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The dynamic kinetic resolution of 3-oxo-4-phenyl-β-lactam by recombinant *E. coli* overexpressing yeast reductase Ara1p

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Abstract—Using a recombinant *E. coli* strain overexpressing yeast reductase Ara1p, we reduced racemic 3-oxo-4-phenyl-β-lactam to *cis*-(3*S*,4*R*)-3-hydroxy-4-phenyl-β-lactam as a single enantiopure product. The dynamic kinetic resolution occurred over the course of fermentation at pH 7. Under the same conditions, 3-oxo-4-(2-thiophenyl)-β-lactam **4** and 3-oxo-4-(2-furyl)-β-lactam **5** were not resolved.

1. Introduction

3-Hydroxy-β-lactams substituted at the 4-position are convenient precursors of β-amino-α-hydroxy esters, the key fragments of biologically important compounds such as α-hydroxy-β-amino acids,[1,2] peptidic enzyme inhibitors,[3] and pharmaceuticals, in particular the anti-cancer drug Taxol.[4] Enantiomerically pure 3-hydroxy-β-lactams have been prepared via asymmetric synthesis employing either a combination of chiral imine and achiral ketenes,[5,6] achiral imines and chiral ketenes,[7,8] or the direct cyclization of ketene–imine in the presence of a chiral catalyst.[9] Biocatalytic approaches include lipase-catalyzed resolution[10–12] and baker's yeast reduction.[13]

The biological activity of a compound often depends on the correct absolute configuration of all the stereogenic centres in the molecule. Constructing a molecule with only two stereogenic centres of the required absolute configuration can be an arduous undertaking. There are two main access routes to enantiomerically pure or enriched compounds: the resolution of racemates and the asymmetrization of prochiral or *meso*-compounds. Biocatalysts have been successfully used in both strategies.[14,15] The kinetic resolution of a racemic mixture is a well-travelled route to optically active compounds; its major disadvantage is that the maximum yield of the desired enantiomer is theoretically limited to 50%. Efforts to overcome this limitation and to obtain compounds with high enantiomeric purity, as well as high yields, have led to the growing interest in dynamic kinetic resolution (DKR), a method that combines enzymatic kinetic resolution with the in situ racemization of the substrate.[16]

The use of biocatalysis in the preparation of enantiopure compounds is an attractive alternative to other chemical methods. Over the years, the enzyme-promoted kinetic resolution of racemates has been extensively explored while the number of reported chemoenzymatic DKRs has been increased.[14–16] Recently, this approach has been successfully applied in microbial reductions[17,18] and oxidations.[19]

Baker's yeast (*Saccharomyces cerevisiae*) is a rich source of reducing enzymes. In addition to metabolic enzymes whose function and physiological substrates are already known, there is a large contingent of enzymes that reduce exogenous ketones. Although several 3-oxo-ketone reductases had been purified and characterized,[20] it was the availability of the yeast genome sequence that allowed the identification of 49 putative reductases.[21] A number of these proteins were found to correspond to the already known reductases; many others, however, open the door to further studies.[21] Recently, 19 of the alleged reductases have been overexpressed in *Escherichia coli* (*E. coli*) as gluthatione (*S*)-transferase fusion proteins, and their ability as enantioselective reducing agents for a series of β-ketoesters has been

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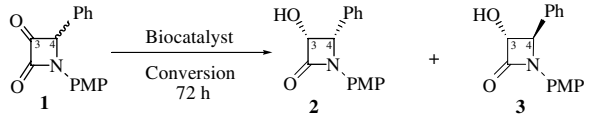
investigated.[22,23] The results of this study have shown that several of these enzymes are highly enantioselective and diastereoselective vis-à-vis 2-substituted, non-cyclic β-ketoesters.[22,23]

2. Results and discussion

We screened several yeast reductases for their ability to reduce 3-keto-β-lactams to the corresponding alcohols and identified several that are suitable for development as reducing agents for these substrates.[24] Among the yeast enzymes which, under screening conditions, reduced 3-oxo-4-phenyl-β-lactam **1** to the corresponding alcohol, reductase Ara1p was found to be efficient and was selected for further studies. The construction and evaluation of the recombinant *E. coli* strain BL21(DE3)/Ara1p (*E. coli*/Ara1p) have already been reported.[25–27] The screening of substrate **1** with *E. coli*/Ara1p gave a mixture of (3*S*,4*R*)- and (3*S*,4*S*)-diastereomers (Scheme 1). Interestingly, these are isomers that are either only minor products or that are not observed in baker's yeast-catalyzed reductions of **1**, thus suggesting that Ara1p is not efficiently expressed under these conditions.



