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Study on the (*R*)-oxynitrilase activity of *Pouteria sapota*

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Abstract—Mamey (*Pouteria sapota*) defatted meal was used to catalyze the enantioselective addition of HCN to α,β-unsaturated aldehydes. Using a biphasic system of diisopropyl ether and citrate buffer (0.1 M, pH 5.0, 10% v/v), the (*R*)-cyanohydrins obtained showed good conversion (from 54 to 98%) and enantiomeric excess (from 74 to 99%).

1. Introduction

Research on enzymatic processes to prepare optically active compounds has been growing constantly in recent years, due to the advantages enzymes have over metallic chiral catalysts, mainly in the preparation of biologically active compounds. Oxynitrilases or hydroxynitrile lyases are enzymes that biocatalyze the enantioselective addition of HCN to aldehydes or ketones to yield cyanohydrins. These compounds are versatile building blocks that have been used in the preparation of pharmaceuticals and agrochemicals. Oxynitrilases have attracted the attention of many researchers, in such way that actually there are very interesting findings in this field.[1–6]

Hydroxynitrile lyases have been found in a wide variety of plant sources, but the selectivity towards the biocatalyzed addition of HCN to carbonyl compounds is not the same, some accept better aromatic rather than aliphatic aldehydes, some biocatalyze the addition of HCN to aldehydes and ketones, others only to aldehydes. Due to this, it can be said that the different sources of oxynitrilases are complementary.[1–5,7,8] So, the study of alternative sources of oxynitrilases becomes important.

In previous work we found that seeds of *Pouteria sapota* (mamey) are a source of (*R*)-oxynitrilase.[7] We found that in aqueous medium the defatted meal of mamey biocatalizes the addition of HCN to aromatic aldehydes with conversions from 24 to 73% and enantiomeric excesses from 34 to 77%. We extended the study to determine the selectivity towards different aldehydes in organic medium. We tested aromatic, heteroaromatic, cinnamaldehyde and aliphatic aldehydes, and except with cinnamaldehyde, the enantiomeric excesses were good (77–98%) (Fig. 1).

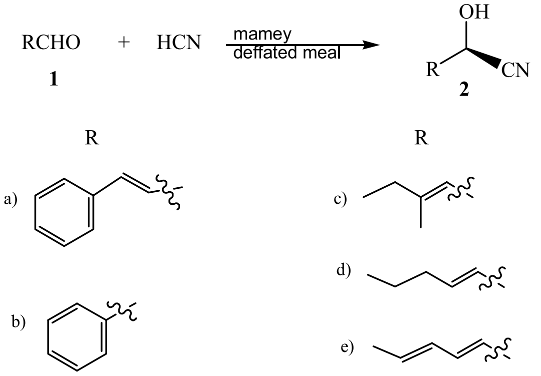

Figure 1.

Due to the promising results with mamey, we extended our study to find the reaction conditions to get the best biocatalytic activity of the defatted meal of mamey seeds, for the addition of HCN to α,β-unsaturated aldehydes. We chose those aldehydes to prepare cyanohydrins which can be used in the preparation of several interesting compounds[9] such as insecticides.[10]

2. Results and discussion

It is well known that oxynitrilases need some water to biocatalyze addition of HCN to aldehydes, but the enantioselectivity of the reaction can be decreased by the chemical reaction that is favored by the aqueous medium. This last reaction can be diminished by lowering the pH and

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Keywords: Oxynitrilases; Cyanohydrins; Mamey.

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temperature of the reaction medium or using proper organic solvents. The aim of this work is to improve the addition of HCN to aldehydes biocatalyzed by mamey meal, the study is divided into several parts to determine the solvent, the acid used in the preparation of the HCN source, concentration of buffer solution for the biphasic system, and the water content of the biphasic system. The reactions for this study were carried out with benzaldehyde and cinnamaldehyde, after analysis of the results the best reaction conditions were applied to other α,β-unsaturated aldehydes.

2.1. Effect of solvent

It is reported that the biocatalytic activity of oxynitrilases is better in biphasic systems, and that organic solvent has an important effect on the yield and enantioselectivity.[2,11,12] To determine the most convenient solvent for the reaction of addition of HCN to cinnamaldehyde (**1a**) to get the corresponding cyanohydrin (**2a**) catalyzed by mamey meal, different solvents were tested to extract the HCN from a buffer solution (KCN/citric acid, pH 5.0, 1 M) and use it as reaction solvent, then the reaction was carried out at 4 °C for 24 h, the results are in Figure 2.

