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BIOSYNTHESIS OF FLAVAN-3-OLS AND OTHER SECONDARY PLANT PRODUCTS FROM (2*S*)-PHENYLALANINE*

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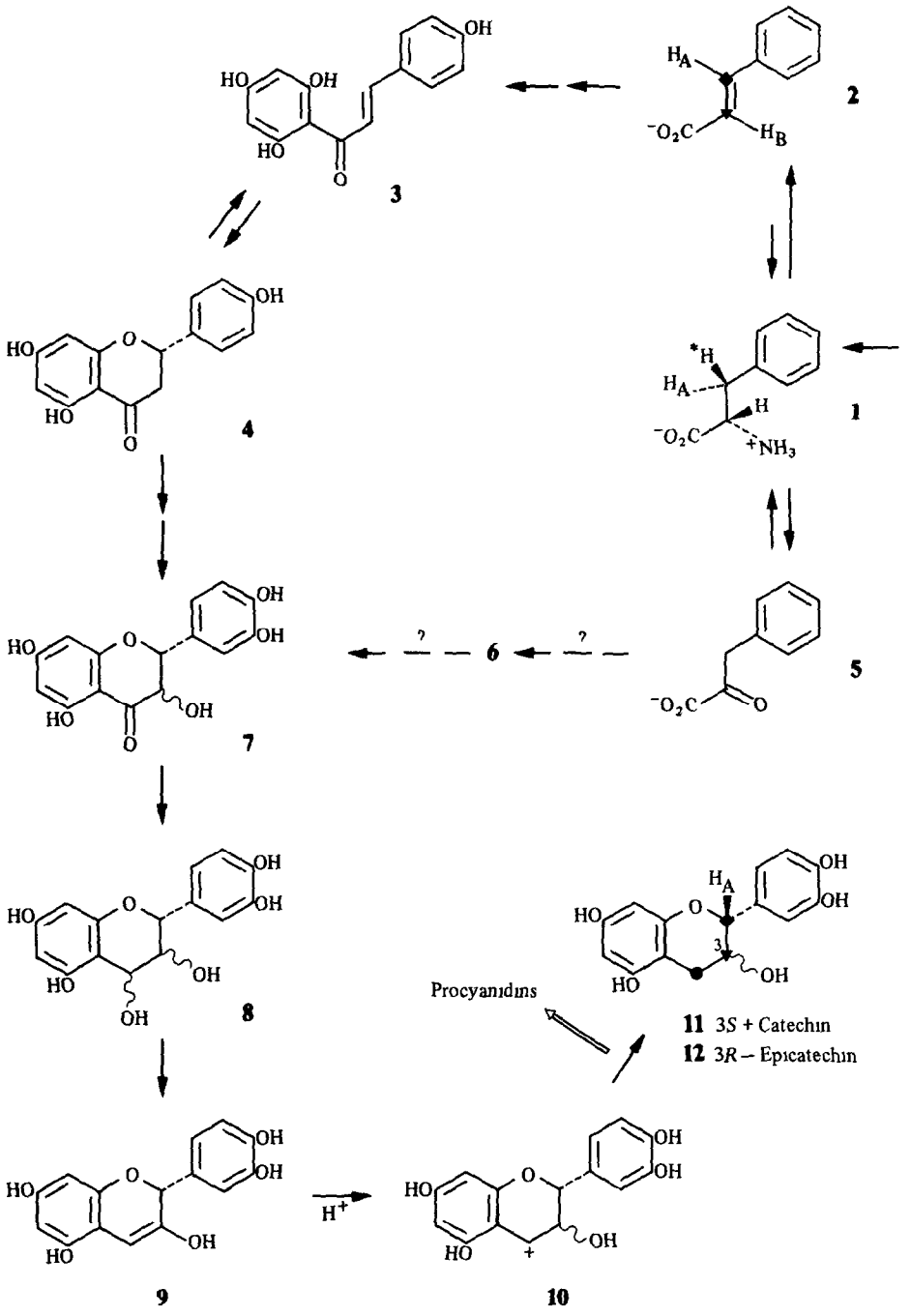
Abstract—(2*S*)-Phenyl[2-^{14}C,3*R*-$^{3}H_1$]alanine and (2*S*)-phenyl[2-^{14}C,3*S*-$^{3}H_1$]alanine have been employed as substrates to study procyanidin and flavan-3-ol biosynthesis Parallel studies with the cyanogenic glucosides prunasin and sambunigrin, Winterstein's acid [(3R)-3-dimethylaminophenylpropionic acid] and tropic acid show these to be derived by stereospecific processes from (2*S*)-phenylalanine New proposals for procyanidin biosynthesis are briefly commented upon