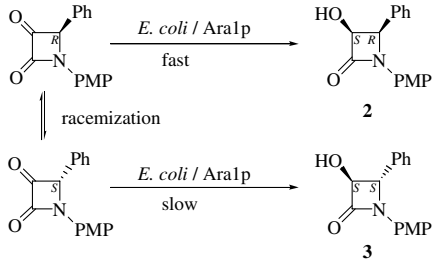
Baker's yeast	>98%	(3*R*,4*S*) 55% ee	1.6 : 1	(3*R*,4*R*) > 99% ee
E. coli / Ara1p (shake-flask)	81%	(3*S*,4*R*) >99% ee	1.4 : 1	(3*S*,4*S*) > 99% ee
E. coli / Ara1p (fermentation)	100%	(3*S*,4*R*) >98% ee		trace

PMP = *p*-methoxyphenyl

Scheme 1.

Since the screening protocol performed in a shake flask is not optimized for maximum conversion, we followed up with a biotransformation in a small fermenter with a constant supply of oxygen and glucose with the pH maintained at 7 throughout the reaction. To our surprise, under these conditions, substrate **1** was completely converted to a single product identified as alcohol (3*S*,4*R*)-**2**. Only traces of the diastereomer (3*S*,4*S*)-**2** could be detected on chiral phase HPLC. The alcohol (3*S*,4*R*)-**2** was isolated in 65% yield and was confirmed by HPLC to be >98% enantiopure. These results indicate that, under fermentation conditions at pH 7, enzymatic reduction is accompanied by in situ racemization of the substrate leading to efficient DKR of substrate **1** (Scheme 2).

Because the active site of an enzyme is chiral, one enantiomer of the substrate is converted to the product at a higher rate than the other. This leads to kinetic resolution. In an ideal case, when conversion of one enantiomer is significantly faster than that of the other, we can obtain an enantiopure product when the reaction



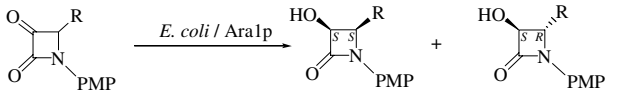
Scheme 2.

is stopped at 50% conversion. When kinetic resolution is accompanied by in situ racemization of the substrate, the slow-reacting enantiomer is continually converted into the fast-reacting enantiomer, which is transformed into a product. Thus, theoretically, DKR is capable of giving the desired enantiomer in 100% yield from the racemic substrate. It has been stated that in order to achieve efficient DKR, the ratio of rate constants for fast and slow reaction (k_F/k_S) should be more than 30 while the rate of racemization of the less reactive enantiomer has to be equal or greater than the rate of conversion of the more reactive enantiomer.[28] Our experiments show that in the Ara1p-catalyzed reduction of **1** under fermentation conditions at constant pH 7, DKR takes place. On the other hand, no significant DKR is observed when this transformation is performed in a shake flask where the levels of oxygen and glucose deviate from the optimal values and pH drops from 7 to approximately 5 as the reaction progresses. Thus, Ara1p-catalyzed reduction of substrate **1** meets the criteria only under fermentation conditions, while the fine-tuning of reaction conditions is necessary to achieve DKR in the reduction of substrate **1**.

To corroborate the racemization of substrate, we monitored, by chiral phase HPLC, the fate of enantiopure (*S*)-**1** stirred in the reaction medium alone and in the reaction medium containing host *E. coli* strain BL21(DE3). The latter experiment was carried out to ascertain that the racemization is not caused by any racemase present in *E. coli*. In both experiments, some racemization was detected (2% after 1 h and 12% after 3 h). However, it was accompanied by depletion of the starting material, probably through hydrolysis and decomposition. After 12 h, all of ketone **1** disappeared. These results explain why despite apparent '100% conversion' low isolated yields have been observed in shake-flask experiments with several reductases. When a reaction is carried out in a shake flask, the quantity of the available enzyme is lower than that under the optimized fermenter conditions. At the same time, the enzyme activity may also be diminished,[†] and racemization of **1** is slower because the pH of the mixture is decreasing from 7 to approximately 5. Under these conditions, DKR is not effective and the yields of alcohols are low because of the loss of starting material through decomposition.

