Electronic Supplementary Information

Phosphorus-Nitrogen Compounds. Part 42. The Comparative Syntheses of 2,4-Ansa(N/O) and Spiro(N/O) Cyclotetraphosphazene Derivatives: Structural Characterizations, Antituberculosis and Cytotoxic Activity Studies

Arzu Binici[a], Aytuğ Okumuş[b], Gamze Elmas*[b], Zeynel Kılıç*[b], Nagehan Ramazanoğlu[c], Leyla Açık[c], Hülya Şimşek[d], Beste Çağdaş Tunalı[e], Mustafa Türk[e], Remziye Güzel[f] and Tuncer Hökelek[g]

[a]Turkey's Health Ministry General Directorate of Public Health, 06100 Ankara, Turkey
[b]Department of Chemistry, Ankara University, 06100 Ankara, Turkey
[c]Department of Biology, Gazi University, 06500 Ankara, Turkey
[d]Faculty of Medicine, Bozok University, 66900 Yozgat, Turkey
[e]Department of Bioengineering, Kırıkkale University, 71450 Yahşihan-Kırıkkale, Turkey
[f]Department of Chemistry, Dicle University, 21280, Diyarbakır, Turkey
[g]Department of Physics, Hacettepe University, 06800 Ankara, Turkey

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Section S1. General Methods

The Cl replacement reactions were made under Ar atmosphere, and the reactions were followed using thin-layer chromatography (TLC) on Merck DC Alufolien Kiesegel 60 B_{254} sheets. The column chromatography was carried out on Merck Kiesegel 60 (230-400 mesh ATSM) silica gel. The melting points were detected with a Gallenkamp apparatus using a capillary tube. The Fourier transform infrared (FTIR) spectra were recorded on a Jasco FT/IR-430 spectrometer in KBr discs and reported in cm^{-1} units. One-dimensional (1D) ^{1}H, ^{13}C and ^{31}P NMR and two-dimensional (2D) heteronuclear single quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC) spectra were monitored on a Bruker DPX FT-NMR (500 MHz) spectrometer ($SiMe_4$ as internal and 85% H_3PO_4 as external standards), operating at 400.13, 100.62 and 161.97 MHz. The NMR spectrometer was equipped with a 5 mm PABBO BB inverse gradient probe and standard Bruker pulse programs[1] were used. HPLC experiments were performed with an Agilent 1100 series HPLC system (Chemstation software) equipped with a G 1311A pump and G 1315B diode array detector monitoring the range of 220–300 nm. A reversible chiral column-(R,R)-whelk-01(250 x 4.6 mm) from Regis Tech. Inc. was used for HPLC. Electron spray ionization-mass spectra (ESI-MS) of all the compounds and high-resolution mass spectra (HRMS) of **3d** and **4d** were determined on the AGILENT 1100 MSD and AGILEND 6224 LC/TOF MS mass spectrometers, respectively. Microanalyses were obtained by the microanalytical service of Ankara University. Microwave-assisted experiments performed with a Milestone Start S system using a weflon^{TM} magnet for THF, toluene and o-xylene. The electrochemical measurements were carried out by Gamry Reference 3000 Workstation (Gamry, USA) with the conventional three-electrode system. Platinum wire, Gold electrode, and $Ag/AgCl/KCl_{(sat)}$ electrodes were used as the counter, working and reference electrodes, respectively. Pt counter electrode, gold working electrode and Ag/AgCl reference electrode were purchased from BAS (USA). Before measurements, electrodes were dried under Ar stream. Thermal stabilities and decomposition properties of the cyclotetraphosphazenes were elucidated by thermogravimetric (TG)-differential thermal analysis (DTA). The TG and DTA curves were scanned by a PYRIS Diamond TG/DTA apparatus in the N_2 atmosphere (platinum crucibles, heating rate: 10 °C/min, mass ~10 mg and temperature range 35-1000 °C).

$N_4P_4Cl_8$ (Otsuka Chemical Co. Ltd.), ferrocenecarboxaldehyde, 4-fluorobenzaldehyde (Aldrich), 3-amino-1-propanol, pyrrolidine, piperidine, morpholine and DASD (Fluka) were purchased.

Reference

(1) Bruker program 1D WIN-NMR (release 6.0) and 2D WIN-NMR (release 6.1).

Section S2. Antimicrobial Activities

This study scrutinized the antimicrobial effects of the cyclotetraphosphazenes in order to determine potential antimicrobial agents for using against some bacteria and fungi. The synthesized compounds were tested for their antimicrobial activity against the eleven bacteria and three yeast strains by the agar diffusion method.[1] For positive control, ampicillin (10 µg/mL) and chloramphenicol (30 µg/mL) were used as the reference antibacterial agents, and ketoconazole (50 µg/mL) was used as a reference antifungal agent. 1,4-dioxane was used as the negative control since it was used as the solvent for all the compounds. G(-) and G(+) bacteria were grown on nutrient broth agar plates and incubated at 37 °C for 24 h. While, the yeast strains were grown in Sabouraud dextrose agar (SDA) medium and incubated at 30 °C for 48 h. After incubation, bacterial suspensions were adjusted to a turbidity of 0.5 McFarland. SDA (for fungal strains) and Mueller Hinton agar (MHA) (for bacterial strains) were mixed with 1% culture suspension and poured into plates. A suspension of the tested microorganism was spread over the surface of agar plates (SDA and MHA). The testing compounds (1665 mM) was added to the wells. The diameter of the inhibition zone was measured in millimeters.

Reference

(1) Erdener Çıralı, D.; Uyar, Z.; Koyuncu, İ.; Hacıoğlu, N. *Appl. Organometal. Chem.* **2015**, *29*, 536-542.

