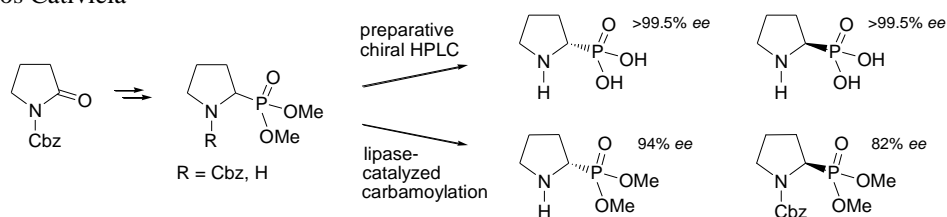


## Graphical Abstract

### Enzymatic and chromatographic resolution procedures applied to the synthesis of the phosphoproline enantiomers

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Alicia Arizpe, María Rodríguez-Mata, Francisco J. Sayago, María J. Pueyo, Vicente Gotor, Ana I. Jiménez, Vicente Gotor-Fernández\*, Carlos Cativiela\*







## Enzymatic and chromatographic resolution procedures applied to the synthesis of the phosphoproline enantiomers

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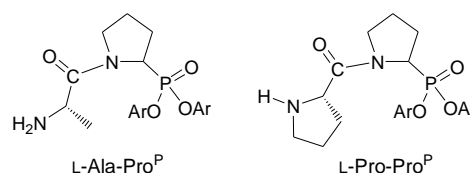
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**Abstract**—The preparation of enantiomerically pure pyrrolidine-2-phosphonic acid (phosphoproline, Pro<sup>P</sup>) has been addressed through the synthesis of adequate racemates and subsequent resolution by independent enzyme-catalyzed and chiral HPLC methods. First, racemic phosphoproline derivatives bearing the adequate protecting groups have been synthesized in excellent global yield starting from inexpensive materials. Preparative HPLC resolution of the *N*-Cbz-protected aminophosphonate on a cellulose-based column has allowed the isolation of optically pure enantiomers in the gram scale. Enzyme-catalyzed alkoxyacylation of the aminophosphonate was studied using different lipases, solvents and carbonates. *Candida antarctica* lipase type A (CAL-A) provided the highest enantioselectivity when combined with benzyl 3-methoxyphenyl carbonate. © 2017 Elsevier Science. All rights reserved.

### 1. Introduction

$\alpha$ -Aminophosphonic acids, the phosphonic counterparts of  $\alpha$ -amino acids in which the carboxylic acid function has been replaced by a phosphonic acid, display a wide variety of biological activities that range from agrochemistry to medicine.<sup>1,2</sup> Of particular relevance in the medicinal field is the finding that small peptides containing a *C*-terminal  $\alpha$ -aminophosphonate are inhibitors of serine proteases.<sup>1–3</sup> A remarkable feature is that such peptide phosphonates do not react with the active site of threonine, cysteine, or aspartyl proteases and are, therefore, specific inhibitors of serine proteases.<sup>4</sup> Moreover, modification of the terminal  $\alpha$ -aminophosphonate moiety and/or the preceding  $\alpha$ -amino acid residue(s) allows for considerable selectivity to target a particular serine protease.<sup>2,3</sup> Thus, dipeptides containing phosphoproline at the *C*-terminus (Xaa-Pro<sup>P</sup>, like those in Figure 1) have been shown to be selective irreversible inhibitors of dipeptidyl peptidase IV (DPP-IV) and closely related serine proteases [DPP-II, DPP8, DPP9, fibroblast activation protein (FAP)].<sup>2,3,5</sup> These serine proteases cleave dipeptide fragments from the *N*-terminus of polypeptide chains with great specificity after a proline residue and are involved in the metabolism of peptide hormones that regulate essential physiological processes.<sup>6</sup> As a consequence, inhibitors of these dipeptidyl peptidases are being considered as novel drug candidates for the treatment

of prevalent diseases like diabetes, immunological disorders, or cancer.<sup>1–3,5,7</sup>



**Figure 1.** Structure of dipeptides shown to be specific inhibitors of the serine protease DPP-IV. Note that the phosphoproline residue is racemic.

A feature common to the inhibition assays of DPP-IV and related serine proteases by phosphoproline-containing dipeptides is the use of diastereomeric mixtures.<sup>5</sup> Thus, for these studies, racemic phosphoproline was coupled to the corresponding *L*- $\alpha$ -amino acid and the mixture of diastereomeric dipeptides formed was used as such for biological evaluation.<sup>5</sup> Only exceptionally have the peptide diastereomers been separated and tested as pure compounds.<sup>5e,g,h</sup> This fact highlights the need for synthetic methodologies that provide access to the phosphoproline enantiomers in an efficient way and sufficient quantities. Ready availability of enantiopure phosphoproline derivatives is also essential for the development of applications for this structurally singular  $\alpha$ -

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aminophosphonic acid (as is proline within proteinogenic amino acids) in other fields such as asymmetric organometallic catalysis<sup>8</sup> and organocatalysis.<sup>9</sup>

Notwithstanding recent advances in the synthesis of  $\alpha$ -aminophosphonic acids,<sup>1,2d,10</sup> the development of efficient methodologies that provide access to optically pure phosphoprolines remains a challenge. Actually, it is a subject of current interest, as evidenced by the fact that about half of the papers describing the isolation of the title compound in enantiopure or enantioenriched form<sup>11</sup> (through either stereoselective syntheses or resolution procedures) have been published in the last few years.<sup>11a-d</sup> Moreover, it is only recently that the absolute configuration of the phosphoprolines enantiomers has been established in an unambiguous manner.<sup>11c</sup> The most successful diastereoselective procedures reported involve the addition of triethyl phosphite to a chiral oxazopyrrolidine bearing a benzotriazol moiety<sup>11g</sup> or the addition of lithium diethyl phosphite to a chiral *N*-sulfinyl imine.<sup>11e</sup> An enantioselective approach has been described involving a Michael reaction between a phosphoglycine imine and benzyl acrylate in the presence of a chiral phase-transfer catalyst to furnish an (*S*)-phosphoprolines precursor with 85% *ee*.<sup>11b</sup> Chemical resolution methods were applied in early studies to isolate (*R*)- and (*S*)-phosphoprolines by coupling the racemate with *L*-leucine and separation of the diastereomeric dipeptides by crystallization, followed by hydrolysis and purification.<sup>11i</sup> In a recent report, the phosphoprolines enantiomers were isolated by acylation with dibenzoyl-*L*-tartaric anhydride, separation of the diastereomeric amides formed by column chromatography and hydrolysis.<sup>11a</sup> Regarding resolution by enzymatic methods, enantiopure (*R*)-phosphoprolines was obtained by kinetic resolution of a  $\delta$ -bromo- $\alpha$ -(chloroacetoxy)butylphosphonate with a protease and further elaboration of the bromide and chloroacetoxy moieties and cyclization.<sup>11c</sup> In the present work, we have addressed the preparation of both phosphoprolines enantiomers by resolution of a cyclic precursor using either enzymatic or chromatographic (chiral HPLC) procedures.