[†] Several reductases studied gave the best conversion at pH 8.

The stringent requirements for a successful DKR are well illustrated when *E. coli*/Ara1p-catalyzed reduction of **1** is compared with the corresponding reductions of the two closely related compounds: 4-(2-thiophenyl)-β-lactam **4**, and 4-(2-furyl)-β-lactam **7**. Here, the results from screening are very similar to those obtained during fermentation (Scheme 3). Under fermentation conditions, both reductions proceeded rapidly (100% conversion in 3 h); however, as the rates of conversion of the (*R*)- and (*S*)-enantiomers were almost identical, no DKR was achieved.



shake-flask	**4**	72 h	93% (3*S*,4*S*) >99% ee	1 : 1	(3*S*,4*R*) > 99% ee
	7	72 h	98% (3*S*,4*S*) >99% ee	1 : 1	(3*S*,4*R*) > 99% ee
fermentation	**4**	3 h	100% (3*S*,4*S*) >98% ee	1 : 1	(3*S*,4*R*) > 99% ee
	7	3 h	100% (3*S*,4*S*) >98% ee	1.2 : 1	(3*S*,4*R*) > 99% ee

Scheme 3.

While the rates of reduction of (*S*)-**4** and (*R*)-**4** are the same, the rate of conversion of (*S*)-**7** is slightly faster than (*R*)-**7**. This is reflected in a slightly higher proportion of the *cis*-alcohol (3*S*,4*S*)-**8** obtained in fermentation. Overall, however, the lack of an efficient DKR in the reduction of both **4** and **7** is a cumulative effect of the low E ratio and a rapid reduction compared to racemization. These points are illustrated in Figures 1–3.

Biocatalytic DKR has been reported for baker's-yeast-catalyzed reductions of β-keto esters.[29–32] α-Monosubstituted β-ketoesters are excellent substrates for yeast reductases and, when the α-substituent is alkyl or aryl group, DKR may be achieved, since racemization is quite rapid under the biotransformation conditions. In the case of substrates that do not undergo rapid racemization at neutral conditions, enzymatic reaction can be combined with base-catalyzed racemization. This approach has been applied in microbial reductions of α-cyano cyclopentanones and cyclohexanones.[33] Furstoss et al. prepared enantiopure lactones by combining microbial Baeyer–Villiger oxidation with a base-catalyzed (pH 9) racemization of the corresponding α-substituted cyclopentanone.[19] A highly efficient preparation of D-phenyl glycines by enzymatic hydrolysis and deracemization of DL-hydantoins, was achieved at pH >8.[34] Racemization of 3-oxo-β-lactams is not obvious; thus the case of DKR of 3-oxo-4-phenyl-β-lactam **1**, reported here, is clearly an example of scientific serendipity.

3. Conclusion

In conclusion, we have prepared *cis*-(3*S*,4*R*)-3-hydroxy-4-phenyl-β-lactam **2** as a single enantiopure product from racemic ketone **1**. The recombinant *E. coli* strain overproducing yeast reductase Ara1p was the 'reducing agent'. The enzymatic DKR occurred during fermentation at pH 7. This short synthesis of the title compound can be readily carried out on a scale of several grams. Parallel experiments with the related substrates, 4-(2-thiophenyl) and 4-(2-furyl) substituted 3-oxo-β-lactams, did not lead to DKR. These results underscore the fact that the successful DKR depends on the ratio of the fast and slow reactions and its relationship to the rate of racemization.

