Phosphorus-Nitrogen Compounds. Part 65. Novel diansa-spiro-cyclotetraphosphazenes: synthesis, characterization, bioactivity and electrochemical properties, fabrication of dye-sensitized solar cell studies

Gürcü Mutlu[a], Aytuğ Okumuş[a], Gamze Elmas*[a], Zeynel Kılıç*[a], Remziye Güzel[b], Büşra Nur Sabah[c], Leyla Açık[c], Hatice Mergen[d] and Tuncer Hökelek[e]

[a]*Department of Chemistry, Ankara University, 06100 Ankara, Türkiye*
[b]*Department of Chemistry, Dicle University, 21280, Diyarbakır, Türkiye*
[c]*Department of Biology, Gazi University, 06500 Ankara, Türkiye*
[d]*Department of Biology, Hacettepe University, 06800 Ankara, Türkiye*
[e]*Department of Physics, Hacettepe University, 06800 Ankara, Türkiye*

Supporting Information

Contents

Section S1. Determination of *in vitro* antimicrobial activity

i. Microorganisms and growth conditions

The microorganisms were obtained from the collections of Gazi University Molecular Biology Culture Collection, Turkey. Eleven bacterial [six G(-), five G(+)] and three fungal strains were used for detection of antimicrobial activity of both cyclotetraphosphazenes (**3** and **4**). G(-) bacterial strains were as follows; *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris* RSKK 96029; G(+) bacterial strains were *Bacillus cereus* NRRL B-371, *Bacillus subtilis* ATCC 6633*, Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Enterococcus hirae* ATCC 9790, and fungal strains were *Candida albicans* ATCC 10231, *Candida krusei* ATCC 6258, *Candida tropicalis* Y-12968. G(+) and G(-) bacteria were grown on nutrient agar (Merck, Germany) plates and incubated at 37 °C for 24 h. Whilst, the yeast strains were grown in Sabouraud dextrose agar (SDA) (Merck, Germany) medium and incubated at 30 °C for 48 h. After incubation, the inoculums of microorganisms were prepared from a single colony in the fresh cultures and suspensions in 0.9% NaCl were adjusted to 0.5 McFarland standard turbidity.

ii. Microdilution assay

The microdilution method was applied to determine the substances having antimicrobial effect on microorganisms exhibited this effect at which concentration.[1] The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungucidal concentration (MFC) values of the compounds were measured by the broth microdilution method[1] in 96-well microliter plates according to CLSI reference method M7-A7[2] for bacteria and M27-A3[3] for yeasts. For antimicrobial testing, 100 µL of medium (Mueller Hinton Broth for bacteria, Saboroud Dextrose Broth for fungi) (Merck, Germany) were added in each well. After adding the three phosphazene derivatives (5000 µM), antimicrobial agents or DMSO to be tested individually to the first wells, serial two-fold dilutions were made in microtitre plates. Five microliters of microorganisms were added to all wells. The microtitre plates were then incubated for 24 h at 37 °C (for bacterial strains) and for 48 h at 30 °C (for fungal strains).

Furthermore, after incubation period, the minimum inhibitory concentration (MIC) values of the compounds were determined by lack visual turbidity. The minimum bactericidal concentration (MBC) and minimum fungucidal concentration (MFC) values of the compounds were designated using subculturing ten microliters volumes from non-turbid wells and spot inoculating onto agar plates [Tryptic Soy Agar (Merck, Germany) for bacteria and Saboroud Dextrose Agar (Merck, Germany) for fungi] and incubated for 24 h at 37 °C (for bacterial strains) and for 48 h at 30 °C (for fungal strains). After incubation, growth was recorded (visible colony formation) and MBCs/MFCs were defined as the lowest concentration without any microorganism growth. The assay was repeated at least three times and average MIC and MBC/MFC values were selected.

iii. Agar well diffusion assay

In this research, the antibacterial and antifungal effects of diansa-spiro-cyclotetraphosphazenes (**3** and **4**) were elucidated by the BACTEC MGIT 960 (Becton Dickinson, Sparks, MD) system using the agar well diffusion assay to identify possible antibacterial and antifungal activities against eleven bacteria and three fungi.[1] For antimicrobial activity testing, a stock solution of the compounds was prepared in DMSO at a concentration of 5000 μM. For positive control, Ampicillin and Chloramphenicol were selected as the reference antibacterial agents, and Ketoconazole was used as a reference antifungal agent. They were the commercially available antibiotics, and were chosen as a control. These antimicrobial agents were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5000 μM. Final concentration of DMSO in the tested cultures was 1%. The stock solutions of the compounds and the antimicrobial agents were stored at 4 °C. DMSO was used as negative control. Before the experiment, all compounds and antimicrobial agents were sterilized by passing through a syringe filter with a pore diameter of 0.2 μm.