INTRODUCTION

The discovery of the chemical nature of the proanthocyanidins [1–4] ('condensed tannins' of the earlier literature) has led to a proliferation of interest in these substances from biochemical, chemical and botanical points of view Based on isotopic tracer experiments with intact shoots of plants a theory for the biogenesis of procyanidins (e g **13**) and the related flavan-3-ols (+)-catechin (**11**) and (−)-epicatechin (**12**) was advanced in 1977 [5–7] (Fig 1) A key role was proposed for the hypothetical intermediate the flav-3-en-3-ol (**9**) itself derived from a flavan-3, 4-diol (**8**) Although the presence of either intermediate (**8**, **9**) in plant tissues could not be directly demonstrated this suggestion was based on three experimental observations, namely (i) the incorporation of the intact C_6–C_3 cinnamate unit (**2**) into the flavan-3-ol skeleton with retention (> 80%) of the hydrogen atom (H_A) at C-3 but the loss (usually > 90%) of the hydrogen atom at C-2 (H_B), (ii) the different extents of isotopic labelling of chain extension and chain termination flavan-3-ol units in procyanidin oligomers, and (iii) the occurrence in some plants of procyanidins in which the chain termination unit and the sole free flavan-3-ol have one configuration at C-3 whilst the chain extension unit has the opposite configuration at this point, e g *Rubus* sp where the flavan-3-ol (−)-epicatechin (**12**) and procyanidin B-4 [**13**, (+)-catechin-(4α → 8)-(−)-epicatechin] so far as can be ascertained occur exclusively in the plant tissue

Recent work [8, 9] on procyanidin biosynthesis, principally by two groups, has prompted us to publish other experimental data relating to this problem and to comment on the new proposals A significant feature of the earlier work [5–7] in which [3-^{3}H]cinnamic acid (**2**, H_A = ^{3}H) was administered to a variety of plants was the small, variable but consistent loss of tritium label from H-2 of the various flavan-3-ol units of the procyanidins It was indicated that this might be explicable if the intermediacy of an α-hydroxychalcone (**6**), derived from phenylpyruvic acid (**5**), were invoked in the biosynthesis of the central flavanonol (**7**) The idea was thought to merit further consideration In order to investigate this possibility experiments have been conducted on the metabolism of several higher plant secondary metabolites including the flavan-3-ols (**11**, **12**) and procyanidins using stereospecifically labelled (2*S*)-phenyl[3*R*-$^{3}H_1$]- and (2*S*)-phenyl [3*S*-$^{3}H_1$]alanine (**14**, **15**) as substrates Previous studies on plant and microbial metabolites, in whole or part organisms, using these substrates or analogues have met with varying success [10–14] Thus Battersby *et al* [10], in a study of the biosynthesis of the alkaloid colchicine in *Colchicum autumnale*, demonstrated unequivocally that (2*S*)-phenylalanine was first metabolised to cinnamic acid by loss of ammonia and stereospecific loss of the 3-pro *S*-proton before incorporation into the alkaloid Conversely in the case of cyclopenin and cyclopenol (metabolites of *Penicillium cyclopium*) the random (> 50%) loss of ^{3}H from (2*S*)-phenyl[3*R*-$^{3}H_1$]- and (2*S*)-phenyl[3*S*-$^{3}H_1$]alanine observed by Kirby and Narayanaswami [11] was explained by assuming that the amino acid is rapidly converted by a transaminase into the α-keto acid which would then lose the isotope randomly by enolisation Analogously using *Bacillus brevis* Parry and Kurylo-Borowska [12] in a study of the mechanism of β-tyrosine (**18**) formation, using **16** and **17** as substrates, obtained uninterpretable results with the whole organism Using the purified mutase enzyme however the results clearly indicated that migration of the amino group from C-2 of α-tyrosine to C-3 leads to loss of the 3-pro *S*-proton and inversion of configuration at C-3 In higher plants loss of ^{3}H isotopic tracer from precursors such as **14** and **15** may be envisaged to occur principally by two processes alternate to those of direct incorporation into a metabolite The first is that of transamination as

