The reactions of $N_3P_3Cl_6$ with monodentate and bidentate ligands: the syntheses and structural characterizations, in vitro antimicrobial activities, and DNA interactions of 4-fluorobenzyl(N/O)spirocyclotriphosphazenes

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Abstract: The Cl replacement reactions of 4-fluorobenzyl(N/O)spirocyclotriphosphazene (2) with excess monoamines led to the formation of 4-fluorobenzylspiro(N/O)tetraaminocyclotriphosphazenes (2a-2d). The partly substituted dispiro 3b and dispiro 3c and fully substituted trispirocyclotriphosphazenes (trans 4a, cis 4c, 4d, and 4e) were obtained, respectively, from the reactions of 2 with one equimolar and two equimolar amounts of diamines, aminoalcohol, and diols. Although efforts were made for the separation of the cis/trans and optical isomers of the dispiro phosphazenes, only one set of diastereomers (RR/RS or SS/SR) of dispiro 3b and dispiro 3c was isolated, respectively. The $^{31}$P NMR spectral data of the other dispiro phosphazenes were evaluated from the $^{31}$P NMR spectra of the reaction mixtures. The reactions of 2 with excess N-methylethylenediamine gave trans 4a as a racemic mixture. While trans 4b (racemic) and cis 4b (meso) occurred from the reaction of 2 with excess N-methyl-1,3-propanediamine, they were not isolated separately. Some of the phosphazenes were screened against bacteria and fungi. The activities of the compounds against anaerobic and microaerophilic gram-negative bacteria were evaluated. It was found that compounds 2, 2b, and trans 4a exhibited tolerable toxic effects on fibroblast cells and had the highest toxicity against MCF-7 cells.

Key words: Monofluorobenzyl(N/O)spirocyclotriphosphazenes, spectroscopy, antimicrobial activity, DNA interaction

1. Introduction

For years, the nucleophilic substitution reactions of hexachlorocyclotriphosphazene, $N_3P_3Cl_6$, with monodentate$^{1,2}$ and bidentate reagents$^3$ have been intensively investigated. The complete substitution reactions of the Cl atoms of $N_3P_3Cl_6$ by primary amines led to the formation of the hexaaminocyclotriphosphazenes.$^4$ The reactions of $N_3P_3Cl_6$ with bidentate reagents afford spiro-, ansa-, dispiro-, trispiro-, spiro-ansa-, spiroansa-spiro-, and spiro-bino-spiro-cyclotriphosphazenes.$^5$ It was observed that diamine and dioxide reagents with trimers usually gave spiro products.$^6-8$ Tetramers with diamines produced spiro and ansa products, as well.$^9$ $N_3P_3Cl_6$, also undergoes regio- and stereoselective reactions. Recently, cyclotriphosphazene derivatives have been attracting much interest because of the potential of their stereogenic properties.$^{10,11}$ Most of the

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chiral cyclotriphosphazene derivatives were obtained by reactions of achiral cyclotriphosphazenes with achiral bidentate reagents. Aminocyclotriphosphazenes have also attracted much consideration for their potential as antibacterial, antifungal, and anticancer agents. Some of the phosphazene derivatives were found to be active on different tumor cells, e.g., HT-29, Hep2, Vero, and DLD1 cells. In addition, the antimicrobial activities of aminocyclotriphosphazenes against various microorganisms were observed.

To the best of our knowledge, there are only two reports on the reactions of bidentate reagents bearing pendant mono and bis (4-fluorobenzyl) precursors in the literature. The present study reports on the replacement reactions of 4-fluorobenzyl(N/O)spirocyclotriphosphazene with monoamines, diamines, aminoa lcohol, and diols (Scheme) for the investigation of their antimicrobial activities against gram-positive, gram-negative, anaerobic, and microaerophilic bacteria and fungi and the DNA interactions of the new 4-fluorobenzyl(N/O)spirocyclotriphosphazenes.

2. Results and discussion
2.1. Chemistry
The condensation reaction of 4-fluorobenzaldehyde with 3-amino-1-propanol resulted in the formation of the intermediate Schiff base N-[E-(4-fluorophenyl)methylidene]-3-(hydroxy)propan-1-amine. The starting difunctional reagent, 3-(4-fluorobenzylamino)-1-propanol, was obtained from the reduction of the Schiff base with NaBH₄ in methanol according to the published procedure. The starting spiro phosphazene derivative, 4-fluorobenzyl(N/O)spirocyclotriphosphazene (2), was also prepared according to the literature. The reactions of 2 with excess monoamines gave the fully substituted cyclotriphosphazenes (2a-2d). The estimated yields of these compounds are in the range of 61%-80%. The dispiro (dispiro 3a and dispiro 3b) and trispiro (trans 4a, trans 4b, and cis 4b) diaminophosphazenes occur from the reactions of 2 with N-methylethylenediamine and N-methylpropanediamine, whereas the reactions of 2 with sodium 2,2,3,3-tetrafluorobutanedioxide and sodium 2,2-dimethylpropanedioxide resulted in the formation of the partly substituted dispiro 3d and dispiro 3e, cis dichloro ansa 3d and cis dichloro ansa 3e, and fully substituted 4d and 4e (Scheme). In addition, the reactions of sodium 1-aminopropane-3-oxide gave dispiro 3c, cis dichloro ansa 3c, and cis 4c. The reactions of 2 with one equimolar amount of N-methylethylenediamine and N-methylpropanediamine produced both partly substituted dispiro 3a and dispiro 3b daminophosphazenes as primary products and the fully substituted trispiro (trans 4a, trans 4b, and cis 4b) compounds as minor products. The reactions of 2 and excess amounts of N-methylethylenediamine and N-methylpropanediamine afforded the mixtures of dispiro 3a and dispiro 3b and trispiro (trans 4a, trans 4b, and cis 4b) products. The compounds dispiro 3a and trans 4b/cis 4b could not be isolated, but the compounds dispiro 3b and trans 4a were obtained from the reaction mixtures. As an example, the 31P NMR spectrum of the mixture of 2 and excess amounts of N-methylethylenediamine is depicted in Figure 1. The spectrum was analyzed, and the results are given in Table 1. The relative yields of dispiro 3a and trans 4a were estimated from the NMR spectrum of the mixture as 45% and 55%, respectively. In addition, the reaction of 2 with one equimolar amount of sodium 3-amino-1-propanoxide with 2 resulted in the formations of cis dichloro ansa 3c and cis 4c. The relative yields of ansa 3c and cis 4c were calculated as 22% and 78%, respectively, from the spectrum of the mixture (Figure 2). When the reactions were made with one equimolar amount of sodium 2,2,3,3-tetrafluorobutanedioxide and 2, the compounds cis dichloro ansa 3d and dispiro 3d were ob-
served in the reaction mixtures, but these compounds were not isolated. The spectrum of the mixture of 2 and one equimolar amount of sodium 2,2,3,3-tetrafluorobutanedioxide is depicted in Figure 3. The relative yields of ansa 3d and dispiro 3d were estimated as 44% and 56%, respectively, from the $^{31}$P spectrum of the reaction mixture. On the contrary, the reactions of one equimolar amount of sodium 2,2-dimethylpropanedioxide with 2 gave dispiro 3e as the major product (64%, calculated from the reaction mixture). The expected product ansa 3e did not occur, and dispiro 3e could not be isolated. The reactions of 2 with excess amounts of sodium 2,2,3,3-tetrafluorobutanedioxide and sodium 2,2-dimethylpropanedioxide afforded trispiro 4d and 4e, respectively, as major products. The expected cis dichloro ansa 3d and cis dichloro ansa 3e were also present as byproducts (relative yields ca. ≤5% from the $^{31}$P NMR spectrum) in the reaction mixture. The compounds trispiro 4d and 4e were isolated in pure form using column chromatography.
The dispiro, ansa, and trispiro phosphazenes may have geometrical and optical isomers and the isomer distributions of these phosphazenes are given in Table 1. The isomer distributions may be rationalized with via stick diagrams as in Figure 4. The choice of the N/O ligand (1) for the preparation of these phosphazenes is very important because it gives solely the restricted spiro structure of the cyclotriphosphazenes. Hence, geometrical and a certain number of optical isomers may arise. The compounds dispiro 3d and dispiro 3e have one
stereogenic P-center, while dispiro 3a, dispiro 3b, and dispiro 3c have two different P centers of chirality with a symmetrically substituted P atom, PCl$_2$. These compounds are expected to form racemic mixtures. The $^{31}$P NMR spectra of the mixtures of dispiro 3a and dispiro 3c indicate that only one diastereomer is present, while dispiro 3b is present as two diastereomeric forms, dispiro 3b and dispiro 3b'. One of them (RR'/SS' or RS'/SR') was obtained using column chromatography. The ansa 3c also has two different P centers of chirality with an unsymmetrically substituted P atom, PON. Only one diastereomer, ansa (2,4-dichloro cis), was observed in the reaction mixture. On the other hand, the phosphazene derivatives ansa 3d, ansa 3e, trans 4a, trans 4b, cis 4b, and cis 4c contain two equivalent P centers of chirality. It is shown by the $^{31}$P NMR spectra that compounds ansa 3d and ansa 3e occur as meso forms. The trans 4a and cis 4c are isolated as racemic and meso forms, respectively (Table 1).

The microanalysis, FTIR, $^1$H NMR, $^{13}$C$^1$H NMR, $^{31}$P$^1$H NMR, HSQC, and HMBC results verified the proposed structure of the compounds (see Section 3). The [M]$^+$ and/or [MH]$^+$ peaks were evaluated in the MS spectra of the compounds.

The $^{31}$P NMR spectral data of the 4-fluorobenzyl(N/O)spirocyclophosphazenes are listed in Table 2. The compounds 2, ansa 3d, ansa 3e, and 4e have the AX$_2$ spin system and appear as one triplet (Pspiro, PA) and one doublet (PX). The partly substituted compounds dispiro 3a, dispiro 3b, dispiro 3b', dispiro 3c, ansa 3c, dispiro 3d, and dispiro 3e have ABX and trans 4b has ABC spin systems, and they give rise to a doublet of doublets (dd) for the P atoms of the phosphazene ring. The spectra of the rest of the phosphazenes give a total of eight signals for AB$_2$ systems. All the $\delta$P-shifts, the coupling constants ($^2$J$_P$), and the $^2$J$_{PP}$/$\Delta \nu$ values were estimated and are listed in Table 2. It is observed that the $\delta$P(spiro)-shifts of all the 4-fluorobenzylspirophosphazenes are shifted to down-field according to compound 2.

