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Original article

Synthesis and antitumor activities of novel thiourea α-aminophosphonates from dehydroabietic acid

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ABSTRACT

A series of novel thiourea α-aminophosphonate derivatives containing DHA structure was designed and synthesized as antitumor agents. Their inhibitory activities against the NCI-H460 (lung), A549 (lung adenocarcinoma), HepG2 (liver) and SKOV3 (ovarian) human cancer cell lines were estimated using MTT assay *in vitro*. The screening results revealed that many compounds exhibited moderate to high levels of antitumor activities against the tested cancer cell lines and that most demonstrated more potent inhibitory activities compared with the commercial anticancer drug 5-fluorouracil. The mechanism of compound **5f** was preliminarily investigated by acridine orange/ethidium bromide staining, Hoechst 33258 staining, JC-1 mitochondrial membrane potential staining, TUNEL assay, DNA ladder assay and flow cytometry, which indicated that the compound can induce cell apoptosis in A549 cells.

1. Introduction

Over the ten decades, the development of novel natural compounds and its derivatives with better medicinal virtue have greatly attracted bioorganic chemists' interest and become a hot research topic in bioorganic chemistry, due to the success of taxol, vinblastine and their related derivatives as anticancer agents [1–5]. In fact, natural products have traditionally been good source of new medicinal leads, and they play an important role in drug discovery, especially in the area of cancer pharmacology, where the fraction of the drugs derived from natural products amounts to 60% [6].

Dehydroabietic acid (DHA), which is a natural occurring diterpenic resin acid, has been found to have properties of increasing the inhibition activity of an anticancer drug in various cells, e.g. cervical carcinoma cells, hepatocellular carcinoma cells, and breast cancer cells, as well as its analogs [7]. Therefore DHA skeleton was chosen as active pharmacal core to screen for new potential antitumor compounds by the introduction of various functional groups [8–11]. In previous work, it has been found that the introduction of sulfocarbamide on ring B and C [8,12], as well as in carboxylic acid group of DHA showed improved antitumor activity [9]. Furthermore, aminophosphonates groups are able to better improve the antitumor activity and many aminophosphonates derivatives have shown good inhibition activities against human tumors [13–16]. However, to the best of our knowledge, the studies on the synthesis, antitumor activities and apoptosis-inducing effects of thiourea α-aminophosphonate derivatives derivated from DHA have not been explored. So in this paper, functional groups thiourea and α-aminophosphonate groups were rationally designed and introduced to the carboxylic acid part of DHA skeleton to offer a series of novel thiourea α-aminophosphonate derivatives containing DHA. Their cytotoxicity *in vitro* against four selected tumor cell lines were evaluated. Results showed that the target compounds can inhibit proliferation of these four tumor cell lines at moderate to high rates. Preliminary investigation on the mode of action of compound **5f** found that it can effectively induce cell apoptosis in A549 cells.

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2. Results and discussion

2.1. Chemistry

Thiourea α-aminophosphonate derivatives bearing DHA structure (compounds **4a**–**p** and **5a**–**o**) were synthesized as outlined in Scheme 1. 7-Oxo-dehydroabietic acid **2** was synthesized by the treatment of dehydroabietic acid with chromic anhydride in the presence of glacial acetic acid [8]. Dehydroabietic chloride was obtained by the condensation of compound **2** with oxalyl chloride, and it was then treated with KSCN to offer dehydroabietic isothiocyanate **3**. α-Aminophosphonate derivatives, which was synthesized according to the literature [17], was treated with compound **3** to offer thiourea α-aminophosphonate derivatives (**4a**–**p**) in good yields. Compounds **5** were finally acquired by the condensation of compounds **4** with hydroxylamine hydrochloride in the presence of ethanol at 80 °C. The structures of thiourea α-aminophosphonate derivatives **4**–**5** were confirmed by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and high resolution mass spectrum (HRMS).

2.2. In vitro cytotoxicity

The *in vitro* cytotoxic potency of thiourea α-aminophosphonate derivatives **4**–**5** were evaluated by MTT assay against NCI-H460, HepG2, A549 and SKOV3 tumor cell lines (with 5-fluorouracil (5-FU) as the positive control). The values of IC_{50}, the effective concentration at which 50% of the tumor cells were inhibited, were calculated to evaluate the antitumor activities. A lower IC_{50} value indicated greater antitumor activity. IC_{50} values of lower than that of control usually implied effective antitumor activity. The tested results were shown in Table 1.

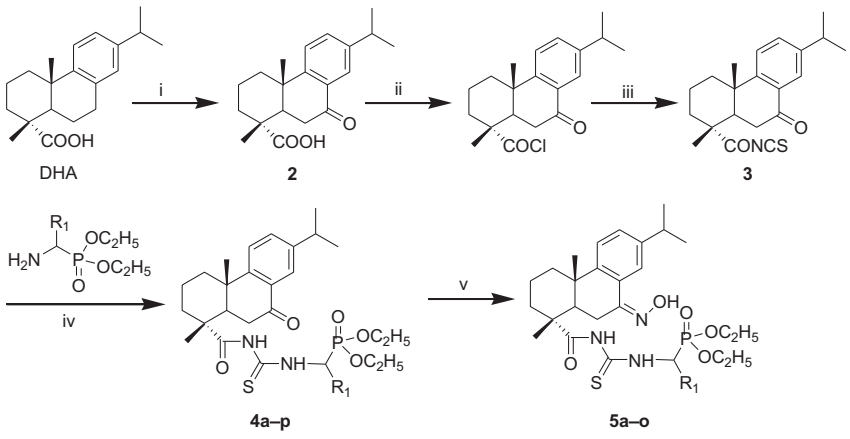
As shown in Table 1, most of thiourea α-aminophosphonate derivatives **4**–**5** displayed much higher inhibitory activity than DHA against the NCI-H460, A549, HepG2 and SKOV3 cell lines, indicating the introduction of thiourea α-aminophosphonate on DHA should markedly improve the antitumor activity. Moreover, the substituents in benzene of compound **4** have important influence on the cytotoxic inhibition and the introduction of electron donor substituents and halogen groups may result in the decrease of cytotoxic inhibition, while substituent groups in benzene of compound **5** also have important effect on the cytotoxic inhibition and the introduction of electron donor substituents and halogen groups may lead to the enhancement of cytotoxic inhibition. In addition, by the comparison of IC_{50} values of compounds **4** with **5**, it could be concluded that C═N–OH group in 7 position of DHA skeleton was an important contributor to their cytotoxic activities.

Table 1 also revealed that, in NCI-H460 assay, all the compounds except **4l** and **4o** exhibited better inhibition than DHA (IC_{50} = 84.53 μM), and even preferable cytotoxic activities than the commercial anticancer drug 5-FU (IC_{50} = 44.04 μM), with IC_{50} in the range of 11.49–41.51 μM, indicating good inhibition activities of these compounds on NCI-H460 cell line. This cytotoxic inhibition screening results demonstrated that the introduction of thiourea α-aminophosphonate on DHA should markedly improve the antitumor activity against NCI-H460 cell line. Among all the compounds, compound **4d** exhibited the best cytotoxicity, with IC_{50} of 11.49 μM.

In A549 assay, all the compounds but **4h** and **5o** exhibited better inhibition than DHA (IC_{50} = 79.46 μM), with IC_{50} in the range of 7.00–49.61 μM. It was worth noting that except compounds **4f**, **4h**, **4l**–**4n**, **5m** and **5o**, other compounds showed more cytotoxic inhibition than 5-FU, indicating potent cytotoxicity of these compounds on A549 cell line. This cytotoxicity screening results implied that the introduction of thiourea α-aminophosphonate on DHA obviously improve the antitumor activity against A549 cell line. Compound **5m** exhibited effective cytotoxicity close to 5-FU, with IC_{50} of 34.70 μM, while compound **5f** showed the best among all the compounds, with IC_{50} of 7.00 μM.

In HepG2 assay, except compounds **4b**, **4c**, **4f**, **4l**–**o** and **5j**, other compounds displayed better cytotoxicity than DHA (IC_{50} = 85.00 μM), with IC_{50} in the range of 8.62–49.32 μM, indicating that the introduction of thiourea α-aminophosphonate on DHA should improve the antitumor activity against HepG2 cell line. Moreover, compounds **4d**, **4e**, **4p**–**5i**, **5k**, **5l**, **5n** and **5o** even demonstrated better cytotoxic inhibition than 5-FU, implying favorable inhibition activities of these compounds on HepG2 cell line. Compounds **5c** and **5g** showed better inhibition on HepG2 cell line than other compounds, with IC_{50} of 8.62 and 9.73, respectively.

In SKOV3 assay, except compounds **4f**, **4i** and **5l**, all the compounds displayed better cytotoxicity than DHA (IC_{50} = 84.00 μM), with IC_{50} in the range of 8.14–45.12 μM, exhibiting that the introduction of thiourea α-aminophosphonate on DHA should improve the antitumor activity against SKOV3 cell line. Moreover, compounds **4c**–**e**, **5a**, **5c**, **5d**, **5f**, **5g**, **5i**, **5k** and **5l** even exhibited better cytotoxic inhibition than 5-FU, showing good inhibition activities of these compounds on SKOV3 cell line. Compound **4p** and **5h** exhibited effective cytotoxicity close to 5-FU, with IC_{50} of 24.59 μM



Scheme 1. Synthetic route to thiourea α-aminophosphonate derivatives containing DHA skeleton. Reagents and conditions: (i) CrO_3, CH_3COOH, r.t.; (ii) oxalyl chloride, CH_2Cl_2, r.t.; (iii) $C_6H_5CH_3$, KSCN, 110 °C; (iv) CH_2Cl_2, r.t.; (v) $NH_2OH{\cdot}HCl$, CH_3CH_2OH, 80 °C.

Table 1
Effect of compounds **4**–**5** against cell viability of different cell lines.

