Stereochemistry of the enzymic lactonisation of *cis,cis*-muconic and 3-methyl-*cis,cis*-muconic acid

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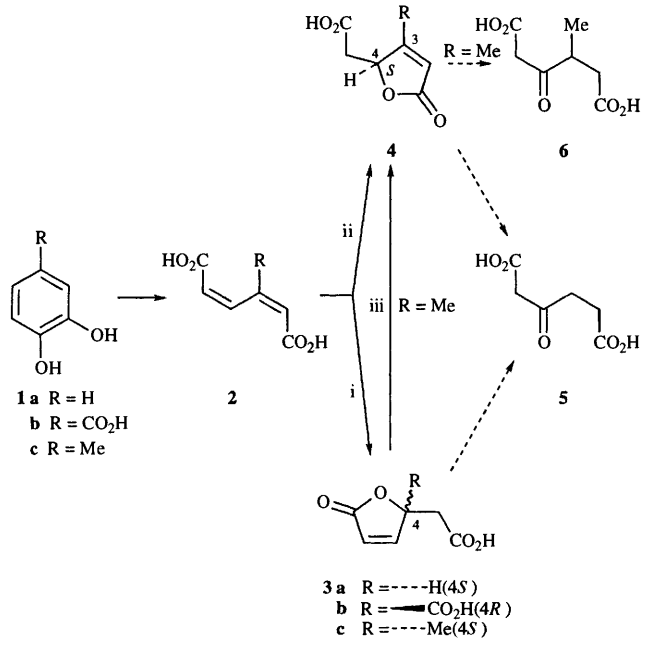
Experiments with 4-methyl[3,5,6-$^2H_3$]pyrocatechol 7 have shown that the product of its enzymic oxygenation, 3-methyl-*cis,cis*-muconic acid 8, undergoes enzymic cyclisation by *syn* addition of carboxy groups to distal double bonds to form the (*S*)-4-methylmuconolactone 9 in the bacterium *Pseudomonas putida* and the (*S*)-3-methylmuconolactone 11 in the fungus *Aspergillus niger*. Similarly, *syn* cyclisation of the *cis,cis*-muconic acid 15 has been shown to occur in *A. niger* to form the (*S*)-muconolactone 16. The relative and absolute stereochemistries of enzymic cycloisomerisation of muconic, 3-carboxymuconic and 3-methylmuconic acid in bacteria and fungi are here compared and discussed.

The muconic acid pathways[1] (Scheme 1) provide key routes in many bacteria and fungi for the dissimilation of benzene derivatives of both biosynthetic and xenobiotic origin. The classical pathways begin with either pyrocatechol **1a** or



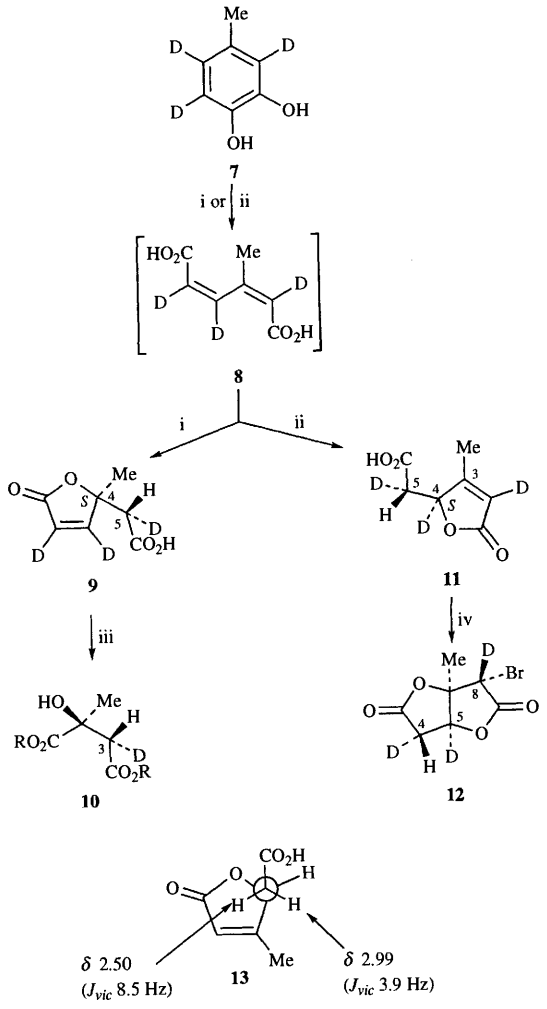
Scheme 1 *Organisms:* i, bacteria, *e.g. Pseudomonas putida* and *Acinetobacter calcoaceticus*; ii, fungi, *e.g. Aspergillus niger* and *Trichosporon cutaneum*; iii, 'specialised' bacteria, *e.g. Alcaligenes eutrophus* and *Rhodococcus rhodocrous*