Section S3. DNA and Compound Interactions

To assess the DNA interaction properties of the compounds, agarose gel electrophoresis technique was performed using pBR322 plasmid DNA.[1] It is investigated that the capacity of the compounds to remove and reverse the supercoiling of closed circular pBR322 plasmid DNA and to induce conformational changes on the DNA helix. Typically, the gel electrophoresis presented two main bands for the untreated plasmid. One strong band shows the covalently closed circular (supercoiled) Form I and one weak band represents the open circular Form II. All the cyclotetraphosphazenes were dissolved in 1,4-dioxane. Electrophoresis was carried out under TAE buffer for 3 h at 60 V.[2] The compounds were incubated with plasmid DNA in an incubator at 37 ^{o}C for 24 h in the dark. The compound/DNA mixtures were loaded onto the 1% agarose gel with a loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol). Electrophoresis was made in 0.05 M Tris base, 0.05 M glacial acetic acid and 1 mM EDTA (TAE buffer, pH = 8.0) for 3 h at 60 V. Afterwards, the gel was stained with ethidium bromide (0.5 µg/mL), visualized under the UV light using a transilluminator (BioDoc Analyzer, Biometra), photographed with a video camera, and saved as a TIFF file. Each experiment was made for three times.

***HindIII* and *BamHI* digestions of the compounds-pBR322 plasmid DNA**

The affinity evaluation between the synthesized compounds and guanine-guanine (GG) and/or adenine-adenine (AA) regions was carried out through restriction endonuclease analysis. All the compound-DNA

adducts were digested with *Bam*HI and *Hind*III enzymes for 1 h at the 37 °C. *Bam*HI enzyme binds at the sequence 5'-G/GATCC-3' and since pBR322 plasmid DNA contains a single sequence of that, cleave this sequence. *Bam*HI then converts supercoiled Form I and open circular Form II to linear form of linear DNA (Form III). *Hind*III recognizes the sequence 5'-A/AGCTT-3' and cleaves this sequence. As a result, *Hind*III converts Form I and Form II to Form III similarly to *Bam*HI. The restricted DNA was run in 1% agarose gel electrophoresis for 2 h at 60 V in TAE buffer.[2]

References

(1) Gümüş, F.; Eren, G.; Açık, L.; Çelebi, A.; Öztürk, F.; Yılmaz, S.; Sakan, R.; Gür, S.; Özkul, A.; Elmalı, A.; Elerman, Y. Synthesis, cytotocity, and DNA interactions of new cisplatin analogues containing substituted benzimidazole ligands. *J. Med. Chem.* **2009**, *52*, 1345-1357.

(2) Sambrook, J.; Fritsch, E. F.; Maniatis, T. Molecular cloning: A laboratory manual. *Cold Spring Harbor,* New York, **1989**.

Section S4. Determination of the cytotoxic activity with WST assay

In this study, the cytotoxicities of the phosphazenes were tested by colorimetric WST-1 assay[1] against normal L929 fibroblast cells (normal cell group) and MCF7 (ATCC® HTB-22) breast cancer cells. Normal L929 mouse fibroblast cells and MCF-7 breast cancer cells were put into flasks containing Dulbecco's Modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640, respectively, with L-glutamine, 10% FCS, and 1% antibiotic, and then were kept in a CO_2 incubator conditioned with 5% CO_2 at 37 °C for 48 h. For harvesting cells, the cell culture medium was removed and the cells were treated with trypsin-EDTA (0.5 mL per flask). Then the cells were transferred into 15 mL Eppendorf tubes and centrifuged at 2.500 rpm for 2 min. After removing the supernatant, the cells were used in the prospective studies. Doxorubicin was used at different concentrations as the positive control. The plates containing DMEM with L-glutamine, 10% FCS, and 1% antibiotic and RPMI with L-glutamine, 10% FCS, and 1% antibiotic were incubated at 37 °C in an incubator for 24 h. Following 24 h incubation under the same conditions, WST-1 (a water-soluble tetrazolium salt) reagent (5 µL) was added into each well. Upon incubation for an additional 4 h, the plates were immediately read in an Elisa Microplate Reader (BioTek, USA) at 440 nm wavelength and the percentage of cell viability of each group was calculated according to definition of the control cell viability as 100%.

Reference

(1) Rzayev, Z. M. O.; Türk, M.; Söylemez, E. A. Bioengineering functional copolymers. XXI. Synthesis of a novel end carboxyl-trithiocarbonate functionalized poly(maleic anhyride) and its interaction with cancer cells. *Bioorg. Med. Chem*. **2012**, *20*, 5053–5061.

Section S5. Cell Proliferation Assay

The xCELLigence System is used for cell-based assays and provides dynamic, real-time, label-free evaluation of proliferation, viability and cytotoxicity of cells.[1,2] This technology was used to determine the proliferation of L929 and MCF-7 cells adapted from the instructions of the supplier (Roche Applied Science, Switzerland). xCELLigence Real-Time Cell Analysis (RTCA) analyzer measures impedance variations in the cell culture media found in E-plate wells. Integrated with gold microelectrodes at the bottom, which is placed in the incubator and determines cell viability and motility against the toxic effect. As the number of cells attached to the electrodes increase, electrode impedance increases. However, impedance is not affected, only by the cell number attached to the electrodes. Impedance measurement is also affected by cell interaction strength with the electrodes and cell morphology. Electrode impedance which represents the biological conditions of observed cells, including the cell number, cell viability, morphology and adhesion degree, is indicated and recorded as Cell Index (CI) values.[3] 100 µL of DMEM was added to the E-plate with 96-wells and placed into xCELLigence for 10 min to stabilize the temperature of the plate and the device. After background reading for 1 min, E-plate was removed from the system and L929 and MCF-7 cells ($5x10^3$ cells/well) were seeded in the plate. The E - plate was placed one more time for incubation and the system ran for 10 min to confirm if the wells are read or not. Then the plate was monitored for cell attachment and growth for 24h. After that, when the cells reached to the growth phase, medium was discarded from the E-plate. Then a final volume of 200µL medium containing different concentrations (200-12.5 µg/mL) of samples (final concentrations of samples are 200 µg/mL presented in Figure 18), was added to cells. Afterward, the plate incubated for approximately 72 h to monitor the cell index and growth status of the cells. Cell index (CI) for real-time dynamic cytotoxicity assessment (n = 3) and slope calculations for the migration assessments were calculated automatically by the RTCA Software Package 1.2 of the RTCA system and in the current study the data analyzed using a slope calculation.

