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SYNTHESIS OF [O-$^{14}CH_3$] SINAPIC ACID

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SUMMARY
[O-$^{14}CH_3$] Sinapic acid was synthesized with a specific activity of 54 mCi/mmol. Radiolabelled was carried out on methyl-3-(4-benzyloxy 3-hydroxy 5-methoxy phenyl) 2-propenoate with high yield, the deprotection of phenolic function and acid function are quantitative.

KEY WORDS : [O-$^{14}CH_3$] Sinapic acid, selective radiolabelling, Lignin biosynthesis.

INTRODUCTION

Lignin biosynthesis (in plants) (figure 1) follows a series of enzymatic reactions which begin with phenylalanine and lead to parahydroxycinnamic alcohols **4** by successively passing through acids **1**, thioesters of coenzyme A **2** and parahydroxycinnamic aldehydes **3** (1)(2). Parahydroxycinnamic alcohols are polymerised by cell wall peroxidases with hydrogen peroxide. It constitutes the lignification (3). Each alcohol is a precursor of lignin structural units.

4a : parahydroxyphenyl units Hp

4b : Guaiacyl units G

4c : Syringyl units S

Proportions of these three units vary in the plant according to tissue, species and variety.

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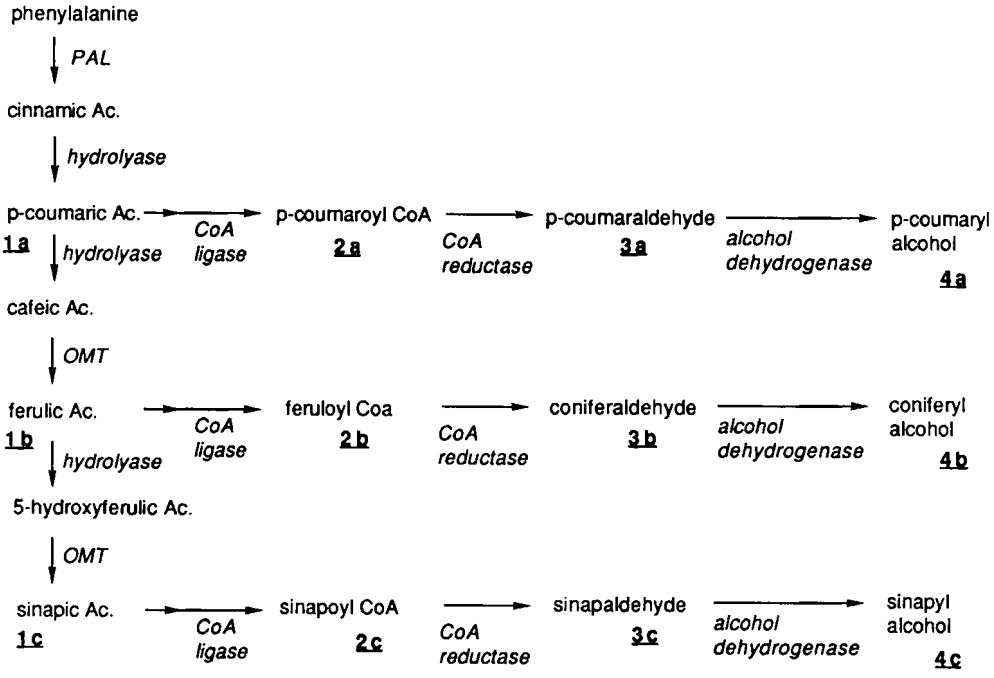
Fig. 1 : LIGNIN BIOSYNTHESIS



In order to study the digestibility of lignins by rumen microorganisms it is necessary to have lignins specifically labelled on the methoxy function of the syringyl units. Incorporation of labelled precursors like phenylalanine or cinnamic acid leads to a global labelling of H, G and S units. Therefore, we have incorporated a radioactive product at a later step in lignin biosynthesis i.e. the step in which sinapic acid **1c** is produced. The precursors most likely to specifically label S units in lignin are :

- Sinapic acid **1c**
- Coenzyme A thioester of sinapic acid **2c**
- Sinapaldehyde **3c**
- Sinapyl alcohol **4c**