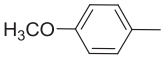
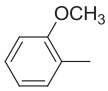
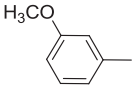
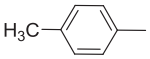
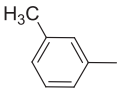
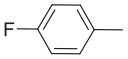
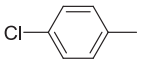
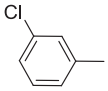
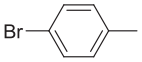
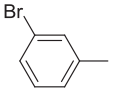
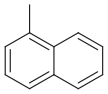
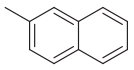
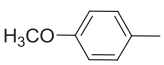
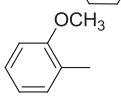
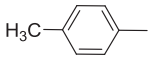
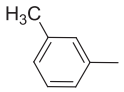
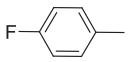
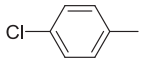
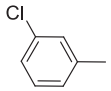
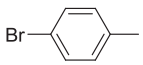
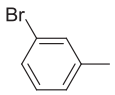
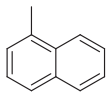
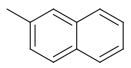
Compounds	IC_{50} (μM)				
	R_1	NCI-H460	A549	HepG2	SKOV3
4a		21.08 ± 0.23	21.59 ± 0.42	46.84 ± 1.02	42.52 ± 0.99
4b		24.47 ± 0.34	26.14 ± 0.24	>50	49.87 ± 0.79
4c		29.52 ± 0.21	19.92 ± 0.56	>50	23.55 ± 0.34
4d		11.49 ± 0.49	14.10 ± 0.04	15.78 ± 0.13	8.41 ± 0.08
4e		24.82 ± 0.58	22.23 ± 0.31	15.15 ± 0.23	11.75 ± 0.11
4f		42.71 ± 1.09	37.11 ± 0.22	>50	>50
4g		22.43 ± 0.78	17.45 ± 0.34	45.42 ± 0.37	30.21 ± 0.35
4h		36.44 ± 0.76	>50	49.32 ± 0.92	45.12 ± 0.46
4i		39.84 ± 0.83	32.95 ± 0.96	37.12 ± 0.55	>50
4j		41.18 ± 0.59	30.30 ± 0.74	39.45 ± 0.52	31.39 ± 0.47
4k		36.11 ± 0.74	22.50 ± 0.47	39.94 ± 0.63	27.86 ± 0.09
4l		>50	44.07 ± 0.34	>50	>50
4m		27.87 ± 0.04	49.61 ± 0.59	>50	36.88 ± 0.58
4n		41.51 ± 1.23	42.46 ± 1.46	>50	49.12 ± 1.08
4o		>50	32.58 ± 1.65	>50	27.31 ± 1.23
4p		19.79 ± 0.68	23.19 ± 0.86	25.15 ± 0.75	24.59 ± 1.21
5a		36.73 ± 0.63	27.02 ± 0.34	27.20 ± 0.28	22.06 ± 0.23
5b		38.45 ± 0.58	23.56 ± 0.47	16.99 ± 0.16	30.78 ± 0.27
5c		18.55 ± 0.18	27.06 ± 0.53	8.62 ± 0.07	17.93 ± 0.18

Table 1 (*continued*)

Compounds	IC_{50} (μM)				
	R_1	NCI-H460	A549	HepG2	SKOV3
5d		22.45 ± 0.13	17.93 ± 0.23	10.91 ± 0.43	8.95 ± 0.08
5e		37.92 ± 0.29	12.93 ± 0.51	12.45 ± 0.21	28.55 ± 0.16
5f		17.74 ± 0.41	7.00 ± 0.04	13.10 ± 0.08	11.38 ± 0.10
5g		19.28 ± 0.15	9.16 ± 0.09	9.73 ± 0.03	19.87 ± 0.21
5h		13.27 ± 0.32	28.87 ± 0.14	17.51 ± 0.13	25.23 ± 0.24
5i		23.83 ± 0.34	9.66 ± 0.08	21.35 ± 0.09	16.57 ± 0.39
5j		29.06 ± 0.45	32.78 ± 0.41	>50	40.20 ± 0.74
5k		28.42 ± 0.52	19.04 ± 0.21	13.23 ± 0.19	12.31 ± 0.17
5l		22.64 ± 0.22	19.02 ± 0.23	26.87 ± 0.27	12.73 ± 0.33
5m		30.17 ± 0.32	34.70 ± 0.34	42.79 ± 0.59	37.71 ± 0.51
5n		37.10 ± 0.53	28.02 ± 0.38	27.91 ± 0.34	40.12 ± 0.44
5o		26.78 ± 0.59	>50	20.91 ± 0.37	40.63 ± 0.53
DHA		84.53 ± 1.11	79.46 ± 1.09	85.00 ± 1.16	84.00 ± 1.21
5-FU		44.04 ± 0.54	34.33 ± 0.23	29.98 ± 0.37	24.43 ± 0.41

and 25.23 μM, respectively, while Compound **4d** displayed the best inhibition on SKOV3 cell line among all the compounds, with IC_{50} of 8.41 μM.

2.3. Preliminary investigation of the apoptosis-inducing effect of target compound *5f*

It is known that apoptosis assay may provide important information to preliminary investigation of the mode of action. Moreover, whether the thiourea α-aminophosphonate derivatives **4**–**5** can induce apoptosis in certain cancer cell lines have not yet been reported so far. Therefore, in the present study, compound **5f**, which exhibited good cytotoxic inhibition in four cell lines and could be used as a good representative of compounds **4**–**5**, was selected and its mechanism of growth inhibition of A549 cells was evaluated.

2.3.1. Fluorescence staining

Changes in the morphological character of A549 cells were studied using acridine orange (AO)/ethidium bromide (EB), Hoechst 33258, JC-1 mitochondrial membrane potential staining and TUNEL assay staining under fluorescence microscopy to estimate whether the growth inhibitory activity of the selected compound was related to the induction of apoptosis.

2.3.1.1. AO/EB staining. AO, which is a vital dye, can stain nuclear DNA across an intact cell membrane, while EB can only stains cells that had lost their membrane integrity. Therefore, after synchronous treating with AO and EB, live cells will be evenly stained as green (in the web version) and early apoptotic cells will be thickly stained as green yellow or show green yellow fragments (in the web version), while late apoptotic cells will be densely stained as orange or display orange fragments and necrotic cells will be stained as orange with no condensed chromatin.

The cytotoxicity of compound **5f** at the concentration of 15 μM against A549 cells from 12 to 24 h was assayed by AO/EB staining, and A549 cells not treated with the **5f** were used as control for 24 h. The results were shown in Fig. 1. As shown in Fig. 1, the A549 cells treated with **5f** from 12 to 24 h had obviously changed. The nuclei markedly stained as yellow green or orange, and the morphology displayed pycnosis, membrane blebbing and cell budding. These phenomena were associated with cell apoptosis.

Based on the above observation, the cells represented with an apoptotic morphology. The complete absence of red cells in compounds **5f** demonstrated that it was associated with very low cytotoxicity. These findings indicate that compounds **5f** could induce apoptosis with low cytotoxicity.

2.3.1.2. Hoechst 33258 staining. Hoechst 33258, which stains the cell nucleus, is a membrane permeable dye with blue fluorescence. Live cells with uniformly light blue nuclei were obviously detected under fluorescence microscope after treatment with Hoechst 33258, whereas apoptotic cells had bright blue nuclei due to karyopyknosis and chromatin condensation. However, the nuclei of dead cells could not be stained. A549 cells treated with compound **5f** at 15 μM from 12 to 24 h were stained with Hoechst 33258. A549 cells not treated with the **5f** was used as control at for 24 h. The results were given in Fig. 2

As shown in Fig. 2, cells not treated with compound **5f** were normally blue (in the web version). It was worth noting that, for **5f** treatment, the cells displayed strong blue fluorescence and indicated typical apoptotic morphology after 12 and 24 h. The observation revealed that compounds **5f** induced apoptosis against A549 cell lines, consistent with the results for AO/EB double staining.

2.3.1.3. Mitochondrial membrane potential staining. In order to further investigate the apoptosis-inducing effect of target compound **5f**, mitochondrial membrane potential changes were designed and detected, using the fluorescent probe JC-1. JC-1, which is a lipophilic cationic dye, can easily pass through the plasma membrane into cells and accumulates in actively respiring mitochondria [18]. There exists close relationship between membrane potential and degree of accumulation of JC-1 in mitochondria, i.e. the degree of accumulation of JC-1 in mitochondria depends on the membrane potential. There is very little accumulation of the dye, when mitochondrial membrane potential is low, and thus the dye exists as a monomer emitting green fluorescence. Accordingly, the accumulation of the dye in mitochondria increases with the increase of mitochondrial membrane potential, while dye forms "J-aggregates" at higher concentrations. Dye aggregation resulted in a shift in the fluorescence emission from green to red. The formation of JC-1 monomer, which is a fast and reversible process, sensitively occurs in specific regions with higher potential, and thus can be used as a sensitive tool for investigating the mitochondrial membrane potential changes. A549 cells treated with compound **5f** at 15 μM from 12 to 24 h were stained with JC-1 and not treated with the compound **5f** were used as control at for 24 h. The results were shown in Fig. 3.

The JC-1 monomer and J-aggregates were excited at 514 nm and 585 nm, respectively, and light emissions were collected at 515–545 nm (green) and 570–600 nm (red). For fluorescence microscopy, Fig. 3 showed that cells not treated with the compound **5f** were normally red (in the web version), while for **5f** treatment, cells showed strong green fluorescence and indicated typical apoptotic morphology after 12 h and 24 h. Therefore, it could be concluded that compound **5f** induced apoptosis against A549 cell line. The results were identical with that of previous experiment of AO/EB double staining and Hoechst 33258 staining.

2.3.2. TUNEL assay

TUNEL (terminal-deoxynucleotidyl transferase meditated nick end labeling) is a common method for identifying apoptotic cells in situ by detecting DNA fragmentation. When the genomic DNA is broken, the exposed 3′-OH at the end of deoxynucleotide transfer as the catalytic plus green fluorescent probes fluorescein (FITC) labeled dUTP, which can be detected by fluorescence microscopy or flow cytometry, as indicated by a green color (in the web version). The results were illustrated in Fig. 4.

For fluorescence microscopy, Fig. 4 revealed that A549 cells treated with the compound **5f** at different time appeared in green (in the web version), indicating that compound **5f** significantly induced apoptosis against A549 cell line. The results were consistent well with the previous experiments.

2.3.3. DNA ladder assay

Apoptosis is a fundamental cellular event during development and is critical for the cytotoxicity induced by radiation or drugs characterized by the cleavage of chromatin DNA into internucleosomal fragments. Fragmentation of chromosomal DNA is the biological hallmark of apoptosis [19] and can be detected by gel electrophoresis [20]. The DNA is cleaved to nucleosome-sized fragments of approximately 180–200 base pairs, which results in the formation of a ladder like pattern when the DNA is subjected to electrophoresis in an agarose gel [21]. This ladder-like pattern is regarded as the biochemical hallmark of apoptosis [22]. Herein, we used agarose gel electrophoresis to detect the DNA fragment. The results were shown in Fig. 5. As shown in Fig. 5, compared with the

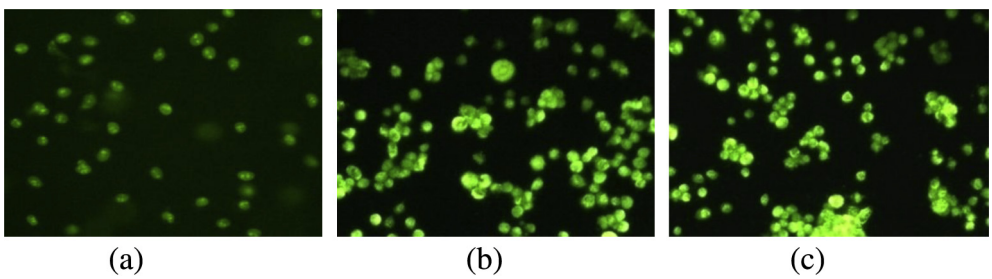


Fig. 1. AO/EB staining of compound **5f** in A549 cells. (a) Not treated with compound **5f** was used as control at for 24 h and (b, c) treatment with compound **5f** (15 μM) for 12 h and 24 h, respectively.