References

(1) Kich, D. M.; Bitencourt, S.; Caye, B.; Faleiro, D.; Alves, C.; Silva, J.; Pinteus, S.; Mergener, M.; Majolo, F.; Boligon, A. A.; Santos, R. C.; Pedrosa, R.; de Souza, C. F.; Goettert, M. I.; Lymphocyte genotoxicity and protective effect of Calyptranthes tricona (Myrtaceae) against H_2O_2-induced cell death in MCF-7 cells , *Molecular and Cellular Biochemistry*, **2016**, 424 (1-2), 35-43.

(2) Roche Diagnostics GmbH. Introduction of the RTCA SP instrument. RTCA SP instrument operator‘s manual, *A. Acea Biosciences*, Inc: **2008**, 14-16.

(3) Ramis, G.; Martinez-Alarcon, L.; Quereda, J. J.; Mendonca, L.; Majado, M. J.; Gomez-Coelho, K.; Optimization of cytotoxicity assay by real-time, impedance-based cell analysis. *Biomed Microdevices*, **2013**, 15, 985–95.

Section S6. Determination of antituberculosis activity

The agar proportion method was performed using Middlebrook 7H10 agar. This is a reference method for drug susceptibility testing of *Mycobacterium tuberculosis* according to CLSI recommendations.[1, 2] The final concentration of each compound in the medium was firstly adjusted to 5, 10, 20, 40 and 80 μg/mL, respectively. The final concentrations were changed for the compounds displaying antituberculosis activity to *M. tuberculosis* H37Rv strain, to detect minimal inhibition concentration (MIC) of compounds. Middlebrook 7H10 medium was prepared and autoclaved at 121°C for 10 minutes. When cooled to 55°C, Middlebrook 7H10 medium was mixed with Middlebrook OADC Enrichment and dissolved compounds, before pouring into the Petri dish bottom divided into four partitions. Quality controls of media were done.

References

(1) Antimycobacterial susceptibility testing for Mycobacterium tuberculosis, Tentative standard M24-T, National Committee for Clinical Laboratory Standards (NCCLS), Wayne, Pa, 1995.

(2) Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes, Approved standard M24-A, National Committee for Clinical Laboratory Standards (NCCLS), Wayne, Pa, 2003.

