Synthesis, spectral characterization and biological evaluation of phosphorylated derivatives of galanthamine

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Original article

Synthesis, spectral characterization and biological evaluation of phosphorylated derivatives of galanthamine

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ABSTRACT

A series of novel phosphorylated derivatives of galanthamine **6–11** and **12–17** were synthesized in two step process with high yields. In the first step galanthamine **1** was reacted with bis (2-chloroethyl) phosphoramidic dichloride **2**/4-nitrophenyl phosphorodichloridate **3** in presence of triethylamine (TEA) in dry tetrahydrofuran (THF) yielded the intermediates **4/5**. They were further reacted with various compounds like 2-aminoethanol, ethyleneglycol, ethylenediamine, 2-aminoethanethiol, 2-hydroxy ethanethiol, monopotassium dihydrogenphosphate to obtain the title compounds **6–11** and **12–17**. The title compounds showed promising antimicrobial, antioxidant activities and was greatly influenced by the presence of different bioactive groups.

1. Introduction

Galanthamine commercially available Reminyl® [1] is an approved AChE inhibitor in USA by the FDA, and in Europe by the European registration bureau for the symptomatic treatment of Alzheimer's disease (AD) [2]. Galanthamine, the naturally occurring alkaloid isolated from the bulbs of different species of the *Amaryllidaceae* family [3] and the Caucasian snow-drop (*Galanthus woronowii*). Its structure was determined on the basis of spectral properties. Galanthamine has been found to possess potent, wide spread spectrum of activities like reversible competitive anti-cholinesterase inhibitor [4], anti-anesthetic [5], analeptic [6], analgesic [7] and antioxidant property [8]. Alzheimer's disease [9–11] is a progressive neurodegenerative disorder and one of the most common causes of mental deterioration in the elderly people. Other major and the most developed class of drugs approved for AD therapy, such as tacrine (Cognex®) [12], donepezil (Aricept®) [13,14], rivastigmine (Exelon®) [15].

These potent biological activities [16] has stimulated great interest in the synthesis of such compounds for extensive studies related to their biological activities. Previously galanthamine sulfur analogs are devoid of noticeable AChE inhibitory activity. In view of these observations and applications of galanthamine, we have focused on the synthesis of a series of novel phosphorylated derivatives of galanthamine by incorporating the bioactive groups attached to phosphorus atom, which fortunately resulted with noticeable antioxidant and antimicrobial activities. The oxidative stress of galanthamine derivatives was assessed by estimating the activities of lipid peroxidation (LPO), reduced glutathione content (GSH), superoxide dismutase (SOD) and catalase (CAT). The obtained results were compared with the natural antioxidants like Vitamin E and Vitamin C. Antimicrobial activity of phosphorylated derivatives of galanthamine have been investigated against Gram-positive bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, and Gram-negative bacteria: *Escherichia coli* and *Klebsiella pneumoniae*. The structures of all the newly synthesized compounds have been established by elemental analyses and spectral data (IR, ^{1}H, ^{13}C, ^{31}P NMR and mass). All the compounds have been screened for their antioxidant and antimicrobial activities.

2. Chemistry

The synthesis of phosphorylated derivatives of galanthamine was carried out by the following process. The hydrobromide salt of galanthamine (Nivalin) was neutralized with ammonium hydroxide to produce pure galanthamine **1** in 89% yield. Galanthamine **1** was reacted with bis (2-chloroethyl) phosphoramidic

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dichloride **2** in the presence of triethylamine (TEA) in dry tetrahydrofuran (THF) at −10 °C for 2 h. The reaction was monitored by thin layer chromatography (TLC). Formation of an intermediate **4** was ascertained after 2 h. Further, the intermediate **4** was reacted with various compounds namely 2-aminoethanol, ethyleneglycol, ethylenediamine, 2-aminoethanethiol, 2-hydroxyethanethiol, monopotassium dihydrogenphosphate to obtain **6–11** in high yields (68–82%). The same protocol was employed for the synthesis of **12–17** by reacting galanthamine **1** with 4-nitrophenyl phosphorodichloridate **3** under same reaction conditions to obtain the intermediate **5**. Further, the intermediate **5** was reacted with the same compounds as shown in Scheme 1 to obtain **12–17** in high yields (68–80%).

3. Pharmacology

The antimicrobial activity of the phosphorylated derivatives of galanthamine has been investigated and their results are presented in Table 1. Antimicrobial activity has been evaluated against Gram-positive bacteria: *B. subtilis*, *S. aureus,* and Gram-negative bacteria: *E. coli*, *K. pneumoniae.* The antioxidant activity was evaluated by Lipid Peroxidation, Reduced Glutathione Content, Superoxide Dismutase and Catalase and their results are presented in Table 2. Chloramphenicol was measured as a standard for antimicrobial activity and Vitamine E and Vitamine C were measured as standards for antioxidant activity respectively.

4. Results and discussion

The chemical structures of all the title compounds **6** –**17** were characterized by IR, ^{1}H, ^{13}C, ^{31}P NMR and APCI-MS studies and their data are presented in the experimental section. Characteristic IR stretching absorptions were observed in the regions 1212–1231 cm^{-1}, 3375–3385 cm^{-1} and 3412–3416 cm^{-1} for P═O [17], N–H [18], O–H [19] respectively. In the ^{1}H NMR spectra of compounds **6–17,** the chemical shifts of aromatic hydrogens of the phenyl ring appeared as doublets in the region δ 6.62–6.70 [20]. The N–H hydrogen resonated as a broad singlet in the region δ 4.72–4.86 and P–O–H protons were resonated as singlets at δ 7.27–7.35. The allylic proton H6 was observed as a broad triplet because H7 and H5β protons are in the same chemical environment with respect to H6 [2]. The H12 protons were observed as doublets in the region 3.19–3.33 and 3.67–3.75 respectively. The H9 proton was observed as a multplet in the region 1.50–1.78. In ^{13}C NMR chemical shifts for compounds **6–17** were observed in their expected regions. ^{31}P NMR signals were observed in the region δ −7.11 to −19.39 ppm [19,21].

