Stereochemistry and Mechanisms of the 3-Carboxymuconate Fungal Pathway in *Neurospora* SY4a

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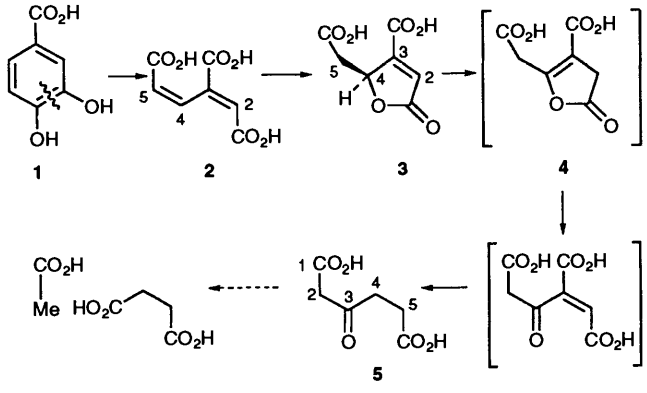
The cyclisation of *cis,cis*-3-carboxymuconic acid **2**, catalysed by the cycloisomerase enzyme of *Neurospora crassa* SY4a, has been shown to occur by *syn* addition of the 1-carboxy group to the 4,5-double bond to give (*S*)-(−)-3-carboxymuconolactone **3**. Thus, the absolute configuration of the lactone **3** was determined by ozonolysis to give (*S*)-malic (L-malic) acid. Furthermore, incubation of trisodium *cis,cis*-3-carboxy-5-deuteriomuconate **9** then ozonolysis of the derived lactone **28** gave (2*S*,3*S*)-3-deuteriomalic acid **29**. This evidence for *syn* addition was confirmed by a complementary incubation of undeuteriated 3-carboxymuconate in deuterium oxide, giving the lactone **31** and hence (2*S*,3*R*)-3-deuteriomalic acid **32**.

The degradation of 3-carboxymuconolactone **3** by the multifunctional enzyme complex of *Neurospora*, to give 3-oxoadipic acid **5**, has been studied with the deuteriated trisodium muconates **27**, **14** and **20**. The overall transformation has been found to involve an intramolecular, suprafacial 1,3-shift of hydrogen (or deuterium) from C-4 in the lactone to C-5 in the oxoadipic acid. The location and stereochemistry of deuterium in the oxoadipic acids **44** and **45** were established by conversion of these acids into the optically active 2-deuteriosuccinic acids **46** and **47**, respectively. The 1,3-shift provides compelling evidence for the formation of the enol lactone **4** as an enzyme-bound intermediate. Successive enzymic hydrolysis and decarboxylation would then complete the biosynthesis of 3-oxoadipic acid **5**.

The muconic acid† pathways[1] provide important routes for the degradation of benzene derivatives by bacteria and fungi present in soil and industrial waste. We report here details[2] of stereochemical and mechanistic studies on the degradation of protocatechuic acid **1**‡ by the fungus *Neurospora crassa* SY4a (Scheme 1).[3] In particular, we have determined the relative and

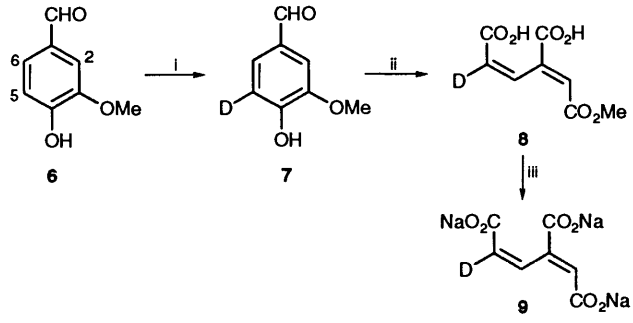


Scheme 1 All steps are catalysed by *Neurospora* enzymes

absolute stereochemistry of the enzymic cyclisation of *cis,cis*-3-carboxymuconic acid **2** to give 3-carboxymuconolactone **3**, and have clarified the pathway leading from the lactone **3** to 3-oxoadipic acid **5**, a precursor of the tricarboxylic acid cycle constituents acetic and succinic acid, which are able to sustain growth of the organism.

Synthesis of Substrates

Deuterium-labelled samples of *cis,cis*-3-carboxymuconic acid **2** were prepared[4] from the correspondingly labelled vanillins by cleavage[5] with sodium chlorite in sulfuric acid (Scheme 2).



