Synthesis of Novel Phosphorylated Guanidine Derivatives from Cyanamide and Their Anti-inflammatory Activity

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A series of novel guanidine derivatives were synthesized in three steps and their anti-inflammatory activities *in vitro* and *in vivo* evaluated. 2-Aminopyridin-3-ol (1) was reacted with thiophosphoryl chloride (2) to give a monochloride (3). It was further reacted with cyanamide to afford the corresponding cyanamine (4), which was subsequently reacted with different heterocyclic amines to form the title compounds (5a–l). The substituent in the guanidine function affected the potency of anti-inflammatory activity. The compounds having benzothiazole, fluorophenyl, and piperazinyl moieties enhanced the anti-inflammatory activity.

Key words phosphorylated guanidine; 2-aminopyridin-3-ol; anti-inflammatory activity

Compounds containing a guanidine functional group exhibit many biological, chemical and medicinal applications.[1)] Guanidine is a ubiquitous group in natural products and plays a key role in many biological activities.[2)] In peptides, guanidine, a residue of L-arginine, exists in the protonated form as guanidinium ions functioning as an efficient recognition moiety of anionic substrates such as carboxylate, phosphate and intonate functionalities.[3)] A wide range of biological activities and chemical applications have been found for guanidine derivatives. These have motivated the development of many reagents for their synthesis and various methods to achieve guanylation by nucleophilic addition of an amine to a cyanamide.[4)] The guanidine derivatives exhibited diverse biological and pharmacological activities such as neuronal Na^+ and Ca^{2+} channel blockers,[5)] glutamate release inhibitors, anti-ischemic agents,[6)] anti-seizure agents,[7)] aderenergic neuron-blocking agents,[8)] human immunodeficiency virus-1 (HIV-1) protease inhibitors,[9)] K^+/ATP channel openers, nitric oxide (NO) synthase inhibitors, influenza neuraminidase inhibitors,[10)] cardiotonic agents,[11)] and histamine H_3 receptor antagonists.[12)] Therefore, the guanidine functional group is an attractive pharmacophore for the development of novel drugs against tumors, hypertension, glaucoma, and inflammation.[13)] We previously reported that phosphorylation of various drugs improves their biological activity.[14)] This finding led us to investigate the anti-inflammatory activities of a novel series of phosphorylated guanidine derivatives. Their anti-inflammatory activities *in vitro* and *in vivo* were compared *versus* those of conventional anti-inflammatory drugs such as aspirin, diclofenac sodium, phenyl butazone, and flufenamic acid.

Results and Discussion

Chemistry The synthesis of new biologically active substituted *N*′-(2-thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)guanidine derivatives was accomplished in straight forward manner as shown in Chart 1. To synthesize target title derivatives, the key intermediate compound **4** was achieved in two steps. First, the monochloride intermediate, 2-chloro-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphole-2-thione (**3**) was synthesized by reaction of 2-aminopyridin-3-ol (**1**) with thiophosphoryl chloride (**2**) in tetrahydrofuran (THF) using triethylamine (TEA) as mild base by stirring at room temperature for 3 h. The formation of monochloride **3** was ascertained by TLC eluting with a 3 : 2 mixture of ethyl acetate and hexane. After completion of the reaction, the resulting triethylamine hydrochloride (Et_3N–HCl) was removed from the reaction mixture by filtration. The filtrate containing the monochoride **3** was used for the next step of the reaction without further purification. Second, cyanamide was added in the presence of TEA to the filtrate and stirred at 30–40°C for 2 h. After completion of the reaction, the mixture was concentrated under vacuum and the residue purified by column chromatography on silica gel (100–200 mesh) using petroleum ether–ethyl acetate (3 : 7) as elutent to obtain the P-cyanamine intermediate **4**. Finally, the title compounds (**5a–l**) were prepared by treatment of **4** with various heterocyclic/aryl amines in the presence of TEA with stirring at 50–60°C for 2 h. All the title compounds were purified by column chromatography on silica gel (100–200 mesh) using petroleum ether–ethyl acetate (2 : 3) as eluent; the title compounds were obtained in excellent yields (71–88%).

The intermediate **4** and all the title compounds were characterized by IR, ^{1}H-, ^{13}C- and ^{31}P-NMR, mass spectra, and elemental analysis. The disappearance of band at 3425 (–O–H) in IR, 10.2 ppm (–OH) in ^{1}H-NMR of 2-aminopyridin-3-ol (**1**) and appearance of bands at 2323 (–CN), 764 (–P=S) in IR, 3.58 ppm (–NH–CN) in ^{1}H-NMR, ^{31}P-NMR and molecular ions confirm the formation of key intermediate compound **4**. Finally, IR bands appeared in the regions of 3472–3436, 3358–3322, and 1670–1624 for N–H, C=N–H, and C=N, respectively. ^{1}H-NMR spectra exhibited peaks at δ 3.93–5.62 (br), 6.89–8.00, and 8.31–10.43 due to guanidine –NH, aromatic protons, and –C=N–H protons, respectively. ^{31}P-NMR chemical shifts were observed in the region −11.8 to 15.2 ppm.[15)] In addition, ^{13}C-NMR, mass spectra, and elemental analysis provided further evidence for confirmation of the title compounds (**5a–l**).

Biological Evaluation. *In Vitro* Anti-inflammatory Activity (a) Inhibition of Albumin Denaturation: The newly synthesized compounds **5a–l** were assayed for their anti-inflammatory activity with respect to inhibition of albumin denaturation.[16)] In this method, the reaction mixture

The authors declare no conflict of interest.

