Journal of Molecular Structure 1236 (2021) 130321

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: www.elsevier.com/locate/molstr

Phosphonoamidates & Phosphopnoamidines: A convenient Synthesis, Spectroscopic Properties, DFT Calculations & Pharmacological Studies

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ARTICLE INFO

Article history:
Received 14 December 2020
Revised 13 March 2021
Accepted 16 March 2021
Available online 22 March 2021

Keywords:
Phosphonoamidates
Phosphonoamidines
Density Functional Theory calculations
Evolution Study
Tautomeric equilibrium
Analgesic & antimicrobial activities
Pain behavior

ABSTRACT

A series of phosphonoamidates and phosphonoamidines derivatives have been prepared with high yields and their conformations were determined. All compounds were characterized by IR, NMR Spectroscopy (^{1}H, ^{13}C, and ^{31}P) and in some cases by elemental analysis and evolution study by ^{31}P-NMR in an external lock with D_2O. A DFT calculations studies was performed to identify the stability of all forms (*Cis* and *Trans*) (*Syn* and *Anti*) at the B3LYP/6-311G** level. The most stable geometry with an *Anti*-conformation. A comparison with the experimental results validates the level of theory.

The biological activity of dimethyl cyclohexyl carbamoylphosphonate (**MAmP**) and diethyl cyclohexyl carbamoylphosphonate (**EAmP**) was evaluated. Only the association of ethyl group with phosphorus in amide gives antimicrobial capacity against *S. aureus*. The evaluation of the analgesic activity following a thermal stimulus showed that the rats treated with the **MAmP** do not present any sensation of pain. The **MAmP** molecule had a dose-dependent significant analgesic activity comparable to morphine effect. The hot plate test showed that the **EAmP** had a significant analgesic effect less than morphine and **MAmP**. The new compounds at low dose treatment cause an increase in the sensation of pain following a chemical stimulation that induces inflammation. So the **EAmP** and the **MAmP** can be morphemic molecules. The morphine mediates a proinflammatory phenotype and induces hyperalgesia.

The **MAmP** was characterized by analgesic activity comparable to morphine and the **EAmP** was characterized by specific antimicrobial activity against *S. aureus*.

1. Introduction

Pain is a costly public health problem, contributing to high health care costs and productivity lost. Pain intensity can be broadly categorized as mild, moderate and severe. The pain can be acute or chronic, including nociceptive state which represents the normal response to noxious insult or injury of tissues such as skin, muscles, visceral organs, joints, tendons, or bones. Neuropathic pain may be initiated or caused by a primary lesion or disease in the somatosensory nervous system. And inflammatory pain results in activation and sensitization of the nociceptive pain pathway by a variety of mediators released at a site of tissue inflammation [1,2]. The pain is treated by opioid or non-opioid drugs and prevented by local anesthesia essentially by the amide drugs.

It is evident that this class of compounds possesses a broad spectrum of important biological and pharmaceutical activities such as anesthetic [2], anti-inflammatory [3], analgesic [3], antitumor [4], antituberculosis [5], anticonvulsive [6], antifungal [7], insecticidal [8], commonly used in pediatric anesthesia including lidocaine, bupivacaine, and ropivacaine [9-14].

Considering the importance of amides in organic chemistry and pharmaceutical industry, the aim of the present work was to syn-

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https://doi.org/10.1016/j.molstruc.2021.130321
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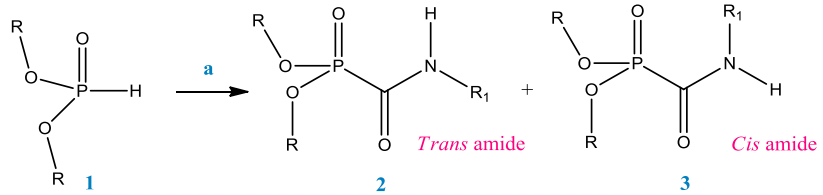
Table 1
Synthesis of compounds **2** and **3**



2,3	R	R^1	(*Trans: Cis*) (%)
a	Me	Ph	95: 5
b	Me	*c*-C_6H_{11}	70: 30
c	Me	*p*-Cl-Ph	100: 0
d	Et	*c*-C_6H_{11}	75: 25
e	Et	*p*-Cl-Ph	95: 5

thesize a series of phosphonoamidates **2** & **3,** the corresponding phosphonoamidines **4** & **5** and evaluate their analgesic effect in rat experimental model of pain behavior and explore antioxidant and antimicrobial activities of two of them, dimethyl cyclohexylcarbamoyl phosphonate (**MAmP)** and diethyl cyclohexylcarbamoyl phosphonate (**EAmP)**.

Phosphonoamidates and Phosphonoamidines can exist in more two tautomeric forms. It is therefore of interest for us to prove that the most stable form of the synthesized products. The density functional theory (DFT) was carried out to investigate the tautomerism of all component at the B3LYP/6-311G** level of theory in gas phase. According with experimental study and given that the energies of the most stable forms obtained in the theoretical study was similar.

2. Results and discussion

2.1. Chemistry

2.1.1. Preparation of phosphonoamidates 2a-e and 3a-e

We have reported the synthesis of a series of phosphonothioamidates by reaction of dialkylphosphites and isothiocyanates in the presence of strong base with high yields [14-16]. Recently, under a nitrogen atmosphere, the dimethylphosphite was stirred at room temperature for 4 h without any solvent and any catalyst with the phenyl isothiocyanate in the presence of triethyl amine [17a-b]. This method was not unsuccessful; the reaction time is very slow.

We now report the synthesis of a new series of phosphonoamidates **2, 3** from isocyanates and dialkyl phosphites **1** in the presence of t-BuOK in anhydrous THF followed by acid hydrolysis at room temperature. Compounds **2,3a-e were** obtained in high yields as a mixture of Cis and Trans conformation, with Trans-amide as the major and favored product in general [14]. Table 1 summarizes the proportionality of the conformations. Interestingly, the treatment of dimethylphosphite with p-chloro phenylisocyanate in the presence t-BuOK and THF affords the phosphonoamidates in Trans conformation only.

The reaction was monitored by ^{31}P-NMR spectroscopy. In the case of **2c** and **3c,** the signal of dimethylphosphite in anhydrous THF appears at 10.17 ppm in the ^{31}P-NMR spectrum. Phosphite upon treatment with *t*-BuOK, the ^{31}P-NMR spectrum shows the appearance of new signals between 154.32 ppm corresponding to potassium dimethyl phosphite. The addition of *p*-chlorophenyliocyanate to potassium dimethyl phosphate melds the formation of a mixture of potassium (*E*)-*N*-(*p*-chlorophenyl) (dimethoxyphosphoryl) formimidate and (*p*-chlorophenyl) ((dimethoxyphosphoryl)carbonyl)amide. This is confirmed by the presence of two signals in the range 10.15 and 2.54 ppm assigned to the two anions. After acid hydrolysis, two signals appearing at 0.04 and -2.12 ppm were obtained corresponding to compounds **2c** and **3c** (Fig. 1).