4. Experimental

4.1. General procedure for the biotransformations with recombinant *E. coli* strains in a shake flask[26]

Fresh plates of engineered *E. coli* strains were streaked from the appropriate frozen stock and incubated at 30 °C until the colony was 1–2 mm in size (normally 24 h). A single colony was used to inoculate 10 mL

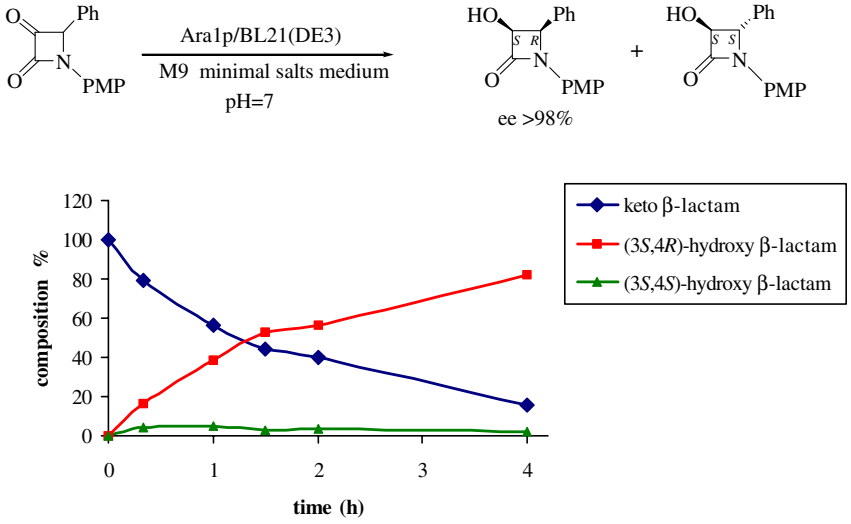


Figure 1. Fermentation of 3-oxo-4-phenyl-β-lactam **4** with *E. coli*/Ara1p.

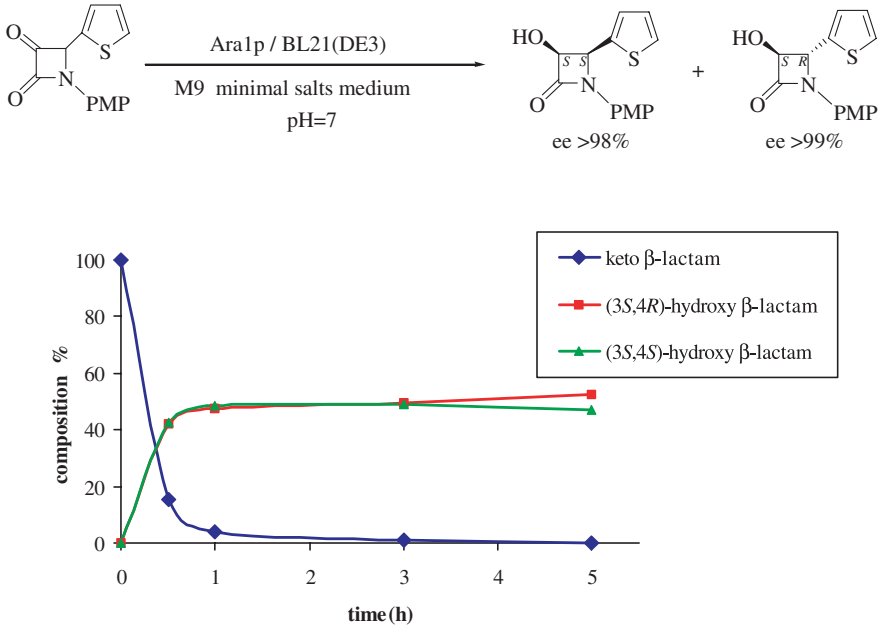


Figure 2. Fermentation of 3-oxo-4-(2-thiophenyl)-β-lactam **4** with *E. coli*/Ara1p.

