A C G publications

Org. Commun. 8:1 (2015) 1-8

organic communications

Synthesis of chiral phosphonoacetamides and their toxic effects on *Paramecium sp*

Samia Guezane Lakoud[*1], Marc Lecouvey[2], Houria Berrebah[3] and Nour-Eddine Aouf[4]

[1]*Chemistry Department, Sciences Faculty, Badji Mokhtar-Annaba University, BP 12 El Hadjar, 23000, Algeria*

[2]*Laboratoire CSPBAT, FRE 3043 CNRS, University of Paris 13, 74 rue Marcel Cachin, 93017 Bobigny Cedex, France*

[3]*Laboratory of Cell Toxicology, Badji-Mokhtar University, Algeria.*

[4]*Laboratory of Applied Organic Chemistry, Badji-Mokhtar University, El-Hadjar, Annaba, Algeria*

(Received October 1, 2013; Revised December 10, 2013; Accepted March 12, 2014)

Abstract: Three chiral phosphonoacetamides were prepared by an alternative method. For this purpose, 2-(diethoxyphosphoryl)acetic acid was prepared from ethyl 2-bromoacetate by treatment of $P(OEt)_3$ followed by saponification of the ester with K_2CO_3. BOP activated amidation of the 2-(diethoxyphosphoryl)acetic acid with (S)-amino acids gave the corresponding phosphonoaceteamides. Growth inhibition of two phosphonoacetamides on *Paramecium sp.* were studied.

Keywords: Paramecium *sp*; Organophosphorus; Phosphonoacetamide; phosphorylation; coupling reaction.

1. Introduction

Organophosphorus compounds are important class in industrial, agricultural, and medicinal chemistry owing to their biological and physical properties[1], they have been used as insecticides and chemical warfare agents are known to cause potent neurotoxic effects in humans and animals[2]. In recent years, a variety of phosphonic acid derivatives such as hydroxyphosphonates[3,4], aminophosphonates[5-7], amidophosphonates[8-10] have been synthesized by diverse methods. The compounds containing the phosphonate derivatives have been also used as chiral pesticides[11], peptidomimetics[12], enzyme inhibitors[13, 14], herbicides[15], insecticides[16], fungicides[17], and antiviral agents[18]. Additionally, they have been used in the preparation of catalytic antibodies for a variety of reactions[19].

The presence of phosphoryl-carboxamide[20] is responsible for evaluation of the potential biological activity of phosphonoacetamides compounds. For example, the compound **1** derived

* Corresponding author: E-mail: g11_samia@yahoo.fr; Tel: 21352715507

from proline is known of its antibacterial properties, the alaphosphine **2** is an herbicide and fungicide powerful and the compound **3** is a dipeptide and used for the treatment of type II diabetes [21] .

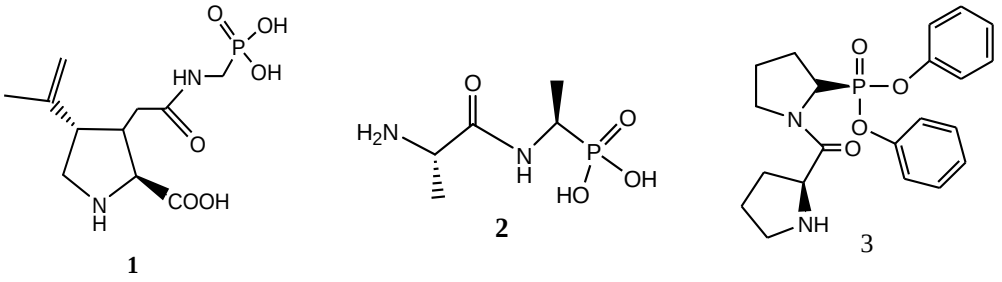

Figure 1. Some compounds containing phosphoryl-carboxamide having a biological activity.

In our previous work we synthesized phosphonoacetamides derivatives by two methods (Arbuzov and Becker reactions) (Figure 3)[22]. The unicellular ciliatea of the genus *Paramecium* belong to the most often studied protists and their use has been proposed as bioethical and excellent test for standardized laboratory procedures to evaluate the environmental quality, and the effects of xenobiotic compounds on a simple alternative biosystem[23,24]. The protozoon in general is sensitive to environmental perturbations such as the occurrence of xenobiotic compounds[25]. The sensitivity of protozoa is due to their simple eukaryotic single-cell organism organization which exposes their receptors to the external environment, making them responds directly to environmental stimuli.

