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Chemoenzymatic enantioselective route to get (+) and (–) 4-acetoxy-azetidin-2-one by lipase-catalysed kinetic resolution and their applications

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1 **Chemoenzymatic enantioselective route to get (+) and (-) 4-acetoxy-**
2 **azetidin-2-one by Lipase-catalysed kinetic resolution and their**
3 **applications.**

4
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11 209 9528; fax +39 051 209 9456;

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15
16 **Highlights**

- 17
- 18 • Kinetic resolution of azetidinones by Lipases
 - 19 • *Pseudomonas fluorescens* lipase promoted the resolution by transesterification
 - 20 • Synthesis of enantiopure 4-acetoxy-azetidinones.
 - 21 • Synthesis of enantiopure *N*-methylthio-4-acetoxy-azetidinones

22

23 Abstract

24

25 4-Acetoxy-azetidin-2-one is an extremely useful intermediate widely applied for the synthesis of
26 several biologically active β -lactam compounds. However, it is available as a racemic mixture that
27 could limit its application in the synthesis of enantiopure products. Herein we evaluated the use of
28 lipases in a kinetic resolution (KR) process to finally obtain 4-acetoxy-zetid-2-one as separated
29 pure enantiomers. From a preliminary screening on a set of commercial enzymes, *Pseudomonas*
30 *fluorescens* emerged as the most suitable lipase that allowed to obtain good conversions and
31 excellent enantiomeric excesses. On the enantiomerically pure 4-acetoxy-azetidin-2-ones some
32 nucleophilic substitutions and *N*-thio-alkylation reactions were tested in order to evaluate the
33 stereochemical integrity at the C-4 position.

34

35 Keywords:

36 Biocatalysis, Kinetic resolution, Lactams, Azetidinones, Lipases, 4-acetoxy-azetidinone, Single-
37 enantiomer drugs.

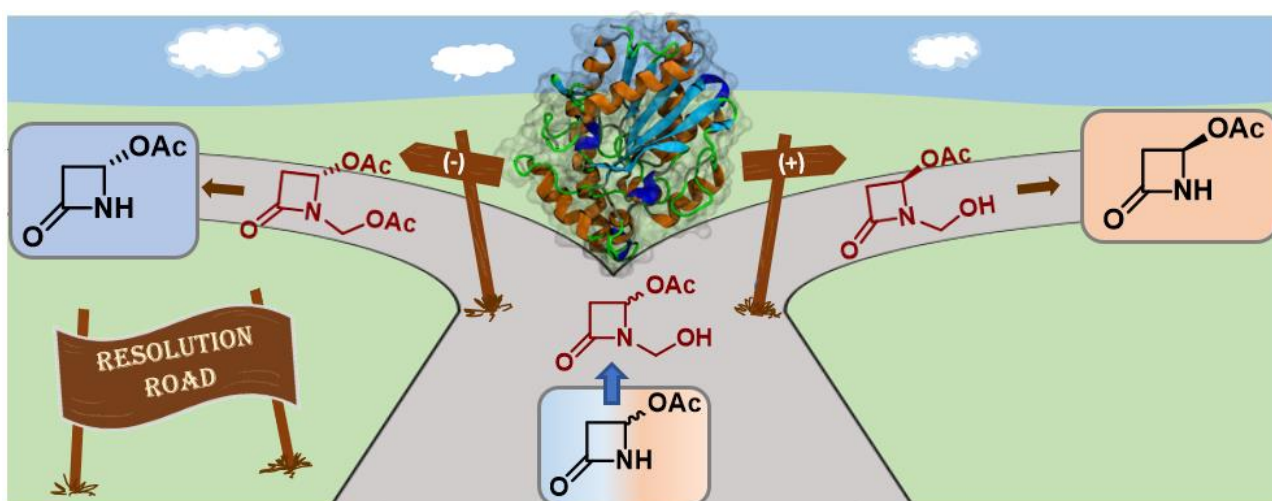
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40 Graphical Abstract

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44

45 Introduction

46 β -Lactam compounds have been generating an increasing interest in medicinal chemistry thanks to
47 their varied biological activities and versatility as intermediates for the synthesis of other biologically
48 active compounds [1-3]. β -Lactams are widely known and exploited as antibiotics and inhibitors of
49 β -lactamases [4]. As a structure-based classification, they fall into two main groups: bicyclic
50 compounds such as penicillins, cephalosporins, carbapenems or clavams, and monocyclic
51 compounds, such as Aztreonam, the only clinically available monobactam antibiotic [5].

52 Monocyclic β -lactams are considered unique structures because of different substituents on the
53 ring that could address different biological activities [6, 7]. Accordingly, over the last 20 years great
54 interest has been focused on the synthesis of properly substituted monocyclic derivatives that
55 showed various pharmacological properties as anticancer, antidiabetic, anti-tubercular, anti-
56 inflammatory, antiparkinsonian and anti-HIV activities [8-10]. Moreover, azetidinones were found
57 promising as cholesterol absorption inhibitors, as thrombin, chymase Cathepsin K inhibitors, as
58 human tryptase vasopressin V1a antagonists [9, 10], and as ligands of integrin receptors able to
59 modulate cell adhesion and signalling [11, 12]. As a contribution in the development of new
60 antimicrobial agents against resistant bacteria, we synthesized 4-alkylidene-azetidinones, a new
61 group of monocyclic β -lactams that were functionalized with phytochemical polyphenolics and
62 explored as dual-target antibacterial- and antioxidant compounds [13-15]. Moreover, the
63 introduction of the so-called β -lactam synthon method by Ojima [16], has further contributed to a
64 greatly increased interest in the synthesis of monocyclic β -lactams as useful intermediates in organic
65 synthesis [17, 18].

66 As useful β -lactam intermediates, (3*R*,4*R*)-4-acetoxy-3-[(1*R*)-1-(*tert*-butyldimethylsilyloxy)-ethyl]-
67 azetidin-2-one (**A**) and 4-acetoxy azetidin-2-one (**rac-1**) are commercially available and have been
68 widely used as starting materials for the synthesis of important bioactive compounds [19] (Figure
69 1). Intermediate **A** is available as enantiopure compound, **rac-1** instead is purchased only as a
70 racemic mixture. Although the great usefulness of **rac-1** as starting material in the synthesis of new
71 bioactive β -lactam compounds [20-27], this reactant could only provide racemic derivatives
72 (selected examples in Figure 1). Since it has been well attested that two enantiomeric drugs could
73 give different pharmacologic responses and diverse pharmacokinetic, pharmacodynamic and
74 therapeutic profiles, or could even reveal adverse effects [28, 29], the possibility to gain access to
75 the enantiopure form of 4-acetoxy-azetidinone **rac-1** would be of utmost importance in the
76 synthesis of stereo-chemically defined bioactive β -lactams [30]. To the best of our knowledge, only
77 (+)4-acetoxy-azetidinone has been reported and obtained by chiral recognition process upon
78 separation of chiral host-guest inclusion complexes [31].

