Engineered dehydrogenase biocatalysts for non-natural amino acids: efficient isolation of the D-enantiomer from racemic mixtures

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With a view to their use in the kinetic resolution of racemic non-natural amino acids, five variants of the enzyme L-phenylalanine dehydrogenase, the wild-type enzyme from *Bacillus sphaericus* and four active-site mutants, have been tested with a range of amino acids. In each case, the rates of reaction with 0.2 mM L-amino acid and with the racemic mixture at 0.4 mM were compared, so that the starting concentration of the active substrate was kept constant. Although the D-amino acids are not substrates, they were inhibitory in all cases. The extent of inhibition, however, varied greatly from compound to compound and among the mutants. With the N145L mutant and DL 4-O-Me-Phe, the equimolar D-enantiomer gave 83.2% inhibition, and with the wild-type enzyme there was 86.7% inhibition with racemic norleucine. By contrast, with these same substrates the N145V mutant showed less than 9% and 24% inhibition respectively. The N145A mutant was selected for use with DL-4-Cl-Phe. The pH was decreased from the enzyme's optimum of 10.4 to 9.5 to minimise breakdown of the coenzyme NAD^+, and the coenzyme was recycled by molecular oxygen with the assistance of a commercial diaphorase. Reaction on a 200 μmole scale in 20 ml ethanolamine HCl buffer, pH 9.5, with 25 μg N145A enzyme and 100 μg diaphorase, was monitored by chiral HPLC. The L-isomer was removed to an extent of >99% after 40 h, with the D-isomer peak undiminished. The pure D-isomer was isolated from the reaction mixture in 85% overall yield after ion-exchange chromatography.

Introduction

The synthesis of amino acids in pure L- and D- forms is a major interest in the pharmaceutical field. Amino acids can be used as chiral synthons in multistep reactions, being easy to derivatise at the carboxylic moiety as well as at the amino group. The chiral amino acid can then induce chirality in a subsequent reaction with a non-chiral substrate, conferring a specific spatial geometry on the new molecule, and, where new stereocentres are introduced, their configuration can be influenced.[1] Natural L-amino acids are easy to obtain from biological sources, but the production of the corresponding D- forms is more challenging. Non-natural amino acids (*e.g. tert*-leucine, phenylglycine) are in demand for the synthesis of novel drugs,[2,3] and, through combinatorial chemistry, libraries of new compounds may be created. In this last context, neither enantiomer is usually readily available, and the resolution of racemic mixtures[4] or asymmetric synthesis[5] are the only options.

Enzymatic approaches are limited by the lack of enzymes that would naturally accept non-natural amino acids as substrates. However, a few industrial processes have optimised the biocatalytic synthesis of optically pure non-natural amino acids, such as the method for L- (or D-) *tert*-leucine using leucine dehydrogenase,[6,7] which shows low but useable activity with the non-natural substrate. A kinetic resolution method for preparing D-amino acids has been described,[8] utilising the enantioselectivity of penicillin-G acylase in the cleavage of N-phenacetyl amino acid derivatives. Its application has so far been reported only for biological amino acids, and apart from the requirement for prior derivatisation, it is also relevant that very variable ee values were reported, depending on the target amino acid chosen. An alternative enzymatic route to D-amino acids is offered by the recently reported D-specific amino acid dehydrogenase.[9]

Our strategy is focused on the creation of new biocatalysts through site-directed mutagenesis to shift and broaden the substrate specificity of natural enzymes. We have previously reported the successful application of mutants of phenylalanine dehydrogenase from *Bacillus sphaericus* in the synthesis of non-natural L-amino acids starting from the corresponding 2-oxo-acids.[10,11] Given the stringent enantioselectivity of PheDHs, these catalysts may also be used for the efficient kinetic resolution of the D- and L- enantiomers. Without any requirement for derivatisation, a single biocatalyst thus offers a route to both enantiomers at high ee. In this paper, we report the selectivity of four PheDH mutants in dealing with racemic mixtures of non-natural amino acids and an example of kinetic resolution involving recycling of the cofactor.