protocatechuic acid **1b**, each formed by oxygenative transformation of a variety of benzenoid precursors. In both bacteria and fungi, pyrocatechol **1a** is cleaved by a catechol 1,2-dioxygenase (E.C.1.13.11.1) to *cis,cis*-muconic acid **2a**, which undergoes enzymic cycloisomerisation to give (*S*)-muconolactone **3a** [≡**4a**] and thence 3-oxoadipic acid **5**, a metabolic source of succinic and acetic acids. However, the pathways leading from protocatechuic acid **1b** diverge after 3-carboxy-*cis,cis*-muconic acid **2b**; in bacteria (procaryotes) (step i) (*R*)-4-carboxymuconolactone **3b** serves as an intermediate for the eventual formation of 3-oxoadipic acid **5**, whereas in fungi (eucaryotes) (step ii) (*S*)-3-carboxymuconolactone **4b** fulfils the same role.

Until recently, it was believed that complete oxidative degradation of 4-methylpyrocatechol **1c** by a 3-methylmuconate pathway could occur only in fungi. For example, Powlowski and Dagley[2] found that the fungus (yeast) *Trichosporon cutaneum* transformed *p*-cresol and 4-methylpyrocatechol **1c** through the muconic acid **2c** into (−)-3-methylmuconolactone (step ii), recently shown[3] to be the *S* enantiomer **4c**, and thence into (−)-4-methyl-3-oxoadipic acid **6**, a metabolic source of pyruvic and acetic acids. In the bacterial genus *Pseudomonas*,[4] however, cycloisomerisation of 3-methyl-*cis,cis*-muconic acid **2c** gives (step i) (*S*)-4-methylmuconolactone[3] **3c**, characteristically as a dead-end metabolite. However, the discovery that the bacterium *Alcaligenes eutrophus* JMP 134[5] and certain nocardioform actinomycetes (bacteria), including *Rhodococcus rhodocrous* N75,[6] could metabolise 4-methylpyrocatechol **1c** to the characteristically *fungal* lactone **4c** led to the identification of a new type of enzymic transformation (step iii). In all these 'specialised' bacteria, cycloisomerisation of the 3-methylmuconic acid **2c** gives initially the characteristically bacterial lactone **3c**, but this is converted by a 4-methylmuconolactone methyl-isomerase[7] into the 'fungal' lactone **4c**, thereby overcoming the bacterial 'block'.

To initiate a general study of the stereochemistry and mechanism of the enzymic reactions of the methylmuconate pathways, we devised[3,8] non-enzymic syntheses of disodium 3-methyl-*cis,cis*-muconate, (+)-4-methylmuconolactone **3c** and (−)-3-methylmuconolactone **4c** and determined the absolute configurations of the lactones. We now report[8] the relative stereochemistry of cycloisomerisation (lactonisation) of the 3-methylmuconic acid **2c** in the bacterium *Pseudomonas putida* (ATCC 12633) (step i) and the fungus *Aspergillus niger* (step ii). Further, the relative stereochemistry of cyclisation of the unsubstituted *cis,cis*-muconic acid **2a** in the latter organism has been determined[9] to complete the set of 6 cycloisomerisations. In all three cases, *syn* addition of a carboxy group to the distal double bond was observed.

