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THE STABILITY OF THE 1′,4′-DIOLS OF ABSCISIC ACID

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Key Word Index—1′,4′-*trans*-diol of ABA; 1′,4′-*cis*-diol of ABA; 1′,4′-dihydroabscisic acid; epimerization; abscisic acid.

Abstract—The 1′,4′-*cis* and 1′,4′-*trans*-diols of abscisic acid (ABA) are unstable in acidic conditions: they interconvert and oxidize to ABA. When the diols were held in acidic (pH 4) [^{18}O]H_2O the oxygen atoms of both the 1′- and 4′-hydroxyl groups exchanged with the medium with retention and inversion of configuration. Thus the (+)-[2-^{14}C]-*trans*-diol was epimerized to the (+)-[2-^{14}C]-*cis*-diol and to the (−)-[2-^{14}C]-*cis*-diol. Similarly, the [1′-^{18}O]*cis* and [1′-^{18}O]*trans*-diol lost ^{18}O during epimerization. The *trans*-diol was the more stable by about 30% and was also less prone to oxidation to ABA. Significant epimerization of the *cis*-diol occurred below pH 6 while an equivalent reaction of the *trans*-diol occurred below pH 5.

INTRODUCTION

The 1′,4′-*trans*-diol of abscisic acid (**2**) has been identified as a metabolite of exogenous abscisic acid (ABA) supplied to pea seedlings [1] and has been isolated from the phytopathogenic fungus, *Botrytis cinerea*, in which it may act as a precursor of ABA [2]. However, it has not been identified as an endogenous constituent of higher plants, although its epimer, the 1′,4′-*cis*-diol of ABA (**3**), has been isolated from immature seeds of *Vicia faba* [3].

Investigations into the metabolic role and biological activity of the diols of ABA have been hampered by their instability [4]. In solution the *cis*- and *trans*-diol of ABA interconvert [5] and also autoxidize to ABA [6]. This work investigates the conditions under which the diols autoxidize and interconvert and the effect of the interconversion on the stereochemistry of the products.

RESULTS

Stability of 1′,4′-*diols of ABA*

The recovery of the *cis*- and *trans*-diols from aqueous solutions was poor due to their oxidation to ABA and to their interconversion. The *cis*-diol was particularly unstable in acidic solutions and a large proportion was converted into ABA and *trans*-diol. The effect of pH on the isomerization and oxidation of each ^{14}C-labelled diol was investigated between pH 2.5 and 12 and separating the products by TLC after 3 days. The *cis*-diol (Fig. 1a) was relatively stable at high pH but below pH 6 oxidation to ABA occurred readily and isomerization to the *trans*-diol was highest at pH 3.6.

The *trans*-diol (Fig. 1b) was more stable than the *cis*-diol at low pH with little isomerization above pH 4.8. Below pH 4.8 oxidation and isomerization increased and at pH 2.5 the *trans*-diol was converted into a mixture containing ABA (57%), *cis*-diol (16%) and *trans*-diol (27%).

The site of inversion during interconversion of the diols

The interconversion of the 1′,4′-*cis*- and 1′,4′-*trans*-diols was analysed by mass spectrometry using ^{18}O and ^{2}H. A mass spectrum of the *trans*-diol of ABA methyl ester **5** does not usually show a molecular ion [1, 7] because the molecule dehydrates readily, although this was attributed to dehydration at the C-4′-position it had not been established. CIMS of the 4′-*O*-acetyl methyl-*trans*-diol showed that the 4′-*O*-acetyl group was lost as acetic acid giving ions at *m/z* 263 and 61. The two most abundant ions: *m/z* 61 and 231, contained oxygen atoms derived exclusively from the 4′- and 1′-hydroxyl groups respectively so these fragment ions were used to determine the proportion of ^{18}O in the 4′- and 1′-hydroxy groups of isomerized materials.