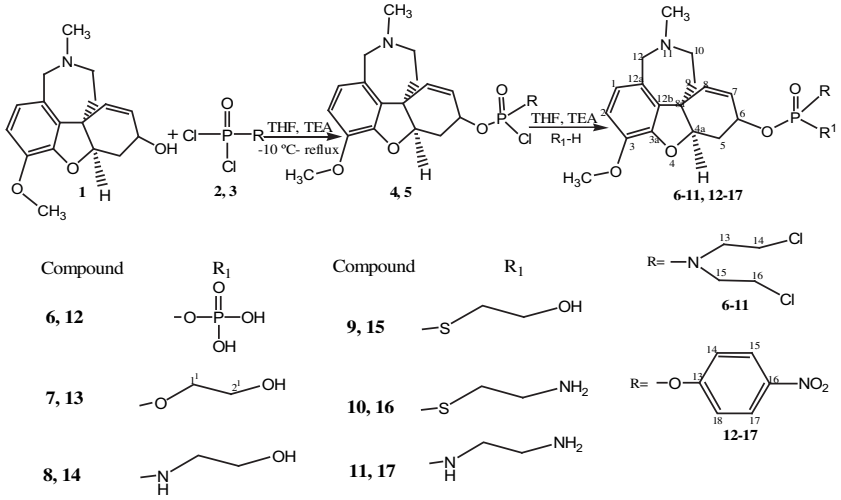
As shown in Table 4 most of the compounds showed high activities for CAT, SOD, LPO and GSH inhibition compared to galanthamine. The potency of these enzymes inhibition was mainly influenced by the fragments attached to the phosphorylated galanthamine. The fragments bis (2-chloroethyl) amine and 2-hydroxyethanethiol linked to phosphorus led to a great change in CAT, SOD, LPO and GSH inhibitory potency (*i.e*-**9**). Bis (2-chloro ethyl) amine and 2-hydroxyethanethiol moieties were found to be the most potent fragments for the enzymes inhibition. N and S chains of phosphorus atom in compound **9** showed high inhibitory potency when compared with other moieties.

Attachment of different moieties to phosphorus atom could affect the ability of the molecule to interact with the peripheral and active cites of the enzymes simultaneously and there by influence the CAT, SOD, LPO and GSH inhibitory potency. The bis (2-chloro ethyl) amino fragment series (i.e **6–11**) compounds **6**, **8** and **9** showed high inhibitory potency for CAT, SOD, LPO and GSH when compared to **7** and **10.** However, the same trend was not observed in the 4-nitrophenoxy series (*i.e* **12–17**) compounds **14** and **15** showed high inhibitory potency of GSH and SOD respectively when compared to compounds **12**, **13**, **16** and **17**.

The significant loss in potency was observed when 4-nitrophenoxy moiety as R fragment in series attached to phosphorus atom with different R_1 moieties when compared to bis (2-chloro ethyl) amino fragment as R series with different R_1 moieties, suggested that the two free chlorine atoms of aliphatic chain are highly reactive when compared with the nitro group attached to an aromatic ring, which led to a slight drop in enzyme inhibitory potency of derivatives containing 4-nitrophenoxy moiety.

Replacement of the terminal R_1 fragments in the phosphorylated derivative series **6–11** and **12–17** shows significant inhibitory potency when phosphorus atom directly attached with N and S aliphatic chains with terminal O–H group when compared with O, S, N directly attached to phosphorus atom without having terminal O–H group.

The phosphorylated derivative **9** showed significant MIC against all represented micro organisms similarly derivative **8** exhibited significant MIC against BS, SA, KP micro organisms. Minimum Inhibitory Concentration (MIC) in microbiology is the lowest concentration of an antimicrobial that will inhibit the visible growth of microorganism after over night incubation. These results



Scheme 1. Synthesis of title compounds **6–17**.

Table 1
Minimum inhibitory concentrations (MIC in μg/ml) of the compounds and galanthamine against the respective microorganisms.

	Gal	Chlo	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
BS	0.18	0.15	0.16	0.28	0.11	0.13	0.17	0.12	0.19	0.25	0.21	0.10	0.09	0.15
SA	1.0	0.20	0.38	0.42	0.29	0.21	0.55	0.49	0.76	1.09	0.81	0.29	0.22	0.75
KP	0.62	0.13	0.48	0.57	0.15	0.59	0.51	0.33	0.28	0.39	0.42	0.36	0.14	0.38
EC	0.7	0.25	0.42	0.50	0.35	0.26	0.28	0.55	0.46	0.67	0.44	0.34	0.23	0.62

Abbreviations: Gal – galanthamine Bs – *B. subtilis*; Sa – *Staphylococcus aureus*; Kp – *Klebsiella pneumoniae*; Ec – *E. coli*. Chlo – chloramphenicol.

clearly indicate that phosphorus atom directly attached to sulfur and nitrogen with their aliphatic chains ending with OH and Cl were found to possess promising MIC when compared with other moieties at their ends. The derivatives **6**, **15** and **16** also showed high MIC values for BS and SA respectively, which indicates that their activity was enhanced by the presence of terminal –OH group.

5. Conclusion

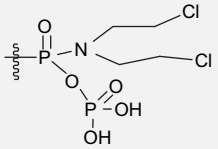
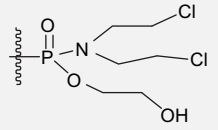
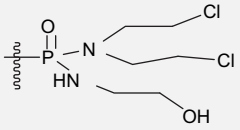
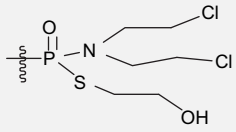
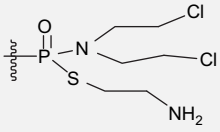
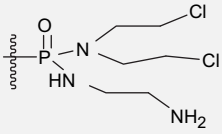
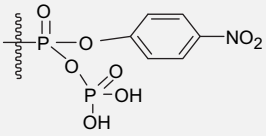
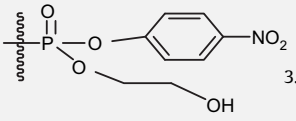
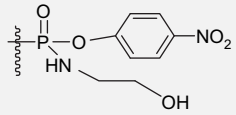
In summary, a series of novel phosphorylated galanthamine derivatives were synthesized and evaluated their antimicrobial and antioxidant properties. Structure activity studies showed that the antioxidant and antimicrobial potency was mainly influenced by the functional groups at the end of the aliphatic chain, as well as the nature of atom attached directly to phosphorus atom and the two different types of R fragments attached to phosphorus atom, which provided additional sites of interactions between the inhibitor and the enzyme, which constitutes a key element for enhanced affinity. The results suggest that the compounds **6**, **8**, **9**, **14**, **15** showed promising antioxidant activities with different R_1 fragments containing terminal –OH groups and also with different R fragments (i.e.) bis (2-chloroethyl) amino and 4-nitrophenoxy moieties. Majority of the derivatives (6–17) exhibited higher antimicrobial activity and lower MIC values when compared to galanthamine and chloramphenicol. The compounds **6**, **8**, **9**, **15**, **16** exhibited higher antimicrobial activities when compared with other derivatives. The results provided a foundation for future design and development of antioxidant, antimicrobial compounds and also more potent drugs for AD.

