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8'-ACETYLENE ABA: AN IRREVERSIBLE INHIBITOR OF ABA 8'-HYDROXYLASE[1]

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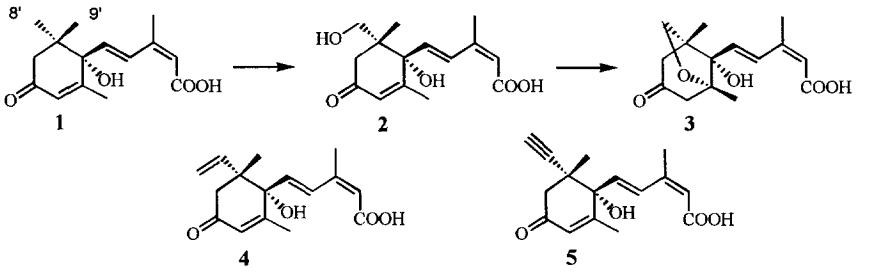
Abstract: (+)-8'-Acetylenic ABA has been synthesized through the ligand assisted nucleophilic addition of a Grignard reagent to quinol **6** and is shown to be the first known irreversible inhibitor of ABA 8'-hydroxylase. A series of ABA analogues has been prepared using this procedure. © 1997 Elsevier Science Ltd.

(+)-(*S*)-Abscisic acid (ABA, **1**) is a plant hormone that regulates a wide range of processes involved in plant growth, including development and germination of seeds, transpiration, growth inhibition and adaptive responses to environmental stress.[2] Recently, there has been progress in understanding the ABA signal transduction pathway,[3] and in cloning ABA biosynthetic enzymes.[4] ABA metabolizing enzymes[5,6] are less well understood. Regulating levels of ABA in plants through controlling ABA catabolizing enzymes (either through chemical or genetic means) is a means to produce more stress tolerant plants or plants with altered seed germination and composition traits.

Towards this goal, we have been developing ABA analogues as plant growth regulators that are altered at the 8'-carbon, the main site of ABA metabolism. Application of ABA itself as a plant growth regulator has been limited by its rapid catabolism in plants, principally through oxidation of the 8'-methyl group to 8'-hydroxy ABA **2**, which then further cyclizes to biologically inactive phaseic acid[7] (PA, **3**). We have previously reported that 8',9'-trideuteromethyl ABA[8] is more persistent in plant cell cultures due to a primary isotope effect and is more potent than ABA in inhibiting germination of cress seeds. Following the same reasoning, biologically active 8'-altered analogues (methoxy methyl,[9] ethyl,[10] and trifluoromethyl[11]) have been reported in the last two years; however, the syntheses are long and often give mixtures of 8' and 9' substituted products. We have recently reported the synthesis and biological activity of 8'-methylene ABA **4**, which shows exceptionally high biological activity and a prolonged lifetime over ABA.[12] The short preparation (6 steps from 2,6-dimethylphenol) of analogue **4** increases the potential for 8'-substituted ABA's as commercial plant growth regulators. ABA 8'-hydroxylase is believed to be a cytochrome P450 monooxygenase, for which olefins and acetylenes are known inhibitors;[13] however, compound **4** did not act as an irreversible inhibitor. Therefore we undertook to prepare compounds with acetylene groups replacing the 8'-carbon of ABA as potential irreversible inhibitors of the 8'-hydroxylase enzyme. We report the improved synthesis of several 8'-substituted analogues, including the first report of the synthesis of 8'-acetylene ABA **5** and biological evidence of the irreversible inhibition of the 8'-hydroxylase in vivo by **5**.



Synthesis

We previously prepared 8'-methylene ABA **4** through the copper catalyzed addition of vinylmagnesium bromide to the lithium anion of quinol **6**[14] at –78 °C. The reaction proceeds in high yields and addition occurs only at the 8'-position, through direction by the hydroxyl group. However, it is limited to very reactive Grignard reagents, as increased temperatures required for less active Grignards (i.e., EtMgBr) results in the formation of epoxide **7**. Previous examples of ligand assisted nucleophilic addition of quinol alkoxides with alkyl or aromatic Grignard reagents (non-copper catalyzed) have been reported,[15] however, for our quinol, the literature conditions again results in the formation of the epoxide. We decided to examine the reaction directly on the free alcohol, as has been shown with the addition of organoaluminium reagents to quinol systems,[16] to avoid the adverse reactivity of the lithium anion of **6** at higher temperatures. We have found that the regioselective addition of Grignard reagents to quinol **6** occurs directly on the free alcohol, giving exclusively 1,4-addition to the face of the ring containing the hydroxyl group (the 8'-position) in high yields. Under these conditions[17] a variety of groups (alkyl, alkenyl, alkynyl) can be added to **6**, producing ABA analogues with a range of 8'-substituents (see Table 1).