79

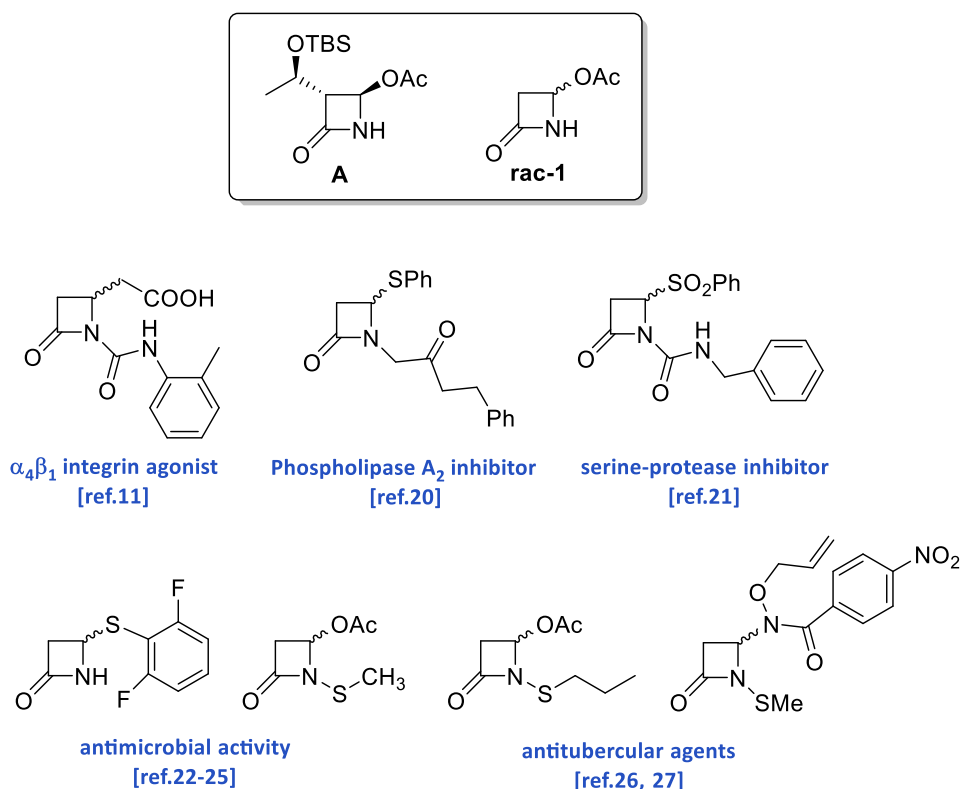


Figure 1. 4-acetoxy-azetidiones **A**, **rac-1**, and some selected racemic bioactive β -lactam compounds obtained from **rac-1**.

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81

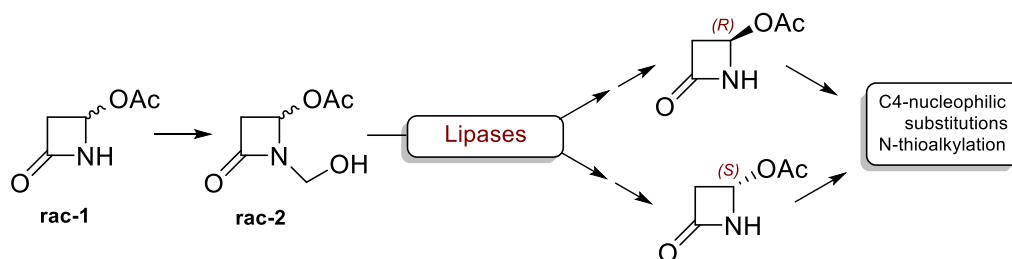
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83

84 Enzymatic kinetic resolution (KR) is a leading approach among the biocatalytic methods for the
 85 conversion of racemates into single enantiomers, and lipases proved to be a powerful tool for the
 86 obtainment of enantiopure fine chemicals and pharmaceutical compounds [32, 33].

87 The aim of the present work is to establish a chemoenzymatic route starting from the commercially
 88 available **rac-1** to obtain the two enantiomers of 4-acetoxyazetididin-2-one from the racemic **rac-2** by
 89 means of a kinetic resolution by lipases, and to preliminary test the single enantiomers in some
 90 typical reactions as C4 substitutions and N-thioalkylation in order to evaluate the stereochemical
 91 integrity of the corresponding products (Figure 2).

92



93

94

95 **Figure 2.** Enzymatic kinetic resolution on functionalized 4-acetoxy-azetidinone and
96 evaluation of the stereochemical outcome in C4 and N-functionalization.

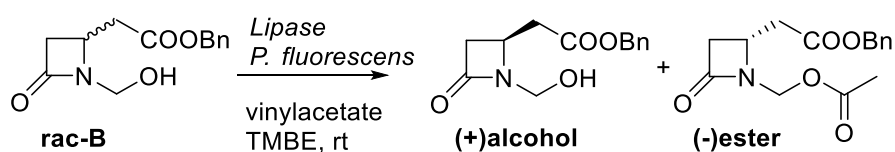
97 We thus chose to take advantage of the *N*-hydroxymethyl functionalization of the beta-lactam
98 which has been already explored in the asymmetric acylation of the primary alcohol. Nagai *et al.*
99 first reported the application of this functionalization in the kinetic resolution of some *N*-
100 hydroxymethyl-4-aryl-azetidinones by *Burkholderia cepacia* and *Candida antarctica* B lipases in
101 transesterification with vinylacetate or vinylbutyrate obtaining good results in term of yields of
102 single enantiomers and enantiomeric excesses [34, 35]; the same process was then further exploited
103 by Fülöp, Kanerva and coworkers. [36-40]. This strategy was also applied on other *N*-hydroxymethyl-
104 azetidinones bridged in bicyclic systems on the C3 and C4 of the β -lactam ring by means of Lipase,
105 among which also Lipase AK (*Pseudomonas sp.*), with trifluoroethyl butyrate or vinyl butyrate in
106 various solvents with good enantioselectivities [41-44].