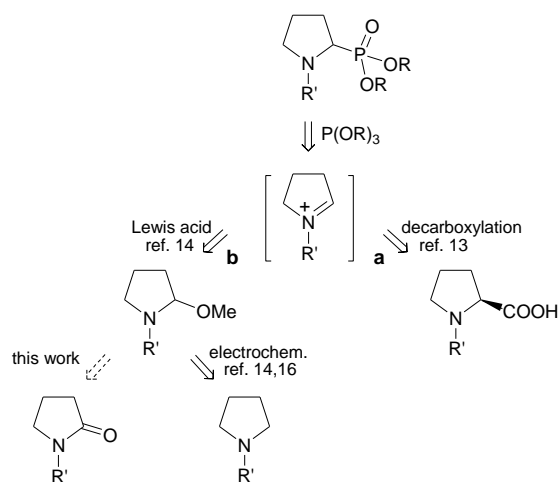
## 2. Results and Discussion

### 2.1. Synthesis of racemic phosphoprolines derivatives

The success of resolution procedures applied to the production of enantiomerically pure compounds relies, to a large extent, on the stage of the synthetic route selected for enantiomer separation. At best, the transformations performed on the valuable enantiomerically pure material obtained should be reduced to a minimum and proceed with high yields under conditions that do not compromise the chiral integrity of the stereogenic centers. With this idea in mind, we envisaged the resolution of a racemic phosphoprolines precursor in which the pyrrolidine ring is already formed and the amino and phosphonic acid functions are already present and, if necessary, protected with groups that can be cleaved smoothly at a final step. This would allow the isolation of the target enantiopure  $\alpha$ -

aminophosphonic acid with minimal transformation after the resolution step.

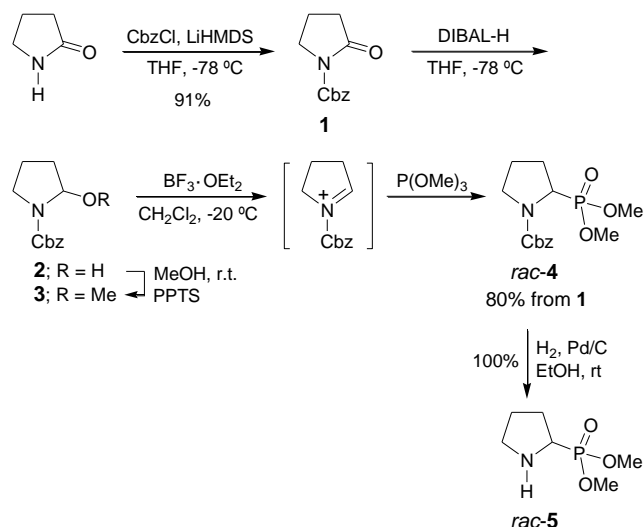
Accordingly, the first aim of the work was the preparation of the appropriate racemic phosphoprolines precursor in an efficient manner. Although racemic phosphoprolines (and small peptides containing it) has been obtained by cyclization of open-chain derivatives,<sup>5h,12</sup> most of the reported routes make use of starting compounds with a preformed pyrrolidine ring.<sup>13-15</sup> In the majority of the latter cases, the phosphonate moiety was introduced by reaction of an alkyl/aryl phosphite with an iminium ion<sup>13,14</sup> (Scheme 1), which in turn was generated either by decarboxylation of *N*-substituted prolines under a variety of conditions<sup>13</sup> (Scheme 1, path a) or by addition of a Lewis acid to *N*-protected 2-methoxypyrrrolidines<sup>14</sup> (Scheme 1, path b). The latter compounds can be prepared by electrochemical methoxylation (Scheme 1), as first reported by Shono.<sup>14,16</sup>



**Scheme 1.** Reported routes for the synthesis of racemic phosphoprolines through an intermediate iminium ion. The contribution of the present work is indicated.

We have proposed<sup>17</sup>  $\gamma$ -lactams as alternative starting compounds for the convenient preparation of the abovementioned 2-methoxylated pyrrolidines (Scheme 1). The use of lactams as precursors of cyclic  $\alpha$ -aminophosphonic acids offers several advantages. First, it avoids the use of electrochemical methods, which is not straightforward in laboratories of synthetic organic chemistry. Moreover, lactams can be easily transformed into the desired  $\alpha$ -aminophosphonates without isolation of any synthetic intermediate.<sup>17</sup> Lactams of different ring size and bearing diverse substituents are commercial or easily available, thus allowing access to a wide variety of cyclic  $\alpha$ -aminophosphonic acids. Most importantly, transformation of the lactam carbonyl into a methoxy group ensures full regiochemical control when substituents are present, whereas electrochemical methoxylation may afford mixtures of 2- and 5-methoxylated pyrrolidines. These advantages have already been demonstrated in the synthesis of substituted phosphoprolines analogues.<sup>17</sup>

Accordingly, pyrrolidin-2-one (Scheme 2) was the substrate of choice for the preparation of the racemic phosphoprolin derivative to be subsequently resolved by enzymatic or chromatographic methods. The presence of an *N*-acyl group is a prerequisite for subsequent transformation into an  $\alpha$ -aminophosphonate as outlined in Scheme 1. An acyl substituent of the carbamate type, namely the benzyloxycarbonyl (*Z* or Cbz) group, was selected for this purpose because final deprotection to liberate the amino function can be accomplished under mild conditions. Moreover, the aromatic unit in Cbz should facilitate UV detection in the HPLC experiments.



**Scheme 2.** Synthesis of racemic phosphoprolin derivatives to be used in resolution processes.

The Cbz group was introduced by reaction of pyrrolidin-2-one with lithium bis(trimethylsilyl)amide and benzyl chloroformate (Scheme 2) to provide the *N*-Cbz protected pyrrolidinone **1**. Compound **1** is also available from commercial sources albeit at a higher price than inexpensive pyrrolidin-2-one. The carbonyl group in **1** was reduced with diisobutylaluminium hydride to give **2**. The unstable hemiaminal was immediately treated with catalytic pyridinium *p*-toluenesulfonate (PPTS) in methanol to form the methoxyaminal **3**, which was not isolated but directly reacted with trimethyl phosphite in the presence of boron trifluoride-diethyl ether. The latter step proceeded via an intermediate *N*-acyliminium and provided the desired *N*-Cbz-protected  $\alpha$ -aminophosphonate *rac*-**4** in 80% overall yield from **1** (Scheme 2). This result highlights the utility of  $\gamma$ -lactams as precursors of the pyrrolidine-2-phosphonate moiety. In particular, the preparation of **3** from lactam **1** proceeds in a much superior yield to that reported for the synthesis of similar methoxylated *N*-carbamoyl pyrrolidines by electrochemical methods.<sup>14,16</sup>

The synthetic route in Scheme 2 allowed us to isolate the phosphoprolin derivative *rac*-**4** in high overall yield and multi-gram quantities from inexpensive pyrrolidin-2-one and with minimal purification of intermediate compounds (none purification step if starting from commercial **1**).