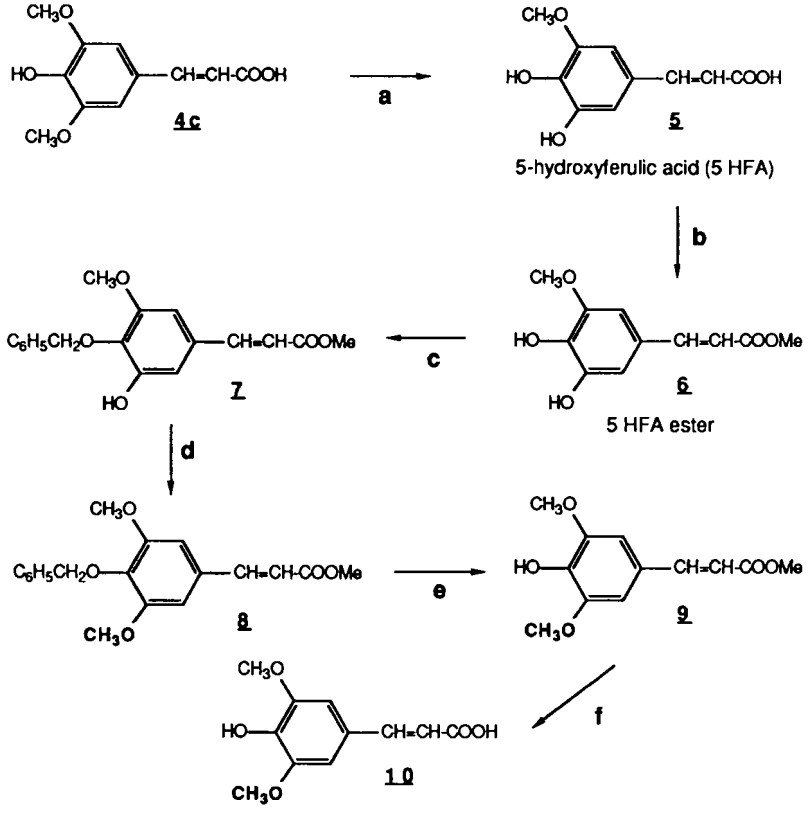
Among these four precursors, sinapaldehyde and CoA thioester synthesis is known (4) but difficult and radioactive synthesis would be unfeasible. Terashima (5) incorporated tritied sinapyl alcohol in young Eucalyptus stem and showed that the resultant lignin was different from natural lignin in both structure and distribution. Therefore, alcohol incorporation was not considered. It seems that sinapic acid is the best precursor for a specific labelling of S units. Terashima demonstrated specific labelling of lignin S units using sinapic acid as precursor in the dark, whereas, in the light, labelling was found also in G units. Grand (6) has obtained a specific labelling using phenylalanine in poplar xylem in the dark. The specific activity of sinapic acid labelled with ^{14}C on one or two methoxyl functions must be optimised in order to avoid incorporation of too much product and disturbing plant metabolism.

Furthermore, for digestibility studies, we need approximately 1 mCi of [^{14}C] sinapic acid (10), hence the reason published methods of synthesis were not suitable (5)(7)(8)(9). With an enzymatic synthesis, the authors (8) obtained only 22 µCi of [O-$^{14}CH_3$] sinapic acid (SA = 45 mCi/mmol). With a chemical synthesis starting of methyl gallate, the authors (9) obtained a specific activity less than 0.35 mCi/mmol with four steps after labelling. Consequently, we have devised a new synthesis based on selective deprotection and protection of the phenol and acid functions of sinapic acid.

RESULTS AND DISCUSSION

We propose a method of synthesis (pathway a) in which the deprotection of sinapic acid 4 leads to 5-hydroxyferulic acid 5.

Pathway a :