In this study, we aimed at an alternative method for preparation of phosphonoacetamides (figure 2) via BOP activated amidation of 2-(diethoxyphosphoryl)acetic acid and its study on growth of *Paramecium sp.*

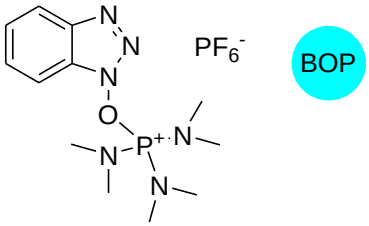

Figure 2. (Benzotriazol-1-yloxy) tris(dimethylamino) phosphonium hexafluorophosphate

2. Results and discussion

2.1. Chemistry

In our previous study, chiral phosphonoacetamides derivatives were synthesized by phosphorylation using the Arbuzov reaction as thermal way or the Michaelis–Becker reaction as anionic way after the acylation reaction of amino esters with chloroacetyl chloride (Figure 3)[22].

In the present method, we first phosphorylated ethyl 2-bromoacetate with triethylphosphite ($P(OEt)_3$) to give ester **7**. Potassium carbonate mediated hydrolysis of ester **7** afforded (diethoxyphosphoryl)acetic acid **8**. BOP activated amidation of acetic acid **8** with amino acids **4a-c** gave phosphonoacetamides (*S*)-**6a-c** as oily products. The activating agent BOP has the advantage of giving good yield; it can reach up to 80% in reaction time relative progress as in our case. Furthermore, it shows an absence of racemization at the asymmetric carbon of the aminoesters[26] (Figure 4).

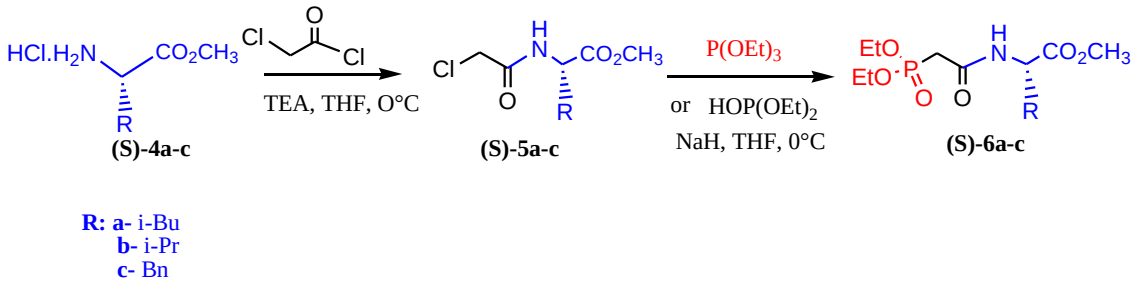

Figure 3. Synthesis of chiral phosphonoacetamide by Michaelis-Becker or Arbuzov reactions[22]

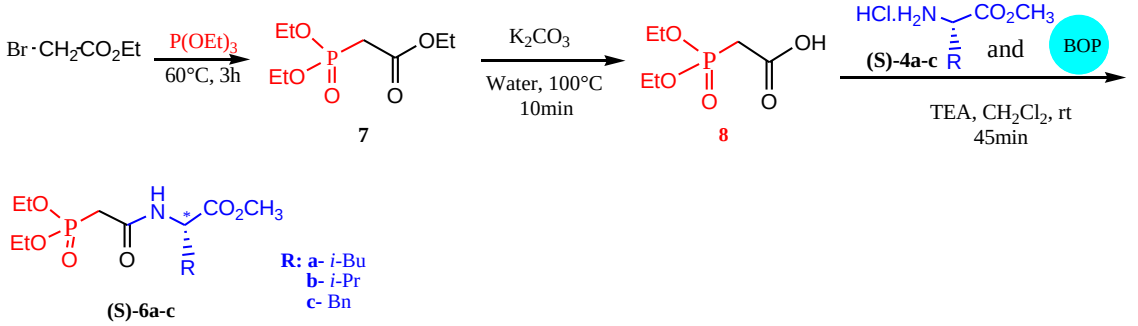

Figure 4. Synthesis of chiral phosphonoacetamide using the coupling agent BOP

2.2. Toxicity Test

In the first time, we confirmed the inhibitory effect of the compounds **6b** and **6c** compared with commercially compounds as Chlorpyrifos Ethyl.