The interconversion of [1′-^{18}O]-*labelled* 1′,4′-*diols of ABA*

The incubation of Me-[1′-^{18}O]*cis*- and Me-[1′-^{18}O]-*trans*-diol at pH 3.5 for 72 hr showed that the 1′-hydroxyl group is exchangeable with the medium (Table 1). Recovered Me-[1′-^{18}O]*trans*- diol had lost 3.04% of its ^{18}O whereas the *cis*-diol produced by isomerization had lost 26.3% of its ^{18}O. Recovered Me-*cis*-diol had lost 8.3% of its ^{18}O while the Me-*trans*-diol formed had lost 11.2% of the 1′-^{18}O.

The stability of the 4′-*hydrogen atom during interconversion of the diols*

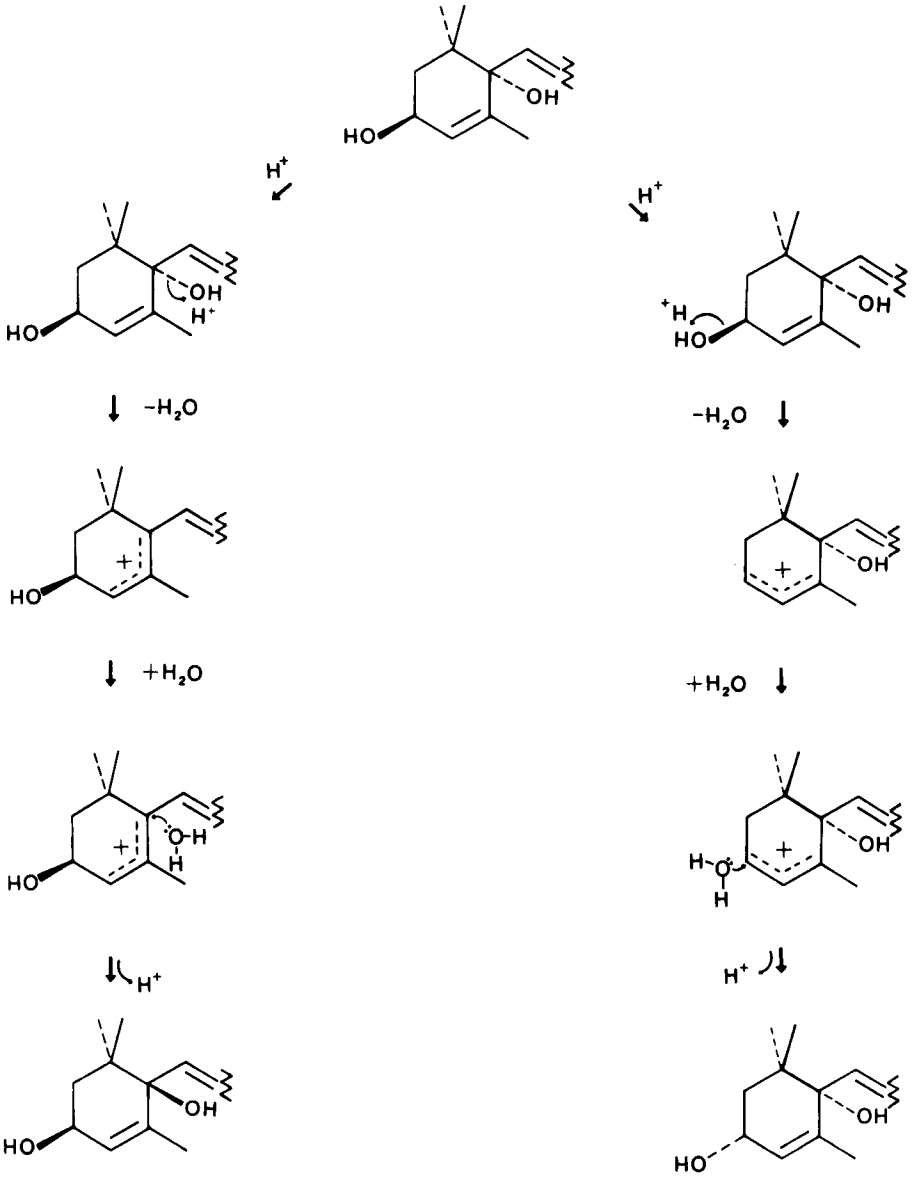
No undeuterated material was detected in the mass spectra of the Me-*cis*- and Me-*trans*-diol of ABA labelled with ^{2}H at C-4′ (98 atom% 4′-^{2}H), after 72 hr in acidic solution. The 4′-hydrogen atom, therefore, is not lost during the interconversion of the diols.