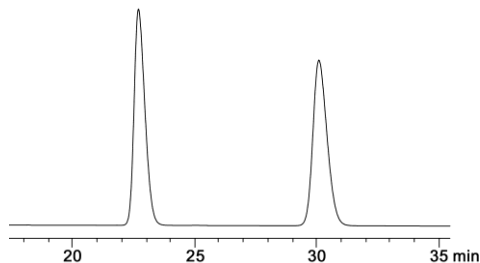
Compound *rac*-**4**, with protected amino and phosphonic acid functions, is a suitable candidate for preparative HPLC resolution. For the enzymatic assays, *rac*-**4** was quantitatively transformed into *rac*-**5** by catalytic hydrogenation under standard conditions (Scheme 2). This aminophosphonate should not be stored for long periods of time and was prepared in small quantities.

## 2.2. HPLC resolution

Based on our previous experience in the preparative-scale HPLC enantioseparation of protected non-natural  $\alpha$ -amino acids,<sup>18</sup> we addressed the resolution of *rac*-**4** by chromatographic methods using the polysaccharide-based columns Chiralpak® IA,<sup>19a</sup> IB,<sup>19b</sup> and IC.<sup>19c</sup> These stationary phases combine the excellent chiral recognition ability typical of polysaccharide-derived phases with high chemical stability.<sup>19–21</sup> The latter feature stems from the covalent bonding of the chiral selector to the silica matrix, which makes these so-called *immobilized* Chiralpak® columns compatible with all organic solvents<sup>19–21</sup> at variance with their *non-immobilized* counterparts, for which only hydrocarbon/alcohol mixtures can be used. This distinctiveness may not be important for analytical assays but is most often crucial for preparative purposes.<sup>18,21</sup> In fact, the success of all our previously reported preparative separations on immobilized polysaccharide-based columns<sup>18</sup> relied on the use of chloroform or dichloromethane to dissolve the sample at very high concentrations (typically, 500 mg of sample per mL of solvent or above) and, in most cases, also on the presence of chloroform, ethyl acetate, acetone, or *tert*-butyl methyl ether (TBME) in the eluent. All preparative HPLC enantioseparations previously carried out in our laboratories<sup>18</sup> were performed on  $\alpha$ -amino acids of the carboxylic type, the present work being the first occasion on which the preparative HPLC resolution of an  $\alpha$ -amino acid of phosphonic nature is undertaken. It is worth mentioning that a recent HPLC analysis<sup>22</sup> of almost 30  $\alpha$ -aminophosphonic acids with different structures using two chiral cinchona-based zwitterionic phases, Chiralpak® ZWIX(+) and ZWIX(–), reached baseline enantioseparation in both columns for all compounds tested except for phosphoprolin and its six-membered ring homologue, which were not separated.

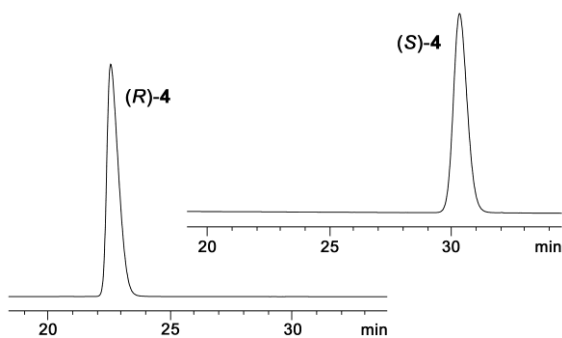
The resolution of *rac*-**4** was first tested at the analytical level using 250 × 4.6 mm Chiralpak® IA, IB and IC columns and eluting with *n*-hexane/2-propanol mixtures. None or very poor resolution was observed on the IA or IB stationary phases whereas Chiralpak® IC showed excellent enantiodiscrimination ability. The effect produced by the addition of a third component to the elution mixture was evaluated for the IC column. Among those tested, TBME proved highly detrimental to the enantioseparation, whereas acetone had the opposite effect. Moreover, elution with *n*-hexane/acetone instead of ternary *n*-hexane/2-propanol/acetone mixtures further improved the selectivity and resolution factors, and was also beneficial to the sample solubility. The analytical conditions finally selected

for further extension to the preparative-scale resolution of *rac*-**4** were eluted with a 72:28 mixture of *n*-hexane/acetone on Chiralpak<sup>®</sup> IC. The chromatographic profile obtained under these conditions is shown in Figure 2.



**Figure 2.** HPLC analytical resolution of *rac*-**4**. Column: Chiralpak<sup>®</sup> IC (250 × 4.6 mm). Eluent: *n*-hexane/acetone 72:28. Flow rate: 1.0 mL/min. UV detection: 210 nm.

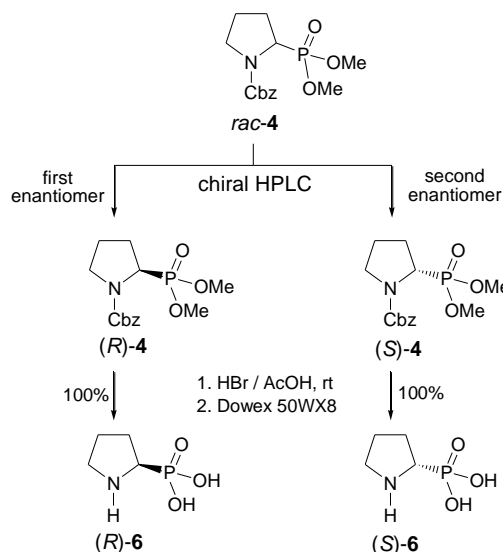
The optimal conditions established at the analytical level for the resolution of *rac*-**4** (Figure 2) were then extended to the preparative scale. For this purpose, a 250 × 20 mm Chiralpak<sup>®</sup> IC column was used and elution was performed with *n*-hexane/acetone 72:28 at 18 mL/min flow rate. The racemate (1.85 g) was dissolved in chloroform at very high concentration (1 g/mL) and 150  $\mu$ L aliquots of this solution were injected successively. More than 900 mg of each enantiomer were thus isolated and both of them were found to be optically pure (Figure 3). This means that 98% of the starting racemic material was recovered in enantiomerically pure form after a single passage through the column.



