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Original article

Synthesis and *in vitro* study of pseudo-peptide thioureas containing α-aminophosphonate moiety as potential antitumor agents

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ABSTRACT

Twenty pseudo-peptide thioureas **IIa**–**l** containing α-aminophosphonate moiety were synthesized from the reaction of chiral α-amino carboxamide derivatives **Ia**–**c** with *O*,*O*′-dialkylisothiocyanato(phenyl) methylphosphonate **5**. The synthesized compounds were completely characterized by elemental analysis, physical and spectral (IR, ^{1}H NMR, ^{13}C NMR) data. According to the preliminary studies on antitumor activities, compounds **IIa**–**l** could inhibit tumor cells PC3, Bcap37 and BGC823. These compounds displayed low to high activity by MTT assays. Among them, *L*-**IIk**, *D*-**IIa** and *D*-**IIe** were identified as potent inhibitors, with IC_{50} values ranging from 4.7 to 11.2 μM according to *in vitro* assay.

1. Introduction

Peptides are among the most versatile bioactive molecules e.g. many peptide hormones and analogous short peptides exert their action by binding to membrane receptors [1]. Peptides and their derivatives may also exhibit a broad spectrum of biological activities such as antimicrobial [2], antiviral, and anticancer activities [3]. However, most natural peptides are composed of *L*-form α-amino acids and because of the ubiquitous prevalence of peptidases, they have limited biostability, and consequently low bioavailability. To overcome this problem, stable and at the same time biologically active pseudo-peptides have been developed. These novel compounds open up new perspectives in drug design by providing an entire range of highly specific and non-toxic pharmaceuticals. With growing application on their synthesis and bioactivity, chemists and biologists in recent years have directed considerable attention on the research of pseudo-peptide derivatives mimicking the pharmacophore and thus the activity of the original peptide [4,5]. As isosteres of peptides, phosphonopeptides containing a transition state analogue of the hydrolysis of the amide bond represent another attractive approach for the preparation of proteolytically stable peptides [6]. In addition to increased stability, incorporation of a phosphonate moiety into the peptide sequence also provides access to additional binding interactions within the transition state conformation of the enzyme/substrate complex [7]. A wide range of phosphonopeptides have been used to design very effective protease inhibitors [8–10]. However, since there are only a limited number of economically viable chemicals available for practical application in biological science or agriculture [11], a great deal of scope still lies ahead for further research in this field. In this context, in order to find broad spectrum biologically active pseudo-peptide thiourea, we have previously described preparation of certain chiral thiourea derivatives containing α-aminophosphonate moiety with significant antiviral activity [12]. Herein, we further turned our attention to prepare novel compounds with enhanced antitumor activities by incorporating α-aminophosphonate moiety at the 1 or 3-position of pseudo-peptide thiourea. The primary aim of this study is to synthesize the title compounds and study their antitumor activities for the development of a new inhibitor to PC3, Bcap37 and BGC823 cells. To the best of our knowledge, this is the first report on the synthesis and antitumor activity of pseudo-peptide thioureas containing α-aminophosphonate moiety.

Abbreviations: ^{1}H NMR, ^{1}H Nuclear Magnetic Resonance; ^{13}C NMR, ^{13}C Nuclear Magnetic Resonance; ^{31}P NMR, ^{31}P Nuclear Magnetic Resonance; ^{19}F NMR, ^{19}F Nuclear Magnetic Resonance; MTT, [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide] assay; PC3, Prostate cancer; BGC823, Human gastric cancer; Bcap37, Breast cancer cell lines; ADM, Adriamycin.

