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Synthesis and antitumor activities of novel rhein α-aminophosphonates conjugates

Gui-yang Yao [a,c,†], Man-yi Ye [a,†], Ri-zhen Huang [a], Ya-jun Li [a], Ying-ming Pan [a], Qing Xu [b], Zhi-Xin Liao [c,*], Heng-shan Wang [a,*]

[a] State Key Laboratory Cultivation Base for the Chemistry and Molecular Engineering of Medicinal Resources, School of Chemistry & Pharmaceutical Science of Guangxi Normal University, Guilin 541004, PR China
[b] College of Pharmacy, Guilin Medical University, Guilin 541004, PR China
[c] Department of Pharmaceutical Engineering, School of Chemistry and Chemical Engineering, Southeast University, Nanjing 211189, PR China

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ABSTRACT

Several rhein α-aminophosphonates conjugates (**5a**–**5q**) were synthesized and evaluated for in vitro cytotoxicity against HepG-2, CNE, Spca-2, Hela and Hct-116 cell lines. Some compounds showed relatively high cytotoxicity. Especially, compound **5i** exhibited the strongest cytotoxicity against Hct-116 cells (IC_{50} was 5.32 μM). All the synthesized compounds exhibited low cytotoxicity against HUVEC cells. The mechanism of compound **5i** was preliminarily investigated by Hoechst 33258 staining, JC-1 mitochondrial membrane potential staining and flow cytometry, which indicated that the compound **5i** induced apoptosis in Hct-116 cancer cells. Cell cycle analysis showed that these compound **5i** mainly arrested Hct-116 cells in G1 stage. The effects of **5i** on the activation of caspases expression indicated that **5i** might induce apoptosis via the membrane death receptor pathways. In addition, the binding properties of a model analog **5i** to DNA were investigated by methods (UV–vis, fluorescence, CD spectroscopy and FRET-melting) in compare with that of rhein. Results indicated that **5i** showed moderate ability to interact ct-DNA.

Improving the targeting of chemotherapeutic drugs to specific cells and organs is one of the emerging trends toward the development of new targeted anticancer therapies.[1] Colorectal cancer (CRC) is the third most commonly diagnosed type of cancer and the third leading cause of death from cancer.[2] However, only a few drugs are currently used in clinical for CRC treatments, including oxaliplatin,[3] leucovorin,[4] 5-FU and irinotecan.[5,6] In addition, the clinical application of traditional drugs was limited responsible for the serious adverse effects or the drug resistance. Thus, there is a need to develop more effective, less toxic and CRC cell-targeting drugs for clinical use.

Rhein, a main constituent of rhubarb, is a well-characterized anti-inflammatory agent which has been used for the treatment of inflammatory diseases such as ostarthritis, diabetic nephropathy, etc.[7] It has also been reported to display inhibitory effects on the proliferation of human breast, lung, NPC,[8] CNS, glioma cancer cells and Ca Ski cells.[9] Especially, it showed cytotoxic activity against human colon cancer cells (Hct-116 and COLO 320DM; IC_{50} were 135 μM and 62.5 μM, respectively).[10] Besides, it could induce apoptosis of Hct-116 cells via activation of the intrinsic apoptotic pathway. Although various synthetic analogs of rhein have been reported for the purpose of cytotoxicity evaluation,[11] structure modifications aimed for cancer cells targeting colorectal cancer has rarely been studied.

Recently, Berlicki and his co-workers suggested that the N–C–P molecular fragment and its chemistry could offer many possibilities for structural modifications,[12] which have resulted in broad biological relevance. As important classes of N–C–P contain molecular, α-aminophosphonates and their derivatives is an important class of compounds that exhibit intriguing biological activities,[13] especially anti-tumor and inhibitors of enzyme related to tumorgenesis and invasions.[14] However, there are far fewer reports about improving synergistic effect by α-aminophosphonates which was targeted certain cancer cells, especially colon cancer. Recently, it was found that aminophosphonate groups could enhance the antitumor activity of natural compound in our laboratory.[15] As part of our series of research work, rhein α-aminophosphonate conjugates were designed in our resent study to establish new strategy toward colon cancer therapy see Scheme 1.

Herein, we report (a) the synthesis of rhein α-aminophosphonic acids conjugates; (b) DNA binding of one potent conjugate **5i**; (c)

* Corresponding authors. Tel./fax: +86 0773 2120958.
E-mail addresses: zxliao@seu.edu.cn (Z.-X. Liao), whengshan@163.com (H. Wang).
† Co-first author: These authors contributed equally to this work.

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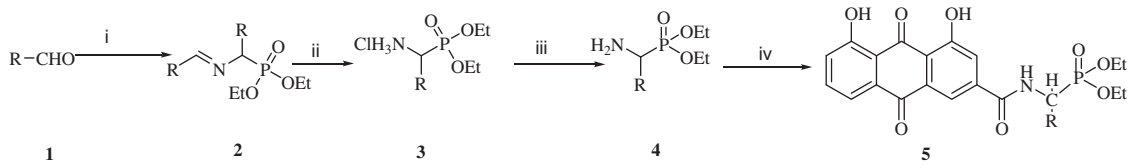

Scheme 1. General synthetic route for compounds **5a**–**5p**. Reagents and conditions: (i) NH_4Ac, $HPO(OC_2H_5)_2$; (ii) (i) HCl(aq), (iii) NaOH(aq); (iv) rhein, EDAC, HOBt, DMSO, rt.

in vitro antitumor activity and cell selectivity of synthesized conjugates; (d) the mechanism of the novel conjugate **5i** killed Hct-116 cells.

O,*O*'-dialky{[*N*-(phenylmethylene)-α-amino]-α-(substituted phenyl)methyl}phosphonates **2**, obtained by reacting substituted benzaldehyde **1** with ammonium acetate and *O*,*O*'-dialkyl phosphite, were converted to *O*,*O*'-dialkyl α-amino-[α-(substituted phenyl)methyl] phosphonate **4** via hydrolysis.[16] The α-aminophosphonates **4** were then coupled with rhein to provide rhein α-aminophosphonic acids conjugates **5** in satisfactory yields.