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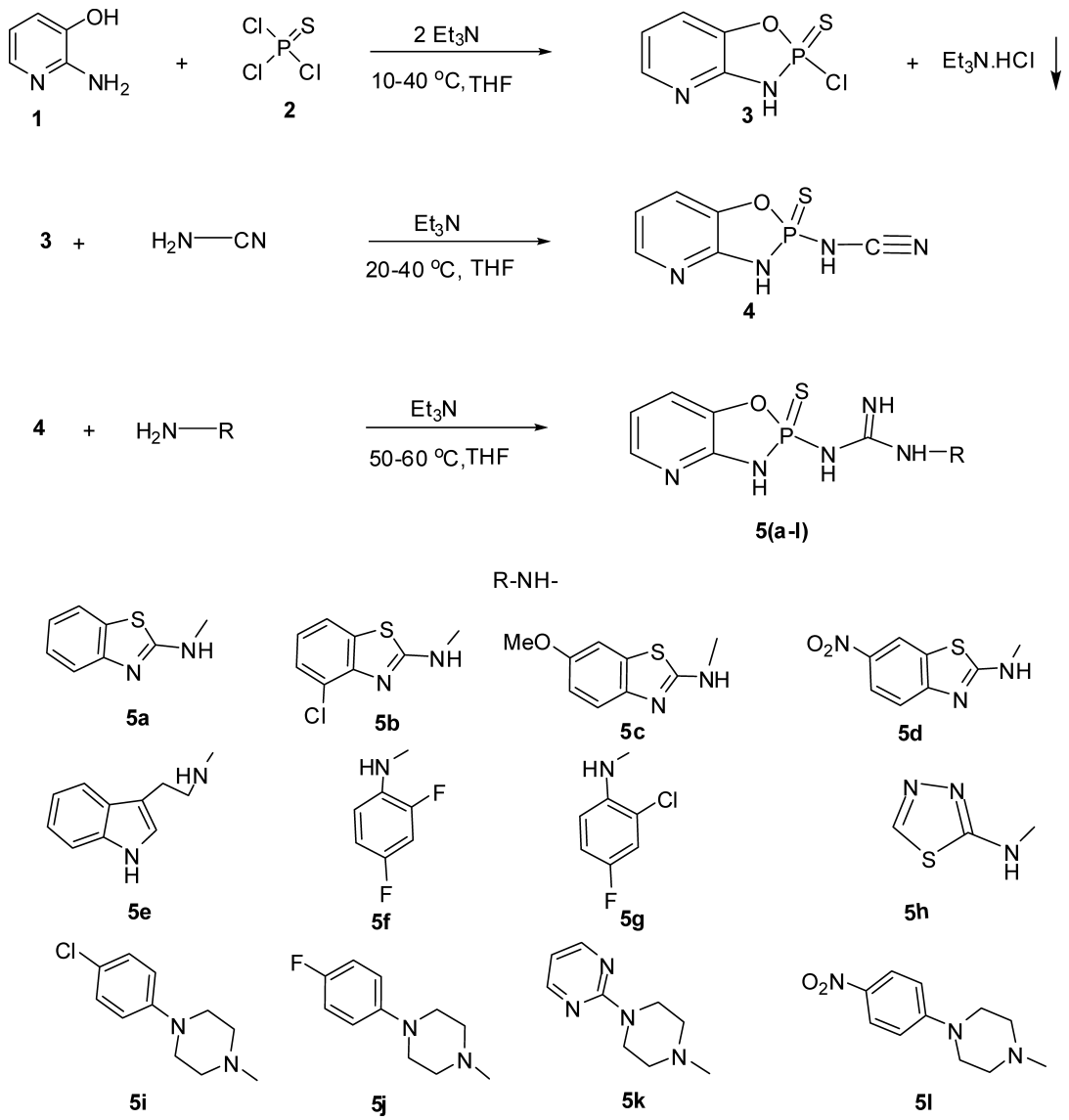

Chart 1

comprised test compounds at different concentrations and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted to 7 using small amount of 1 N HCl. The samples were incubated at 37°C for 20 min then heated at 57°C for 20 min. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:

$$\text{percentage inhibition} = \frac{(\text{absorbance control} - \text{absorbance sample})}{\text{absorbance of control}} \times 100$$

Denaturation of protein is a well-documented cause of inflammation. Phenylbutazone, flufenamic acid (anti-inflammatory drugs), *etc.*, have shown dose-dependent ability to reduce thermally induced protein denaturation. As part of the investigation on the mechanism of the anti-inflammatory activity, ability of extract to inhibit protein denaturation was studied.

It was effective in inhibiting heat-induced albumin denaturation at different concentrations as shown in (Table 1, Fig. 1). Maximum inhibition, 85.92±1.48%, was observed at 0.8 μg/mL. Flufenamic acid, a standard anti-inflammatory drug, showed maximum inhibition of, 85.36±0.56% at a concentration of 0.2 μg/mL. In this bioassay **5a**, **5b**, **5g**, and **5j** compounds exhibited promising percent inhibition compared with that of standard.

Membrane Stabilization Test (a) Preparation of Red Blood Cells (RBCs) Suspension: Fresh whole human blood (10 mL) was collected and transferred to heparinized centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min then washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline.

(b) Heat-Induced Hemoloysis: The reaction mixture (2 mL) consisting of 1 mL of test drug solution and 1 mL of 10% RBCs suspension, was added to the test tube.[17] Aspirin was taken as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in a water bath at 56°C for 30 min. At the end of the incubation, the tubes were cooled under tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants taken at 560 nm. The experiments were performed in triplicate

Table 1. *In Vitro* Anti-inflammatory Activity of Title Compounds (**5a–l**)

S. No.	Compounds	Dose (μg/mL)	Mean OD	S.D.	% Inhibition
1	NC	RO Water	2.509	0.143	—
2	PC1	0.1	1.004	0.017	59.97
3	PC1	0.2	0.367	0.028	85.36
4	**5a**	0.1	1.008	0.015	59.81
5	**5a**	0.2	0.570	0.016	77.27
6	**5b**	0.1	1.002	0.005	60.05
7	**5b**	0.2	0.708	0.014	71.79
8	**5c**	0.1	1.260	0.038	49.79
9	**5c**	0.2	0.863	0.044	65.62
10	**5d**	0.1	1.139	0.018	54.59
11	**5d**	0.2	0.842	0.035	66.43
12	**5e**	0.1	1.292	0.049	48.52
13	**5e**	0.2	1.021	0.037	59.31
14	**5f**	0.1	1.294	0.035	48.44
15	**5f**	0.2	0.993	0.025	60.44
16	**5g**	0.1	0.972	0.015	61.27
17	**5g**	0.2	0.605	0.006	75.90
18	**5h**	0.1	1.101	0.045	56.12
19	**5h**	0.2	0.767	0.031	69.43
20	**5i**	0.1	1.066	0.038	57.53
21	**5i**	0.2	0.766	0.010	69.47
22	**5j**	0.1	0.861	0.018	65.70
23	**5j**	0.2	0.594	0.030	76.31
24	**5k**	0.1	1.102	0.051	53.28
25	**5k**	0.2	0.881	0.046	68.41
26	**5l**	0.1	1.314	0.038	54.64
27	**5l**	0.2	0.984	0.034	67.85

NC=phenyl butazone; PC1=flufenamic acid. PC1=0.1 μg/mL value should be given 59.97 and 0.2 μg/mL value should be given 85.36.