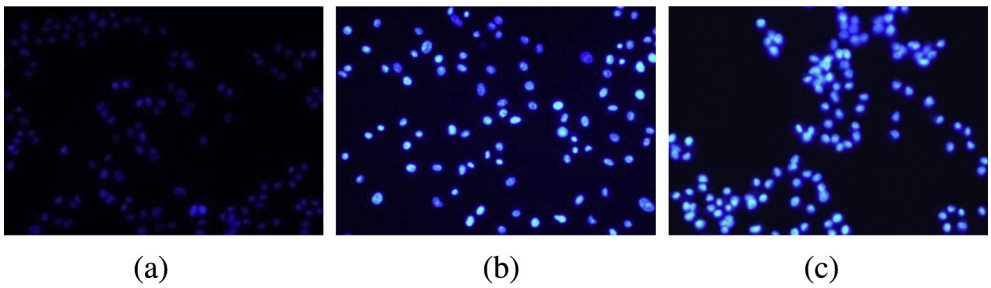


Fig. 2. Hoechst 33258 staining of compound **5f** in A549 cells. (a) Not treated with compound **5f** was used as control at for 24 h and (b, c) treatment with compound **5f** (15 μM) for 12 h and 24 h, respectively.

control (lane 1), the addition of compounds **5f** (lane 2, 3 and 4) lead to obvious ladder-like pattern bands, indicating that compounds **5f** can induce apoptosis against A549 cell lines. The result was well consistent with that of fluorescence staining and TUNEL assay.

2.3.4. Flow cytometry

The apoptosis ratios induced by compound **5f** in A549 tumor cells were quantitatively determined by flow cytometry. Four quadrant images were observed by flow cytometric analysis: the Q1 area represented damaged cells appearing in the process of cell collection, the Q2 region showed necrotic cells and later period apoptotic cells, the Q3 area showed normal cells, and the early apoptotic cells were located in the Q4 area. The results were given in Fig. 6.

Fig. 6 revealed that compound **5f** could induce apoptosis in A549 cells. Apoptosis ratios (including the early and late apoptosis ratios) for compound **5f** were obtained after 12 h of treatment at the concentration of 20 μM and 25 μM. The apoptosis of A549 cells treated with compound **5f** increased gradually in a concentration manner. The apoptosis ratios of compound **5f** measured at different concentration points were found to 19.7% (20 μM) and 51.7% (25 μM), respectively, while that of control was 0.6%. The results evidently illustrated that compound **5f** suppressed cell proliferation by inducing apoptosis.

3. Conclusions

DHA is an important natural product with antitumor bioactivity. In this study, a series of thiourea α-aminophosphonate derivatives from DHA were designed and synthesized, and their cell growth inhibition activities against the NCI-H460, A549, HepG2 and SKOV3 cell lines were evaluated using MTT assay. The *in vitro* antitumor activities screening revealed that all the compounds except **4l** and **4o** exhibited better inhibition activities than the commercial anticancer drug 5-FU on NCI-H460 cell line, with IC_{50} in the range of 11.49–41.51 μM, and all the compounds except compounds **4f**, **4h**, **4l**–**n**, **5m** and **5o** showed more cytotoxic inhibition than 5-FU on A549 cell line, with IC_{50} in the range of 7.00–32.95 μM, while compounds **4d**, **4e**, **4p**–**5i**, **5k**, **5l**, **5n** and **5o** demonstrated better cytotoxic inhibition than 5-FU on HepG2 cell line, with IC_{50} in the range of 8.62–27.91 μM, respectively. In addition, compounds **4c**–**4e**, **5a**, **5c**, **5d**, **5f**, **5g**, **5i**, **5k** and **5l** displayed better cytotoxic inhibition than 5-FU on SKOV3 cell line, with IC_{50} in the range of 8.41–23.55 μM. The apoptosis-inducing activity of representative compound **5f** in A549 cells was investigated by AO/EB staining, Hoechst 33258 staining, JC-1 mitochondrial membrane potential staining, TUNEL assay, DNA ladder assay and flow cytometry. This compound showed clear cell apoptosis inducing effects, with its inducing ratios of 19.7% (20 μM) and 51.7% (25 μM) for 12 h, respectively. Further studies of the specific mechanisms of these compounds in human malignant tumors are currently underway.

4. Experimental

4.1. General

All the chemical reagents and solvents used were of analytical grade. Compound 7-oxo-dehydroabietic acid (**2**) was synthesized according the literature [8]. α-Aminophosphonate derivatives was synthesized according to the literature [17].

4.2. Instrumentation

NMR spectra were recorded on a Bruker AV-500 NMR spectrometer. Mass spectra were determined on a FTMS ESI spectrometer.

4.3. Synthesis

*4.3.1. General procedure for compounds **4a**–**p***

Compound **2** (1 mmol) added to CH_2Cl_2 (15 mL) was stirred at 0 °C and oxalyl chloride (1.5 mmol) was dripped into the mixture and stirred at room temperature for 6 h. After the reaction, the solvent and excess oxalyl chloride was evaporated under reduced pressure. Toluene (15 mL) and KSCN (1.2 mmol) were added to the

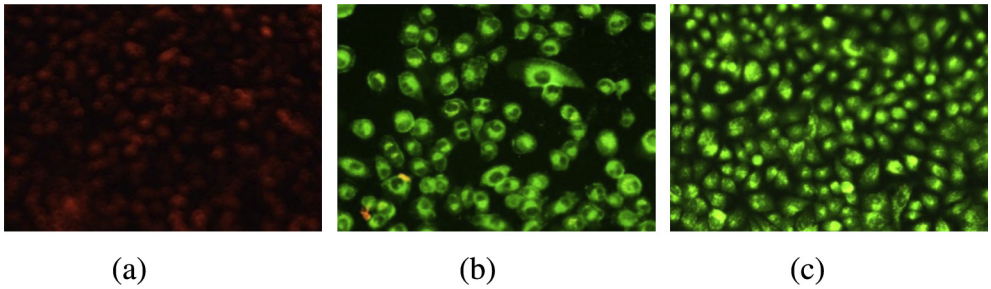


Fig. 3. JC-1 mitochondrial membrane potential staining of compound **5f** in A549 cells. (a) Not treated with the **5f** was used as control at for 24 h and (b, c) treatment with compound **5f** (15 μM) for 12 h and 24 h, respectively.

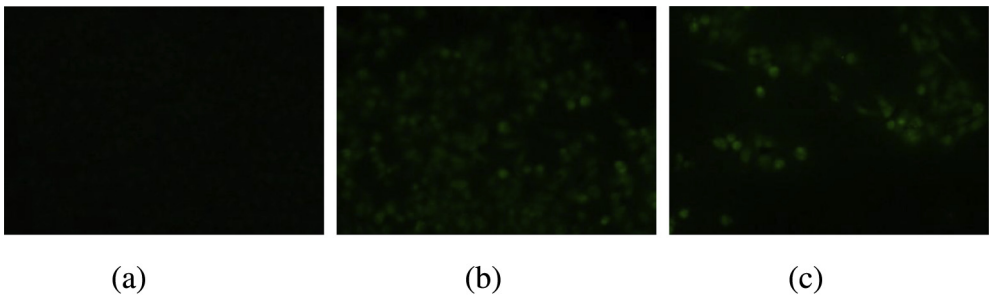


Fig. 4. TUNEL assay of compound **5f** in A549 cells. (a) Not treated with the **5f** was used as control for 24 h and (b, c) treatment with compound **5f** (15 μM) for 12 h and 24 h, respectively.

mixture and stirred at 110 °C for 12 h. After the reaction, the solvent was evaporated under reduced pressure, and the crude product was purified by chromatography on silica gel eluted with petroleum ether/ethyl acetate (v:v = 10:1) to offer compound **3** in 88.25%. Compound **3** (1 mmol) and α-aminophosphonate derivatives (1.2 mmol) were added to CH_2Cl_2 (15 mL) the mixture was stirred at room temperature for 3 h. After the reaction was completed, the solvent was evaporated under reduced pressure, and the crude product was purified by chromatography on silica gel eluted with petroleum ether/ethyl acetate (v:v = 3:1) to offer compounds **4a**–**p**.

*4.3.1.1. Compound (**4a**).* According to the general procedure, compound **3** was treated with diethyl 1-amin phenyl methyl phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4a**.

Yields 88.21%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.52 (s, 1H, NH), 8.60 (s, 1H, NH), 7.86 (dd, *J* = 3.7, 2.2 Hz, 1H), 7.40 (d, *J* = 2.4 Hz, 2H), 7.39–7.35 (m, 3H), 7.32–7.27 (m, 2H), 6.08–6.01 (m, 1H, N–CH–P), 4.02–3.91 (m, 4H, 2× O$\underline{CH_2}$CH$_3$), 2.95–2.90 (m, 1H), 2.73–2.67 (m, 2H), 2.45–2.37 (m, 2H), 1.77–1.66 (m, 5H), 1.42 (d, *J* = 1.1 Hz, 3H, CH_3), 1.27 (d, *J* = 2.9 Hz, 6H, 2× CH_3), 1.25 (dd, *J* = 5.2, 1.5 Hz, 6H, 2× CH_3), 1.21–1.17 (m, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.70, 180.43, 178.23, 152.44, 147.28, 132.80, 130.64, 128.78, 128.71, 128.36, 128.17, 125.23, 123.27, 63.60, 63.37, 57.41, 56.18, 47.50, 43.40, 43.28, 37.60, 37.43, 37.19, 36.97, 36.72, 33.62, 23.81, 23.73, 18.12, 16.34, 16.29, 16.24. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.81. HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{43}N_2O_5PS$ [M + H$^+$]: 599.27085; found: 599.27323.