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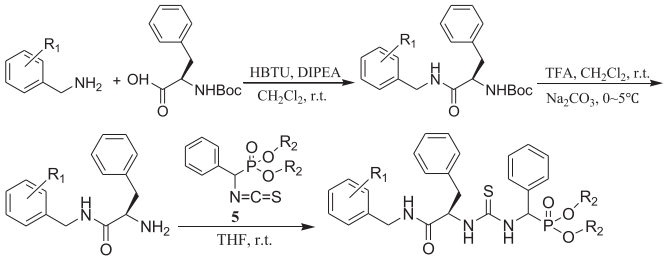


Cmpd	R_1	R_2	Cmpd	R_1	R_2	Cmpd	R_1	R_2	Cmpd	R_1	R_2
L-**IIa**	H	Et	*L*-**IIb**	H	*n*-Pr	*L*-**IIc**	H	*i*-Pr	*L*-**IId**	H	*n*-Bu
D-**IIa**	H	Et	*D*-**IIb**	H	*n*-Pr	*D*-**IIc**	H	*i*-Pr	*D*-**IId**	H	*n*-Bu
L-**IIe**	*o*-F	Et	*L*-**IIf**	*o*-F	*n*-Pr	*L*-**IIg**	*o*-F	*i*-Pr	*L*-**IIh**	*o*-F	*n*-Bu
D-**IIe**	*o*-F	Et	*D*-**IIf**	*o*-F	*n*-Pr	*D*-**IIg**	*o*-F	*i*-Pr	*D*-**IIh**	*o*-F	*n*-Bu
L-**IIi**	*p*-F	Et	*L*-**IIj**	*p*-F	*n*-Pr	*L*-**IIk**	*p*-F	*i*-Pr	*L*-**III**	*p*-F	*n*-Bu

Fig. 1. Synthetic pathway to target compounds **IIa**–**l**.

2. Results and discussion

2.1. Chemistry

The synthetic route designed for the pseudo-peptide thiourea analogues containing α-aminophosphonate moiety **IIa**–**l** is summarized in Fig. 1. Boc-protected intermediate amide was first generated from the reaction of phenylalanine and substituted benzylamine in presence of *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) at room temperature. Chiral α-amino carboxamide derivatives **Ia**–**c** were then obtained through deprotection of Boc with trifluoroacetic acid as shown in the figure. The desired pseudo-peptide thiourea analogues **IIa**–**l** containing α-aminophosphonate moiety were prepared by the addition of racemic *O*,*O*′-dialkylisothiocyanato(phenyl)methylphosphonate **5** to **Ia**–**c** in THF at room temperature.

The key intermediate *O*,*O*′-dialkylisothiocyanato(phenyl)methylphosphonate **5** was prepared according to the literature procedure [12]. In order to improve the yield, the existing method for conversion of **4** into **5** was further modified by using Bis(trichloromethyl) carbonate (BTC) instead of phosphorus oxychloride as shown in Fig. 2. The results obtained with BTC were compared with those obtained with phosphorus oxychloride (Table 1). As could be observed from the table, the yields phosphonate **5** in the former case from different alkyl substituents ranged from 67.4–78.6%. The other intermediates involved in the preparation of **5** were obtained as depicted in Fig. 2 [12].

Table 1
Comparison of the yields of **5a**–**d** in two different methods.

Compound	R_2	Yield (%)	
		$POCl_3$[a]	BTC[b]
5a	Et	60.3	78.6
5b	*n*-Pr	61.5	70.5
5c	*i*-Pr	54.2	67.4
5d	*n*-Bu	57.8	75.1

[a] The reported yields [12].
[b] The yields of isolated products.

