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FORMATION OF SUBSTITUTED TRUXILLIC AND TRUXINIC ACIDS IN PLANT CELL WALLS—A RATIONALE

A. B. HANLEY,* W. R. RUSSELL and A. CHESSON

Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB2 9SB, U.K.

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Key Word Index—Gramineae; truxillic acid; truxinic acid; photodimerization.

Abstract—The formation of phenolic cyclobutane dicarboxylic acids (truxillic and truxinic acids) in plant cell wall materials was investigated using model compounds. Two possible mechanisms were considered—cyclodimerization initiated by light or by a free radical process. Diesters formed by reaction of cinnamic acid with a series of diols had a range of distances separating the double bonds and, upon irradiation with ultraviolet light, gave dimerization products. Dimerization did not occur when peroxidase was added to the diesters to mimic phenolic coupling reactions leading to the formation of lignin *in vivo*. A minimum separation of two cinnamyl moieties and an optimal relative orientation was observed which can be used to predict substitution patterns for unsaturated phenolic residues on the sugar backbone in plant cell walls.

INTRODUCTION

A range of cyclobutane derivatives [truxillic and truxinic acids (**1a–f**)] has been isolated from graminaceous cell wall material [1, 2], which apparently arise from the cyclodimerization of substituted cinnamic acids. The dimerization process could arise from the photocatalysed [2+2] cyclodimerization of *p*-coumaroyl and feruloyl groups esterified to arabinoxylan residues in the walls, a mechanism previously demonstrated for cinnamic acid [3]. A strict requirement for such a reaction to take place is that the reacting alkenyl groups should be held within *ca* 4 Å of one another [3]. The apparent lack of appropriate crystallinity is an obstacle to dimerization by this mechanism for ferulic acid [4, 5]. However, Morrison *et al.* [6] have recently demonstrated that both ferulic and *p*-coumaric acids and, indeed mixtures of the two acids, can be induced to dimerize under appropriate conditions.

Various isomeric forms of the cyclobutane products can occur depending upon the relative orientations of the starting materials. If the two molecules are in the same relative orientation then head-to-head dimers result, while if they are in the opposite orientation, tail-to-head dimers are produced. A further level of complexity arises from the type of double bond present in the putative monomers. If both monomer units are in the *E* conformation then *trans* addition will occur (**1a–c**), while *Z* conformation will result in *cis* (**1d–f**) dimerization. Mixed *Z* and *E* products could also be formed. The relative orientation of the aromatic ring and the carboxyl moiety in the product will provide evidence for the nature of the starting monomers. Hartley *et al.* suggest that while the dimers obtained most commonly are those derived from cycloaddition of two *E*, *E* cinnamic acid derivatives, a significant number of monomers must be in the *Z* conformation since *cis* products are also obtained and this can be rationalized if photocatalysed *E–Z* transitions of the monomers occur *in vivo* [6].

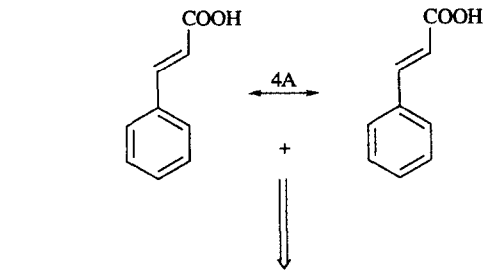
We have investigated the formation of cyclodimers by preparing model compounds from the esterification of cinnamic acid to a series of diols (**2a–d**). We then attempted to induce cyclodimerization by UV irradiation and by incubating the model compounds with peroxidase thus mimicking the biological conditions necessary for the production of lignin. In this way a model for the formation of this particular type of lignin–carbohydrate complex (LCC) can be tested.