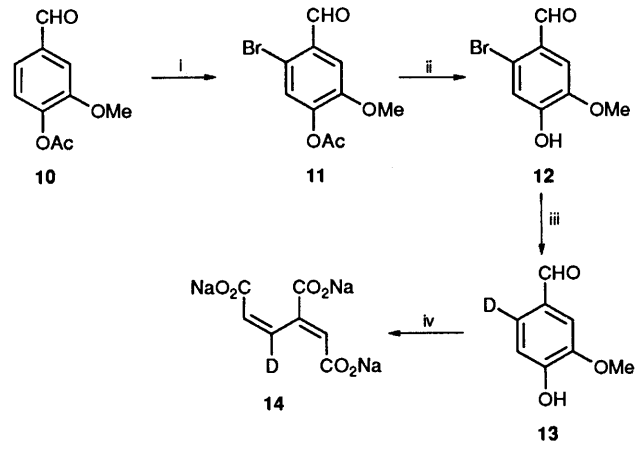
Scheme 2 *Reagents and conditions:* i, Et_3N in D_2O at *ca.* 100 °C; ii, $NaClO_2$–H_2SO_4–H_2O; iii, NaOH–H_2O

Thus, base-catalysed exchange[6] of vanillin **6** in deuterium oxide gave exclusively the 5-deuterio derivative **7**, which was cleaved to yield the methyl ester **8**. This was hydrolysed with sodium hydroxide to form the trisodium salt **9** required for enzymic experiments. Generally, trisodium carboxymuconates are indefinitely stable at ambient temperatures,§ whereas the *cis,cis*-tricarboxylic acids isomerise rapidly[4] in aqueous solutions to give 2-*cis*,4-*trans*-3-carboxymuconic acids, which cyclise only slowly to give 3-carboxymuconolactones. Supplies of (±)-3-carboxymuconolactone [(±)-**3**], required for control experiments, were best prepared by keeping solutions of the trisodium *cis,cis*-muconate in trifluoroacetic acid at room temperature until lactonisation was complete (^{1}H NMR control).

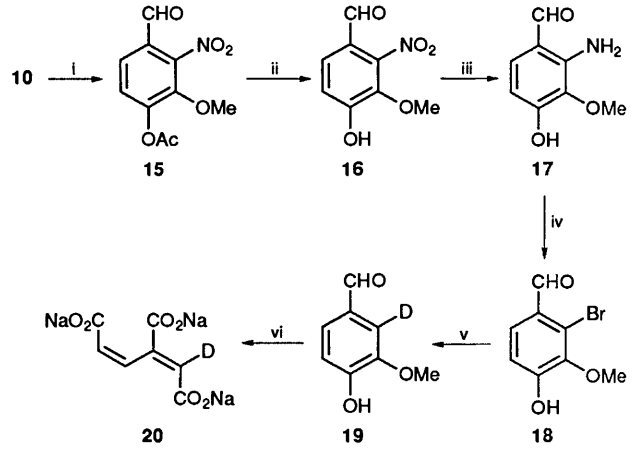
The 4-deuteriomuconate **14** was prepared from *O*-acetylvanillin **10**, as outlined in Scheme 3. Debromination of 6-bromovanillin **12** with zinc in alkaline deuterium oxide gave the required 6-deuteriovanillin **13**. However, the deuterium content of this derivative was only *ca.* 50%, even though the deuterium oxide was highly enriched (>99%) and care was taken to exclude moisture from the reaction mixture. Nevertheless, the deuteriovanillin **13** was converted, as before, into the

† Hexa-2,4-dienedioic acid.
‡ 3,4-Dihydroxybenzoic acid.

§ A sample of trisodium *cis,cis*-3-carboxymuconate prepared in 1966 and stored at room temperature is still stereochemically pure.



Scheme 3 *Reagents and conditions:* i, Br_2–NaOAc–HOAc; ii, KOH–H_2O; iii, Zn–NaOD–D_2O; iv, as in Scheme 2 (ii and iii)