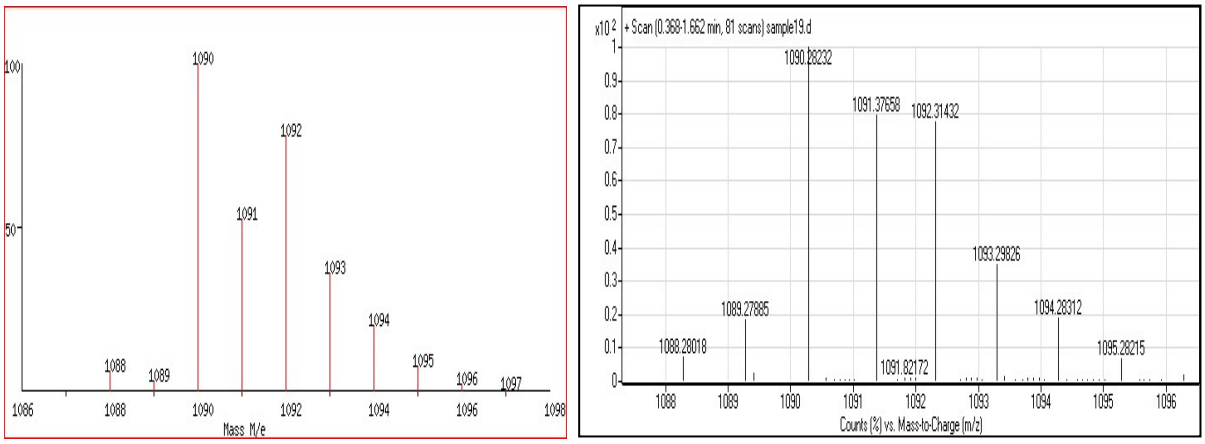


Fig. S1 The experimental HRMS and simulated HRMS spectrum of **3d.**

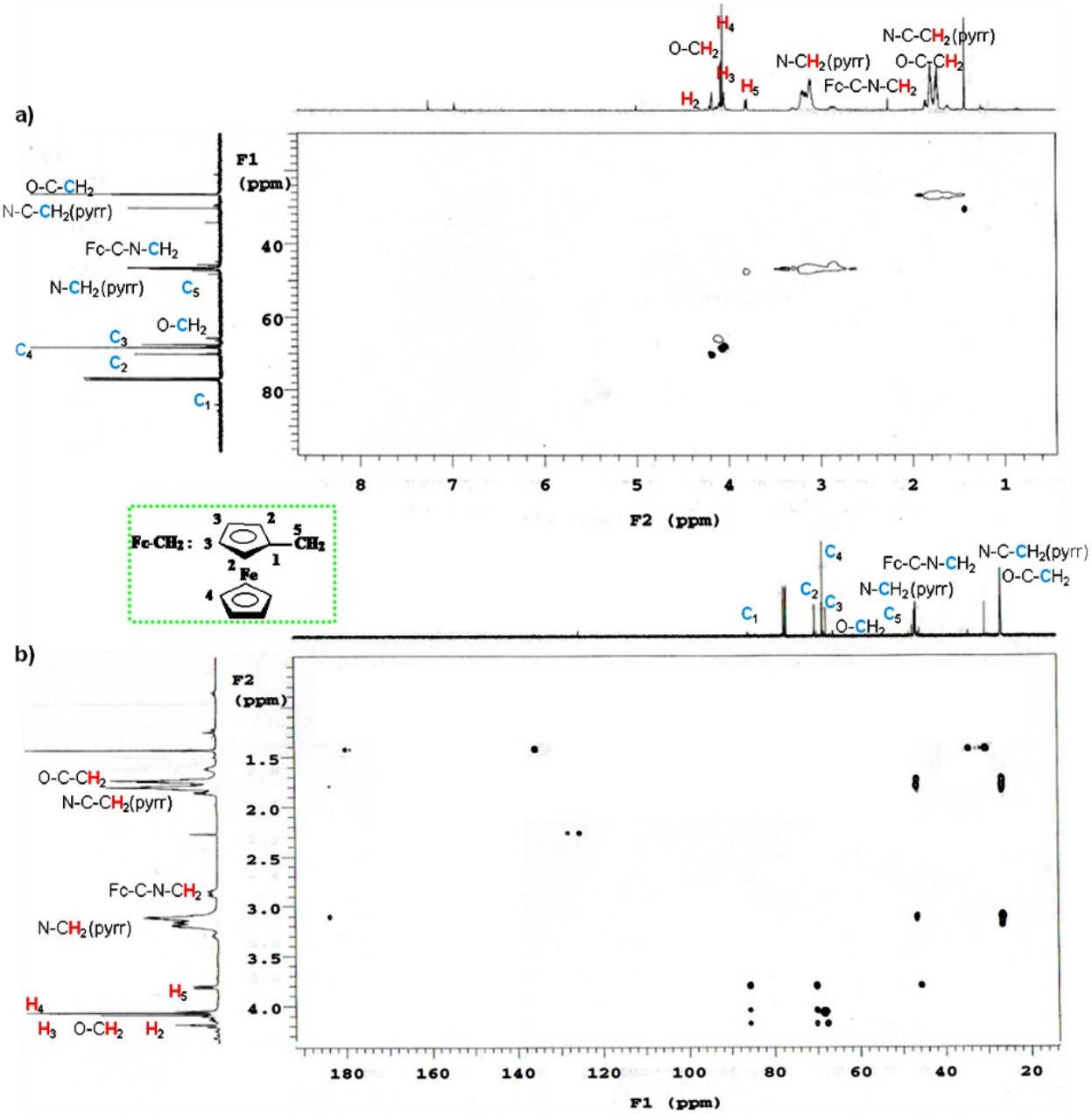
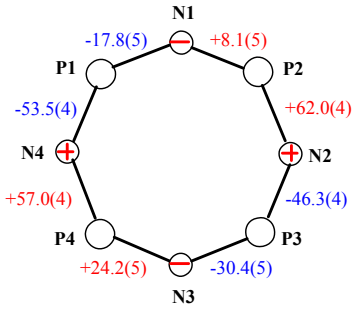
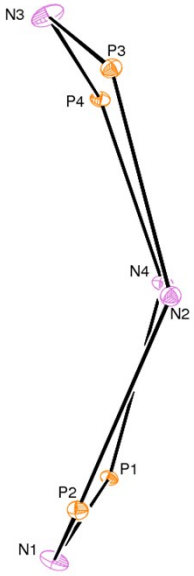


Fig. S2 The (**a**) HMBC and (**b**) HSQC spectra of **3a**.

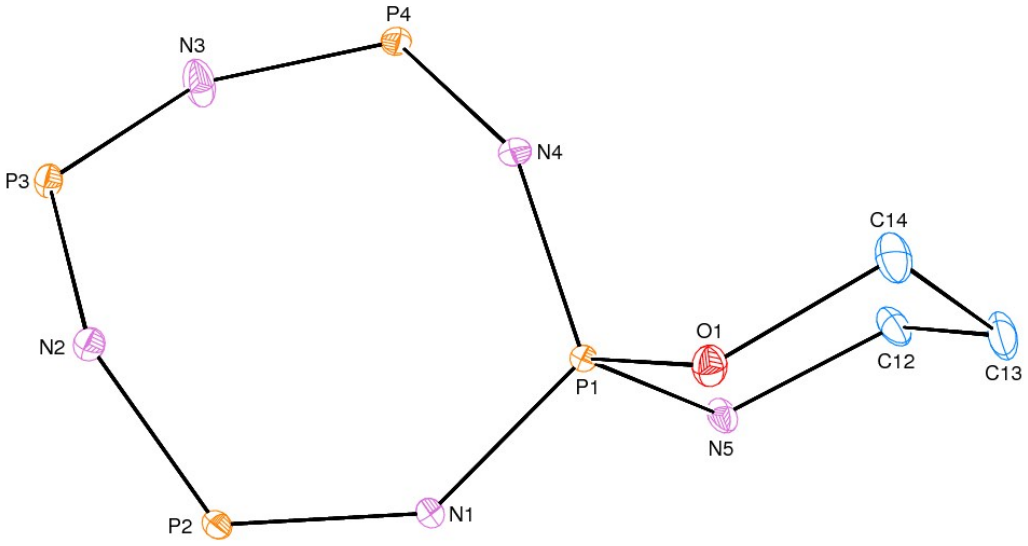


4

Fig. S3 The shape of the phosphazene ring in **4** with torsion angles (deg) given.



(a)



(b)

Fig. S4 The conformations of (**a**) the tetramer ring and (**b**) the six-membered spiro ring of **4**.