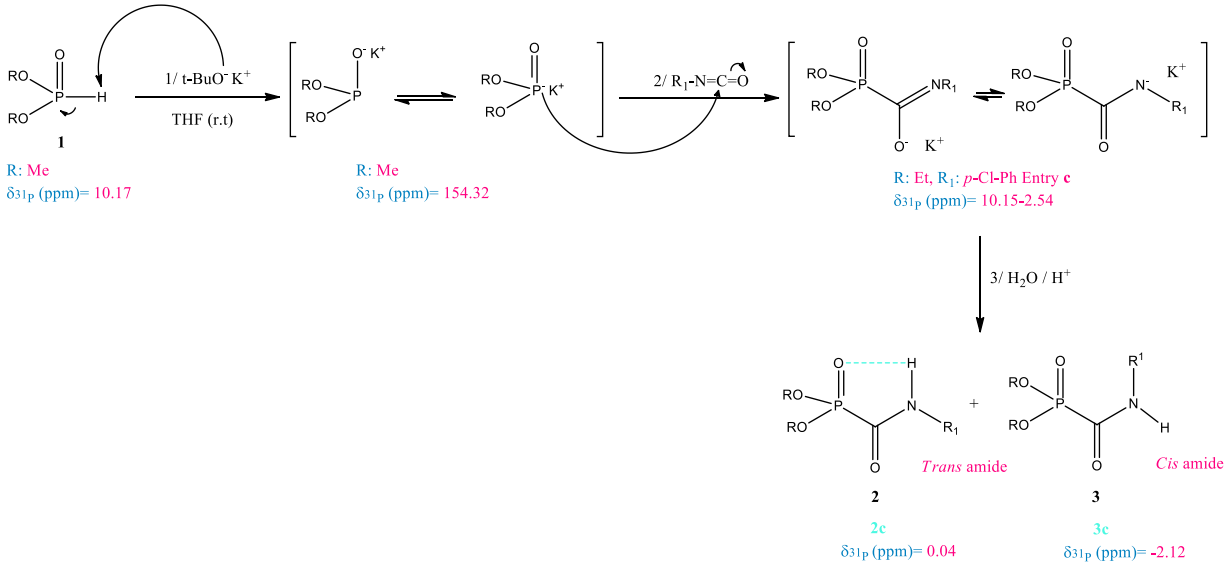
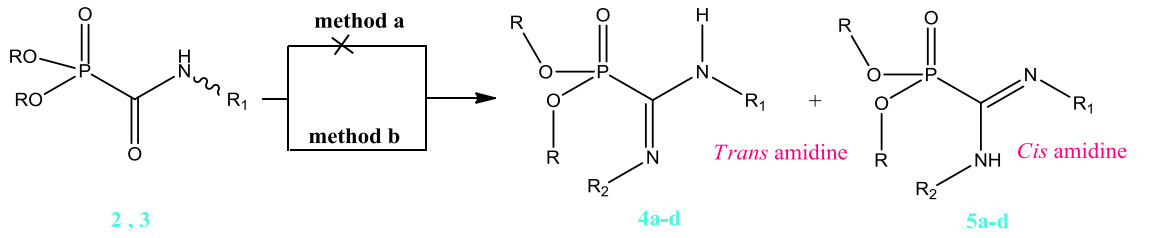


Fig. 1. Reaction mechanism of phosphonoamidates **2** and **3**

Table 2
Synthesis of compounds **4** and **5**



4,5	R	R^1	R^3	(*Syn: Anti*) (%)
a	Me	Ph	Bn	76: 24
b	Me	c-C_6H_{11}	Bn	83: 17
c	Me	*p*-Cl-Ph	Bn	85: 15

reaction conditions: Method a: NH_2R_2/EtOH/ r.t Method b: NH_2R_2/EtOH/ Ac_2O/ Δ/ 4h

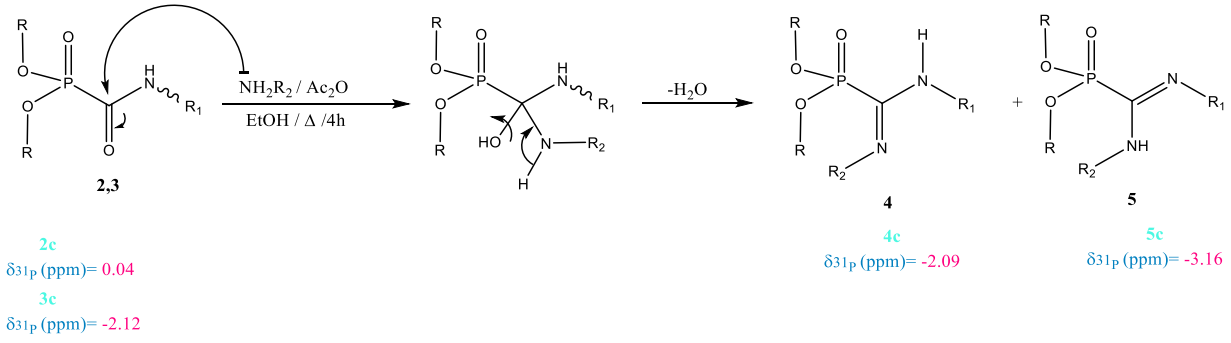


Fig. 2. Reaction mechanism of phosphonoamidines **4** and **5**

Phosphonoamidates were found to be present as mixtures of rotational isomers about CO-NH bond. The ^{1}H chemical shifts of **2, 3 a-e** indicated the presence of two doublets at 8.86-8.78 ppm for **2,3** (entry a), characterizing NH group. The ^{13}C NMR spectrum of the phosphonoamidates **2,3** shows a doublet signal at 155.92-164.26 ppm with a coupling constant of 227Hz, which is typical for a C=O group.

2.1.2. Preparation of phosphonoamidines 4,5a-c

Recent work in our laboratory has shown that various phosphoamidines series were obtained by reaction of phophonothioamidates with primary amines in absolute ethanol at room temperature for 1 hour [14-15]. This method was not unsuccessful when phosphonoamidates reacted with primary amines. Phophonoamidines **4,5a-c** has been synthesized with good yields by using the phosphonoamidate **2,3** with primary amines (Table 2). In connection with current work on the chemistry, we attempted a similar series of reaction, which is described in the present paper.

In this part, with the aim of detecting the reaction intermediates by ^{31}P-NMR for compounds **4,5** (**Entry c**) the signals of **2c** and **3c** in absolute ethanol appeared at 0.04 and -2.12ppm. After addition of primary amines during 4h in reflux, the appearance of two signals at -2.09 and 3.16ppm, confirms the phosphonoamidines structure with *Syn* and *Anti* conformations (Fig. 2).

The ^{13}C-NMR spectra in $CDCl_3$ of the compounds amidines showed a doublet signal at 175-174 ppm with a coupling constant of 224 Hz, which is typical for a -N=C-N fragment and 120-132 ppm, which is typical for a C_{arom} fragment. Two signals were detected in the ^{31}P-NMR spectrum for all compounds confirming the presence of two isomers **4, 5 a-e** with a *Syn* and *Anti* conformations.