6. Experimental protocols

6.1. Chemistry

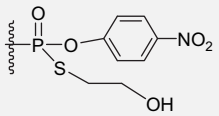
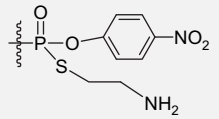
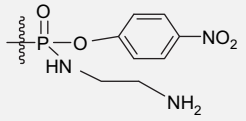
Chemicals were procured from Sigma–Aldrich, Merck and Lancaster, and were used as such without further purification. All solvents used for spectroscopic and other physical studies were reagent grade and were further purified by literature methods [22]. Melting points (m.p.) were determined using a calibrated thermometer by Guna Digital Melting Point apparatus. They expressed in degrees centigrade (°C) and are uncorrected. Infrared Spectra (IR) were obtained on a Perkin-Elmer Model 281-B spectrophotometer. Samples were analyzed as potassium bromide (KBr) disks. Absorptions were reported in wave numbers (cm^{-1}). ^1H and ^{13}C NMR spectra were recorded as solutions in DMSO-d_6 on a Bruker AMX 400 MHz spectrometer operating at 400 MHz for ^1H, 100 MHz for ^{13}C and 161.9 MHz for ^{31}P NMR. The ^1H and ^{13}C chemical shifts were expressed in parts per million (ppm) with reference to tetramethylsilane (TMS) and ^{31}P chemical shifts to 85% H_3PO_4. Optical rotations (in degrees, °) were recorded in methanol on a Perkin-Elmer Model 241 polarimeter at the sodium D line. APCI mass spectra were recorded on a Jeol SX 102 DA/600 Mass spectrometer.

Table 2
EC_{50} values for galanthamine and its derivatives along with standard antioxidants.

S.No	Compound	CAT (μg/mL)	LOP (μg/mL)	SOD (μg/mL)	GSH (μg/mL)
Galanthamine	–	5.06	4.93	3.84	4.21
6		2.18	1.98	1.26	2.68
7		2.21	2.87	1.98	2.02
8		1.52	1.42	1.28	1.03
9		1.67	1.33	1.62	1.11
10		2.66	2.91	2.31	2.66
11		2.73	2.88	2.77	2.43
12		2.92	3.33	2.69	2.15
13		3.28	3.17	3.28	2.82
14		4.94	2.81	2.57	1.08

(*continued on next page*)

Table 2 (*continued*)

S.No	Compound	CAT (μg/mL)	LOP (μg/mL)	SOD (μg/mL)	GSH (μg/mL)
15		4.25	2.64	1.55	1.49
16		2.03	1.86	1.74	1.56
17		1.81	1.99	1.91	1.89
Vitamin E	–	1.48	1.22	1.03	0.99
Vitamin C	Ascorbic acid	1.37	1.02	0.99	0.98

Abbreviations: LPO – lipid peroxidation; GSH – reduced glutathione content; SOD – superoxide dismutase; CAT – catalase.

Elemental analyses were performed by Central Drug Research Institute, Lucknow, INDIA.

*6.1.1. Procedure for the preparation of galanthamine **1***

Galanthamine hydrobromide (6.80 g, 18.5 mmol) was dissolved in water (400 mL), the solution was cooled to 0 °C and ammonium hydroxide (70 mL) was slowly added. The reaction mixture was stirred for 30 min at 0 °C, and then for 2 h at ambient temperature. Ether (200 mL) was added, followed by addition of solid NaCl results in formation of two layers. The aqueous layer was extracted with ether (3 × 200 mL). The extract was washed with brine solution, dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to get crude galanthamine free base and then purified by silica gel column chromatography eluting with acetone: methanol (95:5) to afford pure galanthamine **1** as white crystals (4.52 g, 85% yield), m.p. $[\alpha]_D^{25}$ f114.4°, IR (KBr); (O–H) 3400 cm^{-1}.

Table 3
Antimicrobial activity of the phosphorylated derivatives of galanthamine **6**–**17**.

Samples	Concentration (mg/mL)	Inhibition zone (in mm)			
		BS	SA	KP	EC
Galanthamine	1.0	1.65	1.39	1.83	1.47
	2.0	2.25	2.66	2.21	2.02
6	1.0	2.89	1.75	2.08	1.88
	2.0	4.49	4.65	3.68	4.08
7	1.0	2.36	2.43	2.69	1.65
	2.0	4.89	5.02	4.76	3.18
8	1.0	1.75	1.69	2.78	1.72
	2.0	3.46	4.78	4.45	3.86
9	1.0	1.53	1.47	3.03	1.55
	2.0	3.01	3.09	6.46	2.21
10	1.0	1.90	2.38	2.35	1.59
	2.0	4.82	4.92	4.65	2.43
11	1.0	1.84	1.67	2.50	2.12
	2.0	3.90	3.51	4.80	4.90
12	1.0	1.63	2.09	2.73	2.22
	2.0	3.78	4.56	5.45	4.97
13	1.0	1.57	1.42	1.72	2.05
	2.0	3.71	4.27	4.96	5.01
14	1.0	1.81	1.53	2.61	1.70
	2.0	4.16	3.94	5.44	4.44
15	1.0	1.62	1.82	1.93	1.26
	2.0	3.06	4.91	5.01	4.38
16	1.0	1.14	1.32	1.57	1.76
	2.0	3.03	4.47	6.33	4.84
17	1.0	1.53	2.42	2.33	1.62
	2.0	4.91	5.09	6.11	3.73
Chloramphenicol	1.0	1.02	2.67	3.62	2.02
	2.0	2.99	3.08	4.02	3.27

Abbreviations: Bs – *B. subtilis*; Sa – *Staphylococcus aureus*; Kp – *Klebsiella pneumoniae*; Ec – *E. coli*.

Table 4
Antioxidants in brain homogenates of young rats using galanthamine derivatives **6**–**17**.

