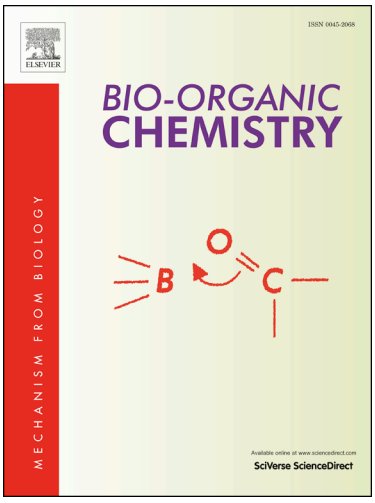

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Chiral β-lactam-based integrin ligands through Lipase-catalysed kinetic resolution and their enantioselective receptor response.

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Graphical abstract

Highlights

Kinetic resolution of azetidinones by Lipases

Burkholderia cepacia lipase promoted the resolution in transesterification

Synthesis of enantiopure β-lactams targeting integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$

Enantiospecific response of (*S*)-azetidinones in promoting cell adhesion

Abstract

Obtainment and testing of pure enantiomers are of great importance for bioactive compounds, because of the assessed implications of enantioselectivity in receptor-mediated responses. Herein we evaluated the use of biocatalysis to obtain enantiomerically pure β-lactam intermediates further exploited in the synthesis of novel integrin ligands as single enantiomers. From a preliminary screening on a set of commercially available hydrolases, *Burkholderia Cepacia* Lipase (*BCL*) emerged as a suitable and highly performing enzyme for the kinetic resolution of a racemic azetidinone, key intermediate for the synthesis of novel agonists of integrins. Upon optimization of the biocatalytic protocol in terms of enzymes, acylating agents and procedures, the two β-lactam enantiomers were obtained in excellent enantiomeric excesses (94% and 98% ee). Synthetic elaborations on the separated enantiomers allowed the synthesis of four chiral β-lactams which were evaluated in cell adhesion assays on Jurkat cell line expressing $\alpha_4\beta_1$ integrin,

and K562 cell line expressing $\alpha_5\beta_1$ integrin. Biological tests revealed that only (*S*)-enantiomers maintained the agonist activity of racemates with a nanomolar potency, and a specific enantio-recognition by integrin receptors was demonstrated.

Keywords: Biocatalysis, Kinetic resolution, Integrins, Cell adhesion, lactams, Azetidinones, Agonists

1. Introduction

Since the early discovery by A. Piutti of the enantioselectivity in receptor-mediated biological events [1, 2], the demand for chiral molecules as active pharmaceutical ingredients (APIs) has continuously increased by the pharmaceutical industry because enantiomers could have dramatically different biological activities such as pharmacokinetic, pharmacology or toxicology [3, 4]. Two enantiomers of a molecule could in fact display different spatial distributions on the same receptor or even bind two diverse receptors being responsible of other biological pathways. Thus, it is of paramount importance to know the biological response of single stereoisomers and to separately obtain two enantiomers of a new bioactive molecule. To meet this challenge, a variety of technologies could be applied to the synthesis of chiral APIs, with biocatalysis playing a prominent role [5, 6].

Biocatalysis has nowadays a widespread application in the synthesis of chiral compounds and offers multiple advantages such as the reduction of side-products and synthetic steps because the milder reaction conditions do not require protection/deprotection of sensible functional groups [7, 8]. Enzymes are highly selective catalysts in terms of chemo-, regio-, and stereo-selectivity, and highly specific toward substrates and reactions [9]. Biocatalysis indeed has many attractive features in the context of green chemistry and sustainable development: enzymes derive from renewable resources and are biocompatible, biodegradable, and inherently non-hazardous [10].

Hydrolases are the most studied enzymes and largely used in organic chemistry because of their stability and their no need of cofactors [11, 12]. In addition, these enzymes are commercially available and exploitable not only for hydrolysis or alcoholysis, but also for the reverse condensation reaction. In particular, lipases (EC 3.1.1.3) have gained attention due to their robustness, wide tolerance to different substrates, stability to organic solvents and the possibility to work at high temperatures [13, 14]. Moreover, lipases are inexpensive and available from multiple sources, mainly of microbial origin [15]. Lipases catalyse various processes such as esterification, transesterification, and hydrolysis and are particularly applied to the synthesis of enantiopure compounds *via* kinetic resolutions (KR) [16-18]. Notwithstanding the intrinsic limitation of a maximum theoretical yield of 50% for a single enantiomer, KR processes have special relevance in the development of new bioactive compounds, allowing access to both enantiomers separately so that their specific activities could be singularly acquired.

Recently, we reported the synthesis of a library of new β-lactam compounds (azetidinones) that were specifically designed by a structure-based strategy to target specific classes of integrin receptors [19, 20]. The biological activities of the new azetidinone derivatives were deeply evaluated by investigating their

effects on isolated receptors, integrin-mediated cell adhesion, and cell signalling. Among the new derivatives, some ligands were very active and showed interesting affinities toward specific integrin families. Some molecules were synthesized as pure enantiomers, other as racemic mixtures, and among the latter emerged some very interesting ligands with nanomolar range activities (Fig. 1). Integrins are transmembrane receptors, composed of α and β subunits that bind to specific ligands mediating cell-cell and cell-extracellular matrix interactions. These abilities allow integrins to regulate crucial cellular functions, such as adhesion, migration, growth and survival [21]. Their physiological importance is underlined by several types of pathologies in which integrins play a pivotal role; therefore, integrins are attractive pharmacological targets for the development of new drugs [22, 23].