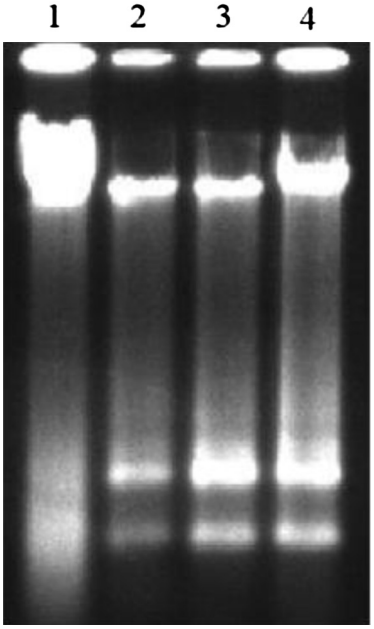


Fig. 5. DNA ladder assay of compound **5f**. Compound **5f** induced apoptosis-associated DNA fragmentation in A549 cells. The electrophoretic gel images of total cellular DNA extracted from compound **5f** treated A549 cells and normal cultural A549 cells. A549 cells were treated with compound **5f** at 10 μM, 20 μM and 30 μM, respectively. The lane 1 was a normal cultural A549 cells as control, and lane 2 was treated with compound **5f** at 10 μM; lane 3 was treated with compound **5f** at 20 μM; lane 4 was treated with compound **5f** at 30 μM, respectively.

*4.3.1.2. Compound (**4b**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (4-methoxy phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4b**.

Yields 82.56%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.46 (s, 1H, NH), 8.58 (s, 1H, NH), 7.86 (t, *J* = 2.5 Hz, 1H), 7.42 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.36–7.32 (m, 2H), 7.28 (dd, *J* = 8.2, 1.4 Hz, 1H), 6.91–6.86 (m, 2H), 5.96 (m, 1H, N–CH–P), 4.11–3.89 (m, 4H, 2× O$\underline{CH_2}$CH$_3$), 3.78 (s, 3H, OCH$_3$), 2.96–2.89 (m, 1H), 2.73–2.64 (m, 2H), 2.40–2.34 (m, 2H), 1.84–1.65 (m, 5H), 1.42 (d, *J* = 4.9 Hz, 3H, CH_3), 1.28 (dd, *J* = 9.9, 4.6 Hz, 6H, 2× CH_3), 1.25–1.24 (m, 6H, 2× CH_3), 1.20 (dd, *J* = 14.0, 7.0 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.65, 180.24, 178.04, 147.28, 132.81, 129.52, 129.47, 125.21, 123.27, 114.27, 114.19, 63.63, 63.35, 56.71, 55.48, 55.27, 47.47, 43.39, 43.24, 37.42, 37.25, 37.12, 36.96, 33.62, 23.80, 23.74, 18.11, 16.43, 16.39, 16.33. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.77. HRMS (*m*/*z*) (ESI): calcd for $C_{33}H_{45}N_2O_6PS$ [M + H$^+$]: 629.28142; found: 629.28198.

*4.3.1.3. Compound (**4c**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (2-methoxy phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4c**.

Yields 80.28%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.69 (s, 1H, NH), 8.57 (s, 1H, NH), 7.88 (d, *J* = 2.1 Hz, 1H), 7.44–7.42 (m, 1H), 7.30–7.28 (m, 3H), 6.96 (dd, *J* = 23.1, 14.7, 2H), 6.48–6.41 (m, 1H, N–CH–P), 4.15–3.96 (m, 4H, 2× O$\underline{CH_2}$CH$_3$), 3.92 (s, 3H, OCH$_3$), 2.94 (dd, *J* = 13.8, 6.9 Hz, 1H), 2.76–2.64 (m, 2H), 2.49–2.38 (m, 2H), 1.89–1.67 (m, 5H), 1.44 (d, *J* = 5.1 Hz, 3H, CH_3), 1.31–1.29 (m, *J* = 5.4, 4.8 Hz, 6H, 2× CH_3), 1.28–1.26 (m, 6H, 2× CH_3), 1.24–1.18 (m, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.54, 179.63, 177.53, 157.42, 132.74, 129.58, 125.18, 123.33, 123.27, 120.97, 120.90, 111.34, 63.26, 63.10, 55.86, 53.86, 52.63, 47.34, 43.35, 43.23, 37.39, 37.26, 37.13, 37.01, 36.73, 33.62, 23.81, 23.75, 18.12, 16.41, 16.37, 16.28. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.68. HRMS (*m*/*z*) (ESI): calcd for $C_{33}H_{45}N_2O_6PS$ [M + H$^+$]: 629.28142; found: 629.28247.

*4.3.1.4. Compound (**4d**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (3-methoxy phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4d**.

Yields 81.42%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.58 (s, 1H, NH), 8.60 (s, 1H, NH), 7.88 (t, *J* = 2.0 Hz, 1H), 7.44 (dd, *J* = 8.1, 2.1 Hz, 1H), 7.30–7.21 (m, 2H), 7.03–6.97 (m, 2H), 6.86 (t, *J* = 8.6 Hz, 1H), 6.06–

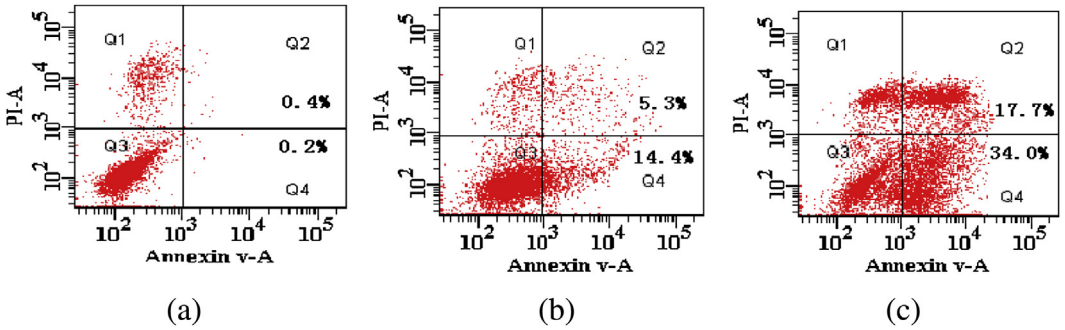


Fig. 6. Apoptosis ratio detection of compound **5f** by Annexin V/PI assay. (a) A549 cells were not treated with **5f** for 12 h. (b, c) A549 cells were treated with compound **5f** at 20 μM and 25 μM for 12 h, respectively.

5.99 (m, 1H, N–CH–P), 4.14–3.94 (m, 4H, 2× OCH_2CH_3), 3.82 (d, J = 7.4 Hz, 3H, OCH_3), 2.99–2.91 (m, 1H), 2.74–2.65 (m, 2H), 2.46–2.38 (m, 2H), 1.83 (dd, J = 17.7, 7.0 Hz, 5H), 1.45 (d, J = 5.6 Hz, 3H, CH_3), 1.32–1.28 (m, 6H, 2× CH_3), 1.28–1.26 (m, 6H, 2× CH_3), 1.23 (dd, J = 11.6, 4.5 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.75, 180.47, 178.09, 159.82, 152.67, 147.25, 133.02, 130.19, 129.60, 125.22, 123.31, 120.08, 114.16, 113.96, 63.60, 63.36, 57.30, 56.09, 55.27, 47.47, 43.25, 37.42, 37.06, 36.94, 36.68, 33.61, 23.75, 18.10, 16.41, 16.32, 16.15. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.37. HRMS (*m*/*z*) (ESI): calcd for $C_{33}H_{45}N_2O_6PS$ [M + H^+]: 629.28142; found: 629.28194.

*4.3.1.5. Compound (**4e**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (4-methyl phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4e**.

Yields 82.58%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.49 (s, 1H, NH), 8.59 (s, 1H, NH), 7.87–7.84 (m, 1H), 7.42 (dd, J = 8.2, 2.1 Hz, 1H), 7.31–7.26 (m, 3H), 7.16 (dd, J = 11.0, 8.3 Hz, 2H), 6.06–5.97 (m, 1H, N–CH–P), 4.11–3.92 (m, 4H, 2× OCH_2CH_3), 2.97–2.89 (m, 1H), 2.72–2.65 (m, 2H), 2.46–2.36 (m, 2H), 2.33–2.31 (m, 3H, CH_3), 1.86–1.68 (m, 5H), 1.42 (d, J = 5.3 Hz, 3H, CH_3), 1.28 (dd, J = 8.2, 5.7 Hz, 6H, 2× CH_3), 1.26–1.24 (m, 6H, 2× CH_3), 1.23–1.18 (m, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.61, 179.99, 178.14, 152.32, 147.27, 138.15, 132.80, 130.62, 129.44, 128.12, 128.02, 125.21, 123.27, 63.58, 63.33, 57.09, 55.91, 47.48, 43.24, 37.42, 37.25, 36.97, 36.71, 33.62, 23.79, 23.74, 21.17, 18.09, 16.42, 16.38, 16.34. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.68. HRMS (*m*/*z*) (ESI): calcd for $C_{33}H_{45}N_2O_5PS$ [M + H^+]: 613.28650; found: 613.28740.

*4.3.1.6. Compound (**4f**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (3-methyl phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4f**.

Yields 80.21%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.68 (s, 1H, NH), 8.62 (s, 1H, NH), 7.44 (dd, J = 8.1, 1.8 Hz, 1H), 7.32–7.27 (m, 2H), 7.25–7.21 (m, 2H), 7.14–7.06 (m, 1H), 6.05–5.98 (m, 1H, N–CH–P), 4.13–3.92 (m, 4H, 2× OCH_2CH_3), 3.00–2.91 (m, 1H), 2.77–2.69 (m, 2H), 2.49–2.40 (m, 2H), 2.37 (s, 3H, CH_3), 1.83–1.76 (m, 5H), 1.45 (d, J = 5.5 Hz, 3H, CH_3), 1.30–1.28 (m, 6H, 2× CH_3), 1.27 (d, J = 6.9 Hz, 6H, 2× CH_3), 1.25–1.20 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.64, 180.42, 178.06, 152.42, 147.17, 138.39, 132.79, 130.64, 129.23, 129.17, 128.94, 128.68, 128.60, 125.22, 123.27, 63.58, 63.32, 57.41, 56.20, 47.48, 43.37, 43.27, 37.42, 37.26, 37.13, 36.99, 36.72, 33.62, 23.81, 23.73, 18.10, 16.34, 16.28, 16.24. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.61. HRMS (*m*/*z*) (ESI): calcd for $C_{33}H_{45}N_2O_5PS$ [M + H^+]: 613.28650; found: 613.28774.

*4.3.1.7. Compound (**4g**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (4-fluor phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4g**.