107 From these studies, it appeared a certain narrowness in the C4 substituents of the *N*-hydroxymethyl
108 azetidinones that are mainly 4-aryl or bicyclic groups. However, the challenge of our project is to
109 face the resolution of **rac-1** which presents the 4-acetoxy moiety, well known for its reactivity as
110 excellent leaving group and widely used in substitution reactions on the C-4 position of azetidinones
111 [19] as above mentioned. Exploiting also our previous experience in the kinetic resolution by lipases
112 of racemic *N*-hydroxymethyl azetidinones with a carboxymethyl group on the C4 [45], we were able
113 to find the best conditions and to successfully obtain the two separated enantiomers of 4-acetoxy-
114 azetidinone.

115 Results and discussion

116 To approach the resolution of **rac-1**, the possibility to directly perform a lipase-mediated hydrolytic
117 kinetic resolution on the 4-acetoxy ester group was excluded because of an alleged low stability of
118 the corresponding 4-hydroxy-azetidinone which could undergo a ring-opening reaction *via*
119 breakdown of the N-C4 bond [46]. In a previous work, we took advantage of an *N*-hydroxymethyl-
120 functionalization to successfully develop a KR on a racemic β -lactam intermediate for the synthesis
121 of enantiopure integrin ligands (Scheme 1) [45].

122

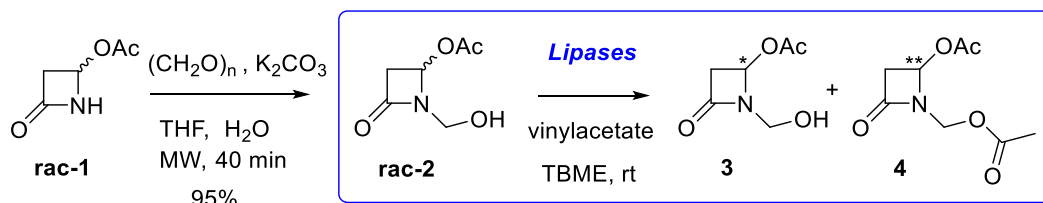


124 **Scheme 1.** Kinetic enzymatic resolution on the β -lactam intermediate **rac-B** previously reported.

125

126 Thus, we applied the same strategy on the racemic 4-acetoxy-azetidinone **rac-1**, which was
 127 accordingly treated with paraformaldehyde and catalytic potassium carbonate under microwave
 128 irradiation to obtain the *N*-methylene-hydroxy derivative **rac-2** in excellent yields (Scheme 2).

129



130

131 **Scheme 2.** Synthesis of *N*-methylene-hydroxy-azetidinone **rac-2** and its enzymatic KR by
 132 transesterification reaction.

133 In order to perform a KR on **rac-2**, some lipases were tested in the transesterification reaction with
 134 vinyl acetate in *t*-butylmethylether (TBME) at room temperature. After filtration of the enzymes,
 135 the crude reaction mixtures were monitored by chiral HPLC analysis for evaluating conversions and
 136 enantiomeric excesses (Table 1).

137

138 **Table 1.** Screening of lipases in KR on **rac-2**.^a

Entry	Lipases (activity) ^b	Solvent	Enzyme (U)	Time (h)	Conv. (%) ^c	3 ee% ^c	4 ee% ^c
1	<i>Burkholderia cepacia</i> (30 U/mg)	TBME	300	20	3.5	6	>99
2	<i>Burkholderia cepacia</i> (30 U/mg)	TBME	300	72	13	24	>99
3	CAL B (10 U/mg)	TBME	75	16	>99	-	0
4	CAL B (10 U/mg)	TBME	75	6	84	>99	22
5	CAL B (10 U/mg)	TBME	75	2	65	>99	65
6	CAL B (10 U/mg)	TBME	75	1	54	78	60
7 ^d	<i>Pseudomonas fluorescens</i> (20 U/mg)	dry THF	223	72	39	>99	70
8	<i>Pseudomonas fluorescens</i> (20 U/mg)	THF	223	72	44	>99	82
9	<i>Pseudomonas fluorescens</i> (20 U/mg)	CH ₃ CN	223	72	44	>99	81
10	<i>Pseudomonas fluorescens</i> (20 U/mg)	Toluene	223	72	46	>99	79

11	<i>Pseudomonas fluorescens</i> (20 U/mg)	TBME	223	72	51	>99	91
12 ^e	<i>Pseudomonas fluorescens</i> (20 U/mg)	TBME	223	24	53	84	>99
13	<i>Pseudomonas fluorescens</i> (36 U/mg)	TBME	48	48	55	89	96
14	<i>Pseudomonas fluorescens</i> (309 U/mg)	TBME	150	23	55	>99	90
15	<i>Pseudomonas fluorescens</i> (309 U/mg)	TBME	117	8	52	>99	90

139 ^a Reaction conditions: **rac-2** (0.063 mmol), vinyl acetate (0.38 mmol), TBME (1.5 mL), enzyme (U in table), rt.

140 ^b Activity of the commercial enzymes.

141 ^c Determined by chiral HPLC analysis on the crude.

142 ^d Reaction conducted under N₂ atmosphere.