The aim of the present work is to establish an enzymatic route to obtain single enantiomers of the most active integrin ligands previously developed [19]. Since the β-lactam intermediate **1** was a common building block for the synthesis of all the racemic derivatives, we studied a biocatalytic strategy for obtaining both its enantiomers. Subsequent synthetic elaborations on the two enantiomers of **1** provided 4 enantiopure integrin ligands, whose biological effects were then evaluated in cell adhesion assays on Jurkat cell line expressing $\alpha_4\beta_1$ integrin, and on K562 cell line expressing $\alpha_5\beta_1$ integrin, and compared to the racemic analogues (Scheme 1).

2. Results and Discussion

Initial studies were focused on performing KR directly on β-lactam **1** as ethyl, benzyl or *t*butyl esters (**1a-c**). A small panel of lipases, esterases and proteases, such as Candida Antarctica lipase A (CAL-A), Candida Antarctica lipase B (CAL-B), Pig liver esterase (PLE) and α-chymotrypsin from bovine pancreas were screened (Table 1). Racemic esters **1a-c** were prepared from the commercially available 4-acetoxy-azetidinone with a Reformatsky reagent as previously reported [19, 20].

The enzymatic KR on the three esters **1a-c** was not satisfactory. With CAL A, CAL B, or PLE the process mainly was not chemo-selective, since these enzymes hydrolysed the amide or the ester functions or both, giving mixtures of by-products (entries 1, 2, 5-6, 10, and 11), with a final poor enantiomeric ratio of the unreacted esters. In selected cases the reaction turned to be chemoselective but with no or poor enantioselection (entries 3-4, 7, and 9, respectively) or very low conversion (entry 12). Only α-chymotrypsine provided reasonable results with benzyl ester **1b**, if considering both conversion and enantiomeric ratio (entry 8).

Detection and isolation of the acid **2** from crude mixtures was a difficult issue in all cases because of its no UV absorption above 200 nm and a high solubility in water. Furthermore, when isolated from the aqueous phase through lyophilization, esterification of **2** to afford the ester **1b** (a necessary step to complete the synthesis of the final integrin ligands) failed.

We then changed the synthetic strategy and turned our attention to an alternative substrate to get an enzymatic KR based on a transesterification process instead of a hydrolysis. Kaman *et al.* reported the KR of some β-lactam intermediates with an oxymethylene residue on the β-lactam nitrogen atom [24].

Following this strategy, the racemic compound **1b** was derivatized with an oxymethylene group using paraformaldehyde and catalytic potassium carbonate under sonication (scheme 2) [25]. A screening of some reaction parameters (amounts of paraformaldehyde and K_2CO_3) led to optimized conditions and racemic β-lactam **5** was obtained in quantitative isolated yields with 6% K_2CO_3 in a mixture of THF/H_2O = 11:1 (Scheme 2).

The first screening for the KR of the racemic compound **5** was carried out in *t*butylmethylether (TBME) as solvent with vinyl acetate as acyl donor at room temperature. In general, the use of vinyl esters as acyl donors could prevent the formation of competitive reactions, since the co-product vinyl alcohol tautomerizes to acetaldehyde with a shift toward formation of the products, hence avoiding hydrolysis or nucleophilic attack on the newly formed ester. A panel of commercially available lipases was screened in order to find the best enzyme in terms of activity and stereoselectivity in the formation of the acylated product **6** and in the enantio-enriched substrate **7** (Table 2). The variability in the enzyme amount used in this screening derives from different commercial enzymes formulations. Some of the tested lipases (lipase from porcine pancreas - crude type II, *Rhizopus niveus*, *Candida lipolytica* and lipase from wheat germ) appeared totally inactive with no conversion after 4 days and were not included in the table. The same negative response was observed with α-chymotrypsin and pig liver esterase (crude extract) that were tentatively tested. Lipase from *Candida Antarctica A* showed a higher conversion compared to other enzymes, but no selectivity (Table 2, entry 1). Other lipases (Table 2, entries 2-9) instead displayed low conversions (6-19%) but in some cases interesting enantiomeric excesses: with porcine kidney acylase and *Mucor miehei* lipase the ee% of product **6** was 84 and 87, respectively (Table 2, entries 6, 7). Lipase from *Aspergillus oryzae* displayed a 57% conversion in 4 days (entry 11), but at the same time *Burkholderia cepacia* lipase (*BCL*)(entry 12) afforded higher enantiomeric excess values for the enantioenriched substrate **7**. Lipase from *Pseudomonas fluorescens* (entry 13) displayed a higher conversion in shorter times than *BCL* (24 h *vs* 4 days), demonstrating a high affinity and a good stereospecificity for the substrate **7** that was obtained with a 91 ee%.

From data in Table 2, *BCL* and *Pseudomonas fluorescens* emerged as the most promising enzymes for the KR of the racemic in terms of both conversion and ees **5** and were thus selected for further evaluations. Specifically, a study was carried out for determining the evolution of the enantiomeric excess of substrate and product *vs* conversion; accordingly, the biocatalytic reactions were set up with *BCL* or *Pseudomonas fluorescens* lipase in TBME and monitored over the time. Vinyl acetate and vinyl butyrate were examined as acyl donors in this study, and conversion plots are reported in Figure 2.

