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Enzyme Catalysed Formation of (*S*)-Cyanohydrins Derived from Aldehydes and Ketones in a Biphasic Solvent System

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Abstract

By employing a vigorously stirred two phase system aqueous buffer/organic solvent and using the hydroxynitrile lyase from *Hevea brasiliensis* as biocatalyst enantiopure *(S)*-cyanohydrins from aliphatic, unsaturated, aromatic and heteroaromatic aldehydes and methyl alkyl and methyl phenyl ketones are obtained in high yield and in general 98-99% enantiomeric excess.

Keywords: Cyanohydrins; Enzymes and Enzyme Reactions; Stereospecificity

1. Introduction

Enantiomerically pure cyanohydrins are valuable chiral building blocks in organic synthesis [1]. Among the known methods for cyanohydrin formation, the use of hydroxynitrile lyases is probably the most promising approach to this class of chiral synthons [2]. In the last decade, a number of different hydroxynitrile lyases have been investigated with respect to their biological properties [3,4,5] and the range of carbonyl compounds accepted as substrates [6,7,8,9,10,11,12,13,14].

The synthesis of (*S*)-cyanohydrins catalysed by the (*S*)-hydroxynitrile lyase from *Hevea brasiliensis* is well established [15,16,17]. Although many aliphatic as well as aromatic aldehydes were converted into the corresponding cyanohydrins with excellent enantiomeric purities, in several cases less satisfying results were obtained. Here low product yield together with unsatisfactory enantiopurities were observed. Moreover, the preparation of ketone derived cyanohydrins by applying the aqueous reaction system described [18] was still found to be inadequate.

By employing a vigorously stirred two phase reaction system, chiral cyanohydrins could be prepared in excellent enantiomeric purity and high yield. In particular, this procedure allowed the preparation of (*S*)-ketone cyanohydrins in good chemical yield and optical purity. This is of

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synthetic importance because chiral 2-hydroxy-2-methyl alkyl and aryl nitriles can be used for the synthesis of biologically active compounds and several chiral natural products [19,20,21,22,23].

In context to the transhydrocyanation of several aliphatic and aromatic aldehydes, the addition of small amounts of diethyl ether as the organic phase to the aqueous buffer system has already been described [24]. Here 2-hydroxy-2-propanenitrile was employed as the cyanide donor and the *(R)*-hydroxynitrile lyase from almonds (*Prunus amygdalus*) was used.

To date, reported enzyme mediated methods for the preparation of cyanohydrins derived from ketones include the (*R*)-hydroxynitrile lyase (*Prunus amygdalis)* catalysed addition of hydrogen cyanide to ketones [10,11,23,25] as well as a synthesis using a (*S*)-specific enzyme from *Manihot esculenta* [14]. The preparation of *(S)*-ketone cyanohydrins by a transhydrocyanation reaction between a racemic ketone cyanohydrin and various aldehydes has also been reported using the *(R)*-hydroxynitrile lyase prepared from apple meal [26] and almond [26,27]. Enzymes other than lyases, for example the application of a microbial esterase from *Pichia miso* for the kinetic resolution of acetates of ketone cyanohydrins, have also reported [22]. Among the nonenzymatic catalytic asymmetric methods investigated, the trimethylsilylcyanation of acetophenone in the presence of a chiral catalyst prepared from 3,3-dimethyl-1,2,4-butanetriol and titanium isopropoxide applying high pressure [28] and the diastereoselective alkylation of a chiral phosphate auxiliary derived from pseudoephedrine have been reported [29].

2. Results and Discussion

The (*S*)-specific hydroxynitrile lyase from *Hevea brasiliensis*, which is also available by overexpression in *Pichia pastoris* [30], turned out to be sufficiently stable under the conditions chosen which rendered immobilisation unnecessary. The volume of the aqueous phase could be equal or higher than that of the organic solvent used. In principle, the only restriction in the type of the organic solvent employed is its immiscibility with water. However, it was found that carboxylic esters and long chain or branched dialkyl ethers such as methyl *t*-butyl ether (MTBE) and diisopropyl ether (DIPE) were the most suitable solvents for this enzymatic reaction.

The formation of cyanohydrins by this method, employing aldehydes as substrates, is summarised in Table 1. In addition, the results are compared with earlier experiments [17], where aqueous buffer was used.