Figure 5 and 6 illustrates the evolution of cell growth as a function of time (in minutes than hours) and increasing a concentration of xenobiotic; methyl isopropylacetate and methyl benzylacetate derivatives (**6b** and **6c** respectively). First, the results show normal growth for the control cells at the first 30th minutes with an optical density (OD) 0.127 for the first one (**6b**) and 0.144 for the second (**6c**). Meanwhile, with the treated cells, it shows the significant inhibitory effect ($P < 0.001$ and $P \leq 0.005$) of the growth of this microorganism and upon contact with the xenobiotic (**6b** and **6c** compounds respectively), with an OD of 0.05 for 10 µmol L^{-1}, and 0.03 for the highest concentration at the 30 minutes for the compound **6b**, and with an OD of 0.126 for 10 µmol L^{-1}, and 0.06 for the highest concentration at the same time for the compound **6c**.

In the hours' time, we observed the same significant inhibitory effect of ($P \leq 0.001$) of growth from the first hour of treatment until the sixth hour for both compounds. Meanwhile, the evolution growth of control cells shows a normal growth with an exponential growth phase between the first and the third hour, and a progressive decrease of the growth from the fourthhour.

The percentages of response in *Paramecium* treated for the cultures exposed by different concentrations of **6b** and **6c** compounds after 6 h of exposure are positive.

We observe response percentages of 68%, 55% and 77% respectively of 10, 20 and 30 µmol L^{-1} for the compound **6b**, and 18%, 30% and 24% respectively of 10, 20 and 30 µmol L^{-1} for the compound **6c** as shown in Figure 7. It can be said that the positive evolution of the

response percentage confirm the growth inhibition of the treated paramecia regardless of the cell concentration. But, for the control cells, the percentage of response is always negative, reflecting a normal growth.

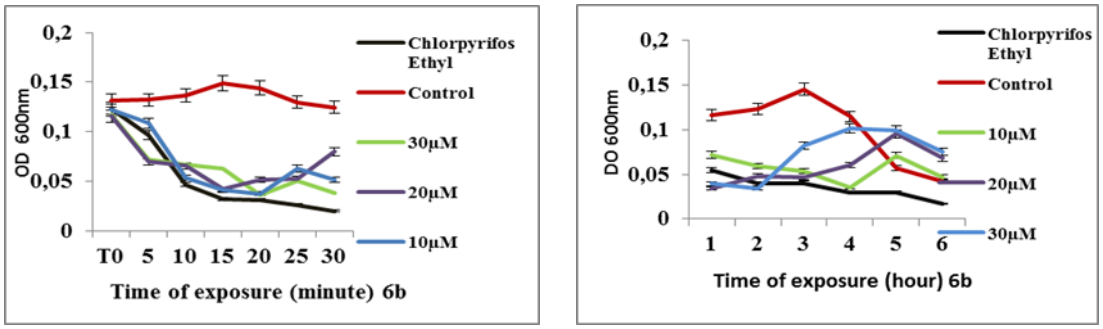

Figure 5. Evaluation of cell growth of *Paramecium* treated with increasing concentrations of 6b compounds for the short and the long times. Each data point represents the average of three independent essays ± standard error

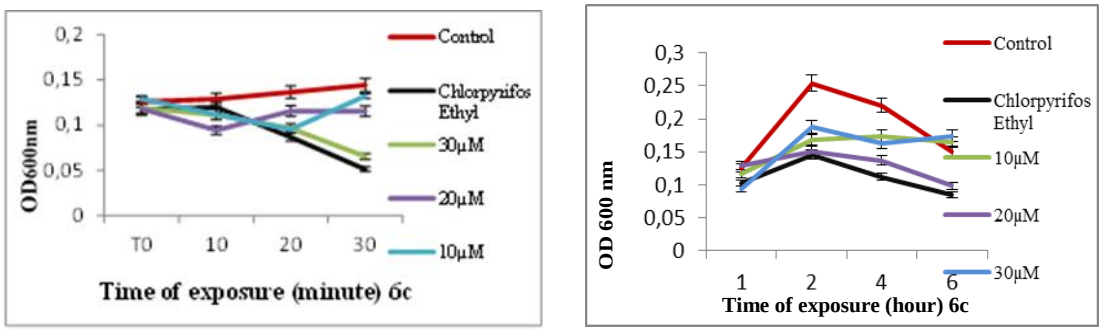

Figure 6. Evaluation of cell growth of *Paramecium* treated with increasing concentrations of 6c compound for the short and the long times (hour). Each data point represents the average of three independent essays ± standard error)

