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Individual stereoisomers of phosphinic dipeptide inhibitor of leucine aminopeptidase

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Abstract—Individual stereoisomers of the phosphinic pseudodipeptide hPheψ[P(O)(OH)CH_2]Phe were obtained by stereoselective liquid chromatographic separation as N- and C-terminally protected derivative on quinidine carbamate modified silica stationary phase. The stereoisomeric purity, exceeding 95% for each fraction, was determined before and after deprotection using two independent methods. The absolute configuration was rationally assigned by application of enantiomerically pure phosphinic acid substrates in the synthetic procedure and correlation with biological activity of the products. Substantial differences in inhibition of leucine aminopeptidase by the individual isomers revealed novel insights into potency of the recently developed and remarkably effective compound.

The design and construction of targeted bioactive molecules requires to take into consideration their strictly stereoselective mode of action. Chirality is, in the most cases, the key factor in the safety and efficacy of many drug products.[1,2] Usually only one enantiomer is responsible for the desired activity, whereas its counterpart could be inactive, possess some activity of interest, be an antagonist of the active enantiomer or have a separate activity that could be either desirable or undesirable. Only in a few cases specific compositions of a racemate or an enantiomeric pair demonstrated a synergistic effect.[1,2] Similarly, diastereoisomers of unique three dimensional structure, basically determined by the configuration of chiral elements such as stereogenic centres present in the molecule, are able to approach the receptors' binding site and interact efficiently, whereas the other forms are discriminated. Apparently, this is particularly valid for peptides and their various chemically modified analogues. Among the latter, phosphinic acid pseudopeptides represent isosters with a scissile bond replaced by non-hydrolysable –PO_2HCH_2– moiety. Tetrahedral phosphinates resemble the high-energy transition state of the peptide bond hydrolysis[3–5] and consequently, they have been extensively applied for effective and selective regulation of activity of various proteases, particularly metallo-dependent ones.[6–10] For these purposes phosphinic peptides were frequently assayed in the form of their pure diastereoisomers, but rarely was it the result of achievements in development of stereoselective strategies for their preparation.[11] Elegant asymmetric hydrogenation of the appropriate phosphinic dehydrodipeptide to obtain D-Ala-D-Ala analogue, antibacterial agent acting as D-Ala-D-Ala ligase inhibitor, is one such example.[12] The extended pseudopeptides containing only one modified amide bond are usually obtained by elongation of a suitably protected dipeptide building block with chiral amino acids using standard peptide synthesis methods. Then, individual diastereoisomers are readily separated upon conventional purification of a final product on reverse phase HPLC (for example, see Ref. 13).

The situation is more complicated in the case of phosphinic acid dipeptides. Possessing two non-defined

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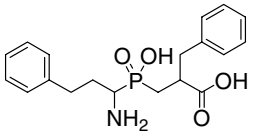
asymmetric carbon atoms, obtained in non-stereoselective manner and lacking a chiral auxiliary, they represent mixture of two enantiomeric pairs which cannot be separated using non-chiral techniques. Recently, we have reported rationally designed phosphinic dipeptide analogues of such type as effective inhibitors of bizinc cytosolic leucine aminopeptidase (LAP, EC 3.4.11.1).[14] Broad interests in LAP are directed towards detailed evaluation of its mechanism of hydrolytic action, biological significance and potential medical importance (for recent references on mechanistic, physiological and pathological implications of LAP activity see Refs. 15 and 16). All these accounts stimulate continuous interest in agents regulating the protease activity.[17] Phosphinic dipeptides, although tested as stereoisomeric mixtures, were ranked amongst the most potent low-molecular weight inhibitors of LAP reported so far. For example, homophenylalanyl-phenylalanine (compound **1**, hPheψ[PO_2HCH_2]Phe, Fig. 1) homophenylalanyl-tyrosine and leucyl-leucine analogues exhibited K_i's in the low nanomolar range.[14] Furthermore, selected dipeptides were even more successfully employed to inhibit recombinant leucyl aminopeptidase of *Plasmodium falciparum*,[18] a potential target protease for the development of new antimalarials.[19] Excellent kinetic parameters of suppression of the enzyme activity and the growth of the pathogen, both in vitro as well as in vivo, were evidenced.[18] As the result, the study provided new interesting lead compounds for malaria treatment.

In this context, the challenge to synthesize/separate all four pure stereoisomers and to specify their potency seems to be fully justified. Here, we report the results of our studies on resolution of the four isomers of hPheψ[PO_2HCH_2]Phe by combination of synthetic protocols and stereoselective liquid chromatographic methods. We also compare their activity towards leucine aminopeptidase depending on the absolute configuration. To the best of our knowledge, this represents very first example of such studies on phosphinic acid pseudodipeptides.