It was found that the transformation proceeded most efficiently at temperatures between 5 to 15°C with anhydrous hydrogen cyanide in molar excess. The formation of a stable emulsion in the reaction mixture was found to be of utmost importance for the success of the system.

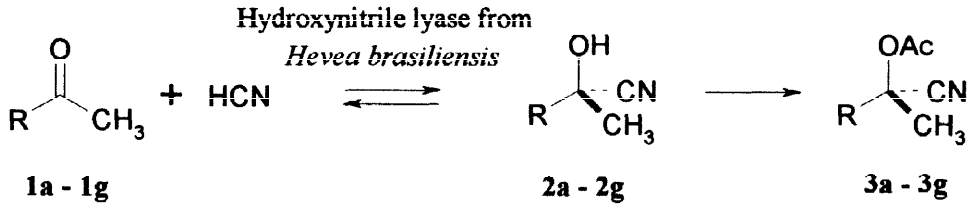
Table 1:
Conversion of aldehydes RCHO into the corresponding cyanohydrins (*S*)-RCH(OH)CN[a]

R-	aqueous citrate buffer, pH 4.5[b]		biphasic reaction system (buffer/MTBE), pH 5.5	
	conversion[c] [%]	e.e.[d] [%]	conversion[c] [%]	e.e.[e] [%]
Ph	67	99	97	99
(*E*)-PhCH=CH	0	0	93	98[e]
3-PhOPh	9	99	99	99
$PhCH_2OCH_2$	n.d.[f]	0	92	12
c-C_6H_{11}	94	99	95	99
2-furyl	55	98	95	98
3-furyl	61	99	98	98
3-thienyl	67	98	98	99
$CH_3(CH_2)_4$	n.d.	84	97	98
CH_2=CH	38	94	92	98
(*E*)-$CH_3(CH_2)_4$CH=CH	62	99	96	99
$CH_3(CH_2)_2$C≡C	43	80	62	98

a: determination of absolute configurations reported in [17]; *b*: data from [17]; *c*: determined by GC analysis of the corresponding acetates [17] or by UV-spectroscopy; *d*: determined by chiral GC analysis [17] of the acetates *e*: determined by chiral HPLC analysis of the TBDMS ether [17]; *f*: not determined

This was achieved by the sequential addition of the carbonyl compound, dissolved in the appropriate organic solvent, together with the aqueous enzyme preparation and, after a stable emulsion had been formed upon efficiently stirring, the hydrogen cyanide. In addition, the special shape of the reaction vessel and a stirrer capable of high rotational frequency contributed to the positive outcome of the reaction.

As can be seen from Table 1, the enantiomeric purity and the chemical yield is, in almost all cases, excellent. The only exception seems to be benzyloxyacetaldehyde, which is obviously not a good substrate for the *Hevea* hydroxynitrile lyase [17]. However, some enantioselectivity could be achieved in comparison to the aqueous reaction system previously used. The somewhat lower isolated yield of 62% for the hex-2-ynal derivative is due to the decomposition of this compound during purification. *(E)*-2-Hexadecenal, trifluoroacetone and cyclohexyl phenyl ketone could not be converted into their corresponding cyanohydrins. This is due to the steric bulkiness of the substrates and to the strong hydration in case of the trifluoroketone.



Scheme 1

For ketones, the experimental procedure in the biphasic system described above as well as the procedure in aqueous buffer solution, was investigated.

In aqueous medium the preparation of cyanohydrins was carried out in 0.1 molar sodium citrate buffer solution at pH 3.75 to 4.0 at low temperature (0-5°C).

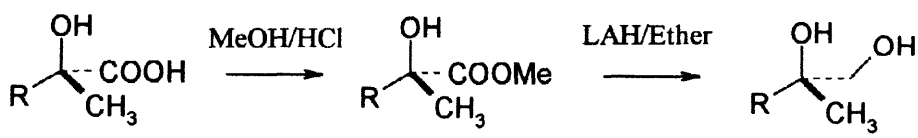
The absolute configuration of compound **2b** was determined by comparing the observed optical rotation with literature values[31]. However, in general, the specific rotation was too low for the other products to allow an accurate assessment. Therefore, the cyanohydrins were converted into their corresponding O-acetylated derivatives which exhibited higher specific rotations. The results are given in Table 2.

