Chemistry of a Colour Test for Abscisic Acid

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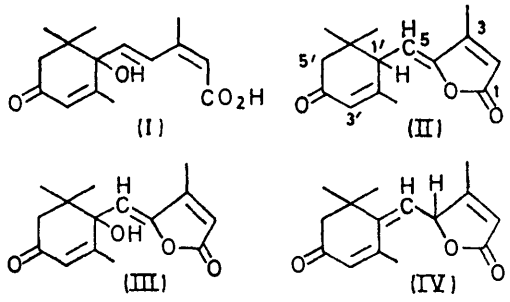
Abscisic acid and its methyl ester are converted by acid-catalysed dehydration into neutral products, one of which has been identified as an unsaturated γ-lactone. It gives an intense violet-red colour with alkalis, which fades as the lactone is hydrolysed. This reaction is a useful qualitative test for abscisic acid.

When abscisic acid (I) is heated with a mixture of formic and concentrated hydrochloric acids, the major product is the lactone (II), which in the presence of alkalis gives an intense, characteristic, violet-red colour lasting some seconds or minutes (depending on the concentrations of lactone and alkali). The mass spectrum of (II) resembles that of abscisic acid below *m/e* 246, except that the peak at *m/e* 111 representing the side-chain of the latter is absent; and the i.r., u.v., and ^{1}H n.m.r. spectra show the expected similarities with those of a lactone (III) reported by Ohkuma.[1] The u.v. spectrum and the chemical shift of the methine H signal, δ 3·41 p.p.m., exclude structure (IV), that of the likely first product of dehydration; the isolated lactone (II) can arise from this presumed intermediate by an allylic rearrangement. The lactone (II) is optically inactive when made from (+)-abscisic acid. (We thank Dr. D. R. Robinson for this result.)



The mixed formic–hydrochloric acid gives a good yield of lactone (II). With other strong acids, the reaction is more complex and at present little understood, although lactone (II) is always one of the products. Thus, hot formic acid alone converted abscisic acid into a mixture of neutral products, separated by t.l.c. into three major components, A (R_F 0·25), B (0·4), and C (0·55), with pronounced streaking between them. A, B, C, and the area between them were coloured violet-red when the chromatogram was sprayed with aqueous sodium hydroxide. Component B was the lactone (II), but A and C were unstable and were largely converted into (II) on attempted isolation. A single peak, corresponding to lactone (II), was observed when the mixture was analysed by g.l.c. The structures of A and C are not yet known; they appear to be lactones, C at least an isomer of (II). A similar pattern of products, often with additional components, was seen when other reagents were used: hot aqueous-alcoholic mineral acids, boron trifluoride–ether (which reacted only if diluted with, *e.g.* benzene), sulphonic acid ion-exchange resins in hot dichloromethane, molten oxalic acid, *etc*; weaker acids such as hot acetic acid effected no dehydration. Methyl abscisate behaved similarly, being converted in good yield into the lactone (II) if heated with a formic–hydrochloric acid mixture, and in rather lower yield and accompanied by other products if formic acid alone was used. The 2-*trans*-isomer of abscisic acid is also readily decomposed by hot formic acid—not, however, into neutral products but into at least five as yet unidentified acids, one of which gives a strong violet-red colour with alkalis.

The colour of alcoholic solutions of lactone (II) when aqueous sodium hydroxide is added is due to the appearance of an absorption band at 577 nm, which we attribute to the formation of the extended delocalised anion (V) by removal of a proton from C-1′. For comparison, the open-chain, unsubstituted anion $-O\cdot[CH{=}CH]_4\cdot CHO$ absorbs at 547·5 nm in dimethylformamide.[2] An analogous reaction (pink colour, $\lambda_{max.}$ 507 nm, λ_{infl} 570 nm) is given by a mixture of methyl 1′-deoxyabscisate (VI) and its 2-*trans*-isomer. If dilute (10^{-4}—10^{-3}M) solutions of lactone (II) are used, the colour develops slowly, then fades as the lactone ring is hydrolysed (*cf.* below). For reasons which are not quite clear, a strong colour is obtained only in the presence of water; sodium ethoxide in dry ethanol gives but a faint colour, which intensifies when water is added. It was found that a high absorbance reading at 577 nm could be obtained by adding 0·12 ml of 2N-aqueous sodium hydroxide to a few micrograms of lactone (II) in 2·5 ml ethanol; the colour reached its maximum intensity (ε 11,800, based on weight of lactone) in *ca.* 10 min, before fading slowly. The by-products A and C mentioned above give similarly coloured solutions, with $\lambda_{max.}$ ~577 nm, and it is probable that they give rise to the same anion as does lactone (II).