a - BBr_3, CH_2Cl_2, O°C, 1hour

b - Amberlite Resin, IR 120, MeOH, reflux 18 h

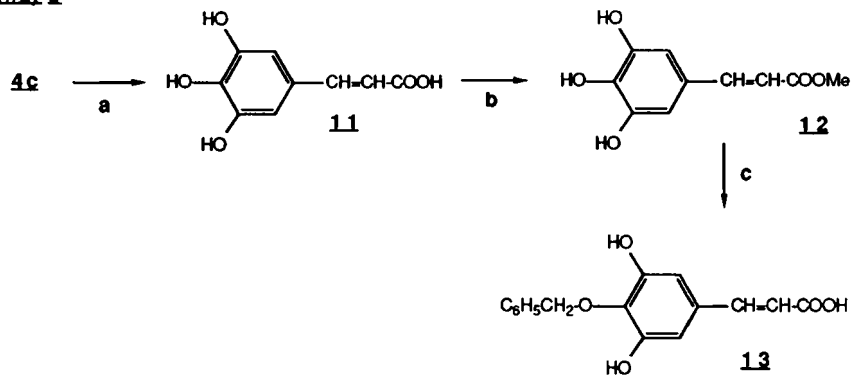
c - $C_6H_5CH_2Br$, K_2CO_3, CH_3COCH_3, -78°C, 18h

d - $^{14}CH_3I$, K_2CO_3, CH_3COCH_3, 20°C, 5h

e - 1) Me_3SiI, CH_2Cl_2, 20°C, 20 mn, 2) MeOH

f - 1) C_2H_5OH, NaOH 1M, reflux 18 h, 2) HCl 2M

Pathway **b**



a - BBr_3, CH_2Cl_2, O°C, 1h

b - Amberlite Resin, IR120, MeOH, reflux 18h

c - $C_6H_5CH_2Br$, K_2CO_3, CH_3COCH_3, -78°C, 18h

By the action of boron tribromide (11), added in stoichiometric quantities, we obtain 5-HFA **5** in 50% yield and 13% trihydroxycinnamic acid **11**. An excess of boron tribromide, added to react with the 37% residual sinapic acid, increases the yield of trihydroxycinnamic acid **11** to the detriment of 5-hydroxyferulic acid **5**. The methyl ester of 5-HFA **6** was prepared using an Amberlite Resin IR 120 and methanol (12), an excess of resin gave the ester with a 57% yield. The classical esterification is performed at room temperature ; however, it was necessary to reflux in methanol for 18 hours, although a significant amount of acid did not react. The ester could not be prepared by classical methods, via an acid chloride for example. In fact, it could not be synthesized due to polymerisation of hydroxycinnamic acid with thionyl chloride (13) ; a phenol function and a conjugated ethylenic system allows easier polymerisation reactions. The protection of the phenol function at the para position of the 5-HFA ester **6** with a benzyl group is classically performed in acetone with potassium carbonate (14). It is necessary to introduce benzyl chloride at -78°C to obtain the highest yield of para O-benzyl component **7** (65%). If the reaction is performed at 0°C, the di-O-benzyl component (meta and para) is dominant. However, only the para O-benzyle of 5-HFA ester **7** can be further methylated at the meta position. These three steps to obtain **7**, the precursor to the [^{14}C] labelling, gave a total yield of 18%.

It is not possible to reverse the order of steps **a** and **b** because the methoxyl deprotection by BBr_3 breaks the ester linkage. We have also examined a second means of synthesis (pathway **b**) using trihydroxycinnamic acid **11** to see if it could be favorable. Deprotection of the two methoxyl functions at the meta position of sinapic acid, to obtain trihydroxycinnamic acid was possible with good yields, but esterification and benzylation gave low yields. The total yield of these three steps was lower than 10%, it was therefore decided to use the benzyl ester **7** obtained from 5-HFA **5** as the precursor to labelling.

The three following steps **d**, **e**, and **f**, were first studied with non radioactive methyl iodide to optimise the yields. The methylation of **7** was quantitative (15), the debenzylation by trimethylsilyl iodide (16) was achieved in a 87% yield. Saponification of the sinapic ester was

also quantitative (10). We could observe that the three reactions with non radioactive components were either quantitative or with good yields. Only the debenzylation needed purification.

Labelling Steps.

During labelling, yields of the three last steps **d**, **e**, and **f** decreased significantly (42% versus 87% for unlabelled sinapic acid) . Working with very small quantities and adsorption of products onto the glass may explain this result. However, the 42% chemical yield was considered good, so, this method of synthesis is relatively easy to perform and yields 450 µCi of [O-$^{14}CH_3$]. Sinapic acid with a very high specific activity (54 mCi/mmole) higher than those obtained in previously published methods (5)(7)(8)(9).

This method of [^{14}C] labelling of a methoxyl function at the meta position of an aromatic ring can be applied to polyphenolic alcohols, aldehydes, esters and acids and may be useful in various studies related to the role of lignin S or G units.