*Part 8 in the series "Plant Proanthocyanidins" For part 7 see Gupta, R K and Haslam, E (1981) *J Chem Soc Perkin Trans* 1, 1148

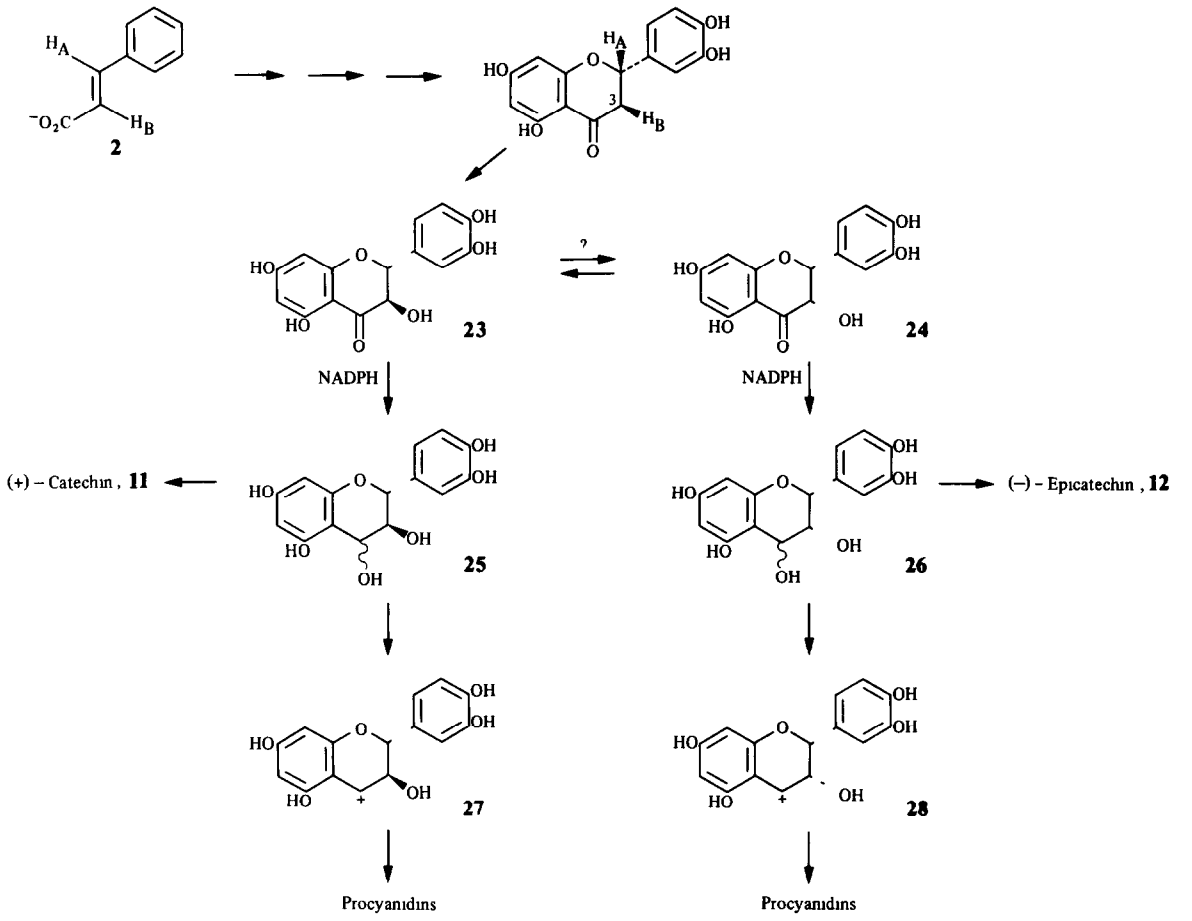


Scheme 1 Biosynthesis of procyanidins (see refs [5–7])