BCL showed the best results, allowing the obtainment of the enriched substrate **7** in good ee% at a 60% conversion, whereas the ee% of the ester products was good only using vinyl butyrate to give enantio-enriched butyrate **8** at around 40% conversion. Conversely, *Pseudomonas Fluorescens* led to worse results than *BCL*: vinyl acetate provided a lower selectivity compared to vinyl butyrate, and enantiomeric excesses in the 40-60% conversion range were not satisfying for both substrate and products. Encouraged by the good results achieved with *BCL*, we further investigated the influence of anhydrides as acyl donors *versus* the corresponding vinyl esters on the enantioselectivity of the KR with *BCL* (Table 3). Anhydrides are not so commonly used in kinetic resolutions, but some literature papers reported the use of these agents as acyl donors in the resolution of primary and secondary racemic alcohols [26].

All reactions were conducted in thermostat at 37 °C and stopped after a fixed time of 5.5 hours by filtration of the enzyme and analysed by chiral HPLC. The temperature enhancement from 25 °C (rt) to 37 °C with vinyl acetate allowed a 64% conversion in 5.5 hours (Table 3, entry 1), while a lower value (50%) was found in 4 days at room temperature (see Table 2, entry 12). Moreover, vinyl acetate provided a higher reaction rate among the tested acyl donors (Table 3, entry 1 *vs* 2-4) whereas acetic anhydride displayed too low conversions for being further considered (Table 3, entry 3). Butyric anhydride showed very good results in terms of both selectivity and conversion (Table 3, entry 4); in this case a classic kinetic resolution could be performed: stopping the reaction at 50% conversion allowed to obtain good enantiomeric excesses for both substrate and product, together with the maximum yield (around 50%). Unfortunately, the use of butyric anhydride as acylating agent released an equivalent of butyric acid which was difficult to separate from the enantiomerically enriched substrate **7** and product **8**. For this reason, considering both reaction outcome and product isolation, the results obtained with vinyl butyrate were considered the most promising to allow a scale-up process.

A preparative KR was therefore carried out with vinyl butyrate and *BCL* in order to isolate a larger amount of both the enantio-enriched substrate **7** and the ester **8** with the highest ee values (Scheme 3) [27]. A two-steps enzymatic resolution process [9] was adopted: in the first step the enzymatic transesterification with *BCL* and vinyl butyrate in TBME was stopped at a 38% conversion, the enzyme was filtered off, and the enantio-enriched substrate **7** was separated from product **8** by flash-chromatography obtaining compound **8** with 90% ee and 33% of isolated yield.

The enriched substrate **7** obtained in the first step was then re-processed in a second step under the same conditions (*BCL*, vinyl butyrate in TBME) up to 67% conversion; after the work-up, the reaction mixture was finally purified to isolate substrate **7** with a good enantiomeric excess (94%) and 35% of isolated yields. By the two-steps procedure good amounts of **7** and **8** were thus obtained and allowed determination of the optical rotatory powers (Scheme 3).

The compounds **(+)7** and **(-)8** were then further elaborated to obtain the two enantiomers **(+)9** and **(-)9**, intermediates required for the synthesis of the final chiral integrin ligands. For this purpose, it was necessary to eliminate the oxymethylene residue on the β-lactam nitrogen atom. Some oxidative conditions were tested ($NaClO/NaClO_2$, $TEMPO/NaIO_4$,) [28, 29], but only $KMnO_4$ [30] was successful: reaction on **(+)7** was effective with a complete conversion and good yields affording **(+)9** in 16 hours without any purification needed (Scheme 4).

Transformation of **(-)8** required the hydrolysis of the butanoate ester, but the use of some chemical procedures (K_2CO_3 in methanol, TEA under MW irradiation, trifluoroacetic acid in CH_2Cl_2) to selectively hydrolyse the butanoate ester over the benzylic one, failed, with detection of partial hydrolysis of the benzylic ester or formation of by-products. It was then decided to exploit again the selectivity of the biocatalysis with *BCL* under hydrolytic conditions (Scheme 4).

The process was carried out in MilliQ water at 37 °C with a minimal amount of acetonitrile to dissolve **(-)8** (H_2O/CH_3CN=11:1). The progress of the reaction was again monitored through chiral HPLC. As expected, the system showed a perfect selectivity toward the aliphatic ester *vs* the benzylic one, and alcohol **(-)7** was obtained from ester **(-)8** in only one hour and good isolated yields. Remarkably, a further increase in ee% was obtained: from 90% of ester **(-)8** to a 98% ee of alcohol **(-)7**, due to the enantiomeric selection by the enzyme. Treatment of **(-)7** with $KMnO_4$ in CH_3CN afforded, also in this case, the chiral β-lactam **(-)9** in good yields. The enantiomeric excesses of β-lactams **(+)9** and **(-)9** were completely preserved, as detected by chiral HPLC analysis after optimization of the analytical conditions (see Supplementary Material, Figure S1).
It was then necessary to assign the absolute configuration to the separated enantiomers **(+)9** and **(-)9**, and this was achieved through chemical correlation with a known reference compound(*S*)-2-(4-oxoazetidin-2-yl)acetic acid, already reported in the literature obtained starting from (*S*)-aspartic acid (scheme 5) [31]. The two enantiomers **(+)9** and **(-)9** were thus submitted to hydrogenolysis to obtain the acids **(-)10** and **(+)10**. When comparing the specific rotatory powers of **(-)10** and **(+)10** with that of the reference compound, it was possible to assign the absolute configurations of our acids as **(-)(*R*)10** and **(+)(*S*)-10** and, consequently, the configurations of the benzylic ester precursors as **(+)(*S*)-9** and **(-)(*R*)-9.** The enantio-preference of *BCL* for the (*R)*-configuration was thus confirmed and in line with what reported in the literature [30].