The assignments of the $\delta$-shifts, multiplicities, and $^2$J$_P$ constants were elucidated from the $^{13}$C and $^1$H
Figure 4. The expected and obtained isomer distributions of the partly and fully substituted phosphazene derivatives.
Table 1. The theoretical and expected stereoisomer distributions of the 4-fluorobenzylspirocyclophosphazenes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Centers of chirality</th>
<th>Stereogenic P atoms (n)</th>
<th>Stereoisomers (2°) (expected)</th>
<th>Chirality (expected)</th>
<th>Chirality (found)</th>
<th>Geometrical isomer (found)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dispiro 3d</td>
<td>One</td>
<td>1</td>
<td>1 2 R S</td>
<td>Racemic (lines 1/2)</td>
<td>Racemic (lines 1/2)</td>
<td>dispiro</td>
</tr>
<tr>
<td>dispiro 3a</td>
<td>Two different (with a symmetrical substituted P atom)</td>
<td>2</td>
<td>1 2 3 4 RR' RR' RS' SR' SS'</td>
<td>Racemic 1 (lines 1/4)</td>
<td>Only one diastereomer was observed in the reaction mixture with 31P NMR.</td>
<td>dispiro</td>
</tr>
<tr>
<td>dispiro 3b</td>
<td>Two different (with a symmetrical substituted P atom)</td>
<td>2</td>
<td>1 2 3 4 RR' RR' RS' SS'</td>
<td>Racemic 1 (lines 1/4)</td>
<td>Two diastereomers were observed in the reaction mixture; one of them was isolated</td>
<td>dispiro</td>
</tr>
<tr>
<td>ansa 3c</td>
<td>Two different (with an unsymmetrical substituted P atom)</td>
<td>2</td>
<td>1 2 3 4 RR' RR' SS' SS'</td>
<td>Racemic 1 (lines 1/4)</td>
<td>Only one diastereomer was observed in the reaction mixture with 31P NMR</td>
<td>ansa (2,4 dichloro cis)</td>
</tr>
<tr>
<td>ansa 3d</td>
<td>Two equivalent (with an unsymmetrical substituted P atom)</td>
<td>2</td>
<td>1 2 3 4 RR RR' RR' RS'</td>
<td>Racemic 1 (lines 1/4)</td>
<td>Meso (lines 2/3, RS=SR)</td>
<td>ansa (2,4 dichloro cis)</td>
</tr>
<tr>
<td>ansa 3e</td>
<td>Two equivalent (with an unsymmetrical substituted P atom)</td>
<td>2</td>
<td>1 2 3 4 RR RR' SR SS</td>
<td>Racemic 1 (lines 1/4)</td>
<td>(from 31P NMR)</td>
<td>trans NN'-di(methyl)</td>
</tr>
<tr>
<td>trans 4a</td>
<td>Two equivalent (with an unsymmetrical substituted P atom)</td>
<td>2</td>
<td>1 2 3 4 RR RR' RS' SR</td>
<td>Racemic 1 (lines 1/4)</td>
<td>Racemic 1 (lines 1/4)</td>
<td>trans NN'-di(methyl)</td>
</tr>
<tr>
<td>trans 4b</td>
<td>Two equivalent (with an unsymmetrical substituted P atom)</td>
<td>2</td>
<td>1 2 3 4 RR RR' RS' SR</td>
<td>Racemic 1 (lines 1/4)</td>
<td>(from 31P, 13C, and 1H NMR)</td>
<td>cis NN'-di(methyl)</td>
</tr>
<tr>
<td>cis 4b</td>
<td>Two equivalent (with an unsymmetrical substituted P atom)</td>
<td>2</td>
<td>1 2 3 4 RR RR' RS' SR</td>
<td>Racemic 1 (lines 1/4)</td>
<td>(from 31P, 13C, and 1H NMR)</td>
<td>cis NN'-di(methyl)</td>
</tr>
<tr>
<td>cis 4c</td>
<td>Two equivalent (with an unsymmetrical substituted P atom)</td>
<td>2</td>
<td>1 2 3 4 RR RR' RS' SR</td>
<td>Racemic 1 (lines 1/4)</td>
<td>(from 31P, 13C, and 1H NMR)</td>
<td>cis NN'-di(methyl)</td>
</tr>
</tbody>
</table>

NMR spectra (HSQC and HMBC experiments for 4d) of all the new 4-fluorobenzyl(N/O)spirocyclotriphosphazenes and are presented in Section 3. The signals of all the carbon atoms are interpreted in the 13C NMR spectra of all the 4-fluorobenzylspirophosphazenes. As expected, in the 13C spectra of the tetraaminocyclotriphosphazenes (2a–2d), the geminal substituents show two small separated peaks for NCH2, NCH2CH2, NCH2CH2CH2, and OCO. The 3JFC coupling constants of these compounds (2a–2d) arise to triplets of the NCH2CH2 carbons because of the second-order effects that have been previously observed for some cyclophosphazene derivatives.20 The 3JPC values were calculated using the external transitions of the peaks. The coupling constants of 1JFC, 2JFC, 3JFC, and 4JFC are very helpful for the interpretations of the phenyl carbons. In addition, the 1JFC, 2JFC, 3JFC, and 4JFC of trans 4b/cis 4b were found to be paired signals, indicating that trans 4b and cis 4b occurred in the presence of a diastereomeric mixture. The δC-shifts of OCH2CF2 carbons were observed at 114.10 ppm (1JFC 256.1 Hz, 2JFC 27.2 Hz) for ansa 3d, 111.55 ppm (1JFC 256.9 Hz, 2JFC 29.1 Hz) for dispiro 3d, and 111.67 ppm (1JFC 256.9 Hz and 2JFC 27.6 Hz) and 114.23 ppm (1JFC 256.9 Hz, 2JFC 27.6 Hz) for 4d. These values are very decisive for the CF2 groups. The δC-shift of OCH2C of 4e is also very significant and it is found at 31.92 ppm (3JPC 5.3 Hz).

The assignments of the phenyl protons of the phosphazenes were estimated using the coupling constants of 3JFH and 4JFH. The average values of 3JFH and 4JFH were calculated as 8.9 Hz and 5.7 Hz, respectively. The average of δH-shifts of NCH2CH2, NCH2, and OCH2 spiro protons of the phosphazenes were found as 1.86 ppm, 3.01 ppm, and 4.30 ppm, respectively, compared to the values (1.70 ppm, 2.80 ppm, and 3.70 ppm)
of the \( \delta \)H-shift of the free amine ligand. In addition, the \( \delta \)H-shift of the protons of Ar\( \text{CH}_2 \text{N} \) of the free amine ligand was observed at 3.71 ppm. It is smaller (ca. 3.90 ppm) than those of compounds 2 and 2a-2d. The \(^1\)H NMR spectra of dispiro 3b, dispiro 3c, dispiro 3d, ansa 3d, trans 4a, trans 4b, cis 4b, and cis 4c are considerably complicated because of the aliphatic hydrogens are diastereotopic. However, the protons of Ar\( \text{CH}_2 \text{N} \) in dispiro 3c, dispiro 3b, trans 4a, and trans 4b create the doublet of doublets due to the geminal H-1 and vicinal P-31 couplings. Hence, these protons are not equivalent to each other and the average values of \( ^3J_{PH} \) and \( ^2J_{HH} \) are 8.0 and 14.6 Hz, respectively. The N\( \text{CH}_3 \) and C\( \text{CH}_3 \) protons of the diamino (dispiro 3b, trans 4a, and trans 4b) and trispiro (4e) compounds were observed as doublets (the average value of \( ^3J_{PH} \) is 12.0 Hz) and a singlet, respectively. The \( \delta \)H-shifts of O\( \text{CH}_2 \) spiro protons of the phosphazenes were observed in the range of 3.75–4.41 ppm, and the average \( ^3J_{PH} \) value, 13.5 Hz, was very large.

The characteristic stretching band (\( \nu_{N-H} \), 3402 cm\(^{-1}\), broad) observed for 3-(4-fluorobenzylamino)-1-propanol disappeared in the IR spectra of 2 and 2a-2d. In the IR spectra of dispiro 3b, dispiro 3c, trans 4a, trans 4b, cis 4b, and cis 4c, broad \( \nu_{N-H} \) peaks were observed in the range of 3201–3163 cm\(^{-1}\), indicating hydrogen bond formation. All the phosphazenes exhibit intense stretching vibrations between 1202–1256 cm\(^{-1}\) and 1141–1198 cm\(^{-1}\), attributed to the \( \nu_{P=N} \) bonds of the trimeric phosphazene skeletons.21 The asymmetric and symmetric vibrations of \( \nu_{P\text{CCl}} \) emerge for 2, dispiro 3b, and dispiro 3c of 580–575 and 545–531 cm\(^{-1}\). In addition, the \( \nu_{\text{COC}} \) stretching frequencies of morpholine (2c) and DASD-substituted (2d) phosphazenes were observed at 1055 and 1053 cm\(^{-1}\), respectively.

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Compound} & \text{Spin system} & \text{P}_A & \text{P}_B & \text{P}_C & \text{P}_X & \text{2}J_{PP} \text{(Hz)} \\
\hline
2 & AX_2 & 9.06 & - & - & 33.22 & 50.2 \\
2a & AB_2 & 21.17 & 18.98 & - & - & 44.5 \quad 0.08 \\
2b & AB_2 & 20.20 & 22.18 & - & - & 46.2 \quad 0.10 \\
2c & AB_2 & 19.95 & 21.45 & - & - & 47.8 \quad 0.13 \\
2d & AB_2 & 19.66 & 21.18 & - & - & 50.2 \quad 0.14 \\
\text{dispiro 3a} & ABX & 27.38 & 25.94 & - & - & 15.40 \quad 2J_{AB}: 48.6; 2J_{AX}: 55.9; 2J_{BX}: 51.0 \\
\text{dispiro 3b} & ABX & 15.08 & 17.76 & - & - & 24.94 \quad 2J_{AB}: 53.5; 2J_{AX}: 45.0; 2J_{BX}: 37.7 \\
\text{dispiro 3b'} & ABX & 15.05 & 17.20 & - & - & 26.12 \quad 2J_{AB}: 52.2; 2J_{AX}: 47.4; 2J_{BX}: 40.1 \\
\text{dispiro 3c} & ABX & 13.31 & 14.25 & - & - & 24.94 \quad 2J_{AB}: 58.1; 2J_{AX}: 54.1; 2J_{BX}: 49.5 \\
\text{ansa 3c} & ABX & 20.72 & 18.55 & - & - & 35.74 \quad 2J_{AB}: 58.3; 2J_{AX}: 46.2; 2J_{BX}: 38.9 \\
\text{dispiro 3d} & ABX & 15.10 & 14.50 & - & - & 26.96 \quad 2J_{AB}: 77.7; 2J_{AX}: 48.6; 2J_{BX}: 80.2 \\
\text{ansa 3d} & AX_2 & 13.76 & - & - & - & 28.20 \quad 62.4 \\
\text{dispiro 3e} & ABX & 14.75 & 8.91 & - & - & 25.72 \quad 2J_{AB}: 71.7; 2J_{AX}: 48.6; 2J_{BX}: 75.3 \\
\text{ansa 3e} & AX_2 & 35.75 & - & - & - & 31.56 \quad 55.9 \\
\text{trans 4a} & ABX & 27.15 & 26.80 & - & - & 15.59 \quad 2J_{AB}: 49.4; 2J_{AX}: 55.9; 2J_{BX}: 53.3 \\
\text{trans 4b} & ABC & 21.09 & 21.74 & 22.39 & - & 2J_{AB}: 41.3; 2J_{AC}: 38.9; 2J_{BC}: 36.4 \\
\text{cis 4b} & AB_2 & 20.49 & 21.80 & - & - & 38.9 \quad 0.11 \\
\text{cis 4c} & AB_2 & 19.13 & 17.37 & - & - & 58.3 \quad 0.14 \\
4d & AB_2 & 18.28 & 20.25 & - & - & 78.4 \quad 0.22 \\
4e & AX_2 & 19.47 & - & - & - & 14.15 \quad 68.8 \\
\hline
\end{array}
\]