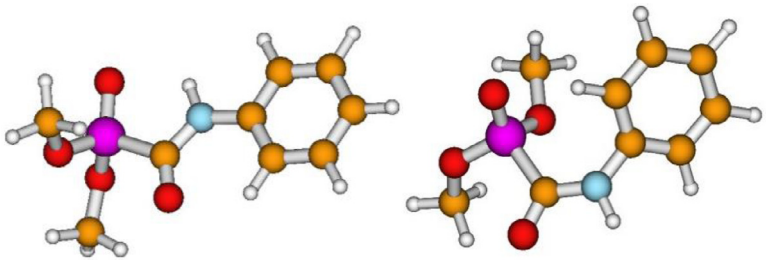
3. Computational Details

All electronic structure calculations in this work were carried out using the Gaussian 09 software package. All geometries were evaluated at the B3LYP [18,19] in conjunction with the 6-311G** basis according to the CPCM model implemented in Gaussian 09 [20,21]. Vibrational analyses confirmed that all reactants intermediates and products have zero imaginary frequencies, and that transition states are true first-order saddle points on the potential energy surface. The Gauss View program [22] was used for visualization of the structure and simulation of the vibration spectra. Intrinsic reaction coordinate (IRC) calculations were followed to check the energy profiles connecting each transition state (TS) through a vibration analysis to the expected reactants and products [23]. The optimization of each structure was considered converged with a maximum gradient less than 0.0001. No frozen coordinates and no symmetry restriction were used (Fig. 3).

4. Theoretical study

4.1. Phosphonamidates conformations

Different conformations of phosphonoamidates in the dihedral angle is around 180°C was calculated at the B3LYP with the 6-311G** basis in gase phase. A graph represented all conformations with different energies Fig. 4. The minimizations showed that for those with the *Anti*-conformation (when the dihedral angle is around 180°C) the potiential energy is at its lowest equal to 0.390 kcal/mol, therefore the most stable. This is due to the CO groups being the farthest awey from each other in the molecules.



E(*Trans*)= -1046.9280236 u.a E(*Cis*)= -1046.9150074 u.a

$$E(Trans) < E(Cis)$$

$$\Delta E = E(Trans)\text{-}E(Cis) = 0.0130162 \text{ u.a} = 8.16 \text{ kcal/mol}$$

Fig. 3. DFT calculations of the compound **2a** / **3a**

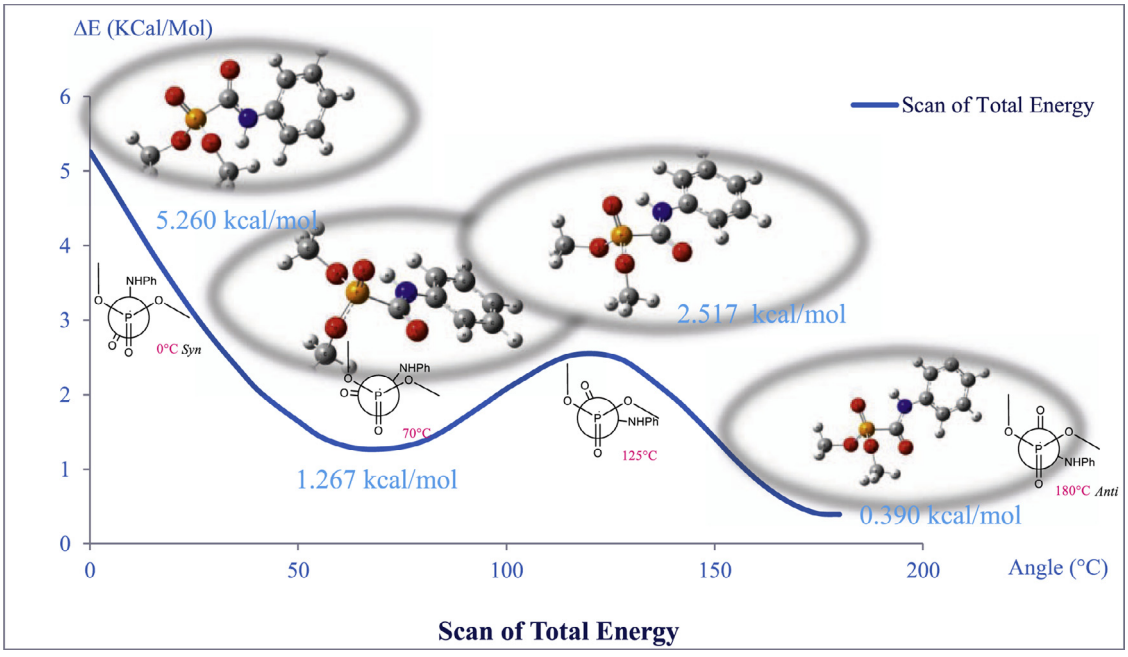


Fig. 4. Newman projections with the different conformations of phosphonoamidates **2a** containing P(O)-C(O)-NH-.

4.2. Tautomerism

In theory, amides can exist in two tautomer forms. To identify the most conformer of these phosphoamidates synthesized, the spectroscopic studies were not concluding. For this reason, it's interesting to study the tautomeric equilibrium using a different approach. In order to observe possible effects of the substituent on tautomeric equilibrium, we performed quantum mechanics calculations, for different components **2**-**3**, in gas phase using CPCM model to obtain thermodynamic parameters such as Gibbs free energy ΔG, enthalpy ΔH and zero point energy (ZPE) corrected total energy ΔE+ZPE for all tautomers. The results are summarized in Table 3. Are the same of amidines can exist in more two tautomer forms? The Fig **5** summarized the tautomeric equilibria of the phosphonoamidines derivatives. The IRC plots showed that the phosphonoamidines **4a** with *Trans-amidine* conformation was the most stable one.

4.3. Biological activities testing

4.3.1. Evaluation of the antibacterial and antifungal activities

It was reported that amide derivatives have antibacterial activity [8]. In this work, after incubation for 24 h at 37°C, the results showed that the compounds **2,3 a, c** and **e** do not inhibit fungal multiplication of *Candida parapsilosis* and do not have antibacterial activity. In addition only the 2,3d compound (**EAmP**) has a specific antimicrobial activity against *S. aureus* proved by the inhibition zone appeared around the disc depot at the different increasing concentrations from 10 to 40 mg/mL. But the **MAmP** phosphonamidate derivatives **(2,3b)** do not reduce or eliminate the bacterial proliferation of the *S. aureus, P. aerogunosa* and *E. coli.*

It appears that the association of methyl group with phosphorus in amide molecule does not give antimicrobial activity. However the association of ethyl group with phosphorus in amide gives antimicrobial capacity against *S. aureus.*