MATERIALS AND METHODS

General : [^{14}C] Methyl iodide (1,08 mCi) was obtained from the CEA (France) and had a specific activity of 54 mCi/mmole. Reagents were purchased from Aldrich and solvents from SDS. Before use, the solvents were distilled and conserved over appropriate molecular sieves. Reactions were followed by thin layer chromatography (TLC) (MERCK SiO_2 60 F_{254}, 0.2 mm thickness). Reverse phase (RP) TLC was performed on MERCK RP 18, F_{254}-S, 0.25 mm thickness plates. The plates were visualized under UV at 254 nm and 350 nm or with 10% phosphomolybdic acid in ethanol. AMICON Silica (70-200 Mesh) was used for column chromatography and AMICON Silica C18 (90-130 mesh) for RP HPLC. Preparative HPLC was performed on a JOBIN-YVON apparatus. Infrared spectra were recorded on a PERKIN-ELMER 883 spectrophotometer - NMR spectra were recorded on BRUKER AC-80 apparatus in CD_3-CO-CD_3 or $CDCl_3$.

3-(3,4-dihydroxy 5-methoxyphenyl)2-propenoic acid 5 or 5-hydroxyferulic acid or 5-HFA

Sinapic acid (450 mg, 2 mmoles) was dissolved in 15 ml of dried (freshly distilled) methylene chloride, under argon. Boron tribromide (2.2 ml, 2.2 mmoles) was then added dropwise with constant stirring at -78°C. The solution was further stirred at 0°C for 1h and 3 ml 1M HCl was added to obtain pH 3. Methylene chloride was removed from the biphasic solution by vacuum evaporation. The aqueous solution was not evaporated to dryness to avoid 5-HFA polymerisation. The mixture (7 ml) was purified by HPLC (50 g, C18 silica, in a 40 mm diameter colomn, Eluent : H_2O/MeOH 70/30, Flow 1ml/mn). Fractions were analysed by TLC and those containing 5-HFA were vacuum evaporated to remove methanol and freeze dried. 5-HFA **5** (210 mg, 1 mmole) was obtained in a 50% yield and trihydroxycinnamic acid **11** (51 mg, 0.26 mmole) in a 13% yield.

5 $C_{10}H_{10}0_5$, M = 210

Rf (C_{18} silica, H_2O/MeOH : 70/30) = 0.25

NMR ^{1}H (CD_3COCD_3) : 3.89 (3H, s, OCH3), 6.33 (1H, d, J = 15.8 Hz, CH), 6.83 (1H, d, J = 1.9 Hz, ϕ), 6.89 (1H, d, J = 1.9 Hz, ϕ), 7.54 (1H, d, J = 15.8 Hz, CH).

11 $C_9H_8O_5$, M = 196

Rf (C_{18} Silica, H_2O/MeOH 70/30) : 0.58

IR (KBr, ν cm^{-1}) : 3415 (OH), 1667 (C=O), 1619 (C=C), 1597 (ϕ), 1516 (ϕ), 980 (δ H-C=C-H)

RMN ^1H (CD_3COCD_3) : 6.23 (1H, d, J = 15.9 Hz, CH), 6.73 (2H, s, ϕ), 7.48 (1H, d, J = 15.9 Hz, CH)

Methyl-3-(3,4-dihydroxy 5-methoxyphenyl)2-propenoate 6 or 5-HFA ester

To 5-HFA 5 (180 mg, 0.86 mmole) in 10 ml of methanol, 800 mg of Amberlite resin IR 120 (Aldrich) was added. The mixture was heated under reflux and stirred for 16 hours. The resin was then filtered off and the solution evaporated to dryness. The mixture was purified by column chromatography (20 g AMICON Silica 70-200 Mesh, 2 cm column diameter). The eluent was CH_2Cl_2/AcOEt (95/5) and fractions were analysed by TLC. The fractions containing product were combined and the eluent evaporated, leaving 96 mg 5-HFA ester 6 in a 57% yield.