Results and discussion

Tables 1 and 2 present data under standard assay conditions for the oxidative deamination reaction of wild-type phenylalanine dehydrogenase (WT PheDH) and mutated variants with a series of derivatives of L-phenylalanine (Phe) substituted at position 4 (**1–4** and the corresponding D-enantiomers (**7–10**), and aliphatic amino acids (L-norleucine **5**, L-cyclohexylalanine **6**, and the racemate

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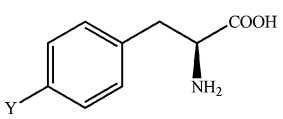
Table 1 Activity of WT PheDH and mutants with 0.2 mM amino acid substrate. Assays were performed at pH 10.4 in Gly–NaOH buffer by following the production of NADH spectrophotometrically at 340 nm. The table shows specific activities (in brackets) with pure L-amino acids, and also relative activities (first number) as a percentage of the activity with L-Phe in each case

Amino acids	WT	N145A	N145L	N145V	N145I
L-Phe	100 (7.5 U mg^{-1})	100 (35 U mg^{-1})	100 (17.5 U mg^{-1})	100 (20 U mg^{-1})	100 (16.4 U mg^{-1})
4-F-L-Phe (**1**)	52 (3.9 U mg^{-1})	129 (45.2 U mg^{-1})	172 (30.1 U mg^{-1})	265 (53 U mg^{-1})	168 (27.6 U mg^{-1})
4-Cl-L-Phe (**2**)	11 (0.8 U mg^{-1})	123 (43.1 U mg^{-1})	204 (35.7 U mg^{-1})	112 (22.4 U mg^{-1})	130 (21.3 U mg^{-1})
4-Me-L-Phe (**3**)	5 (0.4 U mg^{-1})	82 (28.7 U mg^{-1})	124 (21.7 U mg^{-1})	67 (13.4 U mg^{-1})	69 (11.3 U mg^{-1})
4-OMe-L-Phe (**4**)	3 (0.2 U mg^{-1})	105 (36.8 U mg^{-1})	20 (3.5 U mg^{-1})	146 (29.2 U mg^{-1})	14 (2.3 U mg^{-1})
L-Norleucine (**5**)	5.9 (0.4 U mg^{-1})	9.4 (3.3 U mg^{-1})	20.6 (3.6 U mg^{-1})	25 (5 U mg^{-1})	14.6 (2.4 U mg^{-1})
L-Cyclohexyl-alanine (**6**)	9.8 (0.7 U mg^{-1})	17.3 (6.1 U mg^{-1})	13.7 (2.4 U mg^{-1})	32.5 (6.5 U mg^{-1})	12.2 (2 U mg^{-1})

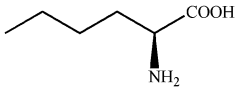
Table 2 Activity of WT PheDH and mutants with racemic amino acids. Each amino acid was present at 0.4 mM concentration *i.e.* 0.2 mM of each enantiomer. The first number in each case gives the activity as a percentage of that with the corresponding pure L-amino acid at 0.2 mM (Table 1). Reactions were performed at pH 10.4 in Gly–NaOH buffer by following the production of NADH at 340 nm spectrophotometrically. The racemic form of **6** could not be obtained and therefore it has not been included in the table

Amino acids	WT	N145A	N145L	N145V	N145I
DL-Phe	77 (5.8 U mg^{-1})	41 (14.4 U mg^{-1})	52 (11.2 U mg^{-1})	64 (12.8 U mg^{-1})	39 (6.4 U mg^{-1})
4-F-DL-Phe (**7**)	77 (3 U mg^{-1})	60 (27.1 U mg^{-1})	54 (16.3 U mg^{-1})	52 (27.6 U mg^{-1})	52 (14.4 U mg^{-1})
4-Cl-DL-Phe (**8**)	83 (0.7 U mg^{-1})	50 (21.6 U mg^{-1})	29 (10.4 U mg^{-1})	43 (9.6 U mg^{-1})	26 (5.3 U mg^{-1})
4-Me-DL-Phe (**9**)	66 (0.3 U mg^{-1})	70 (21.1 U mg^{-1})	31 (6.7 U mg^{-1})	54 (7.2 U mg^{-1})	36 (4.1 U mg^{-1})
4-OMe-DL-Phe (**10**)	95 (0.2 U mg^{-1})	65 (23.9 U mg^{-1})	17 (0.6 U mg^{-1})	91 (26.6 U mg^{-1})	60 (1.4 U mg^{-1})
DL-Norleucine (**11**)	13.3 (0.1 U mg^{-1})	58 (1.9 U mg^{-1})	72.2 (2.6 U mg^{-1})	76.7 (3.8 U mg^{-1})	68.3 (1.6 U mg^{-1})