Table 2:
Formation of cyanohydrins RC(OH)(CH_3)CN and acetates RC(OAc)(CH_3)CN of methyl ketones in aqueous buffer

	cyanohydrins **2a-2g**			cyanohydrin acetates **3a-3g**			
R-		isolated yield [%]	e.e. [%]		e.e.[a] [%]	$[\alpha]_D^{20}$	$[\alpha]_{546}^{20}$
$(CH_3)_2CH$	**2a**	30	75	**3a**	52	-16.8	-20.8
$CH_3CH_2CH_2$	**2b**	30	82	**3b**	45	-8.5	-10.0
$(CH_3)_2CHCH_2$	**2c**	13	78	**3c**	88	-25.1	n.d.[b]
$(CH_3)_3C$	**2d**	49	83	**3d**	78	-40.5	-48.1
$PhCH_2$	**2g**	27	89	**3g**	72	-18.1	-20.9

a: The optical rotation measurements were carried out based on separate reactions, thus leading to different e.e. values for **3a-3g** in contrast to the values depicted on the left side of Table 2; however no change in the e.e. values were found for the acetates **3** prepared from their cyanohydrins **2**; *b:* not determined

On this basis, the *(S)*-configuration was assigned to acetates **3b** and **3c** [22]. To date, the specific rotation of acetates **3d** and **3g** have not been published. Although the optical rotation for **3a** is reported, the low reported e.e. (9 %) [22] implies that this value should be considered with caution. Consequently, the absolute configurations of **2a**, **2d** and **2g** were assigned employing other methods. The configuration of **2a** was assigned to be *(S)* after hydrolysis to the corresponding carboxylic acid [32]. Compound **2g** was transformed into the literature reported diol [33] in a three step sequence, as shown in Scheme 2.



2a, 2g **4g** **5g**

Scheme 2

The determination of the enantiomeric excess was accomplished by chiral GC of the acetates, except for compound **2g** where it was determined by resolution of the diastereomeric MPTA-esters on a nonchiral stationary phase.

With respect to the two phase reaction protocol it was taken into consideration that in parallel to the enzyme catalysed reaction the non-enzymatic cyanohydrin formation also takes place although at a slow rate at this low pH value. Therefore, short reaction times were necessary. Taking this into account, conditions were chosen which allowed the reaction to come to completion in not more than 15 minutes. This could be achieved by further optimisation of parameters such as pH value, amount of enzyme, the phase ratio ($V_{organic}/V_{aqueous}$) and the concentration of the ketone in the organic solvent. As a result, slightly different reaction conditions, depending on the ketone to be converted, were applied. They are summarised in Table 3. The adjusted conditions allowed, for example, the preparation of acetophenone cyanohydrin **2g** in excellent enantiopurity. The low yield of **2g** is due to the unfavorable equilibrium position of cyanohydrins derived from aromatic ketones [34].

Table 3:
Conversion of ketones $RCOCH_3$ to enantiomerically enriched (*S*)-$RC(OH)(CH_3)CN$ (**2**)

R-	cyanohydrin	pH value	enzyme amount IU/mmol	$V_{organic}/V_{aqueous}$	concentration of ketone mg/ml	conversion[a] [%]	e.e. [%]
$(CH_3)_2CH$	**2a**	4.0	750	0.75/1	210	99	98
$CH_3CH_2CH_2$	**2b**	3.9	1200	1/1	200	99	74
$(CH_3)_2CHCH_2$	**2c**	4.5	600	0.75/1	250	86	99
$CH_3(CH_2)_3$	**2e**	4.0	600	0.75/1	200	59	99
Ph	**2f**	3.75	750	0.75/1	190	40	99

a: determined by IR spectroscopy

In conclusion by employing a vigorously stirred two phase system aqueous buffer/organic solvent and using the hydroxynitrile lyase from *Hevea brasiliensis* as biocatalyst enantiopure *(S)*-cyanohydrins from aliphatic, unsaturated, aromatic and heteroaromatic aldehydes and methyl alkyl and methyl phenyl ketones are obtained in high yield and in general 98-99% enantiomeric excess.