The overall procedure to obtain individual stereoisomers of phosphinic acid analogues of homophenylalanyl-phenylalanine (**1**) is outlined in Scheme 1. Partially, it was also described in our previous paper dealing with the application of a chiral stationary phase

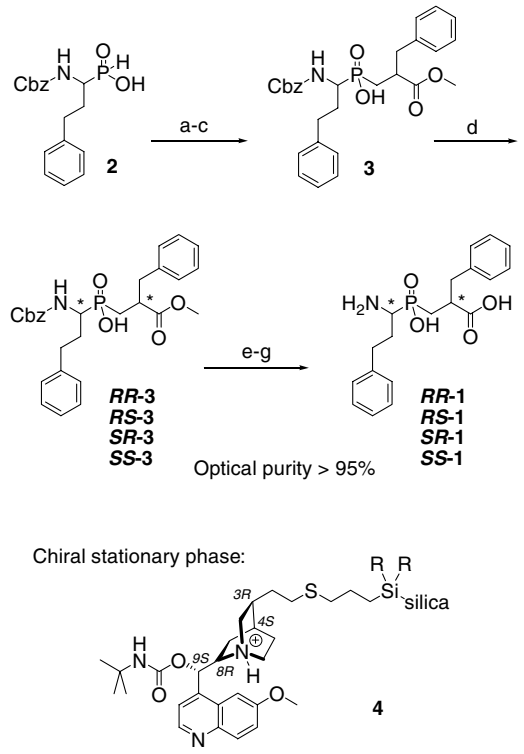


1, hPheψ[PO_2HCH_2]Phe

K_i = 66 nM (*porcine kidney* LAP) [14]
K_i = 13.2 nM (rec. *P. falciparum* LAP) [18]

(activity of the mixture of four stereoisomers)

Figure 1. The structure of the phosphinic acid analogue of homophenylalanyl-phenylalanine and inhibition constants towards porcine kidney and recombinant *Plasmodium falciparum* leucine aminopeptidase.



Scheme 1. Synthesis and separation of the stereoisomers of hPheψ[PO_2HCH_2]Phe phosphinic dipeptide analogue. Reagents and conditions: (a) hexamethyldisilazane, 100–110 °C, 3 h; (b) H_2C=CH(CH_2Ph)COOMe, 80–90 °C, 3 h; (c) MeOH; (d) stereoselective HPLC on a prototype of the commercial CHIRALPAK QD-AX (10 μm) (250 × 20 mm id) (Chiral Technologies Europe) with stationary phase **4**, mobile phase acetonitrile/0.25 M formic acid (80:20, v/v) (pH of the mixture adjusted to 4.0 with ammonia), ambient temperature, flow rate 12 mL/min, elution order: *R*,*R*-**3** < *R*,*S*-**3** < *S*,*R*-**3** < *S*,*S*-**3**; (e) bromotrimethylsilane, 10 equiv, 3 d, rt; (f) H_2O, then concd HCl; (g) HPLC purification, gradient 20:80 (0′) → 40:60 (20′) (v/v, acetonitrile/water, both containing 0.1% of trifluoroacetic acid), t_R = 17.8 min.

based on a quinidine derivative (**4**, Scheme 1) in HPLC and CEC (capillary electrochromatography) separation and purity analysis of the individual phosphinic dipeptide stereoisomers.[20]

The *N*-benzyloxycarbonyl (Cbz) protected phosphinic pseudodipeptide methyl ester (**3**) was obtained in a non-stereoselective manner by addition of the appropriate acrylate to the suitably blocked phosphinic acid (**2**) as described previously.[14] The final material showed two ^{31}P NMR resonance signals of not equal integration, varying in the range 3:2–2:1 depending on the batch. It was the result of more significant loss of the up-field shifted enantiomeric pair in the purification process (liquid chromatography/crystallization). Normally unwanted (although straightforward), this was fortunate in our case and greatly facilitated matching enantiomeric and diastereomeric relationships (see below for the details). The individual stereoisomers of the protected peptide were obtained by preparative stereoselective chromatography on a prototype of the commercial CHIRALPAK QD-AX column (Chiral Technologies Europe, Illkirch, France).[20] The extensive optimization

of the elution conditions did finally allow collection of the four isomer fractions with sufficient purity. Newly elaborated, superior CEC system using the same chiral selector gave confirmation of their satisfactory stereoisomeric purity, exceeding 95% for each of the four fractions corresponding to the individual stereoisomers.[20]