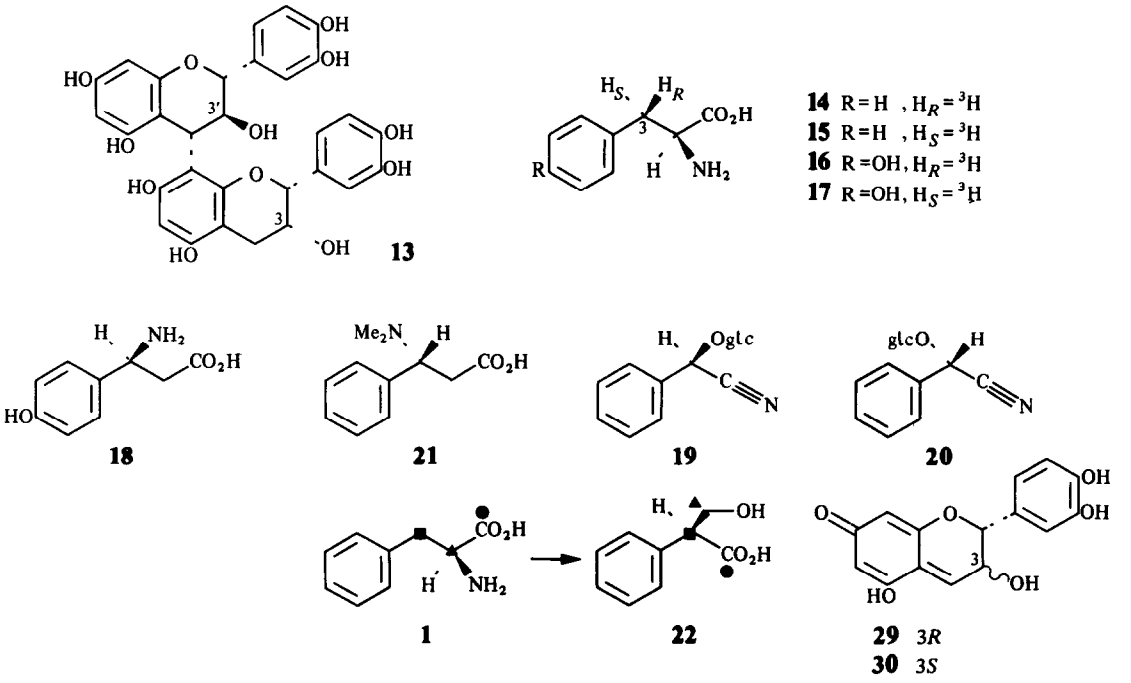
alluded to above (Kirby and Narayanaswami [11]) The second is reversible exchange of the 3-pro *S*-proton by PAL [15, 16] Where one or both of these processes can occur it may well mask the underlying stereospecificity of any secondary metabolic reaction

RESULTS AND DISCUSSION

The substrates (2*S*)-phenyl[3*R*-$^3H_1$]- and (2*S*)-phenyl [3*S*-$^3H_1$]alanine (**14** and **15**) were synthesized by published procedures [10], admixed with (2*S*)-phenyl[2-^{14}C]alanine and their isotopic content at the 3-pro *R* and 3-pro *S* positions determined by measuring the change in $^3H/^{14}C$ ratio on treatment with PAL and conversion to cinnamic acid This analysis showed **14** to contain 88 % of its tritium in the 3-pro-*R* position and **15** to contain 83 % of its tritium in the 3-pro-*S* position The substrates were used to study the biosynthesis of (−)-epicatechin (**12**) in *Aesculus* × *carnea*, *A hippocastanum* and *Prunus laurocerasus* and (+)-catechin (**11**) in *Salix caprea* and *Taxus*



Scheme 2 Procyanidin biosynthesis (see ref [8])



baccata by methods previously described [5–7] The results are shown in Tables 1 and 2 They depict a fairly consistent pattern with an apparently random loss of (at least half in some cases) the ^3H isotopic tracer Small changes are observed with changing length of exposure to the substrate