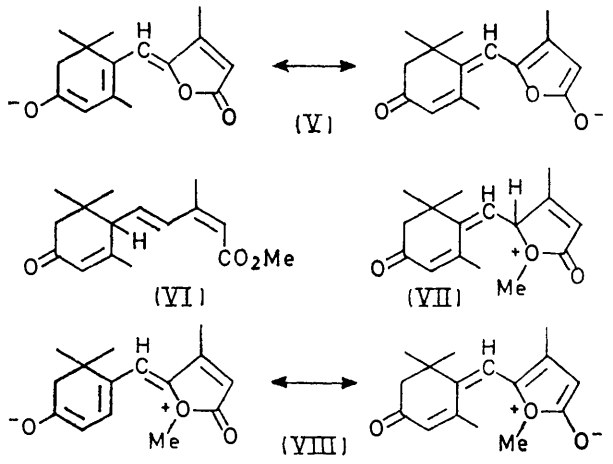
An interesting incidental observation was the appearance, during the first few minutes of heating, of a relatively weak but distinct violet-red colour when methyl abscisate was heated with formic acid. This colour (maximal absorption at 560 nm in formic acid) was similar to that given by lactone (II) in the presence of strong *bases*, suggesting a chromophore of the same type as that discussed above. If (VII) is invoked as an intermediate in the demethanolation, removal of a proton from C-4 by formate acting as a base, in competition with

[1] K. Ohkuma, *Agric. and Biol. Chem.* (*Japan*), 1965, **29**, 262; 1966, **30**, 434.

[2] S. S. Malhotra and M. C. Whiting, *J. Chem. Soc.*, 1960, 3812.

removal of the methyl group, could give small concentrations of the zwitterion (VIII) which would account for the transient colour.

We hoped to devise a simple spectrophotometric assay for abscisic acid based on this colour reaction. Although

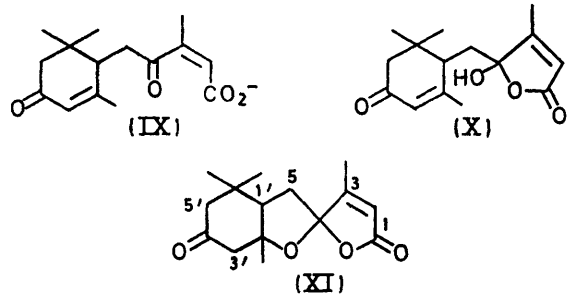


hydrolysis and fading could not be avoided (in non-hydroxylic solvents, *e.g.* using sodium hydride in tetrahydrofuran or dimethyl sulphoxide, a violet precipitate and colourless supernatant were obtained), microgram amounts of the pure lactone (II) could be estimated by standardising the measurements and using solutions of known concentration for calibration. However, the total dehydration products from abscisic acid behaved differently when alkali was added (even if a formic–hydrochloric acid mixture was used for the dehydration); the colour ($\lambda_{max.}$ still at 577 nm) appeared instantaneously, the initial rate of fading was very rapid and no meaningful absorbance value could be measured. This is due to the presence in the mixture of minor by-products, among them the component A mentioned above, which react more rapidly with the base and appear to give a relatively more intense colour than does lactone (II). An assay in which lactone (II) is isolated by t.l.c. and estimated spectrophotometrically or by g.l.c. would be practicable but time-consuming, dependent on a constant yield of the lactone and therefore unsatisfactory. As a sensitive qualitative test for abscisic acid or esters, the reaction is useful. The sample to be tested is heated with formic acid, which is then removed *in vacuo*; the residue is transferred to a depression in a white tile with ether or ethanol; and a drop of aqueous-ethanolic sodium hydroxide is added. A test on 0·1 μg of pure abscisic acid gives a distinct, transient violet-red colour, and somewhat larger amounts of the hormone can be readily detected in this way in fractions of plant extracts that are not themselves too strongly coloured. The colour obtained with 2-*trans*-abscisic acid is similar but less intense. We have not yet met any other compound in the acidic fraction of plant extracts which gives a positive reaction.