Parameters	LPO(μg/mL)	GSH(μg/mL)	SOD(μg/mL)	CAT(μg/mL)
Galanthamine	70.68 ± 1.46	4.69 ± 0.25	29.37 ± 0.62	283.44 ± 6.32
6	74.86 ± 2.32	4.72 ± 0.74	28.14 ± 0.35	278.33 ± 5.65
7	68.24 ± 3.69	4.67 ± 0.92	29.02 ± 0.65	291.27 ± 10.52
8	72.32 ± 1.76	4.73 ± 0.87	29.86 ± 0.97	287.59 ± 7.67
9	71.23 ± 2.38	4.92 ± 1.73	29.55 ± 1.16	285.32 ± 4.76
10	72.39 ± 2.09	5.02 ± 1.44	30.55 ± 1.43	285.43 ± 5.27
11	72.46 ± 1.23	4.84 ± 1.91	30.29 ± 1.76	285.48 ± 4.39
12	72.17 ± 1.94	4.22 ± 1.83	29.55 ± 1.42	287.38 ± 4.88
13	72.11 ± 1.79	5.21 ± 1.62	29.41 ± 1.39	286.43 ± 4.62
14	72.48 ± 1.68	4.72 ± 1.90	29.37 ± 1.47	284.32 ± 4.21
15	72.77 ± 1.47	4.77 ± 1.64	29.69 ± 1.86	288.58 ± 4.47
16	75.49 ± 1.36	5.08 ± 1.72	29.85 ± 1.71	292.83 ± 4.89
17	75.92 ± 1.28	5.06 ± 1.88	29.73 ± 1.66	295.92 ± 4.75
Ascorbic acid	88.06 ± 1.28	31.34 ± 0.74	Unaffected	385.46 ± 5.25
Vitamin E	82.78 ± 1.43	43.62 ± 1.54	32.30 ± 1.04	403.27 ± 4.76

Abbreviations: LPO – lipid peroxidation; GSH – reduced glutathione content; SOD – superoxide dismutase; CAT – catalase.

*6.1.2. Procedure for the preparation of compound **6***

To a stirred solution of galanthamine (**1**, 0.200 g, 0.876 mmol) in dry tetrahydrofuran (THF) (17 mL) was added bis (2-chloroethyl) phosphoramidic dichloride (**2**, 0.2269 g, 0.876 mmol) at −10 °C in the presence of triethylamine (0.0886 g, 0.876 mmol) (TEA). After completion of the addition, the reaction mixture was stirred for 2 h at 0 °C. The reaction progress was monitored by thin layer chromatography (TLC) acetone: methanol (95: 5). After completion of the reaction, it was filtered to remove triethylamine hydrochloride. The filtrate was reacted with mono potassium dihydrogen phosphate (0.1191 g, 0.876 mmol) in THF at reflux temperature for 12 h. The progress of the reaction was monitored by TLC acetone: methanol (95: 5). After completion of the reaction, solvent was removed in a rota-evaporator to obtain crude product. It was purified by silica gel column chromatography eluting with acetone: methanol (95:5) mixture to afford the title compound, 2-dihydrogenphosphato (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4a*H*-4-oxa-11-azacyclohepta [*de*] fluoren-6-yl) bis (2-chloroethyl) amino phosphate **6**. The same experimental procedure was adopted for the preparation of the remaining title compounds **7**–**17**.

*6.1.2.1. Synthesis of 2-dihydrogenphosphato (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de] fluoren-6-yl) bis (2-chloroethyl) amino phosphate **6***. Yield: 78%; m.p.151–153 °C; $[\alpha]_D^{25}$ −117.2°; IR (KBr) 3415 (O–H), 1228 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.50 –1.59 (m, 2H, H9), 2.39 (s, 3H, NMe), 2.51–2.60 (m, 1H, H5α), 2.62–2.78(m, 1H, H5β), 2.99–3.10 (m, 2H, H10), 3.23(d, 1H, J = 13.6 Hz, H12α), 3.26 (t, 4H, J = 13.6 Hz, H14, H16), 3.62 (t, 4H, J = 13.6 Hz, H13, H15), 3.67(d, 1H, J = 14.9 Hz, H12β), 3.82 (s, 3H, OMe), 4.07–4.11 (m, 1H, H4a), 4.62 (br t, 1H, J = 6.0 Hz, H6), 6.00 (d, 1H, J = 10.4 Hz, H7), 6.06 (d, 1H, J = 10.4 Hz, H8), 6.62 (d, 1H, J = 8.4 Hz, H1), 6.66 (d, 1H, J = 8.0 Hz, H2), 7.27 (s, 2H, P–OH) ppm; ^{13}C NMR (DMSO-d_6) δ 30.1(C-9), 33.9(C-5), 41.8(C-14), 42.2(NMe), 48.3(C-13), 55.9(C-8a), 56.1(OMe), 56.0(C-10),

60.7(C-12), 62.2(C-6), 88.8(C-4a), 111.4(C-2), 122.2(C-1), 127.8(C-12a), 127.6 (C-7), 129.4(C-8), 133.2(C-12b), 144.2(C-3), 145.9(C-3a); ^{31}P (DMSO-d_6) δ −7.11, −19.39; APCI-MS m/z (%) 571[$MH^{+\bullet}$]; Anal. Calcd. $C_{21}H_{30}Cl_2N_2O_8P_2$: C 44.15, H 5.29, N 4.90; Found: C 44.11, H 5.27, N 4.89.

6.1.2.2. Synthesis of 2-hydroxyethoxy (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de] fluoren-6-yl) bis (2-chloroethyl) amino phosphate ***7***. Yield: 82%; m.p.172–174 °C; $[\alpha]_D^{25}$ −117.8°; IR (KBr) 3416 (O–H), 1231 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.60 –1.66(m, 2H, H9), 2.40 (s, 3H, NMe), 2.70–2.79 (m, IH, H5α), 2.80–2.86 (m, 1H, H5β), 3.14–3.20 (m, 2H, H10), 3.26(d, 1H, J = 13.9 Hz, H12α), 3.32 (t, 4H, J = 13.6 Hz, H14, H16), 3.61 (t, 4H, J = 13.6 Hz, H13, H15), 3.70(d, 1H, J = 15.2 Hz, H12β), 3.80 (s, 3H, OMe), 3.85–3.88 (m, 2H, H2′), 3.99 (t, 2H, J = 15.2 Hz, H1′), 4.09–4.14 (m, 1H, H4a), 4.62 (br t, 1H, J = 5.5 Hz, H6), 5.02 (s, 1H, OH)6.03 (d, 1H, J = 10.4 Hz, H7), 6.05 (d, 1H, J = 10.4 Hz, H8), 6.64 (d, 1H, J = 8.4 Hz, H1), 6.67 (d, 1H, J = 8.0 Hz, H2) ppm; ^{13}C NMR (DMSO-d_6) δ 28.4(C-9), 34.7(C-5), 41.5(NMe), 43.5(C-14), 46.9(C-13), 54.1(OMe), 55.4(C-8a), 56.7(C-10), 61.3(C-2′), 63.2(C-12), 67.3(C-1′), 74.2(C-6), 86.1(C-4a), 114.8(C-2), 119.4(C-1), 126.4(C-12a), 127.3(C-7), 131.4(C-8), 134.9(C-12b), 144.8(C-3), 146.5(C-3a); ^{31}P (DMSO-d_6) δ −14.59; APCI-MS m/z (%) 535[$MH^{+\bullet}$] $C_{23}H_{33}Cl_2N_2O_6P$: C 51.60, H 6.21, N 5.23; Found: C 51.55, H 6.18, N 5.22.