Interconversion of the diols in $[^{18}O]H_2O$

The 1′,4′-diols of ABA methyl ester in $[^{18}O]H_2O$ (97.2 atoms% ^{18}O), pH 3.5, exchanged ^{18}O into the 1′- and 4′-hydroxyl groups (Table 2). ^{18}O from water was readily exchanged with the 4′-hydroxyl group of Me-*cis*-diol (84% 4′-^{18}O) but not the 1′-hydroxyl. The Me-*trans*-diol produced from Me-*cis*-diol in $[^{18}O]H_2O$ had 76.3% ^{18}O in the 4′- and 11.7% ^{18}O in the 1′-hydroxyl. Thus, both the 1′- and 4′-hydroxyl groups are involved when the Me-*cis*-diol is isomerized to the Me-*trans*-diol. The Me-ABA (19.5% 4′-^{18}OH) produced reflected the proportion of ^{18}O in the Me-*trans*-diol. When Me-ABA was subjected to the same conditions ^{18}O exchanged into the 4′-ketone (87% 4′-oxygen exchanged) but no ^{18}O could be detected at C-1′.

Inversion of the 1′*-hydroxyl group during interconversion of the diols*

The isomerization of the (+)- and (−)-Me-[2-^{14}C]-*trans*-diol was investigated to determine whether the 1′- and/or 4′-hydroxyl groups were inverted during isomerization. The products formed in acidic solution were separated by HPLC on optically-active Pirkle columns. Thus, the *cis*-diol produced from the (+)- or (−)-*trans*-diol could be resolved into its enantiomers to determine whether inversion at the C-1′- or C-4′-positions had occurred. The (−)-Me-[2-^{14}C]*trans*-diol was converted into a mixture which contained 81.4% Me-*trans*-diol, 4.6% Me-ABA, 7.9% (+)-Me-*cis*-diol and 6.1% (−)-Me-*cis*-diol. The (+)- and (−)-*cis*-diols were identified by their HPLC retention times and their UV spectra. The



Scheme 1.

(+)-Me-[2-^{14}C]*trans*-diol produced a mixture with 75% Me-*trans*-diol, 3.1% Me-ABA, 10.2% (+)-Me-*cis*-diol and 11.7% (−)-Me-*cis*-diol. The isomerization of the diols, therefore involves inversion at C-1′ and C-4′.

DISCUSSION

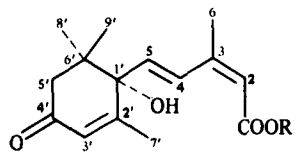
The 1′,4′-*cis*-**3** and 1′,4′-*trans*-diol of ABA (**2**) exhibit biological activity but this was attributed to their oxidation to ABA [4] and their susceptibility to oxidation in air has been reported [6]. The present work has shown that they are also interconverted. The interconversion and oxidations occur to a slight extent at all pH values measured but are rapid at low pH values. The inversion at C-1′ is surprising because of the chemical unreactivity of the tertiary hydroxyl group, the absence of exchange of the 1′-hydroxyl of ABA with [^{18}O]H_2O and the apparent absence of (−)-*R*-ABA in plants.

The interconversion of the diols in acid may be initiated by the protonation of one of the hydroxyl groups (Scheme 1) followed by the elimination of water to yield a carbocation, which is attacked by water to reform a diol by deprotonation. In the absence of any side reactions, either diol would eventually give the same equilibrium mixture of isomers. The position attacked by water would not necessarily be inverted by exchange with the medium. This was observed in [^{18}O]H_2O (Table 1).