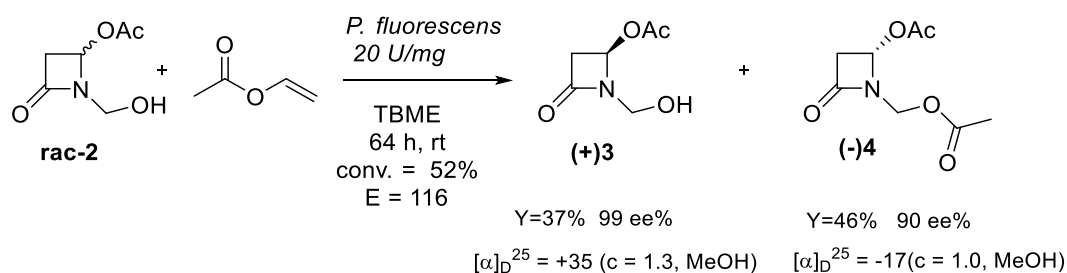
143 ^e T=40°C

144

145 Lipase from *Burkholderia cepacia* or *Candida antarctica B* (CAL B) which achieved very good results
 146 with other *N*-hydroxymethyl-azetidiones [38-39], behaved differently on **rac-2**: *Burkholderia*
 147 *cepacia* gave low conversions with poor ee % of the unreacted alcohol **3**, even after 72 h (Table 1,
 148 entries 1 and 2); on the contrary, CAL B showed high conversions, but null or poor
 149 enantioselectivities in the ester **4** even at lower conversions (Table 1, entries 3-6). The best results
 150 were obtained with *Pseudomonas fluorescens* lipase and some conditions were then studied (Table
 151 1, entries 7-15). Three *Pseudomonas fluorescens* lipase preparations with different activities (20, 36,
 152 and 309 U/mg) were tested, and on changing activity, units and reaction time, satisfactory
 153 conversions (45-55%) and good to excellent ee% for both **3** and **4** were obtained (Table 1, entries
 154 11-13-15). On changing the reaction solvent, THF, acetonitrile and toluene gave good results for the
 155 obtainment of **3** with high enantiomeric excess, but low ee% were detected for **4** (Table 1 entries 7-
 156 10). TBME was confirmed as the best solvent to obtain good ee% for both **3** and **4** (Table 1, entry
 157 11). When we tested the resolution in anhydrous THF under N₂ atmosphere to check an eventual
 158 influence of moisture or water, the reaction was slower and with lower ee% for compound **4** (Table
 159 1, entry 7 vs 8). Also in a tentative exploration at 40 °C the process was faster but with lower ee% of
 160 the unreacted enantiomer **3** (Table 1, entry 12), thus confirming the room temperature as the best
 161 condition.

162 The conditions of Table 1 entry 11 were then applied in a preparative KR in order to isolate discrete
 163 amounts of **3** and **4**.

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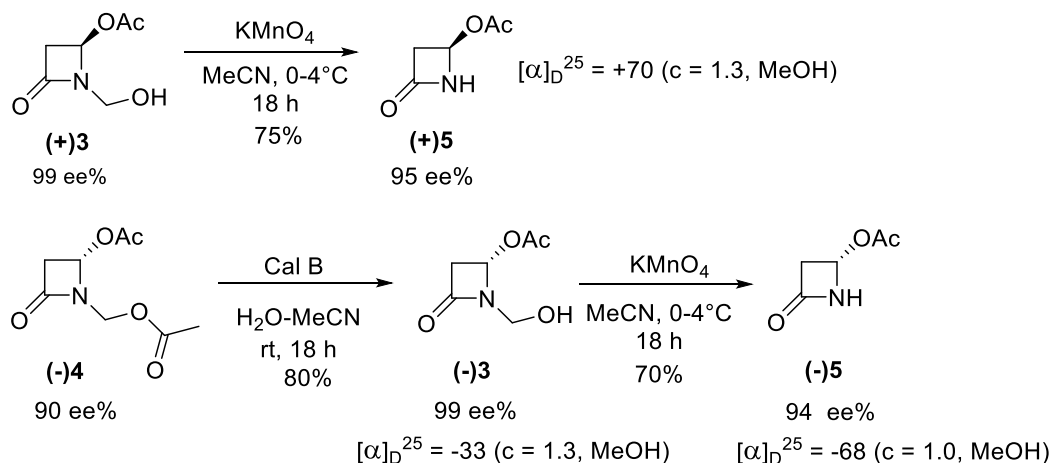
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170 The reaction was stopped at a 52% conversion: the two products were isolated and separated by
 171 flash-chromatography, characterized and analysed by chiral HPLC analysis and polarimetry; their
 172 specific optical rotations and enantiomeric excesses resulted as **(+)**3**** (99 % ee) and **(-)**4**** (90 % ee)
 173 with an excellent enzymatic selectivity (E) of 116 (Scheme 3).

174 Concerning the absolute configuration of the two enantiomers, it can be only tentatively attributed
 175 according to the enantio-preference of lipases on some C-3 unsubstituted β -lactam compounds
 176 bearing primary alcohols with a β -stereocenter [45, 38]. As above mentioned for the kinetic
 177 resolution on the racemic β -lactam **rac-B** [45], *P. fluorescens* gave (*S*)-ester as the preferred
 178 enantiomer (Scheme 1). In that case the absolute configuration was assigned by comparison with
 179 an already known chiral azetidinone obtained from (*S*)-aspartic acid [47]. On assuming the same (*S*)-
 180 enantio-preference by *P. fluorescens* for the C4 substituent, it could be tentatively assigned a (*S*)
 181 configuration to the **(-)**4**** ester as the preferred enantiomer also in this KR.

182 Alcohol **(+)**3**** and ester **(-)**4**** were then further elaborated in order to eliminate the substituents on
 183 the β -lactam nitrogen atom and to obtain the final separated enantiomers **(+)**5**** and **(-)**5**** (Scheme 4).

184



185

186 **Scheme 4.** Synthesis of β -lactam enantiomers **(+)**5 and **(-)**5. Isolated yields, ee%, and
 187 specific optical rotation powers are reported.

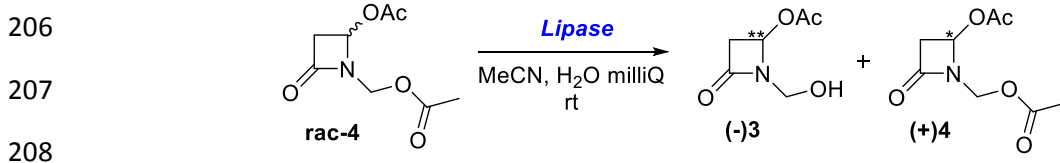
188

189 For this purpose, it was necessary to eliminate the *N*-hydroxymethyl group on **(+)**3 and different
 190 conditions were tested: NH₄OH (25%)/MeOH, KMnO₄/acetone-H₂O, and KMnO₄/MeCN [48]. Only
 191 oxidative conditions were effective, and the use of KMnO₄ in acetonitrile allowed to isolate
 192 compound **(+)**5 in satisfactory yields. As mentioned in the introduction, the (+)4-acetoxy-
 193 azetidinone **(+)**5 has been already reported in the literature, as an enantiopure compound obtained
 194 by means of host-guest inclusion complexes [29]. However, we observed a higher specific rotation
 195 for **(+)**5 [α]_D²⁵ = +70 (c = 1.3, MeOH) than that reported in the literature [31]: [α]_D²⁵ = +6.5 (c = 0.68,
 196 MeOH), but the latter has a lower concentration and its enantiomeric purity as ee% by chiral
 197 chromatography was not reported.