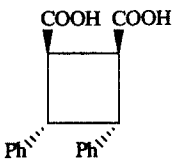
RESULTS AND DISCUSSION

The model compounds chosen were the diesters formed from a series of primary diols in which the separation between the propenyl moieties increased by one methylene group for each successive model compound (**2a–d**). Some consideration was given to preparing diesters using the corresponding unsaturated diols (e.g. 2-butene-1,4-diol). However, the likelihood of *trans–cis* isomerization when UV irradiation was taking place would make control and quantification of the effects observed difficult.

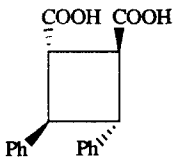
Diesters were prepared by refluxing a mixture of the appropriate diol (1.1 mol equiv.) with cinnamic acid

*Author to whom correspondence should be addressed, at: MAFF Food Science Laboratory, Norwich Research Park, Colney, Norwich, Norfolk NR4 7UQ, U.K.





1a



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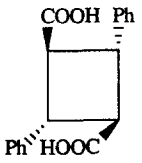

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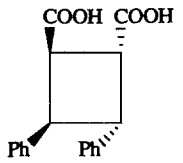

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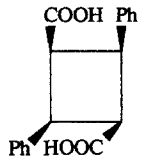

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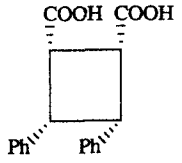

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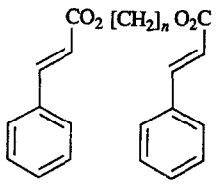

2a $n=2$
2b $n=3$
2c $n=4$
2d $n=5$

(2 mol equiv.) in toluene under Dean and Stark conditions. When esterification was attempted by refluxing in toluene in the presence of a trace of concentrated sulphuric acid without removal of water then quantitative yields of the monoesters were obtained. Attempts to use 1,3-dicyclohexylcarbodimide as coupling agent in ethyl acetate gave only the *N*-acylurea and no ester was detected.

The structures of the diesters were confirmed by NMR spectroscopy. In the ^{1}H NMR spectrum, a distinctive feature was the pair of coupled doublets at *ca* 6.5 ppm and 7.8 ppm ($J = 16$ Hz) corresponding to the hydrogens attached to the propenyl moiety. In the case of the ester with butane-1,4-diol (**2c**), the symmetrical nature of the product removed much of the fine structure of the linking methylene groups and two singlets with fine structure at *ca* 1.9 ppm and *ca* 4.3 ppm were observed corresponding to the protons attached to the carbons β and α to the ester linkage, respectively. The mass spectra of the diesters showed the expected molecular and fragment ions.

Each of the four diesters, **2a–d**, was taken up in acetone and irradiated in a UV irradiation cell for 2 hr. The products were purified by flash chromatography and examined by NMR. The most significant feature of the NMR spectra in the case of **2b** and **2c** was the loss of the resonances at 6.5 ppm and 7.8 ppm which were previously assigned to the propenyl moieties. This suggested that cyclodimerization had indeed taken place. In the case of **2a** and **2d**, no cyclodimerization product was formed. In both cases the only detectable products (TLC) were starting material and some polymeric material which remained on the baseline. Prolonged irradiation of both **2b** and **2c** led to increased formation of the polymeric product. The breakdown of material after prolonged irradiation is consistent with the earlier report of Balan *et al.* [7] who found that the photodimerization of substituted cinnamic acid derivatives gave rise to photounstable dimers which, after prolonged irradiation, polymerized to higher M_r products which remained on the baseline. Although unstable dimers may be formed, this does not provide proof for cyclodimerization product formation.

In the second series of experiments, each of the diesters was incubated with peroxidase and hydrogen peroxide. It has previously been demonstrated that, under similar conditions, lignin model compounds can be formed from cinnamyl alcohols via free radicals [8]. Although prolonged incubations of up to 16 hr were carried out, no detectable cyclobutane-type products were detected. The only compounds observed were the apparent polymers (or similar compounds) detected previously (*vide infra*) and starting material. This would suggest that free radical-mediated formation of truxillic and truxinic acids catalysed by a peroxidase enzyme does not occur although the source of peroxidase (horseradish) is not directly comparable to the situation *in vitro* in Gramineae.