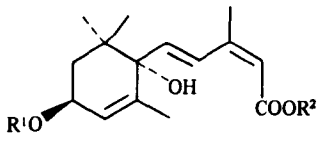
Interconversion of the diols with inversion at C-1′ provides a mechanism by which (−)-ABA may be produced within the plant. This could occur by a sequence such as (+)-ABA→(+)-*cis*-diol→(−)-*trans*-diol→(−)-ABA. However, because the diols occur at concentrations of 1–10% of those of ABA and because only a small percentage of the diols would be expected to isomerize at cellular pH, it can be predicted that only a very small proportion of the ABA in a plant would be (−) and it would be almost undetectable by existing methods. (−)-ABA, however, would be expected to accumulate as the glucose ester of ABA [8] in plants which contain relatively large quantities of diols.

The oxidation of the 1′,4′-diols to ABA could occur by hydride transfer. The carbocation derived from a diol could abstract a hydride ion from the 4′-position of another molecule of diol, converting it into ABA and itself yielding a desoxy diol. However, no product corresponding to a desoxy diol was found in solutions of the diols which had oxidized to ABA. Alternatively, the oxidation of the diols may involve a free-radical reaction.

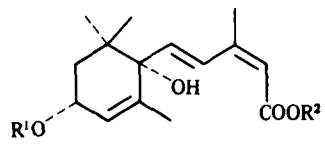
The finding that the diols interconvert and are oxidized more readily at acid than at basic pHs indicates that the isolation and analysis of the diols should be performed under slightly basic conditions.



	R
1	H
4	Me



	R¹	R²
2	H	H
5	H	Me
7	Ac	Me



	R¹	R²
3	H	H
6	H	Me
8	Ac	Me

EXPERIMENTAL

HPLC. Effluent was monitored with an HP 1040A Diode Array Detector (Hewlett-Packard, Waldbronn, F.R.G.). Radioactive compounds were detected with a continuous-flow HPLC radioactivity monitor. The column eluent was mixed with scintillant PPO (9 g/l) in Triton X-100–toluene–MeOH (30:67:15) at a ratio of 1:3 (eluent: scintillant) with a Precision Splitter Mixer (Reeve Analytical, Glasgow). The eluent–scintillant mixture was pumped to a Precision Radioactivity Monitor (Reeve Analytical) fitted with a homogeneous flow cell. Chromatograms of radiolabelled compounds were recorded on an HP-85B personal computer and were integrated and plotted using INT/143 software (Reeve Analytical).

Resolution of Me-1′,4′,-cis-*diol of ABA and the preparation of* (+)-S- *and* (−)-R-*ABA.* The reduction of Me-ABA to the Me-1′,4′-diols and resolution of racemic Me-1′,4′-*cis*-diol of ABA was as previously described [9] except for the following improvements: *RS*-1′,4′,-*cis*-diol Me ester was resolved by HPLC on a 4.6 (i.d.) × 250 mm Pirkle covalent *R*-phenylglycine column connected in series with 2 Pirkle Type 1-A columns (Regis, Morton Grove, IL, U.S.A.) with the mobile phase: isopropanol-hexane (2:23) 2.0 ml/min. The *S*-enantiomer had a retention time of 33.5 min and the *R*-enantiomer 36.5 min. The Me-1′,4′-diols of ABA were oxidized to Me-ABA using pyridinium chlorochromate on alumina [10]. The sample to be oxidized was dissolved in 1 ml hexane (dried by the addition of a molecular sieve) and a three-fold excess of pyridinium chlorochromate was added with stirring for 2 hr.