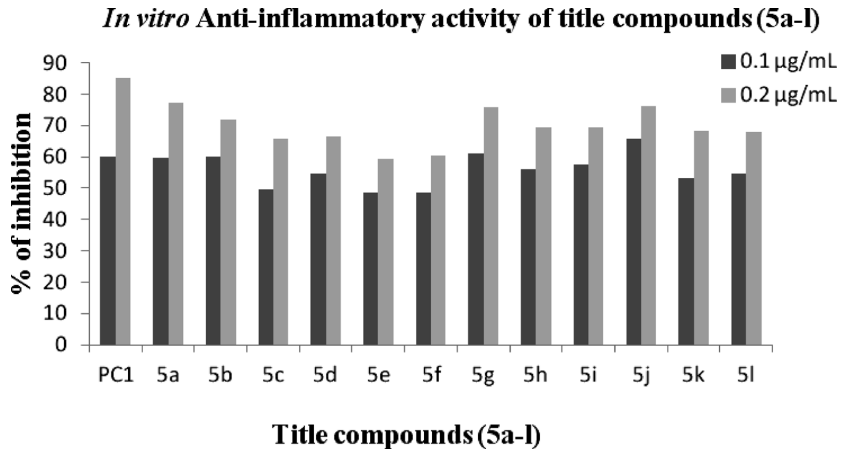

Fig. 1. *In Vitro* Anti-inflammatory Activity of Title Compounds (**5a–l**)

and presented as (Table 2, Fig. 2). Percent membrane stabilization activity was calculated by the following formula:

$$\text{percentage inhibition} = \frac{(\text{absorbance control} - \text{absorbance sample})}{\text{absorbance of control}} \times 100$$

***In Vivo* Anti-inflammatory Activity** (a) Acute Toxicology Studies: Acute toxicology study was performed on the hybrid compounds according to the Organisation for Economic Cooperation and Development (OECD) guidelines for Testing of Chemicals, No. 423 "Acute Oral Toxicity—Acute Toxic Class Method," adopted December 17, 2001. For compounds **5a** to **5i** mortalities were found at 5 mg/kg body weight (bw) during 24 h observation. The common clinical signs observed in the treated animals were dullness, piloerection and recumbency with **5a** to **5l** compounds 50 mg/kg bw treated animals during first 4 h the of observation. Abnormalities were not detected during the observation of gross pathology for all the animals.

(b) Anti-inflammatory Studies: *In vivo* anti-inflammatory activity was performed by carrageenan-induced paw edema and cotton pellet-induced granuloma models. Doses were selected based on the acute toxicity studies.

(c) Carrageenan Induced Paw Oedema: The test was conducted to determine the anti-inflammatory activity of the hybrid compounds.[18] The animals were pretreated with hybrid

Table 2. *In Vitro* Anti-inflammatory Activity of Title Compounds (**5a**–**l**) by the Membrane Stabilization Method

S. No.	Compounds	Dose (μg/mL)	Mean OD±S.D.	% Inhibition
1	NC	RO Water	2.297±0.117*	—
1	PC1	0.1	0.866±0.056*	60.28
2	PC1	0.2	0.724±0.034*	79.18
3	**5a**	0.1	0.971±0.049*	57.73
4	**5a**	0.2	0.678±0.048*	72.50
5	**5b**	0.1	0.961±0.028*	58.15
6	**5b**	0.2	0.664±0.018*	73.09
7	**5c**	0.1	1.192±0.016*	48.12
8	**5c**	0.2	0.875±0.022*	61.89
9	**5d**	0.1	1.108±0.009*	51.78
10	**5d**	0.2	0.929±0.034*	59.56
11	**5e**	0.1	1.455±0.045*	36.66
12	**5e**	0.2	1.152±0.025*	49.83
13	**5f**	0.1	1.856±0.008*	29.18
14	**5f**	0.2	1.641±0.034*	48.57
15	**5g**	0.1	0.955±0.039*	58.41
16	**5g**	0.2	0.547±0.041*	76.19
17	**5h**	0.1	1.017±0.005*	55.74
18	**5h**	0.2	0.844±0.019*	73.27
19	**5i**	0.1	0.962±0.036*	58.12
20	**5i**	0.2	0.768±0.012*	66.58
21	**5j**	0.1	0.863±0.018*	56.44
22	**5j**	0.2	0.401±0.013*	68.53
23	**5k**	0.1	0.822±0.013*	64.24
24	**5k**	0.2	0.754±0.011*	68.12
25	**5l**	0.1	0.822±0.013*	65.28
26	**5l**	0.2	0.724±0.012*	68.12

PC1, salicylic acid; *$p<0.05$.

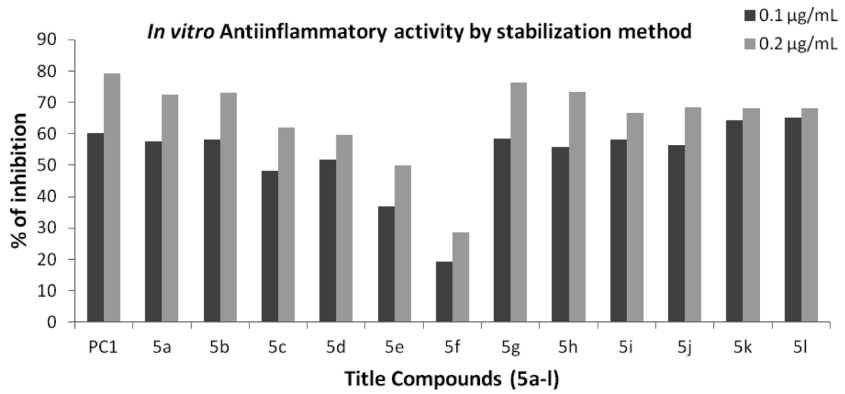

Fig. 2. *In Vitro* Anti-inflammatory Activity of Title Compounds (**5a**–**l**) by the Membrane Stabilization Method

compounds or diclofenac sodium 1 h before carrageenan injection. The animals were injected with 100 μL of 1% carrageenan (0.5% carboxymethyl cellulose (CMC)) solution into the sub-plantar region of right hind paw. Paw volume was measured by dislocation of the water column in a plethysmometer after carrageenan application at 0, 1, 3, 6 and 12 h after test compound stimulus. Reduction in the paw volume compared with the vehicle-treated (0.5% CMC) control animals was considered as anti-inflammatory response; the results are shown in (Table 3, Fig. 3).

(d) Cotton Pellet-Induced Granuloma: The test was performed on Wistar rats using cotton pellet-induced granuloma method.[19] The rats were anesthetized under light ether and an incision was made on the lumbar region by curved scissors; a subcutaneous tunnel was made and a sterilized cotton pellet (100±1 mg) inserted with blunted forceps in that area. All the animals received either hybrid compounds, diclofenac sodium or vehicle (0.5% CMC) orally depending on their respective grouping for 7 d consecutively from the day of cotton pellet insertion. On day 8, animals were anesthetized again and cotton pellets removed, dried, and weighed. The results are shown in (Table 4, Fig. 4).