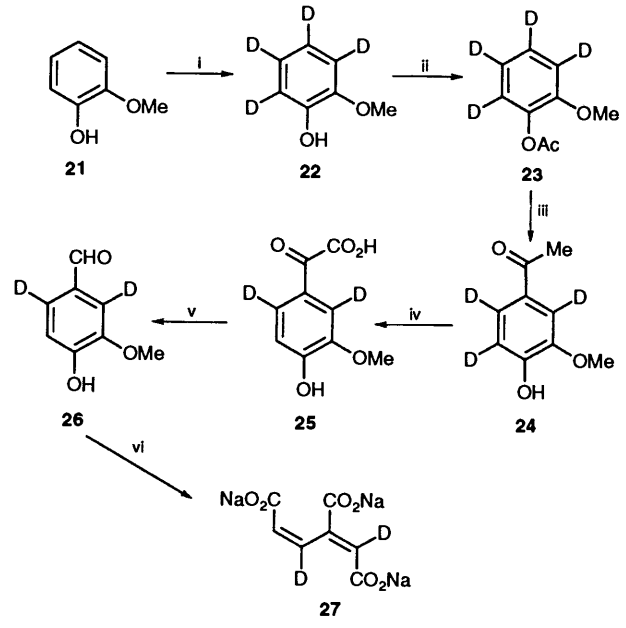


Scheme 4 *Reagents and conditions:* i, fuming HNO_3 at $\leqslant -12$ °C; ii, NaOH–H_2O; iii, $Fe(OH)_2$; iv, $NaNO_2$–HBr–H_2O then CuBr; v, Zn–NaOD–D_2O; vi, as in Scheme 2 (ii and iii)

deuteriomuconate **14**, since the 50% deuterium content of the latter was adequate for the proposed enzymic experiment. Recently, we have prepared[7] 6-deuterioprotocatechuic aldehyde (**13**; H replacing Me) from the corresponding bromo compound by treatment with deuterium gas and triethylamine in tetrahydrofuran over a palladium–carbon catalyst; very likely, similar conditions might effect the transformation **12** → **13** efficiently. A similar strategy was adopted for the synthesis of the 2-deuteriomuconate **20** (Scheme 4). This time, the reductive debromination of 2-bromovanillin **18** with zinc took place with a high (92%) incorporation of deuterium, a finding that makes the related, consistently inefficient deuteriation **12** → **13** all the more surprising.

Finally, the 2,4-dideuteriomuconate **27** was prepared from guaiacol **21**, as shown in Scheme 5. Deuterium was conveniently lost from position 5 of the acetophenone **24** during oxidation with nitrobenzene under alkaline conditions. The acid-catalysed exchange of guaiacol **21** was carried out with sufficient deuterium oxide to produce a deuterium content (80–85%) in the product **22** that was adequate for the enzymic experiment with the substrate **27**.

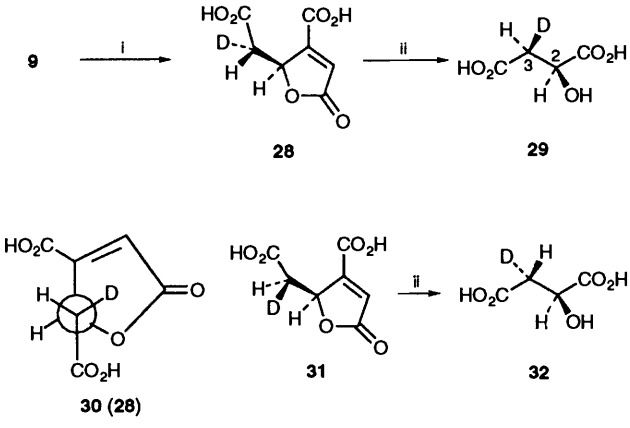
Throughout all the foregoing labelling experiments, the regiospecificity of deuterium labelling was readily checked by ^1H NMR spectroscopy. The deuterium content was determined by ^1H NMR spectroscopy and mass spectrometry. The undeuteriated forms of all the compounds in Schemes 2–5 were known compounds.



Scheme 5 *Reagents and conditions:* i, DCl–D_2O at *ca.* 100 °C; ii, Ac_2O–pyridine; iii, $AlCl_3$ in $PhNO_2$; iv, $PhNO_2$–KOH–H_2O; v, 170 °C in 4-$MeC_6H_4NMe_2$; vi, as in Scheme 2 (ii and iii)

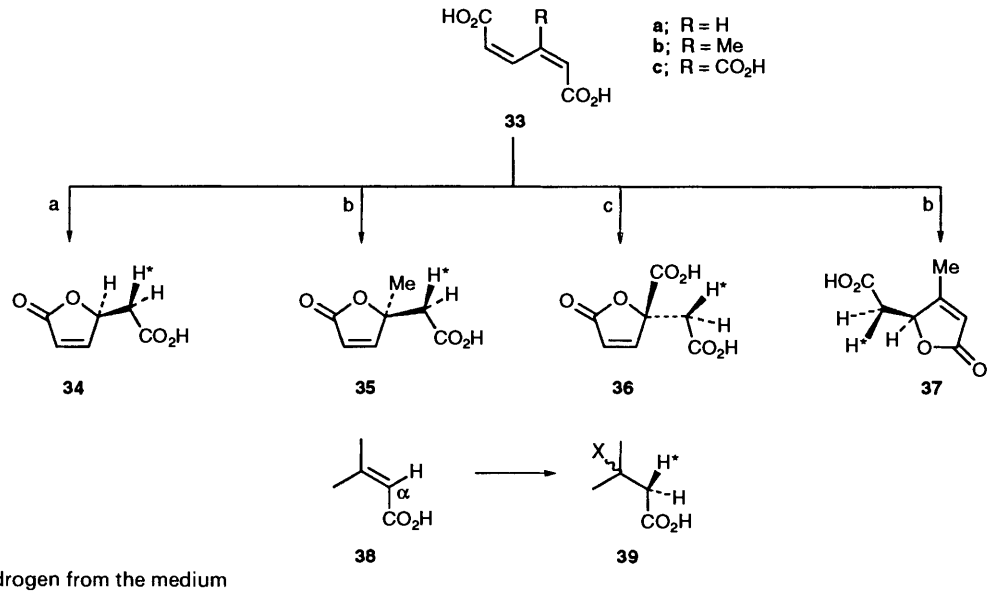
Enzymic Formation of 3-Carboxymuconolactone