The deuteriated 4-methylpyrocatechol **7** (Scheme 2), prepared from 4-methylpyrocatechol **1c** by exchange in DCl–D_2O (4 mol dm^{-3}) at 85 °C, was fed to a culture of *P. putida* (ATCC 12633), the bacterium used by Ornston and Stanier[10] in their classical studies on the muconate **2a** and carboxymuconate **2b** pathways. The ^1H NMR spectrum [200 MHz; $(CD_3)_2CO$] of the resulting 4-methylmuconolactone **9** showed that highly stereoselective cyclisation had occurred, as expected. The only



Scheme 2 *Reagents and conditions:* i, *Pseudomonas putida* culture; ii, *Aspergillus niger* culture; iii, O_3 in CH_2Cl_2, −70 °C then HNO_3–H_2O, 85 °C; iv, Br_2 in CH_2Cl_2–aq. $NaHCO_3$, 20 °C

strong signal† in the spectrum apart from the methyl singlet (δ 1.54) was a triplet δ 2.76 ($J_{H,D}$ 2.3 Hz) for 5-H, whereas the undeuteriated lactone,[4,3b] **3c** gave doublets at δ 2.78 and 2.91 (J_{gem} 15.7 Hz) for the 5-methylene group. Further, the ^2H spectrum (30.7 MHz; $CDCl_3$; ^1H decoupled) of the deuterio lactone **9** showed singlets of similar intensity at δ_D 2.94 (5-D), 6.11 (2-D) and 7.71 (3-D) (*cf.* δ_H 2.92, 6.06 and 7.66 for the corresponding signals from **3c** in $CDCl_3$). The product **9** was degraded[11] to determine the configuration at C-5. Successive treatment with ozone and nitric acid gave the (*S*)-citramalic acid **10** (R = H), which was then esterified with diazomethane. The ^1H NMR spectrum of the dimethyl ester **10** (R = Me) corresponded closely with that reported[11] for synthetic material of unambiguously determined relative configuration; δ(200 MHz; $CDCl_3$) 1.42 (s, Me), 2.64 (t, $J_{H,D}$ 2.2 Hz, 3-H) and 3.66 and 3.77 (2 × s, MeO). The undeuteriated dimethyl ester **10** (H replacing D) gave doublets at δ 2.95 and 2.66 (J_{gem} 16.3 Hz) for the 3-methylene group.

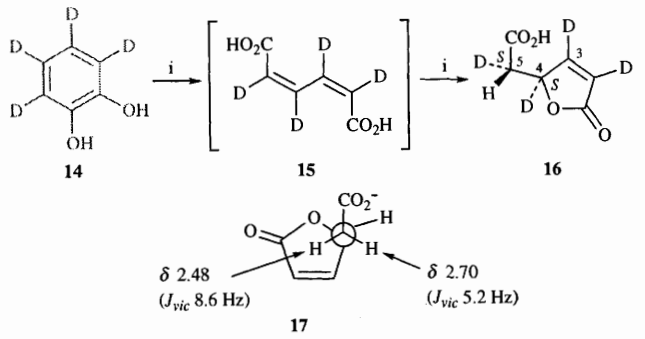
Unexpectedly, the 4-methyl lactone **9** was accompanied by a substantial amount (*ca.* 8% of the mixture) of racemic, deuteriated 3-methylmuconolactone. The ^1H NMR spectrum (see below) of this minor product showed it to be a mixture of 5*R* and 5*S* (±)-**11** diastereoisomers (*ca.* 3:1). Presumably, this must have arisen from non-enzymic cyclisation of the 3-methylmuconic acid **8** formed *in vivo* from the pyrocatechol **7**. Indeed, we have shown[3b,8] that 3-methyl-*cis*,*cis*-muconic acid **2c** in D_2O at pD *ca.* 6.5 gives (^1H NMR control) approximately equal amounts of (±)-3-methylmuconolactone (±)-**4c** and 3-methyl-2-*cis*,4-*trans*-muconic acid at room temperature. The concurrent, non-enzymic formation of the 'fungal' lactone (±)-**4c** during the enzymic cyclisation **2c**→**3c** in a *bacterial* culture may have a bearing on the evolution of the bacterial enzyme able to catalyse the conversion **3c**→**4c** (step iii) efficiently. That is, if antecedents of the 'specialised' bacteria had acquired a pathway for the conversion of the by-product (±)-**4c** into metabolically useful fragments, then evolutionary pressure might account for the emergence of a methylmuconolactone methyl-isomerase in the modern organisms.