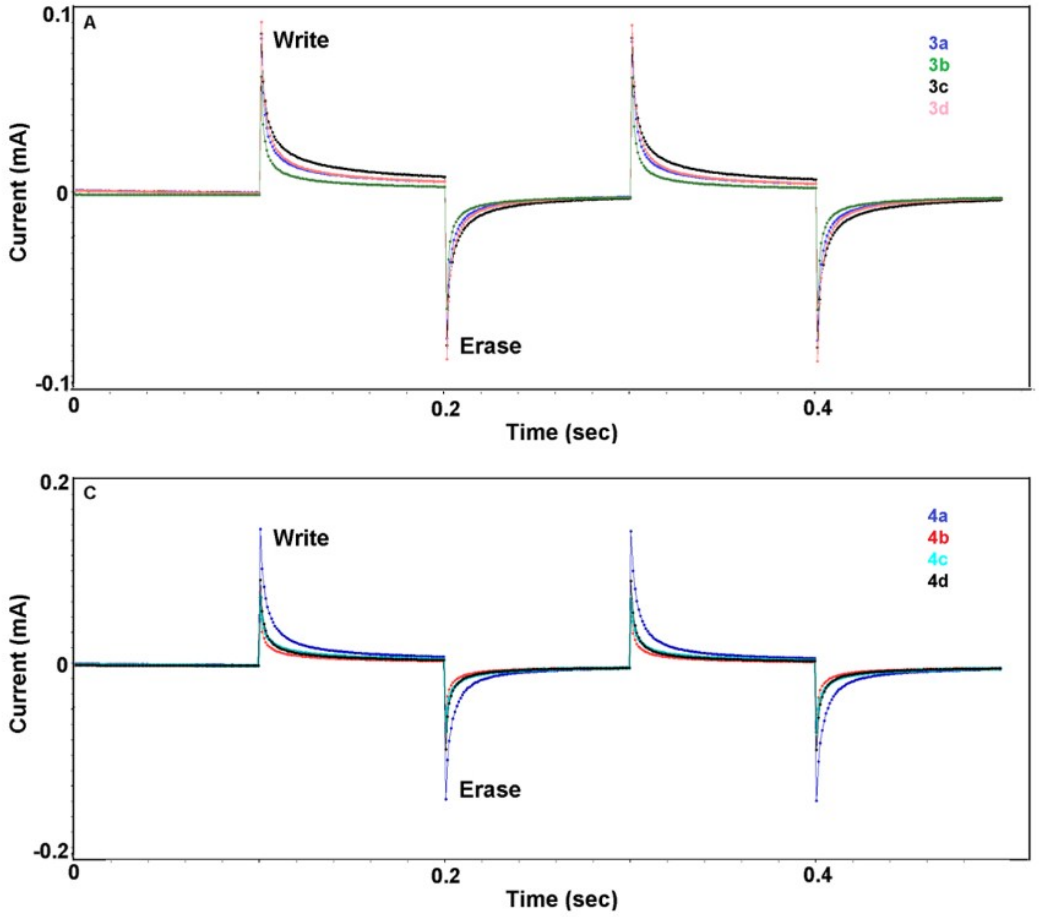


Fig. S5 OCPA response of gold electrode including mono-ferrocenylphosphazenes.

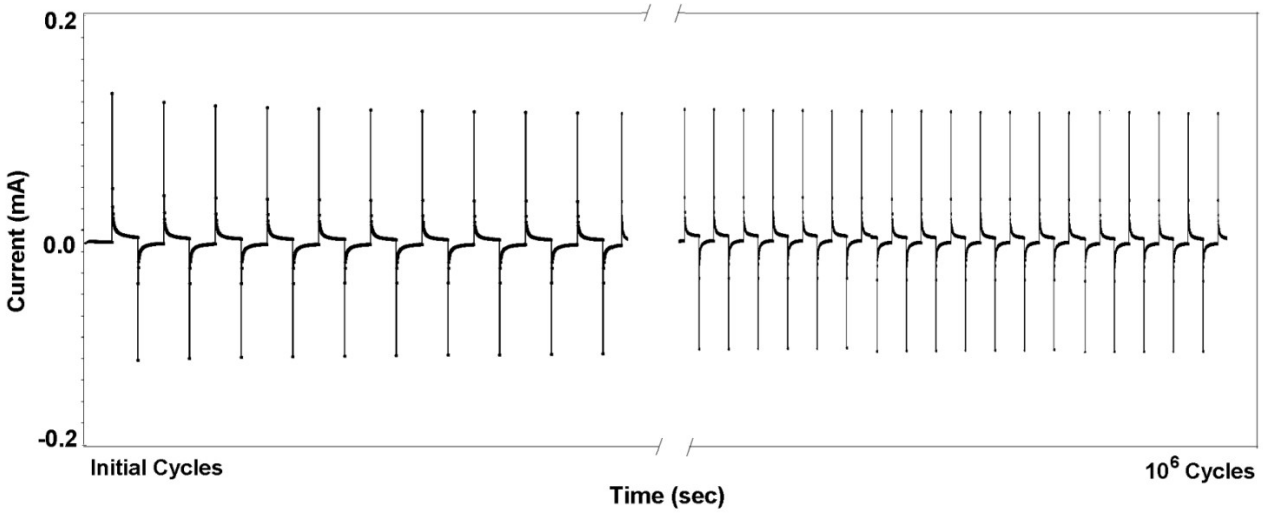
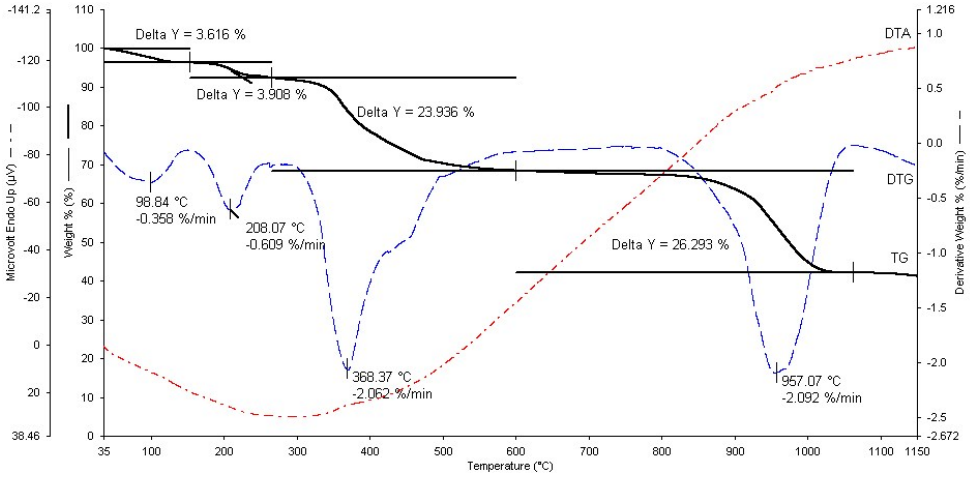