Interconversion of 1′,4′-cis-*diol of ABA and* 1′,4′-trans-*diol of ABA.* [2-^{14}C]-*cis*-Diol (25.6 mCi/mmol, 8 000 dpm) or [2-^{14}C]-*trans*-diol (25.6 mCi/mmol 8 000 dpm) were added to 10 ml 0.1 M Na citrate-Pi buffer at pH values between 2.4 and 7.2 or 0.1 M NaOH-Pi buffer (pH 7.2 to 12) in increments of 0.6 pH unit. The solns were left at 20° for 72 hr and 1′,4′-*cis*-diol, 1′,4′-*trans*-diol and ABA (1 mg each) added to each sample, which was then partitioned against Et_2O (twice). If necessary, the aqueous residue was acidified to pH 4 with oxalic acid and then re-extracted with Et_2O. The radioactivity remaining in the aqueous residue was less than 1% in all cases. The combined Et_2O

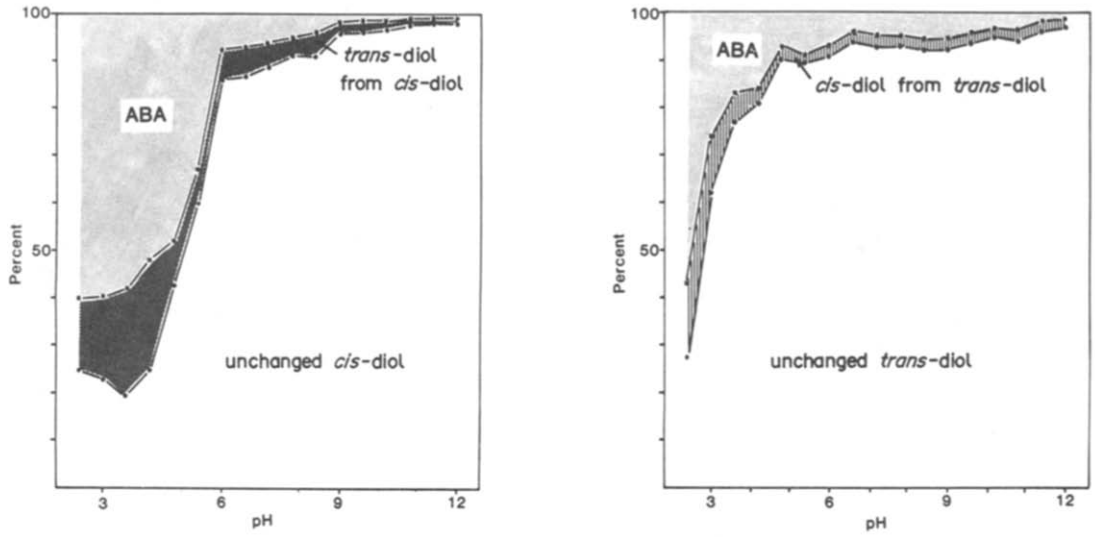


Fig. 1. Effect of pH on the stability of the 1′,4′-diols of ABA. The products of [2-^{14}C]-*cis*-diol of ABA and [2-^{14}C]-*trans*-diol of ABA after 72 hr in solutions buffered at pH 2.4 to 12 were separated by TLC and the radioactivity determined by liquid scintillation counting.

Table 1. Isomerization of [1′-^{18}O]*cis*- and [1′-^{18}O]*trans*-diol of ABA methyl ester at pH 3.5

	Starting material		After incubation			
	[1′-^{18}O]*trans*-diol		*trans*-diol		*cis*-diol	
	m/z	%	*m/z*	%	*m/z*	%
Fragment	61	100	61	100	61	100
containing 4′-OH	63	0.35	63	0.37	63	0.30
Fragment	231	17.75	231	17.52	231	7.27
containing 1′-OH	233	12.34	233	11.48	233	3.14
% 1′-^{18}O	41.0		39.6		30.2	
% starting material	100.0		96.5		73.7	
loss of 1′-^{18}O	—		3.4%		26.3%	
	[1′-^{18}O]*cis*-diol		*cis*-diol		*trans*-diol	
	m/z	%	*m/z*	%	*m/z*	%
Fragment	61	100	61	100	61	100
containing 4′-OH	63	0.22	63	0.23	63	0.19
Fragment	231	6.89	231	6.81	231	16.82
containing 1′-OH	233	4.63	233	4.11	233	9.37
% 1′-^{18}O	40.3		37.6		35.8	
% starting material	100.0		91.7		88.8	
Loss of 1′-^{18}O	—		8.3%		11.2%	