Yields 80.42%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.49 (s, 1H, NH), 8.60 (s, 1H, NH), 7.87 (d, J = 2.1 Hz, 1H), 7.44–7.38 (m, 3H), 7.29 (d, J = 8.2 Hz, 1H), 7.11–7.07 (m, 2H), 6.03–5.98 (m, 1H, N–CH–P), 4.12–3.94 (m, 4H, 2× OCH_2CH_3), 2.98–2.93 (m, 1H), 2.72–2.64 (m, 2H), 2.43–2.34 (m, 2H), 1.90–1.72 (m, 5H), 1.43 (s, 3H, CH_3), 1.29–1.26 (m, 6H, 2× CH_3), 1.25 (dd, J = 5.3, 1.6 Hz, 6H, 2× CH_3), 1.21 (t, J = 7.1 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.69, 180.37, 178.36, 161.66, 152.41, 132.86, 130.57, 129.99, 129.94, 125.20, 123.30, 115.80, 115.62, 63.67, 63.44, 56.58, 55.39, 47.53, 43.42, 43.30, 37.44, 37.26, 37.07, 36.93, 36.70, 33.62, 23.78, 23.74, 18.08, 16.41, 16.35, 16.31. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.22. HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{42}FN_2O_5PS$ [M + H^+]: 617.26143; found: 617.26187.

*4.3.1.8. Compound (**4h**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (2-fluor phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4h**.

Yields 81.23%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.56 (s, 1H, NH), 8.60 (s, 1H, NH), 7.86 (dd, J = 3.9, 2.1 Hz, 1H), 7.42 (m, J = 8.2, 2.1 Hz, 1H), 7.34–7.32 (m, 1H), 7.30–7.26 (m, 2H), 7.18–7.06 (m, 2H), 6.37–6.30 (m, 1H, N–CH–P), 4.16–3.96 (m, 4H, 2× OCH_2CH_3), 2.97–2.89 (m, 1H), 2.75–2.64 (m, 2H), 2.46–2.34 (m, 2H), 1.86–1.72 (m, 5H), 1.43 (d, J = 3.6 Hz, 3H, CH_3), 1.29 (dd, J = 12.2, 4.9 Hz, 6H, 2× CH_3), 1.26–1.24 (m, 6H, 2× CH_3), 1.21 (dd, J = 13.8, 6.9 Hz, 3H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 197.64, 180.51, 178.15, 152.35, 147.32, 132.86, 130.58, 130.06, 130.00, 129.95, 125.24, 123.29, 115.89, 115.64, 63.66, 63.48, 56.63, 55.37, 47.54, 43.32, 37.45, 37.26, 37.10, 36.71, 33.63, 23.82, 23.74, 18.11, 16.40, 16.32, 16.22. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.56. HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{42}FN_2O_5PS$ [M + H^+]: 617.26143; found: 617.26163.

*4.3.1.9. Compound (**4i**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (3-fluor phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4i**.

Yields 85.52%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.53 (s, 1H, NH), 8.64 (s, 1H, NH), 7.94–7.82 (m, 1H), 7.45 (dd, J = 8.2, 2.1 Hz, 1H), 7.36 (dd, J = 13.0, 5.6 Hz, 1H), 7.31 (d, J = 8.2 Hz, 1H), 7.23 (d, J = 7.7 Hz, 1H), 7.19–7.12 (m, 1H), 7.03 (q, J = 8.5 Hz, 1H), 6.02–5.98 (m, 1H, N–CH–P), 4.16–3.99 (m, 4H, 2× OCH_2CH_3), 2.95 (dd, J = 13.9, 6.9 Hz, 1H), 2.76–2.68 (m, 2H), 2.49–2.38 (m, 2H), 1.88–1.71 (m, 5H), 1.46 (dd, J = 4.8 Hz, 3H, CH_3), 1.32–1.29 (m, 6H, 2× CH_3), 1.28–1.27 (m, 6H, 2× CH_3), 1.27–1.23 (m, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.55, 180.69, 178.29, 152.32, 147.28, 132.81, 130.60, 130.17, 125.26, 125.21, 123.29, 123.25, 115.47, 115.29, 63.70, 63.52, 57.01, 55.75, 47.55, 43.46, 37.43, 37.28, 37.10, 36.69, 36.72, 33.62, 23.80, 23.72, 18.11, 16.37, 16.33, 16.31. ^{31}P NMR (200 MHz, $CDCl_3$) δ 18.82.

HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{42}FN_2O_5PS$ [M + H^+]: 617.26143; found: 617.25993.

*4.3.1.10. Compound (**4j**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (4-chloro phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4j**.

Yields 84.37%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.50 (s, 1H, NH), 8.62 (s, 1H, NH), 7.86 (d, J = 2.1 Hz, 1H), 7.42 (dd, J = 8.2, 2.1 Hz, 1H), 7.36–7.33 (m, 2H), 7.33–7.30 (m, 2H), 7.28 (d, J = 8.2 Hz, 1H), 5.96 (m, 1H, N–CH–P), 4.13–3.94 (m, 4H, 2× O$\underline{CH_2}CH_3$), 2.98–2.93 (m, 1H), 2.70 (dd, J = 7.1, 4.0 Hz, 2H), 2.46–2.34 (m, 2H), 1.86–1.74 (m, 5H), 1.43 (s, 3H, CH_3), 1.29–1.26 (m, 6H, 2× CH_3), 1.26–1.24 (m, 6H, 2× CH_3), 1.23 (d, J = 5.0 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.48, 180.55, 178.38, 152.34, 147.31, 134.29, 132.86, 132.24, 130.57, 129.58, 129.54, 128.91, 125.21, 123.31, 63.73, 63.51, 56.72, 55.43, 47.55, 43.42, 37.44, 37.21, 36.96, 36.71, 33.63, 23.81, 23.75, 18.08, 16.42, 16.37, 16.32. ^{31}P NMR (200 MHz, $CDCl_3$) δ 18.96. HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{42}ClN_2O_5PS$ [M + H^+]: 633.23188; found: 633.22879.

*4.3.1.11. Compound (**4k**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (2-chloro phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4k**.

Yields 85.54%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.64 (s, 1H, NH), 8.64 (s, 1H, NH), 7.89 (d, J = 5.8 Hz, 1H), 7.41 (m, 3H), 7.35–7.29 (m, 2H), 7.26 (dd, J = 6.3, 4.6 Hz, 1H), 6.52–6.45 (m, 1H, N–CH–P), 4.19–3.90 (m, 4H, 2× O$\underline{CH_2}CH_3$), 2.99–2.91 (m, 1H), 2.72 (dd, J = 14.5, 7.9 Hz, 2H), 2.49–2.38 (m, 2H), 1.92–1.73 (m, 5H), 1.45 (d, J = 3.4 Hz, 3H, CH_3), 1.37–1.30 (m, 6H, 2× CH_3), 1.28–1.26 (m, 6H, 2× CH_3), 1.22–1.17 (m, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.38, 180.52, 180.02, 152.38, 147.23, 134.35, 132.79, 132.25, 130.65, 129.80, 128.95, 127.36, 125.22, 123.24, 63.71, 63.53, 54.35, 53.13, 47.52, 47.48, 43.46, 43.26, 37.41, 37.25, 37.13, 36.96, 36.73, 33.61, 23.79, 23.72, 18.10, 16.35, 16.31, 16.20. ^{31}P NMR (200 MHz, $CDCl_3$) δ 18.73. HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{42}ClN_2O_5PS$ [M + H^+]: 633.23188; found: 633.23291.

*4.3.1.12. Compound (**4l**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (3-chloro phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4l**.

Yields 86.55%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.51 (s, 1H, NH), 8.64 (s, 1H, NH), 7.97–7.81 (m, 1H), 7.44 (dd, J = 8.2, 2.2 Hz, 1H), 7.41 (dd, J = 11.9, 1.6 Hz, 1H), 7.35–7.31 (m, 2H), 7.31–7.29 (m, 2H), 6.02–5.94 (m, 1H, N–CH–P), 4.14–3.96 (m, 4H, 2× O$\underline{CH_2}CH_3$), 2.97–2.91 (m, 1H), 2.72 (m, 2H), 2.48–2.36 (m, 2H), 1.93–1.70 (m, 5H), 1.45 (d, J = 5.7 Hz, 3H, CH_3), 1.31–1.28 (m, 6H, 2× CH_3), 1.27–1.25 (m, 6H, 2× CH_3), 1.26–1.22 (m, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.40, 180.70, 178.27, 152.50, 147.20, 135.65, 134.55, 132.84, 130.61, 129.92, 128.47, 126.43, 125.19, 123.28, 63.75, 63.54, 56.89, 55.68, 47.54, 43.33, 37.44, 37.26, 37.08, 36.96, 36.70, 33.62, 23.82, 23.75, 18.08, 16.39, 16.34, 16.32. ^{31}P NMR (200 MHz, $CDCl_3$) δ 18.78. HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{42}ClN_2O_5PS$ [M + H^+]: 633.23188; found: 633.23221.

*4.3.1.13. Compound (**4m**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (4-bromo phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4m**.

Yields 89.27%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.68 (s, 1H, NH), 8.62 (s, 1H, NH), 7.86 (dd, J = 4.7, 2.1 Hz, 1H), 7.48 (dd, J = 12.6, 8.3 Hz, 2H), 7.42 (dd, J = 8.2, 2.1 Hz, 1H), 7.30–7.27 (m, 3H), 6.01–5.94 (m, 1H, N–CH–P), 4.11–3.95 (m, 4H, 2× O$\underline{CH_2}CH_3$), 2.93 (dd, J = 7.5, 6.3 Hz, 1H), 2.72–2.65 (m, 2H), 2.43–2.35 (m, 2H), 1.87–1.65 (m, 5H), 1.42 (d, J = 4.8 Hz, 3H), 1.27 (d, J = 6.5 Hz, 6H, 2× CH_3), 1.25–1.24 (m, 6H, 2× CH_3), 1.21 (t, J = 6.9 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.50, 180.46, 178.35, 152.33, 132.84, 131.92, 131.84, 130.59, 129.89, 129.84, 125.24, 125.21, 123.29, 63.69, 63.55, 56.81, 55.60, 47.55, 43.44, 43.35, 37.45, 37.27, 37.08, 36.99, 36.72, 33.62, 23.78, 23.73, 18.09, 16.40, 16.35, 16.31. ^{31}P NMR (200 MHz, $CDCl_3$) δ 18.79. HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{42}BrN_2O_5PS$ [M + H^+]: 677.18137; found: 677.17973.

*4.3.1.14. Compound (**4n**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (3-bromo phenyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4n**.