The deuteriated pyrocatechol **7** was also fed to a mutant strain of *A. niger* known[12] to accumulate (*S*)-3-methylmuconolactone **4c**. The ^1H NMR spectrum [200 MHz; $(CD_3)_2CO$] of the resulting deuterio lactone **11** again showed, as expected, that highly stereoselective cyclisation had occurred. The methyl singlet, δ 2.13, was accompanied by only one other strong signal, a triplet δ 2.98 ($J_{H,D}$ 2.3 Hz) for 5-H, whereas the undeuteriated lactone[5,6] **4c** gave signals at δ 2.50 (dd, *J* 16.2 and 8.5 Hz) and 2.99 (dd, *J* 16.2 and 3.9 Hz) for the 5-methylene group. The corresponding, complementary ^2H NMR spectrum (30.7 MHz; Me_2CO) was especially informative. As expected, strong signals were observed at δ 2.51 ($J_{D,H}$ 2.3 Hz, 5-D), 5.25 (s, 4-D) and 5.87 (s, 2-D). However, a weak doublet (*J ca.* 2 Hz) was also detected having the chemical shift, δ 2.98, expected for the C-5 epimer of the major product **11**. The intensity of this weak signal was *ca.* 3% that of the strong signal at δ 2.51. It appears therefore that, as in *P. putida*, non-enzymic cyclisation of the muconic acid **8** gives the racemic forms of **11** and its C-5 epimer as minor products of the substrate **7**. In agreement with this interpretation, we had earlier observed[3b] that (*S*)-3-methylmuconolactone **4c** isolated from cultures of *A. niger* contained a small quantity of the racemic form.

The NMR spectra of the major product **11** imply the relative stereochemistry shown if the undeuteriated lactone **4c**, like 3-carboxymuconolactone[13] **4b**, exists predominantly in the conformation **13**, with the carboxy and lactone residues antiperiplanar. This conclusion was confirmed unambiguously by conversion[3,8] of the lactone **11** into the rigid bromo dilactone **12**. The ^1H NMR spectrum [200 MHz; $(CD_3)_2CO$] of the undeuteriated bromo dilactone showed signals at δ 2.92 (ddd, *J* 18.7, 1.0 and 0.7 Hz, 4-$H_{pro\text{-}R}$) and 3.36 (dd, *J* 18.7 and 4.9 Hz, 4-$H_{pro\text{-}S}$) for the 4-methylene protons. Unambiguous assignment of these signals follows from the near-zero, vicinal coupling between the *trans* protons 4-$H_{pro\text{-}R}$ and 5-H; in the crystal structure[3] the torsion angle H(5)–C(5)–C(4)-$H_{pro\text{-}R}$ (4) is 99°. The ^1H spectrum of the deuterio dilactone **12** showed strong signals at δ 1.78 (s, Me) and 2.89 (t, $J_{H,D}$ 2.8 Hz, 4-$H_{pro\text{-}R}$), and the complementary ^2H spectrum showed strong signals at δ 3.34 (d, $J_{D,H}$ 2.9 Hz, 4*S*-D), 4.84 (s, 8-D) and 5.33 (s, 5-D). Therefore, the bromo dilactone **12** and the lactone **11** have the relative stereochemistry shown; the absolute configuration follows from that determined[3] for (*S*)-3-methylmuconolactone **4c**.

At the time these studies were undertaken,[9] the stereochemistry of enzymic cycloisomerisation of the unsubstituted muconic acid **2a** had not been determined in a fungus. Consequently, the deuteriopyrocatechol **14**, prepared by exchange of pyrocatechol in DCl–D_2O, was fed to *A. niger* to afford the deuteriomuconolactone **16** (Scheme 3). Chari *et al.*[14] have published the ^1H NMR spectrum of (4*S*,5*R*)-[2,5-$^2H_2$]muconolactone. From their data the following assignments can be made for the ^1H NMR spectrum (200 MHz; D_2O, pD 7–8; standard Bu^{t}OD, δ 1.22) of the undeuteriated muconolactone **3a** (≡**4a**) (our data are given); δ 2.48 (dd, *J* 15.5 and 8.6 Hz, 5-$H_{pro\text{-}S}$) and 2.70 (dd, *J* 15.5 and 5.2 Hz, 5-$H_{pro\text{-}R}$). The deuteriomuconolactone **16** gave, as expected, only one strong signal; the chemical shift δ 2.68 (br s, 5-$H_{pro\text{-}R}$) established the relative configuration shown. Like 3-methylmuconolactone **4c** (Scheme