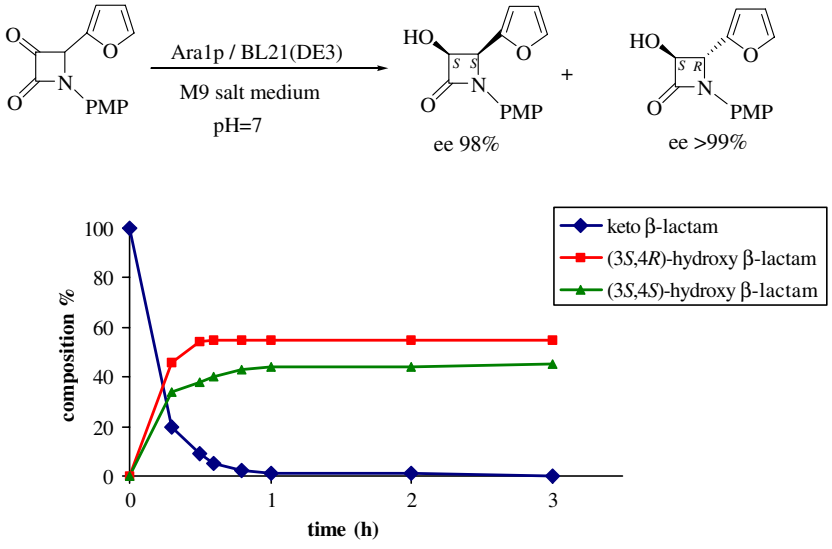


Figure 3. Fermentation of 3-oxo-4-(2-furyl)-β-lactam **7** with *E. coli*/Ara1p.

LB-ampicillin medium in a 50 mL Erlenmeyer flask. The culture was incubated at 200 rpm at 30 °C on a rotary shaker overnight, and 0.25 mL of this culture was used to inoculate a 25 mL fresh LB-ampicillin medium in a 150 mL baffled flask under the same conditions until it reached an OD_{600} of 0.5 (~3 h). Then, IPTG was added to a final concentration of 1 mM. The substrate (25 mg) was added along with β-cyclodextrin (1 equiv if needed). The culture was shaken at 30 °C and sampled periodically for GC and chiral phase HPLC analysis. The analytical samples were prepared by mixing 300 μL of the reaction mixture with 300 μL of ethyl acetate. After vortex mixing for 1 min, the sample was spun in a microcentrifuge for 1 min, then 200 μL of the organic layer was collected and dried under nitrogen. The residue was dissolved in 200 μL of *iso*-propanol and the resulting solution passed through a Cameo 3 N syringe filter (0.22 micro, 3 mm diameter). One microlitre of sample was used for GC analysis (where applicable) and 20 μL used for HPLC analysis.

4.2. Biotransformation with resting cells[35]

Fresh plates were streaked from a frozen stock on solid LB medium supplemented with 50 μg/mL ampicillin. A single colony was used to inoculate a 10 mL LB medium with 50 μg/mL ampicillin. The culture was incubated overnight with shaking (200 rpm) at 30 °C. One litre of

LB medium containing 4 g/L glucose and 50 μg/mL ampicillin in two 500 mL baffled culture flasks were inoculated with 5 mL precultures, separately. The culture was grown at 30 °C with shaking at 200 rpm. When the OD_{600} value reached 0.6, IPTG was added to a final concentration of 0.10 mM. Growth was continued until the D_{600} value reached 4.0. The cells were harvested by centrifugation, and the cell pellet suspended in 250 mL of M9 minimal salt medium lacking nitrogen. To this was added 200 mg of β-lactams (ground with 200 mg β-cyclodextrin when necessary). The biotransformation was carried out at 32 °C with aeration and vigorously stirring. Ten millilitres of a 20% glucose solution was added every 5 h to the reaction mixture. The pH was controlled at 7.0 with a 3 M NaOH solution. The reaction mixture was sampled periodically and analyzed by both GC and HPLC as described above. When the reaction reached complete conversion, the mixture was saturated with NaCl and the cells removed by centrifugation. Then, the reaction mixture was extracted with ethyl acetate and the organic layers combined, washed with brine, and dried over magnesium sulfate. The solvent was removed under vacuum. The diastereoisomeric mixture could be readily separated by column chromatography on silica gel eluting with hexane and ethyl acetate (4:1 to 3:1 to 2:1) to yield the pure product.

4.3. Compounds isolated from *E. coli*Ara1p fermentation reactions

The syntheses and characterizations of ketones and alcohols discussed here have been reported earlier.[24,36] Full characterizations, including HRMS analyses were performed for (3*R*,4*S*)-**2**, (3*R*,4*R*)-**5**, (3*R*,4*S*)-**6**, (3*R*,4*R*)-**8**, and (3*R*, 4*S*)-**9**.[37] Their enantiomers discussed here showed identical IR and NMR spectra (Supplementary Material). Optical rotations for all compounds were measured in CH_2Cl_2 (*c* 1.0).