**Figure 3.** HPLC analytical profile of the resolved enantiomers of **4** (conditions: see Figure 2). The configuration assigned to each enantiomer is shown.

Next, the protecting groups in the resolved enantiomers of **4** were removed by treatment with a commercial solution of hydrogen bromide in acetic acid at room temperature and the resulting phosphoprolines hydrobromides were neutralized by ion-exchange chromatography (Scheme 3). Comparison of the specific rotation measured for the free amino acids (**6**) with the values reported in the literature<sup>11c</sup> allowed us to assign an *R* configuration to the phosphoprolines enantiomer derived from the first eluted enantiomer of **4** (Figure 3, Scheme 3). Accordingly, an *S* stereochemistry was assigned to the more strongly retained **4** enantiomer. It is worth mentioning that the absolute

configuration of phosphoprolines was an unresolved issue until Hammerschmidt *et al.* assigned it unambiguously.<sup>11c,23</sup>



**Scheme 3.** Preparation of enantiopure phosphoprolines by chromatographic (chiral HPLC) resolution.

### 2.3. Enzymatic resolution

Biocatalysis has received much attention in the last decades due to its potential in the production of enantiomerically pure compounds under mild reaction conditions.<sup>24,25</sup> However, there are very few examples<sup>26</sup> of biocatalytic procedures applied to the resolution of  $\alpha$ -aminophosphonic acid derivatives. In fact, most of the works reporting on the access to these compounds by enzymatic methods are based on the resolution of  $\alpha$ -hydroxy- or  $\alpha$ -acyloxyphosphonates<sup>11c,27,28</sup> (enzyme-catalyzed acylation or hydrolysis reactions, respectively) followed by chemical transformations to replace the  $\alpha$  OH/OCOR substituent by an amino group and, if necessary, to elaborate the side chain. This is the case of Hammerschmidt's work mentioned in the Introduction<sup>11c</sup> that describes the highly enantioselective protease-catalyzed kinetic resolution of an  $\alpha$ -(chloroacetoxy)phosphonate (99% *ee*) and subsequent transformation into (*R*)-phosphoprolines.

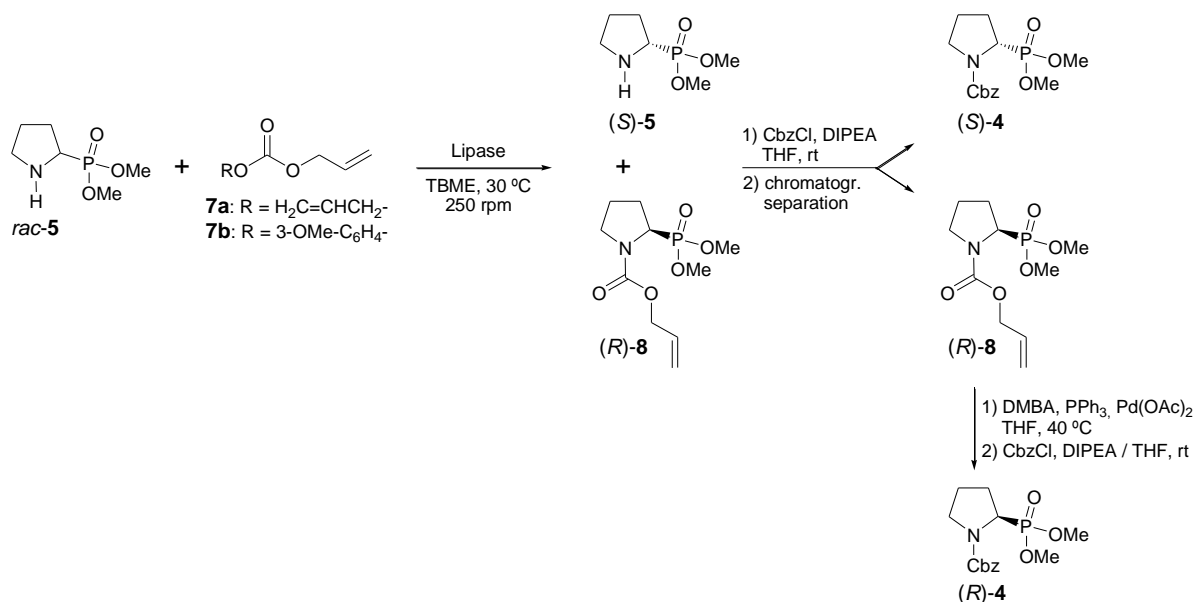
To the best of our knowledge, none of the scarce examples of biocatalytic procedures applied to the resolution of  $\alpha$ -aminophosphonic acid derivatives<sup>26</sup> involves a compound in which the amino group is part of a cycle, as is the case in phosphoprolines. This fact, together with our experience in the enzymatic resolution of  $\alpha$ - and  $\beta$ -amino esters containing an azacycle,<sup>29</sup> prompted us to explore the kinetic resolution of the phosphoprolines precursor *rac*-**5** by carbamylation (alkoxycarbonylation) reactions. Hydrolases were selected for this purpose, since they have been shown to provide access to *N*-heterocyclic amines and amino acid derivatives with high stereocontrol.<sup>29,30</sup>

As starting point, and based on our previous work on the resolution of cyclic  $\alpha$ - and  $\beta$ -amino esters,<sup>29</sup>

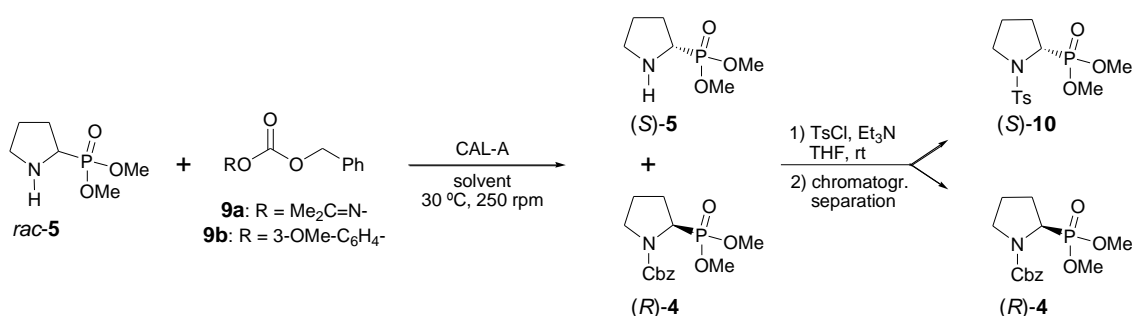
alkoxycarbonylation of the amino function in *rac*-**5** was attempted using *Candida antarctica* lipase type A (CAL-A) and the allyl carbonates **7a-b** (Scheme 4). Unfortunately, the reaction with diallyl carbonate (**7a**, 2.5 equiv.) did not proceed in any extension after 3 days at 30 °C using TBME as solvent. For this reason, the more reactive allyl 3-methoxyphenyl carbonate (**7b**)<sup>31a</sup> was tested under similar conditions and other lipases were included in the screening. Using carbonate **7b**, the highest activities were observed for *Candida antarctica* lipase type B (CAL-B) and *Pseudomonas cepacia* (PSL IM), which promoted the reaction but in a non-selective manner, while CAL-A was found to catalyze the allyloxycarbonylation of aminophosphonate *rac*-**5** to yield the unreacted substrate (*S*)-**5** with 90% *ee* and the allyl carbamate (*R*)-**8** with 20% *ee* (Scheme 4) after 92 h of reaction. These *ee* values were determined by HPLC analysis in an indirect manner since none of these compounds can be easily detected by UV absorbance. Moreover, separation of these compounds by silica-gel column chromatography was not possible due to instability observed for amine **5** under these conditions. For these reasons, prior to HPLC assays, the crude obtained from the enzymatic reaction was treated with benzyl chloroformate to give a mixture of optically active carbamates (*S*)-**4** and (*R*)-**8** (Scheme 4). Laborious column chromatography on silica gel was necessary to separate these carbamates as they showed similar *R<sub>f</sub>* values. The allyloxycarbonyl group in (*R*)-**8** was then exchanged by a benzyloxycarbonyl moiety through a two-step procedure that involved treatment with *N,N*-dimethylbarbituric acid