All of compounds **5** were obtained as yellowish solids after column chromatography. Their structures were fully characterized by ^{1}H NMR. For example, the corresponding ^{1}H NMR spectrum showed the two methyleneoxy (OCH_2CH_3) groups attached with phosphorus appears as three multiplets at 3.30–4.30. The chemical shifts of the two methyl (OCH_2CH_3) hydrogens were different due to the low rate of environmental exchange caused by the slow rotation of the P–C bond. The carbonyl group leads to a downfield shift of the two phenolic hydroxyls. The structures of all the compounds were further confirmed by ^{13}C NMR, ^{31}P NMR, EI–MS and elemental analysis.[17]

The in vitro antitumor activities of all of these compounds were evaluated by MTT assay against HepG-2, CNE, Spca-2, Hela and Hct-116 cell lines, using 5-fluorouracil (5-FU) as the positive control. The tested results were shown in Table 1. As shown in Table 1, the IC_{50} values of most of the conjugates were lower than that of rhein. **5i** exhibited better cytotoxic effects than other compounds. These results demonstrated that large aromatic ring (e.g., **5l** and **5q**) as R substituent showed low cytotoxicity and meta-substituent as R is favorable to the cytotoxicity. As for Hct-116 cells, in the series of compounds (**5a**, **5b** and **5c)** substituted at the R position with bromo group derivatives, compound **5c** showed the strongest inhibitory activity with IC_{50} 40.18 ± 3.56 μM. Based on the above observation, it could be summarized that methoxy, methyl, fluoro and chloro groups at meta position in benzene group of α-aminophosphonate moiety may have important influence on the antitumor activity, while naphthalene and anthracene exhibited negative effect. In addition, **5i** exhibited similar activities compared to 5-fluorouracil which is clinical drug.[18] Moreover, compared our previous work,[15b] the antitumor activities of rhein α-aminophosphonates which have the H at α-position were similar to the rhein α-aminophosphonates which have the methyl at α-position.

These results showed that rhein α-aminophosphonate conjugates had selective cytotoxicity toward Hct-116 among the cell lines. In comparison, activities of compounds against normal cells (HUVEC) were also examined (Table 1). The results indicated that the anti-proliferative activity of some compounds against cancer cells was much higher than against normal cells.

DNA is generally the primary intracellular target of anticancer drugs,[19] and the interaction of small molecules with DNA plays an important role in many biological processes.[20] Recently, there is a great interest on the binding of small molecules with DNA owing to their possible applications as new cancer therapeutic agents.[21] In addition, DNA appears to be the major cellular target for most anthraquinone-based structures which is generally accepted by DNA-interactive drugs.[22] Furthermore, anticancer drugs targeting DNA can inhibit cell cycle progression and/or induce apoptosis by activation of cell cycle checkpoint in response to DNA damage.[23] However, to our best knowledge, little research has been devoted to the mechanism of rhein derivative and their DNA-binding properties. Because compounds **5** are isostructural, **5i** was selected as a representative to investigate their binding properties to DNA. To this end, several analytical methods, including UV–vis, fluorescence, circular dichroism spectroscopies and FRET-melting were performed.

Table 1
IC_{50}[a] values (μM) of rhein and derivatives **5a**–**5q** towards five selected tumor cell lines and normal cell lines for 48 h

Compd	IC_{50}(μM)						
	R	HepG-2	CNE	Spca-2	Hela	Hct-116	HUVEC
5a	*p*-Ph-Br	94.24 ± 5.69	42.47 ± 3.23	88.17 ± 7.12	98.24 ± 7.21	65.45 ± 6.51	>100
5b	*o*-Ph-Br	88.32 ± 7.14	55.68 ± 4.25	85.47 ± 6.23	79.63 ± 5.14	48.93 ± 4.12	>100
5c	*m*-Ph-Br	62.37 ± 2.17	40.12 ± 3.14	75.36 ± 4.89	78.23 ± 6.32	40.18 ± 3.56	>100
5d	*p*-Ph-F	59.38 ± 4.29	34.57 ± 2.31	24.31 ± 2.17	95.36 ± 4.56	57.82 ± 4.56	>100
5e	*o*-Ph-F	71.25 ± 3.69	67.59 ± 4.28	79.89 ± 6.89	94.25 ± 4.34	36.56 ± 5.67	>100
5f	*p*-Ph-Cl	87.34 ± 6.29	55.79 ± 3.18	89.47 ± 8.73	87.47 ± 5.13	61.19 ± 3.12	>100
5g	*m*-Ph-Cl	71.95 ± 2.37	45.56 ± 2.17	76.98 ± 8.27	73.24 ± 6.14	22.36 ± 2.12	>100
5h	*o*-Ph-Cl	39.19 ± 1.02	67.98 ± 5.71	28.27 ± 2.14	35.23 ± 2.23	48.20 ± 2.34	>100
5i	*m*-Ph-OCH_3	22.08 ± 1.30	24.14 ± 1.28	28.27 ± 3.44	19.34 ± 1.58	5.32 ± 0.75	>100
5j	*o*-Ph-OCH_3	30.01 ± 2.31	28.56 ± 1.87	14.97 ± 3.26	25.64 ± 2.59	8.37 ± 0.88	>100
5k	Ph	39.25 ± 2.09	34.28 ± 2.97	27.36 ± 1.89	38.19 ± 2.57	17.79 ± 1.23	>100
5l	2-Naphthalene	>100	>100	>100	>100	>100	>100
5m	*p*-Ph-OCH_3	44.36 ± 2.16	42.85 ± 3.47	38.94 ± 1.87	40.12 ± 3.47	22.06 ± 2.35	>100
5n	*m*-Ph-CH_3	62.22 ± 5.22	74.25 ± 2.37	67.68 ± 1.65	73.34 ± 6.89	45.35 ± 3.36	>100
5o	*p*-Ph-CH_3	77.86 ± 2.22	79.38 ± 6.24	74.36 ± 6.87	88.95 ± 4.68	54.32 ± 4.54	>100
5p	*m*-Ph-F	56.83 ± 2.34	49.22 ± 3.41	55.41 ± 4.32	64.83 ± 5.14	16.66 ± 3.40	>100
5q	9-Anthracene	>100	>100	>100	>100	>100	>100
Rhein		>100	>100	28.31 ± 1.40	>100	>100	89.74 ± 6.40
5-FU		29.98 ± 0.37	40.21 ± 1.98	42.20 ± 2.14	35.34 ± 2.72	5.78 ± 0.85	56 ± 4.40

[a] IC_{50} values are presented as the mean ± SD (standard error of the mean) from three separated experiments.

The UV–vis absorption spectroscopy was primarily employed to probe the binding modes of **5i** to the calf thymus DNA (ct-DNA). Rhein belongs to the same class of coplanar anthraquinones as daunorubicin and mitoxantrone, which have been in clinical use over 30 years for the treatment of various tumors and were used as DNA targeting agents.[24] Their function as non-covalent DNA binders is generally believed to be essential for their activity. The inherent absorbance of **5i** allowed us to investigate its interaction with ct-DNA by ultraviolet absorption spectrum. As shown in Figure 1, although the absorbance of **5i** changed upon addition of DNA, the mother compound (rhein) did not seem to have any change (Fig. b, ESI). This indicated that DNA was one potential target of **5** owing to the dianionic character of N–C–P molecular fragment. The DNA binding constant K_b was calculated by non-linear fitting according to the Equations (**1a**, ESI), the value of K_b for **5i** was found to be $1.03 \times 10^4\ M^{-1}$.