Statistical Analysis Statistical analysis was performed Graph Pad InStat version 3.0 for Windows 95, Graph Pad Software, San Diego, CA, U.S.A. The anti-inflammatory activity by membrane stabilization (*in vitro*), paw edema, cotton pellet (*in vivo*) and percent inhibition of albumin denaturation, was compared *versus* negative control group by one-way analysis of variance (ANOVA) with Dunnett's *post*-test; a value of $p<0.05$ was considered significant.

Table 3. *In Vivo* Anti-inflammatory Activity of Title Compounds (**5a–l**) by the Carrageenan Induced Paw Oedema Method

Group	Dose (mg/kg)	Paw volume									
		0 h		1 h		3 h		6 h		12 h	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Control	CMC	1.92	0.12	1.95	0.08	2.00	0.13	1.83**	0.14	1.67**	0.10
PC	100	1.83^{ns}	0.08	1.57**	0.08	1.35**	0.10	1.17**	0.12	1.08**	0.08
5a	5	1.82^{ns}	0.08	1.70**	0.13	1.57**	0.14	1.38**	0.13	0.98**	0.15
5b	5	1.83^{ns}	0.10	1.70**	0.11	1.50**	0.11	1.38**	0.10	0.93**	0.16
5c	5	1.87^{ns}	0.08	1.75**	0.05	1.57**	0.05	1.42**	0.08	1.15**	0.08
5d	5	1.85^{ns}	0.08	1.77**	0.05	1.60**	0.09	1.43**	0.08	1.17**	0.05
5e	5	1.82^{ns}	0.08	1.72**	0.08	1.57**	0.08	1.43**	0.08	1.17**	0.10
5f	5	1.87^{ns}	0.05	1.82**	0.04	1.63**	0.05	1.48**	0.04	1.23**	0.08
5g	5	1.85^{ns}	0.05	1.70**	0.09	1.58**	0.10	1.43**	0.05	1.13**	0.10
5h	5	1.88^{ns}	0.04	1.72**	0.08	1.57**	0.08	1.42**	0.08	1.17**	0.10
5i	5	1.85^{ns}	0.08	1.68**	0.08	1.53**	0.05	1.40**	0.06	1.17**	0.05
5j	5	1.83^{ns}	0.08	1.65**	0.05	1.52**	0.08	1.33**	0.08	0.92**	0.12
5k	5	1.81^{ns}	0.07	1.64**	0.08	1.51**	0.08	1.32**	0.08	0.91**	0.09
5l	5	1.80	0.06	1.63	0.09	1.50	0.07	1.31	0.07	0.90	0.08

One-way ANOVA with Dunnett's *post*-test was performed, $p<0.05$ was considered statistically significant. ** $p<0.01$, $^{ns}p>0.05$.

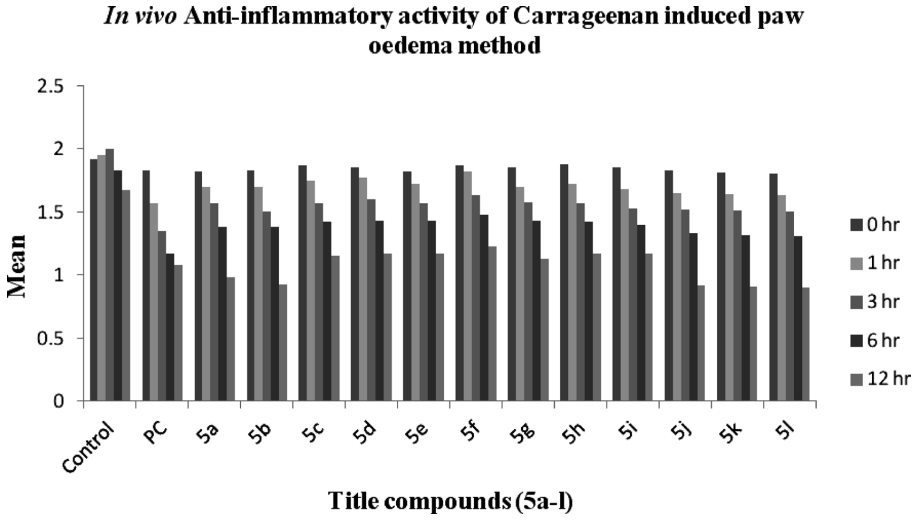

Fig. 3. *In Vivo* Anti-inflammatory Activity of Title Compounds (**5a–l**) by the Carrageenan Induced Paw Oedema Method

Table 4. *In Vivo* Anti-inflammatory Activity of Title Compounds (**5a–l**) by the Cotton Pellet-Induced Granuloma Method

Group	Dose (mg/kg bw)	Granuloma dry weight (mg)		% inhibition
		Mean	S.D.	
Control	0.5% CMC	63.67	1.37	—
PC	100	29.67**	1.21	58.40
5a	5	31.17**	2.56	51.05
5b	5	30.50**	1.87	52.09
5c	5	38.17**	1.47	40.05
5d	5	37.83**	5.04	40.58
5e	5	32.83**	3.54	48.43
5f	5	39.50**	1.64	37.96
5g	5	36.17**	1.60	43.19
5h	5	37.50**	1.87	41.10
5i	5	34.67**	1.21	45.55
5j	5	27.17**	2.23	54.33
5k	5	27.16**	2.24	36.46
5l	5	27.15**	2.25	35.18

Control, 0.5% CMC (carboxymethyl cellulose); PC, positive control (dichlofenac), **5a–5l**, title compounds. One-way ANOVA with Dunnett's *post*-test was performed, $p<0.05$ was considered statistically significant. ** $p<0.01$.