Fig. S6 Current response validating the **4b** compound memory switching for 10^6 cycles.

Table S1 Energy levels of mono-ferrocenylphosphazene compounds

Comp.	E_{ox} vs. Ag/AgCl (V)	HOMO level (eV)	E_{red} vs. Ag/AgCl (V)	LUMO level (eV)	ΔEp (V) (E_{ox}-E_{red})	I_{pc}/I_{pa}	Slope of (log I_a-log υ)	D cm^2s^{-1}
3	0.52	-4.92	0.42	-4.82	0.10	0.62	0.46	$2.63x10^{-5}$
3a	0.48	-4.88	0.41	-4.80	0.07	0.69	0.49	$6.05x10^{-5}$
3b	0.48	-4.88	0.41	-4.81	0.07	0.67	0.49	$6.29x10^{-5}$
3c	0.50	-4.90	0.43	-4.82	0.07	0.69	0.46	$6.34x10^{-5}$
3d	0.49	-4.89	0.43	-4.83	0.06	0.70	0.49	$9.14x10^{-5}$
4	0.52	-4.92	0.42	-4.81	0.10	0.63	0.47	$2.67x10^{-5}$
4a	0.47	-4.87	0.41	-4.80	0.06	0.72	0.52	$6.70x10^{-5}$
4b	0.48	-4.88	0.37	-4.76	0.11	0.67	0.43	$6.68x10^{-5}$
4c	0.47	-4.87	0.39	-4.78	0.08	0.70	0.47	$8.94x10^{-5}$
4d	0.46	-4.86	0.38	-4.78	0.08	0.75	0.44	$9.94x10^{-5}$



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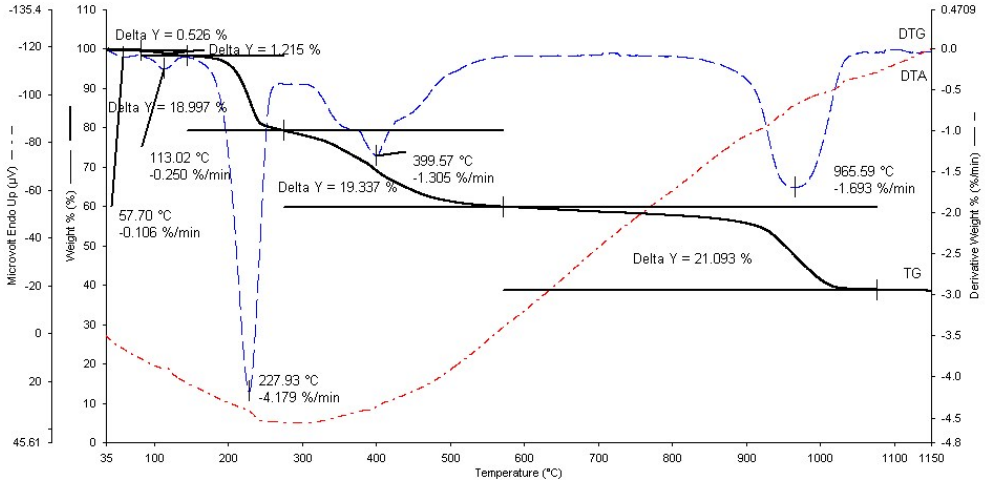