In order to establish whether the overall reaction from enantiomer pure *N*-Boc-phenylalanine leads to any loss of optical purity through partial racemization in presence of base, enantiomeric excess (ee) values of *L*-**Ia** and diastereoisomeric excess (de) values based on the stereoconfiguration of amino acid amide part of final product *L*-**IIa** obtained from *L*-*N*-Boc-phenylalanine were determined. While Boc-deprotected product (free amine) showed an ee of 99.4%, the target compound *L*-**IIa** arising through nucleophilic addition of *L*-**Ia** on α-phosphateisothiocyanate was obtained in 98.5% de. Thus, as expected, stereochemical configuration at α-carbon atom of the acid was practically unaffected and this synthetic transformation from chiral α-amino acid could be applied to a wide range of compounds without undergoing any significant loss of optical activity. Since both *L*- and *D*-isomers of *N*-Boc-phenylalanine are easily accessible, it provides a useful route to obtain both the enantiomeric forms of carboxamide **Ia**–**c** which are useful synthons to a variety of pharmaceuticals. The overall process for novel (*L*)/(*D*)-pseudo-peptide thioureas **IIa**–**l** is convenient, high yielding and atom-economic.

The yield of target compound **IIa**–**l** depended both on the absolute configuration of initial *N*-Boc-phenylalanine and nature of substituents present in the phenyl ring and phosphonate moiety respectively. The structures of all the compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR spectra, and elemental analysis. The IR spectra of products **IIa**–**l** exhibited absorption bands at 3281–3289 cm^{-1}, indicating the presence of amidic N–H. The stretching frequency at 1653–1684 cm^{-1} was assigned to C=O vibrations and the characteristic absorptions at 1207–1224 cm^{-1} and 1005–1024 cm^{-1} were attributed to the presence of P=O and the P–O–C group, respectively. In ^{1}H NMR spectra, all phenyl protons showed multiplet at 7.49–6.71 ppm. The main characteristic of the ^{1}H NMR spectra of **IIa**–**l** is the presence of high-frequency downfield broad singlet δ_H 8.84–8.43 for amidic N–H protons. The broad doublets at δ_H 8.12–7.97 presumably arise due to the presence of deshielded N–H proton of thiourea linked to the phosphonate moiety and benzene ring through an intervening carbon atom. While the broad doublets at δ_H 6.45–6.32 are assigned to the N–H proton of thiourea directly attached to the

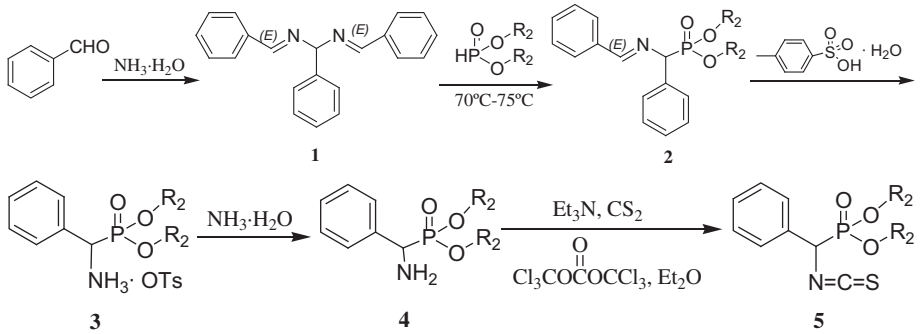
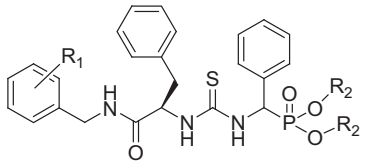


Fig. 2. Synthetic route to the intermediates **5a**–**d**.

Table 2
Growth inhibition of selected cell lines.