Section S2. Evaluation of the DNA and compound interactions

To determine the DNA-binding activities of diansa-spiro-cyclotetraphosphazenes (**3** and **4**), the interactions of both compounds with the plasmid DNA separately were investigated by agarose gel electrophoresis.[1] Stock solutions of both compounds were prepared in DMSO at a concentration of 5000 μM and used within 1 h. After that, aliquots of decreasing concentrations of phosphazenes ranging from 5000 to 312.5 μM in Tris-EDTA buffer, and the 0.5 μg/μL pBR322 plasmid DNA (Thermo Scientific) in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA was added to compounds and the mixtures were incubated at 37°C for 24 h in the dark. Compound/DNA mixtures were loaded onto agarose gel with a loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol). Agarose gel electrophoresis was performed under Tris Acetate-EDTA (TAE) buffer (0.05 M Tris base, 0.05 M glacial acetic acid, and 1 mM Ethylenediaminetetraacetic acid, EDTA, pH=8.0) for 1 h at 70 V. Eventually, the gel was stained with ethidium bromide (0.5 μg/mL) and visualized under UV light using a transilluminator (BioDoc Analyzer, Biometra). The image was captured with a video camera as a TIFF file. Each experiment was repeated three times and the mean values were selected.

Section S3. *Hind*III and *Bam*HI digestions of the compound-plasmid DNA mixture

The affinity evaluation between the diansa-spiro-cyclotetraphosphazenes (**3** and **4**) and adenine-adenine (A/A) and/or guanine-guanine (G/G) regions was performed using restriction endonuclease analysis.[1] Compound/DNA mixtures were incubated for 24 h, and then restricted with 1 U/μL *Hind*III in buffer *Hind*III (Thermo Scientific) and 1 U/μL *BamH*I in buffer *BamH*I (Thermo Scientific) enzymes for 1 h at 37 °C. The restricted DNA was run in 1% agarose gel electrophoresis for 2 h at 70 V in TAE buffer.[4] The gel was stained with ethidium bromide (0.5 μg/mL), and afterward, the gel was viewed with a transilluminator. The image was photographed with a video camera and saved as a Tagged Image File Format (TIFF) file.

*Bam*HI enzyme binds at the sequence 5'-G/GATCC-3'. Since pBR322 plasmid DNA contains a single sequence, it cleaves this sequence. After that, *Bam*HI 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. Hereby, *Hind*III converts Form I and Form II to Form III, similar to *Bam*HI.

Section S4. Cytotoxicity

The cytotoxic activities of the phosphazenes and their encapsulated compounds in nanoparticles were analyzed with MTT assay[5] against MDA-MB-231 breast cancer cells and COS-1 mammalian fibroblast cells. The 96-well cell culture plates were seeded with 1.10^4 cells per well. Cells were left for 24 h of incubation period. The compounds to be tested were dissolved in DMSO (10%) and applied to cells in seven different concentrations (1000, 500, 250, 125, 62.5, 31.25 and 15.62 µM). Cell culture plates were incubated for 24 h (37 °C and 5% CO_2) in the incubator. All concentrations of the samples were applied three times. The solvent DMSO (10%), cisplatin for comparison, DMEM medium (blank) were used as controls. After 24 h, 50 µL MTT (1mg / mL) solution was added to each well. After 2 h of incubation at 37 °C, 100 µL of isopropanol was added to the wells, and the absorbance values of the 96-well plates were read at 570 nm in a microplate reader to determine cell viability. Based on the control groups, the percent viability was calculated by the following formula:

$$Cell\ Viability\% = \frac{Compound\ OD}{Control\ OD} x100$$

The cytotoxicity studies were made in triplicate and the data were given as mean ± standard deviation (SD).