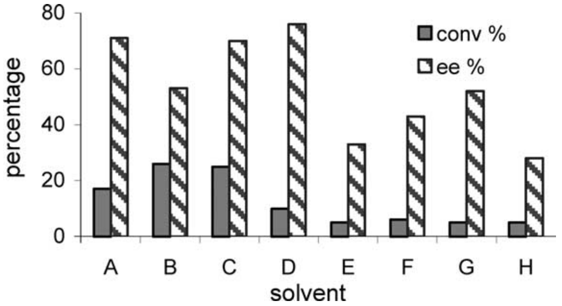

Figure 2. Conversion (determined by ^1H NMR) and enantiomeric excess (determined by HPLC using an OD Chiracel column) of cyanohydrin **2a**, in different solvents. A: diethyether, B: diisopropyl ether, C: methyl *tert*-butyl ether (MTBE), D: ethyl acetate, E: hexanes, F: heptane, G: octane, H: toluene.

From the results in Figure 2, it can be observed that the addition of HCN to cinnamaldehyde catalyzed by mamey defatted meal is not favorable in hydrocarbons such as hexanes (E), heptane (F), octane (G) and toluene (H), since the conversion was less than 10%, and the enantiopurity was also low. When ethyl acetate was used in this reaction the enantioselectivity was the highest (72%), but as with hydrocarbon solvents the conversion was also very low (10%).

By using diethyl ether (A) as solvent, the enantiomeric excess was also high (70%) and the conversion was a little higher (17%) than with the previously mentioned solvents. The best reactivities were obtained with diisopropyl ether (B) and MTBE (C), the conversions were 26 and 25% respectively, but with MTBE the enantiomeric excess was superior (70%).

A decrease in enantiomeric excess can be due to the formation of the racemic cyanohydrin because of the non-biocatalized addition of HCN to the aldehyde. To determine the effect of this unwanted addition, a reaction under the same conditions but without the meal was carried out, and after 24 h no cyanohydrin was detected. We can conclude that the enantiomeric excess of cyanohydrin **2a** is not so high because mamey meal is not very selective towards aldehyde **1a**, under the conditions of reaction tested.

2.2. Effect of the acid used to prepare the KCN/acid buffer

Another important condition in the addition of HCN to aldehydes is the acid used in the preparation of the buffer solution of KCN/acid (pH 5.0, 1 M), which is the source of HCN.[13] Acetic, citric, phosphoric, lactic and tartaric acids were chosen to prepare KCN/acid buffer, the reaction of addition of HCN was performed with benzaldehyde (**1b**) using diisopropyl ether as solvent in microaqueous medium[12,14] and after 24 h of reaction at 4 °C the reaction was analyzed. Although the enantiomeric excess of the obtained mandelonitrile (**2b**) was >99%, with all the acids, the conversion was not the same. From the results in Figure 3 it can be stated that conversion was the highest for citric and phosphoric (93%), than with the other acids. One disadvantage for the use of phosphoric acid is that the product becomes dark and it is difficult to purify.

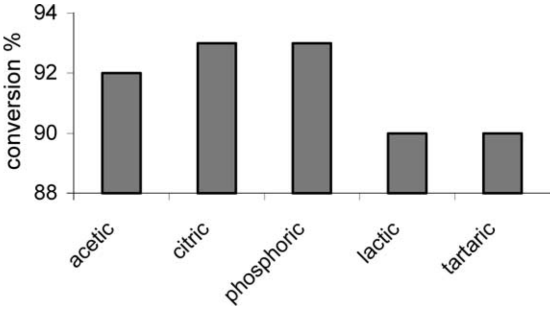

Figure 3. Effect of the acid used to prepare KCN/acid, in the conversion (determined by ^1H NMR) of benzaldehyde (**1b**) to mandelonitrile (**2b**).