The enantiopure compounds **(+)(*S*)-9** and **(-)(*R*)-9** were then separately subjected to those synthetic steps previously optimized on racemic precursors **rac-A** and **rac-B** and necessary for obtaining the chiral integrin ligands [19, 20]. In scheme 6 the synthetic sequence for the synthesis of four enantiomerically pure integrin ligands **(*S*)-11**, **(*R*)-11**, **(*S*)-12**, and **(*R*)-12** is reported. The synthesis of **(*S*)-12**, and **(*R*)-12** comprised four steps: acylation of the β-lactam nitrogen atom with *o*-tolylisocyanate, hydrogenolysis of the benzyl ester obtaining **(*S*)-11**, and **(*R*)-11**, peptidic coupling with glycine benzylester, and final hydrogenolysis to get the terminal carboxylic acid function. The optical purity was monitored all over the intermediates through chiral HPLC analysis and it was consistently maintained up to the final products **(*S*)-11**, **(*R*)-11**, **(*S*)-12**, and **(*R*)-12**.

3. Pharmacological evaluation

The four enantiomers **(*S*)-11**, **(*R*)-11**, **(*S*)-12**, and **(*R*)-12** were finally tested in integrin-mediated cell adhesion assays. Jurkat cell lines expressing leukocyte integrin $\alpha_4\beta_1$ or K562 expressing $\alpha_5\beta_1$ were adopted for the biological evaluations, since the corresponding racemic derivatives **rac-A** and **rac-B** were selective agonists of $\alpha_4\beta_1$ or $\alpha_5\beta_1$, respectively [19, 20]. Interestingly, only β-lactams **(*S*)-11** and **(*S*)-12** showed a concentration-dependent enhancement of cell adhesion in fibronectin-mediated tests, whilst **(*R*)-11** and **(*R*)-12** were completely inactive (Figure 3). In details, compound **(*S*)-11** showed an EC_{50} of 21 nM towards $\alpha_4\beta_1$ integrin, maintaining the same potency as previously found for **rac-A** (12.9 nM vs $\alpha_4\beta_1$ integrin, Figure 1); moreover, compound **(S)-12** displayed a comparable potency towards $\alpha_5\beta_1$ integrin (EC_{50} = 35 nM vs $\alpha_5\beta_1$ integrin, Figure 3) in accordance with that obtained for **rac-B** (9.9 nM vs $\alpha_5\beta_1$ integrin, Figure 1). The

potency of the racemic compounds was thus not impaired by the presence of inactive (*R*)-enantiomers. The apparent decrease in EC_{50} numerical values for the (*S*)-enantiomers could be due to a not complete optical purity (94% ee). Noteworthy, the (*S*)-enantiomers maintained the agonist activity of the racemates, and a specific enantio-recognition by integrin receptors was then demonstrated.

A comparison between the two active enantiomers **(*S*)-11** and **(*S*)-12** and some β-lactam derivatives previously reported [19], leads to the observation that they are among the most potent agonists for $\alpha_4\beta_1$ or $\alpha_5\beta_1$ integrin, respectively, and that the integrin activity *vs* the (*S*) configuration at the C-4 of the azetidinone is consistent with that observed for chiral (*S*)-β-lactam integrin ligands obtained from L-aspartic acid [19].

3. Conclusion

Biocatalysis has become nowadays a standard tool to produce fine chemicals and its application in the pharmaceutical field heavily dominates. In fact, the use of enzymes in APIs synthesis is becoming an alternative way to obtain enantiomerically pure products leading to many advantages such as mild work conditions, reduction of extrasteps and less or no toxic wastes.

Having in hands a series of new β-lactam compounds being active as integrin ligands [19], most of which racemic at the C4 position of the ring, it was necessary to determine how two enantiomers could differently interact with the receptor in terms of activity as agonists or antagonists, and potency. In this work we evaluated the use of enzyme catalysis to obtain the two enantiomers of a β-lactam intermediate **7**, precursor of novel integrin ligands. Among a series of Lipases, *Burkholderia Cepacia* Lipase (*BCL*) was selected as the most suitable enzyme for the kinetic resolution of the intermediate **7**. After optimization of the biocatalytic protocol (enzymes and acylating agents) and of the synthetic procedures, the two enantiomers **(+)*S*-9** and **(-)*R*-9** were obtained in excellent enantiomeric excesses. Assignment of the absolute configurations was assessed by chemical transformation into a known compound. Synthetic elaboration of **(+)*S*-9** and **(-)*R*-9** allowed the obtainment of four chiral β-lactams **(*S*)-11**, **(*R*)-11, (*S*)-12**, and **(*R*)-12** which were finally evaluated in cell adhesion assays. Pharmacological tests on Jurkat cell line expressing $\alpha_4\beta_1$ integrin and K562 cell line expressing $\alpha_5\beta_1$ integrin, revealed that the activity as agonists with a nanomolar potency was fully maintained only in case of (*S*)-enantiomers, while (*R*)-derivatives were completely inactive, thus revealing an important stereochemical requirement for further developments of the new β-lactam-based integrin ligands.