The 5-deuteriomuconate **9** was incubated with a crude preparation[3] of the cycloisomerase enzyme from *N. crassa* (Scheme 6). The resulting (−)-lactone **28** was examined by ^1H



Scheme 6 *Reagents and conditions:* i, *Neurospora* cycloisomerase; ii, O_3 in EtOAc then H_2O

NMR spectroscopy. It was at once apparent that cyclisation had occurred highly stereoselectively. The spectrum of the undeuteriated lactone **3** showed a well separated ABX system for the methylene and methine protons with allylic coupling between 4- and 2-H; δ[$(CD_3)_2CO$] 2.79 (dd, *J* 8.1 and 16.8), 3.24 (dd, *J* 3.3 and 16.8) and 5.59 (ddd, *J* 8.0, 3.3 and 2.2 Hz). The deuteriated lactone **28** gave signals corresponding to only one diastereoisomer; δ 3.24 (dt, J_{HH} *ca.* 3 and J_{HD} *ca.* 3 Hz) and 5.59 (multiplet). In contrast, when the substrate **9** was cyclised in trifluoroacetic acid the resulting lactone showed signals, at δ 2.78 and 3.24, for two diastereoisomers in approximately equal amounts. If the lactone **28** exists predominantly in the conformation **30**, with the large carboxy group and lactone residue (C-3) antiperiplanar, then the relative configuration shown would explain the small vicinal coupling (*ca.* 3 Hz) observed for the methylene proton, δ 3.24. Nevertheless, both the relative and absolute configuration of the lactone were determined unambiguously by degradation. Ozonolysis of the



H* represents hydrogen from the medium

Scheme 7

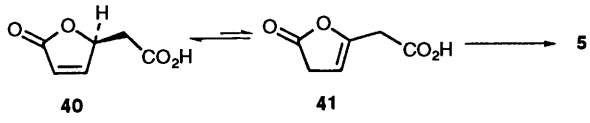
lactone **28** and decomposition of the ozonide simply with water gave the (2*S*,3*S*)-[3-^{2}H]malic acid **29** directly. The relative configuration **29** was deduced from the small vicinal coupling constant (*J* 3.0 Hz) observed in alkaline deuterium oxide, since (2*S*,3*R*)-[3-^{2}H]malic acid **32**, the diastereoisomer formed enzymically from fumaric acid in deuterium oxide, shows[8] the alternative, larger vicinal coupling (*J* 9.7 Hz). The same degradation was then carried out on the undeuteriated (−)-lactone **3**. The derived (2*S*)-malic acid (L-malic acid) had $[\alpha]_D^{15}$ −23.6 (*c* 1.5 in pyridine), essentially the same value being obtained in two separate experiments. This value is somewhat lower than that for pure L-malic acid, $[\alpha]_D$ −28.6. Very likely, partial racemisation had occurred during degradation of the lactone **3**; however, the 4*S* configuration of the lactone **28** is not in doubt.

A complementary enzymic experiment was then carried out. Unlabelled trisodium *cis,cis*-3-carboxymuconate was incubated in deuterium oxide with the crude cycloisomerase preparation from *N. crassa*. This was done to provide an independent check on the foregoing experiment, and to test whether all the olefinic protons in the substrate were retained in the product. The ^{1}H NMR spectrum of the product **31** was, as expected, complementary to that of the lactone **28**, *i.e.* a signal for the 4-H was observed at δ 2.79 having the larger (*J ca.* 8 Hz) vicinal coupling, and there was no indication that deuterium had entered the molecule elsewhere. Further, the deuteriomalic acid **32** obtained by ozonolysis gave the expected, larger (*J* 9.7 Hz), vicinal coupling constant. In conclusion, enzymic cyclisation of the carboxymuconic acid **2** in the fungus *N. crassa* occurs with *syn* addition of the carboxyl group to the 4,5-double bond to give the (*S*)-lactone **3**.