The obtained materials were treated with an excess of bromotrimethylsilane (10-fold excess) in anhydrous CH_2Cl_2 to remove both protecting groups in one step without using alkaline conditions. Free pseudodipeptides were obtained after evaporation of volatile components and subsequent hydrolysis of the residue with water and concd HCl. The crude products were finally purified by means of standard reverse phase HPLC. After this step and before enzymatic assays their stereoisomeric purity was additionally verified. A method developed for analysis of the enantiomeric excess of α-aminophosphonates (phosphorus analogues of amino acids) was used for this purpose. The method relies on ^{31}P NMR analysis of diastereomeric inclusion complexes formed between analytes bearing hydrophobic side chains and cyclodextrins.[21] In our case, treatment of the hPheψ[PO_2HCH_2]Phe stereoisomeric mixture (**1**) with 10-fold excess of α-cyclodextrin gave satisfactory separation for all four ^{31}P NMR resonance signals allowing their reliable integration (Fig. 2, bottom trace). On the basis of the integrated signals it may be concluded that the isomers with signals at 45.08 and 44.90 ppm as well as at 44.48 and 44.37 ppm represent pairs of enantiomers. Furthermore, the analysis of individual stereoisomers by ^{31}P NMR confirmed their high stereoisomeric purity of over 95%, which did not change during deprotection (Fig. 2, upper traces). This successful application of using cyclodextrins and a spectroscopic technique that is simple and straightforward to interpret definitely opens new perspectives to determine the stereoisomeric composition of compounds bearing more than one asymmetric centre.

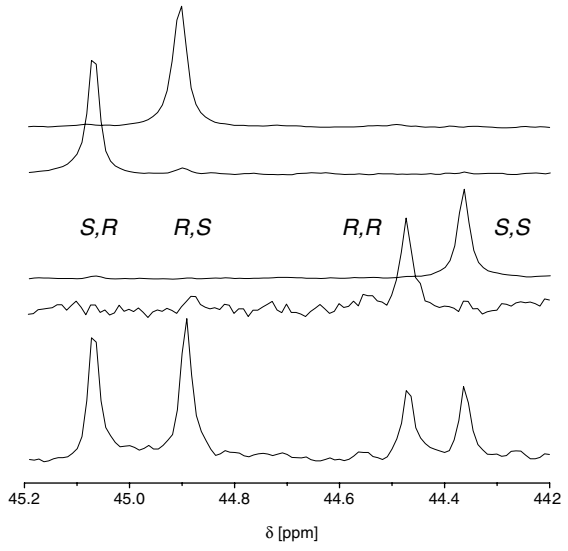
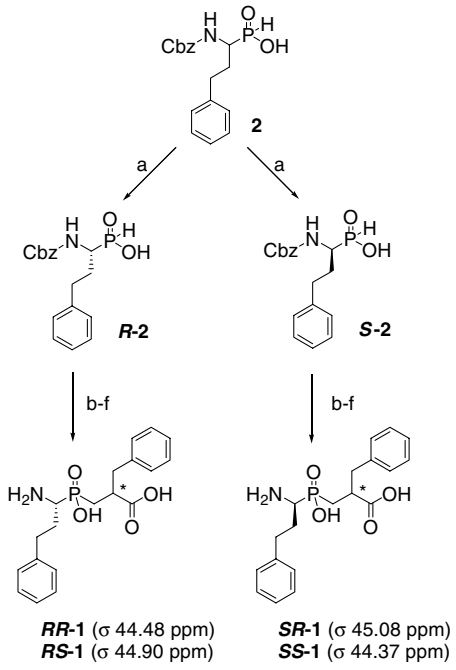


Figure 2. ^{31}P NMR spectra of the stereoisomeric mixture of hPheψ[PO_2HCH_2]Phe (**1**) (bottom trace) and separated individual isomers with the absolute configuration defined (four upper traces). Conditions of the experiments: 10-fold excess of α-cyclodextrin, D_2O/NaOD, pD 12.

To define the absolute configurations of the pseudodipeptide samples additional experiments needed to be performed. First of all, this was accomplished via the synthesis of the substrate – Cbz-protected phosphinic acid (**2**) in the optically pure forms. Usually, it proceeds very efficiently by crystallization of diastereomeric salts of **2** with enantiomeric α-methylbenzylamines as described by Baylis et al.[22] Surprisingly, although various crystallization conditions were tested, we were not able to achieve higher levels of enrichment than 70% of enantiomeric purity. Thus, we returned to stereoselective liquid chromatography. The chiral acid **2** was preparatively resolved into its antipodes using conditions that had been developed in one of our previous studies.[23] As the result, *R*-**2** (equivalent to the relative configuration L) that was eluted first from the quinidine carbamate column was isolated with enantiomeric purity of 99%, while *S*-**2** (equivalent to the relative configuration D), which eluted as second peak, had an enantiomeric purity of 95%. Both were separately converted to the target compound **1** by the same procedure as described above for the racemic mixture. Two batches of the final phosphinic dipeptide were obtained, each containing two diastereoisomers with the defined configuration of the N-terminal amino acid part (Scheme 2). The *R*,*R*/*R*,*S*-**1** pair exhibited two signals at 44.90 and 44.48 ppm, whereas the *S*,*R*/*S*,*S*-**1** pair at 45.08 and 44.37 ppm in ^{31}P NMR experiments with the use of α-cyclodextrin.