6.1.2.3. Synthesis of 2-hydroxy ethylamino (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de] fluoren-6-yl) bis (2-chloroethyl) amino phosphate ***8***. Yield: 76%; m.p.180–182 °C; $[\alpha]_D^{25}$ −125.1°; IR (KBr) 3412 (O–H), 3385 (N–H), 1228 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.52–1.57 (m, 2H, H9), 2.38 (s, 3H, NMe), 2.70–2.81 (m, 1H, H5α), 2.84–2.89 (m, 1H, H5β), 2.96–3.08 (m, 2H, H10), 3.19 (s, 1H, NH), 3.24 (d, 1H, J = 13.5 Hz, H12α), 3.28 (t, 4H, J = 13.6 Hz, H14, H16), 3.49 (q, 2H, H1′), 3.48–3.54 (m, 2H, H2′), 3.60 (t, 4H, J = 13.6 Hz, H13, H15), 3.75(br d, 1H, J = 15.4 Hz, H12β), 3.84 (s, 3H, OMe), 4.07–4.12 (m, 1H, H4a), 4.65 (br t, 1H, J = 6.6 Hz, H6), 6.01 (dd, 1H, J = 10.4 Hz, H7), 6.07 (d, 1H, J = 10.4 Hz, H8), 6.62 (d, 1H, J = 8.4 Hz, H1), 6.64 (d, 1H, J = 8.0 Hz, H2), ppm; ^{13}C NMR (DMSO-d_6) δ 28.4(C-9), 32.3(C-1′), 34.7(C-5), 42.9(C-14), 43.5(NMe), 47.2(C-13), 56.2(OMe), 55.4(C-8a), 56.7(C-10), 61.3(C-2′), 65.1(C-12), 74.5(C-6), 86.5(C-4a), 114.3(C-2), 119.2(C-1), 126.3(C-12a), 127.1(C-7), 131.5(C-8), 134.8(C-12b), 144.1(C-3), 146.5(C-3a); ^{31}P (DMSO-d_6) δ −14.56; APCI-MS m/z (%) 534[$MH^{+\bullet}$]; Anal. Calcd. $C_{23}H_{34}Cl_2N_3O_5P$: C 51.69, H 6.41, N 7.86; Found: C 51.65, H 6.39, N 7.83.

6.1.2.4. Synthesis of 2-hydroxy ethylmercapto (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de] fluoren-6-yl) bis (2-chloroethyl) amino phosphate ***9***. Yield: 72%; m.p.147–149 °C; $[\alpha]_D^{25}$ −127.6°; IR (KBr) 3411 (O–H), 1230 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.56–1.62 (m, 2H, H9), 2.38 (s, 3H, NMe), 2.72–2.81 (m, 1H, H5α), 2.71 (t, 2H, J = 15.6 Hz, H1′), 3.02–3.09 (m, 1H, H5β), 3.12–3.17 (m, 2H, H10), 3.19 (d, 1H, J = 13.6 Hz, H12α), 3.28 (t, 4H, J = 13.6 Hz, H14, H16), 3.60 (t, 4H, J = 13.6 Hz, H13, H15), 3.71(d, 1H, J = 15.2 Hz, H12β), 3.76 (s, 3H, OMe), 3.81–3.87 (m, 2H, H2′), 4.09–4.15 (m, 1H, H4a), 4.70 (br t, 1H, J = 5.9 Hz, H6), 5.04 (s, 1H, OH), 6.02(dd, 1H, J = 10.4 Hz, H7), 6.07 (d, 1H, J = 10.4 Hz, H8), 6.60 (d, 1H, J = 8.4 Hz, H1), 6.63(d, 1H, J = 8.0 Hz, H2), ppm; ^{13}C NMR (DMSO-d_6) δ 28.4(C-1′), 32.7(C-9), 35.9(C-5), 41.5(C-14), 42.9(NMe), 46.3(C-13), 54.3(C-8a), 56.5(C-10), 60.5(OMe), 61.3(C-2′), 66.2(C-12), 74.6(C-6), 86.3(C-4a), 113.4(C-2), 119.3(C-1), 126.5(C-12a), 127.4(C-7), 131.4(C-8), 134.8(C-12b), 144.2(C-3), 145.3(C-3a); ^{31}P (DMSO-d_6) δ −7.82; APCI-MS m/z (%) 551[$MH^{+\bullet}$].

6.1.2.5. Synthesis of 2-amino ethylmercapto (3-amino-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de] fluoren-6-yl) bis (2-chloroethyl) amino phosphate ***10***. Yield: 68%; m.p.148–150 °C; $[\alpha]_D^{25}$ −125.0°; IR (KBr) 3385 (N–H), 1231 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.59–1.65 (m, 2H, H9), 2.39 (s, 3H, NMe), 2.69–2.75 (m, 1H, H5α), 2.73 (t, 2H, J = 15.6 Hz, H1′), 2.98–3.04 (m, 2H, H2′), 2.91–3.02(m, 1H, H5β), 3.10–3.14 (m, 2H, H10), 3.16 (d, 1H, J = 13.2 Hz, H12α), 3.19 (s, 2H, NH_2), 3.28 (t, 4H, J = 13.6 Hz, H14, H16), 3.64 (t, 4H, J = 13.6 Hz, H13, H15), 3.71 (d, 1H, J = 15.2 Hz, H12β), 3.84 (s, 3H, OMe), 4.07–4.11 (m, 1H, H4a), 4.61 (br t, 1H, J = 6.2 Hz, H6), 6.01 (dd, 1H, J = 10.4 Hz, H7), 6.07 (d, 1H, J = 10.4 Hz, H8), 6.63(d, 1H, J = 8.4 Hz, H1), 6.67 (d, 1H, J = 8.0 Hz, H2), ppm; ^{13}C NMR (DMSO-d_6) δ 28.3(C-1′), 31.2(C-9), 34.7(C-5), 37.7(C-2′), 41.1(C-14), 43.1(NMe), 47.1(C-13), 54.9(C-8a), 56.2(OMe), 56.7(C-10), 61.3(C-12), 62.5(C-6), 86.9(C-4a), 114.8(C-2), 119.3(C-1), 126.8(C-7), 127.4(C-12a), 131.3(C-8), 134.2(C-12b), 144.4(C-3), 146.5(C-3a); ^{31}P (DMSO-d_6) δ −12.46; APCI-MS m/z (%) 550[$MH^{+\bullet}$].