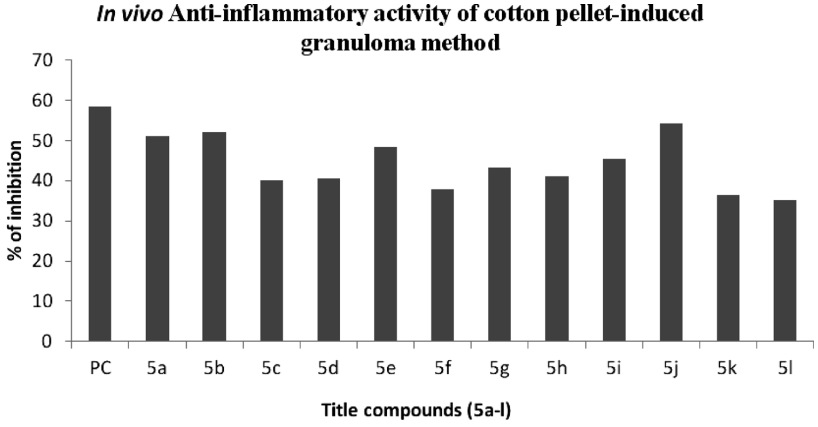

Fig. 4. *In Vivo* Anti-inflammatory Activity of Title Compounds (**5a–l**) by the Cotton Pellet-Induced Granuloma Method

Conclusion

Synthesis of novel phosphorylated guanidine derivatives containing various bioactive amines as substitutents in the guanidine function was accomplished in high yields. Based on the data obtained from the carrageenan-induced paw edema and cotton pellet-induced granuloma, all the title compounds showed good to excellent anti-inflammatory activity compared with control (diclofenac). In particular, compounds **5a**, **5b**, **5g**, and **5j** showed potent anti-inflammatory activity in *in vitro* and *in vivo* models. This enhanced activity may be due to the presence of benzothiazole, chlorobenzothiazole, fluoro-chlorophenyl, and fluorophenylpiperazinyl substituents in the guanidine function. Selected compounds of the present series will be further optimized *in vitro* and *in vivo* and investigated to explore the pharmacological mechanism of anti-inflammatory activity.

Experimental

Chemistry Chemicals were purchased from Sigma-Aldrich, Merck, and Lancaster, and were used without further purification. All solvents used for spectroscopic and other physical studies were reagent grade and were further purified by literature methods.[20] IR spectra were recorded as KBr pellets on a Perkin Elmer 283 unit. ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer operating at 400 MHz for ^{1}H-, 100 MHz for ^{13}C-, and 161.9 MHz for ^{31}P-NMR in DMSO-d_6 and referenced to tetramethylsilane (TMS) (^{1}H and ^{13}C) and 85% H_3PO_4 (^{31}P). LC-MS spectra were recorded on a Jeol SX 102 DA/600 mass spectrometer. Elemental analyses were performed on a Thermo Finnigan Instrument at University of Hyderabad, Hyderabad, India.

Synthesis of 2-Chloro-2,3-dihydro-2λ^5-pyrido[2,3-*d*]-[1,3,2]oxazaphosphole-2-thione 3 2-Aminopyridin-3-ol (**1**) was reacted with thiophosphoryl chloride (**2**) in the presence of Et_3N in THF to get a monochloride, 2-choloro-3*H*-[1,3,2]-oxazaphospholo[4,5-*b*]pyridine-2-sulfide **3** after stirring at room temperature for 2 h. Formation of monochloride **3** was ascertained by TLC analysis run in 3 : 2 mixture of ethyl acetate and hexane. TEA hydrochloride was removed from the reaction mixture by filtration. The compound was characterized by spectral analysis. Yield: 88%; mp 118–120°C. IR (KBr) cm^{-1}: 789 (P=S), (P-N). ^{1}H-NMR (DMSO-d_6) δ: 7.83–6.97 (3H, m, Ar-H), 3.49 (1H, s, P-NH). ^{13}C-NMR (DMSO-d_6) δ: 136.8 (C-2), 117.5 (C-3), 126.2 (C-4), 140.2 (C-5), 151.4 (C-6). ^{31}P-NMR (DMSO-d_6) δ: −6.4. *m*/*z* (%): 207 (100) [$M^{+\cdot}$+1], 209 (50), 187 (25), 169 (21). *Anal.* Calcd for $C_5H_4ClN_2OSP$: C, 29.07; H, 1.95; N, 13.56. Found: C, 29.15, H, 1.91; N, 13.45.

Synthesis of [(2-Thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*]-[1,3,2]oxazaphosphol-2-yl)amino]methanenitrile 4 The filtrate containing **3** was used for the next step of the reaction without further purification. It was further reacted with cyanamide (H_2N–CN) in the presence of Et_3N at 20–40°C for 2 h to afford the corresponding cyanamine (P(S)–NH–CN) **4**. The reaction progress was monitored by TLC analysis using ethyl acetate and hexane (7 : 3). Yield: 85%; mp 126–128°C. IR (KBr) cm^{-1}: 3362 (C=NH), 768 (P=S), 982 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 7.83–6.97 (3H, m, Ar-H), 3.93 (1H, s, Ar-NH) 3.49 (1H, s, P-NH). ^{13}C-NMR (DMSO-d_6) δ: 136.8 (C-2), 117.5 (C-3), 126.2 (C-4), 140.2 (C-5), 151.4 (C-6), 156.2 (C-11). ^{31}P-NMR (DMSO-d_6) δ: −11.8. *m*/*z* (%): 213 (100) [$M^{+\cdot}$], 212 (51), 204 (16), 195 (28). *Anal.* Calcd for $C_6H_4N_4OSP$: C, 33.97; H, 2.38; N, 26.41. Found: C, 33.85, H, 2.41; N, 26.31.

General Procedure for Preparation of *N*-Substituted *N*′-(2-Thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)guanidine Derivatives (5a–l) The intermediate **4** was further treated with heterocyclic/aryl amines in THF in the presence of Et_3N at 50–60°C for 2 h to form title compound **5a–l**. Identification of the product and completion of the reaction were monitored by TLC using ethyl acetate–hexane (4 : 1) as eluent. After completion of the reaction, the mixture was concentrated in a rota-evaporator and the residue purified by column chromatography on silica gel (100–200 mesh) using petroleum ether–ethyl acetate (2 : 3) as eluent. The structure of the title compounds (**5a–l**) were established by spectral and elemental analysis.

N-(1,3-Benzothiazol-2-yl)-*N*′-(2-thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxaza phosphol-2-yl)guanidine (**5a**): This compound was prepared in 80% yield by the protocol described in the general procedure. mp 165–167°C. IR (KBr) cm^{-1}: 3380 (N–H), 3342 (C=NH), 1624 (C=N), 756 (P=S), 914 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 8.31 (1H, s, C=NH), 7.83–6.97 (7H, m, Ar-H), 5.41 (1H, s, Ar′-NH), 3.93 (1H, s Ar-NH) 3.49 (1H, s, P-NH). ^{13}C-NMR (DMSO-d_6) δ: 138.2 (C-2), 117.2 (C-3), 124.8 (C-4), 139.4 (C-5), 148.2 (C-9), 162.7 (C-11), 172.2 (C-13), 151.6 (C-15), 116.4 (C-16), 126.0 (C-17), 124.6 (C-18), 120.2 (C-19), 128.4 (C-20). ^{31}P-NMR (DMSO-d_6) δ: 11.1. *m*/*z* (%): 362 (100) [$M^{+\cdot}$], 361 (52), 260 (41), 152 (35),

89 (21). *Anal.* Calcd for $C_{13}H_{11}N_6OS_2P$: C, 43.09; H, 3.06; N, 23.19. Found: C, 43.07, H, 3.04; N, 23.17.