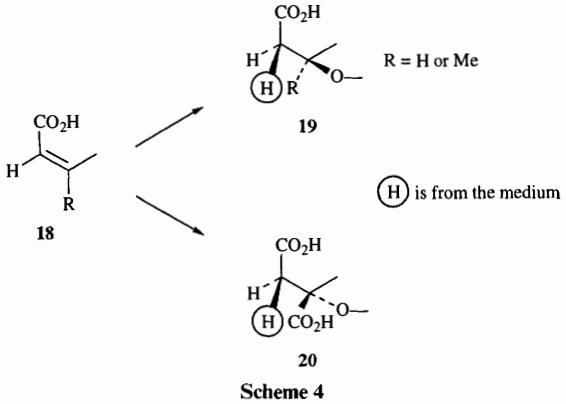
† Weak ^1H signals arose from the products of partially deuteriated 4-methylpyrocatechol.



Scheme 3 *Conditions:* i, *Aspergillus niger* culture

2), muconolactone **4a** appears to exist predominantly in the conformation **17** in solution. Better resolved spectra were obtained for solutions in $(CD_3)_2CO$ (see Experimental section), but the data for D_2O were used to correlate with those reported by Chari *et al.*[14]

The three muconic acids **2a–c** undergo enzymic cycloisomerisation in bacteria (procaryotes) and fungi (eucaryotes). The stereochemistry of each of the 6 possible classes of cyclisation has now been determined for at least one representative organism. Firstly, Avigad and Englard[15] showed that the parent muconic acid **2a** cyclises in *P. putida* by *syn* addition of a carboxy group to the distal double bond to give (*S*)-muconolactone **3a** (≡**4a**). Details of the experiments have not apparently been published, but their finding was recently confirmed by Chari *et al.*[16] Next, the analogous *syn* cyclisation of 3-carboxymuconic acid **2b**, to give (*S*)-3-carboxymuconolactone **4b**, was observed in the fungus *Neurospora crassa*.[13,17] In contrast, the same muconic acid **2b** was found to give (*R*)-4-carboxymuconolactone **3b** in *P. putida* and *Acinetobacter calcoaceticus* by *anti* addition to the double bond.[16] Finally, in this paper, we have reported the *syn* cyclisations **2c**→**3c** in *P. putida*, **2c**→**4c** in *A. niger* and **2a**→**3a** also in *A. niger*. A different example of the last, *syn* cyclisation has been independently observed in the fungus (yeast) *Trichosporon cutaneum*.[18] Thus, five of the six classes of cycloisomerase-catalysed reactions proceed with the same relative and absolute stereochemistry, *i.e.* by *syn* addition, **18**→**19**, of a carboxy group to the distal double bond from the face **18** shown in Scheme 4. Exceptionally, cyclisation of the carboxymuconolac-



Scheme 4

tone **2b** in *P. putida* and *A. calcoaceticus* involves *anti* addition, **18**→**20**. However, in all six classes the lactone methylene group is created in the same stereochemical sense, with a proton from the medium adding to the *re* face of the double bond. Whether by coincidence or evolutionary connection this methylene stereochemistry is observed in other HX additions to acrylic acids, *e.g.* the hydration of fumaric acid. The possible evolutionary origins of the various muconic acid cycloisomerases have been recently discussed,[19] as has the remarkable similarity in structure between the bacterial muconate cycloisomerase and mandelate racemase.[20]

Experimental

^1H NMR spectra at 90 and 200 MHz were obtained with Perkin-Elmer R34 and Bruker WP 200 spectrometers, respectively, and ^2H spectra at 30.7 MHz with the latter instrument. *J* Values are in Hz. Conditions for the bromolactonisation **11**→**12** have been reported elsewhere.[3,8]

The deuteriated pyrocatechols 7 and 14

Freshly distilled thionyl chloride (2.38 g, 20 mmol) was added dropwise to ice-cooled deuterium oxide (10 cm^3), with stirring under dry nitrogen. The mixture was allowed to warm up to room temp. 4-Methylpyrocatechol **1c** (992 mg, 8 mmol) was added to the mixture which was then heated at 85 °C for 2 h before being evaporated. The ^1H NMR spectrum of the residue showed that nuclear deuteriation was *ca.* 90% complete. Repetition of the exchange reaction gave the deuteriopyrocatechol **7** containing >95% aryl deuterium (^1H NMR control). The deuteriopyrocatechol **14** was prepared similarly.