DL-norleucine **11**). These reactions are exceptionally sensitive to pH and the experiments are strictly performed at pH 10.4 and at 25 °C. For each of the amino acids, an activity of at least 5 U mg^{-1} (L-norleucine with N145V) was measured with one or more of the mutated enzymes, and for several substrates activities up to 10-fold higher than this were obtained (*e.g.* 53 U mg^{-1} for **1** and 36.8 U mg^{-1} for **4**). Owing to the low concentration of the substrates (0.2 mM in case of pure L-amino acids and 0.4 mM for the racemic mixtures), consistent through the tables and imposed by the low water solubility of some of the amino acids, the measured activities are certainly sub-optimal in some cases. For example, the specific activity of WT PheDH with L-phenylalanine under these conditions is only 7.5 U mg^{-1}, much lower than that of the mutants, but this is explained by the much higher K_m of WT PheDH for L-phenylalanine,[11] and clearly, for this particular enzyme protein, much higher activity would be achievable by increasing the substrate concentrations. It interesting to note, however, that our WT enzyme behaves as expected with L-norleucine, for example, even at this low concentration of substrate; the retained activity of 5.9%, compared to L-phenylalanine at the same concentration, correlates quite well with that reported by Asano *et al.*[12] whereby the same enzyme shows a retention of activity of 3.9% at 10 mM concentration.



Y= F **(1)**
Cl **(2)**
Me **(3)**
OMe **(5)**



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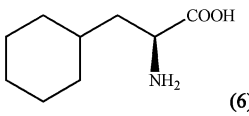

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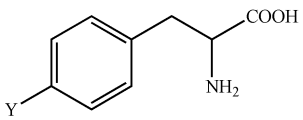
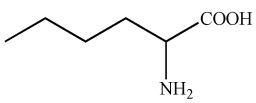
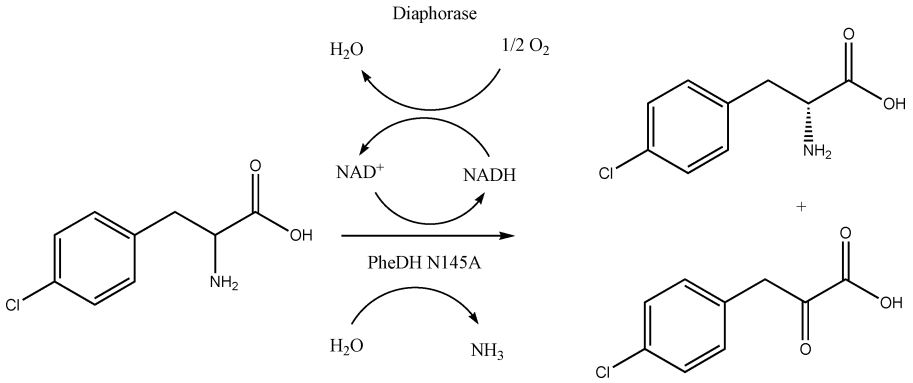


Y= F **(7)**
Cl **(8)**
Me **(9)**
OMe **(10)**



(11)

The data in Table 2, reporting reaction rates measured with the amino acids added at double the concentration but as racemic mixtures (0.2 mM each enantiomer), clearly show that the D-enantiomer is invariably inhibitory (all the relative activities are referred to the figure for the corresponding pure L-amino acid), in some cases quite potently so. Thus, for example, with 0.2 mM L-4-OMe-Phe and N145L, the rate with an equal mixture of the D-enantiomer is 6 times lower than the rate with the pure L-form, and the unmutated enzyme (WT) shows over 7-fold inhibition by the D-enantiomer of norleucine. This is an important factor to consider in any kinetic resolution, since, as the reaction proceeds, competitive inhibition inevitably becomes more severe. For each compound tested, the range of inhibition seen with the five different biocatalysts was considerable. Thus with 4-Cl-Phe, the inhibition ranged from 17% (WT) to 74% (N145I) and for 4-OMe-Phe from 4.8% (WT) to 83.2% (N145L). With Phe and with nearly all the 4-substituted analogues tested, WT PheDH stands out as showing the least inhibition by the D-enantiomer (with the 4-Me analogue, both N145A (30.3%) and WT PheDH (34%) show much less inhibition than the other mutants). Thus the unmutated enzyme shows the least tendency to accept the D-enantiomer, even as an unreactive inhibitory ligand. This pattern is sharply reversed in the case of the aliphatic substrate, for which