For comparative purposes the concomitant assimilation of the isotopically labelled amino acids was also followed in the experiments with *P laurocerasus* into the cyanogenic glucoside [17] prunasin (**19**) as well as (−)-epicatechin (**12**) (Table 3), and in *T baccata* into (3*R*)-3-dimethylamino-3-phenylpropionic acid (**21**, Winterstein's acid, a component of the taxine group of alkaloids [18]) (Table 4) In the case of the latter metabolite it was normally isolated, after elimination of the dimethylamino group, as cinnamic acid Although the results with (2*S*)-phenyl[2-^{14}C, 3*S*-$^3H_1$]alanine (**15**), where tritium retention was predicted, are generally less compelling than those obtained with the (3*R*) stereoisomer—probably for the reasons noted above—the data quite clearly demonstrate that in *P laurocerasus* and *T baccata* the biosynthesis of prunasin (**19**) and Winterstein's acid (**21**) respectively proceed by stereospecific processes from (2*S*)-phenylalanine They contrast sharply with the results obtained in the same experiments (Tables 1 and 2) for (−)-epicatechin and (+)-catechin, where the loss of ^3H isotope from **14** and **15** appears to be random In conjunction with these experiments an analogous study of the biosynthesis of sambunigrin (**20**), [19] was conducted in *Sambucus nigra* (Table 5) Sambunigrin is a cyanogenic glucoside diastereoisomeric with prunasin (**19**) and epimeric at the benzylic centre The result of these experiments fully compliment those of Conn and his collaborators [20] with the diastereoisomeric cyanogenic glucosides dhurrin and taxiphyllin In all four examples now investigated hydroxylation at the benzylic centre occurs

Table 1 Incorporation of (2*S*)-phenyl[2-^{14}C, 3*R*-$^3H_1$]alanine (**14**) into (+)-catechin (**11**) and (−)-epicatechin (**12**)

Plant	Duration of experiment (hr)	Incorporation of ^{14}C (%)	Loss of ^3H (%)	^3H at C-2
(+)-Catechin (**11**)				
S caprea	48	0 06	44	
S caprea	96	0 05	39	
T baccata	144	0 03	40 5	
T baccata	144	0 02	43 2	
(−)-Epicatechin (**12**)				
A × *carnea*	48	0 12	63	0 91
A × *carnea*	144	0 14	64	0 88
P laurocerasus	120	0 08	52	0 85
P laurocerasus	120	0 11	68	0 90
P laurocerasus	48	0 09	46	0 85
P laurocerasus	48	0 09	54	0 81

All substrates administered as solutions to cut stems of plant material

Table 2 Incorporation of (2*S*)-phenyl (2-^{14}C, 3*S*-$^3H_1$]alanine (**15**) into (+)-catechin (**11**) and (−)-epicatechin (**12**)

Plant	Duration of experiment (hr)	Incorporation of ^{14}C (%)	Loss of ^3H (%)	^3H at C-2
(+)-Catechin (**11**)				
S caprea	48	0 07	43	
S caprea	144	0 04	42	
T baccata	144	0 04	58	
T baccata	144	0 03	47	
(−)-*Epicatechin* (**12**)				
A × *carnea*	72	0 10	55	0 88
A × *carnea*	120	0 14	61	0 85
P laurocerasus	120	0 09	48	0 82
P laurocerasus	120	0 08	49	0 81
P laurocerasus	48	0 09	47	0 90
P laurocerasus	48	0 08	47	0 81

All substrates administered as solutions to cut stems of plant material

Table 3 Incorporation of (2*S*)-phenyl[2-^{14}C, 3*S*-$^{3}H_1$]- and (2*S*)-phenyl[2-^{14}C,3*R*-$^{3}H_1$]alanine into prunasin (**19**) in *P laurocerasus*

Expt number	Duration of experiment (hr)	Incorporation of ^{14}C (%)	Loss of ^{3}H (%)
2*S*-Phenyl[2-^{14}C,3R-$^{3}H_1$]alanine (**14**)			
1	120	0 3	83
2	120	0 5	91
3	48	0 4	89
4	48	0 7	93
(2*S*)-Phenyl[2-^{14}C,3*S*-$^{3}H_1$]alanine (**15**)			
1	120	0 8	36
2	120	0 8	35
3	48	0 2	29
4	48	0 5	24

Table 4 Incorporation of (2*S*)-phenyl[2-^{14}C,3*S*-$^{3}H_1$]- and (2*S*)-phenyl[2-^{14}C,3*R*-$^{3}H_1$]alanine into (3*R*)-3-dimethylamino-3-phenylpropionic acid (**21**) in *T baccata*