At the outset of the present investigations, only one stereochemical study on the muconate pathways had been reported. Avigad and Englard[9] showed that cyclisation of *cis,cis*-muconic acid **33a** in the bacterium *Pseudomonas putida* occurred by *syn* addition to give the parent (*S*)-muconolactone **34** (Scheme 7; H* represents hydrogen from the medium). This finding is stereochemically analogous to the cyclisation **2** → **3** in the fungus *N. crassa*. Further, we have recently shown[7] that the enzymic cycloisomerisation **33a** → **34** takes place in the same way in a fungus, *Aspergillus niger*. Again, the 3-methylmuconic acid **33b** is likewise cyclised by *syn* addition,[10] in *P. putida* to give the (*S*)-lactone **35** and in the fungus *A. niger* to give the isomeric (*S*)-lactone **37**. Thus, 5 distinct, enzymic lactonisation reactions occur with the same relative and absolute stereochemistry. Yet, in the bacterium *P. putida cis,cis*-3-carboxymuconic acid **33c** (≡**2**) unexpectedly gave the (*R*)-lactone **36** by *anti* addition to the 2,3-double bond.[11] However, in all six enzymic reactions there is a single feature of stereochemical conformity; the newly created methylene group (see **39**) is formed by α-protonation of an acrylic acid **38** from the *re* face. Whether by chance or genetic connection, the same stereochemical outcome is found for other examples of the enzymic addition of HX to acrylic acids[12] (but not to their co-enzyme A esters). For example, fumarase-catalysed hydration of 2-deuteriofumaric acid gives, by *anti* addition, the deuteriomalic acid **29**.

Enzymic Conversion of 3-Carboxymuconolactone 3 into 3-Oxoadipic Acid 5

It was known[3] from the outset that cell-free extracts and purified proteins of *N. crassa* and other fungi[13] catalyse the conversion of the muconic acid **2** into 3-oxoadipic acid **5** *via* the lactone **3**. No intermediates have been detected in the transformation **3** → **5** and all attempts to fractionate the catalytic protein into species having distinct enzymic activities have been fruitless.[14] We reasoned that the enol lactone **4** and the derived β-keto acid (Scheme 1) might act as enzyme-bound



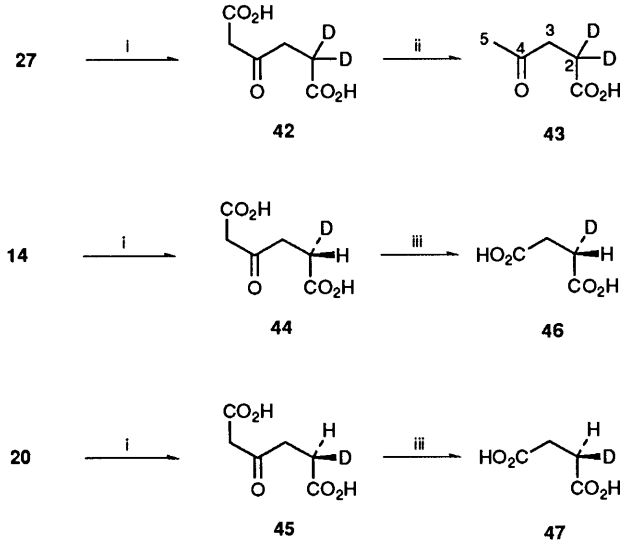
Scheme 8 Enzymic reactions in bacteria and fungi

intermediates, especially since enzymic degradation of muconolactone **40** itself involves the corresponding enol lactone **41** (Scheme 8). To test this idea, and to determine the stereochemistry of formation of the 5-methylene group in the product **5**, a set of deuteriated trisodium muconates **27**, **14** and **20** was incubated with a cell-free preparation of *N. crassa* (Scheme 9). A preliminary experiment was carried out so that the site of any deuterium in the derived 3-oxoadipates could be determined by ^{1}H NMR spectroscopy. Treatment of 4-oxopentanoic acid (laevulinic acid), obtainable from 3-oxoadipic acid **5** by acid-catalysed decarboxylation, with

Table 1 Optical rotations, $[\alpha]^{20}$ (10^{-1} deg cm^2 g^{-1}) (*c* 2, MeOH), of (*S*)-(+)-**46** and (*R*)-(−)-2-deuteriosuccinic acid **47** compared with standard [15] values

λ/nm	46[a]	47[a]	Standard[b]
263	+9.9	−10.15	10.1
270	+7.7	−8.0	7.9
278	+6.3	−6.3	6.3
286	+5.2	−5.0	5.1
294	+4.2	−4.1	4.1
303	+3.7	−3.35	3.35
313	+3.0	−2.8	2.8
323	+2.4	−2.3	2.3
333	+1.9	−1.9	1.9

[a] $[\alpha]^{20}$ Values corrected for deuterium content (see text). [b] [α] Values taken from ORD curves displayed in ref. 15.