IIa-l

Compound[a]	R_1	R_2	Inhibition ratio(%)[b]		
			Bcap37[c]	PC3[d]	BGC823[e]
***L*-IIa**	H	Et	16.9 ± 10.9	20.1	18.3 ± 8.1
***L*-IIb**	H	*n*-Pr	35.4 ± 4.7	33.5	35.7 ± 4.7
***L*-IIc**	H	*i*-Pr	24.4 ± 4.1	50.1	27.9 ± 4.0
***L*-IId**	H	*n*-Bu	34.5 ± 6.5	22.8	21.1 ± 11.6
***L*-IIe**	*o*-F	Et	25.9 ± 4.7	19.3	30.1 ± 3.8
***L*-IIf**	*o*-F	*n*-Pr	44.7 ± 9.2	18.1	11.1 ± 26.3
***L*-IIg**	*o*-F	*i*-Pr	44.4 ± 18.1	26.8	47.9 ± 5.0
***L*-IIh**	*o*-F	*n*-Bu	32.1 ± 7.8	22.5	30.1 ± 21.7
***L*-IIi**	*p*-F	Et	26.1 ± 11.7	27.9	22.9 ± 7.4
***L*-IIj**	*p*-F	*n*-Pr	24.0 ± 16.4	56.7	21.0 ± 6.3
***L*-IIk**	*p*-F	*i*-Pr	53.1 ± 9.2	58.6	89.1 ± 1.2
***L*-III**	*p*-F	*n*-Bu	48.4 ± 6.7	19.7	26.1 ± 9.5
***D*-IIa**	H	Et	52.3 ± 4.3	56.9	55.7 ± 10.0
***D*-IIb**	H	*n*-Pr	49.1 ± 7.2	30.8	24.4 ± 11.5
***D*-IIc**	H	*i*-Pr	22.9 ± 8.4	30.5	29.0 ± 10.9
***D*-IId**	H	*n*-Bu	46.6 ± 8.9	21.7	22.7 ± 12.9
***D*-IIe**	*o*-F	Et	70.2 ± 3.9	73.1	30.1 ± 3.8
***D*-IIf**	*o*-F	*n*-Pr	17.2 ± 4.8	30.6	11.1 ± 26.3
***D*-IIg**	*o*-F	*i*-Pr	27.8 ± 8.1	14.3	51.6 ± 5.0
***D*-IIh**	*o*-F	*n*-Bu	34.0 ± 7.4	18.0	30.1 ± 21.7
***D*-IIi**	*p*-F	Et	34.0 ± 8.6	32.4	44.5 ± 12.6
***D*-IIj**	*p*-F	*n*-Pr	12.6 ± 8.4	30.6	53.9 ± 0.0
***D*-IIk**	*p*-F	*i*-Pr	27.0 ± 5.8	34.8	20.8 ± 14.7
***D*-III**	*p*-F	*n*-Bu	35.8 ± 7.3	33.6	33.2 ± 4.2
ADM[f]					93.8 ± 2.1[g]

[a] These compounds were tested as the free base.
[b] The known numbers of cells (2.0×10^4) were incubated for 24 h in a 5% CO_2 incubator at 37 °C in the presence of different concentrations of test compounds. After 24 h of drug incubation, the MTT solution was added, supernatant was discarded, 100 ml DMSO was added in each well and absorbance was recorded at 595 nm by ELISA reader; determined by three separate experiments and each was performed in triplicate.
[c] Breast cancer.
[d] Prostate cancer.
[e] Stomach cancer.
[f] The standard compound used for comparison of activity.
[g] The value was determined by using our assay protocol.

asymmetric carbon atom, another adjacent doublet at δ_H 6.33–6.12 appeared due to the C–H proton of N–C–P. The multiplet at δ_H 5.28–5.14 was assigned to the C–H of N–C–C group. The typical low intense carbon resonance frequencies at δ_c 183.8–182.9 and δ_c 171.2–170.4 in the ^{13}C NMR spectra of **IIa–l** also confirmed the presence of C=S and C=O double bond respectively. As expected, fluorine and phosphorus resonance appeared at δ_F −118.8 to 115.2 and δ_P 20.3–22.6 in their corresponding fluorine and phosphorus NMR spectra, respectively. According to the ^{31}P NMR spectra of all target compounds, phosphorus atom of the phosphonate moiety was coupled to adjacent CH.