4.4. (3*R*,4*S*)-(+)-3-Hydroxy-1-(4-methoxyphenyl)-4-phenyl-2-azetidinone[38]

White crystals; mp: 222–229 °C; $[\alpha]^{20} = +192.0$; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.42 (1H, d, $J = 9.0$, O*H*), 3.75 (3H, s, OCH_3), 5.18 (1H, dd, $J = 5.2$, $J = 9.0$, HOC*H*), 5.26 (1H, d, $J = 5.1$, NC*H*), 6.80 (2H, d, $J = 9.0$, Ar*H*), 7.29 (2H, d, $J = 9.1$, Ar*H*), 7.32–7.42 (5H, m, Ar*H*); ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.47 (O*C*H$_3$), 62.28 (N*C*H), 76.69 (O*C*H), 114.49, 118.85, 127.45, 128.97, 129.19, 156.54, 166.25 (*C*O); HRMS: $C_{16}H_{15}NO_3$ (M+), calc.: 269.1052, found: 269.1052.

4.5. (3*S*,4*R*)-(−)-3-Hydroxy-1-(4-methoxyphenyl)-4-phenyl-2-azetidinone 2

Isolated yield 65%; ee >98%; $[\alpha]^{20} = -170.0$ (Lit. $[\alpha]^{20} = -184.6$[12]); IR and NMR spectra as above.

4.6. (3*R*,4*R*)-(+)-3-Hydroxy-1-(4-methoxyphenyl)-4-(thiophen-2-yl)-2-azetidinone

White crystals; mp: 178–179 °C; $[\alpha]^{20} = +172.6$; IR ($CHCl_3$) γ_{max}/cm^{-1} 3299, 3012, 2961, 2907, 2831, 1714, 1513, 1252, 1113, 1027; ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.88 (1H, d, $J = 9.1$, O*H*), 3.76 (3H, s, OCH_3), 5.18 (1H, dd, $J = 5.1$, $J = 9.0$, NC*H*), 5.51 (1H, d, $J = 5.1$, OHC*H*), 6.81 (2H, d, $J = 9.1$, Ar*H*), 7.07 (1H, dd, $J = 3.5$, $J = 5.0$, Ar*H*), 7.13 (1H, d, $J = 2.9$, Ar*H*), 7.32 (2H, d, $J = 9.0$, Ar*H*), 7.36 (1H, dd, $J = 1.2$, $J = 5.0$, Ar*H*); ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 55.45 (O*C*H$_3$), 58.67 (N*C*H), 77.53 (OH*C*), 114.41, 118.88, 126.69, 127.29, 127.74, 130.26, 136.55, 156.58; HRMS: $C_{14}H_{13}NO_3S$ (M+), calc.: 275.0616, found: 275.0614.

4.7. (3*S*,4*S*)-(−)-3-Hydroxy-1-(4-methoxyphenyl)-4-(thiophen-2-yl)-2-azetidinone 5

White crystals (X-ray crystal structure);[24] 29% yield; mp: 178–179 °C; $[\alpha]^{20} = -171.0$ (Lit. $[\alpha]^{20} = -115.0$[12]); IR and NMR spectra as above.

4.8. (3*R*,4*S*)-(−)-3-Hydroxy-1-(4-methoxyphenyl)-4-(thiophen-2-yl)-2-azetidinone

White crystals (X-ray crystal structure);[24] mp: 98–99 °C; $[\alpha]^{20} = -43.5$; IR ($CHCl_3$) γ_{max}/cm^{-1} 3352, 2921, 2836, 1728, 1512, 1249, 1138; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.72 (3H, s, OCH_3), 4.67 (1H, s, O*H*), 4.84 (1H, s, NC*H*), 5.13 (1H, s, HOC*H*), 6.71 (2H, d, $J = 8.5$, Ar*H*), 6.99 (1H, t, $J = 3.5$, Ar*H*), 7.12 (1H, s, Ar*H*), 7.17 (2H, d, $J = 8.5$, Ar*H*), 7.28 (1H, d, $J = 5.0$, Ar*H*); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.35 (O*C*H$_3$), 61.82 (N*C*H), 84.51 (OH*C*H), 114.27, 119.16, 126.06, 126.29, 127.34, 129.96, 139.62, 156.62, 166.44 (*C*O); HRMS: $C_{14}H_{13}NO_3S$ (M+), calc.: 275.0616, found: 275.0616.