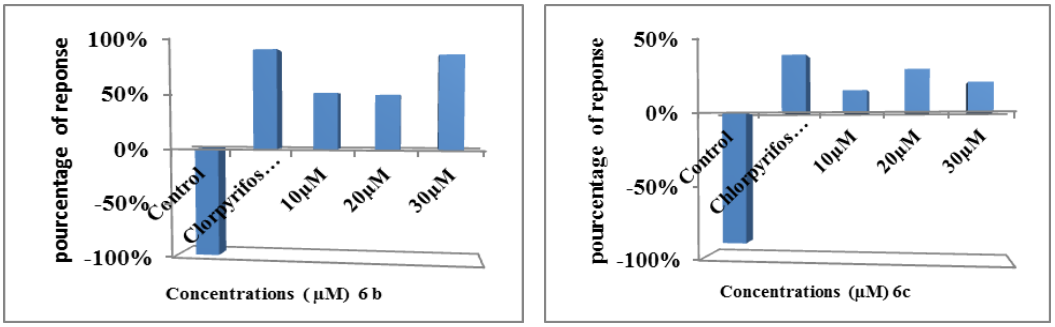

Figure 7. Evolution of the response percentage of *Paramecium sp*. in the presence of the different concentrations of 6b and 6c compounds)

Protists are ubiquitous eukaryotic cells in the aquatic and terrestrial environment, characterized by a short life cycle, rapid multiplication and normal behavior may be affected by the presence of pollutants. Evaluation of cytotoxic effects of a xenobiotic can be performed using different parameters, including cell growth in microorganisms that reflects the state of cell metabolism.

Thus, our results show that the different concentrations of phosphonoacetamide result in an inhibition of the cell growth of Paramecium *sp*. The tested xenobiotic is toxic to a microorganism which is manifested by inhibition of cell growth. This brings us to confirm the influx of xenobiotics inside the cells, despite the presence of the cell membrane that forms a barrier against the massive entry of xenobiotics but is still permeable.

In addition, for the three concentrations tested (10, 20, and 30 μmol L^{-1}), the high value of optical density and the positive evolution of the response percentages confirms the growth inhibition of the treated *Paramecium sp*., regardless of the cell concentration.

2.2.1. Materials and methods

Culture conditions:

Paramecium sp. cell lines were established by single cell isolation in the Cell Toxicology Laboratory at the University of Annaba (Algeria). Cells were grown according to the usual procedures[27] in lettuce medium bacterized by *Klebsiella pneumoniae*, supplemented with 0.8 μg mL^{-1} of *β*-sitosterol (Merck [1]). Culture temperature was 27 ± 3°C. The populations studied were derived from single autogamous cells.

Cell growth, optical density and percentage of response:

Our results were performed by measuring of optical density at wavelength λ=600 nm as function of time at different concentrations (10, 20 and 30 μmol L^{-1}) and by calculation of the percentage of response, these studies were made to evaluate the toxicity of the xenobiotic via the inhibition of cell growth of *paramecium sp* after 6h of exposure.

An optical density (OD) of high value indicates an inhibition of growth, than the low value indicates the stimulation of growth.

A percentage of positive value indicates an inhibition of growth, while negative value indicates a stimulation of growth[28] according to the equation

$$RP = 100.\frac{CN-EN}{CN}$$

Where; RP is the protozoa responses percentage (%); CN is the cell control number (cell mL^{-6}), and EN is treated cells number (cell mL^{-6}).

Statistical analysis

All the experiments were repeated three times, and the results were expressed as average and standard error (SE) values. Statistical analysis was performed by the STUDENT test to compare the means of two populations using data from two independent samples[29]. The *α*- level for significant differences was set at $P \leq 0.05$.