* The numbering shown in formulæ (II) and (XI), and used for n.m.r. results, is based on that of abscisic acid.

A spectrofluorimetric assay [3] for abscisic acid is based on the fact that it gives green-fluorescing spots on t.l.c. plates that have been sprayed with dilute sulphuric acid and heated. Similarly, during our dehydration experiments, the reaction mixtures usually acquired an intense green fluorescence. The origin of this is not clear, since lactone (II) does not fluoresce (even when re-heated with acids), and only *blue*-fluorescing minor by-products have been detected when the products were analysed by t.l.c.

The fading of alkaline solutions of lactone (II) is accompanied by hydrolysis of the lactone ring, and nothing can be extracted from the faded solutions with ether. After acidification, however, a new lactone which is isomeric with abscisic acid can be isolated. Its spectroscopic properties indicate structure (XI); the i.r. spectrum shows that hydroxy-groups are absent and there are no n.m.r. signals ascribable to hydrogens attached to oxygen-bearing carbon atoms. Presumably lactone (II) is hydrolysed to a keto-acid salt (IX and tautomers) which cyclises, *via* a lactol (X), when the solution is acidified and worked up. The formation of phaseic acid [4] provides an analogy for the step (X) ⟶ (XI). When heated with a mixture of formic and concentrated hydrochloric acids, the spiro-lactone (XI) regenerates the dehydrated lactone (II), detected by the characteristic violet-red colour with alkalis (the spiro-lactone gives a bright yellow colour), by the reappearance of an absorption band at 277 nm, and by t.l.c. analysis.



EXPERIMENTAL

M.p.s are corrected. Thin-layer chromatography was on Kieselgel-GF_{254} (Merck) developed in 3 : 2 (v/v) light petroleum–EtOAc. Abscisic acid and derivatives were racemic. A sample of methyl 1′-deoxyabscisate (mixture of 2-*cis*- and 2-*trans*-isomers) was kindly provided by Dr. M. Anderson, Shell Research Ltd. We thank Mrs. J. Tucker for mass spectra (A.E.I. MS-9) and Mr. D. M. Barnett for p.m.r. spectra (Varian HA-100).

2,5-Dihydro-4-methyl-5-(2,6,6-trimethyl-4-oxocyclohex-2-enylmethylene)furan-2-one (II)*.—(*a*) Abscisic acid (200 mg), formic acid (2 ml), and concentrated hydrochloric acid (0·3 ml) were heated at 95° for 30 min, and then evaporated under reduced pressure at 95°. The residue was taken up in ether and washed with aqueous $NaHCO_3$ (acidification and re-extraction of which yielded negligible amounts of material). Removal of the ether, decolouris-

[3] R. Antoszewski and R. Rudnicki, *Analyt. Biochem.*, 1969, **32**, 233.