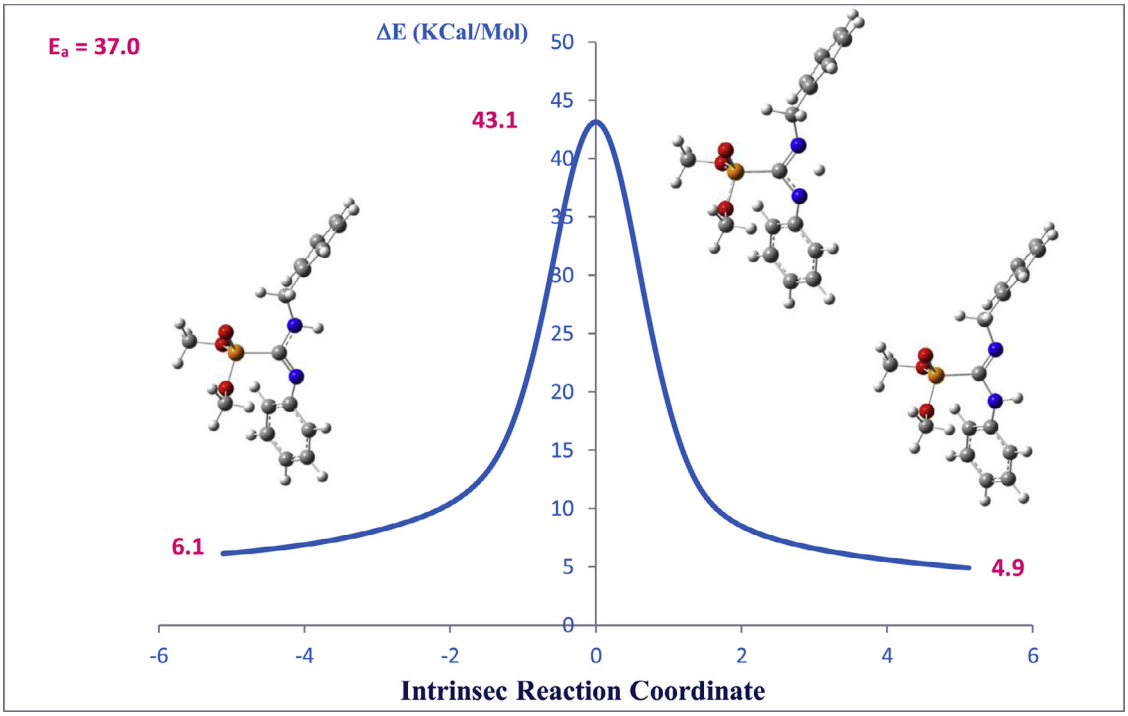


Fig. 5. IRC plot for the transition state TS obtained during the tautomericequilibrium of phosphonoamidinesderivatives (**4a, 5a**)

Table 3
Correction of zero point energy, thermal and free energies of the tautomeric forms of phosphonoamidates derivatives **2** and **3**

Entry 2,3		ΔE+ZPE (kcal/mol)	ΔH (kcal/mol)	ΔG (kcal/mol)
2a	** ***Cis***	6.136	6.238	5.960
3a	** ***Trans***	0	0	0
2b	** ***Cis***	4.396	4.218	5.433
3b	** ***Trans***	0	0	0
0 2c	** ***Cis***	6.296	6.378	6.013
3c	** ***Trans***	0	0	0
2d	** ***Cis***	4.462	4.378	5.094
3d	** ***Trans***	0	0	0
2e	** ***Cis***	6.610	6.654	6.423
3e	** Trans	0	0	0

* ΔE+ZPE: Zero point corrected total energy; Δ*H*: enthalpy; Δ*G*: Gibbs free energy. They are in kcal/mol.

In this work other biological tests were applied to look for other pharmacological activities of the compound **EAmP (2,3d)** and also to show the effect due to the replacement of ethyl phosphonoamidates **(2,3d)** by the methyl simple group **(2,3b).**

4.3.2. Evaluation of the antioxidant capacity

Antioxidant compounds are used in various fields, such as the food industries, to protect the organic molecules from oxidation and pharmacotherapy to protect against carcinogenesis [24]. The obtained results showed that the **MAmP** and the **EAmP** at increasing concentrations from 1 to 20 mg/mL did not reduce the DPPH. The presence of the phosphorus atom with the ethyl or methyl group did not give an antioxidant capacity to amide molecule. These results are conforming to the literature which proves that amides have no antioxidant effect [25].

4.3.3. Evaluation of the antalgic effect

According to the literature review, some amide derivatives have an analgesic activity, such as, barbiturates and hydantoins [3,6]. Our results prompted to evaluate the analgesic activity of the newly synthesized phosphonoamidates amide derivatives **2,3b** (**MAmP**) and **2,3d** (**EAmP).**

4.3.3.1. Hot plate test. The measurement of the latency time marked by the pain sensation following a thermal stimulation (48°C) showed significant increase ($p<0.05$) from 75s in the control group to 434 s in the group treated with morphine at a dose of 10 mg /kg. The **MAmp** treatment significantly reduced the sensation of pain, in a dose dependent manner and comparable to that of morphine at the same doses. The behavior of the rat treated with **MAmP** during this test was normal and comparable to that of morphine with indifference to the temperature of the hot plate (Fig. 6). In this case, the **MAmP** can be a morphemic molecule. Morphine is a strong opioid used in the acute pain that acts in the central nervous system (spinal and supra spinal). Its chemical structure is close to endogenous opioid substances such as enkephalins, endorphins, and dynorphins [26-27]. The existence of the ethyl group instead of methyl in the EAmP molecule gives a significant analgesic propriety proved by the increase of the latency time to 146.2 s ($p<0.05$).

4.3.3.2. Formalin Test. The formalin test consists in evaluating the pain intensity in the treated groups with the **MAmP** and **EAmP** by measuring the time spent in each score during the early phase (first 5 minutes) and the late phase (last 10 minutes from 50 to 60 min) of inflammation. The obtained results were compared to the control rats and the morphine effect.

-The early phase (0-5min): The obtained results showed that the control group after administration of nociceptive agent spent 90s at a score 0 which included the latency time and were characterized by the absence of pain. They were followed by 55s at a score 1 characterized by low pain, 110s at a score 2 characterized by moderate pain and rats spent 50 s in score 3 characterized by severe pain proved by licking, biting and shaking the affected paw (Fig. 5). The pretreatment with morphine at 5mg/kg for 15 min decreased slightly the nociception proved on one hand by the reduction of

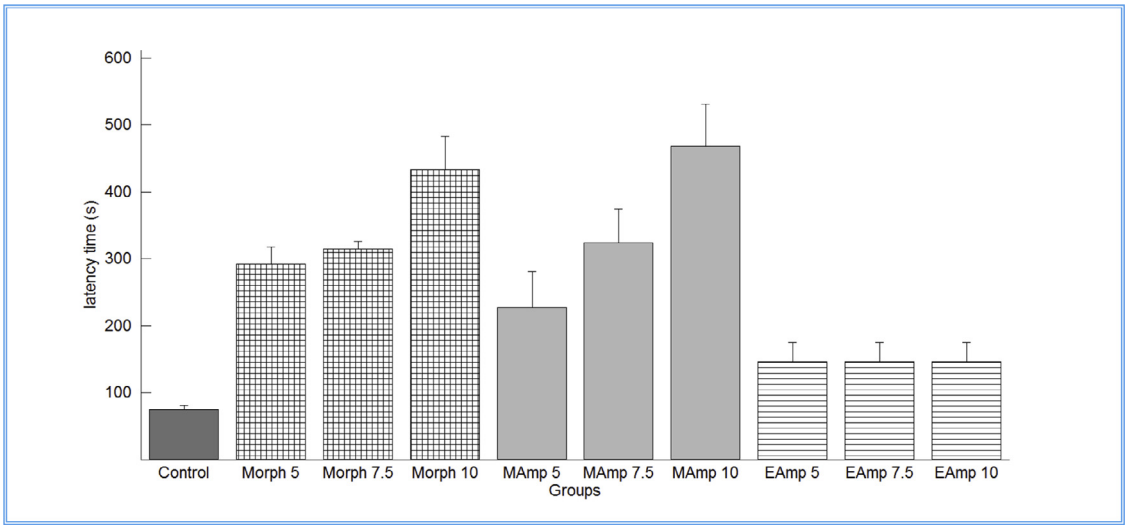


Fig. 6. Analgesic activity of EAmP and MAmP was evaluated by hot plate test noting the latency time of pain perception.