6 $C_{11}H_{12}O_5$, M = 224

Rf (SiO_2,CH_2Cl_2/AcOEt : 95/5) = 0.45

IR ($CHCl_3$ M/20, ν cm^{-1}) : 3553 (OH), 1707 (C=O), 1637(C=C), 1612 (ϕ), 1515 (ϕ), 979 (δ H-C=C-H)

NMR ^1H (CD_3COCD_3) : 3.72 (3H, s, CH_3O ester), 3.88 (3H, s, CH_3O), 6.34 (1H, d, J = 15.9 Hz, CH), 6.84 (1H, d, J = 1.9 Hz, ϕ), 6.88 (1H, d, J = 1.9 Hz, ϕ), 7.52 (3H, d, J = 15.9 Hz, CH)

Methyl-3-(4-benzyloxy 3-hydroxy 5-methoxyphenyl)-2-propenoate 7

In a flask, under agon, 5-HFA ester 6 (110 mg, 0.49 mmole), potassium carbonate (690 mg, 5 mmoles) and 10 ml acetone (dried over K_2CO_3) were stirred at -78°C. Benzyl bromide, (58 µl, 0.49 mmole) was added over 10 mn and the reaction mixture further stirred for 16 hours. Potassium carbonate was filtered and washed with acetone and the acetone removed under vacuum. The residue (160 mg) was purified by column chromatography (20 g Amicon Silica, 130-200 mesh, 2 cm diameter, eluent : petroleum ether/ethyl acetate 70/30). The monobenzyl product 7 (100 mg) was isolated in a 65% yield and the dibenzyl product (12 mg) in a 4% yield.

7 $C_{18}H_{18}O_5$, M = 314

RF(SiO_2, petroleum ether / ethyl acetate: 70/30) : 0.30

IR (KBr, ν cm^{-1}) : 3434 (OH), 1700 (C=O), 1635(C=C), 1589 (ϕ), 1509 (ϕ), 982 (δ -H-C=C-H)

NMR ^1H ($CDCl_3$) : 3.78 (3H, s, CH_3O ester), 3.89 (3H, s, CH_3O), 5.10 (2H, s, CH_2), 5.87 (H, s, OH), 6.31 (1H, d, J = 15.9 Hz, CH), 6.64 (1H, d, J = 1.9 Hz, ϕ), 6.75 (1H, d, J = 1.9 Hz, ϕ), 7.37 (5H, s, ϕ), 7.56 (1H, d, J = 15.9 Hz, CH).

Methyl-3-(3,4-dibenzyloxy 5-methoxyphenyl) 2-propenoate 14

$C_{25}H_{24}O_5$, M = 404

Rf (SiO_2, petroleum ether / ethyl acetate : 70/30) : 0.37

RMN ^1H ($CDCl_3$) : 3.79 (3H, s, CH_3O ester), 3.85 (3H, s, CH_3O), 5.07 (2H, s, CH_2 benzyl meta), 5.10 (2H, s, CH_2 benzyl para), 6.31 (1H, d, J = 15.9 Hz, CH), 6.78 (2H, m, ϕ), 7.37 (10H, m, ϕ benzyl), 7.58 (H, d, J = 15.9 Hz, CH)

Methyl-3-(4-benzyloxy 3,5-dihydroxyphenyl) 2-propenoate 13

$C_{17}H_{16}O_5$, M = 300

Rf (SiO_2, petroleum ether/ethyl acetate : 60/40) = 0.34

IR (KBr, ν cm^{-1}) : 3499 (OH), 3318 (OH), 1685 (C=O), 1637 (C=C), 1600, 1528(ϕ), 972 (δ C=C).

NMR ^1H (CD_3OD) : 3.76 (3H, s, CH_3O ester), 5.11 (2H, s, CH_2 benzyl), 6.28 (1H, d, J = 15.9 Hz, CH), 6.59 (2H, s, ϕ), 7.33 (5H, m, ϕ benzyl), 7.46 (1H, d, J = 15.9 Hz, CH)

Methyl-3-(4-benzyloxy 3,5-dimethoxyphenyl) 2-propenoate 8
or parabenzyl methyl sinapate

Methyl iodide, $^{14}CH_3I$ (1.08 mCi, 0.02 mmole) with a specific activity of 54 mCi/mmole was purchased from CEA in a sealed vial. The vial was dipped in liquid nitrogen and the glass seal pierced with a sharpened steel rod, under argon. Potassium carbonate (40 mg, 0.29 mmole) and 7 (6.3 mg, 0.02 mmole) in solution in 500 µl acetone were added. The solution was then stirred with a vortex stirrer for 20 hours at room temperature. Initially being yellow, the solution was colourless at the end of the reaction. TLC analyses showed a total reaction. The K_2CO_3 was filtered off with a 0.45 µm Acrodisc. The mixture was recovered in a 10 ml flask, the vial washed several times with acetone and the solvent evaporated under reduced pressure