Expt number	Duration of experiment (hr)	Incorporation of ^{14}C (%)	Loss of ^{3}H (%)
(2*S*)-Phenyl[2-^{14}C,3*R*-$^{3}H_1$]alanine (**14**)			
1	144	0 013	91
2	144	0 014	89
(2*S*)-Phenyl[2-^{14}C,3*S*-$^{3}H_1$]alanine (**15**)			
1	144	0 014	33
2	144	0 020	33

Table 5 Incorporation of (2*S*)-phenyl[2-^{14}C,3*S*-$^{3}H_1$]- and (2*S*)-phenyl[2-^{14}C,3*R*-$^{3}H_1$]alanine into sambunigrin (**20**) in *S nigra*

Expt number	Duration of experiment (hr)	Incorporation of ^{14}C (%)	Loss of ^{3}H (%)
(2*S*)-Phenyl[2-^{14}C,3*R*-$^{3}H_1$]alanine (**14**)			
1	120	0 10	24
2	120	0 20	18
(2*S*)-Phenyl[2-^{14}C,3*S*-$^{3}H_1$]alanine (**15**)			
1	120	0 15	85
2	120	0 10	84

Substrates administered as solutions through cut stems

with retention of configuration (i e loss of H_R in prunasin, loss of H_S in sambunigrin)

In the case of Winterstein's acid (**21**) it is interesting to note the similarities with the tyrosine-2,3-mutase of *B brevis* mentioned previously [12] In this work our observations showed that cinnamic acid (both 2 and 3-^{14}C labelled) was incorporated into **21** some 10–100 times less efficiently than (2*S*)-phenylalanine It is tempting, although not proven, to assume that a similar 2,3-shift of an amino group occurs in the synthesis of **21** from phenylalanine In this case however the 3-pro *R* proton is lost stereospecifically (Table 4) and there is an inversion of configuration at C-3

The demonstration of the effectiveness of both substrates (**14, 15**) as probes for stereospecific replacement/displacement reactions at the benzylic carbon atom of (2*S*)-phenylalanine led us to finally examine the case of tropic acid (**22**) Elegant work by Leete has shown [21–23] the relationship of the various carbon atoms in the precursor (2*S*)-phenylalanine (**1**) to those in the biosynthetic product tropic acid (**22**) and has proved that the rearrangement involves an intramolecular migration of the carboxyl group of **1** [23] Both **14** and **15** were administered in solution to *Datura stramonium* plants by the wick procedure and the tropic acid isolated as its methyl ester The tropic acid was also dehydrated to give atropic acid [24] and thus determine the amount of the residual ^{3}H label at the benzylic centre The results are shown in Table 6 Once again, with the provisos already indicated, the results point to a stereospecific loss of H_R in (2*S*)-phenylalanine and inversion of the configuration at the benzylic centre as the carboxyl group migrates—entirely analogous to the case of Winterstein's acid (**21**) noted above The processes involved in these conversions may well be similar in type and initiated by abstraction (oxidative?) of the 3-pro *R* hydrogen

The results obtained utilising **14** and **15** as biosynthetic tracers to elucidate further facets of procyanidin metabolism (Tables 1 and 2) are, in the context of the evidence obtained in the cases of prunasin (**19**), sambunigrin (**20**), (3*R*)-3-dimethylamino-3-phenylpropionic acid (**21**) and tropic acid (**22**) (Tables 3–6) quite distinct They show, irrespective of precursor, an apparently random loss of ^{3}H They are in agreement with earlier results obtained utilising (2*S*)-phenyl[2-^{14}C, $^{3}H_1$, 3-$^{3}H_2$]alanine where over two thirds of the ^{3}H was lost in the conversion to flavan-3-ols and procyanidins As such they permit no unequivocal solution to the problem posed above They suggest that in procyanidin metabolizing tissues there is a rapid equilibration of the 3-pro *R* and 3-pro *S* hydrogen atoms in phenylalanine—probably catalysed by a transaminase—which is in direct contrast to other modes of metabolism of the amino acid in the same tissues These

Table 6 Incorporation of (2*S*)-phenyl[2-^{14}C,3*S*-$^{3}H_1$]- and (2*S*)-phenyl[2-^{14}C,3*R*-$^{3}H_1$]alanine into tropic acid (**22**) in *D stramonium*