2.2. Evaluation of antitumor activities

Some potential chiral thiourea derivatives incorporating phosphonate moiety were identified for cancer treatment by studying their anticancer activities on 3 human tumor cells. All the compounds in the series **IIa–l** and commercial drugs ADM (adriamycin) and PD153035 [6,7-dimethoxy-*N*-(3-bromophenyl)-4-aminoquiazoline] [13] were evaluated for their anti-proliferation activities against three types of human cancer cell lines, breast cancer, prostate cancer and stomach cancer cells as shown in Table 2.

Among the studied compounds, ***L*-IIc** (R_1 = H, R_2 = *i*-Pr), ***D*-IIa** (R_1 = H, R_2 = Et), ***D*-IIe** (R_1 = *o*-F, R_2 = Et), ***L*-IIj** (R_1 = *p*-F, R_2 = *n*-Pr) and ***L*-IIk** (R_1 = *p*-F, R_2 = *i*-Pr) exhibited moderate inhibitory activities of 50.1%, 56.9%, 73.1%, 56.7% and 58.6%, respectively against PC3-cell at 10 μM concentration. The rest of the compounds, however, did not indicate any significant inhibition against PC3-cell. The compounds ***D*-IIe** (R_1 = *o*-F, R_2 = Et), ***L*-IIk** (R_1 = *p*-F, R_2 = *i*-Pr), ***D*-IIa** (R_1 = H, R_2 = Et) and ***D*-IIb** (R_1 = H, R_2 = *n*-Pr) also displayed potential antitumor bioactivities of 70.2%, 53.1%, 52.3% and 49.1% respectively against Bcap37 cells at 10 μM. From Table 2 it is evident that compounds ***L*-IIk** and ***D*-IIa** bearing an electron withdrawing fluorine atom at the 4-position of the aromatic ring showed good antitumor activities with inhibition rate ranging from 55.7% to 89.1% against BGC823 cell lines. Remarkably, these values were comparable to the inhibition rate exhibited by ADM (adriamycin) (98%). From the data, it may also be derived that the cell growth inhibition bioactivity of ***L*-IIk** has appreciable effect on BGC823 cells, being better than the commercial analogue PD153035.

In addition, selected chiral compounds ***D*-IIe**, ***D*-IIa**, ***L*-IIk,** ***L*-IIj** and ***L*-IIc** which displayed good antitumor activities were bioassayed further to investigate their efficacies at different concentrations with ADM and PD153035 serving as the commercial controls. As shown in Table 3, the inhibition effects of these compounds against PC3 were significant. The IC_{50} values of ***D*-IIe**, ***D*-IIa**, ***L*-IIk,** ***L*-IIc** and ***L*-IIj** were 6.7, 11.2, 17.2, 19.5 and 22.1 μM, respectively. Among these compounds, ***D*-IIe** showed highest antitumor activity, comparable to that shown by PD153035 (IC_{50} = 13.7 μM) against PC3. Also an interesting observation noted was that the activity displayed by ***L*-IIk** in terms of the average IC_{50} value (4.7 μM) against BGC823 was also comparable to that of PD153035 (IC_{50} = 6.9 μM). This finding appears to be beneficial to establish SAR of title pseudo-peptide thiourea analogues.