The binding ability of **5i** to ct-DNA was primarily investigated by competitive binding in which they served as an intercalative binding probe in competition with GelRed. GelRed, which is environmentally safe and ultra-sensitive for DNA staining, is a newly developed DNA intercalator to replace the classic DNA intercalator EB. Furthermore, both GelRed and EB bound with ct-DNA emit characteristic fluorescence at 590 nm upon 350 nm UV light excitation.[25]

In competitive binding experiments, GelRed and ct-DNA solutions were pre-incubated for 30 min to ensure sufficient interactions. The concentration ratio of GelRed to DNA was set at [GelRed]/[DNA] = 1:10 to ensure sufficient binding sites of DNA for GelRed. The emission spectras of the GelRed-ctDNA system in the absence and presence of **5i** were shown in Figure 2. GelRed-DNA binary solution system gave a characteristic fluorescence emission at around 590 nm when excited at 350 nm, indicating that GelRed molecules intercalated between the adjacent base pairs of DNA and sufficiently prevented fluorescence quenching by polar solvent molecules. The presence of **5i** considerably quenched the fluorescence emission of GelRed with the saturation state achieved at the [**5i**]/[GelRed] ratio of 8:1.

The quenching ability of **5i** to GelRed fluorescence can be quantitively estimated by their respective quenching constants, K_q, which were derived from the Stern–Volmer quenching equation. The K_q value for **5i** was 1.66×10^3, exhibiting similar intensive intercalation to DNA as rhein. Meanwhile, it can also be confirmed that the intercalative binding mode of **5i** to DNA was similar to GelRed.[26]

The circular dichroism (CD) is a useful technique to assess whether the nucleic acids undergo conformational changes as a result of complex formation or changes in environmental conditions.[27] As indicated in Figure 3, the CD spectra of ct-DNA

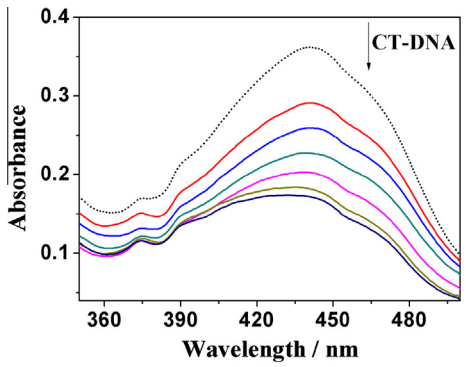

Figure 1. UV–vis absorption spectra of complex **5i** in the absence (—) and presence (—) of ct-DNA with increasing [DNA]/[**5i**] ratios in the range from 1:1 to 6:1.

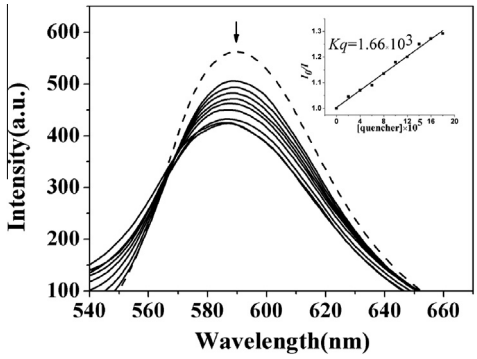

Figure 2. Fluorescence emission spectra of GelRed bound with ct-DNA ([DNA] = 2.0×10^{-4} M, [GelRed] = 2.0×10^{-5} M) in the absence (dash line) and presence (solid lines) of **5i** with [**5i**]/[GelRed] ratios range from 1:1 to 9:1. Inset: linear fitting for quenching constant K_q based on Stern–Volmer equation.

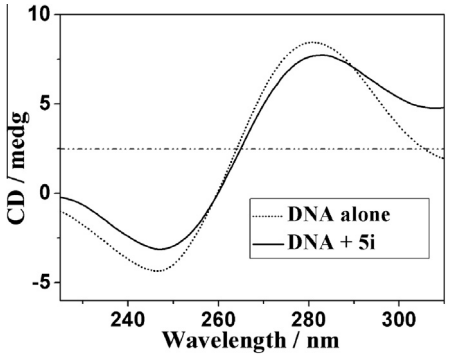

Figure 3. CD spectra of ct-DNA (2 mL solution, 1.5×10^{-4} M) in the absence and presence of **5i** (1.5×10^{-5} M).

(1.5×10^{-4} M) showed a positive absorption peak at 279 nm and a negative absorption peak at 248 nm due to π–π base stacking and right-hand helicity, respectively. This was consistent with the characteristic B conformation of DNA.[28]

Upon the addition of **5i** at a [**5i**]/[DNA] ratio of 1:10, the CD absorption of ct-DNA showed an obvious decrease in the intensities of both the negative and positive absorption bands. The percentage decreases in the maximal DNA positive and negative absorption by **5i** were 8.5% and 28.0%, respectively. It suggested that **5i** might intercalate between the neighbouring base pairs of ct-DNA mainly due to the aromatic planarity of the anthraquinone skeleton. The decrease in the intensities of both positive and negative bands can usually be observed in the intercalative binding of small molecules to DNA.[29]

Fluorescence signals may be created by various means to detect DNA hybridization during genotyping and similar assays.[30] The simplest method is to use an intercalating dye (e.g., SYBR Green I) that is highly specific for double-stranded DNA. Fluorescence resonance energy transfer (FRET) is a powerful tool for detecting spatial relationships between macromolecules, one use of which is the tracking of DNA hybridization status.[31]

Melting of the duplex structures was followed by fluorescence method (FRET measurements) and the FRET experiments are reported here (Fig. 4). The FRET results indicated that the rhein derivative (ΔT_m values was 3.1 °C) had higher stabilizing ability than the mother compound (rhein) (ΔT_m values was 2.0 °C) (Fig. c, ESI), which suggested that the dianionic character of N–C–P molecular fragment provided an important contribution of the rhein conjugates to interact with double-stranded (duplex) DNA. Based on the UV–vis, fluorescence, circular dichroism spectroscopies and

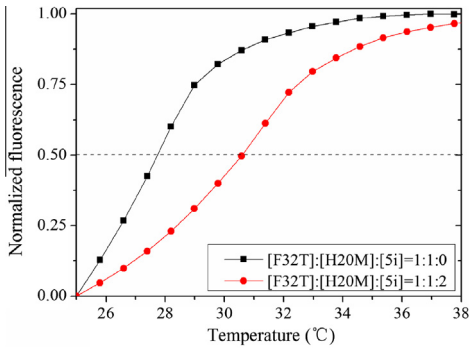

Figure 4. Representative FRET-melting profiles of F32T (0.5 μM) without and with increasing concentration of compound **5i**.