[4] B. V. Milborrow, *Chem. Comm.*, 1969, 966.

ation with charcoal in ethanol, and recrystallisation from ether–methanol gave the *lactone* (II) (107 mg) as colourless needles, moderately soluble in ether, m.p. 130—131·5° (Found: C, 72·7; H, 7·1. $C_{15}H_{18}O_3$ requires C, 73·1; H, 7·4%); $\lambda_{max.}$ (EtOH) 277 nm (ε 22,400), shoulders at 238 (ε 10,000) and 233 (ε 9500) nm; $\nu_{max.}$ (CS_2) 1783vs, 1750infl, and 1666vs cm^{-1}; δ ($CDCl_3$)* 1·01 and 1·10 (each 3 H, s, 6′, 6′-di-CH_3), 1·92 (3H, d, *J* 1·5 Hz, 2′-CH_3), 2·21 (3H, d, *J* 1·5 Hz, 3-CH_3), 2·18 and 2·41 (AB, each 1H, d, *J* 17 Hz, 5′,-5′-H_2), 3·41 (1H, d, *J* 11 Hz, 1′-H), 5·18 (1H, d, *J* 11 Hz, 5-H), 5·92 (1H, broad, 2-H), and 6·05 p.p.m. (1H, broad, 3′-H); *m/e* 246 (rel. int. 8%; M^+), 231 (1·5), 190 (100), 162 (3·5), 147 (2·5), 134 (12·5), 119 (2·3), 106 (3), 91 (5·5), metastables at 146·7 (246 ⟶ 190), 138·1 (190 ⟶ 162), 110·9 (162 ⟶ 134), 105·7 (134 ⟶ 119), 94·9, 83·8 (134 ⟶ 106), 78·1 (106 ⟶ 91), 76·2. The *2,4-dinitrophenylhydrazone* had m.p. 226—228°, $\lambda_{max.}$ (EtOH) 381 and 270 nm, $\nu_{max.}$ (Nujol) 1770 cm^{-1}.

(*b*) Abscisic acid (653 μg), formic acid (0·1 ml), and concentrated hydrochloric acid (0·01 ml) were heated at 100° for 30 min, and then evaporated *in vacuo*. The residue was separated by t.l.c. and the major u.v.-absorbing band, coinciding with a marker of lactone (II), was scraped off and eluted with ethanol; the yield was estimated spectrophotometrically at 81%.

(*c*) Methyl abscisate (425 μg) was treated exactly as in (*b*), above. The yield of lactone (II) was 86% (spectrophotometric); it gave the correct u.v. and mass spectral characteristics and gave a violet-red colour with aqueous-alcoholic sodium hydroxide.

2,2′,3,3a,4,5,5′,6,7,7a-*Decahydro*-3′,4,4,7a-*tetramethylbenzo*[b]*furan*-2-*spiro*-2′-*furan*-5′,6-*dione* (XI)*.—The lactone (II) (42 mg) in ethanol (2 ml) and N-aqueous NaOH (1 ml) was left at room temperature until the initial intense violet colour had faded to a pale red (~1 h). Partial evaporation, dilution with water, and ether-extraction yielded <0·5 mg of material. Acidification of the aqueous layer and further extraction gave an oil (44 mg) from which the *lactone* (XI) (30 mg) was obtained as colourless needles, m.p. 146—148°, by crystallisation from ether (Found: C, 68·2; H, 7·9. $C_{15}H_{20}O_4$ requires C, 68·2; H, 7·6%); the u.v. spectrum (EtOH) showed strong end-absorption only; $\nu_{max.}$ (CCl_4) 1779vs, 1721vs cm^{-1}; δ (CCl_4)* 1·07 and 1·20 (each 3H, s, 6′,6′-di-CH_3), 1·60 (3H, s, 2′-CH_3), 2·00 (3H, d, *J* 1·5 Hz, 3-CH_3), 2·1—2·7 (7H, complex), and 5·85 p.p.m. (1H, *J* 1·5 Hz, 2-H); *m/e* 264 (rel. int. 37%; M^+), 249 (21), 231(2), 222(2), 220(3), 208(14), 207(100), 190(4), 189(4), 166(12), 165(37) (metastable for 207 ⟶ 165 at 131·6), 164(15), 154(21), 152(12), 151(18), 124(18), 122(18), 111(18), 110(8), 99(12), 96(26), 95(15), 83(24), 68(24), 55(12), 43(44).

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* See footnote on page 920.