198 The **(-)**5 enantiomer was obtained from **(-)**4 with a two steps procedure: ester hydrolysis to get
 199 intermediate **(-)**3 followed by oxidative cleavage of the oxymethylene group. Concerning the
 200 hydrolysis, biocatalysis fulfilled the requirement of a high regioselectivity among the two acetates
 201 present on **(-)**5. The effectiveness of lipases to satisfy this requirement was preliminary evaluated
 202 on the racemic ester **rac-4** (Table 2), easily obtained from **rac-2** with acetic anhydride and
 203 triethylamine, and used as racemic standard for chiral HPLC analyses (see Supplementary Material).

204

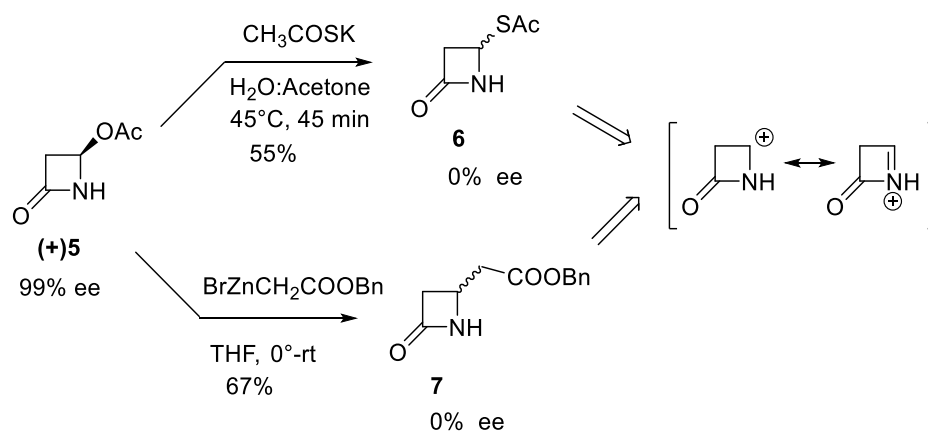
205 **Table 2.** Screening of lipases for enzymatic hydrolysis on **rac-4**.^a



Entry	Lipases (U/mg) ^b	Enzyme amount (U)	Time (h)	T (°C)	Conv. (%) ^c	(-)3 ee% ^c	(+)4 ee% ^c
1	<i>Burkholderia cepacia</i> (30 U/mg)	180	144	rt	30	>99	40
2	<i>Pseudomonas fluorescens</i> (309 U/mg)	326	21	rt	25	>99	24
3 ^d	<i>CAL B</i> (10 U/mg)	100	3	rt	55	>99	66
4 ^d	<i>CAL B</i> (10 U/mg)	100	24	rt	99	>99	-
5 ^e	<i>CAL B</i> (10 U/mg)	35	24	rt	95	96	99 ^f
6 ^{d, e}	<i>CAL B</i> (10 U/mg)	100	3	60	95	>99	99 ^f

7 ^{d, e}	<i>CAL B</i> (10 U/mg)	100	1	60	58	>99	30
209	^a Reaction conditions: rac-4 (0.06 mmol), H ₂ O/MeCN 11:1 (2.5 mL), enzyme (U in table), rt.						
210	^b Activity of the commercial enzymes.						
211	^c Determined by chiral HPLC analysis on the crude.						
212	^d Reaction conditions: rac-4 (0.174 mmol), H ₂ O/MeCN 11:1 (3 mL), enzyme (U in table), rt.						
213	^e The recovery of the organic fraction is around 60%.						
214	^f The ester (+) 4 was detected only in traces in the chiral HPLC analysis						
215	In this ester hydrolysis, <i>Burkholderia cepacia</i> and <i>Pseudomonas fluorescens</i> lipases gave						
216	unsatisfactory results with low conversions even for longer reaction time (Table 2, entries 1 and 2).						
217	<i>CAL B</i> was instead successful and furnished a 55% conversion and excellent enantiomeric excess						
218	(>99 %) for the alcohol 3 in only 3 hours (Table 2, entry 3). This result is quite interesting: the						
219	excellent ee for the product (-) 3 >99% at a conversion exceeding the 50% (i.e. 55 %) and a lower ee						
220	(66%) for the ester (+) 4 catches a glimpse of a possible racemization of the reactant at the C-4						
221	position that could be temporarily ascribed to the tentative formation of a C-4 cation (see for						
222	instance Scheme 5), thus revealing the possibility of a prospective dynamic kinetic resolution under						
223	controlled conditions. On extending the hydrolysis for 24 h with <i>CAL B</i> , the conversion was						
224	quantitative, only the alcohol 3 was isolated as single enantiomer with ee >99 %, but no traces of						
225	the ester 4 were isolated in the organic fraction (Table 2, entry 4). This could probably due to a						
226	hydrolysis of the β-lactam ring in the ester (+) 4 and its further degradation in the aqueous phase						
227	due to a longer reaction time. The hydrolysis with <i>CAL B</i> was further examined at 60 °C at short						
228	reaction time, 1 and 3 h (Table 2, entries 6 and 7) obtaining 95% and 58% conversion, respectively.						
229	The product 3 was isolated with high ee%, but with a poor recovery in the organic extract (60%) with						
230	traces (entry 6) or low amounts (entry 7) of the ester. This result confirms that harsher reaction						
231	conditions could give significant amounts of hydrolysis of the β-lactam ring [49-52] leading to a yield						
232	drop.						
233	The hydrolysis was repeated with <i>CAL B</i> on the enantiomerically enriched ester (-) 4 (Scheme 4). The						
234	process was carried out in MilliQ water with a small amount of acetonitrile necessary to dissolve (-)						
235	4 (H ₂ O/CH ₃ CN = 11:1). Alcohol (-) 3 was obtained in an 80% yield with a 99% optical purity;						
236	remarkably, its enantiomeric excess was greatly enriched if compared to that of the starting ester (-)						
237	4 (90% ee). The final oxidation of (-) 3 with KMnO ₄ in acetonitrile afforded chiral (-)4-acetoxy-						
238	azetidinone (-) 5 in good yields (Scheme 4). Careful attention should be paid for a cold aqueous work-						
239	up of the crude because lowering of the enantiomeric excess was otherwise observed, and an ee =						
240	88% was reached instead of 94% (see supplementary information for HPLC analyses).						
241	Introduction or transformation of functional groups on C4 position of azetidinones is a common step						
242	in the synthesis of β-lactam-based compounds [19]. Once obtained the enantiomerically pure 4-						
243	acetoxy-azetidinones (+) 5 and (-) 5 , some nucleophilic substitution reactions were tested on these						
244	substrates. We then examined the stereochemical outcome in two C4 substitution reactions						
245	(Scheme 5) on enantiomer (+) 5 , with potassium thioacetate and with Reformatsky reagent						