3. Experimental

TLC was performed on MERCK silica gel 60 F_{254} plates. For column chromatography MERCK silica gel (230 - 400 mesh) was used. Optical rotations were measured on a PERKIN ELMER 341 polarimeter at 589 and 546 nm (cell length 10 cm; concentrations are given in g/100 ml). Proton and carbon NMR spectra were recorded in $CDCl_3$ on a VARIAN Gemini 200 spectrometer at 200 and 50.3 MHz, resp. Shifts are given in ppm and are calibrated to the

solvent. GC-analyses were carried out on a HEWLETT PACKARD 5890 Series II plus GC using a 25 m X 0.25 mm HP 5 capillary column (0.32 μm film thickness) with nitrogen as carrier gas. Chiral GC was performed on a SHIMADZU GC 14A using a 25 m X 0.32 mm Chirasil™-Dex-CB capillary column (0.25 μm film thickness) with hydrogen as carrier gas; both equipped with FID.

The enzyme used for all experiments in this investigation was a crude cytosolic fraction of the recombinant protein derived from genetically modified *Pichia pastoris*. Cell disruption and centrifugation was carried out in order to remove whole cells and cell debris. The enzyme activity was measured using a kinetic assay based on mandelonitrile fission, monitored by UV spectroscopy and was determined to be 5000 IU ml^{-1}.

General procedure for the synthesis of (S)-cyanohydrins derived from aldehydes

20 ml (1250 units ml^{-1}) of an aqueous solution of the enzyme (i. e. 1000 units $mmol^{-1}$) was cooled to 15°C and the pH was adjusted to 5.0 - 5.5. To this solution the aldehyde (25 mmol), dissolved in methyl *t*-butyl ether (20 ml), was added. Freshly prepared hydrogen cyanide (5 ml, 126 mmol) was added and the reaction vessel was tightly sealed. The biphasic reaction mixture was vigourously stirred for 15 min. During this time the mixture formed an emulsion, which proved to be necessary for a short reaction time. The layers were separated and extracted 3 times with methyl *t*-butyl ether (20 ml each time) and the organic phase was dried over anhydrous sodium sulfate. The organic solvent was removed and the residue was purified by silica gel chromatography using cyclohexane/ethylacetate (5 - 9/1). Trace amounts of anhydrous HCl were added to the eluent to assure the stability of the cyanohydrin during the chromatographic purification step. For the determination of the enantiomeric excess the cyanohydrin was acetylated by a standard procedure (AcCl, pyridine, in methylene chloride, 0.5 hours, r. t.) and subsequently used for the chiral analysis.

Preparation of (S)-ketone cyanohydrins **2a - 2f** *in biphasic system*

An aqueous solution of the enzyme (24 ml; 1700 units ml^{-1}) was cooled to 0°C and the pH was adjusted to 3.75 - 4.5 with 5 mmolar solution of sodium citrate (pH 3.5). The ketone (80 mmol) dissolved in methyl *t*-butyl ether or diisopropyl ether (20 ml) was added. The reaction mixture was energically stirred (2000 min^{-1}) for 1-3 minutes until a stable emulsion had formed. After addition of freshly prepared anhydrous hydrogen cyanide (10.8 g, 400 mmol) the reaction vessel was hermetically sealed and the emulsion was stirred for another 5 minutes. The addition of organic solvent to the reaction mixture resulted in the phase separation. The aqueous layer was extracted another 3 times using methyl *t*-butyl ether or diisopropyl ether (each time 25 ml). The organic phase was then dried over anhydrous sodium sulfate. After removal of the solvent, the residual oil was purified over silica gel using cyclohexane/ethylacetate (8/1); Table 3.

Preparation of (S)-ketone cyanohydrins **2a - 2d** *and* **2g** *in aqueous citrate buffer*

The ketone was suspended or dissolved in 0.1 molar sodium citrate buffer (10 ml) at pH 3.8 - 4.0 and the enzyme preparation was added. The solution was chilled to 0°C . Potassium cyanide (260 mg, 4 mmol) was dissolved in cold 0.1 molar citric acid (ca. 35 ml) and the pH of the solution was adjusted to 3.8 - 4.0 with 1 N sodium hydroxide. This solution was added to the solution containing the ketone and the enzyme via a dropping funnel in a strictly closed system under stirring within 20 min.The reaction was stirred under cooling for one hour. The reaction mixture was extracted three times with diethyl ether (3 x 20 ml). A centrifugation step had to be carried out in some cases in order to get separation of the layers. The organic layer was collected, dried and the ether removed under reduced pressure. The crude product was purified by silica gel chromatography with petroleum ether/ethyl acetate 6/1 as eluent.