Yields 88.49%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.52 (s, 1H, NH), 8.64 (s, 1H, NH), 7.89 (d, J = 2.0 Hz, 1H), 7.56 (d, J = 1.8 Hz, 1H), 7.48–7.43 (m, 2H), 7.38 (d, J = 7.8 Hz, 1H), 7.31 (d, J = 8.2 Hz, 1H), 7.27–7.25 (m, 1H), 6.01–5.94 (m, 1H, N–CH–P), 4.15–4.00 (m, 4H, 2× O$\underline{CH_2}CH_3$), 2.99–2.93 (m, 1H), 2.76–2.70 (m, 2H), 2.49–2.38 (m, 2H), 1.84–1.80 (m, 5H), 1.47 (s, 3H, CH_3), 1.31–1.29 (m, 6H, 2× CH_3), 1.28–1.26 (m, 6H, 2× CH_3), 1.25 (d, J = 2.0 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.47, 180.50, 178.10, 152.37, 147.33, 134.09, 133.14, 132.74, 130.65, 129.59, 128.99, 127.77, 125.23, 124.82, 123.23, 63.74, 63.61, 56.80, 47.48, 43.47, 43.23, 37.41, 37.28, 37.15, 36.97, 36.74, 33.62, 26.92, 23.79, 23.72, 18.10, 16.36, 16.21, 16.16. ^{31}P NMR (200 MHz, $CDCl_3$) δ 18.78. HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{42}BrN_2O_5PS$ [M + H^+]: 677.18137; found: 677.17949.

*4.3.1.15. Compound (**4o**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (1-naphthyl methyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4o**.

Yields 85.32%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.73 (s, 1H, NH), 8.60 (s, 1H, NH), 8.30 (dd, J = 8.4, 6.1 Hz, 1H), 7.88–7.81 (m, 3H), 7.63–7.57 (m, 2H), 7.53–7.46 (m, 2H), 7.42 (m, 1H), 7.28 (dd, J = 8.2, 2.2 Hz, 1H), 6.94–6.88 (m, 1H, N–CH–P), 4.14–3.63 (m, 4H, 2× O$\underline{CH_2}CH_3$), 2.98–2.90 (m, 1H), 2.75–2.63 (m, 2H), 2.41–2.36 (m, 2H), 1.81 (d, J = 10.0 Hz, 5H), 1.41 (d, J = 7.4 Hz, 3H, CH_3), 1.31–1.26 (m, 6H, 2× CH_3), 1.26–1.23 (m, 6H, 2× CH_3), 1.00–0.94 (m, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.57, 180.00, 178.30, 152.60, 147.31, 133.80, 132.79, 131.40, 130.65, 129.05, 128.77, 126.65, 125.96, 125.41, 125.22, 123.72, 123.64, 123.27, 63.68, 63.41, 53.47, 52.25, 47.47, 43.39, 43.24, 37.43, 37.24, 37.15, 36.94, 36.71, 33.63, 23.84, 23.75, 18.08, 16.44, 16.34, 16.09. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.72. HRMS (*m*/*z*) (ESI): calcd for $C_{36}H_{45}N_2O_5PS$ [M + H^+]: 649.28650; found: 649.28771.

*4.3.1.16. Compound (**4p**).* According to the general procedure, compound **3** was treated with diethyl 1-amin (2-naphthyl methyl methyl) phosphonate and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **4p**.

Yields 85.76%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.65 (s, 1H, NH), 8.63 (s, 1H, NH), 7.91–7.89 (m, 3H), 7.87–7.81 (m, 2H), 7.57 (dd, J = 8.5, 1.5 Hz, 1H), 7.51–7.47 (m, 2H), 7.46–7.42 (m, 1H), 7.31 (d, J = 8.1 Hz, 1H), 6.24–6.17 (m, 1H, N–CH–P), 4.15–3.94 (m, 4H, 2× O$\underline{CH_2}CH_3$), 2.99–2.92 (m, 1H), 2.73 (dd, J = 16.7, 8.9 Hz, 2H), 2.50–2.37 (m, 2H), 1.91–1.68 (m, 5H), 1.45 (d, J = 6.5 Hz, 3H, CH_3), 1.32–1.28 (m, 6H, 2× CH_3), 1.27–1.26 (m, 6H, 2× CH_3), 1.25–1.19 (m, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.58, 180.24, 177.96, 152.35, 147.20, 133.14, 132.82, 131.04, 130.63, 128.65, 128.55, 128.24, 128.16, 127.67, 127.49, 126.34, 125.68, 125.21, 123.29, 63.66, 63.43, 57.55, 56.34, 47.50, 43.37, 43.28, 37.46, 37.26, 37.14, 36.98, 36.71, 33.63,

23.82, 23.75, 18.09, 16.45, 16.40, 16.36. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.43. HRMS (*m*/*z*) (ESI): calcd for $C_{36}H_{45}N_2O_5PS$ [M + H^+]: 649.28650; found: 649.28765.

*4.3.2. General procedure for compounds **5a–o***

Compounds **4** (1 mmol) and hydroxylamine hydrochloride were added to ethanol (15 mL) and the mixture was stirred at 80 °C for 8 h. After the reaction was completed, the solvent was evaporated under reduced pressure, and the crude product was purified by chromatography on silica gel eluted with petroleum ether/ethyl acetate (v:v = 3:1) to obtain compounds **5**. The structures were confirmed by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR (see Supporting information).

*4.3.2.1. Compound (**5a**).* According to the general procedure, compound **4a** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5a**.

Yields 85.58%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.60 (s, 1H, NH), 8.64 (s, 1H, NH), 7.76–7.68 (m, 1H), 7.44 (t, *J* = 7.1 Hz, 2H), 7.37 (dd, *J* = 15.1, 7.5 Hz, 2H), 7.31 (dd, *J* = 8.7, 3.6 Hz, 1H), 7.26–7.19 (m, 2H), 6.11–6.03 (m, 1H, N–CH–P), 4.14–3.96 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.96–2.85 (m, 1H), 2.75–2.60 (m, 2H), 2.35–2.27 (m, 2H), 1.89–1.53 (m, 6H), 1.48–1.45 (m, 3H, CH_3), 1.28–1.25 (m, 6H, 2× CH_3), 1.25–1.20 (m, 6H, 2× CH_3), 1.13 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.48, 178.63, 155.28, 148.31, 147.01, 133.47, 128.75, 128.55, 128.46, 128.33, 128.22, 128.17, 122.76, 122.69, 63.66, 63.51, 57.36, 56.15, 47.44, 41.35, 41.23, 37.80, 37.64, 36.76, 36.58, 33.72, 24.05, 23.78, 22.98, 18.05, 16.48, 16.37, 16.27. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.56. HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{44}N_3O_5PS$ [M + H^+]: 614.28175; found: 614.27985.

*4.3.2.2. Compound (**5b**).* According to the general procedure, compound **4b** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5b**.

Yields 81/36%; ^{1}H NMR (500 MHz, DMSO): δ 11.29 (s, 1H, NH), 8.63 (s, 1H, NH), 7.73 (dd, *J* = 7.8, 1.1 Hz, 1H), 7.38 (dd, *J* = 11.0, 4.2 Hz, 2H), 7.27–7.12 (m, 2H), 6.90 (t, *J* = 8.3 Hz, 2H), 6.01–5.95 (m, 1H, N–CH–P), 4.10–3.95 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 3.79 (s, 3H, OCH_3), 2.99–2.82 (m, 1H), 2.78–2.59 (m, 2H), 2.39–2.21 (m, 2H), 1.99–1.52 (m, 6H), 1.46 (d, *J* = 6.0 Hz, 3H, CH_3), 1.27–1.25 (m, 6H, 2× CH_3), 1.24–1.20 (m, 6H, 2× CH_3), 1.13 (d, *J* = 2.0 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.37, 178.65, 159.57, 155.27, 155.12, 148.32, 146.95, 129.52, 128.56, 128.47, 125.46, 122.75, 122.60, 114.05, 63.61, 63.39, 56.66, 55.44, 55.27, 47.41, 41.33, 41.20, 37.79, 37.64, 36.75, 36.58, 33.71, 24.03, 23.78, 22.97, 18.04, 16.47, 16.40, 16.35. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.85. HRMS (*m*/*z*) (ESI): calcd for $C_{33}H_{46}N_3O_6PS$ [M + H^+]: 644.29232; found: 644.28937.

*4.3.2.3. Compound (**5c**).* According to the general procedure, compound **4c** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5c**.

Yields 80.37%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.37 (s, 1H, NH), 8.58 (s, 1H, NH), 7.75 (dd, *J* = 12.5, 1.7 Hz, 1H), 7.33–7.27 (m, 2H), 7.27–7.19 (m, 2H), 6.99–6.87 (m, 2H), 6.47 (m, 1H, N–CH–P), 4.19–3.95 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 3.91 (s, 3H, OCH_3), 2.96–2.83 (m, 1H), 2.69 (m, 2H), 2.36–2.27 (m, 2H), 2.15–1.51 (m, 6H), 1.45 (d, *J* = 6.0 Hz, 3H, CH_3), 1.28–1.26 (m, 6H, 2× CH_3), 1.25–1.16 (m, 6H, 2× CH_3), 1.13 (d, *J* = 2.4 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.84, 177.98, 157.30, 155.36, 148.43, 146.92, 129.60, 128.59, 128.12, 122.80, 122.70, 122.01, 120.92, 111.32, 63.32, 63.16, 55.86, 53.84, 52.61, 47.35, 41.33, 41.12, 37.77, 37.63, 36.75, 36.58, 33.72, 30.94, 24.03, 23.78, 23.01, 18.06, 16.54, 16.48, 16.30. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.82. HRMS (*m*/*z*) (ESI): calcd for $C_{33}H_{46}N_3O_6PS$ [M + H^+]: 644.29232; found: 644.28961.

*4.3.2.4. Compound (**5d**).* According to the general procedure, compound **4e** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5d**.

Yields 89.68%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.56 (s, 1H, NH), 8.62 (s, 1H, NH), 7.73 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.35–7.30 (m, 2H), 7.24 (m, 1H), 7.22–7.13 (m, 3H), 6.08–5.97 (m, 1H, N–CH–P), 4.18–3.90 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.96–2.85 (m, 1H), 2.76–2.61 (m, 2H), 2.33 (s, 3H, CH_3), 2.32–2.25 (m, 2H), 1.80–1.62 (m, 6H), 1.47 (d, *J* = 6.1 Hz, 3H, CH_3), 1.28–1.26 (m, 6H, 2× CH_3), 1.25–1.20 (m, 6H, 2× CH_3), 1.14 (d, *J* = 2.1 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.34, 178.55, 155.27, 148.30, 146.99, 138.06, 130.41, 129.47, 128.52, 128.42, 128.25, 128.12, 128.02, 122.74, 122.67, 122.58, 63.58, 63.38, 57.10, 55.89, 47.42, 41.34, 41.24, 37.79, 37.65, 36.75, 36.57, 33.72, 30.94, 24.04, 23.77, 22.96, 21.18, 18.05, 16.47, 16.37, 16.28. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.76. HRMS (*m*/*z*) (ESI): calcd for $C_{33}H_{46}N_3O_5PS$ [M + H^+]: 628.29740; found: 628.29663.

*4.3.2.5. Compound (**5e**).* According to the general procedure, compound **4f** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5e**.