The optically pure abscisic acid analogues, which were required for the enzyme inhibition assay, were prepared by first separating the enantiomers of the racemic methyl ester products of the Grignard additions by HPLC using a Chiracel OD column (>99% *ee*), followed by base hydrolysis to the acids.

The facial selectivity of the addition was verified through comparison of spectral data to published data of known compounds, and by analyzing the metabolism of (+)-8'-trideuteromethyl ABA[18] (8'-CD_3-ABA) in maize suspension-culture cells. In this system, we have shown that (+)-ABA is transformed to (-)-PA.[7] 8'-CD_3-ABA was found to be oxidized at the trideuteromethyl group to 8'-CD_2-PA, the product being confirmed by LC-MS.[19] Had the Grignard addition occurred at the 9' position, the labelled PA would still contain 3 deuterium atoms.

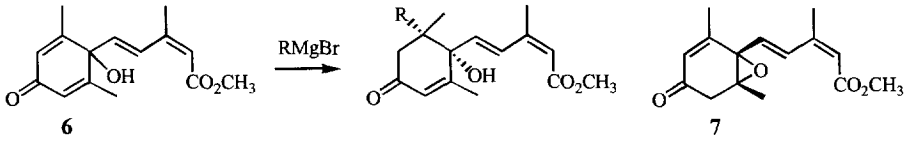


Table 1. Products from Addition of Grignard Reagents to Quinol **6**

Reagent[a] RMgX	Method	Product[b] 8'-R-ABA	% Yield
H_2C=CHMgBr	A	H_2C=CH-[12]	58
HC≡CMgBr	C	HC≡C	78
CH_3C≡CMgBr	C	CH_3C≡C	95
CD_3MgI	C	CD_3-	50 (69[c])
PhMgBr	B	Ph-	40 (56[c])
CH_3OCH_2MgCl[20]	B	CH_3OCH_2-[9]	70
CH_3CH_2MgBr	B	CH_3CH_2-[10]	77
CH_3CH_2MgCl	B	CH_3CH_2-	25
$PhCH_2MgCl$	A, B or C	$PhCH_2$-	<5

[a] unless otherwise noted, all reagents were purchased from Aldrich®

[b] new analogues show spectral data consistent with structure, known analogues show spectral data consistent with previously published data (ref given)

[c] yield based on recovered starting material

Inhibition of ABA 8'-Hydroxylase

We have developed a three stage assay in maize suspension-culture cells (variety Black Mexican Sweet)[6] to evaluate irreversible inhibitors of ABA 8'-hydroxylase. Initially, ABA 8'-hydroxylase activity is induced by ABA treatment of the cells, which are then washed and in the second stage, treated with the potential inhibitor and cycloheximide (to stop further protein synthesis). During this time, an irreversible inhibitor will inactivate some or all of the induced enzyme. The analogue is then removed and the amount of active enzyme remaining is analyzed by addition of (+)-ABA as substrate for the enzyme.[21] The enzymatic conversion of ABA to PA is measured by HPLC[7] and the amount of PA present compared to a control which contained no analogue during the second stage of the experiment. If the enzyme has been partially inactivated, the amount of PA produced will be correspondingly lowered. (+)-8'-Methylene ABA[12] and (+)-8'-methylacetylene ABA[22] did not show any inactivation of the enzyme (data not shown), however (+)-8'-acetylene ABA,[23] the analogue with a terminal acetylene, inactivates about 60% of the 8'-hydroxylase (0.14 μmoles of PA produced/g fresh weight, vs 0.35 μmoles of PA for the control). The (-)-8'-acetylene ABA (0.36 μmoles of PA produced/ g fresh weight), included for comparison, has no effect.

We have recently developed a cell-free in vitro assay utilizing extracted microsomes as a concentrated source of the enzyme, to more fully characterize the 8'-hydroxylase enzyme. Our future research will be aimed at identifying the ABA 8'-hydroxylase through irreversible binding of the radiolabelled (+)-8'-acetylene ABA to the enzyme.