(S)-2-hydroxy-2,3-dimethylbutanenitrile (2a)
Colorless oil, yield 30%; e.e. 75%; e.e. 95% [11] for *(R)*-enantiomer.
^{1}H NMR δ (ppm): 1.07 (t, 6H, $(CH_3)_2$); 1.55 (s, 3H, CH_3); 1.91 (m, 1H, CH); 3.01 (br s, 1H, OH). ^{13}C NMR δ (ppm): 121.6, 72.68, 37.77, 24.96, 17.23.

(S)-2-hydroxy-2-methylpentanenitrile (2b)
Colorless oil, yield 30%; e.e. 82%; e.e. 80% [27] for *(S)*-enantiomer.
0.99 (t, 3H, CH_3); 1.45 - 1.62 (m, 2H, CH_2); 1.59 (s, 3H, CH_3); 1.72 (m, 2H, CH_2); 3.46 (br s, 1H, OH). ^{13}C NMR δ (ppm): 122.33, 68.84, 44.02, 27.79, 17.80, 13.97.

(S)-2-hydroxy-2,4-dimethylpentanenitrile (2c)
Colorless oil, yield 13%; e.e. 78%; e.e. 99% [26] for *(S)*-enantiomer.
^{1}H NMR δ (ppm): 0.94 and 1.15 (2d, 6H, $(CH_3)_2$); 1.62 (s, 3H, CH_3); 1.74 (d, 2H, CH_2); 1.84-2.33 (m, 1H, CH); 3.02 (br s, 1H,OH);

(S)-2-hydroxy-2,3,3-trimethylbutanenitrile (2d)
White solid, m.p. 98 - 99°C (n-heptane), yield 49%; e.e. 83%;
^{1}H NMR δ (ppm): 1.10 (s, 9H, $(CH_3)_3$); 1.57 (s, 3H, 2-CH_3); 2.51 (br. s, 1H, OH).
^{13}C NMR δ (ppm): 122.10, 75.60, 37.96, 24.79, 23.31.

(S)-2-hydroxy-2-methylhexanenitrile (2e)
Colorless oil; e.e. 99%; e.e. 97% [26] for *(S)*-enantiomer; e.e. 95% [27] for *(S)*-enantiomer.
^{1}H NMR δ (ppm): 0.95 (t, 3H, CH_3); 1.25 - 1.58 (m, 4H, CH_2-CH_2); 1.62 (s, 3H, CH_3); 1.72-1.82 (m, 2H, CH_2); 2.70 (s, 1H, OH); ^{13}C NMR δ (ppm): 121.52, 68.81, 41.51, 27.73, 26.37, 22.49, 13.87.

(S)-2-hydroxy-2-phenylpropanenitrile (2f)
Colorless oil; e.e. 99%; e.e. 98% [26] for *(S)*-enantiomer.
^{1}H NMR δ (ppm): 1.87 (s, 3H, CH_3); 3.49 (br m, 1H, OH); 7.36 - 7.65 (m, 5H, phenyl);
^{13}C NMR δ (ppm): 140.77, 129.20, 129.00, 124.53, 121.5, 70.72, 31.11.

(S)-2-hydroxy-2-methyl-3-phenylpropanenitrile (2g)
Colorless oil, yield 27%; e.e. 89%; e.e. 60% [28] for *(S)*-enantiomer.
^{1}H NMR δ (ppm): 1.67 (s, 3H, CH_3); 2.76 (s, 1H, OH); 2.98 and 3.12 (2d, 2H, CH_2); 7.38 (m, 5H, phenyl). ^{13}C NMR δ (ppm): 133.63, 130.51, 128.94, 128.08, 120.08, 68.68, 47.67, 27.42.

Acetylation of (S)-ketone cyanohydrins

Cyanohydrin (5 mmol) was dissolved in methylene chloride (2 ml). Acetyl chloride (25 mmol) and pyridine (25 mmol) were added and the reaction mixture was stirred at r. t. for several hours. The reaction was washed with 5% hydrochloric acid, with saturated sodium bicarbonate solution and finally distilled water. After drying over anhydrous sodium sulfate and evaporation of the solvent the crude acetates were purified by silica gel chromatography using cyclohexane/ethyl acetate (5 - 9/1) as the eluent. The e.e. did not change during this procedure. The cyanohydrins used for the acetylation were prepared according to the aqueous reaction protocol.