Culture conditions and feeding of pyrocatechols

Pseudomonas putida. A strain of *P. putida* (ATCC 12633), obtained from NCIMB Ltd. (Torry Research Station, Aberdeen), was maintained on slants of 1% (w/v) agar containing 1% (w/v) yeast extract and grown on the mineral salts medium (pH 6.8) described by Ornston and Stanier[10] supplemented with sodium benzoate (10 mmol dm^{-3}). Conical flasks (250 cm^3) containing the medium (100 cm^3) were inoculated with aq. suspensions of the bacteria and shaken at 160 rev. min^{-1} for 3 d at 30 °C. Flasks containing fresh culture medium were inoculated with the 3 day-old culture and incubation was continued, as before, for 20 h. The deuteriopyrocatechol **7** (100 mg) in sterile water was added to 10 flasks (containing 1 dm^3 culture medium in total) and incubation was continued for 5 h, during which time the pyrocatechol had all been consumed ($FeCl_3$ colour test). A second batch of the pyrocatechol (150 mg) was then added and, after a further 19 h, was followed by a final batch (150 mg). The incubation was terminated 5 h later. The medium was adjusted to pH 7.5 with aq. ammonia and extracted with diethyl ether (3 × 250 cm^3) to remove any non-acidic material. The medium was then acidified with phosphoric acid to pH 2.5, saturated with sodium chloride and extracted with diethyl ether (5 × 250 cm^3). The combined extracts were dried ($MgSO_4$) and evaporated to give the 4-methylmuconolactone **9** as an oil (290 mg). Continuous extraction of the aq. layer with diethyl ether gave a mixture (63 mg) judged by ^1H NMR spectroscopy to contain the lactone **9** (49 mg) and deuteriated (±)-3-methylmuconolactone (14 mg) (see main text for the ^1H and ^2H NMR spectra of the metabolites).

Aspergillus niger. A mutant strain EM 32 of *A. niger*,[12] lacking the enzyme muconolactone isomerase and known to accumulate (−)-3-methylmuconolactone **4c**, was maintained at 4 °C on potato dextrose agar slopes. Inocula were prepared by shaking the slopes with sterile water, containing a trace of Tween 80 detergent, to form conidial suspensions. The organism was grown on a glucose–mineral salts medium containing $(NH_4)_2SO_4$ (0.5 g), KH_2SO_4 (1 g), $MgSO_4·7H_2O$ (50 mg), the trace elements solution (1 cm^3) described below, glucose (1.8 g) and deionised water (1 dm^3). The trace elements solution contained $FeSO_4·7H_2O$ (540 mg dm^{-3}), $MnSO_4·4H_2O$ (400 mg dm^{-3}), $ZnSO_4·7H_2O$ (200 mg dm^{-3}), $CuSO_4·5H_2O$ (40 mg dm^{-3}), $CoCl_2·6H_2O$ (40 mg dm^{-3}), KI (300 mg dm^{-3}), $Na_2MoO_4·2H_2O$ (50 mg dm^{-3}), $CaCl_2·2H_2O$ (6.62 g dm^{-3}) and NaCl (10 g dm^{-3}). The culture medium was adjusted to pH 5.5 with 1 mol dm^{-3} aq. sodium hydroxide and autoclaved at 120 °C. Conical flasks (250 cm^3) containing the medium (100 cm^3) were inoculated and shaken at 160 rev. min^{-1} at 30 °C. The consumption of glucose was monitored colourimetrically[21] (λ_{max} 487 nm) by treatment of aliquots with phenol and conc.