2.3. Structure–activity relationship (SAR)

Within the limits of experimental error, the title compounds with *D*-configuration displayed better antitumor activities against Bcap37 compared to their respective *L*-counterparts e.g. ***D*-IIa** (52.3%) > ***L*-IIa** (16.9%), ***D*-IIe** (70.2%) > ***L*-IIe** (25.9%), ***D*-IIi** (34.0%) > ***L*-IIi** (26.1%). Furthermore, the compound bearing a fluorine atom at the 4-position of the benzyl ring linked to the nitrogen atom of the amide (R_1 = *p*-F) showed good antitumor activities with inhibition rate ranging from 55.7 to 89.1% against BGC823 cell lines. With regard to the alkyl substituents (R_2) in the phosphonate part of the studied compounds, *D*-diastereoisomers with diethyl substituents (R_2 = Et) displayed significant enhancement towards inhibition against Bcap37, PC3 and BGC823 cells in comparison with *D*-*O,O′*-dialkylphosphonates derived from *n*-propyl, isopropyl or *n*-butyl groups. In particular, the compound ***D*-IIe** (R_1 = *o*-F, R_2 = Et) displayed most promising result amongst all. The *L*-diastereoisomers of *O,O′*-diisopropylphosphonate e.g. ***L*-IIk** (R_1 = *p*-F, R_2 = *i*-Pr), ***L*-IIc** (R_1 = H, R_2 = *i*-Pr), ***L*-IIg** (R_1 = *o*-F, R_2 = *i*-Pr), on the other hand, revealed higher antitumor activities than the *L*-diastereoisomers of phosphonates derived from ethyl, *n*-propyl or butyl groups (R_2 = Et, *n*-Pr, Bu). Thus, the nature of stereochemical configuration (*D* or *L*) of the amino acid amide part, type of substituents (R_1) in the aromatic ring as well as alkyl substituents (R_2) in the phosphonate moiety of the title compounds appear to be the key factors in controlling the antitumor activity. While phosphonates derived from branched alkyl chains were preferred over straight-chain

Table 3
Cytotoxic activity of promising compounds in PC3 and BGC823 cell at different concentrations of drug exposure by MTT assay.

Compound	1 μM[a]	5 μM[a]	10 μM[a]	20 μM[a]	IC_{50} (μM)[b]	Cell line
***L*-IIc**	2.7 ± 0.2	12.8 ± 1.4	21.8 ± 3.2	50.14 ± 4.9	19.5 ± 2.1[d]	PC3
***L*-IIj**	18.1 ± 4.1	19.8 ± 4.2	28.0 ± 1.9	51.2 ± 2.3	22.1 ± 1.9[d]	
***L*-IIk**	15.8 ± 2.1	17.3 ± 1.9	31.4 ± 3.1	66.7 ± 3.0	17.2 ± 2.1[d]	
***D*-IIa**	10.4 ± 2.1	21.2 ± 0.9	56.9 ± 3.1	73.8 ± 4.0	11.2 ± 0.9[d]	
***D*-IIe**	4.9 ± 0.9	45.6 ± 3.1	73.1 ± 2.1	97.2 ± 0.2	6.7 ± 0.1[d]	
ADM (adriamycin)[c]	36.4 ± 2.0	89.4 ± 2.1	98.0 ± 3.1	100	3.3 ± 0.8[d]	
PD153035[c]					13.7 ± 1.4[d]	
***L*-IIk**	30.6 ± 2.1	70.1 ± 1.1	89.1 ± 2.2	100	4.7 ± 0.04[e]	BGC823
PD153035[c]					6.9 ± 1.1[e]	

[a] The data represented the mean of three experiments in triplicate and were expressed as means ±SD; Only descriptive statistics were done in the text.
[b] The IC_{50} value was defined as the concentration at which 50% survival of cells was observed. The results are listed in the table.
[c] Used as a positive control.
[d] IC_{50} values on PC3.
[e] IC_{50} values on BGC823.

alkyls for *L*-diastereoisomers, simple diethyl derived phosphonates gave better result with *D*-diastereoisomers. Therefore, for ideal activity, R_1 should be a strong electron withdrawing group and for *L*-diastereoisomers R_2 should be *i*-Pr, whereas for *D*-diastereoisomers R_2 needs to be Et. Although our studies indicate the existence of a definite relationship of antitumor activity with the nature of substituents and type of configuration within the compound, the role of steric, electronic and hydrophobic effects at physiological pH on structure–activity relationships could not be successfully ascertained due to lack of structural diversity.