Yields 88.65%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.68 (s, 1H, NH), 8.65 (s, 1H, NH), 7.73 (dd, *J*v7.3, 1.5 Hz, 1H), 7.29–7.24 (m, 3H), 7.24–7.18 (m, 2H), 7.13 (d, *J* = 4.9 Hz, 1H), 6.09–6.01 (m, 1H, N–CH–P), 4.14–3.93 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.97–2.83 (m, 1H), 2.77–2.64 (m, 2H), 2.36 (d, *J* = 6.0 Hz, 3H), 2.33 (dd, *J* = 11.4, 4.1 Hz, 2H), 2.09–1.52 (m, 6H), 1.47 (d, *J* = 5.2 Hz, 3H, CH_3), 1.30–1.26 (m, 6H, 2× CH_3), 1.26–1.20 (m, 6H, 2× CH_3), 1.14 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.50, 178.59, 155.09, 148.20, 146.97, 138.40, 133.35, 129.18, 128.96, 128.65, 128.41, 125.19, 122.74, 122.62, 122.56, 63.64, 63.42, 57.31, 56.10, 47.44, 41.37, 41.27, 37.79, 37.65, 36.77, 36.58, 33.72, 24.06, 23.79, 22.97, 21.47, 18.05, 16.47, 16.37, 16.31. ^{31}P NMR (200 MHz, $CDCl_3$): δ 19.71. HRMS (*m*/*z*) (ESI): calcd for $C_{33}H_{46}N_3O_5PS$ [M + H^+]: 628.29740; found: 628.29663.

*4.3.2.6. Compound (**5f**).* According to the general procedure, compound **4g** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5f**.

Yields 81.57%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.69(s, 1H, NH), 8.66 (s, 1H, NH), 7.75 (s, 1H), 7.47–7.37 (m, 2H), 7.28–7.20 (m, 2H), 7.06–6.98 (m, 2H), 6.06–5.98 (m, 1H, N–CH–P), 4.16–3.97 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.98–2.84 (m, 1H), 2.77–2.62 (m, 2H), 2.36–2.27 (m, 2H), 1.74 (dd, *J* = 57.2, 26.4 Hz, 6H), 1.48 (s, 3H, CH_3), 1.31–1.27 (m, 6H, 2× CH_3), 1.24 (dd, *J* = 16.1, 9.0 Hz, 6H, 2× CH_3), 1.15 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.55, 178.67, 163.62, 161.64, 155.21, 148.37, 147.02, 130.00, 129.50, 128.60, 128.12, 122.81, 122.63, 115.79, 115.62, 63.69, 63.57, 56.58, 55.36, 47.48, 41.41, 37.61, 36.77, 36.60, 33.73, 29.69, 24.03, 23.77, 22.99, 18.03, 16.45, 16.36, 16.32. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.36. HRMS (*m*/*z*) (ESI): calcd for $C_{32}H_{43}FN_3O_5PS$ [M − H^+]: 630.25668; found: 630.25848.

*4.3.2.7. Compound (**5g**).* According to the general procedure, compound **4h** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5g**.

Yields 80.65%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.69 (s, 1H, NH), 8.66 (s, 1H, NH), 7.75 (s, 1H), 7.47–7.37 (m, 2H), 7.28–7.20 (m, 2H), 7.06 (t, *J* = 8.4 Hz, 2H), 6.03–5.97 (m, 1H, N–CH–P), 4.16–3.97 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.98–2.84 (m, 1H), 2.77–2.62 (m, 2H), 2.36–2.27 (m, 2H), 1.74 (m, 6H), 1.48 (s, 3H, CH_3), 1.31–1.27 (m,

6H, 2× CH_3), 1.24 (dd, J = 16.1, 9.0 Hz, 6H, 2× CH_3), 1.15 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.70, 178.74, 163.60, 161.75, 155.23, 148.31, 146.99, 130.01, 129.96, 129.44, 128.59, 128.13, 122.71, 115.82, 115.64, 63.68, 63.50, 56.57, 55.35, 47.44, 41.20, 37.76, 36.72, 36.57, 33.71, 30.93, 24.02, 23.87, 23.75, 22.96, 18.04, 16.45, 16.36, 16.27. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.72. HRMS (m/z) (ESI): calcd for $C_{32}H_{43}FN_3O_5PS$ [M + H^+]: 632.27223; found: 632.27136.

*4.3.2.8. Compound (**5h**).* According to the general procedure, compound **4i** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5h**.

Yields 88.85%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.57 (s, 1H, NH), 8.67 (s, 1H, NH), 7.75 (dd, J = 9.9, 1.8 Hz, 1H), 7.37–7.31 (m, 1H), 7.25–7.21 (m, 3H), 7.16 (m, 1H), 7.05–6.97 (m, 1H), 6.09–6.01 (m, 1H, N–CH–P), 4.14–4.01 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.92–2.82 (m, 1H), 2.76–2.63 (m, 2H), 2.43–2.27 (m, 2H), 2.03–1.55 (m, 6H), 1.47 (dd, J = 13.2, 5.0 Hz, 3H, CH_3), 1.29–1.26 (m, 6H, 2× CH_3), 1.25 (dd, J = 6.4, 3.0 Hz, 6H, 2× CH_3), 1.14 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.47, 178.81, 163.87, 161.57, 155.19, 148.49, 146.87, 136.01, 132.85, 130.18, 128.55, 123.89, 122.74, 115.43, 63.76, 63.61, 56.93, 55.72, 47.49, 43.37, 41.38, 41.30, 37.75, 37.62, 36.75, 36.60, 33.72, 24.04, 23.77, 22.99, 18.05, 16.45, 16.39, 16.34. ^{31}P NMR (200 MHz, $CDCl_3$) δ 18.90. HRMS (m/z) (ESI): calcd for $C_{32}H_{43}FN_3O_5PS$ [M + H^+]: 632.27223; found: 632.27087.

*4.3.2.9. Compound (**5i**).* According to the general procedure, compound **4j** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5i**.

Yields 89.75%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.58 (s, 1H, NH), 8.67 (s, 1H, NH), 7.74 (d, J = 9.3 Hz, 1H), 7.37–7.32 (m, 4H), 7.30–7.20 (m, 2H), 6.06–5.98 (m, 1H, N–CH–P), 4.16–3.92 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.91 (dd, J = 8.8, 4.9 Hz, 1H), 2.69 (dd, J = 13.1, 4.8 Hz, 2H), 2.32–2.26 (m, 2H), 2.19–1.67 (m, 6H), 1.47 (d, J = 4.6 Hz, 3H, CH_3), 1.31–1.26 (m, 6H, 2× CH_3), 1.24 (dd, J = 9.2, 5.8 Hz, 6H, 2× CH_3), 1.14 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.78, 178.78, 155.21, 148.21, 147.11, 134.28, 132.19, 129.56, 128.93, 128.54, 128.24, 122.77, 122.66, 122.58, 66.76, 66.65, 56.68, 55.47, 47.47, 41.39, 41.25, 37.78, 37.62, 36.75, 36.58, 33.72, 24.05, 23.79, 22.98, 18.04, 16.46, 16.37, 16.35. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.05. HRMS (m/z) (ESI): calcd for $C_{32}H_{43}ClN_3O_5PS$ [M + H^+]: 648.24278; found: 648.23962.

*4.3.2.10. Compound (**5j**).* According to the general procedure, compound **4k** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5j**.

Yields 80.36%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.67 (s, 1H, NH), 8.61 (s, 1H, NH), 7.68 (dd, J = 5.1, 1.8 Hz, 1H), 7.38 (m, 2H), 7.30–7.24 (m, 2H), 7.23–7.17 (m, 2H), 6.55–6.48 (m, 1H, N–CH–P), 4.19–3.88 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.92–2.85 (m, 1H), 2.66–2.62 (m, 2H), 2.34–2.24 (m, 2H), 1.74–1.62 (m, 5H), 1.45 (d, J = 3.5 Hz, 3H, CH_3), 1.31–1.26 (m, 3H, CH_3), 1.24 (dd, J = 6.9, 3.1 Hz, 6H, 2× CH_3), 1.17–1.15 (m, 3H, CH_3), 1.13 (d, J = 1.3 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.54, 178.65, 154.91, 148.09, 146.76, 132.25, 129.77, 129.34, 129.01, 128.71, 128.18, 127.21, 122.72, 122.34, 63.71, 63.60, 54.34, 53.07, 47.53, 41.55, 41.40, 37.77, 37.61, 36.81, 36.57, 33.72, 24.07, 23.79, 23.47, 23.98, 18.07, 16.44, 16.22, 16.18. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.48. HRMS (m/z) (ESI): calcd for $C_{32}H_{43}ClN_3O_5PS$ [M–H^+]: 646.22713; found: 646.22931.

*4.3.2.11. Compound (**5k**).* According to the general procedure, compound **4l** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5k**.

Yields 88.72%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.59 (s, 1H, NH), 8.69 (d, J = 8.3 Hz, 1H, NH), 7.72 (dd, J = 6.7, 1.7 Hz, 1H), 7.42 (dd, J = 11.2, 1.3 Hz, 1H), 7.35–7.28 (m, 3H), 7.23–7.19 (m, 2H), 6.06–5.98 (m, 1H, N–CH–P), 4.15–3.99 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.96–2.85 (m, 1H), 2.76–2.63 (m, 2H), 2.39–2.27 (m, 2H), 2.14–1.58 (m, 6H), 1.47 (d, J = 4.8 Hz, 3H, CH_3), 1.31–1.26 (m, 6H, 2× CH_3), 1.24 (dd, J = 5.9, 4.8 Hz, 6H, 2× CH_3), 1.14 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.85, 178.94, 154.91, 148.19, 146.93, 135.60, 129.93, 128.52, 128.30, 126.37, 122.76, 122.56, 122.47, 63.85, 63.65, 56.88, 55.57, 47.52, 41.37, 36.76, 36.57, 33.72, 30.95, 24.07, 23.78, 23.64, 22.97, 18.04, 16.43, 16.34, 16.27. ^{31}P NMR (200 MHz, $CDCl_3$) δ 18.91. HRMS (m/z) (ESI): calcd for $C_{32}H_{43}ClN_3O_5PS$ [M + H^+]: 648.24278; found: 648.23950.

*4.3.2.12. Compound (**5l**).* According to the general procedure, compound **4m** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5l**.