Expt number	Duration of experiment (hr)	Incorporation of ^{14}C (%)	Loss of ^{3}H (%)
(2*S*)-Phenyl[2-^{14}C,3*R*-$^{3}H_1$]alanine (**14**)			
1	192	0 02	88
2	192	0 02	91
3	192	0 03	93
4	192	0 07	93
(2*S*)-Phenyl[2-^{14}C,3*S*-$^{3}H_1$]alanine (**15**)			
1	192	0 02	30
2	192	0 01	35
3	192	0 01	35
4	192	0 01	33

Substrates were administered through cotton wool wicks into the stems of *D stramonium* plants

results suggest—but do not prove—the possibility of alternative pathways of biosynthesis of the flavanonol intermediate **7**, such as were proposed earlier [5–7] They also raise once again the question of whether PAL is an obligatory enzyme in flavonoid biosynthesis [26, 27]

Recent proposals by Hemingway and Foo [9] and by Stafford [8] (Fig 2) have suggested modifications to the original proposal for the biosynthesis of procyanidins [5–7] Stafford [8] has provided enzymic evidence in favour of the proposition that the key stereochemistry at the 3-position of the flavan-3-ols is fixed at the stage of the dihydroquercetin intermediate (**7**) Hemingway and Foo [9] have ingeniously demonstrated the feasibility of the procyanidin oligomerization reaction occurring irreversibly from the flavan-3, 4-diol intermediate (such as **8**) *via* the base catalysed generation of quinone-methide intermediates (e g **29**, **30**, tautomeric forms of the flav-3-en-3-ol) Because of this chemical relationship of the flav-3-en-3-ol (**9**) and the quinone methides (**29**, **30**) the Hemingway and Foo suggestions [9] can, with suitable elaboration, satisfy all the criteria listed earlier (see Introduction—i, ii, iii) They thus permit an entirely reasonable alternative biosynthetic pathway to that formulated earlier [5–7] The Stafford proposals [8] (Fig 2) can accommodate the evidence (i, ii) cited previously if it is assumed by analogy [27] that the chalcone ⇌ flavanone isomerase acts by *anti* addition to the chalcone double bond, that the hydroxylation at C-3 then occurs by replacement of the proton H_B with *retention* of configuration—typical of biological hydroxylation at saturated carbon—to give dihydroquercetin (**23**) and that the unknown flavanonol isomer (**24**) is then generated, as necessary, by the action of an epimerase (Fig 2) A crucial feature of the original hypothesis [5–7] devised to satisfy the criterion (iii) noted above was the ability to generate both chain termination units (flavan-3-ol, **11** and **12**) and chain extension units (carbocation, **10**) with *opposite* stereochemistry at C-3 simultaneously in one and the same reaction It is, at present, difficult to readily conceive of the satisfaction of the criterion (iii) under the new Stafford proposals (Fig 2) unless it is assumed that formation of the flavan-3-ols and the flavan-3-ol-4-yl carbocations (**27**, **28**) take place at quite different sites in the cell and that the two processes are not linked as the two stages in one reaction as envisaged in the original hypothesis [5–7] Moreover to satisfy (iii) it is necessary also to assume that under some circumstances one flavan-3,4-diol may only be converted to the flavan-3-ol, whilst the other isomeric diol may only be converted to the carbocation Clearly these intriguing problems and those outlined earlier will best be resolved by more detailed work at the enzymic level

EXPERIMENTAL

Radioactive substrates (2*S*)-Phenyl[3*R*-$^3H_1$]alanine (**14**) and (3*S*)-phenyl[2*S*-$^3H_1$]alanine (**15**) were synthesised by published procedures [10] The former had a sp act 2 8 Ci/mg and the latter 1 0 Ci/mg In biosynthetic experiments the tritiated amino acid was admixed with (2*S*)-phenyl[2-^{14}C]alanine (Amersham Radiochemical Centre) to give an amino acid with $^3H/^{14}C$ ratio of 5 1 to 15 1 as appropriate Measurements of radioactivity were as previously described [5–7]