246 BrZnCH₂COOBn, previously applied for the synthesis of integrin ligands with a β-lactam scaffold [10,
247 11].



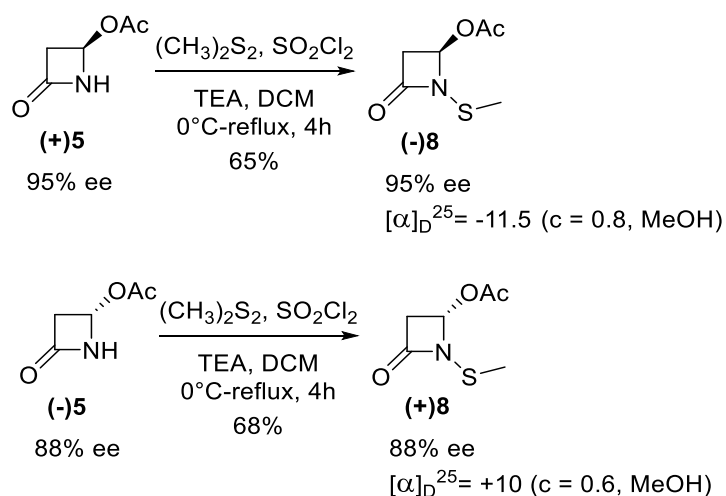
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249 **Scheme 5.** Nucleophilic substitution reactions at C4 position of the enantiomerically
250 pure 4-acetoxy-azetidinone (**(+)5**).

251

252 However, starting from enantiopure compound (**(+)5**), the C4 substitution products **6** and **7** were
253 obtained as racemic mixtures (chiral HPLC analyses in Supplementary information), confirming the
254 mechanism of this substitution as an addition-elimination pathway *via* the short-living intermediate
255 1-azetin-2-one (Scheme 5), as determined by Gavina *et al.* [53].

256 As a further stereochemical investigation, the (**(+)5**) and (**(-)5**) enantiomers were considered for a
257 functionalization on the nitrogen atom of the β-lactam ring (Scheme 6). For this purpose, we
258 selected a *N*-thioalkylation reaction, since *N*-alkyl-thio-4-acetoxy-azetidinones demonstrated to
259 have interesting antibacterial activities [24, 26] and were also successfully applied in the
260 development of new antibacterial functional materials [25]. Enantiomers (**(+)5**) and (**(-)5**) were
261 separately subjected to a *N*-thiolation reaction using dimethyl disulfide in the presence of sulfonyl
262 chloride and triethylamine (TEA) in DCM (Scheme 6), according to a previously reported procedure
263 [25].



264

265

Scheme 6. *N*-thiomethylation on enantiopure 4-acetoxy-azetidinone **(-)****5**.

266 Compounds **(-)****8** and **(+)****8** were isolated by flash chromatography and then analysed by chiral HPLC
 267 (see Supplementary Material). The chromatographic analysis showed the preservation of the ee at
 268 95% and 88%, as that of the precursors **(+)****5** and **(-)****5**, respectively, thus confirming that the *N*-
 269 thioalkylation reaction did not affect the configuration at C4 of the β-lactam. The optical rotations
 270 of the *N*-methylthio derivatives **(-)****8** and **(+)****8** have opposite signs compared to the starting
 271 compounds **(+)****5** and **(-)****5**, however, optical power is an inherent property of a molecule, and a
 272 change in sign between different molecules does not entail an inversion of the configuration [54,
 273 55].

274

275 **Conclusion**

276 The syntheses of several biologically active β-lactam-based compounds share as starting material
 277 the 4-acetoxy-azetidin-2-one, a cheap and readily available compound, but marketed as a racemic
 278 mixture. Given the effect of chirality on biological activity and the need to synthesize enantiopure
 279 β-lactams for pharmacological use it would be advisable to obtain 4-acetoxy-azetidin-2-one in its
 280 separated single enantiomers. Biocatalysis is becoming a valid and an increasingly employed
 281 technique for achieving enantiomerically pure products. Among a series of lipases, *Pseudomonas*
 282 *fluorescens* was selected as the most suitable enzyme for performing a kinetic resolution by
 283 transesterification on the *N*-hydroxymethyl β-lactam **rac-2**, giving the corresponding ester **(-)****4** and
 284 the residual starting alcohol **(+)****3** with excellent enantiomeric excesses. The successful ester
 285 hydrolysis of **(-)****4** was obtained with *Candida antarctica lipase B* (*CAL B*) with a significant
 286 enrichment of the enantiomeric excess of the *N*-hydroxymethyl-azetidinone **(-)****3**. The potential
 287 dynamic kinetic resolution of the β-lactam **rac-4** by *CAL B* is currently under investigation. Following
 288 the optimized deprotection steps, 4-acetoxy-azetidin-2-ones **(+)****5** and **(-)****5** were thus obtained as
 289 single enantiomers with excellent optical purities. Nucleophilic substitution reactions were then
 290 studied on the pure enantiomers in order to evaluate their stereochemical outcome. The C4

291 substitution on the enantiomer **(+)**5 with potassium thioacetate or BrZnCH₂COOBn gave the
292 corresponding products **6** and **7** as racemic mixtures, confirming the propensity of 4-acetoxy-
293 azetidinones to undergo a S_N1 mechanism. On the contrary, the C4 configuration is not affected by
294 functionalization of the β-lactam nitrogen atom upon sulfenylation reaction, and optically active *N*-
295 methylthio-4-acetoxy-β-lactams were successfully obtained. With this result we report for the first
296 time the synthesis of an enantiopure *N*-alkylthio-4-acetoxy-azetidinone, hence paving the way to
297 the development of novel chiral β-lactams that could be employed in medicinal chemistry as
298 antimicrobial agents as pure enantiomers instead of racemic mixtures.