\footnote{242.93 MHz \(^{31}\)P\(^{\text{1}}\)H NMR measurements in CDCl\(_3\) solutions at 298 K. Chemical shifts referenced to external H\(_3\)PO\(_4\).}
2.2. Antimicrobial activities

The activities of the parent amine (3-(4-fluorobenzylamino)-1-propanol) and phosphazene derivatives (2, 2a–2d, dispiro 3b, trans 4a, trans 4b/cis 4b, cis 4c, 4d, and 4e) against anaerobic and microaerophilic gram-negative bacteria were determined. The fully substituted phosphazenes trans 4a, trans 4b/cis 4b, cis 4c, 4d, and 4e are active against *Prevotella intermedia* ATCC 25261. The most active one was cis 4c (Table 3).

Most of the known antibiotics target five main mechanisms of microorganisms: DNA replication, RNA synthesis, protein synthesis, cell wall synthesis, and folic acid synthesis.22 Bacterial response to chemicals or antibiotics changes according to the drug chemical structure or the bacterial structure. Reagents have different binding affinities for their targets and different chemical properties that affect their ability to enter the cell.23 On the other hand, gram-negative bacteria are better protected than gram-positive bacteria due to their additional lipopolysaccharide layer.22

The antimicrobial activities of the phosphazenes were evaluated against bacterial and fungal species. The results are listed in Table 4. The compounds trans 4a, cis 4c, 4d, and 4e are more active against *Escherichia coli* ATCC 35218 than chloramphenicol. Compound 4d seems to be effective against *Salmonella typhimurium* ATCC 14028 as much as ampicillin. Some of the phosphazenes exhibit strong anticandidal activity against *Candida albicans* ATCC 10231 and *C. krusei* ATCC 6258. They are especially more efficient than the control antifungal agent ketoconazole for *C. albicans* (Table 4). It is well known that *Candida* species cause fungal infections. Consequently, the tested compounds are the most promising anticandidal derivatives against *C. albicans* and *C. krusei*. However, it is important to note that the bacterial response ought to be different for standard antibiotics and the compounds tested in this study.

2.3. Interaction with plasmid DNA

Figure 5 depicts the electrophoretograms showing the interaction of pBR322 DNA with the compounds at concentrations in the range of 1–10 μM. Lane P displays plasmid DNA as a control that is a mixture of supercoiled form I and singly nicked circular form II. Lanes 1–6 show that the plasmid DNA interacted with increasing concentrations of amine, 2, 2a, 2b, 2c, dispiro 3b, trans 4a, 4d, and 4e. These compounds caused a slight decrease of the mobility of form I. When pBR322 DNA interacts with decreasing concentrations of 4e, there is a commencing decrease in the mobility of the form I DNA at high concentrations of the compound. In the case of trans 4b and cis 4c, form III bands occurred, indicating the cleavage of DNA-compound binding. These results suggest that compounds trans 4b and cis 4c lead to conformational changes in pBR322 DNA.

2.4. *BamHI* and *HindIII* digestion

Figure 6 illustrates the electrophoretograms for the incubated mixtures of plasmid DNA and the compounds, followed by *BamHI* and *HindIII* digestion. When plasmid DNA was digested with *BamHI* and *HindIII* in the absence of the phosphazenes, the linear form III band was observed solely, indicating that plasmid DNA was digested with *BamHI* and *HindIII* at the specific GG site and AA site, respectively. On the other hand, when compounds amine, 2, 2a, 2b, 2c, 2d, dispiro 3b, trans 4a, trans 4b/cis 4b, cis 4c, 4d, and 4e were digested with *BamHI*, only form III bands were observed. The *HindIII* digestions of trans 4a, dispiro 3b, trans 4b/cis 4b, cis 4c, 4d, and 4e create a mixture of form I and form III bands. The results suggest that dispiro 3b, trans 4a, trans 4b/cis 4b, cis 4c, 4d, and 4e can cause a greater conformational change to the DNA than the other phosphazenes, indicating the compound binding to AA nucleotides of DNA.
Table 3. The mean numbers of the measurements of inhibition zones of the compounds, their solvents (DMF), and the amoxicillin used as a control against anaerobic and microaerophilic bacteria.

<table>
<thead>
<tr>
<th>Test bacteria/</th>
<th>Amine 1</th>
<th>2a</th>
<th>2b</th>
<th>2c</th>
<th>trans 4a</th>
<th>trans 4b/cis 4b</th>
<th>cis 4c</th>
<th>4d</th>
<th>4e</th>
<th>Amoxicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. gingivalis</td>
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<tr>
<td>A. actinomycetemcomitans</td>
<td>-</td>
<td>-</td>
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<tr>
<td>ATCC 29323</td>
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</tbody>
</table>

Note: The values are in mm.
Table 4. Antimicrobial activity of the 4-fluorobenzylspirocyclotriposphazenes registered as inhibition zones (mm).

<table>
<thead>
<tr>
<th>Test microorganism</th>
<th>Compounds</th>
<th>Amine 2</th>
<th>2a</th>
<th>2b</th>
<th>2c</th>
<th>2d</th>
<th>dispiro 3b</th>
<th>trans 4a</th>
<th>Trans 4b/cis 4b</th>
<th>cis 4c</th>
<th>4d</th>
<th>4e</th>
<th>Amp</th>
<th>C</th>
<th>Keto</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli ATCC 35218, G (-)</td>
<td>9 ± 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11 ± 1</td>
<td>-</td>
<td>11 ± 1</td>
<td>11 ± 1</td>
<td>-</td>
<td>8 ± 0</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. subtilis ATCC 6633, G (+)</td>
<td>-</td>
<td>9 ± 1</td>
<td>-</td>
<td>11 ± 1</td>
<td>12 ± 1</td>
<td>10 ± 0</td>
<td>10 ± 1</td>
<td>13 ± 0</td>
<td>12 ± 1</td>
<td>14 ± 1</td>
<td>14 ± 1</td>
<td>18 ± 0</td>
<td>24 ± 0</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>S. aureus ATCC 25923, G (+)</td>
<td>-</td>
<td>8 ± 0</td>
<td>-</td>
<td>9 ± 0</td>
<td>9 ± 1</td>
<td>11 ± 1</td>
<td>8 ± 1</td>
<td>11 ± 1</td>
<td>11 ± 0</td>
<td>14 ± 1</td>
<td>10 ± 1</td>
<td>-</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis ATCC 29212, G (+)</td>
<td>10 ± 0</td>
<td>9 ± 0</td>
<td>-</td>
<td>13 ± 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 ± 1</td>
<td>10 ± 1</td>
<td>23 ± 1</td>
<td>21 ± 0</td>
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</tr>
<tr>
<td>S. aureus ATCC 27853, G (-)</td>
<td>9 ± 1</td>
<td>10 ± 1</td>
<td>11 ± 1</td>
<td>12 ± 0</td>
<td>12 ± 1</td>
<td>8 ± 0</td>
<td>14 ± 1</td>
<td>12 ± 1</td>
<td>11 ± 1</td>
<td>14 ± 1</td>
<td>60 ± 0</td>
<td>34 ± 0</td>
<td>NS</td>
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<td></td>
</tr>
<tr>
<td>K. pneumoniae ATCC 13883, G (-)</td>
<td>-</td>
<td>12 ± 1</td>
<td>8 ± 1</td>
<td>-</td>
<td>12 ± 2</td>
<td>11 ± 1</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
<td>16 ± 2</td>
<td>13 ± 1</td>
<td>19 ± 1</td>
<td>38 ± 1</td>
<td>NS</td>
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</tr>
<tr>
<td>E. hirae ATCC 9790, G (+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14 ± 0</td>
<td>16 ± 2</td>
<td>-</td>
<td>NS</td>
<td>32 ± 1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>P. vulgaris RSK 96029, G (-)</td>
<td>-</td>
<td>10 ± 1</td>
<td>13 ± 1</td>
<td>19 ± 1</td>
<td>18 ± 1</td>
<td>19 ± 1</td>
<td>17 ± 0</td>
<td>17 ± 1</td>
<td>22 ± 1</td>
<td>18 ± 2</td>
<td>17 ± 2</td>
<td>NS</td>
<td>NS</td>
<td>11 ± 1</td>
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<tr>
<td>C. albicans ATCC 10231</td>
<td>-</td>
<td>12 ± 0</td>
<td>14 ± 1</td>
<td>13 ± 0</td>
<td>15 ± 0</td>
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<td>11 ± 1</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
<td>18 ± 1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>C. krusei ATCC 6288</td>
<td>-</td>
<td>11 ± 1</td>
<td>14 ± 1</td>
<td>13 ± 1</td>
<td>12 ± 0</td>
<td>11 ± 1</td>
<td>14 ± 1</td>
<td>14 ± 1</td>
<td>13 ± 1</td>
<td>NS</td>
<td>NS</td>
<td>34 ± 2</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5. Gel electrophoretic mobilities of pBR322 DNA after incubation at concentrations ranging from 2500 to 78 μM at 37 °C. Line 1: 2500, line 2: 1250, line 3: 625, line 4: 312.50, line 5: 156.25, line 6: 78 (μM), P: untreated plasmid.

2.5. Evaluation of toxicity results

The cytotoxicity of all the compounds was determined using the WST-1 method, and the results are listed in Tables 5 and 6. The results indicate that all the compounds were less toxic against L929 fibroblast (normal) cells at 25-50 μg/mL concentrations with a duration of 24 h of incubation. Even at 50 μg/mL, more than 50% of the fibroblast cells were viable, except for trans 4a. The viabilities of the cancer cells vary between 8.5% and 93.2% (Table 6). When compound concentrations are more than 100 μg/mL, toxicities of the compounds are increasing against MCF-7 cancer cells. The compounds 2, 2b, and trans 4a are considerably toxic at higher concentrations than the other compounds. Compound 2 is very toxic to the cancer cell line at all the concentrations. It is important to note that the compounds display moderate cytotoxic activity against fibroblast cell lines at low concentrations, but 2, 2b, and trans 4a exhibit strong cytotoxic effects against the MCF-7 cancer cell line at low concentrations.