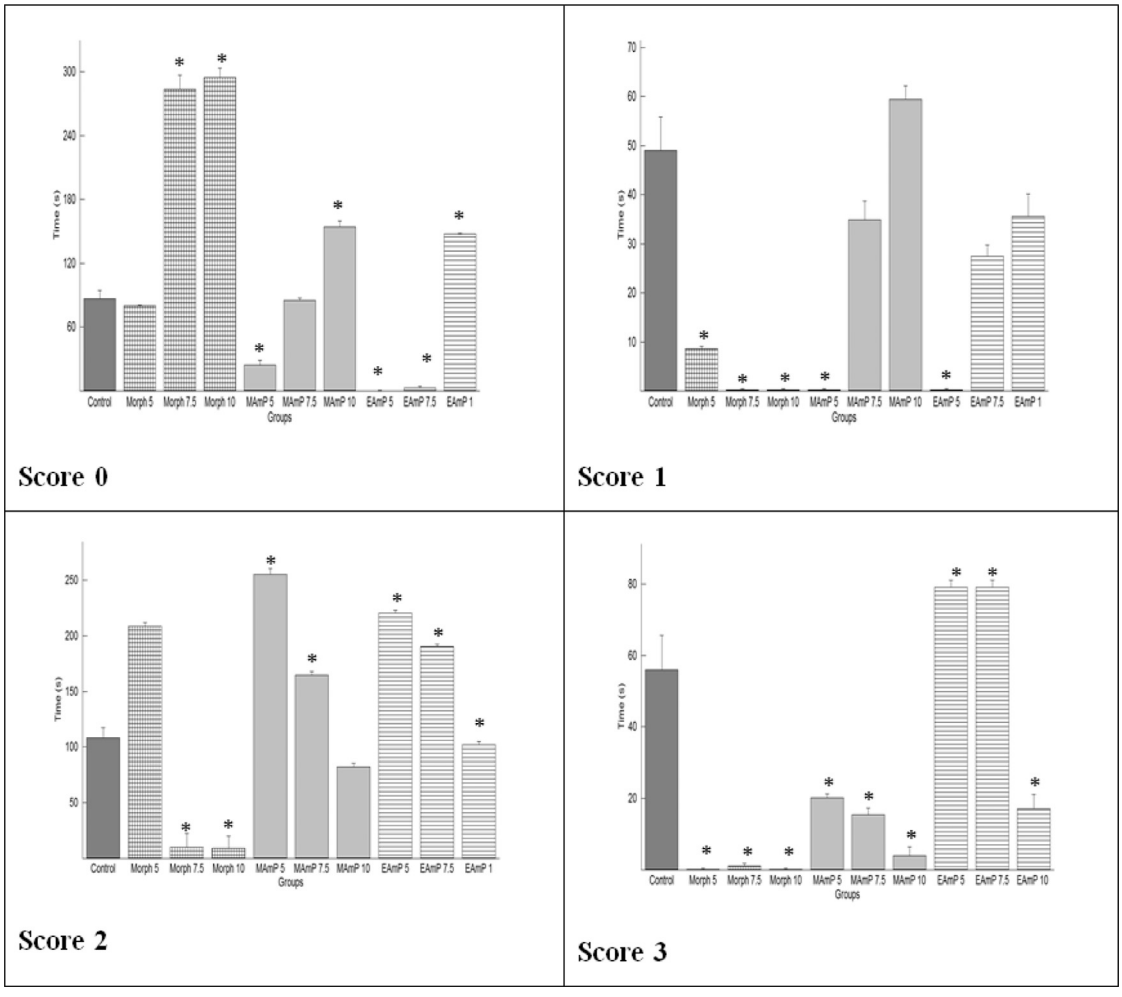


Fig. 7. Evaluation of the anti-nociception effect of the EAmP and the MAmP during the early phase after pain induction by formalin.

the spent time in the score 3 to 8.6 s and on the other hand by the increase of the spent time in the 2 to 211 s ($p<0.05$) (Fig. 7). The increase of the treatment dose with morphine to 7.5 and 10 mg/kg caused a total absence of pain behavior proved by the total spent time of formalin test at score 0 (300 s) and the absence of score 1, 2 and 3. Compared to the control group, pretreatment by **MAmP** at 5 mg/kg doseinduced a decrease of score 3 (20.2 s) and an increase of score 2 (255.4 s). The 7.5 mg/kg dose of **MAmP** induced a decrease of score 3 (15.4 s) and an increase of score 2 (164.8 s). The pretreatment by **MAmP** at 10 mg/kg dose induced a higher increase of score 3 (3.4 s) and an increase of score 0 and 1 (154 s and 59 s respectively) (Fig. 5). This means that **MAmP** has

a moderate analgesic effect compared to that of morphine and has a dependent dose effect.

The pretreatment with **EAmP** at 5 and 7.5 mg//kg induced on one hand a significant decrease of the spent time in the score 0 and 1 and on the other hand an increase in score 2 and 3. This means that **EAmP** at low doses increased the nociception in the case of inflammation induced by formalin injection. The augmentation of dose treatment with **EAmP** to 10 mg/kg induced an increase of the spent time in the score 0 to 147.4 s when the pain was low ($p<0.05$) and a decrease of score 3 (17.2 s) (Fig. 5). This means that **EAmP** at a high dose has a moderate analgesic effect compared to morphine treatment.

4.3.3.3. The late phase (50-60min). 50 min after the pain induction with formalin, the control group spent 80s in score 0, 180s in score 1, 200s in score 2 and 140s in score 3. The last score was characterized by severe pain proved by licking, biting and shaking the affected paw (**Fig. 6**). Similar to the early phase, in the late phase pretreatment with morphine induced total suppression of pain induced by formalin injection. In this case the rat's behavior was normal.

In comparison with the control group, the pretreatment with the **MAmP** at 5 and 7.5 mg/kg decreased the nociception. It was shown firstly by the reduction of the spent time in the scores 3 and secondly by the increase of the spent time in the scores 2 ($p<0.05$) (Fig. 8). The rats' behavior showed that the pain decreased again with the pretreatment with **MAmP** at 10 mg/kg dose. The monitoring of pain evidenced a significant decrease of spent time in score 2 and 3 and a significant increase in score 1 ($p<0.05$) (Fig. 8). **MAmP** has a moderate analgesic effect compared to morphine.

The effect of **EAmP** at late phase was comparable to its effect at early phase. At low doses, it accentuated the pain. This effect was proved by an increase in score2 and a decrease in score 0 and 1. At 10 mg/kg dose **EAmP** induced an increase in score 1 and a decrease in score 2 and 3 (Fig. 8). This confirms that at efficient dose **EAmP** has a moderate analgesic effect compared to morphine that is a strong analgesic.

The early phase of inflammation triggered by the subcutaneous injection of formalin

(5%) was characterized by the release of histamine, responsible for inflammation and the sensation of pain [28]. The newly synthesized amides at low dose cause an increase in the sensation of pain following a chemical stimulation that induces inflammation. So the **EAmP** and the **MAmP** can be pro-inflammatory molecules while a high dose (10 mg / kg) shows a modulation of pain sensation during the early and the late phases of inflammation. This proves that these amides do not have an anti-inflammatory effect. Based on literature data, morphine mediates a pro-inflammatory phenotype in mouse and induces hyperalgesia without involvement of opioid receptor. The above results confirm that the **MAmP** and the **EAmP** could be ranked as opioid compounds [29-34].