Since cinnamaldehyde seems to be the less accepted substrate for mamey oxynitrilase, we decided to do an experiment similar to the one described above, to determine the effect of the acid on the reactivity and enantioselectivity with this aldehyde. Acetic, citric, ascorbic and lactic acids were selected, the mixture was analyzed after 24 h of reaction at 4 °C and the results are shown in Figure 3. The enantiomeric excesses are similar in all the cases (approximately 50%), but the conversion is very low with acetic acid (8%) and practically the same with citric, ascorbic and lactic acids (approximately 25%). Due to the results obtained, we decided to continue our studies with mamey meal using diisopropyl ether and MTBE as solvents, and citric acid for the KCN/acid buffer solution.

2.3. Effect of the concentration of the citrates buffer solution

We evaluated the effect of the concentration of the citrate buffer solution,[13] on the preparation of cyanohydrin **2a** catalyzed by the defatted meal of mamey. The citrate buffer solution in concentrations of 0.02, 0.05, 0.1, 0.15 and 0.5 M (pH 5.0) was added in a ratio of 1% (v/v) to the solution of diisopropyl ether and HCN, then the meal and the aldehyde **1a** were added, after 6 and 12 h at 4 °C the reaction mixture was analyzed.

The enantiomeric excess of the product obtained in all the cases was very high (99%), so the enantioselectivity of the reaction was not affected by the concentration of the buffer solution, but the degree of conversion showed a dependence of the concentration. From Figure 4 it is clear that there is an increase in the conversion as the solution becomes more concentrated until a maximum of activity of the mamey meal is reached, at a concentration of 0.1 M of the buffer solution, then the reactivity is decreased with more concentrated solutions (Fig. 5).

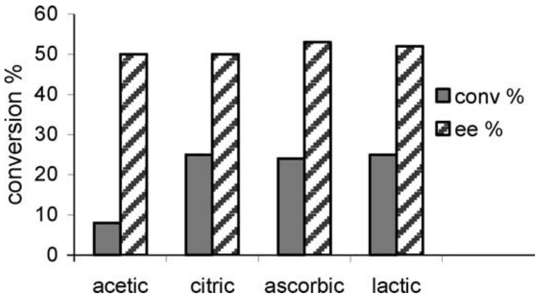

Figure 4. Effect of the acid used to prepare KCN/acid, in the conversion (determined by ^{1}H NMR) and enantiomeric excess (determined by HPLC using an OD Chiracel column) of aldehyde (**1a**) to cyanohydrin (**2a**).

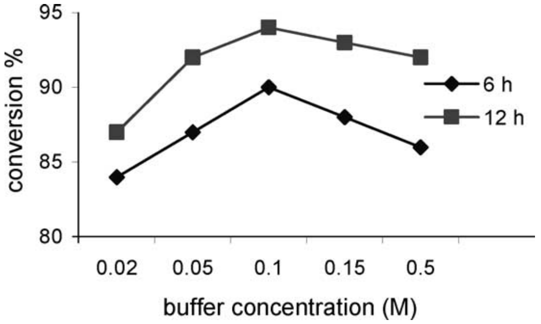

Figure 5. Effect of buffer concentration (M) on the conversion (determined by ^{1}H NMR) of benzaldehyde (**1b**) to mandelonitrile (**2b**), biocatalyzed by mamey meal.

2.4. Effect of the water content of the biphasic system

It is well known that some quantity of water is necessary for the optimum activity of oxynitrilases, but there is not an agreement on the minimum and maximum water content needed for these enzymes to work. It is also known that at high water content the undesirable chemical addition of HCN to aldehydes is a favored reaction, resulting in a lower enantiomeric excess. Some examples are the following: Straathof[15] found that a 50% aqueous phase is the optimum for (*R*)-mandelonitrile production; the same author determined that a 15% aqueous phase is the optimum for (*R*)-4-hydroxymandelonitrile production,[16] Kanerva determined 13.5% as optimum for the resolution of 2-hydroxy-2-phenylpropanenitrile,[17] and Lin reported that higher yields and enantioselectivity in the preparation of several cyanohydrins are obtained in microaqueous medium (0.32%, v/v of water content).[12,14] All these studies were done using almond meal.

Since there are no data about the effect of water content on the oxynitrilase activity of mamey meal, we carried out the following experiment. To a solution of HCN in diisopropyl ether or MTBE were added different quantities of citrate buffer solution, then the mamey meal, followed by cinnamaldehyde (**1a**), the reaction was monitored at 24, 48, 72, and 96 h, at 4 °C.