It can be suggested that the compounds intended for therapeutic use, especially 2, 2b, and trans 4a, can be used at a lower concentration level of ≤25 μg/mL. In this study, compounds 2, 2b, and trans 4a show tolerable toxic effects on the fibroblast cells, but they have the highest toxicity against cancer cells at low
Figure 6. The electrophoretograms for A) BamHI and B) HindIII digested mixtures of pBR322 DNA after treatment with the phosphazenes. Lane P is untreated plasmid DNA and lanes PH and PB are HindIII and BamHI digestion of untreated DNA. The numbers above the lines indicate the compounds digested with the enzymes.

Table 5. Cell viability (%) of L929 fibroblast cells treated with the compounds.

<table>
<thead>
<tr>
<th>Amount of compounds (μg/mL)</th>
<th>Fibroblast cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>85.6</td>
</tr>
<tr>
<td>50</td>
<td>72.4</td>
</tr>
<tr>
<td>100</td>
<td>66.3</td>
</tr>
<tr>
<td>200</td>
<td>34.1</td>
</tr>
</tbody>
</table>

Table 6. Cell viability (%) of MCF - 7 cell treated with the compounds*.

<table>
<thead>
<tr>
<th>Amount of compounds (μg/mL)</th>
<th>MCF-7 cell viability(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>16.2</td>
</tr>
<tr>
<td>50</td>
<td>12.4</td>
</tr>
<tr>
<td>100</td>
<td>10.8</td>
</tr>
<tr>
<td>200</td>
<td>8.5</td>
</tr>
</tbody>
</table>

*Cisplatin was used as a positive control; the IC50 value is 6.02 ± 0.8 μg/mL.

concentration levels. For all the other compounds, no toxicity was seen against normal or cancer cells at an acceptable dose (≤50 μg/mL).
2.6. Conclusions
The partly substituted (2, dispiro 3b, and dispiro 3c) and fully substituted 4-fluorobenzyl(N/O)spirocyclo-
triposphazenes (2a-2d, trans 4a, cis 4c, 4d, and 4e) were synthesized with the aim of understanding
their potentials as antimicrobial and anticancer agents. The spectroscopic and stereogenic properties of these
phosphazenes were primarily investigated using one- and two-dimensional NMR methods. The NMR results
indicate that the compounds obtained from the reactions of 2 with bidentate ligands have geometrical and
optical isomers, except 4d and 4e. There are many difficulties for the separation of all the isomers using
crystallizations and column chromatography. Hence, some of the phosphazene derivatives were observed in
the reaction mixture and characterized spectroscopically. In addition, the aminospirocyclotriphosphazenes are
known as the strong bases. Thus, the 4-fluorobenzyl(N/O)spirocyclotriphosphazenes obtained in this study are
likely to be used as multidentate ligands for transition metal cations, and they appear to also give phosphazenium
salts with bulky acids. The cytotoxic activities of the phosphazenes were evaluated against fibroblast L929 and
MCF-7 cancer cells. Compounds 2, 2b, and trans 4a appear to be more active than the other phosphazenes
against MCF-7 cancer cells. The compounds have weak activity against the tested bacterial strains; however,
the antifungal activity results indicate that some of the phosphazenes (especially 2c and cis 4c) were more
active than ketoconazole against the yeast strain Candida albicans. The interactions of the phosphazenes with
plasmid DNA show that the chemicals interacted with DNA, causing conformational changes. Therefore, we
may predict that compounds dispiro 3b, trans 4a, trans 4b/cis 4b, cis 4c, 4d, and 4e cause inhibition of
DNA or protein synthesis, resulting in cell death. The HindIII restriction digestion results suggest that dispiro
3b, trans 4a, trans 4b/cis 4b, cis 4c, 4d, and 4e can cause double-strand breaking of the DNA, indicating
the compound binding to AA nucleotides of DNA.

3. Experimental
3.1. Material and methods
Before use, all the solvents were dried and distilled using standard methods. 4-Fluorobenzaldehyde, N-
methyl-1,3-propandiamine, N-methyl-1,2-ethandiamine, 2,2-dimethyl-1,3-dihydroxypropane, 2,2,3,3-tetrafluoro-
1,4-dihydroxybutane, 3-amino-1-propanol, pyrrolidine, piperidine, morpholine, DASD (Merck), and N
3
P
3
Cl
6
(Aldrich) were purchased. All the reactions were conducted under an inert atmosphere and tracked using
thin-layer chromatography on Kieselgel 60 B254 sheets. Column chromatography was carried out on silica gel
[Kieselgel 60 (230–400 mesh ATSM)].

The IR spectra of all the 4-fluorobenzylspirophosphazenes were obtained on a Jasco FT/IR-430 spectrom-
eter in KBr disks and reported in cm
-1
 units. The ESI-MS spectra of the 4-fluorobenzylsphosphazenes were
recorded on a Waters 2695 Alliance Micromass ZQ spectrometer. The elemental analyses were carried out using
a LECO CHNS-932 instrument (microanalytical service of Ankara University). The 1D (1
H
 and 13
C
) and 2D
(HSQC and HMBC) spectra were obtained on a Varian Mercury FT-NMR (400 MHz) spectrometer (SiMe
4
as
an internal standard), operating at 400.13 and 100.62 MHz. The spectrometer was fitted with a 5-mm PABBO
BB inverse-gradient probe and Bruker pulse programs24 were used. The 31
P
 spectra of the phosphazenes were
recorded on a Bruker Ascend 600 ULH spectrometer (85% H3PO4 as an external standard), operating at 242.93
MHz.

538
3.2. Preparation of the compounds

3.2.1. Synthesis of 2a

A solution of 2 (0.80 g, 1.75 mmol) was slowly put into a solution of pyrrolidine (1.73 mL, 20.96 mmol) with stirring and refluxing for 36 h in dry THF (150 mL). The oily compound was purified using column chromatography [toluene-THF (1:1)] as the eluent and was afterwards recrystallized from n-hexane. Yield: 0.85 g (75%). Mp: 93 °C. Anal. Calcd. for C_{26}H_{44}ON_{8}FP_3: C, 52.34; H, 7.43; N, 18.78. Found: C, 51.89; H, 7.39; N, 18.61. ESI-MS (Ir %, Ir indicates the fragment percentage of abundance): m/z 597 ([MH]⁺, 100).

FTIR (KBr, cm⁻¹): ν 3061 (asymm.), 3024 (symm.) (C-H arom.), 1210 (asymm.), 1196 (symm.) (P=N). ¹H (400 MHz, CDCl₃, ppm, in the Scheme the numberings of carbons are presented): δ 6.97 (dd, 2H, 3J_{HH} 8.4 Hz, 3J_{FH} 8.8 Hz, H₂, H₆), 7.40 (dd, 2H, 3J_{HH} 8.4 Hz, 4J_{FH} 5.6 Hz, H₃, H₅), 1.81 (m, 2H, 3J_{HH} 6.0 Hz, 3J_{HH} 5.6 Hz, N-CH₂-C₂H₂ (pyrr)), 1.73 [m, 8H, N-CH₂-C₂H₂ (pyrr)], 1.78 [m, 8H, N-CH₂-C₂H₂ (pyrr)], 2.92 (m, 2H, 3J_{PH} 13.2 Hz, 3J_{HH} 6.0 Hz, N-C₂H₂), 3.09 [m, 8H, N-C₂H₂ (pyrr)], 3.16 [m, 8H, N-C₂H₂ (pyrr)], 3.92 (d, 2H, 3J_{PH} 7.2 Hz, Ar-C₂H₂-N), 4.28 (m, 2H, 3J_{PH} 12.8 Hz, 3J_{HH} 5.6 Hz, O-C₂H₂). ¹³C (100 MHz, CDCl₃, ppm, in the Scheme the numberings of carbons are presented): δ 161.92 (d, 1J_{FC} = 244.6 Hz, C₁), 134.60 (dd, 3J_{PC} = 10.7 Hz, 4J_{FC} = 3.1 Hz, C₄), 130.10 (d, 3J_{PC} = 8.5 Hz, C₃,C₅), 114.83 (d, 2J_{FC} = 21.4 Hz, C₂,C₆), 65.87 (d, 2J_{PC} = 6.8 Hz, O-C₂H₂), 51.27 (s, Ar-C₂H₂N), 46.21 and 46.02 [d, 2J_{PC} = 4.4 Hz and 2J_{PC} = 4.6 Hz, N-C₂H₂ (pyrr)], 45.94 (s, N-C₂H₂), 26.28 and 26.34 [(d, 3J_{PC} = 9.3 Hz and 3J_{PC} = 9.1 Hz, N-C₂H₂-C₂H₂ (pyrr)], 26.60 (d, 3J_{PC} = 3.0 Hz, N-C₂H₂-C₂H₂).

3.2.2. Synthesis of 2b

The experimental procedure was followed as in 2a using 2 (0.80 g, 1.75 mmol) and piperidine (2.07 mL, 20.96 mmol) for 35 h. The raw oily compound was purified using column chromatography [toluene-THF (3:1)] as the eluent and then crystallized from acetonitrile. Yield: 0.85 g (75%). Mp: 93 °C. Anal. Calcd. for C₃₀H₅₂O₁₂N₈FP₃: C, 55.20; H, 8.03; N, 17.17. Found: C, 55.63; H, 8.09; N, 17.01. ESI-MS (Ir %, Ir indicates the fragment percentage of abundance): m/z 653 ([MH]⁺, 100). FTIR (KBr, cm⁻¹): ν 3065 (asymm.), 3040 (symm.) (C-H arom.), 1210 (asymm.), 1196 (symm.) (P=N). ¹H (400 MHz, CDCl₃, ppm, in the Scheme the numberings of protons are presented): δ 6.97 (dd, 2H, 3J_{HH} 8.4 Hz, 3J_{FH} 8.8 Hz, H₂, H₆), 7.38 (dd, 2H, 3J_{HH} 8.4 Hz, 4J_{FH} 5.2 Hz, H₃,H₅), 1.79 (m, 2H, 3J_{HH} 6.0 Hz, 3J_{HH} 5.6 Hz, N-CH₂-C₂H₂), 1.45 [m, 8H, N-CH₂-C₂H₂ (pip)], 1.50 [m, 8H, N-CH₂-C₂H₂ (pip)], 1.42 [m, 8H, N-CH₂-C₂H₂-C₂H₂ (pip)], 2.90 (m, 2H, 3J_{PH} 13.6 Hz, 3J_{HH} 6.0 Hz, N-C₂H₂), 2.99 [m, 8H, N-C₂H₂ (pip)], 3.05 [m, 8H, N-C₂H₂ (pip)], 3.86 (d, 2H, 3J_{PH} 6.8 Hz, Ar-C₂H₂-N), 4.27 (m, 2H, 3J_{PH} 13.2 Hz, 3J_{HH} 5.6 Hz, O-C₂H₂). ¹³C (500 MHz, CDCl₃, ppm, in the Scheme the numberings of carbons are presented): δ 161.96 (d, 1J_{FC}=244.6 Hz, C₁), 134.57 (dd, 3J_{PC} = 11.5 Hz, 4J_{FC} = 3.1 Hz, C₄), 130.03 (d, 3J_{PC} = 7.7 Hz, C₃,C₅), 114.83 (d, 2J_{FC} = 20.7 Hz, C₂,C₆), 65.87 (d, 2J_{PC} = 6.1 Hz, O-C₂H₂), 51.33 (s, Ar-C₂H₂N), 45.42 and 45.23 [s, N-C₂H₂ (pip)], 45.05 (s, N-C₂H₂), 26.64 (d, 3J_{PC} = 3.0 Hz, N-C₂H₂-C₂H₂), 26.38 and 26.32 [(d, 3J_{PC} = 7.6 Hz and 3J_{PC} = 7.6 Hz, N-C₂H₂-C₂H₂ (pip)], 25.12 and 25.01 [(s, N-C₂H₂-C₂H₂ (pip)].
3.2.3. Synthesis of 2c