N-(4-Chloro-1,3-benzothiazol-2-yl)-*N*′-(2-thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)guanidine (**5b**): This compound was prepared in 78% yield by the protocol described in the general procedure. mp 174–176°C. IR (KBr) cm^{-1}: 3412 (N–H), 3348 (C=NH), 1632 (C=N), 724 (P=S), 921 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 8.43 (1H, s, C=NH), 7.83–7.06 (6H, m, Ar-H), 5.2 (1H, s, Ar′-NH), 4.5 (1H, s, Ar-NH), 3.12 (1H, s, P-NH) . ^{13}C-NMR (DMSO-d_6) δ: 137.4 (C-2), 118.4 (C-3), 125.2 (C-4), 138.6 (C-5), 149.4 (C-9), 164.2 (C-11), 173.4 (C-13), 152.4 (C-15), 120.4 (C-16), 127.2 (C-17), 126.2 (C-18), 121.6 (C-19), 129.7 (C-20). ^{31}P-NMR (DMSO-d_6) δ: 12.1. *m/z* (%): 397 (100) [$M^{+\cdot}$+1], 399 (33) [$M^{+\cdot}$+2], 246 (35), 172 (19), 105 (14). *Anal.* Calcd for $C_{13}H_{10}N_6OS_2P$: C, 39.35; H, 2.54; N, 21.18. Found: C, 39.32, H, 2.50; N, 21.14.

N-(6-Methoxy-1,3-benzothiazol-2-yl)-*N*′-(2-thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)guanidine (**5c**): This compound was prepared in 82% yield by the protocol described in the general procedure. mp 181–183°C. IR (KBr) cm^{-1}: 3418 (N–H), 3342 (C=NH), 1624 (C=N), 710 (P=S), 934 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 8.66 (1H, s, C=NH), 8.24–7.08 (7H, m, Ar-H), 5.45 (1H, s, Ar′-NH), 3.94 (1H, s, Ar-NH), 3.06 (1H, s, P-NH), 2.83 (3H, s, O–CH_3). ^{13}C-NMR (DMSO-d_6) δ: 135.2 (C-2), 116.5 (C-3), 125.2 (C-4), 138.6 (C-5), 149.4 (C-9), 164.2 (C-11), 173.4 (C-13), 152.4 (C-15), 120.4 (C-16), 127.2 (C-17), 126.2 (C-18), 121.6 (C-19), 129.7 (C-20) 50.2 (O-Me). ^{31}P-NMR (DMSO-d_6) δ: 12.8. *m/z* (%): 393 (100) [M+1], 361 (25), 281 (19). *Anal.* Calcd for $C_{14}H_{13}N_6OS_2P$: C, 42.85; H, 3.34; N, 21.42. Found: C, 42.75, H, 3.31; N, 21.36.

N-(6-Nitro-1,3-benzothiazol-2-yl)-*N*′-(2-thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)guanidine (**5d**): This compound was prepared in 76% yield by the protocol described in the general procedure. mp 192–194°C. IR (KBr) cm^{-1}: 3445 (N–H), 3348 (C=NH), 1648 (C=N), 726 (P=S), 965 (P-N): ^{1}H-NMR (DMSO-d_6) δ: 10.2 (1H, s, C=NH), 8.32–7.38 (6H, m, Ar-H), 5.62 (1H, s, Ar′-NH), 4.4 (1H, s, Ar-NH), 3.24 (1H, s, P-NH). ^{13}C-NMR (DMSO-d_6) δ: 138.6 (C-2), 118.2 (C-3), 125.4 (C-4), 140.4 (C-5), 152.8 (C-9), 166.4 (C-11), 175.1 (C-13), 158.2 (C-15), 120.4 (C-16), 124.6 (C-17), 144.6 (C-18), 120.6 (C-19), 131.2 (C-20). ^{31}P-NMR (DMSO-d_6) δ: 9.5. *m/z* (%): 406 (100) [$M^{+\cdot}$], 391 (45), 363 (21), 349 (18). *Anal.* Calcd for $C_{13}H_{10}N_7O_3S_2P$: C, 38.33; H, 2.47; N, 24.07. Found: C, 38.30, H, 2.47; N, 24.04.

N-[2-(1*H*-3-Indolyl)ethyl]-*N*′-(2-thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)guanidine (**5e**): This compound was prepared in 74% yield by the protocol described in the general procedure. mp 156–158°C. IR (KBr) cm^{-1}: 3432 (N–H), 3342 (C=NH), 1636 (C=N), 713 (P=S), 945 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 8.4 (1H, s, C=NH), 7.28–6.79 (8H, m, Ar-H), 5.2 (1H, s, Ar′-NH), 4.6 (1H, s, Ar-NH), 3.73 (1H, s, P-NH), 1.17 (2H, t, –CH_2), 3.05–2.54 (2H, m, –CH_2–NH). ^{13}C-NMR (DMSO-d_6) δ: 137.2 (C-2), 114.5 (C-3), 125.2 (C-4), 140.7 (C-5), 150.2 (C-9), 166.6 (C-11), 48.2 (C-13), 22.6 (C-14) 116.2 (C-15), 128.4 (C-16), 118.2 (C-17), 132.2 (C-18), 114.2 (C-19), 121.4 (C-20), 120.5 (C-21), 118.4 (C-22) 127.6 (C-23). ^{31}P-NMR (DMSO-d_6) δ: 6.9. *m/z* (%): 372 (100) [$M^{+\cdot}$], 354 (46), 176 (21). *Anal.* Calcd for $C_{16}H_{17}N_6OSP$: C, 51.61; H, 4.60; N, 22.57. Found: C, 51.58, H, 4.56; N, 22.54.

N-(2,4-Difluorophenyl)-*N*′-(2-thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)guanidine (**5f**): This compound was prepared in 79% yield by the protocol described in the general procedure. mp 182–184°C. IR (KBr) cm^{-1}: 3452 (N–H), 3352 (C=NH), 1638 (C=N), 747 (P=S), 960 (P-N): ^{1}H-NMR (DMSO-d_6) δ: 10.12 (1H, s, C=NH), 7.84–6.58 (6H, m, Ar-H), 5.3 (1H, s, Ar′-NH), 4.8 (1H, s, Ar-NH), 3.34 (1H, s, P-NH). ^{13}C-NMR (DMSO-d_6) δ: 139.4 (C-2), 116.4 (C-3), 125.6 (C-4), 141.5 (C-5), 150.6 (C-9), 165.6 (C-11), 126.4 (C-13), 158.6 (C-14), 106.4 (C-15), 152.6 (C-16), 118.5 (C-17), 124.2 (C-18). ^{31}P-NMR (DMSO-d_6) δ: 14.2. *m/z* (%): 341 (100) [$M^{+\cdot}$]. *Anal.* Calcd for $C_{12}H_{10}F_2N_5OSP$: C, 42.23; H, 2.95; N, 20.52. Found: C, 42.20, H, 2.94; N, 20.48.