The first series of experiments involving photodimerization are consistent with previously published work which states that a separation of *ca* 4 Å is necessary for dimerization to take place [3]. In the case of the diester formed with ethanediol (**2a**), model building suggests that the geometry of the two cinnamyl groupings means that they cannot orientate such that the alkenyl groups are parallel and are able to participate in a 2+2 cycloaddition reaction. Since steric requirements for this reaction include the necessity for the two double bonds to be in the same plane and adjacent to one another, it is not surprising that reaction does not occur in this instance. This becomes clear if a theoretical model is considered. The tetrahedral sp^3 nature of saturated carbon bonding means that, in order to minimize steric interactions in the diester, the aromatic nuclei should be staggered relative to one another. While free rotation about the single C–C bond of the diol can take place, the need to avoid steric interference means that the preferred conformation will tilt the aromatic rings and hence the propenyl moieties

away from one another. Under such circumstances, 2+2 cyclodimerization cannot take place.

In the case of the pentanediol diester the theoretical model suggests that the propenyl moieties cannot be brought close enough together to enable cycloaddition to take place because of the number of methylene groups which separate the cinnamyl groups. Under these circumstances the failure to form a cyclobutane addition product again is not surprising.

Butanediol and propanediol esters form cycloaddition products when irradiated. Models suggest that both diesters conform to the relatively strict conformational requirements necessary for cyclodimerization to take place. In the case of the propanediol diester the ethylenic bond separation varies between *ca* 3.5 Å and 6.5 Å. The butanediol diester has similar molecular constraints with the distance being between *ca* 4.0 Å and 7.0 Å. These results confirm the requirement that a separation of the order of 3–4 Å is necessary for cycloaddition reaction to take place. In plant cell walls the reacting molecules are considered to be esterified to a polysaccharide backbone (arabinoxylan in the case of Gramineae). In a typical sugar, model building suggests that cinnamic acid-type moieties esterified on to adjacent positions in the ring could be separated comfortably by 4 Å but without the aromatic moieties stacking. This would disfavour cyclodimerization. Substitution at the next carbon atom can also permit the ethylenic groups to be separated by *ca* 4–5 Å. However, substitution directly across the ring places the unsaturated groups too far apart for reaction to occur readily. This is principally a consequence of the positional restraints placed upon the molecule by the rigidity of the sugar ring as compared with the rather more free alkyl chain.

Morrison *et al.* [6] have recently investigated the *in vitro* dimerization of *p*-coumaric and ferulic acids and compared the products with the distribution of substituted cyclobutane dimers derived from plant cell walls from (*E*)-*p*-coumaroyl and (*E*)-feruloyl trisaccharides. In this elegant study, the authors noted a similar product distribution between the various isomers in both cases. The major dimers observed were 4,4′-dihydroxy-α-truxillic acid and 4,4′-dihydroxy-3-methoxy-α-truxillic acid, all of which arise from cyclodimerization of substituted *E* cinnamic acids. In more complex systems the yield of cyclobutane dimers was lower and the percentage of isomers other than the simplest ones increased. Clearly this is a consequence of the relative orientation of contributing monomers and the rigidity with which they are held in place.

It is clear from the above experiments and model building that certain substitution patterns are likely to be more advantageous to cyclodimerization than others. If the reaction is indeed light-catalysed, the relative orientations of all the molecules which react together must be such that the separation of the double bonds is between 3.5–4.1 Å and that they are parallel to one another. The high level of conversion of phenolic monomers converted to dimers suggested by Hartley [1] (*ca* 15%) argues against a random orientation. Our results and those of Morrison [6] suggest that the orientational requirements for light-catalysed dimer formation are very high. It is not clear that the traditional picture of cell wall material can explain both sets of results.