Significantly, the *R*,*R*/*R*,*S*-**1** pair appeared much more potent when tested for inhibition of leucine aminopeptidase. This should not be surprising as the protease is responsible for cleavage of the N-terminal amino acids of natural configuration L from polypeptide chains.[24] Similar dependence is observed for the enzyme inhibitors being much more effective in their relative configuration L.[17] Going into details, the fraction corresponding to the ^{31}P NMR signal at 44.90 ppm appeared clearly to be of superior activity to regulate LAP with K_i equal to 45 nM (Table 1). Thus, its configuration can be finally specified as *R*,*S* (equivalent to the relative one L,L) with high level of certainty. The *R*,*R* (L,D)-**1** isomer (44.48 ppm) exhibited six times lower potency. Consequently, the enantiomer of the aforementioned (45.08 ppm, *S*,*R* (D,D)-**1**) was far the weakest inhibitor of LAP with K_i in micromolar range, while the enantiomer of the last mentioned (44.37 ppm, *S*,*S* (D,L)-**1**) was again placed somewhere between the extremes.

Differences in activity of the studied stereoisomers towards leucine aminopeptidase thus proved to be significant indicating strong spatial preference of the enzyme active site, particularly concerning the N-terminal fragment. The closest literature examples to be compared with are represented by phosphinic tripeptide analogues, extended by an additional L-amino acid residue on the C-terminus. Cbz-Pheψ[PO_2HCH_2]-(3-phenylpropyl) Gly-Trp-NH_2 was described as very effective inhibitor of stromelysin 3,[4] while Pheψ[PO_2HCH_2]Leu-Phe



Scheme 2. Synthesis and separation of diastereoisomeric pairs with fixed configuration on the N-terminal asymmetric carbon atom. Reagents and conditions: (a) stereoselective HPLC on a prototype of the commercial CHIRALPAK QD-AX (15 μm) (250 × 16 mm id) (Chiral Technologies Europe) with stationary phase **4** (Scheme 1), mobile phase methanol/50 mM phosphate buffer (80:20; v/v), pH 5.6, flow rate 4.5 mL/min, elution order: *R*-**2** < *S*-**2**; (b) hexamethyldisilazane, 100–110 °C, 3 h; (c) $H_2C{=}CH(CH_2Ph)COOMe$, 80–90 °C, 3 h; (d) MeOH; (e) bromotrimethylsilane, 10 equiv, 3 d, rt; (f) H_2O, then concd HCl. For the conditions of the ^{31}P NMR experiments see Figure 2.

Table 1. Inhibition of leucine aminopeptidase by individual stereoisomers of hPheψ[PO_2HCH_2]Phe phosphinic pseudodipeptide (for the experimental details of the assay see Ref. 14)

Stereoisomer of the hPheψ[PO_2HCH_2] Phe inhibitor	^{31}P NMR: δ [D_2O, ppm][a]	K_i [nM]
R,*R*-**1** (L,D)	44.48	271
R,*S*-**1** (L,L)	44.90	45
S,*R*-**1** (D,D)	45.08	8800
S,*S*-**1** (D,L)	44.37	988

[a] In the presence of 10-fold excess of α-cyclodextrin.

appeared to be a dual neutral endopeptidase (neprilysin)/aminopeptidase N inhibitor.[25] In the first case, the potency of its three out of four stereoisomers is practically indistinguishable, with K_i varying in the range 5–9 nM. Flexible phenylpropyl side chain in the P1′ and L-tryptophan in the P2′ position seem responsible for such privileged binding. Only the *S*,*R*,*S* (D,D,L) form is significantly discriminated (K_i = 50 nM) and thus less potent as inhibitor.[4] For the latter, the absolute configuration of the P1′ residue is the crucial differentiation factor. Diastereoisomers bearing the L leucine isoster were found significantly more efficient than those with D arrangement (roughly two orders of magnitude) in cases of inhibition of both studied enzymes.[25] The absolute configuration in the P1 position was less significant, however, as generally the *R* (L) was slightly more preferred. We believe the recent correlation study on the dipeptide analogue represents an interesting contribution to chemistry and biological activity relationship of phosphinic pseudopeptides.

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