4. Experimental

4.1 Material and Methods

Anhydrous solvents and reagents were obtained commercially and used as received. Deionized water was obtained from a Millipore analytical deionization system (MilliQ). For TLC monitoring Merck 60 F_{254} plates were used and for liquid chromatography Merck silica gel 200-300 mesh was used. ^{1}H and ^{13}C NMR spectra were recorded with an INOVA 400 instrument with a 5 mm probe. All chemical shifts are quoted relative to deuterated solvent signals (δ in ppm and *J* in Hz). FTIR spectra were recorded with Alpha FT IR Bruker spectrometer. Polarimetric analyses were conducted on Unipol L 1000 Polarimeter at 598 nm. The purities

of the target compounds were assessed as being >95% using HPLC-MS. HPLC-MS: Agilent Technologies HP1100 instrument, equipped with a ZOBRAX-Eclipse XDB-C8 Agilent Technologies column; mobile phase: H_2O/CH_3CN, 0.4 mL/min, gradient from 30 to 80% of CH_3CN in 8 min, 80% of CH_3CN until 25 min, coupled with an Agilent Technologies MSD1100 single-quadrupole mass spectrometer, full scan mode from m/z = 50 to 2600, in positive ion mode, ESI spray voltage 4500 V, nitrogen gas 35psi, drying gas flow 11.5mL/min, fragmentor voltage 20 V. Chiral-HPLC: Agilent Technologies 1200 instrument equipped with a diode array UV detector on Daicel Chiralcel columns (0.46 cm I.D. x 25 cm) with HPLC grade isopropanol and *n*-hexane as eluting solvents.

The employed enzymes were: *Burkholderia Cepacia* lipase (*BCL*) powder, ≥30 U/mg by Sigma Aldrich; *Candida antartica* lipase A (CAL-A), CLEA (Cross Linked Enzyme Aggregate), ≥10 U/mg, by Fluka (Sigma Aldrich); Lipase crude from porcine pancreas, 187 U/mg, by Sigma Aldrich; Acylase from porcine kidney, powder, 52 U/mg, by Serva Biochemical; Alpha-chymotripsine from bovine pancreas, powder, 42 U/mg, by Serva Biochemical; Esterase crude from porcine liver, powder, 19 U/mg, by Sigma Aldrich; Lipase from *Aspergillus niger*, powder, 0.94 U/mg, by Fluka; Lipase from hog pancreas, powder, 27 U/mg, by Fluka; Lipase from *Pseudomonas fluorescens*, powder, 36 U/mg, by Fluka; Lipase from *Rhizopus niveus*, powder, 1.7 U/mg, by Fluka; Lipase from *Rhizopus arrhizus*, powder, 2.2 U/mg, by Fluka; Lipase from *Mucor miehei*, powder, 1.4 U/mg, by Fluka; Lipase from *Candida cylindracea*, powder, 2.8 U/mg, by Fluka; Lipase from *Aspergillus oryzae*, powder, 48 U/mg, by Fluka; Lipase from *Penicillium roqueforti*, powder, 0.65 U/mg, by Fluka; Lipase from wheat germ, powder, 0.10 U/mg, by Fluka; Lipoprotein Lipase from *Chromobacterium viscosum*, powder, 2962 U/mg, by Fluka; Lipase from *Mucor javanicus*, powder, 9.9 U/mg, by Fluka; Lipase from *Candida lipolytica*, powder, 1.1 U/mg, by Fluka; Lipase From *Candida antartica B,* immobilized on acrylic resin, 10 U/mg, by Sigma Aldrich.

4.2 Synthesis of racemic β-lactams

Compounds **1a-c** were synthesized as previously reported [19] and their spectroscopic data are in accordance with those reported. Preparation of racemic esters **(±)-6** and **(±)-8** for the optimization of an analytic method by chiral HPLC was reported in the Supplementary Material.

Benzyl 2-(1-(hydroxymethyl)-4-oxoazetidin-2-yl)acetate (**5**)
To a solution of compound **1b** (55 mg, 0.25 mmol, 1 equiv) in THF (0.55 mL), paraformaldehyde (11 mg, 0.38 mmol, 1.5 equiv), K_2CO_3 (2 mg, 0.01 mmol, 0.3 equiv) and water (22 µL) were added. The system was sonicated at room temperature. At completion (4 h, TLC monitoring), the reaction mixture was diluted with EtOAc (2.5 mL), dried on anhydrous Na_2SO_4, filtered and concentrated. The crude was purified by flash-chromatography (cyclohexane/EtOAc 35:65) yielding compound **5** as a colorless oil (62 mg, 99%).
IR (film, cm^{-1}) 3406, 3033, 2953, 1736, 1397, 1175; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.41 – 7.28 (m, 5H), 5.15 (s, 2H), 4.64 (d, J_{AB} = 11.6 Hz, 1H), 4.60 (d, J_{AB} = 11.6 Hz, 1H), 4.16 – 4.04 (m, 1H), 3.15 – 3.08 (dd, *J* = 5.3, 15.0 Hz 1H), 2.86 – 2.71 (m, 2H), 2.70 – 2.64 (dd, *J* = 2.4, 15.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 171.1, 166.6, 135.2, 128.6, 128.5, 128.3, 66.9, 64.1, 47.0, 42.6, 38.7; ESI-MS (R_t = 4.4 min) *m/z* 232 $[M\text{-}OH]^+$.

4.3 Synthesis of enantio-enriched β-lactam compounds

*4.3.1. Enzymatic KR of esters **1a-c** (data Table 1):*
In a glass vial with a screw cap, to a solution of ester **1a-c** (0.1 mmol) in 4 mL of phosphate buffer 0.1 M pH 7.5, the desired enzyme was added (in the amount specified in Table 1). The mixture was stirred at room

temperature under orbital shaking (700 rpm). The reaction was monitored at set time intervals with chiral HPLC (reaction times specified in Table 1) for evaluate the substrate conversion and the enantiomeric excess of unreacted esters **1a-c**. At the end of the reaction, the residual solution was extracted with CH_2Cl_2 (3 × 5 mL), dried on anhydrous Na_2SO_4, filtered and concentrated. The aqueous phase was lyophilized and the ratio of compounds **2**/**3**/**4** was analysed *via* ^{1}H NMR.

