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Organic and biochemical synthesis of monolignol biosynthetic pathway intermediates

1. Organic synthesis of 5-hydroxyferulic acid

Supplies and Reagents:

Malonic acid
3, 4-Dihydroxy-5-methoxy-benzaldehyde
0.1 N HCl
Ethyl acetate
Dichloromethane
Acetone
Sodium sulfate

Protocol:

1. 75 Milligram of malonic acid is mixed with 50 mg of 3, 4-dihydroxy-5-methoxy-benzaldehyde in 1 mL pyridine and 5 μL piperidine, to react for 1 week at room temperature.
2. Acidify the mixture with 0.1 N HCl (20 ml)
3. Extract with ethyl acetate (50 ml ×3)
4. The combined ethyl acetate layers dry over Na_2SO_4 and then evaporate
5. Purify over a silica column (CH_2Cl_2: Et_2O, 4:1, v/v)

2. Organic synthesis of each aldehyde and alcohol

Supplies and Reagents:

3- Hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 3, 4-dihydroxy-5-methoxy-benzaldehyde, 4-hydroxy-3,5-dimethoxy-benzaldehyde
Pyridine
Acetic andydrid
(1,3-Dioxolan-2-yl-methyl)-triphenylphosphosphonium bromide
Dichloromethane
Acetone
Ethyl acetate
Potassium carbonate
18-Crown-6

Sodium bicarbonate
Magnesium sulfate
Sodium chloride
Potassium hydroxide
Potassium phosphate monobasic
Sodium borohydride

Protocol:

1. 50 mM Benzaldehyde and 0.5 M acetic anhydride is mixed with pyridine at room temperature for 48 hours, under N_2. Evaporate the organic layer and get the acetate benzaldehyde
2. Acetate benzaldehyde (5 mmol) and (1,3-dioxolan-2-yl-methyl)-triphenylphosphosphonium bromide (5 mmol) are dissolved in CH_2Cl_2 (80 ml) with vigorous stirring. Solid K_2CO_3 (5 mmol) and 18-crown-6 (0.05 mmol) are added. The reaction mixture is kept at room temperature for 8 hours. The organic phase is separated from the solid phase by filtration. Aqueous HCl (10%, 50 ml) is added to the organic portion and the mixture stirred at room temperature for a further 6 hours. At the end of the reaction, the mixture is diluted with 50 ml H_2O and extracte three times with 100 ml CH_2Cl_2. The combined organic layers are washed with saturated $NaHCO_3$ and saturated aqueous NaCl solution successively, dry over $MgSO_4$ and evaporate under vacuum. The residue is dissolved in a small amount of CH_2Cl_2 and pass through a silica gel column (elute with CH_2Cl_2: EtOAc, 90:10, v/v).
3. Evaporation of the solvent, acetoxy aldehyde is obtained as a white solid
4. Acetoxy aldehyde is dissolved in 100 ml of 0.2 M KOH in 95% EtOH, and stirred for 8 h under N_2. The solvent is removed under vacuum, and the mixture dilute with 50 ml H_2O and extracted with ethyl acetate (50 ml ×3). The combined organic layers are dried over Na_2SO_4 and evaporate to give each aldehyde
5. Acetoxy aldehyde is dissolved in MeOH along with KH_2PO_4, $NaBH_4$ is slowly added at 0 ℃ and mixture stir for 1 hour. Cold water is added slowly and acidifiy the solution to pH 4 with 5% HCl. Remove the MeOH and extract the mixture with CH_2Cl_2 and dry over $MgSO_4$. Evaporate the organic layer. Dissolve in a solution of KOH in 95% EtOH and stirrer for 12 hours at 0 ℃. dilute with water and extract with Et_2O then evaporate the solvent. Purify by silica gel (elute with CH_2Cl_2: Et_2O, 3:1, v/v)

3. Biochemical synthesis of CoA compounds

Supplies and Reagents:

p-Coumaric acid
Caffeic acid

Ferulic acid
5-Hydroxyferulic acid
Sinapic acid
Coenzyme A
Tis-HCl buffer (pH 7.5, 50 mM) containing 2.5 mM MgCl2
ATP

Protocol:

1. Purified P. trichocarpa 4-coumarate: CoA ligase-3 (Ptr4CL3) recombinant protein from Escherichia coli is used.
2. Six milligrams of each acid, 4 mg CoA, and 14 mg ATP are incubated with 0.3 mg purified Ptr4CL3 in a total volume of 40 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 2.5 mM $MgCl_2$ for 60 min at 37 °C.
3. The reaction is terminated by addition of 1.6 g ammonium acetate, and the reaction product is purified using a CHROMABOND C18 solid phase extraction cartridge (Macherey-Nagel).
4. The fractions containing CoA compound are collected and freeze dried.