FRET-melting, it can be concluded that DNA is one potential target of **5i** as expected.

Hoechst 33258 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. On the contrary, the nuclei of dead cells could not be stained. Hct-116 cells which were treated with compound **5i** for 24 h at different concentrations were stained with Hoechst 33258. Hct-116 cells not treated with **5i** were used as control at for 24 h. The results were given in Figure 5. As shown in Figure 5, cells not treated with compound **5i** were normally blue. It was worth noting that, for **5i** treatment, the cells displayed strong blue fluorescence and indicated typical apoptotic morphology at 3 and 5 μM. The observation revealed that compound **5i** induced apoptosis against Hct-116 cells.

Mitochondrial membrane potential changes were also designed and detected to further investigate the apoptosis-inducing effect of target compound **5i**, employing a fluorescent probe JC-1. JC-1 is a lipophilic cationic dye and it can easily pass through the plasma membrane into cells and accumulate in actively respiring mitochondria. Hct-116 cells treated with **5i** for 24 h at different concentrations were stained with JC-1 and not treated with **5i** were used as control at for 24 h. The results were shown in Figure 6. The JC-1 monomer and J-aggregates were excited at 514 and 585 nm, respectively, and light emissions were collected at 515–545 nm (green) and 570–600 nm (red). For fluorescence microscopy, Figure 6 displayed that cells not treated with the compounds were normally red, while for **5i** treatment, cells exhibited strong green fluorescence as that for 5-FU which is known to induce the apoptosis (Fig. d, ESI) and indicated typical apoptotic morphology. Therefore, it could be concluded that compound **5i** induced apoptosis against Hct-116 cell line. The results were identical with that of previous experiment of Hoechst 33258 staining.

It was reported that many natural anthraquinones such as rhein and emodin could induce apoptosis.[32] Several studies have concluded that rhein induced apoptosis in HL-60 cells through the mitochondrial death pathway by causing the loss of mitochondrial membrane potential, cytochrome *c* release, and cleavage of Bid protein. In addition, it inhibited invasion in NPC cells by reducing the expression of matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF).[8] Furthermore, rhein can affect mitochondrial membrane permeability.[33] So, when the mitochondrial membrane potential is lost, ATP production ceases and the cell dies.[34] Recently, some α-aminophosphonic acid derivatives have been evaluated as inhibitors of matrix metalloproteinases MMP-1, MMP-2, MMP-3 and MMP-8.[35] Some derivatives were found to trigger apoptotic cell death in DOHH-2 cells.[36] Based on these facts, we postulated that these conjugates could induce apoptosis via reducing the expression of apoptosis-related proteins.

In the present study, the apoptotic cell rates were determined for the Hct-116 cells after stimulation for 24 h by the compound **5i** at different concentrations. The graphical values of the results were given in Figure 7. From the results, it was found that these compounds stimulated apoptosis for Hct-116 cells compared to negative (unstimulated cells by compounds) controls. Compound **5i** induced apoptosis in 33.5% of the Hct-116 cells in 5 μM. In addition, the apoptosis ratio of 5-FU was assayed to be 17.9% (Fig. e, ESI) under the similar condition. Meanwhile, we investigated the effects on the apoptosis of HUVEC cells. The results showed that the compounds we synthesized had no significant apoptotic effects on HUVEC cells (Fig. a, ESI).

Flow-cytometric analysis further confirmed tumor cell apoptosis as shown in Figure 8. Cytometric profiles of the PI-stained DNA showed cell cycle arrest of Hct-116 cells treated with **5i** for 48 h at different concentrations. As shown in Figure 8, the treatment of Hct-116 cells with compound **5i** enhanced cell cycle arrest at the G1 phase at different concentrations, resulting in concomitant population increase in the G1 phase (45.26% and 49.67%) compared with the control cells (39.08%) and the population of the G2 phase decrease at a certain extent (20.09% and 17.90%) compared with the control cells (24.09%). These results suggested that compound **5i** mainly arrest the cell-cycle of Hct-116 cells in the G1 stage. And the effect of compound 5-FU on the cell cycle of Hct-116 cell was also studied in 5 μM (Fig. f, ESI). It indicated that 5-FU arrest Hct-116 cells in the G1 and S stage, resulting in concomitant population increase in the G1 phase (65.60%) compared with the control cells (39.08%) and the population of the S phase decrease at a certain extent (28.00%) compared with the control cells (23.29%).

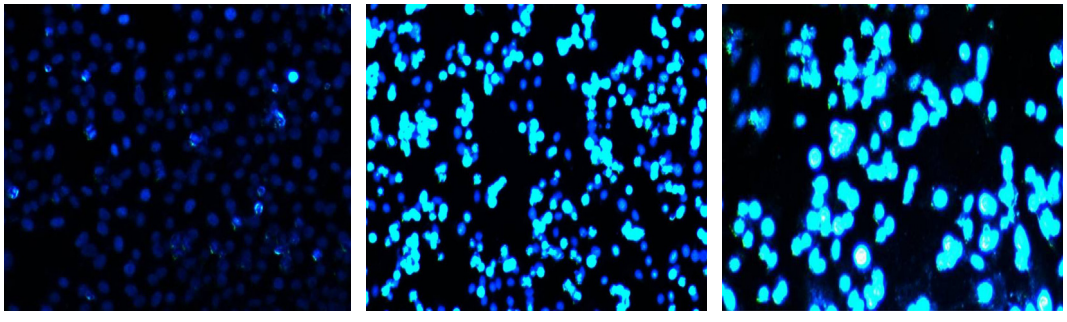

Figure 5. Hoechst 33258 staining of compound **5i** in Hct-116 cells.

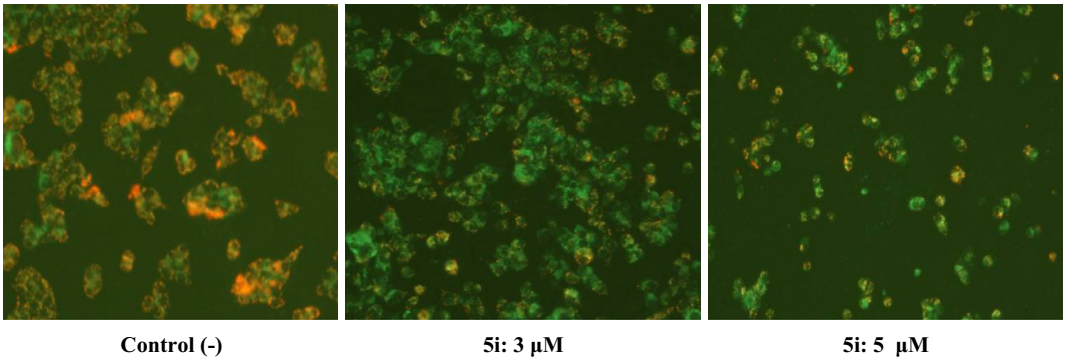

Figure 6. JC-1 mitochondrial membrane potential staining of compound **5i** in Hct-116 cells.