*4.3.2. Enzymatic KR of compound **5***

Method A: in a glass vial with a screw cap, to a solution of alcohol **5** (20 mg, 0.08 mmol, 1 equiv), acyl donor (6 equiv) and solvent (2 mL), the desired enzyme was added (in the amount specified in Tables 2 and 3). The mixture was stirred at room temperature under orbital shaking (700 rpm) – as specified in Table 2 - or at 37 °C under magnetic stirring – as specified in Table 3. The reaction was monitored at set time intervals with chiral HPLC (reaction times specified in Tables 2 and 3) for evaluate the substrate conversion and the enantiomeric excesses of unreacted alcohol **7** and ester products **6** or **8**.

Method B: With the best conditions (TBME as solvent, *BCL* as enzyme, vinyl butyrate as acyl donor), the kinetic resolution was scaled-up: alcohol **5** (150 mg, 0.6 mmol, 1 equiv), vinyl butyrate (457 µL, 3.6 mmol, 6 equiv), TBME (15 mL) and *BCL* (150 mg) were reacted at room temperature in a 30 mL vial with a screw cap. At 38% conversion (chiral HPLC monitoring), solvent was evaporated and the crude was directly purified by flash-chromatography (cyclohexane/EtOAc 40:60) to separate target ester **(-)8** as a colorless oil (60 mg, 33%, ee = 90%, $[\alpha]_D^{20}$ = – 15 (c = 1.0, CH_2Cl_2)) and residual alcohol **(+)7**, which was re-processed in the same reaction conditions. At 67% conversion, work-up and purification were carried out as described above, and alcohol **(+)7** was isolated as a colorless oil (51 mg, 35%, ee = 94%, $[\alpha]_D^{20}$ = + 13 (c = 1.0, CH_2Cl_2)).
Spectroscopic data of **(+)7** and **(-)8** were in fully accordance with those reported for their corresponding racemic analogues **5** and **(±)8** (Supplementary Material).

4.3.3. Enzymatic hydrolysis of compound **(-)8**
In a 10 mL vial with a screw cap, to a solution of ester **(-)8** (200 mg, 0.8 mmol, 1 equiv) CH_3CN and MilliQ H_2O (1:11 ratio, total volume 20 mL), *Burkolderia Cepacia* lipase was added (200 mg). The mixture was stirred at 37 °C in thermostat. At completion (1 h, chiral HPLC monitoring), the aqueous mixture was extracted with EtOAc (3 × 10 mL). The collected organic phases were dried over Na_2SO_4, filtered and concentrated under vacuum. The crude was purified by flash-chromatography (cyclohexane/EtOAc 40:60) to yield target alcohol **(-)7** as a colorless oil (75%, ee = 98%, $[\alpha]_D^{20}$ = – 16 (c = 1.0, CH_2Cl_2)). Spectroscopic data of **(-)7** were in fully accordance with those reported for its corresponding racemic analogue **5**.

4.3.4. Oxidation with $KMnO_4$ of the enantiomers **(+)7** or **(-)7**
To a solution of the alcoholic compound **(+)7** or **(-)7** (1 equiv) in CH_3CN (30 mL/mmol), $KMnO_4$ (6 equiv) was added portionwise at 0°C. The system was then warmed to room temperature and left under stirring overnight. At completion (16 h, TLC monitoring), the reaction mixture was quenched with a saturated solution of $Na_2S_2O_5$ until complete decoloring. The mixture was then filtered and the acetonitrile evaporated under reduced pressure. The residual aqueous solution was then extracted with EtOAc (3 × 10 mL), dried on anhydrous Na_2SO_4, filtered and concentrated. The desired product **(+)9** or **(-)9** was obtained without further purification as a white solid.
(+)9: 91%, ee = 94%, $[\alpha]_D^{20}$ = + 26 (c = 1.0, CH_2Cl_2)
(-)9: 90%, ee = 98%, $[\alpha]_D^{20}$ = – 26 (c = 1.0, CH_2Cl_2).
Spectroscopic data of **(+)9** and **(-)9** were in fully accordance with those reported for their corresponding racemic analogues [19].

*4.3.5. Synthesis of compounds **(S)10**, **(R)10**, **(S)11**, **(R)11**, **(S)12**, and **(R)12***

Compounds **(*S*)10**, **(*R*)10**, **(*S*)11**, **(*R*)11**, **(*S*)12**, and **(*R*)12** were synthesized according to the previously reported procedures [19]; yields and spectroscopic data were in fully accordance with those reported for their corresponding racemic analogues [19].

4.4. Analytical methods

The enantiomeric composition and conversions of azetidinones were determined by chiral HPLC analysis and established at 210 nm. To evaluate the conversion, a chromatographic analysis was conducted also at time 0, i.e. before inserting the enzyme into the reaction; areas of the substrate chromatographic peaks at time 0 were then compared with those, progressively decreased, found over the time.

Samples for HPLC injection were prepared as follows:

Transesterification reactions:

- in case of explorative reactions: 50 µL of the reaction mixture were sampled and the solvent was evaporated under air flux. Then, the mixture was suspended in 350 µL of a solution of *n*-hexane/isopropanol 1:1, filtered through regenerated cellulose syringe filters (diameter = 25 mm, pore diameter = 0.2 µm) and injected in chiral HPLC.
- in case of preparative reactions: 0.5 mL of the reaction mixture was filtered through regenerated cellulose syringe filters (diameter = 25 mm, pore diameter = 0.2 µm); the organic solvent was dried, re-suspended in a solution of *n*-hexane/isopropanol 1:1 and directly injected in chiral HPLC.