(S)-2-Acetoxy-2,3-dimethylbutanenitrile (3a)
Colorless oil, yield 23%; $[\alpha]_D^{20}$ -16.8 (c 2.0, chloroform), e.e. 52%;
^{1}H NMR: δ (ppm): 1.10 (q, 6H, $(CH_3)_2$); 1.69 (s, 3H, CH_3); 2.10 (s, 3H, CH_3CO); 2.13 - 2.34 (m, 1H, CH). ^{13}C NMR: δ (ppm): 168.96, 118.16, 75.79, 36.49, 21.23, 17.10, 16.67.

(S)-2-Acetoxy-2-methylpentanenitrile (3b)
Colorless oil, yield 51%; $[\alpha]_D^{20}$ -8.5 (c 1.94, benzene), e.e. 45% ; $[\alpha]_D^{26}$ -15.0 (c 1.32, benzene) for *(S)*-enantiomer, e.e. 95% [22].
^{1}H NMR: δ (ppm): 0.99 (t, 3H, CH_3); 1.41 - 1.70 (m, 2H, CH_2); 1.73 (s, 3H, CH_3); 1.77 - 2.04 (t, 2H, CH_2); 2.09 (s, 3H, CH_3CO). ^{13}C NMR: δ (ppm): 168.88, 118.83, 71.00, 41.79, 24.60, 21.17, 17.33, 13.75.

(S)-2-Acetoxy-2,4-dimethylpentanenitrile (3c)
Colorless oil, yield 38%; $[\alpha]_D^{20}$ -25.1 (c 1.25, chloroform), e.e. 88%; $[\alpha]_D^{20}$ -10.8 (c 1.07, chloroform) for *(S)*-enantiomer, e.e. 90% [22].
^{1}H NMR: δ (ppm): 1.01 and 1.04 (2d, 6H, $(CH_3)_2$); 1.43 (d, 2H, CH_2); 1.75 (s, 3H, CH_3); 1.83 - 2.04 (m, 1H, CH); 2.09 (CH_3CO).
^{13}C NMR: δ (ppm): 168.87, 119.04, 71.69, 30.36, 25.22, 24.84, 23.68.

(S)-2-Acetoxy-2,3,3-trimethylbutanenitrile (3d)
Colorless oil, yield 35%, $[\alpha]_D^{20}$ -40.5 (c 2.25, chloroform), e.e. 78%;
^{1}H NMR: δ (ppm): 1.11 (s, 9H, $(CH_3)_3$); 1.69 (s, 3H, 2-CH_2); 2.09 (s, 3H, CH_3CO).
^{13}C NMR: δ (ppm): 168.97, 118.10, 78.07, 38.39, 24.66, 21.20, 18.93.

(S)-2-Acetoxy-2-methyl-3-phenylpropanenitrile (3g)
Colorless oil, yield 25%, $[\alpha]_D^{20}$ -18.1 (c 1.7, chloroform), e.e. 72% ;
^{1}H NMR: δ (ppm): 1.71 (s, 3H, CH_3); 2.10 (s, 3H, CH_3CO); 3.26 (q, 2H, CH_2); 7.26 - 7.34 (m, 5H, phenyl). ^{13}C NMR: δ (ppm): 168.93, 133.09, 130.74, 128.58, 127.93, 118.55, 71.81, 44.93, 24.29, 21.25.

(S)-2-methyl-3-phenyl propanediol (5g)
White crystals; yield 62mg (84%); mp. 62 - 63°C (n-pentane); $[\alpha]_D^{20}$ -15.5 (c 1.08, 95% EtOH); e.e. determined by chiral GC after derivatisation to the acetonide; $[\alpha]_D^{20}$ +17.3 (95% EtOH), e.e. 98% for *(R)*-enantiomer [33].
^{1}H NMR: δ (ppm): 1.14 (s, 3H, CH_3); 1.68 (br., 1H, OH); 2.07 (s, 1H, OH); 2.77 and 2.86 (2d, 2H, phenyl-CH_2); 3.43 and 3.46 (2d, 2H, CH_2OH); 7.22 - 7.33 (m, 5H, phenyl).
^{13}C NMR: δ (ppm): 137.0, 130.48, 128.40, 126.69, 72.98, 69.33, 44.69, 23.67.

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