5. Conclusion

Our present work was reporting the synthesis of phosphonoamidates **2,3 a-e** .The latter have interesting structural to synthesis of a new series of phosphonoamidines **4, 5 a-d**. The tautomeric equilibrium was investigated by DFT calculation to determine the most conformation form of the compounds synthesized. The results were confirmed by the IRC calculations and the energetic diagrams established.

A biological activity was studied in this work. The association of methyl group with phosphorus in amide gives a dimethyl cyclohexylcarbamoyl- phosphonate (**MAmP**) molecule characterized by analgesic activity comparable to morphine in thermal stimulation test. The change of alkyl groups of MAmP by ethyl gives a new amide molecule the diethyl cyclohexylcarbamoylphosphonate (**EAmP**) characterized by specific antimicrobial activity against *S. aureus* and low analgesic activity in thermal stimulation test. These newly synthesized amides derivatives at low dose increase pain perception in inflammatory state.

Thus, the present study warrants further investigations involving these novel molecules for possible development of new analgesic and antibiotic drugs which do not have any effect on the behavior.

6. Experimental

6.1. Chemical synthesis

6.1.1. Instruments

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 300, 75 and 121 MHz respectively on a Bruker AC-300 with TMS as internal reference (for ^{1}H and ^{13}C) and H_3PO_4 (for ^{31}P) in $CDCl_3$. Mass spectra were accomplished with an HP 5889A quadripolar spectrometer by electronic impact EI (70 eV) or chemical ionization CI (500 eV) with NH_3 gas. CHN elementary analysis was performed at the INRAP (National Institute of Physico-Chemical Analysis (INRAP) Bio-technopole Sidi Thabet, Tunisia) Perkin Elmer Model: Analyzer 2400 series II CHN.

The study of the evolution of the reaction mixture was performed by ^{31}P NMR used in an external lock with D_2O.

6.1.2. General procedure for the preparation of phosphonoamidates (2,3 a-e)

A solution composed by dialkyl phosphite **1** (0.02 mol, 1.0 equiv) in anhydrous THF (10 mL) was added dropwise to a solution of potassium tert-butoxide (0.025 mol, 1.5 equiv) in anhydrous THF (20 mL). Stirring was continued at room temperature and under a nitrogen atmosphere for 1 h. Isocyanate (0.02 mol, 1.0 equiv) in dry THF (10 mL) was added to the reaction mixture. After 2h of stirring at room temperature, the hydrolysis was performed with concentrated HCl (12N). The aqueous layer was then extracted with $CHCl_3$. After drying with $MgSO_4$, filtration and solvent evaporation, the obtained residue was recrystallized with Ethyl Acetate. The oily and viscous residue was purified by column chromatography on Ether / Petroleum Ether (1:1) to give a pure product.

- ***(2a/3a). dimethyl phenylcarbamoylphosphonate***

White solid; yield= 94%; mp 102°C; ^{1}H NMR δ_H (ppm): **(2a)** 3.80 (d, J= 12 Hz, 6H, CH_3), 7.0-7.62 (m, 5H, H_{arom}), 8.86(d, J= 8.5 Hz, 1H, NH). **(3a)** 3.87 (d, J= 9 Hz, 6H, CH_3), 7.0-7.63 (m, 5H, H_{arom}), 8.87 (d, J= 8.4 Hz, 1H, NH).^{13}C NMR δ_C (ppm): **(2a)** 163.22 (d, J= 227.18Hz, C=O), 120.16-136.50(m, C_{arom}), 54.87 (d, J=6.80Hz, MeO). ^{31}P NMR δ_H (ppm): (2a) = -0.45. (3a) = 0.53. Anal. Calc. for $C_9H_{12}NO_4P$ (229g.mol^{-1}): C, 47.16; H, 5.24; N, 6.11; found C, 47.15; H, 5.33; N, 6.10%.

- ***(2b/3b). dimethyl cyclohexylcarbamoylphosphonate***

White solid; yield= 87%; mp 96.4°C; ^{1}H NMR δ_H (ppm): 0.96-2.32 (m, 10H, $H_{cyclohexyl}$), 3.51 (d, J= 725.1 Hz, 6H, CH_3), 4.79(s, 1H, H_{ipso}), 7.28 (d, J= 8.5 Hz, 1H, NH). ^{13}C NMR δ_C (ppm): 155.70(d, 227.12Hz, C=O), 56.03 (s, C_{ipso}), 50.73(d, J=6.79Hz, MeO), 20.80-37.75(C_{hexyl}). ^{31}P NMR δ_P (ppm): **(2b)** = -0.97. **(3b)** = 2.65. Anal. Calc. for $C_9H_{18}NO_4P$ (235g.mol^{-1}): C, 45.95; H, 7.65; N, 5.97; found C, 46.01; H, 7.65; N, 5.92%.

- ***(2c/3c). dimethyl (p-Chlorophenyl)carbamoylphosphonate***

Yellow solid; yield= 93%; mp 84.8°C; ^{1}H NMR δ_H (ppm): 3.94(d, J= 12.1Hz, 6H, CH_3),6.70-8.20 (m, 4H, H_{arom}), 9.27 (d, J= 8.6