(DMBA), triphenylphosphine and palladium acetate, and subsequent protection with benzyl chloroformate (Scheme 4). The enantioenriched Cbz-protected aminophosphonates (*S*)-**4** and (*R*)-**4** obtained after these transformations and derived, respectively, from the unreacted amine (*S*)-**5** and the allyl carbamate (*R*)-**8** were separately subjected to HPLC analysis for optical purity determination. Their absolute stereochemistries were assigned by comparison with enantiomerically pure samples of (*R*)-**4** and (*S*)-**4** obtained in the preparative-scale HPLC resolution process described above. The enantiomeric excesses determined in this way for the substrate (*ees*) and product (*ee<sub>P</sub>*) were not consistent with a conversion value around 50%. Similar discrepancies were observed when the process was repeated and stopped at different reaction times. This disagreement could be due to the extensive chemical elaboration –including difficult purification– required prior to enantioselectivity determination. In particular, partial racemization of the product during the (*R*)-**8**-to-(*R*)-**4** transformation cannot be discarded.

These difficulties prompted us to consider a change in the approach. Thus, to avoid the inconveniences associated with UV detection and purification of the allyl derivative, we envisaged the possibility of directly introducing a benzyloxycarbonyl group in *rac*-**5**. For this purpose, two benzyl carbonates (**9a-b**) were prepared following reported



**Scheme 4.** Lipase-catalyzed kinetic resolution of *rac*-**5** using allyl carbonates **7a-b**.



**Scheme 5.** CAL-A catalyzed kinetic resolution of *rac*-**5** using benzyl carbonates **9a-b**.

procedures<sup>31b,c</sup> and their reactivity in the benzyloxycarbonylation of *rac*-**5** catalyzed by CAL-A was studied (Scheme 5, Table 1). Carbonate **9a** in TBME turned out to be highly reactive and produced the complete transformation of the starting material into the racemic Cbz-protected derivative *rac*-**4**. On the other hand, the use of benzyl 3-methoxyphenyl carbonate (**9b**) resulted, after 84 h, in the formation of (*R*)-**4** with 82% *ee* and the recovery of the starting material (*S*)-**5** with 94% *ee* (Table 1, entry 1). The enantiomeric excess of both compounds was determined by HPLC after transformation of the remaining (*S*)-**5** into the *N*-tosylated phosphonate (*S*)-**10**, which was cleanly separated from the Cbz-derivative (*R*)-**4** by column chromatography on silica gel (Scheme 5). Enantiopure (*R*)-**4** and (*S*)-**4** obtained by preparative HPLC resolution served as reference for absolute configuration assignments. In an attempt to optimize enantioselectivity, other organic solvents were tested in the CAL-A catalyzed benzyloxycarbonylation of *rac*-**5** with carbonate **9b**, but none of them resulted in improved selectivity. Thus, no conversion was observed in the presence of tetrahydrofuran or 1,4-dioxane. Toluene and diethyl ether led to lower activity than TBME and produced a loss of selectivity in both substrate and product (Table 1, entries 2 and 3).

**Table 1.** Kinetic resolution of *rac*-**5** catalyzed by CAL-A using carbonate **9b** in organic solvents.<sup>a</sup>

Entry	Solvent	<i>t</i> (h)	<i>ee</i> p (%) <sup>b</sup>	<i>ees</i> (%) <sup>b</sup>	<i>c</i> (%) <sup>c</sup>	<i>E</i> <sup>d</sup>
1	TBME	84	82	94	54	35
2	Toluene	96	71	30	30	8
3	Et <sub>2</sub> O	96	29	13	31	2

<sup>a</sup> Enzymatic reaction conditions: 2:1 ratio CAL-A:*rac*-**5** in weight, carbonate **9b** (2.5 equiv.), solvent (0.07 M), 30 °C, 250 rpm.

<sup>b</sup> Determined by HPLC (see experimental part for details).

<sup>c</sup> Conversion,  $c = ees/(ees+ee_p)$ .

<sup>d</sup>  $E = \ln[(1-c) \times (1-ee_p)] / \ln[(1-c) \times (1+ee_p)]$ .

Even if the enantioselectivity reached did not allow the isolation of enantiopure phosphoprolinone by these methods,

it should be emphasized that the present work is, to the best of our knowledge, the first example of a biocatalytic resolution applied to an  $\alpha$ -aminophosphonic acid of cyclic structure.

### 3. Conclusions

An efficient methodology has been developed for the preparation of both phosphoprolinone enantiomers in optically pure form. The procedure involves the synthesis of a racemic phosphoprolinone derivative, with protected amino and phosphonic acid functions, and its subsequent chromatographic resolution by chiral HPLC. The racemic derivative has been synthesized from a commercially available *N*-protected pyrrolidine-2-one in a straightforward manner that provides the desired compound in high overall yield and multi-gram quantities without isolation of any intermediate species. The efficiency of the procedure highlights the great value of  $\gamma$ -lactams as precursors of the pyrrolidine-2-phosphonate moiety. The racemic phosphoprolinone derivative has been subjected to HPLC resolution on a cellulose-based chiral stationary phase. Specifically, a semi-preparative Chiralpak<sup>®</sup> IC column has allowed the enantioseparation of about 2 grams of racemate eluting with a mixture of *n*-hexane/acetone. Final removal of the protecting groups has provided both phosphoprolinone enantiomers in optically pure form. In addition, resolution of the racemic phosphoprolinone has been explored through enzyme-catalyzed alkoxylation reactions. From the hydrolases toolbox, *Candida antarctica* lipase type A (CAL-A) displayed the best activity and enantioselectivity. Different allyl and benzyl carbonates were tested using a variety of organic solvents. Under the optimal conditions, the unreacted aminophosphonate and the corresponding benzyl carbamate were obtained with good enantiomeric excesses (94% and 82% *ee*, respectively). As far as we know, this is the first biocatalytic resolution of a cyclic  $\alpha$ -aminophosphonic acid.