Yields 89.24%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.55 (s, 1H, NH), 8.64 (s, 1H, NH), 7.72 (d, J = 8.5 Hz, 1H), 7.48 (t, J = 7.6 Hz, 2H), 7.30 (t, J = 7.4 Hz, 2H), 7.21 (dd, J = 19.2, 8.0 Hz, 2H), 6.02–5.93 (m, 1H, N–CH–P), 4.17–3.97 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.95–2.80 (m, 1H), 2.67–2.62 (m, 2H), 2.30 (dd, J = 16.2, 9.1 Hz, 2H), 2.04–1.53 (m, 5H), 1.45 (d, J = 4.3 Hz, 3H, CH_3), 1.28–1.24 (m, 6H, 2× CH_3), 1.21 (dd, J = 16.5, 8.6 Hz, 6H, 2× CH_3), 1.12 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.71, 178.88, 155.03, 148.21, 146.91, 132.86, 131.85, 129.90, 129.86, 128.37, 122.76, 122.62, 122.55, 63.77, 63.67, 56.72, 55.51, 47.51, 41.44, 41.27, 37.74, 37.59, 36.75, 36.58, 33.72, 30.94, 24.06, 23.79, 22.97, 18.04, 16.44, 16.36, 16.30. ^{31}P NMR (200 MHz, $CDCl_3$) δ 18.88. HRMS (m/z) (ESI): calcd for $C_{32}H_{43}BrN_3O_5PS$ [M + H^+]: 692.19227; found: 692.18927.

*4.3.2.13. Compound (**5m**).* According to the general procedure, compound **4n** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5m**.

Yields 87.55%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.73 (s, 1H, NH), 8.66 (s, 1H, NH), 7.78–7.72 (m, 1H), 7.55 (d, J = 1.8 Hz, 1H), 7.47–7.42 (m, 1H), 7.38 (d, J = 7.2 Hz, 1H), 7.29–7.16 (m, 3H), 6.01–5.96 (m, 1H, N–CH–P), 4.16–4.00 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 3.00–2.85 (m, 1H), 2.71–2.68 (m, 2H), 2.41–2.28 (m, 2H), 1.91–1.57 (m, 6H), 1.48 (d, J = 5.3 Hz, 3H, CH_3), 1.31–1.27 (m, 6H, 2× CH_3), 1.25 (dd, J = 6.9, 2.4 Hz, 6H, 2× CH_3), 1.14 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.71, 178.78, 155.31, 148.32, 147.00, 135.98, 131.45, 131.18, 131.13, 130.21, 128.66, 128.04, 126.87, 126.83, 122.82, 122.67, 63.79, 63.68, 56.79, 55.58, 47.46, 41.27, 37.61, 36.74, 36.59, 33.72, 30.94, 24.03, 23.78, 22.99, 18.02, 16.47, 16.33, 16.28. ^{31}P NMR (200 MHz, $CDCl_3$) δ 18.88. HRMS (m/z) (ESI): calcd for $C_{32}H_{43}BrN_3O_5PS$ [M + H^+]: 692.19227; found: 692.18982.

*4.3.2.14. Compound (**5n**).* According to the general procedure, compound **4o** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5n**.

Yields 86.35%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.74 (s, 1H, NH), 8.66 (s, 1H, NH), 8.34 (d, J = 8.6 Hz, 1H), 7.85 (dd, J = 16.6, 7.4 Hz, 2H), 7.73 (dd, J = 10.3, 1.6 Hz, 1H), 7.68–7.59 (m, 2H), 7.54–7.46 (m, 2H), 7.26–7.18 (m, 2H), 6.95–6.92 (m, 1H, N–CH–P), 4.17–3.68 (m, 4H, 2× O$\underline{CH_2}$$CH_3$), 2.89 (dd, J = 13.3, 6.7 Hz, 1H), 2.76–2.62 (m, 2H), 2.36–2.26 (m, 2H), 1.92–1.51 (m, 6H), 1.49–1.41 (m, 3H, CH_3), 1.34–1.25 (m, 6H, 2× CH_3), 1.24–1.12 (m, 6H, 2× CH_3), 0.99 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.61, 178.52, 154.95, 148.23, 147.06, 133.79, 131.38, 130.38, 130.30, 129.03, 128.73, 128.42, 126.66, 125.96, 125.39, 125.34, 123.69, 122.73, 122.63, 122.56, 63.69, 63.56, 53.50,

52.21, 47.41, 41.42, 41.31, 37.94, 37.69, 36.78, 36.58, 33.72, 30.94, 24.07, 23.78, 22.97, 18.04, 16.45, 16.08, 16.03. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.84. HRMS (m/z) (ESI): calcd for $C_{36}H_{46}BrN_3O_5PS$ [M + H$^+$]: 664.29740; found: 664.29510.

*4.3.2.15. Compound (**5o**).* According to the general procedure, compound **4p** was treated with hydroxylamine hydrochloride and then purified on silica gel column using petroleum ether/ethyl acetate (v:v = 3:1) to obtain compound **5o**.

Yields 87.74%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 11.70 (s, 1H, NH), 8.67 (s, 1H, NH), 7.84–7.79 (m, 4H), 7.71 (d, J = 11.9 Hz, 1H), 7.60–7.52 (m, 1H), 7.49–7.41 (m, 2H), 7.25–7.12 (m, 2H), 6.23–6.18 (m, 1H, N–CH–P), 4.13–3.91 (m, 4H, 2× O$\underline{CH_2}$CH$_3$), 2.88 (dd, J = 13.7, 6.8 Hz, 1H), 2.76–2.59 (m, 2H), 2.32 (dd, J = 11.6, 6.5 Hz, 2H), 1.86–1.57 (m, 5H), 1.45 (t, J = 8.4 Hz, 3H, CH_3), 1.29–1.23 (m, 6H, 2× CH_3), 1.20 (dd, J = 14.9, 6.4 Hz, 6H, 2× CH_3), 1.12 (d, J = 2.7 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.56, 178.81, 155.10, 148.21, 146.96, 132.93, 130.81, 128.59, 128.42, 128.34, 128.16, 127.65, 127.46, 126.32, 125.70, 122.75, 122.66, 122.58, 63.72, 63.54, 57.49, 56.28, 47.47, 41.37, 41.29, 37.76, 37.61, 36.76, 36.58, 33.71, 30.95, 24.05, 23.97, 22.97, 18.04, 16.46, 16.40, 16.35. ^{31}P NMR (200 MHz, $CDCl_3$) δ 19.54. HRMS (m/z) (ESI): calcd for $C_{36}H_{46}BrN_3O_5PS$ [M + H$^+$]: 664.29740; found: 664.29419.

4.4. In vitro cytotoxicity

The NCI-H460, A549, HepG2 and SKOV3 cell lines used in this study were all obtained from the Institute of Biochemistry and Cell Biology, China Academy of Sciences. All were supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2/95% air at 37 °C. In order to investigate the potential of compounds **4**–**5**, 5-FU, a commercial classical anticancer drug was used as a reference organic drug. Assays of cytotoxicity were determined in 96-well, flat bottomed microtiter plates. The supplemented culture medium with cell lines was added to the wells. Compounds **4**–**5**, and 5-FU were dissolved in the culture medium with 1% DMSO to give various concentrations (1.25, 2.5, 5, 10, 20 mg/mL, respectively). The resulted solutions were subsequently added to a set of wells. Control wells contained supplemented media with 1% DMSO. The microtiter plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air for a further 3 day. Cytotoxic screening by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted. At the end of each incubation period, the MTT solution (10 mL, 5 mg/mL) was added into each well and the cultures were incubated further for 48 h (for the time-dependent cytotoxic effects studies, the treatment time is 24, 48, 72 h, respectively) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. After removal of the supernatant, DMSO (150 mL) was added to dissolve the formazan crystals. The absorbance was read by enzyme labeling instrument with 570/630 nm double wavelength measurement. The cytotoxicity was estimated based on the percentage cell survival in a dose dependent manner relative to the negative control. The final IC_{50} values were calculated by the Bliss method (n = 5). All the tests were repeated in at least three independent experiments.

4.5. AO/EB staining

Cells were seeded at a concentration of 5 × 10^4 cell/mL in a volume of 2 mL on a sterile cover slip in six-well tissue culture plates. Following incubation, the medium was removed and replaced with fresh medium plus 10% fetal bovine serum and supplemented with compound **5f** (15 μM). After the treatment period, the cover slip with monolayer cells was inverted on a glass slide with 20 μL of AO/EB stain (100 mg/mL). Fluorescence was read on a Nikon ECLIPSETE2000-S fluorescence microscope (OLYMPUS Co., Japan).

4.6. Hoechst 333258 staining

Cells grown on a sterile cover slip in six-well tissue culture plates were treated with compounds for a certain range of time. The culture medium containing compounds was removed, and the cells were fixed in 4% paraformaldehyde for 10 min. After being washed twice with PBS, the cells were stained with 0.5 mL of Hoechst 33258 (Beyotime) for 5 min and then again washed twice with PBS. The stained nuclei were observed under a Nikon ECLIPSETE2000-S fluorescence microscope using 350 nm excitation and 460 nm emission.

4.7. Mitochondrial membrane potential staining

JC-1 probe was employed to measure mitochondrial depolarization in A549 cells. Briefly, Cells cultured in six-well plates after indicated treatments were incubated with an equal volume of JC-1 staining solution (5 μg/ml) at 37 °C for 20 min and rinsed twice with PBS. Mitochondrial membrane potentials were monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using a Nikon ECLIPSETE2000-S fluorescent microscope. Mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio.

4.8. TUNEL assay

The TUNEL method was performed to label 3′-end of fragmented DNA of the apoptotic A549 cells. The cells treated as indicated were fixed with 4% paraform phosphate buffer saline, rinsed with PBS, then permeabilized by 0.1% Triton X-100 for FITC end-labeling the fragmented DNA of the apoptotic A549 cells using TUNEL cell apoptosis detection kit. The FITC-labeled TUNEL-positive cells were imaged under a fluorescent microscopy by using 488 nm excitation and 530 nm emission.

4.9. DNA ladder assay

The 15 μL DNA and 3 μL 6× DNA loading buffer mixed samples were added into wells of 1% agarose gel in TAE containing 0.5 μg/mL ethidium bromide. Gel electrophoresis was run at 60 V for 2 h. DNA ladders were finally visualized by a UV light source and documented by photography. DNA extracted from apoptotic cells showed a distinct DNA ladder, and DNA from viable cells stayed on the top of the gel as a high-molecular weight band.

4.10. Flow cytometry

Prepared A549 cells (1 × 10^6 cells/mL) were washed twice with cold PBS and then resuspended gently in 500 μL of binding buffer. Thereafter, cells were stained in 5 μL of Annexin V-FITC and shaken well. Finally, the cells were mixed with 5 μL of PI, incubated for 20 min in the dark and subsequently analyzed using FACSCali-bur (Becton Dickinson).

4.11. Statistical analysis

All statistical analysis was performed with SPSS Version 10. Data was analyzed by one-way ANOVA. Mean separations were performed using the least significant difference method. Each experiment was replicated thrice, and all experiments yielded similar

results. Measurements from all the replicates were combined, and treatment effects were analyzed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.08.055.

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