References

[1] Elmas G., Okumuş A., Kılıç Z., Çelik S. P., Açık L., The spectroscopic and thermal properties, antibacterial and antifungal activity and DNA interactions of 4-(fluorobenzyl)spiro(N/O) cyclotriphosphazenium salts. *J. Turk. Chem. Soc. Sect. A: Chem.* **2017**, *4 (3)*, 993-1016. DOI: 10.18596/jotcsa.316902.

[2] Clinical and Laboratory Standards Institute, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Seventh Edition. Wayne, PA: CLSI, **200**6.

[3] Clinical and Laboratory Standards Institute, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard – Second Edition. Wayne, PA: CLCI, **2002**.

[4] Sambrook J., Fritsch E. F., Maniatis T., Molecular cloning:a laboratory manual Cold Spring Harbor, Cold Spring Harbor, New York, **1989**.

[5] ISO 10993-5. Biological evaluation of medical devices-Part 5: Tests for *in vitro* cytotoxicity. International Organisation for Standardization **2009**.

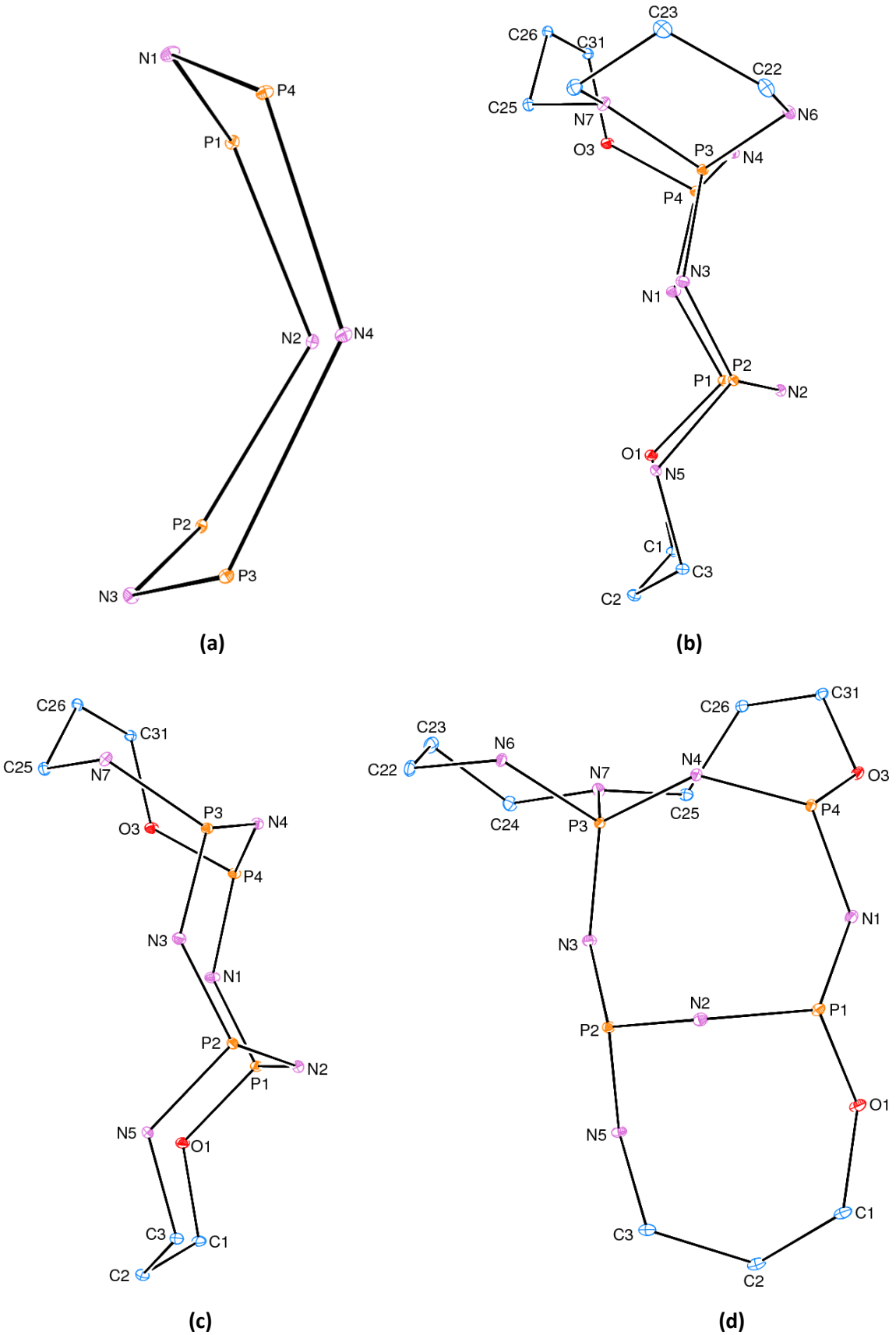


Figure S1. The conformations of (**a**) the tetramer ring, (**b**) the eight-membered 2,4-ansa ring, (**c**) the eight-membered 6,8-ansa ring and (**d**) the six-membered spiro ring of **4.**

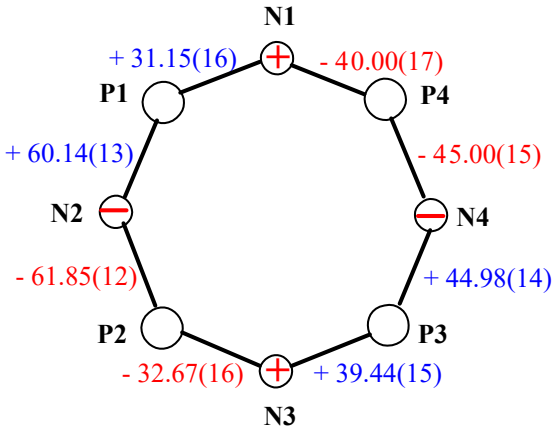


Figure S2. The shape of the cyclotetraphosphazene ring in **4** with torsion angles (deg) given

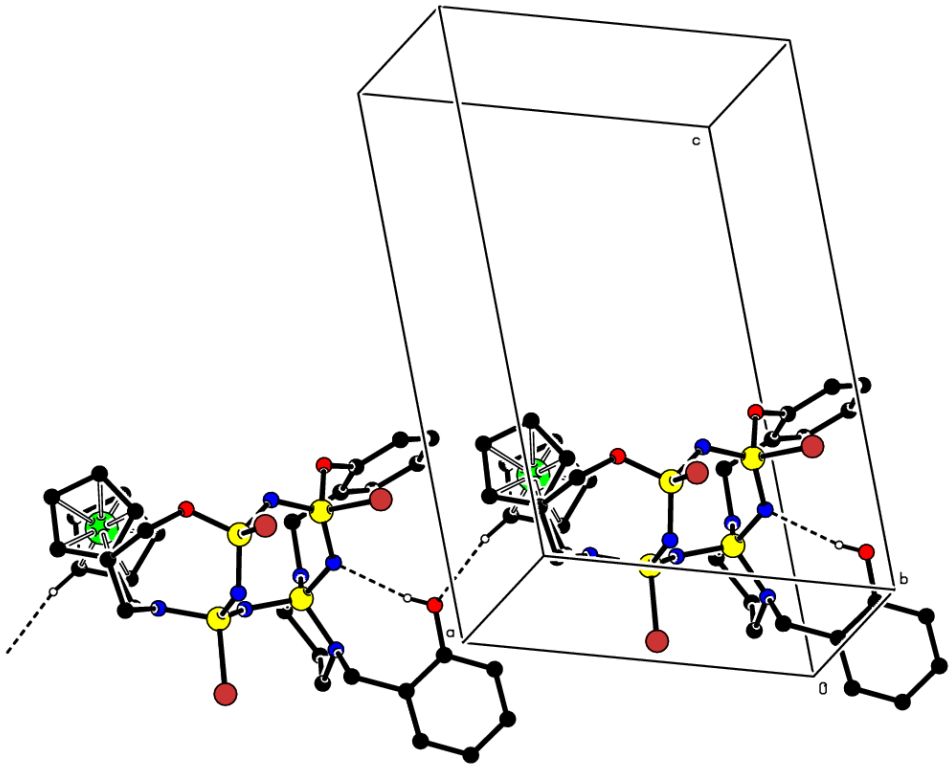
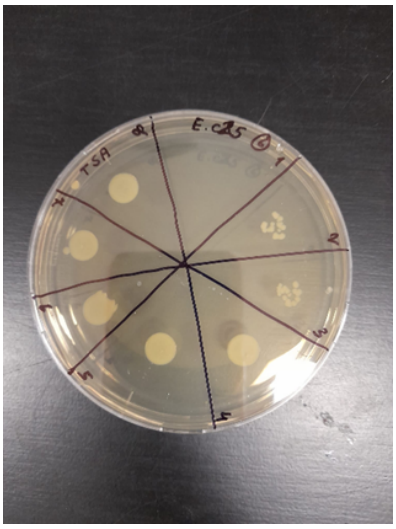
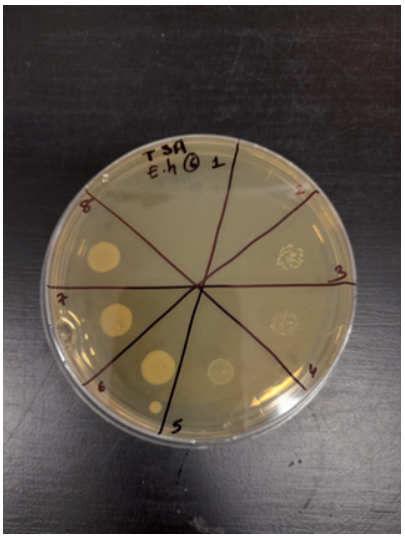