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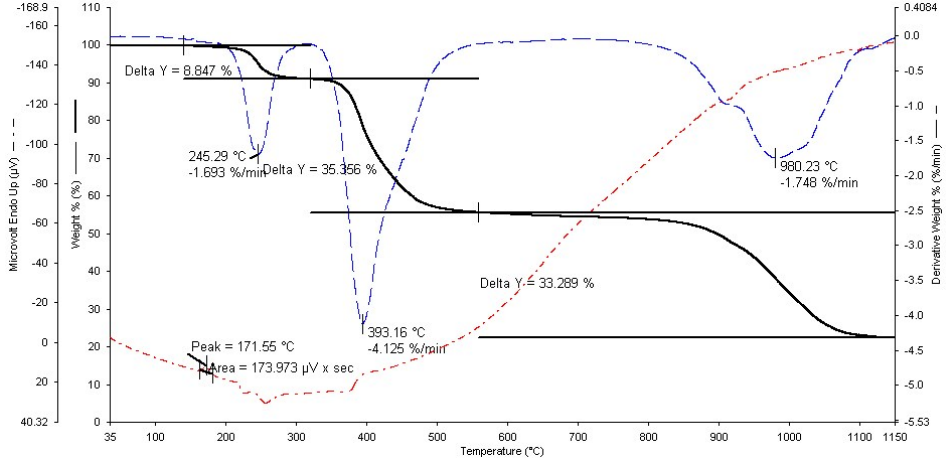
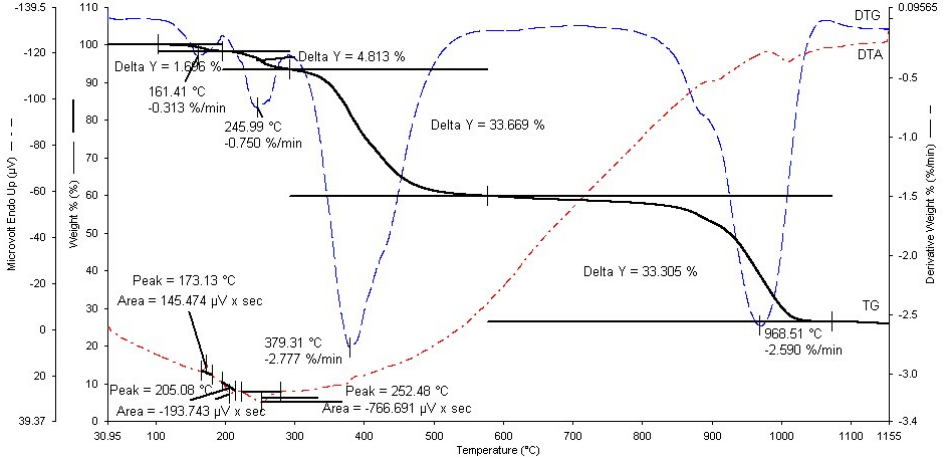
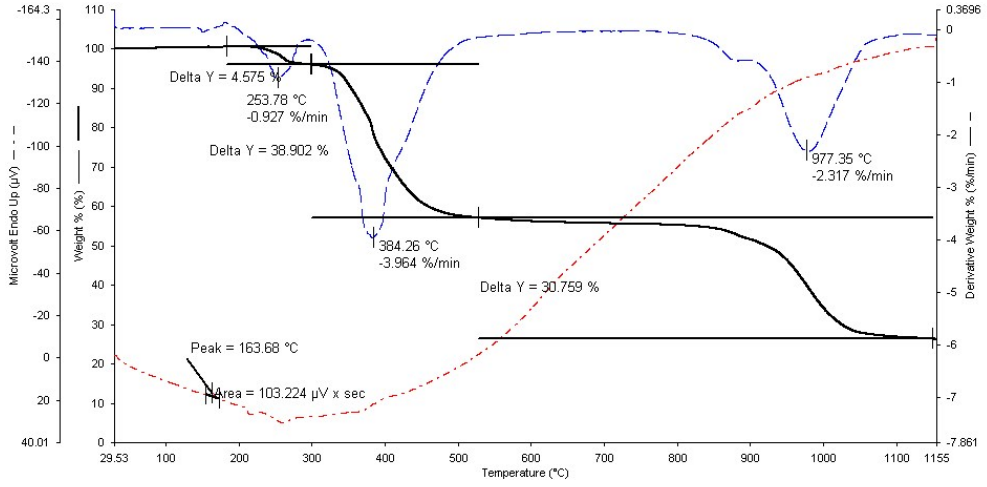


3a

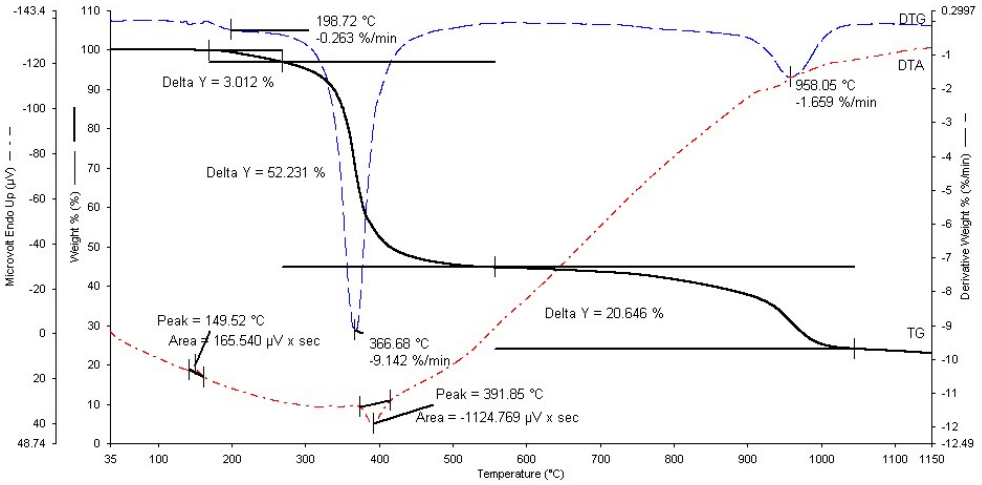


4a

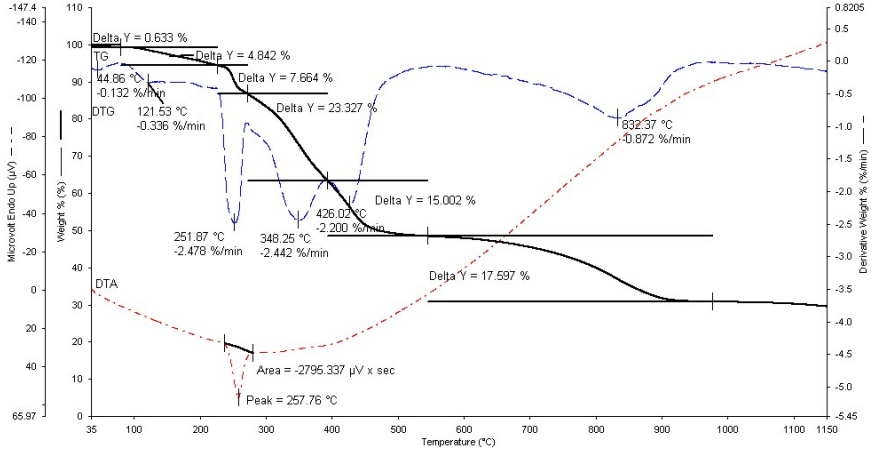
Fig. S7 TG curves of the cyclotetraphosphazenes (**3, 4, 3a** and **4a**).