From the results in Figure 6 it can be stated that the conversion is higher when the reaction is carried out in diisopropyl ether than in MTBE. It is also observed that the conversion increases proportionally with the increase in water content, in such way that after 48 h the maximum conversion (54%) is obtained with 90% of aqueous phase in diisopropyl ether (f), but it is noticeable that after that time the cyanohydrin began to transform into the aldehyde. Waiting longer than 48 h for a higher conversion degree will decrease cyanohydrin concentration, due to the reversibility of the reaction.

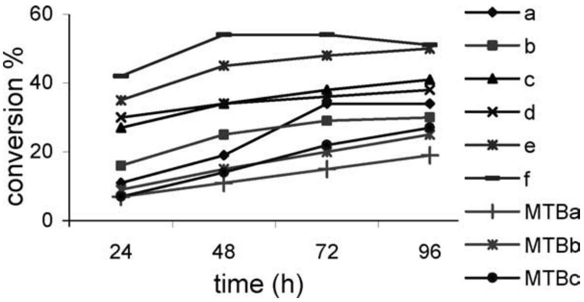

Figure 6. Effect of water content on conversion (determined by ^{1}H NMR) % of aldehyde (**1a**) to cyanohydrin (**2a**) catalyzed by mamey meal. Water content in diisopropyl ether, a: microaqueous medium, b: 1%, c: 5%, d: 10%, e: 50%, f: 90%. Water content in methyl *tert*-butyl ether, MTBa: microaqueous medium, MTBb: 1%, MTBc: 5%.

In the rest of the reactions, an important increase in conversion from the beginning of the reaction until approximately 48 h is observed, but from 72 to 96 h the change in the degree of conversion is minimal, and in some cases there is no change at all (a and b). It is possible that around this time the biocatalyzed reaction has reached an equilibrium. From Figure 7 some interesting facts can be observed: with the highest water content (f, 90% of water in diisopropyl ether) and the lowest (a, microaqueous medium in diisopropyl ether and MTBa, microaqueous medium in methyl *tert*-butyl ether), the enantiomeric excess have the lowest values. In Figure 7 it is noticeable that in almost all the cases the enantiomeric excess reaches a maximum at 48 h and after that time the enantiomeric excess begins to decrease. This can be explained by an important competition between the biocatalyzed and chemical addition of HCN to the aldehyde. In addition, the cyanohydrin began to decompose and racemize, this behavior is similar to that observed for conversion (Fig. 6).

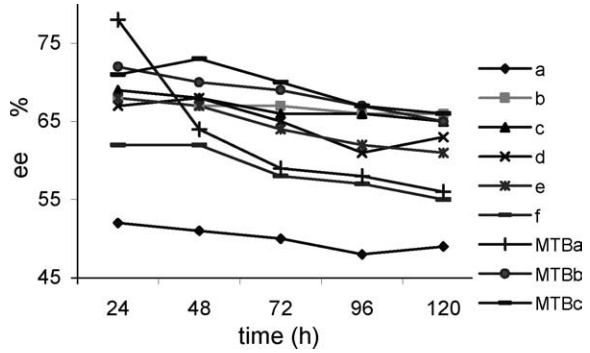

Figure 7. Effect of water content on enantiomeric excess % (determined by HPLC using an OD Chiracel column) of cyanohydrin (**2a**) catalyzed by mamey meal. Water content in diisopropyl ether, a: microaqueous medium, b: 1%, c: 5%, d: 10%, e: 50%, f: 90%. Water content in methyl *tert*-butyl ether, MTBa: microaqueous medium, MTBb: 1%, MTBc: 5%.

The highest enantiopurity is reached after 48 h with MTBE (water content of 5 and 10%, v/v) and diisopropyl ether at water content of 5 and 10%. In the case of diisopropyl ether it is very interesting that with 1, 5, 10 and 50% of water content, enantiomeric excess are very similar at 48 h, but after that time the decrease is much more significant with 10 and 50% of water. This could be because of the competition between biocatalyzed and chemical reaction, which results in racemization of the reaction.

From these results the best conditions for the addition of HCN to cinnamaldehyde biocatalyzed by mamey meal were: the use of diisopropyl ether in a biphasic system, with a water content of 5%, with reaction times not longer than 48 h, at 4 °C.