3. Conclusion

A series of new pseudo-peptide thioureas **IIa**–**l** containing α-aminophosphonate moiety were synthesized in high yield under mild conditions by the addition of racemic *O,O′*-dialkyl isothiocyanato(phenyl)methylphosphonate **5** to chiral α-amino carboxamide derivatives in THF. The synthetic method from chiral *N*-Boc-phenylalanine practically does not affect the stereochemical configuration of α-carbon bearing the amino group. The synthesized compounds **IIa**–**l** could inhibit tumor cells PC3, Bcap37 and BGC823 and showed low to high activity by MTT assays. Among them, *L*-**IIk**, *D*-**IIa** and *D*-**IIe** were found as potent inhibitors, with IC_{50} values ranging from 4.7 to 11.2 μM by *in vitro* assay. The present work demonstrates that the antitumor activity of pseudo-peptide thioureas was significantly improved through introduction of suitably substituted stereoisomer with *Ds.*-configuration. The *D*-diastereoisomers, in general, displayed enhanced activity compared to their corresponding *L*-diastereoisomers. Influence of different substituents, minor structural modification and steric parameters on structure–activity relationships for identifying lead bioactive compound would be taken up in our future investigation.

4. Experimental section

4.1. Analysis and instruments

The melting points of the products were determined on a XT-4 binocular microscope (Beijing Tech Instrument Co., China) and were not corrected. The IR spectra were recorded on a Bruker VECTOR22 spectrometer in KBr disks. ^1H and ^{13}C NMR (solvent DMSO-d_6) spectra were recorded on a JEOL-ECX 500 NMR spectrometer at room temperature using TMS as an internal standard. Elemental analysis was performed on an Elementar Vario-III CHN analyzer. UV spectra were recorded on a VARIAN Cary-50 spectrometer using a cell path length of 1 cm. BIO-RAD, Model 680 Microplate Reader. The reagents were all of analytical grade or chemically pure. Analytical TLC was performed on silica gel GF254.

4.2. Preparation of intermediates ***Ia*–*c***

Phenylalanine (2.65 g, 0.01 mol, 1.00 equiv.) and *O*-benzotriazol-1-yl-*N,N,N′,N′*-tetramethyl- uronium hexafluorophosphate (HBTU, 3.80 g, 0.01 mol, 1.00 equiv.) were loaded into an oven-dried round-bottomed flask equipped with a magnetic stir bar, rubber septum, and argon inlet. Anhydrous dichloromethane (50 mL) was then added. After 3 min, anhydrous DIPEA (2.58 g, 0.02 mol, 2.0 equiv) and benzylamine (0.011 mol, 1.10 equiv.) were sequentially added and the reaction mixture was stirred at room temperature for 2–4 h. During the process, the state of the solution was seen to change from slightly heterogeneous to homogeneous confirming the consumption of HBTU. And the reaction was followed by TLC (developing solvent: 4% MeOH/CH_2Cl_2, *V/V*). The reaction mixture was poured into a separatory funnel containing 1 N HCl (50 mL), and was then partitioned between dichloromethane and aqueous hydrochloric acid solution. The organic layer was washed with 1 N HCl (3 × 40 mL), dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. The resulting light yellow oil was transferred to a 100-mL flask and redissolved in dichloromethane (40 mL). Trifluoroacetic acid (10 mL) was then added in one portion. After 2–4 h, the mixture was slowly partitioned with dichloromethane and chilled, saturated aqueous sodium carbonate solution (50 mL) was added. The aqueous layer was extracted with dichloromethane (3 × 40 mL), and the combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. The crude product was purified by thin layer chromatography (TLC) on a silica gel (developing solvent: 4% MeOH/CH_2Cl_2, *V/V*) to give the intermediates **Ia**–**c**. The structure was confirmed by ^1H NMR, ^{13}C NMR, IR, and elemental analysis (see the Supporting Information).