4. Organic synthesis of isotope labeled acids

Supplies and Reagents:

[$^{13}C_3$]-Malonic acid
3- hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 3, 4-dihydroxy-5-methoxy-benzaldehyde, 4-hydroxy-3,5-dimethoxy-benzaldehyde
0.1 N HCl
Ethyl acetate
Chloroform
Acetone
Na_2SO_4

Protocol:

1. 75 Milligram of [$^{13}C_3$]-malonic acid is mixed with 50 mg of each benzaldehyde in 1 mL pyridine and 5 μL piperidine, to react for 1 week at room temperature.
2. Acidify the mixture with 0.1 N HCl (20 ml)
3. Extract with ethyl acetate (50 ml ×3)
4. Collect the ethyl acetate, dry over Na_2SO_4 and then evaporate

5. Purify over a silica column (CH_2Cl_2: Et_2O, 4:1, v/v)

5. Biochemical synthesis of CoA compounds

Supplies and Reagents:

[$^{13}C_2$] p-Coumaric acid
[$^{13}C_2$] Caffeic acid
[$^{13}C_2$] Ferulic acid
[$^{13}C_2$] 5-Hydroxyferulic acid
Coenzyme A
Tis-HCl buffer (pH 7.5, 50 mM) containing 2.5 mM $MgCl_2$
ATP

Protocol:

1. Purified P. trichocarpa 4-coumarate: CoA ligase-3 (Ptr4CL3) recombinant protein from Escherichia coli is used.
2. Six milligrams of each acid, 4 mg CoA, and 14 mg ATP are incubated with 0.3 mg purified Ptr4CL3 in a total volume of 40 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 2.5 mM $MgCl_2$ for 60 min at 37 °C.
3. The reaction is terminated by addition of 1.6 g ammonium acetate, and the reaction product is purified using a CHROMABOND C18 solid phase extraction cartridge (Macherey-Nagel).
4. The fractions containing CoA compound are collected and freeze dried.

6. Biochemical synthesis of p-coumaroyl shikimic acid and caffeoyl shikimic acid compounds

Supplies and Reagents:

p-Coumaroyl-CoA
Caffeoyl-CoA
Shikimic acid
Potassium phosphate buffer (pH 7, 50 mM)

Protocol:

1. E. coli produced and purified P. trichocarpa hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase-6 (PtrHCT6) recombinant protein is used.
2. Six milligrams of p-coumaroyl-CoA or caffeoyl-CoA combined with 2 mg shikimic acid are incubated with 29 mg PtrHCT6 in a volume of 20 mL of potassium phosphate buffer (pH 7) for 20 min at 30 °C, followe by ethyl acetate extraction (three times, 20 mL each). The organic layer is then dry over Na_2SO_4 and evaporate to give the product

7. Biochemical synthesis of aldehyde compounds

Supplies and Reagents:

[$^{13}C_2$] p-Coumaroyl-CoA
[$^{13}C_2$] Caffeoyl-CoA
[$^{13}C_2$] Feruloyl-CoA
[$^{13}C_2$]5-Hydroxyferuloyl-CoA
Potassium phosphate buffer (pH 6, 50 mM)

Protocol:

1. E. coli produced and purified P. trichocarpa cinnamoyl-CoA reductase (CCR) recombinant protein is used.
2. Twelve milligrams of isotope labeled CoA and 2 mM NADPH is incubated with 40 mg CCR in a volume of 20 mL of potassium phosphate buffer (pH 6) for 20 min at 45 °C, follow by ethyl acetate extraction (three times, 20 mL each). The organic layer is then dry over Na_2SO_4 and evaporate to give the product

8. Biochemical synthesis of alcohol compounds

Supplies and Reagents:

[$^{13}C_2$] p-Coumaraldehyde
[$^{13}C_2$] Caffeyl aldehyde
Potassium phosphate buffer (pH 6, 50 mM)

Protocol:

1. E. coli produced and purified P. trichocarpa cinnamyl alcohol dehydrogenase (CAD) recombinant protein is used.
2. Six milligrams of isotope labeled aldehyde and 2 mM NADPH are incubate with 20 mg CAD in a volume of 20 mL of potassium phosphate buffer (pH 6) for 20 min at

25 °C, follow by ethyl acetate extraction (three times, 20 mL each). The organic layer is then dry over Na_2SO_4 and evaporate to give the product