The experimental procedure was followed as in 2a using 2 (0.80 g, 1.75 mmol) and morpholine (1.83 mL, 20.96 mmol) for 36 h. The product was purified using column chromatography [toluene-THF (1:1)] as the eluent and then crystallized from n-hexane. Yield: 0.70 g (61%). Mp: 129 °C. Anal. Calcd. for C_{25}H_{44}O_{5}N_{8}FP_{3}: C, 47.27; H, 6.71; N, 16.96. Found: C, 47.11; H, 6.78; N, 16.83. ESI-MS (Ir %, Ir indicates the fragment percentage of abundance): m/z 661 [(MH)^+], 100. FTIR (KBr, cm^-1): ν 3070 (asym.), 3050 (symm.) (C-H arom.), 1256 (asymm.), 1185 (symm.) (P=N), 1055 (COC). 1 H (400 MHz, CDCl₃, ppm, in the Scheme the numberings of protons are presented): δ 6.98 (dd, 2H, 3J_HH 8.4 Hz, 3J_FF 8.8 Hz, H₂, H₆), 7.34 (dd, 2H, 3J_HH 8.4 Hz, 4J_FF 5.6 Hz, H₃, H₅), 1.81 (m, 2H, 3J_HH 5.6 Hz, 3J_HH 5.6 Hz, N-CH₂-C-H₂), 2.91 (m, 2H, 3J_PH 13.6 Hz, 3J_HH 5.6 Hz, N-C-H₂), 3.09 [m, 16H, N-C-H₂(m)], 3.85 (d, 2H, 3J_PH 7.2 Hz, Ar-C-H₂-N), 4.28 (m, 2H, 3J_PH 12.8 Hz, 3J_HH 5.6 Hz, O-C-H₂), 3.57 [t, 8H, 3J_HH 8.8 Hz, O-C-H₂ (m)], 3.63 [t, 8H, 3J_HH 9.2 Hz, O-C-H₂ (m)]. 13 C (100 MHz, CDCl₃, ppm, in the Scheme the numberings of carbons are presented): δ 162.13 (d, 1J_FC = 245.2 Hz, C₁), 133.66 (dd, 3J_PC = 11.0 Hz, 4J_FC = 2.6 Hz, C₄), 129.86 (d, 3J_FC = 7.8 Hz, C₃, C₅), 115.17 (d, 2J_FC = 21.3 Hz, C₂, C₆), 67.22 [(d, 2J_PC = 7.8 Hz, O-C-H₂ (morph)], 67.21 [(d, 2J_PC = 7.9 Hz, O-C-H₂ (morph)], 66.29 (d, 2J_PC = 7.1 Hz, O-C-H₂), 51.16 (s, Ar-C-H₂N), 45.88 (s, N-C-H₂), 44.73 and 44.62 [s, N-C-H₂ (morph)], 25.63 (d, 3J_FC = 3.8 Hz, N-C-H₂-C-H₂).

3.2.4. Synthesis of 2d

The experimental procedure was followed as in 2a using 2 (0.80 g, 1.75 mmol) and DASD (2.70 mL, 20.96 mmol) for 36 h. The row product was purified using column chromatography [benzene-THF (3:2)] as the eluent and then crystallized from acetonitrile. Yield: 1.02 g (66%). Mp: 228 °C. Anal. Calcd. for C_{38}H_{60}O_{9}N_{8}FP₃: C, 51.58; H, 6.83; N, 12.66. Found: C, 51.32; H, 6.88; N, 12.55. ESI-MS (Ir %, Ir indicates the fragment percentage of abundance): m/z 885 [(MH)^+], 100. FTIR (KBr, cm^-1): ν 3066 (asym.), 3044 (symm.) (C-H arom.), 1287 (asymm.), 1147 (symm.) (P=N), 1053 (COC). 1 H (400 MHz, CDCl₃, ppm, in the Scheme the numberings of protons are presented): δ 6.98 (dd, 2H, 3J_HH 8.4 Hz, 3J_FF 8.8 Hz, H₂, H₆), 7.36 (dd, 2H, 3J_HH 8.4 Hz, 4J_FF 5.6 Hz, H₃, H₅), 1.80 (m, 2H, 3J_HH 6.0 Hz, 3J_HH 5.2 Hz, N-CH₂-C-H₂), 1.59 [t, 8H, 3J_PH 11.2 Hz, 3J_HH 6.0 Hz, N-CH₂-C-H₂(d)], 1.67 [t, 8H, 3J_PH 10.8 Hz, 3J_HH 5.2 Hz, N-CH₂-C-H₂(d)], 2.92 (m, 2H, 3J_PH 13.2 Hz, 3J_HH 6.0 Hz, N-C-H₂), 3.16 [m, 8H, N-C-H₂(d)], 3.21 [m, 8H, N-C-H₂(d)], 3.84 (d, 2H, 3J_PH 6.8 Hz, Ar-C-H₂-N), 4.26 (m, 2H, 3J_PH 13.2 Hz, 3J_HH 5.2 Hz, O-C-H₂), 3.91 [s, 8H, O-C-H₂(d)], 3.95 [s, 8H, O-C-H₂(d)]. 13 C (100 MHz, CDCl₃, ppm, in the Scheme the numberings of carbons are presented): δ 160.47 (d, 1J_FC = 243.8 Hz, C₁), 134.03 (dd, 3J_PC = 11.3 Hz, 4J_FC = 2.9 Hz, C₄), 129.30 (d, 3J_FC = 7.8 Hz, C₃, C₅), 114.87 (d, 2J_FC = 21.1 Hz, C₂, C₆), 107.73 and 107.50 (s, O-C=O), 66.03 (d, 2J_PC = 7.0 Hz, O-C-H₂), 64.15 [(s, O-C-H₂ (DASD)], 51.10 (s, Ar-C-H₂-N), 45.96 (s, N-C-H₂), 42.66 and 42.58 [s, N-C-H₂ (DASD)], 35.59 [d, 3J_PC = 7.1 Hz, N-CH₂-C-H₂(DASD)], 35.46 [d, 3J_PC = 6.4 Hz, N-CH₂-C-H₂(DASD)], 26.55 (d, 3J_PC = 3.2 Hz, N-CH₂-C-H₂).

3.2.5. Synthesis of dispiro 3b

A solution of 2 (0.60 g, 1.31 mmol) in dry THF (150 mL) was slowly put into a solution of triethylamine (0.37 mL, 2.62 mmol) and N-methyl-1,3-diaminopropane (0.14 mL, 1.31 mmol) in dry THF (50 mL) at room
temperature. The mixture was stirred for 2 days at room temperature under argon atmosphere. The oily product was purified using column chromatography [toluene-THF (1:1)] as the eluent and then recrystallized from toluene. Yield: 0.32 g (52%). Mp: 147 °C. Anal. Calc. for C_{14}H_{22}N_{6}FO_{3}P_{3}Cl_{2}: C, 35.54; H, 4.69; N, 17.76. Found: C, 35.40; H, 4.70; N, 17.57; ESI-MS (Ir %, Ir indicates the fragment percentage of abundance): m/z 473 ([MH]^+, 100); FTIR (KBr, cm\(^{-1}\)) : \(\nu = 3070\) (asymmetric), 3030 (symmetric) (C-H arom), 1236 (asymmetric), 1183 (symmetric) (P=\(\equiv\)N), 575 (asymmetric), 545 (symmetric) (PCl). \(^1\)H (400 MHz, CDCl\(_3\), ppm, in the Scheme the numberings of protons are presented): \(\delta = 7.00\) (dd, 2H, \(^3\)J\(_{HH}\) 8.4 Hz, \(^3\)J\(_{FH}\) 8.8 Hz, \(H_2,H_6\)), 7.36 (dd, 2H, \(^3\)J\(_{HH}\) 8.4 Hz, \(^4\)J\(_{FH}\) 5.4 Hz, \(H_3,H_5\)), 1.84 (m, 1H, N-CH\(_2\)-CH\(_2\)), 1.97 (m, 1H, N-CH\(_2\)-CH\(_2\)), 1.75 (m, 1H, NH-CH\(_2\)-CH\(_2\)), 1.81 (m, 1H, NH-CH\(_2\)-CH\(_2\)), 3.22 (m, 2H, N-CH\(_2\)), 2.97 (m, 2H, N-CH\(_3\)-CH\(_2\)), 3.72 (dd, 1H, \(^3\)J\(_{FH}\) 6.4 Hz, \(^2\)J\(_{HH}\) 14.4 Hz, Ar-CH\(_2\)-N), 4.15 (dd, 1H, \(^3\)J\(_{FH}\) 11.2 Hz, \(^2\)J\(_{HH}\) 14.8 Hz, Ar-CH\(_2\)-N), 2.41 (m, 1H, N-\(\equiv\)H), 3.10 (m, 2H, NH-CH\(_2\)), 2.54 (d, 3H, \(^3\)J\(_{FH}\) 14.0 Hz, CH\(_3\)), 4.32 (m, 1H, O-CH\(_2\)), 4.40 (m, 1H, O-CH\(_2\)), 13C (100 MHz, CDCl\(_3\), ppm, in the Scheme the numberings of carbons are presented): \(\delta = 162.18\) (d, \(^1\)J\(_{FC}\) = 245.3 Hz, \(C_1\)), 133.19 (dd, \(^3\)J\(_{PC}\) = 9.4 Hz, \(^4\)J\(_{FC}\) = 2.9 Hz, \(C_4\)), 129.99 (d, \(^3\)J\(_{FC}\) = 8.5 Hz, \(C_3,C_5\)), 115.20 (d, \(^2\)J\(_{FC}\) = 21.5 Hz, \(C_2,C_6\)), 67.38 (d, \(^2\)J\(_{PC}\) = 6.9 Hz, O-CH\(_2\)), 50.60 (s, Ar-CH\(_2\)-N), 50.49 (d, \(^2\)J\(_{PC}\) = 3.1 Hz, N-CH\(_3\)-CH\(_2\)), 45.66 (s, N-CH\(_2\)), 41.45 (d, \(^2\)J\(_{PC}\) = 3.1 Hz, NH-CH\(_2\)), 35.47 (s, CH\(_3\)), 27.46 (d, \(^3\)J\(_{PC}\) = 4.6 Hz, N-CH\(_2\)-CH\(_2\)), 26.24 (d, \(^3\)J\(_{PC}\) = 3.8 Hz, NH-CH\(_2\)-CH\(_2\)).