4.9. (3*S*,4*R*)-(+)-3-Hydroxy-1-(4-methoxyphenyl)-4-(thiophen-2-yl)-2-azetidinone 6

Isolated yield 18%; ee >99%; $[\alpha]^{20} = +47.4$; IR and NMR spectra as above.

4.10. (3*R*,4*R*)-(+)-4-(Furan-2-yl)-3-hydroxy-1-(4-methoxyphenyl)-2-azetidinone

White crystals; mp: 158–159 °C; $[\alpha]^{20} = +216.6$; IR ($CHCl_3$) γ_{max}/cm^{-1} 3370, 3120, 2951, 2916, 2847, 2836, 1747, 1510, 1397, 1240, 1119, 1029; ^{1}H NMR (250 MHz, $CDCl_3$) δ 3.20 (1H, d, $J = 9.8$, O*H*), 3.75 (3H, s, OCH_3), 5.21 (1H, dd, $J = 5.0$, $J = 9.8$, OHC*H*), 5.27 (1H, d, $J = 5.1$, NC*H*), 6.43 (1H, dd, $J = 1.9$, $J = 3.3$), 6.53 (1H, d, $J = 3.3$), 6.81 (2H, d, $J = 9.0$), 7.29 (2H, d, $J = 9.0$), 7.48 (1H, d, $J = 1.7$); ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 55.47 (OCH_3), 56.49 (N*C*H), 77.81 (OH*C*H), 110.97, 111.18, 114.38, 118.67, 130.45, 143.93, 147.77, 156.57, 165.44 (*C*O); HRMS: $C_{14}H_{13}NO_4$(M+), calc.: 259.0845, found: 259.0839.

4.11. (3*S*,4*S*)-(−)-4-(Furan-2-yl)-3-hydroxy-1-(4-methoxyphenyl)-2-azetidinone 8[39]

Isolated yield 34%; white crystals (X-ray crystal structure);[24] ee >98%; $[\alpha]^{20} = -215.0$; IR and NMR spectra as above.

4.12. (3*R*,4*S*)-(−)-4-(Furan-2-yl)-3-hydroxy-1-(4-methoxyphenyl)-2-azetidinone

White powder; mp: 96–97 °C; $[\alpha]^{20} = -40.7$; IR ($CHCl_3$) γ_{max}/cm^{-1} 3362, 3124, 2935, 2916, 2837, 1729, 1513, 1250, 1144; ^1H NMR (250 MHz, $CDCl_3$) δ 3.73 (3H, s, OC*H*$_3$), 3.74 (1H, d, $J = 1.9$, NC*H*), 4.92 (1H, dd, $J = 1.9$, $J = 4.8$, OHC*H*), 1.09 (1H, d, $J = 5.0$, O*H*), 6.37 (1H, dd, $J = 1.9$, $J = 3.2$, Ar*H*), 6.46 (1H, d, $J = 3.2$, Ar*H*), 6.73 (2H, d, $J = 9.1$, Ar*H*), 7.20 (2H, d, $J = 9.1$, Ar*H*), 7.39 (1H, d, $J = 1.0$, $J = 1.2$, Ar*H*); ^{13}C NMR (62.9 MHz, $CDCl_3$) δ 55.34 (O*C*H$_3$), 58.98 (N*C*H), 80.62 (OH*C*H), 110.10, 110.68, 114.22, 118.86, 130.27, 143.40, 1478.71, 156.57, 166.69 (*C*O); HRMS: $C_{14}H_{13}NO_4$ (M+), calc.: 259.0845, found: 259.0836.

4.13. (3*S*,4*R*)-(+)-4-(Furan-2-yl)-3-hydroxy-1-(4-methoxyphenyl)-2-azetidinone 9

Isolated yield 40%; ee >99%; $[\alpha]^{20} = +44.8$; IR and NMR spectra as above.

Acknowledgements

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