N-(2-Chloro-4-fluorophenyl)-*N*′-(2-thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)guanidine (**5g**): This compound was prepared in 75% yield by the protocol described in the general procedure. mp 168–170°C. IR (KBr) cm^{-1}; 3452 (N–H), 3352 (C=NH), 1638 (C=N), 734 (P=S), 960 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 8.62 (1H, s, C=NH), 7.84–6.58 (6H, m, Ar-H), 5.44 (1H, s, Ar′-NH), 4.8 (1H, s, Ar-NH), 3.34 (1H, s, P-NH). ^{13}C-NMR (DMSO-d_6) δ: 138.6 (C-2), 118.2 (C-3), 124.8 (C-4), 140.8 (C-5), 151.6 (C-9), 166.2 (C-11), 127.4 (C-13), 138.2 (C-14), 116.2 (C-15), 132.6 (C-16), 119.2 (C-17), 120.6 (C-18). ^{31}P-NMR (DMSO-d_6) δ: 13.8. *m/z* (%): 357 (100) [$M^{+\cdot}$], 236 (54), 195 (21). *Anal.* Calcd for $C_{12}H_{10}ClFN_5OSP$: C, 40.29; H, 2.82; N, 19.58. Found: C, 40.25, H, 2.79; N, 19.52.

N-(1,3,4-Thiadiazol-2-yl)-*N*′-(2-thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)guanidine (**5h**): This compound was prepared in 72% yield by the protocol described in the general procedure. mp 146–148°C. IR (KBr) cm^{-1}: 3462 (N–H), 3342 (C=NH), 1642 (C=N), 744 (P=S), 992 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 8.3 (1H, s, C=NH), 8.86–7.58 (4H, m, Ar-H), 5.46 (1H, s, Ar′-NH), 4.6 (1H, s, Ar-NH), 3.12 (1H, s, P-NH). ^{13}C-NMR (DMSO-d_6) δ: 137.2 (C-2), 117.5 (C-3), 125.2 (C-4), 140.1 (C-5), 152.4 (C-9), 167.2 (C-11), 152.2 (C-13), 148.6 (C-16). ^{31}P-NMR (DMSO-d_6) δ: 15.2. *m/z* (%): 313 (100) [$M^{+\cdot}$]. *Anal.* Calcd for $C_8H_8N_7OS_2P$: C, 30.67; H, 2.57; N, 31.29. Found: C, 30.64, H, 2.52; N, 31.26.

N^1-(2-Thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)-4-(4-chlorophenyl)-1-piperazinecarboximidamide (**5i**): This compound was prepared in 73% yield by the protocol described in the general procedure. mp 122–124°C. IR (KBr) cm^{-1}: 3472 (N–H), 3348 (C=NH), 1638 (C=N), 782 (P=S), 952 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 8.7 (1H, s, C=NH), 8.02–7.68 (7H, m, Ar-H), 4.92 (1H, s, Ar′-NH), 3.42 (1H, s, P-NH), 3.26 (4H, t, *J*=5.8 Hz, CH_2–NAr), 2.98 (4H, t, *J*=4.2 Hz, CH_2–N aliphatic). ^{13}C-NMR (DMSO-d_6) δ: 139.5 (C-2), 116.4 (C-3), 125.6 (C-4), 140.4 (C-5), 151.7 (C-9), 163.5 (C-11), 45.2 (C-13), 44.8 (C-13′), 52.2 (C-14), 52.6 (C-14′), 147.5 (C-16), 116.4 (C-17), 128.6 (C-18), 127.2 (C-19), 128.6 (C-20), 116.2 (C-21). ^{31}P-NMR (DMSO-d_6) δ: 12.4; *m/z* (%): 408 (100) [$M^{+\cdot}$], 378 (62), 340 (54), 287 (36), 181 (19). *Anal.* Calcd for $C_{16}H_{18}ClN_6OSP$: C, 47.00; H, 4.44; N, 20.56. Found: C, 46.98, H, 4.42; N, 20.51.

N^1-(2-Thioxo-2,3-dihydro-2λ^5-pyrido[2,3-*d*][1,3,2]oxazaphosphol-2-yl)-4-(4-fluorophenyl)-1-piperazinecarboximidamide (**5j**): This compound was prepared in 71% yield by the protocol described in the general procedure. mp 132–134°C. IR (KBr) cm^{-1}: 3458 (N–H), 3338 (C=NH), 1642 (C=N), 762 (P=S), 962 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 8.5 (1H, s, C=

NH), 7.98–6.86 (7H, m, Ar-H), 4.72 (1H, s, Ar′-NH), 3.46 (1H, s, P-NH), 3.16 (4H, t, J=6.4 Hz, CH_2–NAr), 2.82 (4H, t, J=4.8 Hz, CH_2–N aliphatic). ^{13}C-NMR (DMSO-d_6) δ: 139.2 (C-2), 115.4 (C-3), 126.2 (C-4), 141.5 (C-5), 150.4 (C-9), 164.6 (C-11), 44.8 (C-13), 43.6 (C-13′), 51.4 (C-14), 51.5 (C-14′), 146.2 (C-16), 115.7 (C-17), 118.8 (C-18), 157.4 (C-19), 118.2 (C-20), 115.6 (C-21). ^{31}P-NMR (DMSO-d_6) δ: 13.4. m/z (%): 392 (100) [$M^{+\cdot}$], 272 (53), 114 (28). *Anal.* Calcd for $C_{16}H_{18}FN_6OSP$: C, 48.97; H, 4.62; N, 21.42. Found: C, 48.92, H, 4.58; N, 21.38.