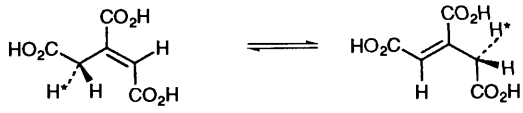


Scheme 9 *Reagents and conditions:* i, *Neurospora* enzymes; ii, HCl–H_2O at 100 °C; iii, NaOH–H_2O at 20 °C

sodium deuteroxide in deuterium oxide gave a pentadeuterio derivative, which showed a broad singlet, δ[$(CD_3)_2CO$] 2.55, for the remaining, C-2 methylene group. In contrast, when the dideuterio-oxoadipic acid **42**, obtained enzymatically from the dideuteriomuconate **27**, was converted into laevulinic acid, the product **43** gave a broad singlet at δ 2.76, arising therefore from a C-3 methylene group. Further, incubation of an equal mixture of dideuteriated **27** and undeuteriated salts gave a 3-oxoadipic acid containing largely dideuterio- and diprotio-species (mass spectrometric analysis); the small amount (*ca.* 10%) of monodeuteriated product corresponded to the monodeuteriated species present in the substrate **27**. Thus, intramolecular migration of deuterium had occurred during the enzymic transformation **27** → **42**.

The same enzyme preparation converted the 4-deuteriomuconate **14** (*ca.* 50% deuteriated) into the monodeuterio-oxoadipate **44**. This was purified by crystallisation then cleaved with aqueous sodium hydroxide to yield the 2-deuteriosuccinic acid **46**, which was crystallised several times. The optical rotation of this acid was compared, point by point within the range 263–333 nm, with the standard data published by Cornforth *et al.*,[15] allowance being made for the 50% deuterium content (see Table 1). The data showed clearly that the product was (*S*)-(+)-2-deuteriosuccinic acid **46**. In a complementary experiment, the 2-deuteriomuconate **20** (92% deuteriated) was converted *via* the oxoadipate **45** into (*R*)-(−)-2-deuteriosuccinic acid **47**, which again had the expected optical rotation within the standard range (Table 1).

These findings show that the enzymic conversion of (*S*)-3-carboxymuconolactone **3** into 3-oxoadipic acid **5** involves an intramolecular suprafacial 1,3-shift of hydrogen. Very likely, the proton at C-4 is removed by a basic group on the enzyme to form either the anion of a 2-hydroxyfuran or, with concerted protonation of the carbonyl group, the 2-hydroxyfuran itself. Reprotonation at C-2 from the same face of the furan ring could then occur, after only a small displacement of the substrate relative to the protonated enzyme, to form the enol lactone **4**. Hydrolysis followed by decarboxylation would complete the sequence of reactions catalysed by the fungal enzyme complex (Scheme 1). Recently,[16] an analogous, suprafacial 1,3-hydrogen shift has been shown, indirectly, to occur in *P. putida* during the interconversion of muconolactone **40** and the corresponding enol lactone **41**. Experiments are planned to discover whether a similar shift is involved in the degradation of the 3-methylmuconolactone **37** in fungi. A somewhat similar example is provided by the enzymic interconversion[17] of *cis* and *trans*-aconitic acid (Scheme 10). The enzyme causes stereospecific



Scheme 10

exchange of one of the methylene hydrogens (H* in Scheme 10) in each substrate, although only a small (*ca.* 4%) intramolecular shift of hydrogen was observed. Presumably, the protonated enzyme undergoes hydrogen exchange with the medium faster than it reprotonates the substrate.

Experimental

General.—^{1}H NMR spectra were obtained at 90 MHz with a Perkin-Elmer R 34 spectrometer and at 100 MHz with a Varian HA 100 spectrometer; *J* values are in Hz. Mass spectra were obtained by EI at 70 eV with AEI MS 12 and MS 9 spectrometers.