Scheme 1 The DL-4-Cl-phenylalanine is subjected to enzymatic deamination. The L-enantiomer is oxidised to the corresponding oxo-acid while the D-enantiomer does not react. The NAD^+ cofactor is reduced to NADH in this process and is reoxidised by molecular oxygen in a coupled reaction catalysed by a commercial diaphorase (from Enzolve Technologies, Dublin).

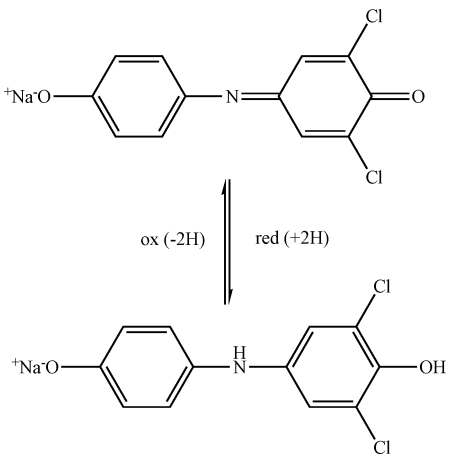
WT PheDH shows much higher inhibition by the unreactive enantiomer than any of the mutant enzymes. This contrasting pattern of behaviour in WT PheDH presumably reflects the fact that it has been evolutionarily perfected for a perfect fit to the aromatic substrate. PheDH from *B. sphaericus*, either in its wild-type form or generated mutants, has not been previously tested either with racemic amino acids, or with pure D-enantiomers. However, Asano *et al.* reported a study on PheDH from *B. badius* (82% similarity with our enzyme),[13] in which they tested the enzyme with a series of substrates and compared activities with L-Phe as a standard. *B. badius* PheDH retained 34% activity with DL-4-F-phenylalanine (**7**), in very good agreement with our results where the WT retains a 40% activity with **7** when compared with L-Phe.

Synthesis of D-4-Cl-phenylalanine

To establish whether these biocatalysts could be used in the racemic resolution of amino acids, one substrate among those tested was selected as an example for further investigations (compound **8**). Using the mutant N145A PheDH, chosen both for its higher activity with that substrate and relatively moderate inhibition (activity of 21.4 U mg^{-1} even in the presence of the equimolar D-enantiomer), we optimised conditions for a successful preparation of D-4-Cl-phenylalanine starting from the cheaper DL-4-Cl-phenylalanine. In view of the expense of the cofactor NAD^+, a coupled recycling system was introduced (Scheme 1), allowing the use of catalytic amounts of this reactant.

Recycling systems reported in the literature mainly utilise NADH oxidases (NOX) and molecular oxygen to re-oxidise the cofactor,[7] yielding H_2O or H_2O_2. Unfortunately, the optimal pH range for these enzymes in terms of both stability and optimal activity is between pH 5 and 7, quite unsuitable for our experiment in view of the high pH optimum of PheDH. The diaphorase used here is much more stable at alkaline pH values.

To maximise the concentration of dissolved oxygen, necessary for the reoxidation of NADH, the reaction was performed in a large vessel with constant orbital shaking. To further facilitate reaction with oxygen, 0.1 mg of 2,6-dichlorophenolindophenol (DCPIP) was added to the mixture as a mediator. DCPIP is a readily autoxidisable dye with an oxidoreduction characterised by colour change (blue and colourless respectively for oxidised and reduced) (Scheme 2).



Scheme 2 Oxidoreduction mechanism of 2,6-dichlorophenolindophenol.