3.2.6. Synthesis of dispiro 3c

The experimental procedure was similar to that of dispiro 3b, using 2 (0.60 g, 1.31 mmol) and sodium 1-aminopropane-3-oxide (1.13 g, 1.31 mmol) and triethylamine (0.37 mL, 2.62 mmol). The product was crystallized from n-hexane. Yield: 0.29 g (49%). Mp: 136 °C. Anal. Calc. for C\(_{13}\)H\(_{19}\)N\(_{5}\)FO\(_2\)P\(_3\)Cl\(_2\): C, 33.93; H, 4.16; N, 15.22. Found: C, 33.61; H, 4.12; N, 15.37; ESI-MS (fragments are based on \(^{35}\)Cl, Ir %, Ir indicates the fragment percentage of abundance): m/z 460 ([MH]^+, 100); FTIR (KBr, cm\(^{-1}\)) : \(\nu = 3070\) (asymmetric), 3030 (symmetric) (C-H arom), 1230 (asymmetric), 1183 (symmetric) (P=\(\equiv\)N), 560 (asymmetric), 544 (symmetric) (PCl). \(^1\)H (400 MHz, CDCl\(_3\), ppm, in the Scheme the numberings of protons are presented): \(\delta = 7.01\) (dd, 2H, \(^3\)J\(_{HH}\) 8.2 Hz, \(^3\)J\(_{FH}\) 8.8 Hz, \(H_2,H_6\)), 7.36 (dd, 2H, \(^3\)J\(_{HH}\) 8.0 Hz, \(^4\)J\(_{FH}\) 5.6 Hz, \(H_3,H_5\)), 1.92 (m, 1H, N-CH\(_2\)-CH\(_2\)), 1.78 (m, 2H, NH-CH\(_2\)-CH\(_2\)), 3.38 (m, 2H, N-CH\(_2\)), 3.02 (m, 2H, NH-CH\(_2\)), 3.80 (dd, 1H, \(^3\)J\(_{PH}\) 7.8 Hz, \(^2\)J\(_{HH}\) 15.0 Hz, Ar-CH\(_2\)-N), 4.04 (dd, 1H, \(^3\)J\(_{PH}\) 9.6 Hz, \(^2\)J\(_{HH}\) 14.8 Hz, Ar-CH\(_2\)-N), 3.12 (m, 1H, N-\(\equiv\)H), 4.42 (m, 2H, O-CH\(_2\)), 4.34 (m, 2H, O-CH\(_2\)), 13C (100 MHz, CDCl\(_3\), ppm, in the Scheme the numberings of carbons are presented): \(\delta = 162.17\) (d, \(^1\)J\(_{FC}\) = 245.3 Hz, \(C_1\)), 132.95 (dd, \(^3\)J\(_{PC}\) = 9.2 Hz, \(^4\)J\(_{FC}\) = 3.1 Hz, \(C_4\)), 130.01 (d, \(^3\)J\(_{PC}\) = 8.5 Hz, \(C_3,C_5\)), 115.22 (d, \(^2\)J\(_{FC}\) = 20.7 Hz, \(C_2,C_6\)), 67.75 (d, \(^2\)J\(_{PC}\) = 6.9 Hz, O-CH\(_2\)), 67.44 ([\(^2\)J\(_{PC}\) = 6.1 Hz, O-CH\(_2\)-(aminoalcohol)], 50.60 (s, Ar-CH\(_2\)-N), 45.74 (s, N-CH\(_2\)), 41.01 (d, \(^2\)J\(_{PC}\) = 3.0 Hz, NH-CH\(_2\)), 27.14 (d, \(^3\)J\(_{PC}\) = 3.8 Hz, N-CH\(_2\)-CH\(_2\)), 25.74 (d, \(^3\)J\(_{PC}\) = 6.1 Hz, NH-CH\(_2\)-CH\(_2\)).

3.2.7. Syntheses of dispiro 3d and ansa 3d

The experimental procedure used for dispiro 3b was similar to that of dispiro 3d and ansa 3d using 2 (0.60 g, 1.31 mmol), triethylamine (0.37 mL, 2.62 mmol), and sodium 2,2,3,3-tetrafluorobutanediox ide (0.27 g, 1.31 mmol) for 3 days. The compound was chromatographed using toluene as the eluent.
dispiro 3d: $^1$H (400 MHz, CDCl3, ppm, in the Scheme the numberings of protons are presented): $\delta$ 7.07 (dd, 2H, $^3$JHH 8.8 Hz, $^3$JHF 9.2 Hz, H2, H6), 7.34 (dd, 2H, $^4$JHH 8.8 Hz, $^4$JHF 4.4 Hz, H3, H5), 1.90 (m, 2H, N-CH2-C-H2), 3.03 (m, 2H, N-CH2), 3.94 (d, 2H, $^3$JPH 9.2 Hz, Ar-C-H2-N), 4.04 (m, 2H, $^3$JPH 14.8 Hz, $^3$JHH 10.4 Hz, O-C-H2), 4.38 (m, 4H, O-C-H2), $^{13}$C (100 MHz, CDCl3, ppm, in the Scheme the numberings of carbons are presented): $\delta$ 162.33 (d, $^1$JFC = 245.3 Hz, C1), 132.23 (dd, $^3$JPC = 9.2 Hz, $^4$JFC = 3.0 Hz, C4), 130.00 (d, $^3$JFC = 7.6 Hz, C3, C5), 115.30 (d, $^2$JFC = 21.4 Hz, C2, C6), 111.55 (d, $^1$JFC = 256.9 Hz, $^2$JFC = 29.1 Hz, O-CH2-CF2), 67.68 (d, $^2$JPC = 6.8 Hz, O-C-H2), 61.45 [(d, $^2$JFC = 34.5 Hz, O-C-H2(diol)], 50.41 (d, $^2$JPC = 3.1 Hz, Ar-C-H2N), 45.57 (s, N-C-H2), 25.96 (d, $^3$JPC = 3.8 Hz, N-CH2-C-H2).

ansa 3d: $^1$H (400 MHz, CDCl3, ppm, in the Scheme the numberings of protons are presented): $\delta$ 7.01 (dd, 2H, $^3$JHH 8.8 Hz, $^3$JHF 9.2 Hz, H2, H6), 7.36 (dd, 2H, $^4$JHH 8.8 Hz, $^4$JHF 5.6 Hz, H3, H5), 1.83 (m, 2H, N-CH2-C-H2), 2.98 (m, 2H, N-CH2), 3.94 (d, 2H, $^3$JPH 9.2 Hz, Ar-C-H2-N), 3.75 (m, 2H, $^3$JPH 14.4 Hz, $^3$JHH 6.8 Hz, O-C-H2), 4.30 (m, 4H, O-C-H2), $^{13}$C (100 MHz, CDCl3, ppm, in the Scheme the numberings of carbons are presented): $\delta$ 162.35 (d, $^1$JFC = 245.3 Hz, C1), 132.34 (dd, $^3$JPC = 8.5 Hz, $^4$JFC = 3.0 Hz, C4), 130.11 (d, $^3$JFC = 8.4 Hz, C3, C5), 115.38 (d, $^2$JFC = 21.5 Hz, C2, C6), 114.10 (d, $^1$JFC = 256.1 Hz, $^2$JFC = 27.2 Hz, O-CH2-CF2), 67.89 (d, $^2$JPC = 6.1 Hz, O-C-H2), 63.62 [(d, $^2$JFC = 33.7 Hz, O-C-H2(diol)], 50.45 (d, $^2$JPC = 3.8 Hz, Ar-C-H2N), 45.65 (s, N-C-H2), 26.01 (d, $^3$JPC = 3.8 Hz, N-CH2-C-H2).

3.2.8. Synthesis of trans 4a

For the synthesis of trans 4a the experimental procedure used for 2a was carried out using 2 (0.80 g, 1.75 mmol) and N-methyl-1,2-diaminoethane (0.91 mL, 10.48 mmol) for 18 h. The product was purified using column chromatography [THF] as the eluent and then crystallized from n-hexane. Yield: 0.51 g (64%). Mp: 110 °C. Anal. Calc. for C16H28N8: FOP3: C, 41.75; H, 6.13; N, 24.34. Found: C, 41.34; H, 6.18; N, 24.11. ESI-MS (Ir %, Ir indicates the fragment percentage of abundance): m/z 461 ([MH]+, 70.0). FTIR (KBr, cm⁻¹): ν 3065 (asymn.), 3026 (symm.) (C-H arom), 1221 (asymn.), 1180 (symm.) (P=N), 573 (asymn.), 550 (symm.) (PCI). $^1$H (400 MHz, CDCl3, ppm, in the Scheme the numberings of protons are presented): $\delta$ 7.01 (dd, 2H, $^3$JHH 8.4 Hz, $^3$JHF 8.8 Hz, H2, H6), 7.36 (dd, 2H, $^4$JHH 8.4 Hz, $^4$JHF 5.6 Hz, H3, H5), 1.83 (m, 1H, N-CH2-C-H2), 1.95 (m, 1H, N-CH2-C-H2), 3.34 (m, 2H, N-CH2), 3.01 (m, 4H, N-CH3-C-H2), 3.80 (dd, 1H, $^3$JPH 7.8 Hz, $^2$JHH 14.6 Hz, Ar-C-H2-N), 4.04 (dd, 1H, $^3$JPH 10.2 Hz, $^2$JHH 15.0 Hz, Ar-C-H2-N), 2.38 (m, 2H, N-H), 3.22 (m, 4H, NH-C-H2), 2.59 (d, 3H, $^3$JPH 12.8 Hz, CH3), 2.57 (d, 3H, $^3$JPH 12.4 Hz, CH3), 4.34 (m, 1H, O-C-H2), 4.40 (m, 1H, O-C-H2), $^{13}$C (100 MHz, CDCl3, ppm, in the Scheme the numberings of carbons are presented): $\delta$ 162.23 (d, $^1$JFC = 245.2 Hz, C1), 132.98 (dd, $^3$JPC = 9.3 Hz, $^4$JFC = 2.9 Hz, C4), 130.02 (d, $^3$JFC = 7.6 Hz, C3, C5), 115.24 (d, $^2$JFC = 21.5 Hz, C2, C6), 67.31 (d, $^2$JPC = 6.9 Hz, O-C-H2), 50.74 (d, $^2$JPC = 3.1 Hz, Ar-C-H2N), 49.25 (d, $^2$JPC = 9.9 Hz, N-CH3-C-H2), 49.21 (d, $^2$JPC = 11.6 Hz, N-CH3-C-H2), 45.43 (s, N-C-H2), 40.11 (d, $^2$JPC = 3.1 Hz, NH-C-H2), 40.10 (d, $^2$JPC = 3.8 Hz, NH-C-H2), 30.44 (d, $^2$JPC = 1.8 Hz, CH3), 30.41 (d, $^2$JPC = 2.3 Hz, CH3), 26.21 (d, $^3$JPC = 4.6 Hz, N-CH2-C-H2).
3.2.9. Syntheses of trans 4b and cis 4b