Trisodium cis,cis-3-*Carboxymuconate and the Derived* (±)-3-*Carboxymuconolactone* [(±)-**3**].—Vanillin **6** was oxidised[5] with sodium chlorite in sulfuric acid to give 1-methyl *cis,cis*-3-carboxymuconate (**8**; H replacing D), which was hydrolysed with sodium hydroxide to yield the required trisodium salt of the acid **2**.[4] This salt was dissolved in trifluoroacetic acid and the progress of concurrent *cis-trans*-isomerisation and lactonisation at room temperature was monitored by ^{1}H NMR spectroscopy (100 MHz). The rate of lactonisation was substantially greater than that of isomerisation to form 2-*cis*,4-*trans*-3-carboxymuconic acid; after 1 h *ca.* 50% of the lactone [(±)-**3**] had formed and only *ca.* 10% of the *cis,trans* acid was present. After 20 h, signals from the *cis,cis* acid were no longer detectable but those from a small amount of the *cis,trans* acid remained. For preparative purposes, solutions of the trisodium salt were kept at room temperature in trifluoroacetic acid for 4–5 days. After this time, the ^{1}H spectrum indicated essentially quantitative formation of (±)-3-carboxymuconolactone; δ 3.05 (dd, *J* 8 and 17, 5-H), 3.51 (dd, *J* 8 and 17, 5-H), 5.80 (m, 4-H) and 7.06 (d, *J* 2, 2-H). The mixture was evaporated and the lactone purified in the usual way.

Preparation of Deuteriated Trisodium 3-*Carboxymuconates.*—The following substrates were prepared by the methods reported in the literature for undeuteriated compounds (with the exception of the derivative **7**), with occasional minor modifications. The quantity and location of deuterium in the vanillins **7**, **13**, **19** and **26** were determined by MS and ^{1}H NMR

analysis. Each deuteriated vanillin was converted into the corresponding trisodium muconate **9**, **14**, **20** and **27** by chlorite oxidation, as described above.

Trisodium cis,cis-3-*Carboxy*-5-*deuteriomuconate* **9** (*Scheme 2*).—Vanillin **6** was heated in deuterium oxide containing triethylamine to afford 5-deuteriovanillin **7**.[6]

Trisodium cis,cis-3-*Carboxy*-4-*deuteriomuconate* **14** (*Scheme 3*).—Vanillin **6** was acetylated with acetic anhydride in pyridine and the product **10** was treated with bromine and sodium acetate in acetic acid, containing a little water, at room temperature to give the bromo acetate **11**, which was hydrolysed with hot aqueous potassium hydroxide to afford 6-bromovanillin[18] **12**. 6-Bromovanillin **12** (4.6 g) was added to sodium deuteroxide [prepared from sodium (1.9 g)] in deuterium oxide (40 cm^3) under nitrogen with stirring at 60–80 °C. Zinc dust (10 g) was added in portions and heating was continued for 25 min. The mixture was diluted with water (50 cm^3) and then filtered. The filtrate was acidified and extracted with diethyl ether to give 6-deuteriovanillin **13**, which was crystallised from light petroleum (b.p. 80–100 °C) (yield 70%, deuterium content *ca.* 50%).

Trisodium cis,cis-3-*Carboxy*-2-*deuteriomuconate* **20** (*Scheme 4*).—*O*-Acetylvanillin **10** was added slowly to fuming nitric acid at $\leqslant -12$ °C. Dilution of the mixture with ice gave the *O*-acetylnitrovanillin **15**, which was hydrolysed with hot aqueous sodium hydroxide to give 2-nitrovanillin[19] **16**. The nitro compound **16** was reduced with ferrous hydroxide, prepared from ferrous sulfate and aqueous ammonium hydroxide, to give the amine[20] **17**. Diazotisation of this amine **17** in hydrobromic acid and decomposition of the resulting diazonium salt with cuprous bromide gave 2-bromovanillin[19] **18**. Reductioin of the bromo compound **18** with zinc in alkaline deuterium oxide, as described for the isomer **12**, gave 2-deuteriovanillin **19** (yield 90%, deuterium content 92%).

Trisodium cis,cis-3-*Carboxy*-2,4-*dideuteriomuconate* **27** (*Scheme 5*).—Freshly distilled thionyl chloride (20 cm^3) was added cautiously to deuterium oxide (150 cm^3), then nitrogen was passed through the resulting solution of deuterium chloride to remove sulfur dioxide. Guaiacol **21** (50 g) was added to the solution and the mixture was heated under reflux, with exclusion of atmospheric moisture, for 60 h. The mixture was cooled and then extracted with diethyl ether (3 × 50 cm^3). The extracts were dried ($MgSO_4$) and evaporated to give the deuteriated guaiacol **22** (46 g) (80–85% $^2H_4$), which was acetylated directly with acetic anhydride in hot pyridine. The resulting acetyl derivative[21] **23** was distilled [b.p. 123–124 °C (13 mmHg)] then isomerised with aluminium trichloride in nitrobenzene at 0 °C to give the acetophenone[21] **24**. Oxidation of the acetophenone **24** with nitrobenzene in aqueous potassium hydroxide at 120 °C gave the impure keto acid **25**, which was directly decarbonylated in *N,N*-dimethyl-*p*-toluidine at 170 °C to yield 2,6-dideuteriovanillin[21] **26**.