The failure of peroxidase to catalyse the production of cyclodimers does not necessarily mean that this mechanism of formation is eliminated from consideration. The rate of lignification and cross-linking varies depending on the age of the plant and the cell type; the complex interplay of enzymes and cellular components makes precise delineation of the mechanism of formation of truxillic and truxinic acids difficult. Linkages through both positions of the propenyl moiety have been postulated in lignin by an oxidative mechanism catalysed by peroxidase. Therefore, dimerization by a free radical route is theoretically possible. The commercial peroxidase used (from horseradish) may not facilitate the reaction due to lack of specificity. Nevertheless, this source has been used previously [8] to produce lignin-type molecules from cinnamyl alcohols and hence radical formation is presumably possible. Further studies are presently underway in order to elucidate the mechanism and stereochemical consequences of the dimerization process in plant cell wall material.

EXPERIMENTAL

Preparation of diesters. Cinnamic acid (2.0 mol equiv.) was suspended in toluene and treated with one of the four diols (1.2 mol equiv.). The mixt. was refluxed using a Dean and Stark trap to remove H_2O for 4 hr. After this time the mixt. was allowed to cool to room temp. and evapd to dryness to afford the crude product as an off-white solid in each case which could either be recrystallized from EtOAc–hexane directly or else purified by flash CC on silica gel, (TLC grade) using EtOAc–hexane (3:7→1:1) as mobile phase. Ethane diol diester: yield 65%. EIMS *m/z* 313 $[M]^+$. ^1H NMR (ppm) ($CDCl_3$): 4.00 (*m*, 4H); 6.50 (*d*, $J=7$ Hz, 2H); 7.50 (*m*, 10H); 7.75 (*d*, $J=7$ Hz, 2H). Propane diol diester: yield 72%. EIMS *m/z* 327 $[M]^+$. ^1H NMR (ppm) ($CDCl_3$): 2.15 (*q*, $J=6$ Hz, 2H); 4.41 (*t*, $J=6$ Hz, 4H); 6.48 (*d*, $J=16$ Hz, 2H); 7.40 (*m*, 6H); 7.53 (*m*, 4H); 7.73 (*d*, $J=16$ Hz, 2H). Butane diol diester: yield 61%. EIMS *m/z* 340 $[M]^+$. ^1H NMR (ppm) ($CDCl_3$) 1.85 (*br s*, 4H); 4.25 (*br s*, 4H); 6.50 (*d*, $J=7$ Hz, 2H); 7.50 (*m*, 10H); 7.75 (*d*, $J=7$ Hz, 2H). Pentane diol diester: yield 55%. EIMS *m/z* 354 $[M]^+$.

Photodimerization products. Photodimerization was carried out using a Hanova UV irradiation cell at 3500 Å. De-aerated Me_2CO solns were irradiated for 2 hr and the product isolated by evapn *in vacuo*. Propyldimer: ^1H NMR (ppm) ($CDCl_3$) 2.05 (*q*, $J=6$ Hz, 2H); 4.60 (*t*, $J=6$ Hz, 4H); 6.05 (*m*, 2H); 6.25 (*m*, 2H); 7.75 (*m*, 10H). Butyldimer: ^1H NMR (ppm) ($CDCl_3$) 1.40 (*m*, 2H); 2.10 (*br s*, 2H); 3.75 (*m*, 2H); 4.10 (*m*, 2H); 4.25 (*br s*, 4H); 7.50 (*m*, 10H). Decoupling the broad singlet at 2.10 ppm led to sharpening of the broad singlet at 4.25 ppm.

Reaction with peroxidase. The diesters (typically 50 mg in Me_2CO) were incubated with peroxidase (Type VI from horseradish, 1000 U) and H_2O_2 (1.2 mol equiv.).

The products were isolated by extraction into EtOAc (× 3) and evapn of the organic extract *in vacuo*.

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