Hydrolysis reactions:

- 0.5 mL of the reaction mixture was extracted with EtOAc (0.5 mL); the organic solvent was dried, re-suspended in a solution of *n*-hexane/isopropanol 1:1 and directly injected in chiral HPLC

In case of kinetic resolution performed on alcohol **5**, we determined the best analytical conditions in order to verify in a single HPLC analysis both the reaction conversion and the enantiomeric excesses of the residual substrate **7** and the formed product **6** or **8** (see Supplementary Material, Figure S2).

Elution conditions:

Compounds **1a-c**: Column: Chiralpak IA, solvent: *n*-hexane/isopropanol 80:20, flow: 0.5 mL/min, temperature: 40 °C.

Compounds **5**-**8**: Column: Chiralpak IC, solvent: *n*-hexane/isopropanol 50:50, flow: 0.5 mL/min from 0 to 18 min, 1 mL/min from 19 min, temperature: 40 °C.

Compound **9**: Column: Chiralpak IC, solvent: *n*-hexane/isopropanol 60:40, flow: 0.5 mL/min, temperature: 40 °C.

4.5. Cell culture

Jurkat E6.1 and K562 cell lines were routinely grown in RPMI-1640 (Thermofisher) and glutamine with 10% FBS. Cells were kept at 37°C under 5% CO_2 humidified atmosphere; 40 h prior to experiments, K562 cells were treated with 25 ng/mL PMA (Phorbol 12-myristate 13-acetate, Sigma Aldrich SRL, Milan, Italy) in order to prompt differentiation and to augment cell surface $\alpha_5\beta_1$ integrin expression. These two cell lines were employed as a suitable model to study integrins as Jurkat cells express $\alpha_4\beta_1$ integrin, while K562 cells express $\alpha_5\beta_1$ integrin. Both cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

4.6. Cell adhesion assays

The assays were performed as previously described in ref. 19, 20, 32, and 33. Briefly, for adhesion assay on K562 cells 96-well plates (Corning Costar, Celbio, Milan, Italy) were coated with fibronectin (FN, 10 µg/mL) overnight at 4 °C. K562 cells were pre-incubated with several concentrations of each compound or with the vehicle (methanol) for 30 min at room temperature, plated (50,000 cells/well) on fibronectin coated wells and incubated at room temperature for 1 h. After three washes with 1% BSA (bovine serum albumin) in PBS (phosphate-buffered saline) to remove non-adherent cells, 50 µL hexosaminidase substrate were added to each well. Afterwards 100 µL of stopping solution were used to stop the enzymatic reaction and absorbance was measured at 405 nm in a Victor^2 Multilabel Counter (PerkinElmer, Waltham, MA, USA). For adhesion assays on Jurkat E6.1 cells, black 96-well plates were coated overnight at 4 °C with VCAM-1 (5 µg/mL). Cells were stained with CellTracker green CMFDA (12.5 µM, 30 min at 37 °C, ThermoFisher). Jurkat E6.1 cells were pre-incubated with various concentrations of each compound or with the vehicle (methanol) for 30 min at 37°C and then plated (500,000/well) on VCAM-1-coated wells for 30 min at 37 °C. After three washes, Jurkat E6.1 cells were lysed with 0.5% Triton X-100 in PBS (30 min at 4 °C) and fluorescence was measured (Ex485 nm/Em535 nm).
Experiments were repeated at least three times and carried out in quadruplicate. Data analysis and EC_{50} values were calculated using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

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Fig. 1. β-Lactam–based ligands evaluated as racemic mixtures, and EC_{50} values as agonists of specific integrins (in brackets).

Fig. 2. KR of racemic β-lactam **5** with *Burkolderia Cepacea* lipase (*BCL*) or *Pseudomonas fluorescen,* vinyl acetate or vinyl butyrate were used as acyldonors. The configurations reported in the scheme for **6**, **7**, and **8** were assigned as described in the text. Plots of enantiomeric excesses of enantio-enriched substrate **7** (blue) and acetate **6** (red) or butyrate **8** (green) vs conversion are depicted.

Fig. 3. Effects of β-lactams **(*S*)-11** and **(*R*)-11** in cell adhesion assays on Jurkat cell line expressing $\alpha_4\beta_1$ integrin, and **(*S*)-12**, and **(*R*)-12** on K562 cell line expressing $\alpha_5\beta_1$ integrin. Concentration-response curve showing the effects of **(*S*)-11**, **(*R*)-11**, **(*S*)-12**, and **(*R*)-12** on cell adhesion are shown. Results are expressed as the number of cells attached ±S.E.M from quadruplicate wells and repeated at least three times. EC_{50} values ± SD are reported on the corresponding curves.

Scheme 1. Synthetic strategy to obtain enantiopure azetidinones to be evaluated as integrin ligands through enzymatic KR.

Scheme 2. *N-o*xymethylene insertion to obtain **5**.

Scheme 3. Results of the kinetic resolution of racemic β-lactam **5**. Reagents and conditions: a) *BCL*, vinyl butyrate, TBME, 37 °C. Isolated yields, ee%, and specific optical rotatory powers are reported.

Scheme 4. Synthesis of β-lactams enantiomers **(+)9** and **(-)9**. Reagents and conditions: a) $KMnO_4$, CH_3CN, 0 °C then rt, 16 h, b) *BCL*, H_2O/CH_3CN 11:1, 37 °C, 1 h. Isolated yields, ee%, and specific optical rotatory powers are reported.