For the preparation of trans 4b and cis 4b the experimental procedure used for 2a was carried out using 2 (0.80 g, 1.75 mmol) and N-methyl-1,3-diaminopropane (1.08 mL, 10.48 mmol) for 15 h. The product was chromatographed using toluene-THF (1:2) as the eluent.

**trans 4b:** $^1$H (400 MHz, CDCl$_3$, ppm, in the Scheme the numberings of protons are presented): $\delta$ 6.97 (dd, 2H, $^3$J$_{HH}$ 8.4 Hz, $^4$J$_{FH}$ 8.8 Hz, H$_2$,H$_6$), 7.38 (dd, 2H, $^3$J$_{HH}$ 8.4 Hz, $^4$J$_{FH}$ 6.4 Hz, H$_5$,H$_{3}$), 1.84 (m, 2H, N-CH$_2$-C$_H_2$), 1.88 (m, 2H, N-CH$_2$-C$_H_2$), 1.73 (m, 8H, NH-CH$_2$-C$_H_2$), 2.96 (m, 2H, $^3$J$_{PH}$ 13.1 Hz, $^2$J$_{HH}$ 6.1 Hz N-CH$_2$), 2.91 (m, 2H, $^3$J$_{PH}$ 13.2 Hz, $^3$J$_{HH}$ 6.0 Hz N-CH$_2$), 3.05 (m, 8H, N-CH$_3$-C$_H_2$), 3.77 (dd, 1H, $^3$J$_{PH}$ 6.4 Hz, $^2$J$_{HH}$ 14.8 Hz, Ar-CH$_2$-N), 3.74 (dd, 1H, $^3$J$_{PH}$ 5.8 Hz, $^2$J$_{HH}$ 14.0 Hz, Ar-CH$_2$-N), 2.30 (m, 1H, N-H), 2.36 (m, 1H, N-H), 3.18 (m, 2H, NH-C$_H_2$), 3.08 (m, 2H, NH-C$_H_2$), 2.44 (d, 3H, $^3$J$_{PH}$ 8.0 Hz, C$_H_3$), 2.50 (d, 3H, $^3$J$_{PH}$ 12.8 Hz, C$_H_3$), 4.20 (m, 1H, O-C$_H_2$), 4.24 (m, 1H, O-C$_H_2$), $^{13}$C (100 MHz, CDCl$_3$, ppm, in the Scheme the numberings of carbons are presented): $\delta$ 160.90 (d, $^1$J$_{FC}$ = 244.6 Hz, C$_1$), 134.34 (dd, $^3$J$_{FC}$ = 10.7 Hz, $^4$J$_{FC}$ = 2.9 Hz, C$_4$), 129.98 (d, $^3$J$_{FC}$ = 7.6 Hz, C$_3$,C$_5$), 114.75 (d, $^2$J$_{FC}$ = 21.4 Hz, C$_2$,C$_6$), 66.39 (d, $^2$J$_{PC}$ = 6.9 Hz, O-C$_H_2$), 50.53 (d, $^2$J$_{PC}$ = 3.1 Hz, Ar-CH$_2$N), 50.97 (s, N-CH$_3$-C$_H_2$), 50.76 (s, N-CH$_3$-C$_H_2$), 45.72 (s, NH-C$_H_2$), 45.59 (s, NH-C$_H_2$), 41.86 (s, N-CH$_2$), 36.25 (s, C$_3$H), 35.87 (s, C$_3$H), 28.20 [d, $^3$J$_{PC}$ = 9.2 Hz, N-CH$_2$-C$_H_2$(amine)], 28.11 [d, $^3$J$_{PC}$ = 9.2 Hz, N-CH$_2$-C$_H_2$(amine)], 26.46 (d, $^3$J$_{PC}$ = 6.9 Hz, N-CH$_2$-C$_H_2$).

**cis 4b:** $^1$H (400 MHz, CDCl$_3$, ppm, in the Scheme the numberings of protons are presented): $\delta$ 6.97 (dd, 2H, $^3$J$_{HH}$ 8.4 Hz, $^4$J$_{FH}$ 8.8 Hz, H$_2$,H$_6$), 7.38 (dd, 2H, $^3$J$_{HH}$ 8.4 Hz, $^4$J$_{FH}$ 6.4 Hz, H$_5$,H$_{3}$), 1.84 (m, 2H, N-CH$_2$-C$_H_2$), 1.88 (m, 2H, N-CH$_2$-C$_H_2$), 1.73 (m, 8H, NH-CH$_2$-C$_H_2$), 2.96 (m, 2H, $^3$J$_{PH}$ 13.1 Hz, $^2$J$_{HH}$ 6.1 Hz N-CH$_2$), 2.91 (m, 2H, $^3$J$_{PH}$ 13.2 Hz, $^3$J$_{HH}$ 6.0 Hz N-CH$_2$), 3.05 (m, 8H, N-CH$_3$-C$_H_2$), 3.94 (dd, 2H, $^3$J$_{PH}$ 7.2 Hz, Ar-CH$_2$-N), 2.42 (m, 2H, N-H), 3.23 (m, 4H, NH-C$_H_2$), 2.57 (d, 3H, $^3$J$_{PH}$ 13.2 Hz, C$_H_3$), 4.34 (m, 2H, O-C$_H_2$), $^{13}$C (100 MHz, CDCl$_3$, ppm, in the Scheme the numberings of carbons are presented): $\delta$ 161.84 (d, $^1$J$_{FC}$ = 244.6 Hz, C$_1$), 134.37 (dd, $^3$J$_{PC}$ = 10.3 Hz, $^4$J$_{FC}$ = 2.8 Hz, C$_4$), 130.03 (d, $^3$J$_{FC}$ = 7.6 Hz, C$_3$,C$_5$), 114.81 (d, $^2$J$_{PC}$ = 20.6 Hz, C$_2$,C$_6$), 66.49 (d, $^2$J$_{PC}$ = 6.9 Hz, O-C$_H_2$), 50.56 (d, $^2$J$_{PC}$ = 3.1 Hz, Ar-CH$_2$N), 50.91 (s, N-CH$_3$-C$_H_2$), 41.87 (d, $^2$J$_{PC}$ = 3.1 Hz, NH-C$_H_2$), 41.89 (s, N-CH$_2$), 36.16 (s, C$_3$H), 28.02 [d, $^3$J$_{PC}$ = 9.3 Hz, N-CH$_2$-C$_H_2$(amine)], 26.50 (d, $^3$J$_{PC}$ = 6.8 Hz, N-CH$_2$-C$_H_2$).

3.2.10. Synthesis of cis 4c

For the preparation of cis 4c the experimental procedure used for 2a was carried out using 2 (0.50 g, 1.09 mmol) and sodium 1-aminopropane-3-oxide (0.79 g, 6.55 mmol) for 12 h. The product was purified using column chromatography (THF) as the eluent and then crystallized from n-hexane. Yield: 0.33 g (66%). Mp: 185 °C. Anal. Calc. for C$_{16}$H$_{26}$N$_6$FO$_3$P$_3$: C, 41.56; H, 5.67; N, 18.17. Found: C, 41.18; H, 5.62; N, 17.99. ESI-MS (Ir %, Ir indicates the fragment percentage of abundance): m/z 463 ([MH]$,^+$, 100.0). FTIR (KBr, cm$^{-1}$): ν 3067 (asymm.), 3040 (symm.) (C-H amin), 1221 (asymm.), 1141 (symm.) (P=N). $^1$H (400 MHz, CDCl$_3$, ppm, in the Scheme the numberings of protons are presented): $\delta$ 6.98 (dd, 2H, $^3$J$_{HH}$ 8.4 Hz, $^4$J$_{FH}$ 9.2 Hz, H$_2$,H$_6$), 7.35 (dd, 2H, $^3$J$_{HH}$ 8.4 Hz, $^4$J$_{FH}$ 5.6 Hz, H$_3$,H$_5$), 1.85 (m, 2H, N-CH$_2$-C$_H_2$), 1.83 (m, 2H, NH-CH$_2$-C$_H_2$), 2.98 (m, 2H, N-CH$_2$), 3.90 (d, 2H, $^3$J$_{PH}$ 7.6 Hz, Ar-CH$_2$-N), 2.78 (m, 2H, N-H), 3.42 (m, 2H, NH-C$_H_2$), 4.26 (m, 2H, O-CH$_2$), $^{13}$C (100 MHz, CDCl$_3$, ppm, in the Scheme the numberings of
carbons are presented): δ 162.07 (d, $^1 J_{FC} = 244.5$ Hz, $C_1$), 134.37 (dd, $^3 J_{PC} = 9.3$ Hz, $^4 J_{FC} = 2.8$ Hz, $C_4$), 130.03 (d, $^3 J_{FC} = 7.8$ Hz, $C_3, C_5$), 115.02 (d, $^2 J_{FC} = 21.4$ Hz, $C_2, C_6$), 67.37 (broad, O-CH$_2$), 50.25 (s, Ar-CH$_2$N), 45.56 (s, N-CH$_2$), 41.35 (d, $^2 J_{PC} = 3.1$ Hz, NH-CH$_2$), 26.38 (s, N-CH$_2$-CH$_2$), 25.87 [s, N-CH$_2$-CH$_2$(aminoalcohol)].

