the *cis, trans* side chain in both the dihydro analog (4) and acetylenic ABA (not shown). The shaded circles are the van der Waals radii of the axial ring hydrogen atoms and the open circles are the van der Waals radii of the C-5 hydrogen, or the electron cloud surrounding the sp hybridized carbons in the side chain.

quirements for MAC 62 monoclonal antibody recognition (Walker-Simmons et al. 1991) may illustrate specificity for the stable, ground state, pseudochair conformation (S)-ABA (Milborrow 1984).

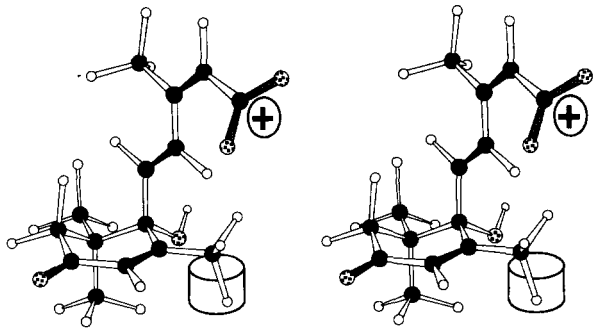


Fig. 4. Stereoview of a hypothetical active site model illustrates the conformation of (S)-ABA bound to the receptor that is linked to the induction of freezing tolerance in cultured Bromegrass cells. The salient features of the model are: 1) the side chain must extend off the upper face of the molecule, which is the α-face of (S)-ABA; 2) the ring conformation is pseudochair with the side chain residing in an axial position; 3) the carboxyl group is rotated toward the ring and occupies a position approximately above the 7′-methyl group; and 4) the space below the C-2′ of (S)-ABA shows steric restriction and cannot be occupied by a bulky group (a methyl or oxygen is not permissible, but a hydrogen atom is).

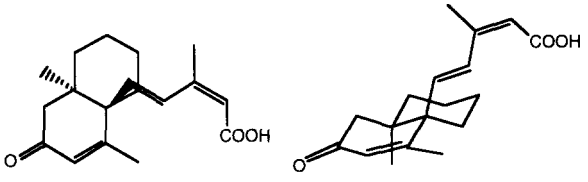


Fig. 5. Conformationally restricted bicyclic analog that could validate the proposal that the side chain must be axial for activity. Alternatively, the second ring could be formed by including C-7′ rather than C-9′.

Use of the Model in Future Testing

The usefulness of any model resides in its ability to predict compounds that could validate, refine, or disprove its features; therefore, the following examples are included to illustrate the use of the model in predicting new analogs that could be used to test two features of the model.

Testing the importance of an axial side chain could be accomplished with a structurally restricted analog such as that in Fig. 5. Inactivity of this compound would not disprove the model, but activity would confirm it. To validate the relationship between activity and sterically bulky groups in the region below the C-2′ of (S)-ABA, future synthetic and screening efforts could concentrate on all combinations of the pure stereoisomers de-methyl analogs, such as those studied by Nagano et al. (1980), and analogs with an additional methyl group at C-2′, as studied by Schubert et al. (1991). If 9′-demethyl-

(R)-ABA was tested, activity would again verify the model, whereas inactivity may relate to the positioning of the two remaining methyl groups for binding rather than invalidation of the model.

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