2.5. Preparation of cyanohydrins from α,β-unsaturated aldehydes

Due to their synthetic utility and because they are precursors of insecticides, we explored the effect of water content on the addition of HCN to α,β-unsaturated aldehydes (**1c**, **1d**, **1e**), catalyzed by mamey meal. According to results from previous experiments, the solvent selected was diisopropyl ether, KCN/citric acid buffer (pH 5.0, 1 N) as HCN source, for the aqueous phase citrate buffer (pH 5.0, 0.1 M), the amounts of the last one were: microaqueous medium (approximately 0.32%), 1, 5, 10 and 50%, v/v. After 48 h at 4 °C the reaction mixture was analyzed, the results are shown in Table 1.

From the results in Table 1, it can be observed that the reactivity and enantioselectivity of the addition of HCN to aldehydes **1c**, **1d**, **1e** catalyzed by mamey meal, increases constantly with the increase in water content in the reaction medium. These facts are in agreement with that shown in Figures 6 and 7. The best results were obtained with 50% water content in the reaction, however, the disadvantage of employing 50% of water is that the recovery of the product becomes difficult, so it is better to use 10% of water since the results obtained were similar to those with 50%.

Although all the aldehydes investigated were α,β-unsaturated (**1a**, **1c**, **1d**, **1e**), except for benzaldehyde (**1b**), the reactivity towards addition of HCN catalyzed by mamey was very different. With cinnamaldehyde (**1a**), the maximum conversion and enantioselectivity were not so high, 54 and 75%, respectively (Figs. 6 and 7). Something similar was observed with 1,4-hexadienal (**1e**), the highest conversion was 69% (Table 1) and enantioselectivity 79% (Table 1).

2-Methyl-2-pentenal (**1c**) and *trans*-2-hexenal (**1d**) were found to be more reactive under the reaction conditions tested and had better enantiopurities, for **2c** 84 and 90%, respectively (Table 1), for **2d** 88 and 98%, respectively (Table 1). For some reason it seems that substrates having more extended conjugated systems become less reactive to the addition of HCN catalyzed by mamey meal, this can explain the less favorable results with substrates **1a** and **1e**. It is worth mentioning that 1,4-addition was not observed in these experiments.

3. Conclusions

From the results obtained we determined that mamey meal is an (*R*)-oxynitrilase source which displays its best biocatalytic activity in the addition of HCN to aldehydes in the following reaction conditions, diisopropyl ether as the solvent, citric acid for the preparation of KCN/acid buffer as HCN source, and a water content of 10% in a biphasic system. Benzaldehyde (**1b**), 2-methyl-2-pentenal (**1c**) and *trans*-2-hexenal (**1d**) proved to be more reactive under the mentioned reaction conditions, whereas cinnamaldehyde (**1a**) and 1,4-hexadienal (**1e**) showed less reactivity.

Table 1. Effect of water content on conversion and enantiomeric excess of cyanohydrins **2c**, **2d** and **2e** of the reaction catalyzed by mamey meal

	Cyanohydrin **2c**		Cyanohydrin **2d**		Cyanohydrin **2e**	
% of water content (v/v)	Conv. %[a]	ee %[b]	Conv. %[a]	ee %[b]	Conv. %[a]	ee %[b]
Microaqueous medium	68	70	37	87	27	52
1	74	77	56	94	51	79
5	77	88	67	98	50	72
10	84	89	62	92	63	70
50	83	90	88	95	69	75

[a] Determined by ^{1}H NMR.

[b] Determined by HPLC using an OD Chiracel column.

4. Experimental

4.1. Chemicals and sources of enzymes

Ripe mamey (*Pouteria sapota*) seeds were obtained from a fresh fruit purchased in a local grocery store. The mamey seeds were ground and washed three times with acetone and once with diisopropyl ether, after filtration by suction the powder was air dried and stored at 4 °C. Commercial aldehydes were purchased from Aldrich.

Optical rotations were measured in a Perkin–Elmer polarimeter model 341. Enantiomeric excesses were determined by HPLC analyses, using a Chiracel OD column using hexanes–isopropanol as eluent in a Hewlett–Packard 1050 series, equipped with a diode array detector. Conversion percentages were determined by NMR. ^{1}H NMR spectra were recorded on a Varian at 400 MHz using $CDCl_3$ as a solvent and TMS as internal reference.