The methyl ester of [1′-^{18}O]*cis*- or [1′-^{18}O]*trans*-diol of ABA was left in pH 3.5 buffer for 72 hr and the products separated by normal phase HPLC. The retention of 1′-^{18}O was measured by mass spectrometry of the O-acetyl derivative. The ions *m/z* 231 ($^{18}O_0$) and 233 ($^{18}O_1$) were used to measure the proportion of ^{18}O in the samples. 2 experiments.

extracts were subjected to TLC on silica gel 60 F_{254}, (Merck) developed in toluene-EtOAc–HOAc (25:15:2.).

Preparation and isomerization of 1′,4′-*Me*-[1′-^{18}O]cis- *and* 1′,4′-*Me*-[1′-^{18}O]trans-*diol of ABA.* [1′-^{18}O]ABA from the sample used by Gray *et al.* [11] was methylated with CH_2N_2. Me-[1′-^{18}O]ABA (500 μg) was reduced with $NaBH_4$ at 0° for 30 min and the diols separated by normal-phase HPLC on an 8 × 250 mm Techsil 1 column eluted with *iso*-PrOH–hexane (1:19) at 4.0 ml/min. R_t s: MeABA, 6.5 min; Me-1′,4′-*trans*-diol of ABA, 7.1 min; Me-1′,4′-*cis*-diol of ABA, 11 min. The diols were left in 0.1 M NaOAc buffer (pH 4) for 72 hr. The products of isomerization were separated by normal phase HPLC with the same conditions used above. The proportion of ^{18}O in the products was measured by mass spectrometry.

Isomerization of the cis- *and* trans-*diols in* [^{18}O]H_2O. (+)-1′,4′*cis*- and (+)-1′,4′-*trans*-Diol methyl ester (200 μg) were dissolved in 30 ml [^{18}O]H_2O (97.2 atoms% ^{18}O; Novachem, South Yarra, Aust.) and HOAc (1 ml) added to give a final pH of 3.5. After 72 hr at 20° the soln was evapd under N_2 and the products chromatographed on three 4.6 (i.d.) × 250 mm Pirkle

Table 2. Isomerization of the Me-*cis*- and Me-*trans*-diol of ABA at pH 3.5 in [^{18}O]H_2O

	Starting material *cis*-diol		After incubation					
			cis-diol		*trans*-diol		ABA	
	m/z	%	*m/z*	%	*m/z*	%	*m/z*	%
Fragment containing	61	100	61	19.00	61	30.99	261	16.04
4'-OH	63	0.20	63	100	63	100	263	46.96
Fragment containing	231	7.75	231	6.14	231	31.34		
1'-OH	233	0.19	233	—	233	4.16		
% 4'-^{18}O	0.2%		84%		76.3%		74.5%	
% 1'-^{18}O	2.4%		—		11.7%			
	Starting material *trans*-diol		After incubation *trans*-diol				ABA	
	m/z	%	*m/z*		%		*m/z*	%
Fragment containing	61	100	61		100		261	20.74
4'-OH	63	0.4	63		29.96		263	8.93
Fragment containing	231	40	231		28.6			
1'-OH	233	0.8	233		0.44			
% 4'-^{18}O	0.4%		23.6%				19.5%	
% 1'-^{18}O	2.0%		1.5%					

The Me-1',4'-*cis*- and Me-1',4'-*trans*-diol of ABA were dissolved in [^{18}O]H_2O adjusted to pH 3.5 with acetic acid, and the products separated by HPLC. Mass spectrometry of the acetylated products was used to measure the proportion of ^{18}O in the 4'-($^{18}O_0$)—*m/z* 61; $^{18}O_1$—*m/z* 63) and 1'-($^{18}O_0$—*m/z* 231; $^{18}O_1$—*m/z* 233) positions.

columns (one covalent *R*-phenyl glycine connected in series with two type 1-A columns) with *iso*-PrOH–hexane (7 : 93) as the mobile phase at 2.0 ml/min. R_ts: Me-*trans*-diol, 28.5 min; Me-ABA, 32.5 min; (+)-Me-*cis*-diol, 46.2 min; (−)-Me-*cis*-diol, 48.3 min.