3-*Oxoadipic Acid* **5**, 4-*Oxopentanoic Acid* (*Laevulinic Acid*), *Succinic Acid and Their Deuteriated Derivatives*.—3-Oxoadipic acid **5** (160 mg), prepared by the literature method,[22] was heated in 3 mol dm^{-3} hydrochloric acid (3 cm^3) at 100 °C for 1 h. The mixture was cooled then extracted with ethyl acetate (3 × 5 cm^3) to give 4-oxopentanoic acid as an oil (100 mg, 86%); $\delta[(CD_3)_2CO$; 100 MHz] 2.15 (s, Me), 2.55 (m, 2-H_2) and 2.76 (m, 3-H_2). When this keto acid was kept in deuterium oxide containing sodium deuteroxide, 3,3,5,5,5-pentadeuterio-4-oxopentanoic acid was formed; $\delta[(CD_3)_2CO$; 100 MHz] 2.55 (br s, 2-H_2). Similarly, the biosynthetic dideuterio-3-oxoadipic acid **42** was converted into 2,2-dideuteriolaevulinic acid **43**; $\delta[(CD_3)_2CO$; 100 MHz] 2.76 (br s, 3-H_2).

3-Oxoadipic acid **5** (500 mg) was kept in water (10 cm^3) containing sodium hydroxide (500 mg) for 0.5 h at room temperature. The solution was acidified with hydrochloric acid then evaporated to dryness. The residue was extracted with acetone to obtain succinic acid (220 mg), which was crystallised from acetone–light petroleum (b.p. 60–80 °C). The 2-deuteriosuccinic acids **46** and **47**, obtained similarly, were recrystallised repeatedly before their optical rotations (Table 1) were measured.

Ozonolysis of 3-*Carboxymuconolactone* **3**.—An excess of ozone was passed into a solution of the lactone **3** in ethyl acetate at 0 °C. The solution was then purged with nitrogen and evaporated to dryness. **CAUTION**—the evaporation was carried out behind a safety screen. Water was added to the residue and the mixture was warmed then again evaporated to dryness. The residue of partially racemic (*S*)-(−)-malic acid had m.p. 115–118 °C [from ethyl acetate–light petroleum (b.p. 60–80 °C)], $[\alpha]_D^{15}$ −23.6 (*c* 1.5 in pyridine). Measurements on reference samples in the same manner gave for (*S*)-(−)-malic acid (L-malic acid), m.p. 101 °C, $[\alpha]_D$ −28.6, and for (±)-malic acid, m.p. 131–132 °C.

Experiments with the Enzymes of Neurospora Crassa.—*Neurospora* strain SY4a (FGSC 621), obtained from the Fungal Genetics Stock Centre, Humboldt State University Foundation, Arcata, California 45521, USA, was grown on the recommended[3] media. Mycelia were frozen with liquid nitrogen and ground with a mortar and pestle. Cell debris were removed from an aqueous extract by low-speed centrifugation. A portion of the supernatant was freeze-dried and reconstituted in deuterium oxide for the enzymic synthesis of the lactone **31**. No attempt was made to fractionate the supernatant solution, which contained *inter alia* the enzymes required for the conversion of *cis,cis*-3-carboxymuconate into 3-carboxymuconolactone and for degradation of the latter to 3-oxoadipate. The disappearance of the muconate, and the formation and disappearance of the lactone, were monitored, as recommended,[3] by UV spectroscopy. Substantial amounts of the lactone accumulated during short-term incubations; the lactone **3** and 3-oxoadipic acid **5** were isolated and purified as described earlier.

(*S*)-(−)-3-Carboxymuconolactone **3** showed $\delta[(CD_3)_2CO$; 100 MHz) 2.79 (dd, *J* 16.8 and 8.1, 5-$H_{pro\text{-}S}$), 3.24 (dd, *J* 16.8 and 3.3, 5-$H_{pro\text{-}R}$), 5.59 (ddd, *J* 8.1, 3.3 and 2.2, 4-H), 6.71 (d, *J* 2.2, 2-H) and *ca.* 10 (br s, CO_2H). The spectra of the deuteriolactones **28** and **31** are described in the main text; geminal deuterium splitting of 5-H was apparent for solutions in hexadeuterioacetone but not in trifluoroactic acid. The spectra of the derived deuteriomalic acids **29** and **32** are also described in the main text. Again, geminal deuterium splitting was apparent in hexadeuterioactone but not in alkaline deuterium oxide; the latter solvent gave larger differences in vicinal coupling constants and methylene chemical shifts and was chosen to facilitate comparison with literature[8] values.

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