Scheme 5. Synthetic elaborations for the assignment of absolute configurations. Hydrogenolysis of **(+)9** and **(-)9** to obtain **(+)10** and **(-)10**. Assignment of the absolute configurations by comparison with the reference compound. Reagents and conditions: a) H_2, Pd/C (10%), THF/CH_3OH 1:1, rt, 2 h.

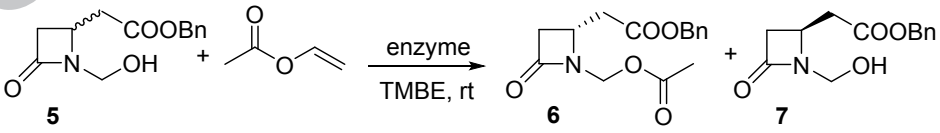
Scheme 6. Synthetic sequence for the obtainment of the enantiomerically pure ligands **(*S*)-11**, **(*R*)-11**, **(*S*)-12**, and **(*R*)-12**. Reagents and conditions: a) K_2CO_3, *o*-tolylisocyanate, CH_3CN, rt, 2 h; b) H_2, Pd/C (10%), THF/CH_3OH 1:1, rt, 2 h; c) EDC, TEA, DMAP, glycine benzylester·PTSA, CH_2Cl_2, 0 °C then rt, 16 h.

Table 1. Enzymatic KR of the racemic compound **1** as ethyl, benzyl, or *t*butyl esters.[a]

			\fx			
Entry	Compound (R)	enzyme	Enzyme amount (U)	Conversion % (time)	**2**/**3**/**4**[b]	**1** er[c]
1	**1a** (Et)	CAL A	45	93 (1h)	40/0/60	60/40
2	**1a** (Et)	CAL B	150	82 (5h)	37/0/63	65/35
3	**1a** (Et)	PLE	170	100 (1h)	100/0/0	-
4	**1a** (Et)	α-chym	84	68 (5h)	100/0/0	-
5	**1b** (Bn)	CAL A	15	95 (1h)	72/0/28	53/47
6	**1b** (Bn)	CAL B	150	97 (24h)	68/0/32	46/54
7	**1b** (Bn)	PLE	34	100 (5h)	100/0/0	-
8	**1b** (Bn)	α-Chym	84	41 (24h)	100/0/0	13/87
9	**1c** (*t*Bu)	CAL A	45	49(1h)	100/0/0	36/63
10	**1c** (*t*Bu)	CAL B	150	36 (24 h)	72/28/0	50/50
11	**1c** (*t*Bu)	PLE	170	43 (1h)	18/82/0	82/12
12	**1c** (*t*Bu)	α-Chym	84	15 (24 h)	traces/0/0	

[a] Reaction conditions: ester **1a-c** (0.1 mmol), PBS 0.1M pH 7.5 (4 mL), enzyme (U in table), rt
[b] Determined by ^{1}H NMR of aqueous phases after solvent extraction and lyophilization.
[c] enantiomeric ratio (er) of the unreacted **1a-c** determined by chiral HPLC.

Table 2. Screening of lipases in the KR of racemic β-lactam **5**.[a]



5 **6** **7**

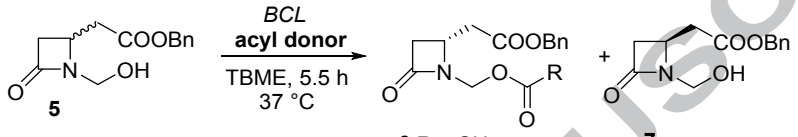
Entry	Lipases	Enzyme amount (U)	Time	Conv. (%)[b]	6 ee%[b]	7 ee%[b]
1	*CaL A* (CLEA)	30	1 h	43	29	28
2	*Mucor javanicus*	60	4 d	6	79	6
3	*Penicillium roqueforti*	6.5	4 d	7	62	6
4	*Aspergillus niger*	4.5	24 h	10	59	8
5	*Rhizopus arrhizus*	22	24 h	14	77	17
6	*Porcine kidney acylase*	300	44 h	15	84	21
7	*Mucor miehei*	14	24 h	18	87	24
8	*Hog pancreas*	135	24 h	19	79	18
9	*Candida cylindracea*	28	24 h	19	44	13
10	*Chromobacterium viscosum*	2962	4 d	26	39	20

11	*Aspergillus oryzae*	200	4 d	57	75	45
12	*Burkholderia cepacia L* (Amano PS)	30	4 d	50	68	89
13	*Pseudomonas fluorescens*	180	24 h	70	31	91

[a] Reaction conditions: compound **5** (20 mg, 1 equiv), TBME (2 mL), vinyl acetate (6 equiv), enzyme (U in table), rt

[b] Determined by chiral HPLC analysis. The configurations reported in the table scheme for **6** and **7** were assigned as described in the text.

Table 3. Kinetic resolution of β-lactam **5** with different acyl donors in TBME at 37 °C.[a]



Entry	Acyl donor	Conversion (%)[b]	7 (ee%)[b]	Products (ee %)
1	Vinyl acetate	64	88	**6** (61)
2	Vinyl butyrate	46	61	**8** (90)
3	Acetic anhydride	12	26	**6** (74)
4	Butyric anhydride	53	91	**8** (87)

[a] Reaction conditions: compound **5** (20 mg, 1 equiv), TBME (2 mL), acyl donor (6 equiv), *BCL* (20 mg), 37°C, 5.5 h

[b] Determined by chiral HPLC analysis and the configurations reported in the table scheme for **6, 7** and **8** were assigned as described in the text.