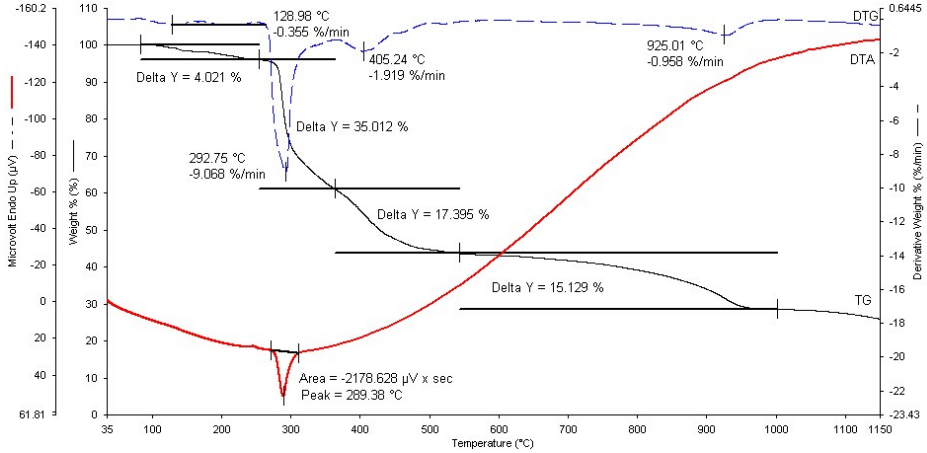
3c



4c



3d



4d

Fig. S8 TG curves of the cyclotetraphosphazenes (**3c, 4c, 3d** and **4d**).

Table S2 Antimicrobial Activities of the Cyclotetraphosphazenes Expressed as Inhibition Zones (mm)

	E. coli ATC 25922	***E. coli ATC 35218***	***E. faecalis ATCC 29212***	***B. subtilis ATCC 6633***	***B. cereus NRRL B-3711***	***P. vulgaris RSKK 96029***	***S. aureus ATCC 25923***	***P. aeruginosa ATCC 27853***	***S. typhimurium ATCC 14028***	***E. hirae ATCC 9790***	***K. pneumaniae ATCC 13883***	***C. krusei ATCC 6258***	***C. albicans ATCC 10231***	***C. tropicalis Y-12968***
3a	11.3±0.5	11.0± 0.0	-	-	-	-	-	-	-	-	-	-	-	-
3b	-	-	11.5±0.5	12.7±0.5	-	11.0±0.0	-	12.3±1.0	-	-	-	-	-	-
3c	-	11.0± 0.0	11.0± 0.0	-	-	-	-	-	-	-	11.0±0.0	-	-	-
3d	24.7±1.0	20.0± 2.0	22.0±3.0	23.3±1.0	23.3±1.5	26.0±1.0	24.3±1.5	17.0±0.0	26.0±0.0	24.0±2.0	22.0±0.0	13.7±1.0	-	-
4	14.0±0.0	13.0±0.0	14.7±0.5	17.7± 0.5	12.0±1.0	13.0±0.0	11.0±1.0	13.0±0.0	13.0±0.0	11.0±2.0	12.7±0.5	-	-	-
4a	-	-	-	-	-	-	-	11.7±0.5	11.7±0.5	-	-	-	-	-
4b	10.3±1.5	12.3±0.5	11.7±1.5	14.3±1.0	11.3±1.0	15.0±0.0	13.7±0.5	13.0±0.0	10.0±0.0	13.7±0.5	12.3±0.5	-	-	-
Amp	18.0±0.0	-	27.0±0.0	23.0±1.0	-	-	44.0±1.0	60.0±0.0	19.0±1.0	9.0±1.0	-	NS	NS	NS
C	25.0±0.0	8.0±0.0	20.0±0.0	21.0±0.0	-	32.0±1.0	24.0±1.0	34.0±0.0	38.0±1.0	22.0±1.0	31.0±1.0	NS	NS	NS
Keto	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	18.0±1.0	11.0±1.0	34.0±2.0

Amp: Ampicillin, C: Chloramphenicol, Keto: Ketokanozol (NS: Not studied)
1,4-dioxane was used as the negative control, and it has no activity for microorganisms used in this study

Table S3 Minimum Inhibitory and Bactericidal Concentrations of the Compounds Against Test Strains (MIC and MBC values are reported in μM)

	E. coli ATCC 25922	***E. coli ATCC 35218***	***E. faecalis ATCC 29212***	***B. subtilis ATCC 6633***	***B. cereus NRRL B-3711***	***P. vulgaris RSKK 96029***	***S. aureus ATCC 25923***	***P. aeruginosa ATCC 27853***	***S. typhimurium ATCC 14028***	***E. hirae ATCC 9790***	***K. pneumaniae ATCC 13883***
	MIC, MBC	**MIC, MBC**	**MIC, MBC**	**MIC, MBC**	**MIC, MBC**	**MIC, MBC**	**MIC, MBC**	**MIC, MBC**	**MIC, MBC**	**MIC, MBC**	**MIC, MBC**
4	-	-	51.5, 51.5	103.0,206.25	-	-	-	-	-	-	-
4b	-	-	-	103.0, 103.0	-	25.75, 25.75	-	-	-	-	-
3d	25.75, 25.75	25.75, 25.75	25.75, 25.75	13.0, 13.0	25.75, 25.75	13.0, 13.0	25.75, 25.75	13.0, 13.0	25.75, 25.75	51.5, 51.5	13.0, 25.75
Amp	>125.0	>125.0	31.25	62.5	31.25	>125.0	62.5	>125.0	62.5	62.5	125.0
C	125, >125	>125.0	62.5, >125	3.91	125, >125	125.0	125.0	>125.0	125.0	62.5, >125	15.63