6.1.2.6. Synthesis of 2-aminoethylamino (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de] fluoren-6-yl) bis (2-chloroethyl) amino phosphate ***11***. Yield: 78%; m.p.152–154 °C; $[\alpha]_D^{25}$ −119.2°; IR (KBr) 3375 (N–H), 1228 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.56–1.65 (m, 2H, H9), 2.40 (s, 3H, NMe), 2.69–2.80 (m, 1H, H5α), 3.02–3.10 (m, 1H, H5β), 3.12–3.17 (m, 2H, H1′), 3.19 (s, 3H, NH), 3.20–3.23 (m, 2H, H2′), 3.25–3.29 (m, 2H, H10), 3.28 (t, 4H, J = 13.6 Hz, H14, H16), 3.32(d, 1H, J = 13.7 Hz, H12α), 3.60 (t, 4H, J = 13.6 Hz, H13, H15), 3.71(d, 1H, J = 15.2 Hz, H12β), 3.84 (s, 3H, OMe), 4.04–4.08 (m, 1H, H4a), 4.63(br t, 1H, J = 5.7 Hz, H6), 5.05 (s, 1H, OH), 6.05 (dd, 1H, J = 10.4 Hz, H7), 6.08 (d, 1H, J = 10.4 Hz, H8), 6.60 (d, 1H, J = 8.4 Hz, H1), 6.64 (d, 1H, J = 8.0 Hz, H2) ppm; ^{13}C NMR (DMSO-d_6) δ 28.2(C-1′), 32.1(C-9), 33.2(C-5), 41.3(C-14), 42.9(C-2′), 44.3(NMe), 46.9(C-13), 55.2(C-8a), 56.2(C-10), 56.7(OMe), 61.2(C-12), 64.2(C-6), 86.7(C-4a), 114.7(C-2), 119.5(C-1), 126.6(C-7) 127.2(C-12a) 131.4(C-8), 134.9(C-12b), 144.3(C-3), 145.1(C-3a); ^{31}P (DMSO-d_6) δ −14.89; APCI-MS m/z (%) 533[$MH^{+\bullet}$].

6.1.2.7. Synthesis of 2-dihydrogenphosphato (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de] fluoren-6-yl) (4-nitrophenyl) phosphate ***12***. Yield: 75%; m.p.161–163 °C; $[\alpha]_D^{25}$ −116.9°; IR (KBr) 3424 (O–H), 1230 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.58–1.69 (m, 2H, H9), 2.39 (s, 3H, NMe), 2.66–2.80 (m, 1H, H5α), 2.89–2.94 (m, 1H, H5β), 3.22–3.29 (m, 2H, H10), 3.34(d, 1H, J = 13.4 Hz, H12α), 3.71 (d, 1H, J = 15.2 Hz, H12β), 3.80 (s, 3H, OMe), 4.09–4.13 (m, 1H, H4a),4.52 (br t, 1H, J = 6.5 Hz, H6), 6.04 (dd, 1H, J = 10.4 Hz, H7), 6.06 (d, 1H, J = 10.4 Hz, H8), 6.64 (d, 1H, J = 8.4 Hz, H1), 6.67 (d, 1H, J = 8.0 Hz, H2), 7.35 (s, 2H, POH) 7.38 (d, 2H, J = 8.0 Hz, H14, H18), 8.10 (d, 2H, J = 7.9 Hz, H15, H17), ppm; ^{13}C NMR (DMSO-d_6) δ 31.3(C-9), 34.7(C-5), 42.9(NMe), 55.4(C-8a), 56.4(C-10), 56.7(OMe),61.3(C-12), 69.8(C-6), 86.1(C-4a), 114.8(C-2), 119.4(C-14 & C-18), 122.7 (C-1), 125.1 (C-15 & C-17),127.4 (C-12a), 131.1 (C-7), 134.2 (C-8), 135.5 (C-12b), 141.2 (C-16), 144.1 (C-3), 146.2 (C-3a), 154.2 (C-13); ^{31}P (DMSO-d_6) δ −7.83, −19.37; APCI-MS m/z (%) 568 [MH^+]; Anal. Calcd. $C_{23}H_{26}N_2O_{11}P_2$: C 48.60, H 4.61, N 4.93; Found: C 48.55, H 4.58, N 4.91.

6.1.2.8. Synthesis of 2-hydroxyethoxy (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de] fluoren-6-yl) (4-nitrophenyl) phosphate ***13***. Yield: 71%; m.p.158–160 °C; $[\alpha]_D^{25}$ −112.4°; IR (KBr) 3416 (O–H), 1231 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.55–1.62 (m, 2H, H9), 2.39 (s, 3H, NMe), 2.70–2.89 (m, 1H, H5α), 2.95–3.08(m, 1H, H5β), 3.15–3.20 (m, 2H, H10), 3.23 (d, 1H, J = 13.6 Hz, H12α), 3.70(d, 1H, J = 15.2 Hz, H12β), 3.72–3.79 (m, 2H, H2′), 3.82 (s, 3H, OMe), 3.95 (t, 2H,, J = 15.2 Hz, H1′), 4.02–4.08 (m, 1H, H4a), 4.61 (br t, 1H, J = 5.4 Hz, H6), 5.06 (s, 1H, OH), 6.00 (dd, 1H, J = 10.4 Hz, H7), 6.06 (d, 1H, J = 10.4 Hz, H8), 6.62 (d, 1H, J = 8.4 Hz, H1), 6.66 (d, 1H, J = 8.0 Hz, H2), 7.40 (d, 2H, J = 8.5 Hz, H14, H18), 8.11 (d, 2H, J = 9.2 Hz, H15, H17), ppm; ^{13}C NMR (DMSO-d_6)

δ 28.8(C-9), 35.9(C-5), 41.1(NMe), 55.2(C-8a), 56.2(C-10), 56.2(OMe), 60.1(C-2′), 61.2(C-12), 67.7(C-1′), 71.9(C-6), 86.0(C-4a), 114.2(C-2), 119.2(C-14 & C-18), 121.8(C-1), 125.0 (C-15 & C-17), 127.2(C-12a), 131.2(C-7), 134.9(C-8), 137.3(C-12b), 141.1(C-16), 144.5(C-3), 146.5(C-3a), 153.6(C-13); ^{31}P (DMSO-d_6) δ −14.35; APCI-MS *m/z* (%) 532 [MH$^{+\bullet}$], Anal. Calcd. $C_{25}H_{29}N_2O_9P$: C 56.39, H 5.49, N 5.26; Found: C 56.32, H 5.46, N 5.24.