## 4. Experimental

### 4.1. General.

*Candida antarctica* lipase type A (CAL-A, immobilized NZL-101, 5.0 U/g) was purchased from Codexis. *Candida antarctica* lipase type B (CAL-B, Novozyme 435, 7300 PLU/g) was a gift from Novo Nordisk Co. *Pseudomonas cepacia* lipase immobilized on diatomite (PSL IM, 943 U/g) was provided by Amano Europe. All reagents were used as received from commercial suppliers without further purification. Dowex® 50WX8 (H<sup>+</sup> form, 50–100 mesh) used for ion-exchange chromatography was purchased from Sigma-Aldrich. Thin-layer chromatography (TLC) was performed on Macherey-Nagel Polygram® SIL G/UV<sub>254</sub> precoated silica gel polyester plates. The products were visualized by exposure to UV light (254 nm), ninhydrin, iodine vapor or ethanolic solution of phosphomolybdic acid. Column chromatography was performed using 60 M (0.04–0.063 mm) silica gel from Macherey-Nagel. Melting points were determined on a Gallenkamp apparatus. IR spectra were registered on Nicolet Avatar 360 FTIR or Perkin-Elmer 1720-XFT spectrophotometers;  $\nu_{\max}$  is given for the main absorption bands. <sup>1</sup>H, <sup>13</sup>C and <sup>31</sup>P NMR spectra were recorded on Bruker AV-400 or DPX-300 instruments at room temperature using the residual solvent signal as the internal standard; chemical shifts ( $\delta$ ) are expressed in ppm and coupling constants ( $J$ ) in Hertz. High-resolution mass spectra were obtained on a Bruker Microtof-Q spectrometer. Optical rotations were measured on JASCO P-1020 or Perkin-Elmer 241 polarimeters. The HPLC systems used for the chromatographic and enzymatic resolution studies are described in the following sections. Carbonates **7b**,<sup>31a</sup> **9a**,<sup>31b</sup> and **9b**<sup>31c</sup> were prepared following reported procedures. Racemic samples of the allyl derivative *rac*-**8** and the tosyl derivative *rac*-**10** were synthesized to be used as references for HPLC and/or TLC analysis in the enzymatic studies.

### 4.2. Synthesis of *N*-(benzyloxycarbonyl)pyrrolidin-2-one, **1**.

A 1 M solution of lithium bis(trimethylsilyl)amide in tetrahydrofuran (23.71 mL, 23.71 mmol) was slowly added to a solution of pyrrolidin-2-one (2.02 g, 23.71 mmol) in anhydrous tetrahydrofuran (60 mL) kept at –78 °C under argon. After stirring at this temperature for 30 minutes, benzyl chloroformate (4.05 g, 3.38 mL, 23.71 mmol) was added dropwise. The reaction mixture was slowly warmed to –40 °C and stirred at this temperature for 3 h. Then, saturated aqueous ammonium chloride (30 mL) was added and the resulting mixture was allowed to warm to room temperature. The two layers were separated and the aqueous phase was further extracted with dichloromethane (2 × 30 mL). The combined organic extracts were dried, filtered, and concentrated. Purification by column chromatography (eluent: hexanes/ethyl acetate 2:1) afforded **1** as an oil, which crystallized upon cooling; white solid (4.74 g, 21.63 mmol, 91% yield). Mp 36–37 °C. Spectroscopic data were in agreement with reported values.<sup>32</sup> This compound is also available from commercial sources.

### 4.3. Synthesis of dimethyl *N*-(benzyloxycarbonyl)pyrrolidine-2-phosphonate, *rac*-**4**.

A 1 M solution of diisobutylaluminum hydride in hexanes (30.70 mL, 30.70 mmol) was slowly added to a solution of **1** (4.48 g, 20.43 mmol) in anhydrous tetrahydrofuran (100 mL) kept at –78 °C under argon. After stirring at this temperature for 2 h, the reaction was treated with saturated aqueous sodium acetate (30 mL) and allowed to warm to room temperature. A 3:1 mixture of diethyl ether and saturated aqueous ammonium chloride (48 mL) was then added and the resulting mixture was stirred until a suspension was formed. The solid was filtered off under reduced pressure and washed with diethyl ether (2 × 20 mL). The organic layer was separated and the aqueous phase was further extracted with diethyl ether (2 × 30 mL). The combined organic extracts were washed with water (20 mL) and brine (20 mL), dried, filtered, and evaporated to provide the hemiaminal **2** as an oil. It was dissolved in methanol (100 mL) and treated with pyridinium *p*-toluenesulfonate (514 mg, 2.04 mmol). After stirring at room temperature for 2 h, triethylamine (884 mg, 1.22 mL, 8.74 mmol) was added. The solvent was evaporated and the crude methoxyaminal **3** thus obtained was dissolved in anhydrous dichloromethane (80 mL) and kept under argon. Trimethyl phosphite (2.53 g, 2.41 mL, 20.43 mmol) was added and the resulting solution was cooled to –20 °C. Boron trifluoride-diethyl ether (2.90 g, 2.59 mL, 20.43 mmol) was added dropwise and the reaction mixture was slowly warmed to room temperature and stirred for 12 h. After quenching with water (20 mL), the two layers were separated and the aqueous phase was extracted with dichloromethane (2 × 30 mL). The combined organic extracts were dried, filtered, and concentrated. Purification by column chromatography (eluent: ethyl acetate/hexanes 9:1) afforded *rac*-**4** as a colorless oil (5.11 g, 16.31 mmol, 80% yield). IR (neat)  $\nu$  1705, 1248, 1028 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.81–1.94 (m, 1H, H<sub>4</sub>), 1.97–2.40 (m, 3H, H<sub>3</sub>+H<sub>4</sub>), 3.37–3.86 (m, 8H, H<sub>5</sub>+OMe), 4.18–4.36 (m, 1H, H<sub>2</sub>), 5.06–5.21 (m, 2H, CH<sub>2</sub>Ph), 7.21–7.44 (m, 5H, Ph). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz)  $\delta$  (duplicate signals are observed for most carbons, an asterisk \* indicates the minor rotamer) 23.5\*, 24.5 (C<sub>4</sub>); 26.9, 27.7\* (C<sub>3</sub>); 46.8, 47.1\* (C<sub>5</sub>); 53.1 (d,  $J$  = 6.9 Hz, OMe); 52.9 (d,  $J$  = 6.5 Hz, OMe); 53.2 (d,  $J$  = 162.3 Hz, C<sub>2</sub>); 67.3, 67.6\* (CH<sub>2</sub>Ph); 127.9 (Ph); 128.1 (Ph); 128.3 (Ph); 128.5 (Ph); 136.6 (Ph); 155.1\*, 155.2 (CO). <sup>31</sup>P NMR (CDCl<sub>3</sub>, 162 MHz)  $\delta$  (duplicate signal, an asterisk \* indicates the minor rotamer) 27.3\*, 27.6. HRMS (ESI) C<sub>14</sub>H<sub>20</sub>NNaO<sub>5</sub>P [M+Na]<sup>+</sup>: calcd 336.0971, found 336.0987.