Preparation of [4'-^{18}O]cis- *and* [4',^{18}O]trans-*diol of ABA.* ABA (1 mg) was dissolved in 50 μl [^{18}O]H_2O to which a small piece of Na metal (*ca* 0.1 mg) had been added. After 16 hr, 50 μl MeOH was added and the [4'-^{18}O]ABA reduced with $NaBH_4$ at 0° for 30 min. The mixture was acidified, extracted with Et_2O and the products in the Et_2O phase were methylated and separated by normal-phase HPLC.

Isomerization of (+)- *and* (−)-[2-^{14}C]trans-*diol.* (+)- and (−)-[2-^{14}C]*trans*-Diol of ABA were prepared by the reduction of (+)- and (−)[2-^{14}C]ABA (25 mCi/mmol, 77 000 dpm) in 35 mM HOAc (100 μl, 72 hr). The products were separated on three Pirkle columns.

4'-O-Acetyl Me-1',4'-*trans*-diol of ABA: CIMS (methane GC) 100 eV, *m/z* (rel. int.): 291 $[M+H-MeOH]^+$ (3), 263 $(M+H-HOAc]^+$ (4), 245 (27), 232 (6), 231 (40), 213 (22), 137 (5), 125 (13), 61 $[HOAc+H]^+$ (100).

4'-*O*-Acetyl Me-1',4'-*trans*-diol of ABA: CIMS (methane GC) −ve ion 100 eV, *m/z* (rel. int.): 262 (16), 261 (26), 260 (100), 258 (5), 244 (8), 243 (20), 230 (14), 228 (12), 147 (5), 146 (18).

4'-*O*-Acetyl Me-1',4'-*cis*-diol of ABA: CIMS (methane GC) 100 eV, *m/z* (rel. int.): 305 $(M+H-H_2O]^+$ (2), 291 $[M+H-MeOH]^+$ (3) 263 $(M+H-HOAc]^+$ (8), 245 (11), 231 (47), 213 (8), 125 (8), 89 (6), 61 (100).

4'-*O*-Acetyl Me-1',4'-[4'-^{18}O]*trans*-diol of ABA: CIMS (methane GC) 100 eV, *m/z* (rel. int.): 353 $[M+29]^+$ (2), 307 $[M+H-H_2O]^+$ (5), 263 $[M+H-H^{18}OAc]^+$ (22), 245 (20), 232 (17), 231 $[M+H-H^{18}OAc-H_2O]^+$ (100), 213 (10), 63 $[H^{18}O-AccH]^+$ (39), 61 (17).

4'-*O*-Acetyl Me-1',4'-[1'-^{18}O]*trans*-diol of ABA: CIMS (methane GC) 100 eV, *m/z* (rel. int.): 265 (3), 263 (5), 233 (11), 232 (3), 231 (18), 213 (2), 125 (4), 89 (10), 61 (100).

4'-*O*-Acetyl Me-1',4'-[^{2}H]*trans*-diol of ABA: CIMS (methane GC) 100 eV, *m/z* (rel. int.): 264 (6), 232 (36), 214 (3), 125 (5), 61 (100).

4'-*O*-Acetyl Me-1',4'-[6-$^2H_3$]*trans*-diol of ABA: CIMS (methane GC) 100 eV, *m/z* (rel. int.): 266 (10), 248 (21), 234 (661), 216 (18), 137 (19), 133 (9), 128 (14), 125 (12), 114 (28), 98 (10), 89 (27), 71 (100), 65 (10), 61 (93).

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