6.1.2.9. Synthesis of 2-hydroxy ethylamino (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de]fluoren-6-yl) (4-nitrophenyl) phosphate ***14***. Yield: 68%; m.p.154–156 °C; $[\alpha]_D^{25}$ −118.0°; IR (KBr) 3426 (O–H), 3381 (N–H), 1212 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.51– 1.69 (m, 2H, H9), 2.39 (s, 3H, NMe), 2.60–2.81 (m, 1H, H5α), 2.94–3.01 (m, 1H, H5β), 3.22 (d, 1H, *J* = 13.8 Hz, H12α), 3.26–3.32 (m, 2H, H10), 3.39 (q, 2H, H1′), 3.58–3.66 (m, 2H, H2′), 3.71 (d, 1H, *J* = 15.2 Hz, H12β), 3.82(s, 3H, OMe), 4.10–4.15 (m, 1H, H4a), 4.86 (s, 1H, NH), 4.65 (br t, 1H, *J* = 6.5 Hz, H6), 5.06 (s, 1H, OH), 6.05 (dd, 1H, *J* = 10.4 Hz, H7), 6.08 (d, 1H, *J* = 10.4 Hz, H8), 6.64 (d, 1H, *J* = 8.4 Hz, H1), 6.68 (d, 1H, *J* = 8.0 Hz, H2), 7.38 (d, 2H, *J* = 9.5 Hz, H14, H18), 8.12 (d, 2H, *J* = 8.7 Hz, H15, H17), ppm; ^{13}C NMR (DMSO-d_6) δ 30.1(C-9), 35.7(C-5), 36.8(C-1′), 41.4(NMe), 54.3(C-8a), 55.7(C-10), 56.4(OMe), 62.4(C-12), 63.2(C-2′), 72.1(C-6), 86.2(C-4a), 113.8(C-2), 119.8(C-14 & C-18), 121.6(C-1), 125.9(C-15 & C-17),127.4(C-12a), 131.9(C-7), 134.5(C-8), 137.2(C-12b), 140.9(C-16), 144.2(C-3) 146.3(C-3a), 153.2(C-13); ^{31}P (DMSO-d_6) δ −12.38; APCI-MS *m/z* (%) 531 [MH$^{+\bullet}$]; Anal. Calcd. $C_{25}H_{30}N_3O_8P$: C 56.49, H 5.69, N 7.91; Found: C 56.44, H 5.67, N 7.88.

6.1.2.10. Synthesis of 2-hydroxy ethylmercapto (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de]fluoren-6-yl) (4-nitrophenyl) phosphate ***15***. Yield: Yield: 78%; m.p.168–170 °C; $[\alpha]_D^{25}$ −119.0°; IR (KBr) 3432 (O–H), 1220 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.49– 1.57 (m, 2H, H9), 2.39 (s, 3H, NMe), 2.66 (t, 2H, *J* = 15.4 Hz, H1′), 2.54–2.72 (m, 1H, H5α), 2.85–2.99 (m, 1H, H5β), 3.18–3.22 (m, 2H, H10), 3.25 (d, 1H, *J* = 13.6 Hz, H12α), 3.70 (d, 1H, *J* = 15.2 Hz, H12β), 3.82 (s, 3H, OMe), 4.01–4.09(m, 2H, H2′), 4.10–4.13 (m, 1H, H4a), 4.64 (br t, 1H, *J* = 6.3 Hz, H6), 5.06 (s, 1H, OH), 6.02 (dd, 1H, *J* = 10.4 Hz, H7), 6.08 (d, 1H, *J* = 10.4 Hz, H8), 6.61 (d, 1H, *J* = 8.4 Hz, H1), 6.62 (d, 1H, *J* = 8.0 Hz, H2), 7.39 (d, 2H, *J* = 8.7 Hz, H14, H18), 8.12 (d, 2H, *J* = 8.2 Hz, H15, H17), ppm; ^{13}C NMR (DMSO-d_6) δ 28.1(C-1′), 34.1(C-9), 35.1(C-5), 42.9(NMe), 55.2(C-8a), 55.7(C-10), 56.7(OMe), 61.3(C-12), 71.9(C-6), 86.1(C-4a), 112.3(C-2),119.4(C-14 & C-18), 121.3(C-1), 126.1(C-15 & C-17), 127.5(C-12a), 130.6(C-7), 134.8(C-8), 137.1(C-12b), 141.9(C-16), 143.8(C-3), 146.45(C-3a), 154.1(C-13); ^{31}P (DMSO-d_6) δ 7.82; LCMS *m/z* (%) 548 [MH$^{+\bullet}$].

6.1.2.11. Synthesis of 2-amino ethylmercapto (3-amino-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de]fluoren-6-yl) (4-nitrophenyl) phosphate ***16***. Yield: 68%; m.p.168–170 °C; $[\alpha]_D^{25}$ −117.7°; IR (KBr) 3376 (N–H), 1222 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.54– 1.78 (m, 2H, H9), 2.40 (s, 3H, NMe), 2.68 (t, 2H, *J* = 15.5 Hz, H1′), 2.62–2.83 (m, 1H, H5α), 2.94–3.09(m, 1H, H5β), 3.07–3.15 (m, 2H,H2′), 3.23 (d, 1H, *J* = 13.6 Hz, H12α), 3.27–3.39 (m, 2H, H10), 3.72 (d, 1H, *J* = 15.2 Hz, H12β), 3.80 (s, 3H, OMe), 4.05–4.11 (m, 1H, H4a), 4.86 (s, 2H, NH_2), 4.62 (br t, 1H, *J* = 6.0 Hz, H6), 6.11 (dd, 1H, *J* = 10.4 Hz, H7), 6.14 (d, 1H, *J* = 10.4 Hz, H8), 6.68 (d, 1H, *J* = 8.4 Hz, H1), 6.70 (d, 1H, *J* = 8.0 Hz, H2), 7.40 (d, 2H, *J* = 9.4 Hz, H14, H18), 8.15 (d, 2H, *J* = 8.6 Hz, H15, H17), ppm; ^{13}C NMR (DMSO-d_6) δ 28.8(C-1′), 35.2(C-9), 36.1(C-5), 41.1(C-2), 42.4(NMe), 53.6(C-8a), 54.5(C-10) 56.2(OMe), 62.6(C-12), 71.4(C-6), 85.4(C-4a), 114.1(C-2), 118.6(C-14 & C-18), 121.7(C-1), 124.8(C-15 & C-17), 126.5(C-12a), 130.1(C-7), 133.7(C-8), 136.8(C-12b), 140.5(C-16), 143.7(C-3), 146.3(C-3a), 155.0 (C-13), ^{31}P (DMSO-d_6) δ−14.12; APCI-MS *m/z* (%) 547 [MH$^{+\bullet}$].