Isotopic tracer experiments Methods for flavan-3-ol and procyanidin containing plants were essentially as described earlier [5–7] for *S caprea Aesculus* sp (+)-Catechin and (−)-epicatechin were finally obtained as their crystalline tetramethyl ethers, crystallized to constant activity (−)-Epicatechin tetramethyl ether was degraded *via* its tosylate to 3,4,5,7-tetramethoxyflav-2-ene in order to determine the ^3H label at C-2 (Tables 1 and 2)

Prunus laurocerasus Samples of (2*S*)- phenyl[3*R*-$^3H_1$]alanine (3–5 mg) or (2*S*)-phenyl[3*S*-$^3H_1$]alanine (6–8 mg) were mixed with (2*S*)-phenyl[2-^{14}C]alanine (10–15 mg) and crystallized from H_2O, pH 5 0 Samples were then dissolved in H_2O (5 ml) *P laurocerasus* shoots (3–4 young leaves, 30 g) were cut and placed in the soln After absorbtion of the soln H_2O was added as required (4–5 days) The shoots were macerated with EtOH (200 ml × 4) and the combined extracts reduced to 30 ml at 30°, diluted with H_2O (200 ml), extracted with petrol (60–80°, 4 × 50 ml) and EtOAc (4 × 50 ml) The EtOAc extract was dried ($MgSO_4$), dissolved in EtOH and chromatographed on Sephadex LH-20 (2 × 30 cm, EtOH) Each fraction (10 ml) was analysed by PC (6% HOAc) Epicatechin was determined as described previously [5–7], prunasin as a mauve spot by spraying with emulsin (0 1% in 0 02 M Pi, pH 7 4) and after 30 min with Na_2CO_3 (10%) the *p*-nitrobenzaldehyde and *o*-dinitrobenzene (0 05 M in $MeOCH_2CH_2OH$) Relevant fractions were combined, (−)-epicatechin was treated as above [5–7] and prunasin (520 mg) was converted to its acetate (Ac_2O–C_5H_5N), mp and mmp 138–139° after crystallization from EtOAc–petrol (60–80°) TLC (EtOAc–hexane, 1 1, R_f 0 3)

Sambucus nigra Procedure as adopted for *P laurocerasus* to give sambunigrin tetra-acetate (260 mg), mp and mmp 125–126° after crystallization from EtOAc–petrol (60–80°) TLC (Et_2O–C_5H_{12}, 9 1, R_f 0 9)

Taxus baccata Procedure as adopted for *P laurocerasus* After extraction of plant material with MeOH and reduction and dilution with H_2O (100 ml) the aq extract was extracted with $CHCl_3$ (3 × 50 ml) The residual aq soln was extracted with EtOAc (4 × 50 ml) and (+)-catechin isolated as above [5–7] The mixture of taxine alkaloids was obtained from the $CHCl_3$ soln by extraction with 0 5 N H_2SO_4 (3 × 50 ml) The acid extract was basified with NH_3 soln and extracted with Et_2O (3 × 20 ml), dried ($MgSO_4$) and evaporation of the Et_2O gave the alkaloids (90 mg, ~ 90%) The powder was redissolved in Et_2O (10 ml), treated with MeI (0 1 ml) for 24 hr at room temp The ppt was removed by filtration, dissolved in EtOH (10 ml), treated with K_2CO_3 (0 2 g in 20 ml H_2O, 2 hr) then NaOH (0 5 M 10 ml) for 4 hr, before acidification (0 5 M HCl) and extraction into Et_2O Evaporation gave cinnamic acid (20 mg) which after dilution (50 mg) was purified *via* its methyl ester (CH_2N_2) TLC (C_6H_6–$CHCl_3$, 9 1, R_f 0 75)

Datura stramonium Radioactive solns were administered by cotton wicks to plants (2–3 months old) The alkaloid fraction was obtained as above *via* solvent extraction for *T baccata* The solids were dissolved in NaOH (10%, 5 ml) and heated under reflux (30 min), extracted with Et_2O (3 × 5 ml), acidified (0 1 M HCl) then extracted with Et_2O (3 × 5 ml) Evaporation gave crude tropic acid (55 mg) which was converted after dilution (80 mg), (CH_2N_2, Ac_2O–C_5H_5N) to give methyl-3-acetoxytropate which was purified by TLC (silica, EtOAc–C_6H_6, 3 7, R_f 0 5) A sample of tropic acid was converted to atropic acid [24]

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