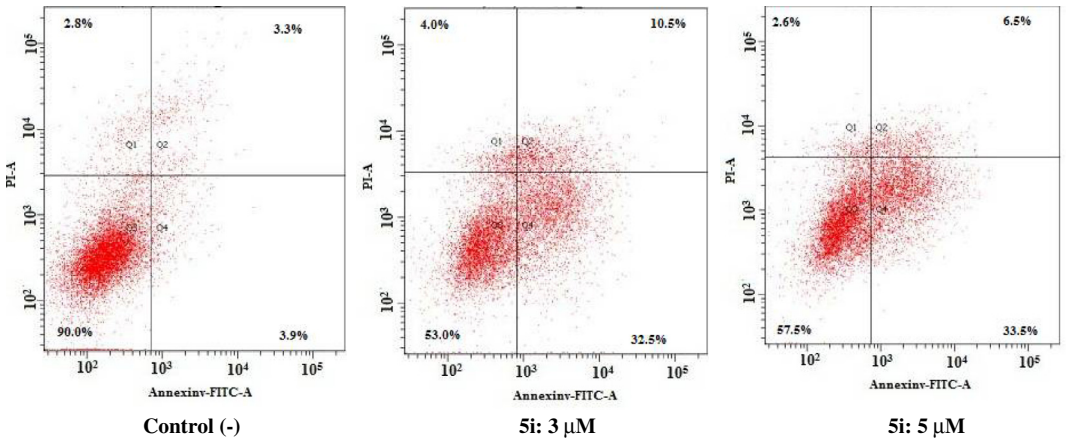

Figure 7. Effect of compound **5i** on apoptosis of Hct-116 cells. Apoptotic cells were analyzed by flow cytometry, after being stained with Annexin V-FITC together with PI. The percentage of cells positive for PI and/or Annexin V-FITC is reported inside the quadrants.

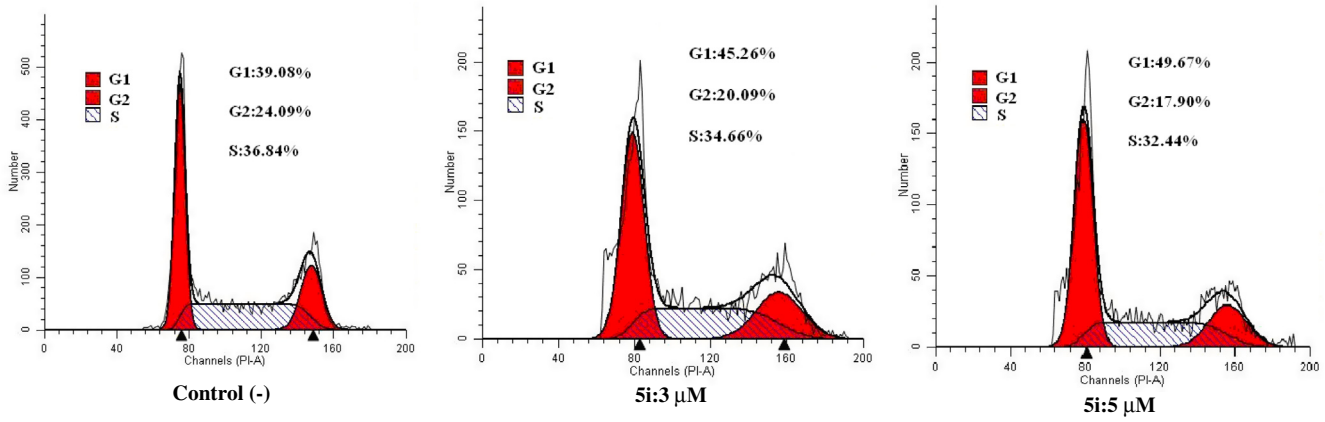

Figure 8. Fluorescence-activated cell sorting analyses based on DNA content of Hct-116 cells treated with **5i** for 48 h and stained by propidium iodide.

Apoptosis can be triggered by several stimuli and is controlled by two major pathways, namely the mitochondrial pathway and membrane death receptor pathway. The membrane death receptor pathway is characterized by the binding between cell death ligands and cell death receptors and the subsequent activation of caspase-8 and caspase-3. The rhein α-aminophosphonate derivatives induced apoptotic pathway was investigated by qRT–PCR analysis of caspase expressions. As showed in Figure 9, compound **5i** treatment showed concentration-dependent effects in caspases-3 and caspases-8, and these effects were concentration-dependent. Therefore, we preliminarily concluded that **5i** induced the apoptosis of Hct-116 cells via the membrane death receptor pathways.

In summary, a series of rhein α-aminophosphonates conjugates were synthesized and the cytotoxic of the rhein derivatives on five cancer cell lines were determined. It was detected that the compound **5i** had selective and significant cytotoxic effects on Hct-116 cells and the toxicity of all conjugates were lower than rhein. Moreover, **5i** exhibited similar activities compared to fluorouracil which was used as clinical drugs. The apoptosis-inducing activity of representative compound **5i** in Hct-116 cells was

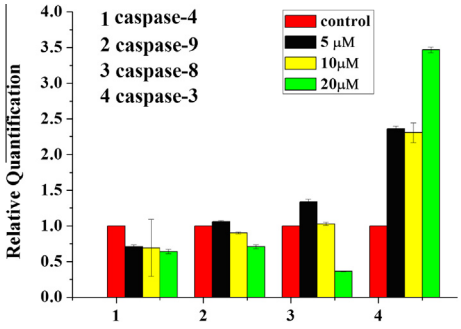

Figure 9. **5i** affected the activities of caspase-4, -9, -8, and -3 of Hct-116 cells.

studied and the results revealed that this compound showed clear cell apoptosis inducing effects. Cell cycle analysis indicated that compound **5i** could arrest Hct-116 cell in G1 stage. The effects of **5i** on the activation of caspases expression indicated that **5i** might induce apoptosis via the membrane death receptor pathways. The binding properties of **5i** to DNA examined by various methods indicated that **5i** interacted with DNA. Therefore, these compounds may be considered as the agents with high potential anti-cancer activity and good candidates for more advanced screening.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.12.030.