299

300 **Experimental**

301 Solvents and reagents were obtained commercially and used as received. Deionized water was
302 obtained from a Millipore analytical deionization system (MilliQ). For TLC monitoring Merck 60 F254
303 plates were used and for liquid chromatography Merck silica gel 200–300 mesh was used. ¹H and
304 ¹³C NMR spectra were recorded with an INOVA 400 instrument with a 5 mm probe. All chemical
305 shifts are quoted relative to deuterated solvent signals (δ in ppm and J in Hz). FTIR spectra were
306 recorded with Alpha FT IR Bruker spectrometer. Polarimetric analyses were conducted on Unipol L
307 1000 Polarimeter at 598 nm. The purities of the target compounds were assessed as being > 95%
308 using HPLC-MS. HPLC-MS: Agilent Technologies HP1100 instrument, equipped with a ZORBAX-
309 Eclipse XDB-C8 Agilent Technologies column; mobile phase: H₂O/CH₃CN, 0.4 mL/min, gradient from
310 30 to 80% of CH₃CN in 8 min, 80% of CH₃CN until 25 min, coupled with an Agilent Technologies
311 MSD1100 single-quadrupole mass spectrometer, full scan mode from m/z = 50 to 2600, in positive
312 ion mode, ESI spray voltage 4500 V, nitrogen gas 35psi, drying gas flow 11.5 mL/min, fragmentor
313 voltage 20 V. Enantiomeric excesses were determined by chiral-HPLC: Agilent Technologies 1200
314 instrument equipped with a diode array UV detector on Daicel Chiralcel column IA (25 cm, I.D. 0.46
315 cm, 5 μm) with HPLC grade isopropanol and *n*-hexane as eluting solvents. Racemic compounds were
316 used for comparison (see supplementary material). The commercially available enzymes used in this
317 work are: *Burkholderia cepacia* lipase (BCL) powder, ≥30 U/mg by Sigma Aldrich; Lipase from
318 *Pseudomonas fluorescens*, powder, 309 U/mg by Fluka; Lipase from *Pseudomonas fluorescens*,
319 powder, 20-36 U/mg by Sigma Aldrich; Lipase from *Candida antarctica* B, immobilized on acrylic
320 resin, 10 U/mg, by Sigma Aldrich; Lipase B from *Candida antarctica*, immobilized on Immobead 150,
321 4.4 U/mg, by Sigma Aldrich.

322

323 *Synthesis of racemic β-lactams*

324 Preparation of racemic esters **rac-4** for the optimization of an analytic method by chiral HPLC is
325 reported in the Supplementary Material.

326

327 *1-(hydroxymethyl)-4-oxoazetidin-2-yl acetate (rac-2)*.

328 To a solution of compound **rac-1** (200 mg, 1.55 mmol, 1 equiv) in THF (3.4 mL), paraformaldehyde
329 (72 mg, 2.48 mmol, 1.6 equiv), K₂CO₃ (2 mg, 0.05 mmol, 0.03 equiv) and water (140 μL) were added.
330 The system was subjected to microwave irradiation at 180 W for 40 minutes. At completion, the
331 reaction mixture was diluted with EtOAc (2.5 mL), dried on anhydrous Na₂SO₄, filtered and
332 concentrated to yield compound **rac-2** as a colorless oil (240 mg, 95%) without further purifications.
333 ¹H NMR (400 MHz, CDCl₃) δ (ppm) 6.01 (dd, *J* = 4.2, 1.2 Hz, 1H), 4.78 (d, *J* = 11.5 Hz, 1H), 4.49 (d, *J* =
334 11.5 Hz, 1H), 3.23 (dd, *J* = 15.3, 4.2 Hz, 1H), 2.96 (dd, *J* = 15.3, 1.2 Hz, 1H), 2.10 (s, 3H). ¹³C NMR (100
335 MHz, CDCl₃) δ (ppm) 171.6, 165.3, 74.5, 64.1, 44.3, 20.7. IR (film, cm⁻¹) = 3425, 2950, 1752, 1649,
336 1378, 1241, 1120, 1043. HPLC-MS (ESI⁺) *t*_r=2.9 min, *m/z*=100 [M-OAc]⁺, 160 [M+H]⁺, 177 [M+H₂O]⁺,
337 182 [M+Na]⁺.

338

339 *Procedure for lipases screening in enzymatic kinetic resolution on rac-2 (see Table 1).*

340 In a glass vial with a screw cap, to a solution of alcohol **rac-2** (10 mg, 0.063 mmol, 1 equiv) and vinyl
341 acetate (45 μL, 0.38 mmol, 6 equiv) in TBME (1.5 mL), the selected enzyme (see Units in Table 1)
342 was added. The mixture was stirred at room temperature. At set time intervals, the substrate
343 conversion and the enantiomeric excesses of unreacted alcohol **(+)**3 and ester product **(-)**4 were
344 monitored by chiral HPLC. Chiral HPLC samples were prepared as follows: 0.5 mL of mixture was
345 filtered through regenerated cellulose syringe filters (diameter = 25 mm, pore diameter = 0.45 μm);
346 the filtrate was then concentrated, re-suspended in a solution of *n*-hexane/isopropanol 1:1 and
347 directly analysed.

348

349 *Preparative enzymatic KR of compound rac-2.*

350 In a glass vial with a screw cap, to a solution of alcohol **rac-2** (164 mg, 1.03 mmol, 1 equiv) and vinyl
351 acetate (0.57 mL, 6.18 mmol, 6 equiv) in TBME (24.5 mL), Amano Lipase from *Pseudomonas*
352 *luorescens* (20 U/mg, 1640 U, 82 mg) was added. The mixture was then kept under magnetic stirring
353 at room temperature and monitored by chiral HPLC. At 52% conversion, after 64 h, the mixture was
354 filtered through regenerated cellulose syringe filters and the organic solvent was removed under
355 reduced pressure. Compounds **(+)**3 and **(-)**4 were separated by flash chromatography
356 (Cyclohexane/EtOAc 1:1 then 35:65); target ester **(-)**4 was obtained as a colorless oil (46%, ee = 90%,
357 [α]_D²⁵ = -17 (c = 1.0, MeOH)) and residual alcohol **(+)**3 was isolated as a colorless oil (37%, ee = 99%,
358 [α]_D²⁵ = +35 (c = 1.3, MeOH)).