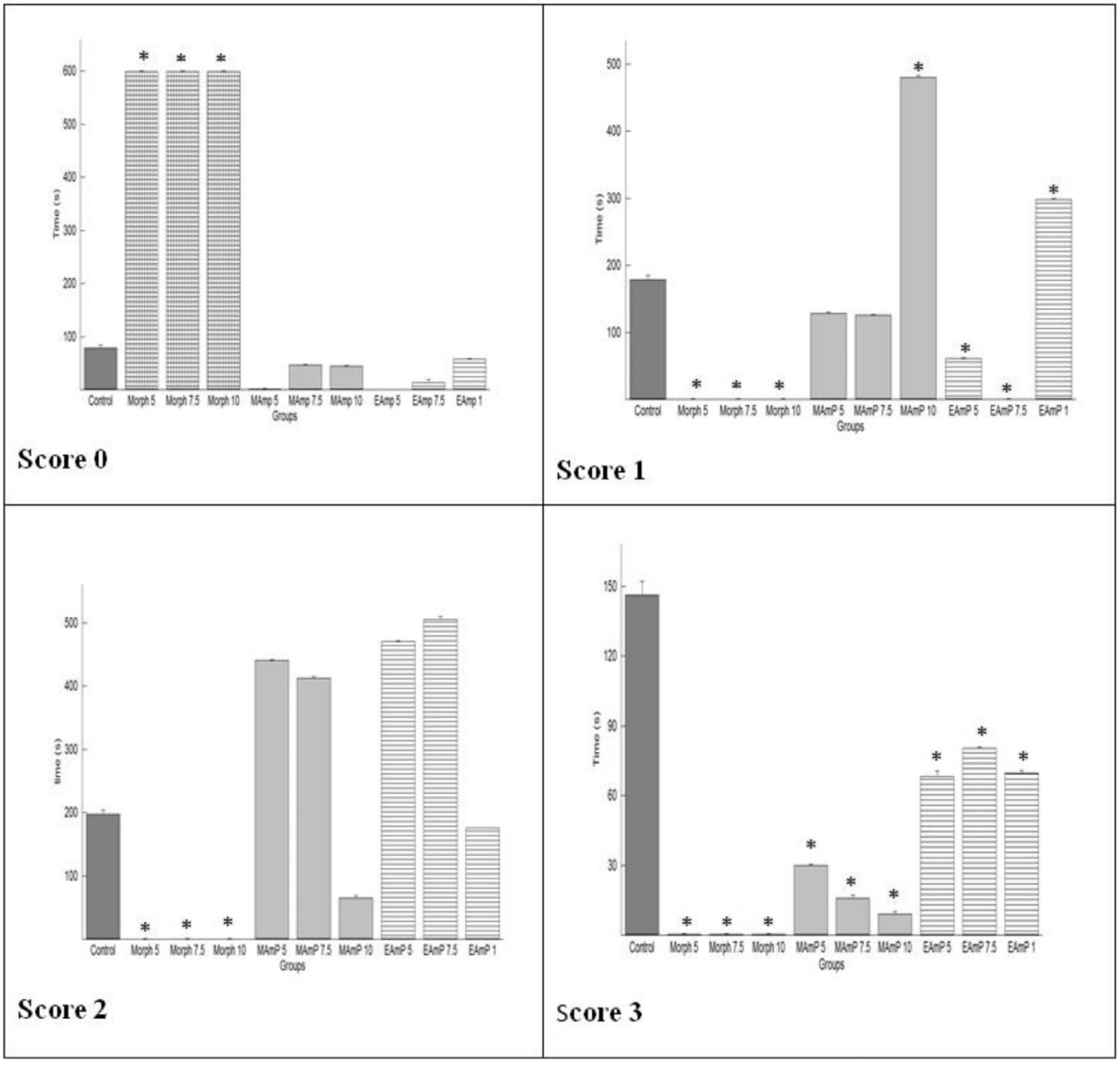


Fig. 8. Evaluation of the anti-nociception effect of the EAmP and the MAmP during the Late Phase after pain induction by formalin.

Hz, 1H, NH). ^{13}C NMR δ_C (ppm): 160.47 (d, 227.94 Hz, C=O), 127.03-136.12 (C_{arom}), 55.23(d, J=6.79Hz, MeO). ^{31}P NMR δ_P (ppm): **(2c)** = 0.02 Anal. Calc. for $C_9H_{11}NO_4ClP$ (263g.mol^{-1}): C, 41.06; H, 4.18; N, 5.32; found C, 42.30; H, 4.17; N, 5.29%.

- ***(2d/3d).diethyl (cyclohexyl)carbamoylphosphonate***

White solid; yield= 82%; mp 68°C; ^{1}H NMR δ_H (ppm): 0.63-2.32 (m, 10H, $H_{cyclohexyl}$), 1.62 (t, J= 9 Hz, 6H, CH_3), 3.96 (d, J=7.1Hz, 2H, CH_2), 4.87 (m, 1H, H_{ipso}), 7.28 (d, J= 8.4 Hz, 1H, NH). ^{13}C NMR δ_C (ppm): 155.46(d, 227.81Hz, C=O), 49.44 (d, J=30.1Hz, C_{ipso}), 58.06 (d, J=6 Hz, CH_2O), 24.22-34.15(C_{hexyl}), 14.52 (d, J= 6.80Hz, CH_3). ^{31}P NMR δ_P (ppm): (**2d**)= -1.25 **(3d)**= -0.33. Anal. Calc. for $C_{11}H_{22}NO_4P$ (263 g.mol^{-1}): C, 50.19; H, 8.36; N, 5.32; found C, 50.24; H, 8.32; N, 5.28%.

- ***(2e/3e). diethyl (p-chlorophenyl)carbamothioylphosphonate***

Yellow solid; yield= 86%; mp 67.2°C; ^{1}H NMR δ_H (ppm): 1.33 (t, J= 9 Hz, 6H, CH_3), 4.18-4.25 (m, 4H, CH_2), 7.20-7.55(m, 4H, H_{arom}), 9.80 (d, J= 8.5 Hz, 1H, NH).^{13}C NMR δ_C (ppm): 164.19 (d, 227.94 Hz, C=O), 129.13-135.54 (C_{arom}), 64.88(d, J=6.80Hz, CH_2), 16.24 (d, J= 6Hz, CH_3). ^{31}P NMR δ_P (ppm): **(2e)**= 0.22 **(3e)**= -2.19. Anal. Calc. for $C_{11}H_{15}ClNO_4P$ (291g.mol^{-1}): C, 45.36; H, 5.15; N, 4.81; found C, 45.32; H, 5.16; N, 4.81 %.

6.1.3. General procedure for the preparation of phosphonoamidines (4,5a-c)

Phosphonoamidate **2,3** (1equiv, 1mmol) and benzyl amine (1equiv, 1mmol) were dissolved in 15mL of absolute ethanol and added at dropwise to glacial acetic acid. Then mixture was refluxed for 4 h. The reaction mixture was dried over Na_2SO_4 and the solvent was removed. The obtained residue was purified by silica gel column chromatography (Ethanol: Petroleum Ether eluent) (8:2).

- ***(4a/5a) (Z)-dimethyl N'-benzyl-N-phenylcarbamimidoylphosphonate***

Yellow solid; yield= 91%; mp 104.6°C; ^{1}H NMR ($CDCl_3$+DMSO, δ_H ppm): 3.94 (d, J=12.9Hz, 6H, CH_3), 4.74 (d, J=12.9Hz, 2H, CH_2), 7.99 (d, J=7.8Hz, 1H, NH), 6.87-7.68 (m, 10H, H_{arom}). ^{13}C NMR δ_C ($CDCl_3$+DMSO, δ_C ppm): 165.46 (d, 148.2Hz, -C=N-), 136.50 (d, J= 137.1Hz, N-C_{ipso}), 128.54 (d, J=10.9Hz, C_{ipso}), 128.11 (s, N-C_{meta}), 127.49 (s, C_{ortho}), 127.16 (s, C_{meta}), 127.10 (s, C_{para}), 127.05(s, N-C_{para}), 122.23 (d, J=Hz, C_{ortho}), 51.97(d, J=6.3Hz, CH_2), 48.17 (d, 6.2Hz, MeO). ^{31}P NMR ($CDCl_3$+DMSO, δ_P ppm): (**3a**) = -5.40 (**4a**)=

0.85. IR ($CHCl_3$, υ cm^{-1}): (**NH**)= 3046.77, (**C=C**)= 1630.21, (**PO**)= 1252.72. Anal. Calc. for $C_{16}H_{19}N_2O_3P$ (318g.mol^{-1}): C, 60.37; H, 5.97; N, 8.80; found C, 60.31; H, 5.96; N, 8.76%.

- ***(4b/5b). (Z)-Dimethyl N'-benzyl-N-cyclohexylcarbamimidoylphosphonate***

Yellow solid; yield= 81%; mp 108.7°C; ^{1}H NMR ($CDCl_3$, δ_H ppm): 0.73-3.08 (m, 11H, H_{hexyl}), 3.45 (d, 10.8Hz, 6H, CH_3), 4.30 (d, J=5.7Hz, 2H, CH_2),7.02-7.73 (m, 5H, H_{arom}), 6.8 (d, J=7.5Hz, 1H, NH). ^{13}C NMR ($CDCl_3$, δ_C ppm): 172.73 (d, 148.5Hz, -C=N-), 137.91 (s, N'-C_{ipso}), 128.60 (d, J=1.3Hz, N'-C_{ortho}), 127.78 (s, N'-C_{meta}), 127.38 (s, N'-C_{para}), 52.72 (s, CH_2), 50.08 (d, J= 6.4Hz, MeO), 42.63 (d, J=6.4Hz, N-C_{ipso}), 31.04 (s, N-C_{ortho}), 24.87 (s, N-C_{para}). ^{31}P NMR ($CDCl_3$, δ_P ppm): (**3c**) = -1.28 (**4c**)= 0.91. IR ($CHCl_3$, υ cm^{-1}): (**NH**)= 2946.84, (C=C)=1630.83, (**PO**)= 1228.18. Anal. Calc. for $C_{16}H_{25}N_2O_3P$ (324g.mol^{-1}): C, 59.25; H, 7.71; N, 8.64; found C, 5922; H, 7.73; N, 8.63%.