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References and Notes

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17. Typical procedure for Grignard reaction: To a cooled solution (-78 °C) of quinol 6 (100 mg) in dry THF (8 mL) and under argon is added 5 equiv of Grignard reagent (typically ~0.5 to 1.0 M in ether). The reaction mixture is left at –78 °C for 30 min. (method A), 2 h at –20 °C (method B) or is stored overnight at –20 °C (method C), then quenched with sat NH_4Cl, extracted into ether (3 30 mL), washed with brine, dried over sodium sulfate and purified by flash chromatography.
18. *(+)-8'-Trideuteromethyl ABA.* $[\alpha]_D^{20}$ + 376° (*c* 0.91 in MeOH). δ_H (500 MHz in CD_3OD) 7.76 (1H, d, *J* = 16.1, H-4), 6.23 (1H, d, *J* = 16.1, H-4), 5.91 (1H, d, *J* = 1, H-3'), 5.74 (1H, s, H-2), 2.51 (1H, d, *J* = 16.9, H-5'), 2.17 (1H, d, *J* = 16.9, H-5'z), 2.03 (3H, d, *J* = 0.9, H-6 or 3'), 1.92 (3H, d, *J* = 0.9, H-6 or 3'), 1.01 (3H, s, H-9'). δ_C (125.77 MHz in CD_3OD) 201.07, 169.42, 166.55, 151.10, 137.94, 129.40, 127.57, 121.66, 119.53, 80.59, 50.58, 42.63, 24.56, 21.24, 19.61. HREIMS at *m/z* 267.1549 (M^+ + 1) ($C_{15}H_{17}D_3O_4$ requires 267.1550).
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21. Under sterile conditions, the cells (0.2 g aliquots in 10 mL culture medium) were treated with (+)-ABA (24 h, 100 μM) to induce the enzyme, washed, and treated with 10 μM cycloheximide and the potential inhibitor (100 μM for 3 h). The analogue was removed by rinsing and the cells were treated with 10 μM cycloheximide and 100 μM (+)-ABA for 24 h. At that time, the PA content of the medium was analyzed by HPLC as previously reported.[7] Values shown are averages of triplicate samples. They have been corrected for residual PA that is formed in the absence of an ABA induction and for PA that is trapped in the cells at the end of the induction and carried over into the final stage of the experiment in which PA formation is measured.
22. *(+)-8'-MethylacetyleneABA.* $[\alpha]_D^{20}$ + 364° (*c* 3.0 in MeOH). FTIR υ_{max} (KBr)/cm^{-1} 3500-3000br (OH), 1670br (CO); δ_H (500 MHz in CD_3OD) 7.78 (1H, d, *J* 16.2, H-4), 6.00 (1H, s, H-3'), 5.95 (1H, d, *J* 16.1, H-4), 5.74 (1H, s, H-2), 2.80, (1H, br s, OH), 2.56 (1H, d, *J* 16.7, H-5'), 2.46 (1H, d, *J* 16.7, H-5'), 2.01 (3H, s, H-6 or 3'), 1.92 (3H, d, *J* 0.9, H-6 or 3'), 1.74 (3H, s, CCC*H*$_3$), 1.24 (3H, s, H-9'). δ_C (125.77 MHz in CD_3OD) 196.24, 170.62, 163.25, 151.21, 133.18, 130.17, 127.52, 118.36, 81.56, 80.59, 78.29, 47.99, 44.36, 23.53, 21.26, 19.25, 3.56. LRMS (electrospray) at *m/z* 311 (M^++Na, 100%), 289 (29, M^++1), 271 (29, M^+-18+1).
23. *(+)- and (-)-8'-Acetylene ABA.* $[\alpha]_D^{20}$ + 317° (*c* 0.51 in MeOH). FTIR υ_{max}(KBr)/cm^{-1} 3500-2500br (OH), 1684 (CO); δ_H (500 Mhz; CD_3OD) 7.81 (1H, d, *J* = 16.2, H-4), 6.03 (1H, s, H-3'), 5.95 (1H, d, *J* = 16.1, H-4), 5.77 (1H, s, H-2), 2.80, (1H, br s, OH), 2.63 (1H, d, *J* = 17.0, H-5'), 2.50 (1H, d, *J* = 16.9, H-5'), 2.27 (1H, s, *H*CC), 2.02 (3H, d, *J* = 0.7, H-6 or 3'), 1.95 (3H, d, *J* = 1.0, H-6 or 3'), 1.31 (3H, s, H-9'). LRMS (electrospray) *m/z* at 297 (M^++Na, 100%), 257 (3.3, M^+-H_2O+1), 275 (1.3, M^++1). (-)-8'-Acetylene ABA gave identical spectral data to the (+)-enantiomer, with the following change: $[\alpha]_D^{20}$ - 297° (*c* 0.78 in MeOH).

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