4.3. Preparation of title chiral compounds ***IIa*–*l***

A solution of *O,O′*-dialkylisothiocyanato(phenyl)methylphosphonate **5** (1 mmol) in tetrahydrofuran (10 mL) was stirred, followed by drop wise addition of chiral amine **Ia**–**c** (1.1 mmol). The reaction mixture was stirred for 1 h at 23 °C, the solvent was removed by evaporation, and the crude product was purified by chromatography on silica using a mixture of petroleum ether and tetrahydrofuran as the eluent to give the title compounds **IIa**–**l** in 84–98% yields (see the Supporting Information).

4.3.1. *O,O′-diethyl(3-(L-1-(benzylamino)-1-oxo-3-phenylpropan-2-yl)thioureido)(phenyl)methyl-phophonate (**L-IIa**)*

White solid, mp 57–59 °C, yield, 95%; $[\alpha]_D^{20} = 11.4°$ ($c = 0.1$, acetone); IR (KBr, cm^{-1}) ν: 3287, 3082, 2978, 2926, 1684, 1522, 1454, 1352, 1211, 1024, 978, 698; ^1H NMR (500 MHz, $CDCl_3$, ppm) δ: 8.76 (br s, 1H, NH), 8.02 (br d, 1H, $J = 18.5$ Hz, NH), 7.29–7.13 (m, 15H, ArH), 6.33 (br d, 1H, $J = 9.4$ Hz, NH), 6.21 (d, 1H, $J = 8.1$ Hz, NCHP),

5.21 (d, 1H, $J = 8.1$ Hz, CH), 4.18–4.14 (m, 2H, NCH_2), 3.96–3.70 (m, 4H, 2 OCH_2), 3.04–3.02(m, 2H, CH_2Ar), 1.28–1.09 (m, 6H, $2CH_3$); ^{13}C NMR (125 MHz, $CDCl_3$, ppm) δ: 183.3, 170.8, 137.6, 137.1, 136.9, 129.4, 128.6, 128.5, 125.5, 64.0, 59.8, 54.3, 43.4, 38.3, 16.2; ^{31}P NMR (200 MHz, $CDCl_3$, ppm) δ: 22.4; Anal. Calcd for $C_{28}H_{34}N_3O_4PS$ (540): C, 62.32; H, 6.35; N, 7.79%. Found: C, 62.53; H, 6.86; N 7.38%.

4.4. MTT assay against cancer cell proliferation

All tested compounds were dissolved in DMSO (1–100 μM solution) and subsequently diluted in the culture medium before treatment of the cultured cells. Tested cells were plated in 96-well plates at a density 2×10^3 cells/well/100 μL of the proper culture medium and treated with the compounds at 1–100 μM for 72 h. In parallel, the cells treated with 0.1% DMSO served as control. An MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium-bromide] assay (Roche Molecular Biochemicals, Cat. No. 11465007001) was performed 4 h later according to the instructions provided by Roche. This assay was based on the cellular cleavage of MTT into formazane which is soluble in cell culture medium. Any absorbance caused by formazan was measured at 595 nm with a microplate reader (BIO-RAD, model 680), which is directly proportional to the number of living cells in culture. Three types of cells were used in these assays, PC3 (prostate cancer), BGC823 (human gastric cancer) and Bcap37 (breast cancer) cell lines, provided by ATCC and cultivated in RPMI 1640 (for PC3, BGC823 and Bcap37) supplemented with 10% fetal bovine serum. Tissue culture reagents were obtained from Gibco BRL [14]. The experiment was performed in triplicate. The percentage cytotoxicity was calculated using the formula.

$$\%\,\text{Cytotoxicity} = \frac{(\text{Control abs} - \text{Blank abs}) - (\text{Test abs} - \text{Blank abs})}{(\text{Control abs} - \text{Blank abs})} \times 100$$

4.5. Statistical analysis

All statistical analyses were performed with SPSS 10. Data were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed using the least significant difference method (LSD test). Each experiment had three replicates and all experiments were run three times with similar results. Measurements from all the replicates were combined and treatment effects analyzed.

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Supporting information

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