3. Experimental

Melting points were determined in open capillary tubes on an Electro thermal apparatus and uncorrected. IR spectra were recorded on a Perkin–Elmer FT-600 spectrometer. Proton nuclear magnetic resonance was determined with a 360 WB or AC 250-MHz Bruker spectrometer using $CDCl_3$ and DMSO-d_6 as a solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported in δ units (ppm). All coupling constants (J) are reported in Hertz. Multiplicity is indicated as s (singlet), d (doublet), t (triplet), m (multiplet), and combination of these signals. Electron ionization mass spectra (30 eV) were recorded in positive mode on a Water MicroMass ZQ. High- resolution mass were measured on a Joel SX 102 mass spectrometer and recorded in FAB positive mode. All reactions were monitored by TLC on silica Merck h60 F254 (Art. 5554) percolated aluminum plates and were developed by spraying with ninhydrin solution.

3.1. Synthesis of 2- diethoxyphosphoryl 2- ethyl acetate 7: 0.1 mole of ethyl acetate bromide was heated to 60° C, and then 1.1equiv of the triethylphosphite was added dropwise under nitrogen. When the addition was complete, the solution is heated to 100 ° C and stirred for 3 hours at the same temperature, we evaporated the reaction mixture to remove the $P(OEt)_3$ excess. The 2-diethoxyphosphoryl 2-methyl acetate was directly obtained as oil in 98% yield, R_f = 0.45 (CH_2Cl_2/MeOH), ^{1}H NMR ($CDCl_3$, 400MHz): δ = 1.29 (t, *J* = 6.50 Hz, 6H, $(CH_3CH_2O)_2P$), 2.85 (d, *J* = 12.50, 2H, CH_2P), 3.75 (s, 3H, OCH_3), 4.0 (m, 4H, $(CH_3CH_2O)_2P$), ^{13}C NMR ($CDCl_3$, 400MHz): 16.50 ($\mathbf{C}H_3CH_2O)_2$ P, 16.50 ($\mathbf{C}H_3CH_2O)_2P$, 35.70 ($\mathbf{C}H_2P$), 54,55($O\mathbf{C}H_3$), 63.75 ($CH_3\mathbf{C}H_2O)_2P$, 63.78 ($CH_3\mathbf{C}H_2O)_2$ P, 176.5 (**C**O-OCH_3), IR (CCl_4, σ cm^{-1}**) :** 1720.5 (C=O), 1230.5 (P=O), 1145.5 (OEt).

3.2. Synthesis of 2- diethoxyphosphoryl 2- carboxylic acid 8: To a solution of potassium carbonate (20 mmol) in 15ml of water, the 2- diethoxyphosphoryl 2- ethyl acetate (15mmol) was added and the mixture was refluxed for 10 min. The solution was washed with ether (2x10ml) then acidified by acid chlorohydrate (6 mol / l) to PH = 2, we evaporated half the water than extracted with dichloromethane (2x45ml). The organic layers were combined, dried over anhydrous $MgSO_4$ and concentrated under vacuum. The acid is obtained as colorless oil with an excellent yield (98%), R_f = 0.3 (CH_2Cl_2/MeOH), ^{1}H NMR ($CDCl_3$, 400MHz): δ = 1.27 (t, *J* = 6.72 Hz, 6H, $(CH_3CH_2O)_2P$), 2.90 (d, *J* = 21.68 Hz, 2H, CH_2P), 4.15 (m, 4H, $(CH_3CH_2O)_2P$), 7.63 (s, 1H, OH), RMN ^{13}C ($CDCl_3$, 400MHz): 16.45 ($CH_3\mathbf{C}H_2O)_2P$, 16.45 ($CH_3\mathbf{C}H_2O)_2$ P, 35.50 ($\mathbf{C}H_2P$), 63.80 ($CH_3\mathbf{C}H_2O)_2$ P, 63.98 ($CH_3\mathbf{C}H_2O)_2$ P, 175.45 (**C**O-OH), IR (CCl_4, σ cm^{-1}**) :** 3350-3500 (O-H), 1620.5 (**C**=O), 1225.5 (P=O), 1198 (OEt).