A further aspect to consider was the instability of NAD^+ at high pH over extended periods. NAD^+ was therefore incubated at different pH values and periodically assayed spectrophotometrically with alcohol dehydrogenase from *Saccharomyces cerevisiae* (see Experimental). In view of the results (not shown), the pH was lowered from 10.4, found to be optimal for activity in the experiments reported above, to 9.5, thus considerably decreasing cofactor breakdown. The overall kinetic resolution was followed by chiral HPLC[10,11] with the pure enantiomers and 2-oxo-acid separately injected as standards. The reaction went to completion (99%) after 40 hours (Fig. 1) without any decline in the peak for the D-enantiomer, confirming that only the L-enantiomer is recognised by the enzyme as a substrate.

The pure D-4-Cl-phenylalanine was recovered from the crude reaction mixture by ion-exchange chromatography with a final yield of 85%.

A second experiment was carried out under the same conditions (substrate and enzyme concentrations) utilising WT PheDH as biocatalyst. It can be noted that, while the mutant N145A PheDH is certainly more active than the unmodified enzyme with the substrate DL-4-Cl-Phe (21.6 U mg^{-1} and 0.7 U mg^{-1} respectively), the WT PheDH is less inhibited by the D-amino acid (12.5%

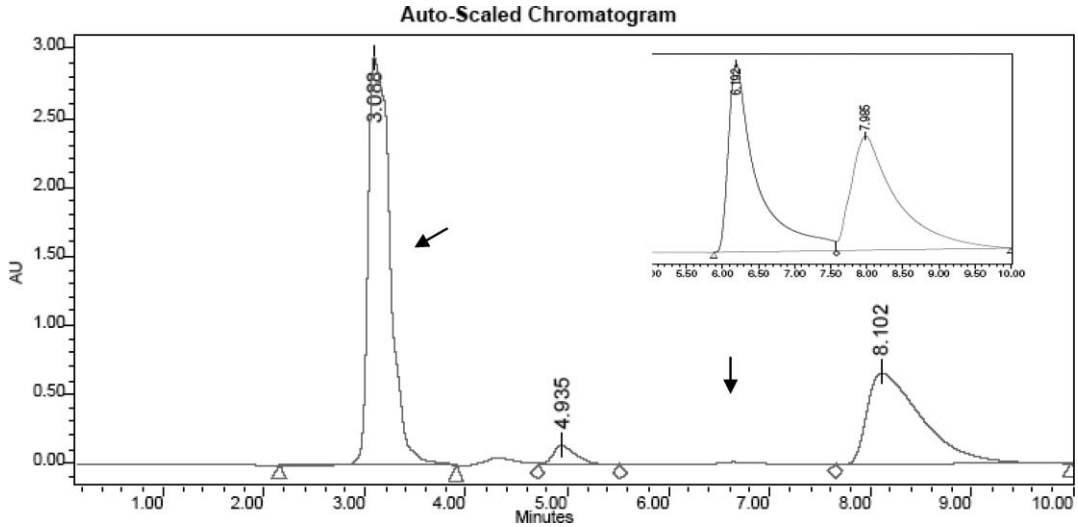


Fig. 1 Chromatogram of the crude reaction mixture after 40 hours. The oxoacid elutes after 3 min (arrow) and the peak at 8.1 min corresponds to the D-enantiomer. The L-enantiomer would be expected at ~6.7 min (arrow), as visible in the inset graph, which shows a close-up of the trace for the DL-starting material.

inhibition *versus* 50% for the mutant). The reaction progressed much more slowly, as expected, reaching only 35% conversion after 40 hours.