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17. (a) *General procedure for the preparation of rhein conjugates* ***5****:* A suspension of aromatic aldehyde (10 mmol), diethyl phosphite (5 mmol) and ammonium acetate (11 mmol) was stirred for 6 h at 70 °C to give the **2**. After that, hydrochloric acid (4 mmol) in 30 mL ether was added to the reaction mixture, which was stirred for 2 h at 0 °C to give the precipitate **3**. The precipitate was filtered and washed with ether (15 mL), and then it was added to 10 mL sodium hydroxide solution (15%) and stirred for 30 min at room temperature. Extraction with dichloromethane (3 × 30 mL) and evaporation of the solvent gave the oils of **4**, which was purified by column chromatography on silica gel with ethyl acetate. To a mixture of rhein and HOBt in DMSO (10 mL), the appropriate EDAC was stirred for 15 min at 0 °C. Then **4** was added dropwise with constant stirring at 0 °C, the mixture was stirred at ambient temperature for another 5 h, then washed with brine, dried with anhydrous sodium sulfate and evaporated. The residue was purified by column chromatography on silica gel with petroleum ether/ethyl acetate (3:1, *V: V*) to give the pure title compounds **5** as yellowish solids.
(b) *Experimental:* NMR spectra were recorded on a BRUKER AVANCE 500 NMR spectrometer in $CDCl_3$. Mass spectra were determined on a BRUKER ESQUIRE HCT spectrometer. **5a**: Yield 81%. mp 221–222 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.17 (t, J = 7.0 Hz, 3H, CH_3), 1.31 (t, J = 6.9 Hz, 3H, CH_3), 3.86–4.21 (m, 4H, OCH_2), 5.77 (dd, J = 9.2, 21.9 Hz 1H, PCH), 7.29–8.62 (m, 9H, Ar-H, 1H, NH), 11.93 (s, 1H, OH), 11.96 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.41, 181.04, 164.60, 161.36, 160.91, 140.89, 137.52, 134.68, 133.49, 133.28, 131.12, 130.70, 130.66, 124.49, 123.03, 121.13, 121.10, 119.38, 118.09, 117.84, 116.12, 62.69, 62.45, 49.36, 16.22, 16.02. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.31. ESI–MS m/z: 610.1 $(M+Na)^+$. Anal. Calcd (for $C_{26}H_{23}BrNO_8P$): C, 53.08; H, 3.94; N, 2.38. Found: C, 53.11; H, 4.17; N, 2.38. Compound **5b**: Yield 87%. mp 197–199 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.09 (t, J = 7.1 Hz, 3H, CH_3), 1.35 (t, J = 7.2 Hz, 3H, CH_3), 3.72–4.26 (m, 4H, OCH_2), 6.31 (dd, J = 9.0, 21.4 Hz 1H, PCH), 7.17–8.22 (m, 9H, Ar-H, 1H, NH), 11.95 (s, 1H, OH),12.00 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 192.63, 180.91, 164.40, 162.75, 162.52, 141.54, 137.69, 134.90, 133.96, 133.41, 133.20, 129.76, 129.61, 127.91, 124.93, 124.68, 123.51, 120.36, 119.97, 118.13, 115.74, 63.91, 63.50, 49.62, 16.39, 16.03. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.33. ESI–MS m/z: 610.1 $(M+Na)^+$. Anal. Calcd (for $C_{26}H_{23}BrNO_8P$): C, 53.08; H, 3.94; N, 2.38. Found: C, 53.10; H, 4.18; N, 2.39. Compound **5c**: Yield 82%. mp 208–209 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.17 (t, J = 7.3 Hz, 3H, CH_3), 1.32 (t, J = 7.2 Hz, 3H, CH_3), 3.76–4.19 (m, 4H, OCH_2), 6.12 (dd, J = 9.8, 21.2 Hz 1H, PCH), 6.97–8.42 (m, 9H, Ar-H, 1H, NH), 11.94 (s, 1H, OH), 11.99 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 192.63, 180.88, 164.81, 162.74, 162.43, 141.56, 137.68, 137.07, 133.85, 133.41, 131.35, 131.25, 130.15, 126.94, 124.89, 123.48, 122.69, 120.33, 118.51, 117.44, 115.74, 63.79, 63.57, 49.81, 16.40, 16.12. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.31. ESI–MS m/z: 610.1 $(M+Na)^+$. Anal. Calcd (for $C_{26}H_{23}BrNO_8P$): C, 53.08; H, 3.94; N, 2.38. Found: C, 53.12; H, 4.16; N, 2.38. Compound **5d**: Yield 85%. mp 197–199 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.14 (t, J = 7.1 Hz, 3H, CH_3), 1.31 (t, J = 7.1 Hz, 3H, CH_3), 3.80–4.21 (m, 4H, OCH_2), 5.77 (dd, J = 9.2, 21.3 Hz 1H, PCH), 7.01–8.22 (m, 9H, Ar-H, 1H, NH), 11.94 (s, 1H, OH), 11.99 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 192.61, 180.89, 164.68, 162.74, 162.46, 161.61, 141.67, 137.69, 133.89, 133.39, 130.61, 130.22, 130.16, 130.11, 124.91, 123.43, 120.33, 118.34, 117.43, 115.72, 115.56, 63.66, 63.40, 49.58, 16.42, 16.12. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.29. ESI–MS m/z: 550.1 $(M+Na)^+$. Anal. Calcd (for $C_{26}H_{23}FNO_8P$): C, 59.21; H, 4.40; N, 2.66. Found: C, 59.12; H, 4.50; N, 2.59. Compound **5e**: Yield 87%. mp 234–236 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.13 (t, J = 7.1 Hz, 3H, CH_3), 1.35 (t, J = 7.0 Hz, 3H, CH_3), 3.84–4.22 (m, 4H, OCH_2), 6.03 (dd, J = 9.2, 21.7 Hz 1H, PCH), 7.10–8.21 (m, 9H, Ar-H, 1H, NH), 11.97 (s, 1H, OH), 12.05 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.41, 181.08, 164.62, 161.36, 160.90, 140.76, 137.52, 133.53, 133.30, 130.52, 130.08, 124.50, 124.43, 123.06, 122.46, 119.38, 118.07,