4.2. General procedure for enzymatic reactions

HCN (1.5 equiv) was extracted from a buffer solution (KCN/citric acid, 1 M, pH 5.0) with the proper solvent (2× 2.5 mL). To this solution was added the citrate buffer solution (0.1 M, pH 5.0); the quantity added to the reaction medium depended on the experiment, then 200 mg of mamey defatted meal were added and stirred for 10 min at room temperature, after this 1 mmol of the aldehyde was added. The reaction mixture was stirred for the necessary time at 4 °C, filtered, dried over sodium sulfate and the solvent was evaporated to dryness. Enantiomeric excess was determined by HPLC using a Chiracel OD column with hexanes–isopropyl alcohol as eluent and conversion percentage was determined by ^{1}H NMR, of the crude product.

4.2.1. (*R*)-(+)-2-Hydroxy-4-phenyl-3*E*-butenenitrile (2a). Conversion 54%, $[\alpha]_D^{22}=+17$ (*c* 1, CH_2Cl_2), ee 72%; lit.:[12] $[\alpha]_D^{13}=+26.1$ (*c* 0.78, $CHCl_3$), ee 69.3%. ^{1}H NMR ($CDCl_3$): $\delta=5.18$ (d, $J=6.0$ Hz, 1H), 6.26 (dd, $J=16.0$, 6.0 Hz, 1H), 6.9 (d, $J=6.0$ Hz, 1H), 7.35 (m, 5H).

4.2.2. (*R*)-(+)-2-Hydroxybenzeneacetonitrile (2b). Conversion 95%, $[\alpha]_D^{22}=+46$ (*c* 1.9, CH_2Cl_2); lit.:[12] $[\alpha]_D^{20}=+47.5$ (*c* 1.89, $CHCl_3$), ee >99%. ^{1}H NMR ($CDCl_3$): $\delta=5.52$ (s, 1H), 7.49 (m, 5H).

4.2.3. (*R*)-(−)-2-Hydroxy-3-methyl-3*E*-hexanenitrile (2c). Conversion 84%, $[\alpha]_D^{22}=-72$ (*c* 3.9, CH_2Cl_2), ee 89%. ^{1}H NMR ($CDCl_3$): $\delta=1.0$ (t, $J=7.6$ Hz, 3H), 1.78 (s, 3H), 2.09 (m, 2H), 3.42 (br, 1H), 4.83 (s, 1H), 5.72 (t, $J=7.6$ Hz, 1H). Configuration was assigned on the basis of mamey meal selectivity, and of elution order of the enantiomers on chiracel OD column using hexanes–ispropanol (95:5) as eluent, (*R*)-enantiomer $t_r=11.37$ min, (*S*)-enantiomer $t_r=11.036$ min.

4.2.4. (*R*)-(−)-2-Hydroxy-3*E*-heptanenitrile (2d). Conversion 88%, $[\alpha]_D^{22}=-60$ (*c* 3.16, CH_2Cl_2), ee 95%; lit.:[9] $[\alpha]_D^{20}=+20.3$ (*c* 0.3, $CHCl_3$), (*S*)-enantiomer, ee 95%. ^{1}H NMR ($CDCl_3$): $\delta=0.92$ (t, $J=7.6$ Hz, 3H), 1.43 (m, 2H), 2.08 (m, 2H), 3.72 (br, 1H), 4.94 (d, $J=6.4$ Hz, 1H), 5.59 (dd, $J=15.5$, 6.4 Hz, 1H), 6.03 (m, 1H).

4.2.5. (*R*)-(−)-2-Hydroxy-3*E*,5*E*-heptadienenitrile (2e). Conversion 79%, $[\alpha]_D^{22}=-52$ (*c* 3.85, CH_2Cl_2) ee 69%; lit.:[18] $[\alpha]_D^{23}=-24.9$ (*c* 1, $CHCl_3$), ee 96%. ^{1}H NMR ($CDCl_3$): $\delta=1.8$ (d, $J=6.45$ Hz, 3H), 3.87 (br, 1H), 4.99 (d, $J=6.0$ Hz, 1H), 5.62 (dd, $J=15.2$, 6.0 Hz, 1H), 5.91 (m, 1H), 6.06 (dd, $J=15.0$, 10.4 Hz, 1H), 6.46 (dd, $J=15.0$, 10.4 Hz, 1H).

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