6.1.2.12. Synthesis of 2-aminoethylamino (3-methoxy-11-methyl-5, 6, 9, 10, 11, 12-hexahydro-4aH-4-oxa-11-azacyclohepta [de]fluoren-6-yl) (4-nitrophenyl) phosphate ***17***. Yield: 80%; m.p.175–177 °C; $[\alpha]_D^{25}$ −116.9°; IR (KBr) 3382 (N–H), 1220 (P═O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.49– 1.64 (m, 2H, H9), 2.41 (s, 3H, NMe), 2.47–2.60 (m, 1H, H5α), 2.85–3,02(m, 1H, H5β), 3.07 (s, 3H, NH), 3.11–3.19 (m, 2H, H1′), 3.20–3.26 (m, 2H, H10), 3.33 (d, 1H, *J* = 13.6 Hz, H12α), 3.35–3.39 (m, 2H, H2′), 3.74 (d, 1H, *J* = 15.2 Hz, H12β), 3.84 (s, 3H, OMe), 4.08–4.16 (m, 1H, H4a), 4.65 (br t, 1H, *J* = 6.3 Hz, H6), 4.86 (s, 2H, NH_2), 6.08 (dd, 1H, *J* = 10.4 Hz, H7), 6.12 (d, 1H, *J* = 10.4 Hz, H8), 6.66 (d, 1H, *J* = 8.4 Hz, H1), 6.69 (d, 1H, *J* = 8.0 Hz, H2), 7.41 (d, 2H, *J* = 9.6 Hz, H14, H18), 8.14 (d, 2H, *J* = 8.6 Hz, H15, H17), ppm; ^{13}C NMR (DMSO-d_6) δ 28.2(C-1′), 34.3(C-9), 35.9(C-5), 41.8(NMe), 42.5(C-2′), 53.2(C-8a), 54.5(C-10), 61.1(C-12), 72.2(C-6), 86.1(C-4a), 113.5(C-2), 119.4(C-14 & C-18), 121.8(C-1), 125.2(C-15 & C-17), 126.3(C-12a), 131.1(C-7), 134.4(C-8), 137.5(C-12b), 141.3(C-16), 143.6(C-3)146.5(C-3a), 153.3(C-13); ^{31}P (DMSO-d_6) δ −14.25; APCI-MS *m/z* (%) 403 [MH$^{+\bullet}$].

6.2. Pharmacology

6.2.1. Preparation of microbial strains and inoculums

Antimicrobial activity of phosphorylated derivatives of galanthamine have been investigated against Gram-positive bacteria: *B. subtilis*, *S. aureus*, and Gram-negative bacteria: *E. coli* and *K. pneumoniae*. A preliminary screening for antimicrobial activity of **6–17** and galanthamine was tested against the microbial cultures. All the bacterial species were cultured in nutrient broth before the antimicrobial activity test performed. The inoculated flasks were incubated at 37 °C for 18 h on a rotary shaker at 150 rpm. The extracts were tested against all the species mentioned above and the observed results showed 90% activity against both Gram-positive and Gram-negative bacteria in the disc-diffusion assay.

6.2.2. Antimicrobial activity screening

Antimicrobial studies were performed according to agar disc-diffusion method [23,24]. To obtain more significant information of antimicrobial potency of galanthamine derivatives **6–17** against *S. aureus*, *B. subtilis*, *K. pneumoniae* and *E. coli*, the following test conditions were applied; all the compounds were dissolved in dimethylformamide (DMF, Merck). Nutrient agar (Oxoid) plates were prepared and dried at 35–36 °C for about 30 min in an incubator. Test strains were spread on solid nutrient agar surface by using sterile spreader. At the same time, absorbent paper discs were placed on agar surface (5 mm for compounds and 6 mm for antibiotic) and impregnated with known concentrations which determined previously by MIC tests (500 μg for each disc). Blank test showed that, DMF used in the preparations of the test solutions does not affect the test organisms, they were inverted and allowed to incubate at 37 °C. The inhibition zone around the disc was calculated edge to edge zone of confluent growth which is usually corresponds to the sharpest edge of the zone and was measured in millimeters. All tests were repeated three times and average data has taken as final result (Table 3).

6.2.3. Evaluation of antioxidant potential

Adult Wistar albino rats (90 days) were used in the study. The rats were fed a standard pellet diet (Hindustan Lever Ltd., India) and were given water ad libitum. The animals were maintained under proper temperature (25–30 °C), ventilation and hygienic conditions. They were exposed to 12 h each of light and dark. The phosphorylated derivatives of galanthamine were used to estimate the antioxidant activity *in vitro*. The oxidative stress was assessed by estimating lipid peroxidation (LPO), reduced glutathione content (GSH) and the activities of superoxide dismutase (SOD)

and catalase (CAT). The results were compared with that of vitamin E and C, the known natural antioxidants. The formula for calculation of antioxidant activities for LOP, SOD, GSH, CAT are as follows.

Inhibition of lipid peroxidation:

$$\%\ \text{inhibition} = \frac{100 \times A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}$$

$$\text{Superoxide Dismutase} = \frac{0.93 \times (Vs/Vc) - 1}{(1.073 - 0.073) \times (Vs/Vc)}$$

here, Vs is the rate of sample containing SOD

Vc is the Average rate of blank sample (SOD = 0)

$$\text{GSH} = \frac{\text{Net Rate} - \text{Intercept}}{\text{Slope}} \times \text{Dilution Factor}$$

here, OD = Optical Density

Brain tissues of rat were homogenized 1:40 (w/v) in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA. Total glutathione (GSH + GSSG) [25] and reduced glutathione (GSH) and the activities of GSH-PX [26] and GST [27] were assayed. Protein content of the homogenates was determined by the method of Lowry et al, [28] and the GSH-PX and GST activities were expressed in terms of units/gram protein. For estimation of ascorbic acid, the tissues were homogenized 1:9 (w/v) in ice-cold 5% trichloroacetic acid. Ascorbic acid assay is based on oxidation of ascorbic acid to dehydroascorbic acid followed by treatment with 2, 4-dinitrophenylhydrazine to form the bis-dinitrophenylhydrazone [29].

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