117.91, 116.16, 115.16, 114.98, 62.75, 62.51, 42.29, 16.21, 15.90. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.24. ESI–MS *m/z*: 550.1 $(M+Na)^+$. Anal. Calcd (for $C_{26}H_{23}FNO_8P$): C, 59.21; H, 4.40; N, 2.66. Found: C, 59.18; H, 4.42; N, 2.57. Compound **5f**: Yield 84%. mp 224–226 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.16 (t, J = 7.0 Hz, 3H, CH_3), 1.32 (t, J = 7.1 Hz, 3H, CH_3), 3.82–4.21 (m, 4H, OCH_2), 5.77 (dd, J = 9.3, 22.4 Hz 1H, PCH),7.26–8.41 (m, 9H, Ar-H, 1H, NH), 11.94 (s, 1H, OH), 11.98 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.43, 181.08, 164.60, 161.36, 160.91, 140.90, 137.53, 134.24, 133.52, 133.31, 132.53, 130.38, 130.33, 128.19, 124.50, 123.02, 119.39, 118.08, 117.88, 116.16, 115.92, 62.73, 62.50, 50.51, 16.26, 16.01. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.34. ESI–MS *m/z*: 566.1 $(M+Na)^+$. Anal. Calcd (for $C_{26}H_{23}ClNO_8P$): C, 57.42; H, 4.26; N, 2.58. Found: C, 57.32; H, 4.36; N, 2.60. Compound **5g**: Yield 80%. mp 194–196 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.16 (t, J = 7.0 Hz, 3H, CH_3), 1.33 (t, J = 7.1 Hz, 3H, CH_3), 3.82–4.21 (m, 4H, OCH_2), 5.72 (dd, J = 8.5, 21.7 Hz 1H, PCH), 7.28–8.24 (m, 9H, Ar-H, 1H, NH), 11.96 (s, 1H, OH), 12.03 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.40, 181.04, 164.59, 161.37, 160.92, 140.83, 137.60, 137.52, 133.49, 133.27, 132.94, 130.00, 128.31, 127.79, 127.33, 124.49, 123.04, 119.38, 118.10, 117.86, 116.11, 62.74, 62.52, 49.44, 16.21, 15.97. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.30. ESI–MS *m/z*: 566.1 $(M+Na)^+$. Anal. Calcd (for $C_{26}H_{23}ClNO_8P$): C, 57.42; H, 4.26; N, 2.58. Found: C, 57.37; H, 4.38; N, 2.51. Compound **5h**: Yield 82%. mp 242–244 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.09 (t, J = 7.0 Hz, 3H, CH_3), 1.35 (t, J = 7.1 Hz, 3H, CH_3), 3.72–4.26 (m, 4H, OCH_2), 6.31 (dd, J = 9.1, 21.4 Hz 1H, PCH), 7.17–8.22 (m, 9H, Ar-H, 1H, NH), 11.95 (s, 1H, OH), 12.00 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 192.65, 180.94, 164.40, 162.78, 162.56, 161.20, 141.51, 137.71, 134.02, 133.42, 133.09, 129.90, 129.55, 129.37, 127.32, 124.96, 123.48, 120.39, 117.97, 117.57, 115.76, 63.88, 63.46, 47.07, 16.40, 16.03. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.33. ESI–MS *m/z*: 566.1 $(M+Na)^+$. Anal. Calcd (for $C_{26}H_{23}ClNO_8P$): C, 57.42; H, 4.26; N, 2.58. Found: C, 57.31; H, 4.43; N, 2.53. Compound **5i**: Yield 87%. mp 176–178 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.13 (t, J = 6.6 Hz, 3H, CH_3), 1.31 (t, J = 6.6 Hz, 3H, CH_3), 3.76 (s, 3H, OCH_3), 3.80–4.18 (m, 4H, OCH_2), 5.77(dd, J = 7.6, 21.7 Hz 1H, PCH), 6.80–8.25 (m, 9H, Ar-H, 1H, NH), 11.94 (s, 1H, OH), 11.99 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.40, 181.01, 164.55, 161.37, 160.93, 159.15, 141.10, 137.50, 136.56, 133.44, 133.25, 129.18, 124.47, 123.03, 120.84, 119.37, 118.14, 117.74, 116.07, 114.28, 113.26, 62.55, 62.39, 55.12, 49.86, 16.29, 16.02. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 20.92. ESI–MS *m/z*: 562.2 $(M+Na)^+$. Anal. Calcd (for $C_{27}H_{26}NO_9P$): C, 60.11; H, 4.86; N, 2.60. Found: C, 60.17; H, 4.96; N, 2.51. Compound **5j**: Yield 90%. mp 228–230 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.10 (t, J = 7.3 Hz, 3H, CH_3), 1.32 (t, J = 7.2 Hz, 3H, CH_3), 3.76–4.19 (m, 4H, OCH_2), 3.98 (s, 3H, OCH_3), 6.12 (dd, J = 9.8, 21.2 Hz 1H, PCH), 6.97–8.15 (m, 9H, Ar-H, 1H, NH), 11.93 (s, 1H, OH), 12.01 (s, 1H, OH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 192.57, 180.89, 163.86, 162.69, 162.55, 157.35, 142.01, 137.62, 133.86, 133.35, 129.74, 129.70, 129.66, 124.89, 123.41, 122.63, 121.06, 120.29, 117.75, 117.34, 111.36, 63.35, 62.96, 55.96, 46.30, 16.41, 16.09. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.31. ESI–MS *m/z*: 562.2 $(M+Na)^+$. Anal. Calcd (for $C_{27}H_{26}NO_9P$): C, 60.11; H, 4.86; N, 2.60. Found: C, 60.01; H, 4.96; N, 2.51. Compound **5k**: Yield 79%. mp 223–225 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.11 (t, J = 7.0 Hz, 3H, CH_3), 1.32 (t, J = 7.2 Hz, 3H, CH_3), 3.85–4.22 (m, 4H, OCH_2), 5.76 (dd, J = 9.3, 22.0 Hz 1H, PCH), 7.17–8.21 (m, 10H, Ar-H, 1H, NH), 11.96 (s, 1H, OH), 12.03 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.43, 181.05, 164.62, 161.36, 160.92, 141.06, 137.51, 135.13, 133.46, 133.29, 128.55, 128.50, 128.17, 127.75, 127.65, 127.31, 124.48, 123.03, 119.37, 118.14, 116.12, 62.52, 62.33, 49.91, 16.23, 15.98. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.30. ESI–MS *m/z*: 532.1 $(M+Na)^+$. Anal. Calcd (for $C_{26}H_{24}NO_8P$): C, 61.30; H, 4.75; N, 2.75. Found: C, 61.20; H, 4.87; N, 2.69. Compound **5l**: Yield 84%. mp 248–250 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 0.85 (t, J = 7.0 Hz, 3H, CH_3), 1.37 (t, J = 7.1 Hz, 3H, CH_3), 3.50–4.30 (m, 4H, OCH_2), 6.60 (dd, J = 9.3, 22.3 Hz 1H, PCH), 7.26–8.33 (m, 12H, Ar-H, 1H, NH), 11.94 (s, 1H, OH), 11.98 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.41, 181.05, 164.49, 161.35, 160.91, 140.86, 137.50, 133.50, 133.28, 133.22, 131.37, 130.84, 128.68, 128.52, 127.42, 126.58, 125.78, 125.18, 124.47, 123.13, 123.03, 119.36, 118.10, 117.84, 116.12, 62.67, 62.33, 45.07, 16.29, 15.82. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.33. ESI–MS *m/z*: 582.2 $(M+Na)^+$. Anal. Calcd (for $C_{30}H_{26}NO_8P$): C, 64.40; H, 4.68; N, 2.50. Found: C, 64.27; H, 4.68; N, 2.43. Compound **5m**: Yield 89%. mp 213–215 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.13 (t, J = 7.2 Hz, 3H, CH_3), 1.32 (t, J = 7.0 Hz, 3H, CH_3), 3.77 (s, 3H, OCH_3), 3.77–4.22 (m, 4H, OCH_2), 5.72 (dd, J = 9.5, 20.1 Hz 1H, PCH), 6.84–8.20 (m, 9H, Ar-H, 1H, NH), 11.94 (s, 1H, OH), 11.97 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.42, 181.06, 164.47, 161.36, 160.93, 158.88, 141.14, 137.50, 133.46, 133.29, 129.90, 129.85, 126.96, 124.47, 123.00, 119.36, 118.12, 117.76, 116.12, 114.46, 113.62, 62.41, 62.26, 55.06, 49.19, 16.25, 16.03. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.98. ESI–MS *m/z*: 562.2 $(M+Na)^+$. Anal. Calcd (for $C_{27}H_{26}NO_9P$): C, 60.11; H, 4.86; N, 2.60. Found: C, 60.02; H, 5.06; N, 2.51. Compound **5n**: Yield 86%. mp 194–196 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.11 (t, J = 7.1 Hz, 3H, CH_3), 1.29 (t, J = 7.0 Hz, 3H, CH_3), 2.30 (s, 3H, Ar-CH_3), 3.75–4.20 (m, 4H, OCH_2), 5.77 (dd, J = 9.4, 20.7 Hz 1H, PCH), 7.06–8.53 (m, 10H, Ar-H, 1H, NH), 11.88 (s, 1H, OH), 11.91 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.42, 181.04, 164.57, 161.36, 160.92, 141.09, 137.51, 137.32, 134.99, 133.44, 133.28, 129.15, 128.37, 128.06, 125.68, 124.47, 123.03, 119.38, 118.16, 117.76, 116.10, 62.50, 62.33, 49.86, 20.95, 16.23, 15.98. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 20.93. ESI–MS *m/z*: 546.1 $(M+Na)^+$. Anal. Calcd (for $C_{27}H_{26}NO_8P$): C, 61.95; H, 5.01; N, 2.68. Found: C, 61.89; H, 5.11; N, 2.59. Compound **5o**: Yield 85%. mp 223–225 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.11 (t, J = 7.1 Hz, 3H, CH_3), 1.29 (t, J = 7.1 Hz, 3H, CH_3), 2.29 (s, 3H, Ar-CH_3), 3.75–4.20 (m, 4H, OCH_2), 5.78 (dd, J = 9.4, 20.6 Hz 1H, PCH), 7.09–8.66 (m, 10H, Ar-H, 1H, NH), 11.86 (s, 1H, OH), 11.89 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.43, 181.04, 164.49, 161.36, 160.92, 141.12, 137.51, 137.02, 137.00, 133.45, 133.29, 132.09, 128.73, 128.48, 128.43, 124.47, 123.01, 119.37, 118.14, 117.75, 116.11, 62.46, 62.28, 49.59, 20.63, 16.24, 16.02. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 21.02. ESI–MS *m/z*: 524.2 $(M+H)^+$. Anal. Calcd (for $C_{27}H_{26}NO_8P$): C, 61.95; H, 5.01; N, 2.68. Found: C, 61.90; H, 5.06; N, 2.75. Compound **5p**: Yield 88%. mp 163–165 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 1.15 (t, J = 7.0 Hz, 3H, CH_3), 1.31 (t, J = 7.1 Hz, 3H, CH_3), 3.85–4.22 (m, 4H, OCH_2), 5.81 (dd, J = 9.4, 22.0 Hz 1H, PCH), 6.95–8.60 (m, 10H, Ar-H, 1H, NH), 11.93 (s, 1H, OH), 11.97 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.40, 181.03, 164.58, 161.37, 160.92, 140.87, 137.85, 137.52, 133.49, 133.27, 130.12, 130.06, 124.74, 124.49, 123.03, 119.38, 118.09, 117.84, 116.10, 115.38, 114.72, 62.70, 62.49, 49.49, 16.21, 15.98. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 20.24. ESI–MS *m/z*: 550.1 $(M+Na)^+$. Anal. Calc. (for $C_{26}H_{23}FNO_8P+H_2O$): C, 59.21; H, 4.40; N, 2.66. Found: C, 59.15; H, 4.62; N, 2.57. Compound **5q**: Yield 83%. mp 205–207 °C. ^{1}H NMR (500 MHz $CDCl_3$) δ (ppm): 0.63 (t, J = 7.1 Hz, 3H, CH_3), 1.42 (t, J = 7.1 Hz, 3H, CH_3), 2.30 (s, 3H, Ar-CH_3), 3.31–4.35 (m, 4H, OCH_2), 7.25–7.27 (m, 2H, Ar-H), 7.35 (dd, J = 8.5, 25.7 Hz 1H, PCH), 7.48–8.89 (m, 12H, Ar-H, 1H, NH), 11.88 (s, 1H, OH), 11.91 (s, 1H, OH). ^{13}C NMR (125 MHz, DMSO) δ (ppm): 191.37, 180.87, 165.43, 161.32, 160.79, 140.92, 137.46, 133.21, 131.30, 131.28, 130.90, 130.38, 129.70, 128.70, 128.67, 127.55, 127.35, 126.63, 126.53, 125.31, 124.98, 124.94, 124.41, 123.85, 123.16, 119.32, 118.31, 117.60, 116.00, 62.58, 62.40, 46.38, 16.26, 15.54. ^{31}P NMR (200 MHz, $CDCl_3$) δ (ppm): 22.34. ESI–MS *m/z*: 632.2 $(M+Na)^+$. Anal. Calcd (for $C_{34}H_{28}NO_8P$): C, 66.99; H, 4.64; N, 2.30. Found: C, 66.92; H, 4.71; N, 2.23.

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