Conclusion

PheDH mutants have proved to be versatile biocatalysts able to accommodate non-natural substrates. The strict enantioselectivity of the unmutated enzyme with the natural substrate, Phe, has been fully retained, but even though the D-enantiomer is never a substrate for the biocatalyst, it can nevertheless be a strong inhibitor. The extent of inhibition (at a fixed substrate concentration) by the D-enantiomer varies across the substrates and mutants, making the optimal pairing of biocatalyst and substrate an important choice. With other enzymatic systems such as lipases and hydrolases, this phenomenon is less common: while product inhibition is well investigated, no direct inhibition studies of the less reactive enantiomer have been reported.[14] D-Amino acid inhibition is, on the other hand, a well exploited phenomenon with peptidases, and novel drugs aimed at inhibition of proteases often do contain D-amino acids.[15,16] In our opinion, the inhibition observed with PheDH mutants can be explained by the broadened substrate selectivity introduced with the mutations: the side chain of an amino acid and the α-carboxyl group are the key binding elements, but even though both enantiomers can be accommodated in the active pocket, correct orientation of the amino group and the proton to be removed, and therefore catalysis, only occurs with the L-isomer. The D-enantiomer effectively competes with the L- for the active site in a reversible manner, and, since it is not removed by the catalytic reaction, over time, the non-reactive molecule produces a somewhat similar effect to the (inhibitory) accumulation of a product. The results obtained here are generic, in the sense that the system could readily be employed in the same way with any suitable pairing of biocatalyst and racemic amino acid mixture *e.g.* from the results in Tables 1 and 2. The biocatalyst efficiently removes 100% of the L-enantiomer, leaving a pure peak of the D-stereoisomer. Although WT PheDH could in principle be used to catalyse the reaction with non-natural substrates, its much lower activity requires a correspondingly much higher ratio of enzyme to substrate for the reaction time to be comparable with that of the mutant. The fact that the WT enzyme seems to be less inhibited by the D-amino acids does not adequately compensate for the low activity. Finally, it can be envisaged that the separated oxoacid could be fed straight back into the reaction under the synthetic conditions described by Paradisi *et al.*[11] to regenerate the separate, pure L-enantiomer, thus completing the resolution of the racemate. The method described here appears to be both robust and versatile.

Experimental

All the amino acids were obtained from Bachem (Switzerland). Laboratory chemicals were reagent-grade and used without further purification. HPLC-grade solvents were used for HPLC separations. NAD^+ (grade II) was purchased from Roche. The wild-type PheDH and the mutant enzymes N145A/V/L/I in which the Asp residue in position 145 in the active pocket of the biocatalyst had been replaced with Ala/Val/Leu/*i*-Leu were over-expressed in *E. coli* TG1 cells and purified as described elsewhere.[17,18] The enzymes were stored as precipitates in 60% ammonium sulfate at 4 °C and desalted before use on PD-10 desalting columns from Amersham Biosciences.

Evaluation of enzyme activity—UV assays

Each amino acid (0.2 mM or 0.4 mM) was dissolved to form a component of a reaction mixture containing KCl (100 mM) and Gly–NaOH buffer (50 mM). The pH was adjusted to 10.4 by adding a suitable amount of NaOH. In view of the instability of NAD^+ at pH 10.4, the coenzyme was prepared as a stock solution in water (125 mM) and 20 μL was added to the reaction mixture immediately before starting each experiment (final concentration of NAD^+ 2.5 mM). A total volume of 1 mL of reaction mixture was incubated at 25 °C. The reaction was followed spectrophotometrically at 340 nm over 1 minute after

adding an appropriate amount of enzyme to achieve an optimally measurable reaction rate (between 0.01–0.03 Abs units per min). Each reaction was carried out in duplicate and the average rate is reported.

Resolution of 4-Cl-phenylalanine

The reaction was performed at pH 9.5 (ethanolamine–HCl, 20 mL, 50 mM) and at room temperature (15–20 °C) in a vessel with a large surface area and shaking or stirring to maximise the exchange with molecular oxygen. The reaction mixture was 5–10 mM in DL-Cl-phenylalanine (**9**), 1 mM in NAD^+, 100 mM in KCl and contained 0.1 mg DCPIP (dichlorophenol indophenol). 25 μg of the biocatalyst (N145A PheDH or WT PheDH) and 0.1 mg diaphorase were added to initiate the reaction, which was monitored by chiral HPLC (CHIROBIOTIC T column, eluent 80 : 20 MeOH–H_2O 1 mL min^{-1}) as reported previously.[10] Upon completion, the reaction mixture was adsorbed onto an ion exchange resin, Dowex 50WX8-200. The resin was washed with distilled water, and eluted with 25% NH_4OH to give D-4-Cl-phenylalanine (78% 15.6 mg). ^{1}H NMR (500 MHz, D_2O, NaOD) δ ppm 2.66 (dd J = 13.56, 7.28 Hz, 1H), 2.79 (dd J = 13.53, 5.50 Hz, 1H), 3.31 (t, J = 6.38 Hz 1H), 7.06 (d, J = 8.12 Hz, 2Ar-H), 7.19 (d, J = 8.14 Hz, 2Ar-H).

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