N^1-(2-Thioxo-2,3-dihydro-2λ^5-pyrido[2,3-d][1,3,2]oxazaphosphol-2-yl)-4-(2-pyrimidinyl)-1-piperazinecarboximidamide (**5k**): This compound was prepared in 77% yield by the protocol described in the general procedure. mp 138–140°C. IR (KBr) cm^{-1}: 3458 (N–H), 3338 (C=NH), 1642 (C=N), 732 (P=S), 1022 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 8.9 (1H, s, C=NH), 8.48–7.12 (6H, m, Ar-H), 4.92 (1H, s, Ar′-NH), 3.48 (1H, s, P-NH), 3.18 (4H, t, J=6.6 Hz, CH_2–NAr), 2.86 (4H, t, J=5.2 Hz, CH_2–N aliphatic). ^{13}C-NMR (DMSO-d_6) δ: 138.6 (C-2), 115.7 (C-3), 125.8 (C-4), 140.6 (C-5), 151.2 (C-9), 165.2 (C-11), 45.2 (C-13), 44.8 (C-13′), 51.2 (C-14), 50.8 (C-14′), 162.5 (C-16), 156.2 (C-18), 116.4 (C-19), 157.8 (C-19). ^{31}P-NMR (DMSO-d_6) δ: 11.7. m/z (%): 376 (100) [$M^{+\cdot}$]. *Anal.* Calcd for $C_{14}H_{17}N_8OSP$: C, 44.68; H, 4.55; N, 29.77. Found: C, 44.64, H, 4.52; N, 29.73.

N^1-(2-Thioxo-2,3-dihydro-2λ^5-pyrido[2,3-d][1,3,2]oxazaphosphol-2-yl)-4-(4-nitrophenyl)-1-piperazinecarboximidamide **(5l)**: This compound was prepared in 81% yield by the protocol described in the general procedure. mp 146–148°C. IR (KBr) cm^{-1}; 3462 (N–H), 3354 (C=NH), 1638 (C=N), 774 (P=S), 968 (P-N). ^{1}H-NMR (DMSO-d_6) δ: 8.58 (1H, s, C=NH), 8.06–7.16 (7H, m, Ar-H), 4.7 (1H, s, Ar′-NH), 3.36 (1H, s, P-NH), 3.28 (4H, t, J=5.6 Hz, CH_2–NAr), 2.82 (4H, t, J=4.2 Hz, CH_2–N aliphatic). ^{13}C-NMR (DMSO-d_6) δ: 137.8 (C-2), 116.5 (C-3), 125.2 (C-4), 140.8 (C-5), 152.4 (C-9), 166.4 (C-11), 45.6 (C-13), 45.2 (C-13′), 51.8 (C-14), 51.2 (C-14′), 156.5 (C-16), 116.4 (C-17), 126.7 (C-18), 139.2 (C-20), 125.7 (C-21), 124.2 (C-21). ^{31}P-NMR (DMSO-d_6) δ: 14.7. m/z: 419 (100) [$M^{+\cdot}$], 301 (67), 194 (39), 138 (25). *Anal.* Calcd for $C_{16}H_{18}N_7O_3SP$: C, 45.82; H, 4.33; N, 23.38. Found: C, 45.76, H, 4.30; N, 23.34.

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References

1) Berlinck R. G. S., *Nat. Prod. Rep.*, **19**, 617–649 (2002).
2) Patti A. D., Kumar N. V., Kokke W. C., Bean M. F., Freyer A. J., De Brosse C., Mai S., Truneh A., Faulkner D. J., Carte B., Breen A. L., Hertzberg R. P., Johnson R. K., Westley J. W., Potts B. C. M., *J. Org. Chem.*, **60**, 1182–1188 (1995).
3) Schmidtchen F. P., Berger M., *Chem. Rev.*, **97**, 1609–1646 (1997).
4) Rogister F., Laeckmann D., Plasman P. O., Van Eylen F., Ghyoot M., Maggetto C., Liégeois J., Géczy J., Herchuelz A., Delarge J., Masereel B., Herchuelz A., *Eur. J. Med. Chem.*, **36**, 597–614 (2001).
5) Maillard M. C., Perlmn M. E., Amitay O., Baxter D., Berlove D., Connaughton S., Fischer J. B., Guo J. Q., Hu L. Y., Mcburney R. N., Nagy P. I., Subbarao K., Yost E. A., Zhang L., Durant G. J., *J. Med. Chem.*, **41**, 3048–3061 (1998).
6) Reddy L. N., Connaughton S., Daly D., Fischer J. B., Goldin S. M., Hu L. Y., Subbarao K., Durant G. J., *Bioorg. Med. Chem. Lett.*, **5**, 2259–2262 (1995).
7) Hu L. Y., Durant G. J., Guo J. Q., Maillard M., Wolcott T., Berlove D., "214th American Chemical Society Meeting," Las Vegas, NV, p. MEDI 32, 1997.
8) Gilman A. G., Goodman L. S., "The Pharmacological Basis of Therapeutics," 6th ed., Macmillan, New York, 1980, pp. 380–390.
9) Jadhav P. K., Woerner F. J., Lam P. Y., Hodge C. N., Eyermann C. J., Man H., Deneker W. F., Bacheler L. T., Rayner M. M., Meck J. L., Erickson viitanen S., Jackson D. A., Calabrese J. C., Schadt M., Change C. H., *J. Med. Chem.*, **41**, 1446–1455 (1998).
10) Kim C. U., Lew W., Williams M. A., Wu H., Zhang L., Chen X., Escarpe P. A., Mendel D. B., Lever W. G., Stevens R., *J. Med. Chem.*, **41**, 2451–2460 (1998).
11) Melero C. P., Medarde M., Feliciano A. S., *Molecules*, **5**, 51–81 (2000).
12) Linney I. D., Buck I. M., Harper E. A., Kalindjian S. B., Pether M. J., Shankley N. P., Watt G. F., Wright P. T., *J. Med. Chem.*, **43**, 2362–2370 (2000).
13) Wagman A. S., Nuss J. M., *Curr. Pharm. Des.*, **7**, 417–450 (2001).
14) Valasani K. R., Alahari J. R., Sanapalli S. R., Chamarthi N. R., Pasupuleti V. R., Sunil K.G., *Eur. J. Med. Chem.*, **45**, 203–209 (2010).
15) Quin L. D., Verkade J. G., "Phosphorus-31 NMR Spectral Properties Compound Characterization and Structural Analysis," VCH Publishers, New York, 1994.
16) Mizushima Y., Kobayashi M., *J. Pharm. Pharmacol.*, **20**, 169–173 (1968).
17) Sadique J., Al-Rqobahs W. A., Bughaith M. F., EL Gindi A. R., *Fitoterapia*, **60**, 525–532 (1989).
18) Winter C. A., Risly E. A., Nuss G. W., *Proc. Soc. Exp. Biol. Med.*, **111**, 544–547 (1962).
19) Goldstein S. A., Shemano L., Demer R., Beiler J. M., *Arch. Int. Pharmacodyn. Ther.*, **165**, 294–301 (1976).
20) Armarego W. L. F., Perrin D. D., "Purification of Laboratory Chemicals," 4th ed., Butterworth Heinemann, Oxford, 1997.