3.3. General procedure for the synthesis of 6a-c: To a solution of amino esters (1.5mmol) in dry CH_2Cl_2 the triethylamine (1.5mmol) was added dropwise at 0°C. We added then successively; a solution of diethoxyphosphoryl carboxylic acid (1.5 mmol) in CH_2Cl_2 (15ml), a solution of BOP (1.5 mmol) in CH_2Cl_2 (7 ml) and a solution of triethylamine (1.5 mmol) in CH_2Cl_2 (5ml), a slightly basic pH is maintained by a gradual addition of TEA. The ice bath was removed and the reaction mixture was stirred at room temperature for 45min. The organic layer is then washed successively with H_2SO_4 2M (3x10ml), a saturated aqueous solution of NaCl (10 ml), aqueous solution of $NaHCO_3$ (3x10ml) and with a saturated aqueous solution of NaCl (10 ml), then dried over anhydrous $MgSO_4$. The residue was evaporated then purified by chromatography over silica gel (gradient elution: hexane and ethyl acetate) to give compound **6** in good yield. *Caution:* This reaction produces HMPA which has been shown to cause nasal cancers in rats.

The spectral data of (S)-methyl 2-[2-diethoxyphosphoryl acetamide] 2-isobutylacetate **6a**, (S)-methyl 2-[2-Diethoxyphosphoryl acetamide] 2-Isopropylacetate **6b** and (S)-methyl 2-[2-diethoxyphosphoryl)acetamide] 2-benzylacetate **6c** is shown in reference 22.

4. Conclusion

In conclusion, we here showed that the synthesis of phosphonoacetamides can be synthesized by different method and good yield. The tested xenobiotics exhibit significant activity compared to Chlorpyrifos Ethyl, they are also cytotoxic, manifested by reduction growth at different concentrations, increasing of generation time, increasing of optical density (OD), and high response percentage of *paramecium sp*. We can conclude that the toxicity being highest for analogue **6b**.

Acknowledgements

This work was generously supported by the (Direction Generale de la Recherche Scientifique et du Développement Technologique, DGRS-DT), Algerian Ministry of Scientific Research, (FNR) and CMEP 08 MDU 729.

References

[1] Hashem**,** S.; Sakineh, E.; Mohammad. M. D. Iron-doped single walled carbon nanotubes as an efficient and reusable heterogeneous catalyst for the synthesis of organophosphorus compounds under solvent-free conditions. *Tetrahedron* **2013**, *69*, 4708-4724.

[2] Marjorie, S. H.; Hong, S. J.; Barhoumi, R.; Burghardt, R. C.; Donnelly, K. C.; Wild, J. R.; Vankatraj, V.; and Tiffany-Castiglioni, E. Neurotoxicity induced in differentiated SK-N-SH-SY5Y human neuroblastoma cells by organophosphorus compounds. *Toxicology and Applied Pharmacology*. **2003**, *186*, 110–118.

[3] Sobhani, S.; Vafaee, A. Efficient one-pot synthesis of β-hydroxyphosphonates: regioselective nucleophilic ring opening reaction of epoxides with triethyl phosphite catalyzed by $Al(OTf)_3$. *Tetrahedron* **2009**, *65*, 7691–7695.

[4] Kolodiazhnyi, O. I. Asymmetric synthesis of hydroxyphosphonates. *Tetrahedron: Asymmetry* **2005**, *16*, 3295-3340.

[5] Kukhar, V. P.; Hudson, H. R.; Aminophosphonic and Aminophosphinic acids.*Chemistry and Biological Activity, John Wiley and Sons, Ltd. Chichester*. **2000**, *205*.

[6] Ordóñez, M. F.; Sayago, J.; Cativiela, C.; Synthesis of quaternary α- aminophosphonic acids. *Tetrahedron* **2012**, *68*, 6369–6412.

[7] Ordóñez, M.; Rojas-Cabrera, H.; Cativiela, C. An overview of stereoselective synthesis of α-aminophosphonic acids and derivatives. *Tetrahedron* **2009**, *65*, 17–49.

[8] Mario, O.; Eugenio, H. F.; Hydree, R. C.; Victoria, L. G. *Tetrahedron Asymmetry.* **2008**, *19*, 2767-1770.

[9] Castelot, D.; Pannecoucke, G.. X.; Quirion, J. C. Diastereoselective synthesis of alpha-substituted beta-amidophosphonates. *Tetrahedron Lett*. **2001**, *42*, 1025-1028.

[10] Ordóñez, M.; Hernández-Fernández, E.; Montiel-Perez, M.; Bautista, R.; Bustos, P.; Rojas-Cabrera, H.; Fernández-Zertuche, M.; García Barradas, O. *Tetrahedron: Asymmetry.* **2007**, *18*, 2427–2436.