sulfuric acid. When the glucose was largely consumed (3 d), the deuteriopyrocatechol **7** (222 mg) in sterile water (2 cm^3) was added to 10 flasks (1 dm^3 culture medium in total) and the mixture readjusted to pH 5.5 with aq. K_2HPO_4. Incubation was continued, as before, and the mixture again adjusted to pH 5.5 after 24 h. After a further 24 h, a second batch (333 mg) of the pyrocatechol **7** was added and incubation was continued for 48 h with occasional readjustment of the mixture to pH 5.5. The cultures were filtered through muslin and the filtrate was concentrated under reduced pressure to 100 cm^3 then adjusted to pH 7.5 with aq. sodium hydrogen carbonate. Extraction with diethyl ether gave mainly deuteriated 4-methylpyrocatechol (115 mg). The aq. layer was acidified with phosphoric acid to pH 2, saturated with NaCl and then continuously extracted with diethyl ether to give the deuterio-3-methylmuconolactone **11** (225 mg) (the ^1H and ^2H NMR spectra are given in the main *text*).

Similarly, the deuteriopyrocatechol **14** (515 mg) gave the deuteriomuconolactone **16** (290 mg); δ_H[200 MHz; D_2O at pD 7–8; standard Bu^tOD, δ 1.22] 2.68 (br s, 5-$H_{pro\text{-}R}$); δ_H[200 MHz; $(CD_3)_2CO$] 2.86 (t, $J_{H,D}$ 2.3). The undeuteriated lactone **3a** (≡**4a**) gave $\delta_H(D_2O)$ 2.48 (dd, J 15.5 and 8.6, 5-$H_{pro\text{-}S}$), 2.70 (dd, J 15.5 and 5.2, 5-$H_{pro\text{-}R}$), 5.50 (ddt, J 8.6, 5.2 and 1.8, 4-H), 6.18 (dd, J 5.8 and 2.0, 2-H) and 7.78 (dd, J 5.8 and 1.5, 3-H); and $\delta_H[(CD_3)_2CO]$ 2.67 (dd, J 16.6 and 7.9, 5-$H_{pro\text{-}S}$), 2.88 (dd, J 16.6 and 5.6, 5-$H_{pro\text{-}R}$), 5.45 (ddt, J 7.6, 5.9 and 1.8, 4-H), 6.18 (dd, J 5.7 and 2.0, 2-H) and 7.81 (dd, J 5.7 and 1.5, 3-H), under the conditions described for the deuterio lactone **16**.

Oxidation[11] of the deuterio-4-methylmuconolactone 9 to the deuteriocitramalic acid 10 (R = H)

The deuterio lactone **9** (246 mg, 1.55 mmol) was ozonised in dichloromethane (75 cm^3) at −70 °C for 4 h. After passage of nitrogen through the solution to remove any excess of ozone it was allowed to warm to room temp. when it was treated with nitric acid (1 mol dm^{-3}; 0.7 cm^3). After removal of dichloromethane by evaporation the residue was treated with conc. nitric acid (2.4 cm^3) and the mixture was heated to 85 °C, when brown fumes were evolved. After 1 min at 85 °C the mixture was allowed to cool to room temp. After 15 h, the mixture was adjusted to pH 3 with conc. aq. ammonia and treated with lead nitrate (1.5 g, 4.5 mmol) in water (2.5 cm^3). The mixture was readjusted with ammonia to pH 6.6 to induce precipitation of a lead salt, which was collected by centrifugation. The supernatant was adjusted to pH 9.0 with ammonia and heated at 50–60 °C to yield a further precipitate of a lead salt. The combined lead salts were washed with water (2 × 10 cm^3) and then suspended in water (5 cm^3) and acidified with a mixture of conc. sulfuric acid (0.25 cm^3, 4.5 mmol) and water (0.5 cm^3). The resulting precipitate of lead sulfate was filtered off and the filtrate was evaporated at *ca.* 25 °C to low volume (*ca.* 2 cm^3). The solution was neutralised with 2 mol dm^{-3} aq. sodium hydroxide and then applied to a column of Amberlite IRA-400 ion-exchange resin in the formate form. The column was washed with water and then eluted with 0.9 mol dm^{-3} aq. formic acid. The eluate (300 cm^3) was evaporated at < 50 °C to afford the deuteriocitramalic acid **10** (R = H) (70 mg), which was converted with diazomethane in diethyl ether into the dimethyl ester **10** (R = Me). The ^1H NMR spectrum of this ester is described in the main text.

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