### 4.4. HPLC resolution of *rac*-**4**: isolation of (*R*)-dimethyl *N*-(benzyloxycarbonyl)pyrrolidine-2-phosphonate and (*S*)-dimethyl *N*-(benzyloxycarbonyl)pyrrolidine-2-phosphonate, (*R*)-**4** and (*S*)-**4**.

The HPLC resolution of *rac*-**4** was carried out on a Waters 600 HPLC system equipped with a 2996 photodiode array detector (used at the analytical level) and a 2487 dual wavelength absorbance detector (used for the preparative-scale resolution). Analytical assays were performed on 250 × 4.6 mm Chiralpak® IA, IB, and IC columns eluting with different binary or ternary mixtures (IA, IB: *n*-hexane/2-

propanol; IC: *n*-hexane/2-propanol, *n*-hexane/2-propanol/chloroform, *n*-hexane/2-propanol/TBME, *n*-hexane/2-propanol/acetone, *n*-hexane/acetone) at flow rates ranging from 0.8 to 1.0 mL/min. The preparative resolution was carried out on a 250 × 20 mm Chiralpak® IC column eluting with *n*-hexane/acetone 72:28 at a flow rate of 18 mL/min. The process was monitored by UV absorbance at 215 nm. The racemate *rac*-4 (1.850 g) was dissolved in chloroform (1.85 mL) and 150 μL aliquots of this solution were injected consecutively every 20 min. Each injection was collected into three separate fractions, with equivalent fractions of successive injections being combined. Evaporation of the first and third fractions provided, respectively, enantiomerically pure (*R*)-4 (909 mg) and (*S*)-4 (905 mg). The second fraction (17 mg) was found to contain a 33:67 mixture of (*R*)-4/(*S*)-4 and was discarded.

(*R*)-4: colorless oil,  $[\alpha]_D^{22} = -60.0$  (*c* 0.53, CHCl<sub>3</sub>).

(*S*)-4: colorless oil,  $[\alpha]_D^{22} = +61.3$  (*c* 0.52, CHCl<sub>3</sub>).

Spectroscopic data were identical to those obtained for *rac*-4.

#### 4.5. Synthesis of (*R*)-pyrrolidine-2-phosphonic acid, (*R*)-phosphoproline, (*R*)-6.

A 33% solution of hydrogen bromide in acetic acid (8 mL) was added to (*R*)-4 (400 mg, 1.28 mmol) and the reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated and the residue was submitted to ion-exchange chromatography on Dowex® 50WX8 (H<sup>+</sup> form, 50–100 mesh) eluting with water. Lyophilization of the aqueous solution afforded (*R*)-6 as a white solid (193 mg, 1.28 mmol, 100% yield); mp 272–274 °C (lit.<sup>11c</sup> 265–270 °C).  $[\alpha]_D^{21} = -49.8$  (*c* 1.1, 1 M NaOH) {lit.<sup>11c</sup>  $[\alpha]_D^{20} = -49.1$  (*c* 1.1, 1 M NaOH)}. Spectroscopic data were in agreement with reported values.<sup>11c</sup>

#### 4.6. Synthesis of (*S*)-pyrrolidine-2-phosphonic acid, (*S*)-phosphoproline, (*S*)-6.

An identical procedure to that described above was applied to transform (*S*)-4 (400 mg, 1.28 mmol) into (*S*)-6 (193 mg, 1.28 mmol, 100% yield); mp 272–274 °C.  $[\alpha]_D^{21} = +51.1$  (*c* 1.1, 1 M NaOH). Spectroscopic data were identical to those obtained for (*R*)-6.

#### 4.7. Synthesis of dimethyl pyrrolidine-2-phosphonate, *rac*-5.

A mixture of *rac*-4 (200 mg, 0.64 mmol) and 10% Pd/C (20 mg) in ethanol (10 mL) was stirred overnight at room temperature under an atmospheric pressure of hydrogen gas. Filtration of the catalyst and evaporation of the solvent provided *rac*-5 as a colorless oil (115 mg, 0.64 mmol, 100% yield). IR (neat)  $\nu$  3315, 1247, 1031 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.61–2.24 (m, 5H, NH+H<sub>3</sub>+H<sub>4</sub>), 2.81–2.89 (m, 1H, H<sub>5</sub>), 2.92–3.00 (m, 1H, H<sub>5</sub>), 3.25–3.32 (m, 1H, H<sub>2</sub>), 3.72 (d, *J* = 10.4 Hz, 3H, OMe), 3.73 (d, *J* = 10.4 Hz, 3H, OMe). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz)  $\delta$  26.0 (d, *J* = 8.1 Hz, C<sub>4</sub>), 26.7 (C<sub>3</sub>), 47.6 (d, *J* = 11.6 Hz, C<sub>5</sub>), 53.0 (d, *J* = 4.0 Hz, OMe), 53.0 (d, *J* = 4.1 Hz, OMe), 53.6 (d, *J* = 164.7 Hz, C<sub>2</sub>). <sup>31</sup>P NMR (CDCl<sub>3</sub>, 162 MHz)  $\delta$  31.1. HRMS (ESI) C<sub>6</sub>H<sub>14</sub>NNaO<sub>3</sub>P [M+Na]<sup>+</sup>: calcd 202.0604, found 202.0613.