3.2.11. Synthesis of 4d

For the preparation of 4d the experimental procedure used for 2a was followed using 2 (0.70 g, 1.53 mmol) and sodium 2,2,3,3-tetrafluorobutanedioxide (0.88 g, 9.17 mmol) for 26 h. The product was purified using column chromatography (THF) as the eluent and then crystallized from toluene. Yield: 0.69 g (71%). Mp: 116 °C. Anal. Calc. for C$_{18}$H$_{20}$F$_{10}$O$_{5}$P$_3$: C, 33.99; H, 3.17; N, 8.80. Found: C, 34.17; H, 3.20; N, 8.71. ESI-MS (m/z 637 ([$\text{MH}^+$], 100.0)). FTIR (KBr, cm$^{-1}$): ν 3068 (asymm.), 3024 (symm.) (C-H arom), 1241 (asymm.), 1187 (symm.) (P=N). $^1$H (400 MHz, CDCl$_3$, ppm, in the Scheme the numberings of carbons are presented): δ 7.01 (dd, 2H, $^3 J_{HH} = 8.4$ Hz, $^3 J_{FH} = 8.8$ Hz, $H_2, H_6$), 7.30 (dd, 2H, $^3 J_{HH} = 8.4$ Hz, $^4 J_{FH} = 6.4$ Hz, $H_3, H_5$), 1.87 (m, 2H, N-CH$_2$-CH$_2$), 2.99 (m, 2H, $^3 J_{FH} = 12.8$ Hz, $^3 J_{HH} = 5.6$ Hz, N-CH$_2$), 3.86 (d, 2H, $^3 J_{FH} = 8.4$ Hz, Ar-C$_2$H$_2$-N), 4.22 (m, 2H, O-CH$_2$), 4.32 (m, 8H, O-CH$_2$), $^{13}$C (100 MHz, CDCl$_3$, ppm, in the Scheme the numberings of carbons are presented): δ 162.25 (d, $^1 J_{FC} = 245.4$ Hz, $C_1$), 132.78 (dd, $^3 J_{PC} = 8.8$ Hz, $^4 J_{FC} = 2.7$ Hz, $C_4$), 129.92 (d, $^3 J_{FC} = 7.7$ Hz, $C_3, C_5$), 115.27 (d, $^2 J_{FC} = 21.4$ Hz, $C_2, C_6$), 111.67 (d, $^1 J_{FC} = 256.9$ Hz, $^2 J_{FC} = 27.6$ Hz, O-CH$_2$-CF$_2$), 114.23 (d, $^1 J_{FC} = 256.9$ Hz, $^2 J_{FC} = 27.6$ Hz, O-CH$_2$-CF$_2$), 67.49 (d, $^2 J_{PC} = 6.9$ Hz, O-CH$_2$), 61.35 [(d, $^2 J_{FC} = 34.8$ Hz, O-CH$_2$(diol)], 50.45(d, $^2 J_{PC} = 2.2$ Hz, Ar-C$_2$H$_2$N), 45.59 (s, N-CH$_2$), 25.13 (d, $^3 J_{PC} = 3.8$ Hz, N-CH$_2$-CH$_2$).

3.2.12. Synthesis of 4e

For the preparation of 4e the experimental procedure used for 2a was followed using 2 (0.70 g, 1.53 mmol) and sodium 2,2-dimethyl-1,3-propanedioxide (1.36 g, 9.17 mmol) for 26 h. The product was purified using column chromatography (THF) as the eluent and then crystallized from toluene. Yield: 0.57 g (72%). Mp: 132 °C. Anal. Calc. for C$_{20}$H$_{32}$N$_4$F$_9$O$_5$P$_3$: C, 46.16; H, 6.20; N, 10.77. Found: C, 46.53; H, 6.27; N, 10.86. ESI-MS (m/z 521 ([$\text{MH}^+$], 100.0)). FTIR (KBr, cm$^{-1}$): ν 3068 (asymm.), 3024 (symm.) (C-H arom), 1230 (asymm.), 1190 (symm.) (P=N). $^1$H (400 MHz, CDCl$_3$, ppm, in the Scheme the numberings of carbons are presented): δ 7.00 (dd, 2H, $^3 J_{HH} = 8.4$ Hz, $^3 J_{FH} = 8.8$ Hz, $H_2, H_6$), 7.36 (dd, 2H, $^3 J_{HH} = 8.4$ Hz, $^4 J_{FH} = 5.6$ Hz, $H_3, H_5$), 1.86 (m, 2H, N-CH$_2$-CH$_2$), 3.01 (m, 2H, N-CH$_2$), 3.90 (d, 2H, $^3 J_{FH} = 8.4$ Hz, Ar-C$_2$H$_2$-N), 0.89 (s, 12H, CH$_3$), 4.05 (m, 2H, $^3 J_{FH} = 12.8$ Hz, $^3 J_{HH} = 6.4$ Hz O-CH$_2$), 3.47 (s, 8H, O-CH$_2$), $^{13}$C (100 MHz, CDCl$_3$, ppm, in the Scheme the numberings of carbons were presented): δ 162.08 (d, $^1 J_{FC} = 244.6$ Hz, $C_1$), 134.57 (dd, $^3 J_{PC} = 10.5$ Hz, $^4 J_{FC} = 3.1$ Hz, $C_4$), 130.04 (d, $^3 J_{FC} = 8.5$ Hz, $C_3, C_5$), 115.05 (d, $^2 J_{FC} = 20.6$ Hz, $C_2, C_6$), 76.15 (s, O-CH$_2$), 70.95 [(s, O-CH$_2$(diol)], 50.39 (s, Ar-C$_2$H$_2$N), 45.61 (s, N-CH$_2$), 31.92 (d, $^2 J_{PC} = 5.3$ Hz, OCH$_2$C), 26.27 (d, $^3 J_{PC} = 3.2$ Hz, N-CH$_2$-CH$_2$), 21.33 (s, CH$_3$).
3.3. Biological assays

3.3.1. Determination of antimicrobial activity

Antibacterial susceptibility experiments were carried out with the BACTEC MGIT 960 system (Becton Dickin-
son, Sparks, MD, USA) using the agar-well diffusion method. The activities of the compounds were evaluated
against gram-positive and gram-negative bacteria and fungi (Table 4). Microorganism strains were obtained
from the collections of the Gazi University Culture Collection, Turkey. Ampicillin (10 μg), chloramphenicol (30
μg) (antibacterial), and ketoconazole (50 μg) (antifungal) were used as the standard antimicrobial agents. The
bacterial cultures were incubated on nutrient agar plates at 37 °C for 24 h for bacteria, and the yeast cultures
were incubated on Sabouraud dextrose agar (SDA) medium at 30 °C for 48 h. After incubation, bacterial
suspensions were adjusted to a turbidity of 0.5 McFarland on Mueller Hinton agar (MHA, for bacterial strains)
or SDA (for fungal strains) mixed with 1% culture suspension and poured into plates. Wells were prepared with
a diameter of 6.0 mm and the solution (50 μL) of the 5000 μM test compound was pipetted into the well. The
diameter of the inhibition zone was measured in millimeters.

3.3.1.1. Preparation of the oral anaerobic and microaerophilic bacteria

The antimicrobial effects of the phosphazenes were also evaluated using Porphyromonas gingivalis ATCC 33277
and Prevotella intermedia ATCC 25261 as anaerobic bacteria and Aggregatibacter actinomycetemcomitans
ATCC 29523 as a microaerophilic bacterium in the Medical Microbiology Laboratory of the Gazi University
Faculty of Dentistry. P. gingivalis and P. intermedia were inoculated onto Colombia agar (Merck, Germany)
plates supplemented with hemin (5 μg/mL), menadione (1 μg/mL), and 5% horse blood and incubated at 37
°C under anaerobic conditions for 5–7 days in an automated anaerobic chamber (Electrotek, United Kingdom)
with an atmosphere of 90% N₂, 5% CO₂, and 5% H₂. A. actinomycetemcomitans was inoculated on trypticase
soy agar supplemented with 5% horse serum and incubated at 37 °C in the incubator with 5% CO₂ for 2–3 days.
After harvesting the bacteria, bacterial suspensions were prepared in sterilized test tubes containing Colombia
broth supplemented with hemin (5 μg/mL) and menadione (1 μg/mL) and the inoculums were adjusted
according to the turbidity of 0.5 McFarland standard. The density of bacterial suspensions was determined
using an ELISA reader (BioTek, Winooski, VT, USA) spectrophotometrically.

3.3.2. Determination of the DNA interaction with the compounds

The interaction of phosphazenes with plasmid DNA was studied by agarose gel electrophoresis. Stock solutions in
DMSO were prepared and used immediately. The 40-μL aliquots of decreasing concentrations of the compounds
ranging from 2500 to 1 μM were incubated with plasmid DNA in the dark at 37 °C for 24 h. The aliquots of the
DNA/compound mixtures were mixed with the loading buffer and loaded onto the agarose gel. Electrophoresis
was carried out in TAE buffer for 3 h at 70 V. The gel was stained with ethidium bromide and visualized
under UV light using a transilluminator, and the image was recorded with a video camera as a TIFF file. The
experiments were repeated three times, and the mean values were estimated.

Determination of BamHI and HindIII restriction enzyme digestion

The compound/plasmid DNA mixtures were incubated for 24 h and then restricted with BamHI or HindIII
enzyme at 37 °C in order to test the phosphazenes binding to DNA. The restricted DNA was run in agarose gel
electrophoresis in TAE buffer. The gel was stained and then viewed with a transilluminator. The electrophoreograms were photographed with a video-camera and saved as a TIFF file.

**Determination of cytotoxicity with WST-1**

The L929 fibroblast and MCF-7 cell lines were obtained from the Tissue and Cell Culture Collection of the Bioengineering Division of Kirikkale University (Kirikkale, Turkey). Cell culture plastic materials were purchased from Corning (USA). The growth medium (DMEM) without L-glutamine supplemented with fetal calf serum and trypsin-EDTA were purchased from Biological Industries (USA). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium monosodium salt (WST-1) was purchased from Roche (Germany). Hoechst 33342 and propidium iodide were purchased from Serva (Israel). Phosphate buffer solution was purchased from Sigma-Aldrich (USA). The WST-1 assay was used to evaluate the cytotoxicity exerted by the phosphazenes.26 L929 fibroblast and MCF-7 cells were planted into 96-well plates at a density of $5 \times 10^3$ cells/well and incubated 12 h in a CO$_2$ incubator at 37 °C. The compound solutions (25, 50, 100, and 200 μg/mL) were cleared with culture medium, added to the wells, and incubated in a CO$_2$ incubator for 48 h. Medium only containing 10% DMSO was added as a control. The cell culture medium in each well was then changed with 100 μL of medium and 15 μL of the WST-1 solution. After incubation for another 4 h at 37 °C in the dark, the wells were read at 440–480 nm with an ELISA plate reader (BioTek), and then the percentages of the viability of the cells were estimated. For the WST-1 assay, the control cell viability was defined as 100%. The samples were evaluated for each group and repeated three times.

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**References**