8 $C_{19}H_{20}O_5$, M = 328

Rf (SiO_2, cyclohexan/ethyl acetate : 7/3) : 0.49

IR ($CHCl_3$ M/20, ν cm^{-1}): 1710 (C=O), 1637(C=C), 1583 (ϕ), 1502 (ϕ), 979 (δ H-C=C-H)

NMR ^1H ($CDCl_3$) : 3.79 (3H, s, CH_3O ester), 3.82 (6H, s, CH_3O), 5.04 (2H, s, CH_2), 6.34 (1H, d, J = 15.9 Hz, CH), 6.76 (2H, s, ϕ), 7.35 (5H, m, ϕ), 7.61 (1H, d, J = 15.9 Hz, CH)

Methyl-3-(4-hydroxy 3,5-dimethoxyphenyl)-2-propenoate 9 or methyl sinapate

In a 10 ml flask containing the labelled product 8 under argon, 2 ml methylene chloride and trimethylsilyl iodide (20 µl, 0.14 mmole) were added. After 15 mn, an orange solution was obtained. This solution became yellow after hydrolysis with 100 µl methanol. The mixture was purified on a silica cartridge (Waters) with methylene chloride/ethyl acetate 90/10 as eluent. Pure methyl sinapate (0.017 mmole) 9 was obtained in a 85% yield.

9 $C_{12}H_{14}O_5$, M = 238

Rf (SiO_2, CH_2Cl_2/ethyl acetate : 9/1) : 0.42

IR ($CHCl_3$ M/20, ν cm^{-1}) : 3534 (OH), 1707 (C=O), 1635 (C=C), 1610 (ϕ), 1513 (ϕ), 979 (δ H-C=C-H)

NMR ^1H (CD_3COCD_3) : 3.73 (3H, s, CH_3O ester), 3.82 (6H, s, CH_3O), 6.24 (1H, d, J= 15.6 Hz, CH), 6.69 (2H, s, ϕ), 7.53 (1H, d, J = 15.6 Hz, CH).

3-(4-Hydroxy 3,5-dimethoxyphenyl)2-propenoic acid 10 or sinapic acid

In a 10 ml flask containing 0.017 mmole labelled methyl sinapate 9, 1 ml ethanol and 5 ml 1M NaOH were added. The solution became yellow and was heated under reflux for 45 mn. The solution was acidified with 4 ml of 2M HCl. The resultant solution was extracted with 3x20 ml ethyl acetate and the organic phase dried with $MgSO_4$. The $MgSO_4$ was filtered and the solvent evaporated. The product was partially purified with a C_{18} Silica cartridge (eluent H_2O/MeOH 1/1) and fractions containing product were evaporated. Sinapic acid was purified by HPLC (C_{18} silica. Eluent : A : H_2O, 0,2% TFA, B : acetonitrile A/B : 76/24, flow 1 ml/mn, λ = 260 nm). [O-$^{14}CH_3$] Sinapic acid (450 µCi, 8.3 µmole) was obtained in 42% yield. The radioactivity of [O-$^{14}CH_3$] sinapic acid was measured by liquid scintillation counting. As dilution by non radioactive material never occurs during the last three steps of the synthesis, the specific activity of sinapic acid is directly related to that of the starting material $^{14}CH_3I$ (54 mCi/mmol).

10 $C_{11}H_{12}O_5$, M = 224 ; Rf (Silica C_{18}, MeOH/$H_2$0 : 5/5) : 0.28

IR (KBr, ν cm^{-1}) : 3400-3347 (OH), 1664 (C=O) ; 1626(C=C), 1594 (ϕ), 1516 (ϕ), 973 (δ H-C=C-H)

NMR ^1H (CD_3OD) : 3.87 (6H, s, $CH_3$0), 6.34 (1H, d, J = 15.6 Hz, CH), 6.88 (2H, s, ϕ), 7.59 (1H, d, J = 15.6 Hz, CH)

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