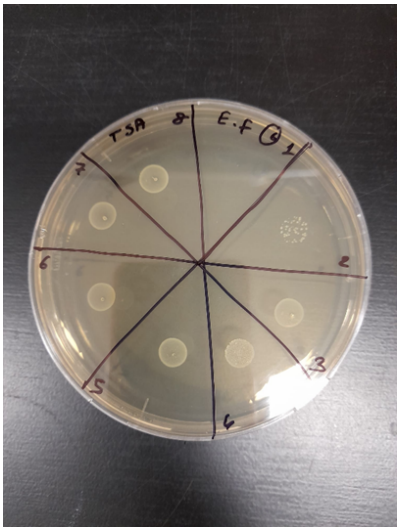
Figure S3. A partial packing diagram of compound **4**. Intramolecular O–H···N and intermolecular C–H···O hydrogen bonds are shown as dashed lines. Nonbonding H atoms have been omitted for clarity.



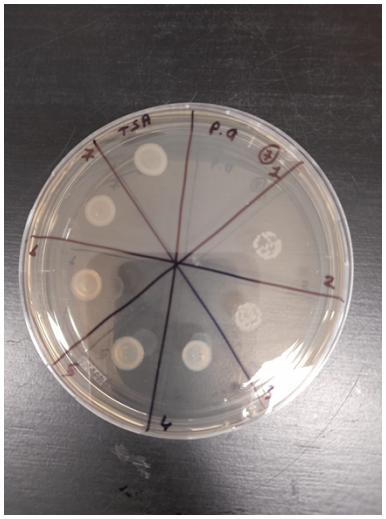
Compound **3** (*E. coli* ATCC 25922)



Compound **3** (*E. hirae* ATCC 9790)

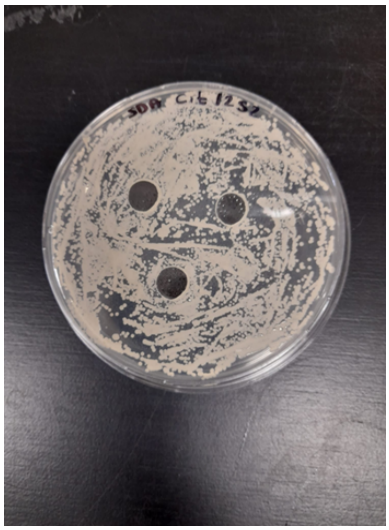


Compound **3** (*E. faecalis* ATCC 29212)



Compound **4** (*P. aeruginosa* ATCC 27853)

Compound **4** (*B. cereus* NRRL B-3711)



Compound **3** (*C. tropicalis* Y-12968)

Figure S4. Antimicrobial and antifungal activity tests of **3** and **4** against the four test strains.