- ***(4g/5g). (Z)-Dimethyl N'-benzyl-N-(4-chloro)phenylcarbamimidoylphosphonate***

Orange oil; yield= 76%; ^{1}H NMR ($CDCl_3$, δ_H ppm): 3.39 (d, J=11.1Hz, 6H, CH_3), 3.87 (s, 2H, CH_2), 6.25-6.70 (m, 9H, H_{arom}), 7.18 (d, J=7.6Hz, 1H, NH). ^{13}C NMR ($CDCl_3$, δ_C ppm): 174.85 (d, J=148.2 Hz, C=N), 162.6 (d, J= 137.1Hz, N-C_{ipso}), 141.6 (d, J=10 Hz, C_{ipso}), 132.71 (d, J=8Hz, N-C_{meta}), 129.9 (s, C_{ortho}), 127. 5 (s, C_{meta}), 127.15 (s, C_{para}), 116.4 (d, J=3.9Hz, N-C_{ortho}), 57.19 (d, J=6.4Hz, CH_2), 53.79 (d, J= Hz, MeO). ^{31}P NMR ($CDCl_3$, δ_P ppm): (**3g**) = -2.09 (**4g**)= -3.16.

6.2. Biological activities assays

6.2.1. Evaluation of antibacterial and antifungal activities

The synthesized compounds have been screened for following activity by the paper-disc method with a diameter of 6.0 mm. The antibacterial activity was determined by disc diffusion method on Mueller-Hinton agar [34]. These were evaluated by measuring the inhibition zone in the presence of increasing concentrations of phosphonoamidates and phosphonoamidines from 10 to 40mg/mL. The antibacterial study was focused on standard bacterial strains of *Staphylococcus aureus* (ATCC29213), *Pseudomonas aerogunosa* (ATCC27859) and *Escherichia coli* (ATCC 25922). The antifungal study was focused on standard strains of *Candida parapsilosis* (CECT13009)

6.2.2. Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay was based on the method reported by Blois [23]. The phosphonoamidate and phosphonoamidine compounds were added at increasing concentrations (from 1 to 40 mg/mL) to 1 mL of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) dissolved in methanol to 100 µM. The mixture was incubated at room temperature for 30 min and then the absorbance of stable DPPH was determined at 517 nm using a UV– visible spectrophotometer type UNIVERSAL 320UV/VIS. The concentration of DPPH was calculated using a standard curve. The free radical scavenging activity was expressed as follows: % Inhibition = ((C0–Csample)/C0) *100

(C0 = Concentration of DPPH in the control and Csample = Concentration of DPPH in the sample

6.2.3. Animals

Male *Wistar* rats were housed in pairs in cages (25 × 50 cm) and maintained at 23°C, 12/12h light/dark cycle under specific pathogen-free conditions. The rats were allowed to acclimatize in the experimental medicine unit for a period of one week before the beginning of the study. During the experiment period, they received a commercial pellet diet and water. Rats weighing 200 g were used for the experiments. All experimental procedures were approved by the Ethics Committee of the Faculty of Medicine of Tunis according to the standards of the International Council for Laboratory Animal Science (ICLAS).

6.2.4. Animal experimentation design

The analgesic activity of newly synthesized phosphonate amide derivatives was evaluated using the formalin test and the hot plate test 15 minutes after an intra-peritoneal injection (IP) to rats divided into 10 groups as follows:

Group C: Control group treated with 0.9% saline solution (IP)

Group morph 5: treated with morphine at 5mg/kg dose (IP)

Group morph 7.5: treated with morphine at 7.5mg/kg dose (IP)

Group morph 10: treated with morphine at 10mg/kg dose (IP)

Group MAmP 5: treated by dimethylcyclohexylcarbamoylphosphonate at 5mg/kg dose (IP)

Group MAmP 7.5: treated by dimethylcyclohexylcarbamoylphosphonate at 7.5mg/kg dose

Group MAmP 10: treated by dimethylcyclohexylcarbamoylphosphonate at 10mg/kg dose (IP)

Group EAmP 5: treated by diethylcyclohexylcarbamoylphosphonate at 5mg/kg dose (IP)

Group EAmP 7.5: treated by diethylcyclohexylcarbamoylphosphonate at 7.5mg/kg dose

Group EAmP 10: treated by diethylcyclohexylcarbamoylphosphonate at 10mg/kg dose (IP)

6.2.5. Hot Plate Test

The Hot plate test is a behavioral model of nociception which is commonly employed to screen analgesic drug effects. During a hot plate test, rats display several noxious-evoked patterns as well as exploratory and self-care responses.

The Hot plate test uses the reflexes of the thermal pain due to the contact of the paws with a heated surface. The rats were placed on a heated plate maintained at a constant temperature of 48°C. During the hot plate test period we noted the latency time before the rat elevated and licked its paws and jumped along with the time when the rat fled out of the heated plate [17,24].

6.2.6. Formalin Test

The formalin test is used to evaluate nociception, applied mainly to rats and mice, and involves moderate continuous pain, generated by the inflamed tissues. In this test, a solution of 5% formalin was injected subcutaneously in a posterior paw of the rat to produce a biphasic pain. During the 60 min of the test, the initial response to the pain (the early phase) was evaluated from 1 to 5 min after the injection of formalin. The response to pain (the late phase) was evaluated 50 min after the pain induction during 10 min [15,16].

To evaluate the rat pain degree we attributed a score as follows:

Score 0 - The injected paw is pressed firmly on the floor and obviously bears the animal's weight. **Score 1 -** The injected paw rests lightly on the floor or wall and is definitely in contact with it, but little if any weight is placed on the paw; during locomotion there is a definite limp. **Score 2 -**The injected paw is elevated and is not in contact with any surface. **Score 3 -** The animal licks, bites, or shakes the affected paw.

6.2.7. Statistical analyses

The results are presented as averages ± standard deviation. All analyses were carried out with Biostat software for Windows. Significant differences between treatment effects were determined by the U Man Whitney test for multiple comparisons with statistical significance when $p < 0.05$.

Credit author statement

Rania Omrani and Ridha Ben Ali: Chemical synthesis, design, characterization and, development of animal model, Pharmacological Studies of Novel crystal of MThmP and statistical studies.

Anis Raddaoui: Evaluation of the antibacterial and antifungal activities

Hedia Hmani: design and molecule characterization

Youssef Arfaoui: Computational studies via Density fuctional Theory (DFT)

MichèleVéronique El May: Development of animal model

Azaiez Ben Akacha: Identification of the molecule structure

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We gratefully acknowledge the Histology, Embryology and Cell Biology Laboratory (UR17ES13), Laboratory Selective Organic & Heterocyclic Synthesis Biological Activity Evaluation (LR17ES01) for their financial support to research project.

We are grateful to Mr. Tarek Ben Ali for his help in revising the English of the manuscript.

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