359

360 *Procedure for lipases screening in the enzymatic hydrolysis on rac-4 (see Table 2).*

361 In a glass vial with a screw cap, to a solution of **rac-4** (12 mg, 0.06 mmol) in CH₃CN and Milli Q H₂O
362 (1:11 ratio, total volume 2.5 mL), the selected enzyme (see Units in Table 2) was added. The mixture
363 was stirred at room temperature under orbital shaking (450 rpm). The conversion and the
364 enantiomeric excesses of **(-)**3 and **(+)**4 were monitored by chiral HPLC at set time intervals. Chiral
365 HPLC samples were prepared as follows: 200 μ L of the mixture were extracted with 0.5 mL EtOAc,
366 organic solvent was dried, re-suspended in a solution of *n*-hexane/isopropanol 1:1 and directly
367 analysed.

368

369 *Preparative enzymatic hydrolysis of compound (-)4.*

370 In a glass vial with a screw cap, to a solution of ester **(-)**4 (102 mg, 0.51 mmol, 1 equiv) in CH₃CN and
371 Milli Q H₂O (1:11 ratio, total volume 17 mL), *Lipase B from Candida Antarctica immobilized on*
372 *Immobead 150* (4.4 U/mg, 224 U, 51 mg) was added. The mixture was stirred at room temperature
373 under orbital shaking (450 rpm) overnight and monitored by chiral HPLC. At reaction completion (18
374 h), the enzyme was filtered and the aqueous mixture was saturated with brine and extracted with
375 EtOAc (3 \times 10 mL). The collected organic phases were dried over anhydrous Na₂SO₄, filtered and
376 concentrated under vacuum. The target alcohol **(-)**3 was obtained without further purification as a
377 colorless oil (65 mg, Y =80%, ee = 99%, [α]_D²⁵ = -33 (c = 1.3, MeOH)). Spectroscopic data of **(-)**3 were
378 in fully accordance with those reported for its corresponding racemic analogue **rac-2**.

379

380 *Oxidation with KMnO₄ to give (+)5 and (-)5.*

381 To a solution of the alcohol **(+)**3 or **(-)**3 (1 equiv) in CH₃CN (30 mL/mmol), KMnO₄ (6 equiv) was
382 added portionwise at 0°C. The reaction flask was maintained at 4°C overnight (refrigerator). The
383 reaction mixture was quenched at 0°C with a saturated solution of Na₂S₂O₅ until complete
384 decoloring. The mixture was then filtered and acetonitrile evaporated under reduced pressure. The
385 residual aqueous solution was then extracted with DCM (3 \times 10 mL). The collected organic phases
386 were dried over anhydrous Na₂SO₄, filtered and concentrated. The desired products **(+)**5 and **(-)**5
387 were obtained without further purification as sticky solid. **(+)**5: Y = 75%, ee = 99%, [α]_D²⁵ = +70 (c =
388 1.3, MeOH); **(-)**5: 70%, ee = 99%, [α]_D²⁵ = -68 (c = 1.0, MeOH). Spectroscopic data of **(+)**5 and **(-)**5
389 were in fully accordance with those reported for their corresponding racemic analogue **rac-1**.

390

391 *Synthesis of S-(4-oxoazetidin-2-yl) ethanethioate (6).*

392 To a solution of CH₃COSK (27 mg, 0.24 mmol, 1.2 equiv) in H₂O (1.5 mL) warmed at 45°C, compound
393 **(+)**5 (25 mg, 0.2 mmol, 1 equiv) dissolved in acetone (0.5 mL) was added dropwise. At completion
394 (50 min, TLC monitoring), acetone was evaporated under reduced pressure and the residual
395 aqueous solution was then extracted with EtOAc (5 \times 10 mL). The collected organic phases were dried
396 over anhydrous Na₂SO₄, filtered and concentrated. Compound **6** was yielded as a yellow oil (16 mg,

397 55%) without further purification. Spectroscopic data were in fully accordance with those reported
398 in literature [24].

399

400 *Synthesis of benzyl 2-(4-oxo-azetidin-2-yl) acetate (7).*

401 In a 25 mL 3-neck flask under inert atmosphere (N₂), Zn powder (203 mg, 3.12 mmol, 8 equiv) and
402 THF (1 mL) were introduced followed by TMSCl (20 μL, 0.155 mmol, 0.39 equiv). After 30 min of
403 stirring the temperature was raised to 30-32 °C and a solution of benzylbromoacetate (247 μL, 1.56
404 mmol, 4 equiv) in THF (2 mL) was slowly added dropwise. After 30 min of stirring the mixture was
405 cooled to rt and decanted. The limpid grey supernatant was slowly added dropwise into a 25 mL
406 flask under nitrogen containing a solution of **(+)**5 (50 mg, 0.39 mmol, 1 equiv) in anhydrous THF (2.2
407 mL) at 0°C. The mixture was stirred at rt for 3 h, quenched with a saturated Seignette salt (potassium
408 sodium tartrate) solution and extracted with EtOAc (3x10 mL). The organic layers were dried on
409 Na₂SO₄, filtered and concentrated in vacuum. The crude was purified by flash chromatography
410 (Cyclohexane/AcOEt = 1:1) yielding **7** as a white solid (57 mg, 67%). Spectroscopic data were in fully
411 accordance with those reported in literature. [11]

412

413 *Synthesis of compounds (+) 8 and (-8).*

414 In a round bottom flask under inert atmosphere (N₂), to a solution of dimethyl disulfide (10 μL, 0.11
415 mmol, 1 equiv) in dry DCM (1 mL), SO₂Cl₂ (13 μL, 0.16 mmol, 1.5 equiv) was slowly added at 0°C.
416 After 10 minutes, **(+)** 5 or **(-)** 5 (14 mg, 0.11 mmol, 1 equiv) dissolved in DCM (1 mL) was added,
417 followed by dropwise addition of TEA (28 μL, 0.22 mmol, 2 equiv). After 10 minutes at 0°C, the
418 reaction mixture was warmed to rt and then refluxed for 4 h. At completion (TLC monitoring), the
419 reaction was quenched with saturated aqueous solution of NH₄Cl and the mixture extracted with
420 DCM (3x5 mL). The collected organic phases were dried over anhydrous Na₂SO₄, filtered and
421 concentrated under vacuum. The desired product was obtained as a yellow oil after purification by
422 flash chromatography on silica gel (Cyclohexane/EtOAc = 7:3). **(+)**8: 13 mg, Y=68%, ee = 88%, [α]_D²⁰
423 = +10 (c = 0.6, MeOH); **(-)**8: 12.5 mg, Y=65%, ee = 95%, [α]_D²⁵ = -11.5 (c = 0.8, MeOH). Spectroscopic
424 data were in fully accordance with those reported in literature [24, 25].

425

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431

432

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