[11] Imran, A.; Gupta, V. K.; Aboul-Enein, H. Y.; challenge for the environmental scientists. *Current science*. **2003**, *84*, 152-156.

[12] Kafarski, P.; Lejczak, B. Biological activity of aminophosphonic acids. *Phosphorus, Sulfur, Sili con Relat. Elem.* **1991**, *63*, 193.

[13] Allen, M. C.; Fuhrer, W.; Tuck, B.; Wade, R.; Wood, J. M. Renin inhibitors, Synthesis of transition-state analog inhibitors containing phosphorus acid derivatives at the scissile bond. *J. Medicinal Chemistry.* **1989**, *32*, 1652.

[14] Logusch, E. W.; Walker, D. M.; Donald, J. F.; Leo, G. C.; Franz, J. E. Synthesis of alpha and gamma-alkyl-substituted phosphinothricins: potent new inhibitors of glutamine synthetase. *J. Org. Chem.* **1988**, *53*, 4069.

[15] Natchev, I.; Liebigs, A.; Herbicidal activity of phosphoryl analogues of glycine. *Ann. Chem*. **1988**, *861*.

[16] Emsley, J.; Hall, D. The Chemistry of Phosphorus, *Harper and Row, London.* **1976**, *494*.

[17] Maier, L.; Sporri, H. Resolution of 1-amino-2-(4-fluorophenyl) ethylphosphonic acid as well as some di-and tripeptides. *Phosphorus, Sulfur, Silicon Relat. Elem.* **1991**, *61*, 69.

[18] Huang, J.; Chen, R. An overview of recent advances on the synthesis and biological activity of α-aminophosphonic acid derivatives. *Heteroatom Chem*. **2000**, *11*, 480.

[19] Weimer, D. F. Synthesis of nonracemic phosphonates. *Tetrahedron* **1997**, *53*, 16609.

[20] Tay, M. K.; About-Jaudet, E.; Collignon, N.; Savignac, P. Phosphonates α-lithies agents de transfert fonctionnel. Preparation de phosphonates α-amides et d'amides α, β insatures, α-substitues. *Tetrahedron* **1989**, *45*, 4415-4430.

[21] Lejczak, B.; Kafarski, P.; Mastalerz, P. Phosphonopeptides-Synthesis and Biological Activity. *Beitrage zur Wirkstofforschung, 25, P.Oehme, H. Lowe & E. Gores Eds., Institut fur Wirstofforschung, Berlin*. **1985.**

[22] Guezane Lakoud, S.; Berredjem, M.; Aouf, N-E. Efficient method for the synthesis of α-amidophosphonate via the Michaelis-Arbuzov reaction. *Phosphorus, Sulfur, and Silicon.* **2012,** *187*, 762–768.

[23] Delmonte Corrado, M. U.; Trielli, F.; Amaroli. A.; Ognibene, M.; Falugi; C. Protists as tools for environmental biomonitoring: Importance of cholinesterase enzyme activities. In Water pollution: New research, ed. A.R. Burk. NY: Hauppaug, *Nova Science Publishers*. **2005**, 181-200.

[24] Delmonte-Corrado. M. U.; Amaroli, A.; Trielli, F.; Falugi, C. Cholinesterase enzyme activity in protists and environmental biomonitoring. *Current Trends in Microbiology*. **2006**, *2*, 123-136.

[25] Amaroli, A.; Trielli, F.; Sifredi, F.; Chessa, M. G.; Delmonte-Corrado, M. U. Nitric oxide production inhibited by xenobiotic compounds in the protozoan Paramecium primaurelia. *Ecological Indicators*. **2010**, *10*, 212–16.
[26] Coutrot, Ph. C.; Grison, C. Charbormier-Gerardin. Synthese de peptides modifies incorporant un motif phosphore n ou c terminal. *Tetrahedron* **1992**, *48*, 9841-9868**.**
[27] Sonneborn, T. M. Methods in Paramecium research. In Methods in cell physiology, ed. D.M. Prescott. **1970,** 241–399. New York: *Academic Press*.
[28] Wong, C. K.; Cheung, MH. Y. Toxicological assessment of coastal sediments in Hong Kong using a flagellate Dunalliella tertiolecta. *Environmental pollution* **1999,** *105*, 175–83.
[29] Dagnelie, P. Theoretical and Applied Statistics: Tome 2. Edition: *DE BOECK and Larcier University, Belgium*. **1999**, p 450.