#### 4.8. General procedure for the enzymatic kinetic resolution of *rac*-5.

To a suspension of *rac*-5 and the lipase (1:2 in weight) in dry solvent (0.07 M) kept under a nitrogen atmosphere was added carbonate **7a-b** or **9a-b** (2.5 equiv.). The system was shaken at 30 °C and 250 rpm until conversion was around 50% (TLC, eluent: ethyl acetate). The enzyme was filtered and washed with tetrahydrofuran (5 × 2 mL). The solvent was evaporated and the crude obtained was elaborated in one of the following ways:

A) Carbonates **7a-b** (see Scheme 4): The crude was treated with benzyl chloroformate in the presence of *N,N*-diisopropylethylamine (similarly to that described in section 4.9) to give a mixture of enantioenriched (*S*)-4 and (*R*)-8. The two carbamates were separated by column chromatography on silica gel (eluent: ethyl acetate). Enantioenriched (*R*)-8 was transformed into (*R*)-4 by treatment with *N,N*-dimethylbarbituric acid, triphenylphosphine and palladium acetate (as described in ref. 33; no purification was performed) and subsequent reaction with benzyl chloroformate in the presence of *N,N*-diisopropylethylamine (similarly to that described in section 4.9). The enantioenriched *N*-Cbz derivatives (*S*)-4 and (*R*)-4 obtained were subjected to HPLC analysis for *ee* determination.

B) Carbonates **9a-b** (see Scheme 5): The crude was treated with tosyl chloride in the presence of triethylamine (as described for the synthesis of *rac*-10) to give a mixture of enantioenriched *N*-tosyl and *N*-Cbz derivatives (*S*)-10 and (*R*)-4. The two compounds were separated by column chromatography on silica gel (eluent: ethyl acetate) and subjected to HPLC analysis for *ee* determination.

HPLC elution times and conditions: (*R*)-4 9.5 min, (*S*)-4 11.8 min; (*S*)-10 18.0 min, (*R*)-10 30.9 min; Hewlett Packard 1100 chromatograph, 250 × 4.6 mm Chiralcel® OJ-H column, 30 °C, *n*-hexane/ethanol 85:15, 0.8 mL/min flow rate, UV monitoring at 210 nm. Samples of *rac*-4, *rac*-10, and enantiopure (*R*)-4 and (*S*)-4 (from the preparative HPLC resolution, see above) were used as references for retention times and absolute configuration assignment.

(*S*)-10:  $[\alpha]_D^{20} = +76.7$  (*c* 1.0, CHCl<sub>3</sub>) for 94% *ee*; spectroscopic data were identical to those obtained for *rac*-10.

#### 4.9. Synthesis of dimethyl *N*-(allyloxycarbonyl)pyrrolidine-2-phosphonate, *rac*-8.

*N,N*-Diisopropylethylamine (74 mg, 99 μL, 0.57 mmol) was added to a solution of *rac*-5 (82 mg, 0.46 mmol) in anhydrous tetrahydrofuran (3 mL) kept at room temperature under an argon atmosphere. The mixture was cooled to 0 °C and allyl chloroformate (69 mg, 60 μL, 0.57 mmol) was added. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solvent was evaporated and the crude was purified by column chromatography (eluent: ethyl acetate) to afford *rac*-8 as a colorless oil (57 mg, 0.22 mmol, 47% yield). IR (neat)  $\nu$  1704, 1648, 1248, 1029 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.80–1.93 (m, 1H, H<sub>4</sub>), 1.95–2.30 (m, 3H, H<sub>3</sub>+H<sub>4</sub>), 3.35–3.59 (m, 2H, H<sub>5</sub>), 3.64–3.80 (m, 6H, OMe), 4.17–4.30 (m, 1H, H<sub>2</sub>), 4.51–4.62 (m, 2H, CH<sub>2</sub>CH=), 5.13–5.20 (m, 1H, CH=CH<sub>2</sub>), 5.22–5.34 (m, 1H, CH=CH<sub>2</sub>),

5.82–5.96 (m, 1H, CH=CH<sub>2</sub>). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) δ (duplicate signals are observed for most carbons; an asterisk \* indicates the minor rotamer) 23.4\*, 24.5 (C<sub>4</sub>); 26.8, 27.6\* (C<sub>3</sub>); 46.7, 46.9\* (C<sub>5</sub>); 52.9 (d, *J* = 6.6 Hz, OMe); 53.1 (d, *J* = 7.2 Hz, OMe); 53.1 (d, *J* = 162.3 Hz, C<sub>2</sub>); 66.1, 66.4\* (CH<sub>2</sub>CH=); 117.5, 117.9\* (CH=CH<sub>2</sub>); 132.8 (CH=CH<sub>2</sub>); 155.0 (CO). <sup>31</sup>P NMR (CDCl<sub>3</sub>, 162 MHz) δ (duplicate signal, an asterisk \* indicates the minor rotamer) 27.3\*, 27.7. HRMS (ESI) C<sub>10</sub>H<sub>18</sub>NNaO<sub>5</sub>P [M+Na]<sup>+</sup>: calcd 286.0815, found 286.0833.

#### 4.10. Synthesis of dimethyl *N*-tosylpyrrolidine-2-phosphonate, *rac*-10.

Triethylamine (12 mg, 17 μL, 0.12 mmol) was added to a solution of *rac*-5 (17 mg, 0.09 mmol) in anhydrous tetrahydrofuran (1 mL) kept at room temperature under an argon atmosphere. The mixture was cooled to 0 °C and tosyl chloride (23 mg, 0.12 mmol) was added. The reaction mixture was allowed to warm to room temperature and stirred for 6 h. After evaporation of the solvent, the residue was purified by column chromatography (eluent: ethyl acetate) to afford *rac*-10 as a white solid (16 mg, 0.05 mmol, 51% yield). Mp 119–120 °C. IR (KBr) ν 1348, 1309, 1263, 1189, 1158, 1037 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 1.35–1.49 (m, 1H, H<sub>4</sub>), 1.52–1.72 (m, 1H, H<sub>4</sub>), 1.89–2.11 (m, 2H, H<sub>3</sub>), 2.39 (s, 3H, Me), 3.34–3.41 (m, 2H, H<sub>5</sub>), 3.77 (d, *J* = 10.5 Hz, 3H, OMe), 3.84 (d, *J* = 10.5 Hz, 3H, OMe), 4.07–4.16 (m, 1H, H<sub>2</sub>), 7.28 (d, *J* = 8.2 Hz, 2H, Ph), 7.69 (d, *J* = 8.2 Hz, 2H, Ph). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz) δ 21.6 (Me), 24.5 (C<sub>4</sub>), 27.0 (C<sub>3</sub>), 49.3 (C<sub>5</sub>), 53.0 (d, *J* = 6.9 Hz, OMe), 54.2 (d, *J* = 7.2 Hz, OMe), 55.3 (d, *J* = 171.2 Hz, C<sub>2</sub>), 127.6 (Ph), 129.9 (Ph), 134.8 (Ph), 144.0 (Ph). <sup>31</sup>P NMR (CDCl<sub>3</sub>, 121 MHz) δ 26.1. HRMS (ESI) C<sub>13</sub>H<sub>20</sub>NNaO<sub>5</sub>PS [M+Na]<sup>+</sup>: calcd 356.0692, found: 356.0721.

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