Abstract: We reported a new method dealing with the synthesis of novel pharmacologically relevant α-aminophosphonate derivatives via a lipase-catalyzed Kabachnik–Fields reaction with yields of up to 93%. The advantages of this protocol are excellent yields, mild reaction conditions, low costs, and sustainability. The developed protocol is applicable to a range of H-phosphites and organic amines, providing a wide substrate scope. A new class of α-aminophosphonate analogues possessing P-chiral centers was also synthesized. The synthesized compounds were characterized on the basis of their antimicrobial activities against E. coli. The impact of the various alkoxy groups on antimicrobial activity was demonstrated. The crucial role of the substituents, located at the aromatic rings in the phenylethoxy and benzyloxy groups, on the inhibitory action against selected pathogenic E. coli strains was revealed. The observed results are especially important because of increasing resistance of bacteria to various drugs and antibiotics.

Keywords: α-aminophosphonates; Kabachnik–Fields reaction; antimicrobial activity
The aim of the work is to develop a metal-free protocol for the preparation of α-aminophosphonate derivatives with P-chiral centers on bacterial strains K12 and R2−R4.

2. Materials and Methods

2.1. Microorganisms and Media

All microorganisms and media were accurately described in detail in the previous work [37–74] and analyzed by a Tukey test.

2.2. General Methods of Synthesis α-Aminophosphonate Derivatives

All the chemicals were described in detail in the previous work [74]. All specific strains, such as Pseudomonas cepacia (PcL) and wheat germ, were provided by Sigma-Aldrich (Merck). The bovine acetone powder was prepared in our laboratory according to the literature procedure [38]. Symmetrical and unsymmetrical H-phosphites were obtained via alcoholysis of the dimethyl phosphate with the appropriate alcohol according to the literature procedure [40–46] (see Supplementary Materials).

3. Results

3.1. Chemistry

Organophosphorus compounds show a variety of relevant biological activities [47–49]. The Kabachnik–Fields reaction is the most efficient method for the formation of carbon–phosphorus bonds using an aldehyde, amine, and H-phosphite. A number of other synthetic approaches have also been reported for the preparation of α-aminophosphonates [7,50–95]. These methods are generally conducted in the presence of various organic and inorganic bases [56–58] as well as Lewis and Bronsted acids, such as zirconium tetrachloride (ZrCl₄), aluminum chloride (AlCl₃), tantalum pentachloride (TaCl₅), or lanthanide triflates [59–63]. Therefore, it is necessary to further develop an efficient one-pot, multicomponent synthesis of α-aminophosphonates that is devoid of these problems and fulfills requirements of the pharmaceutical industry. Enzymes, which are natural catalysts with high catalytic activity, seem to be the best alternative leading to the development of new synthesis methods that meet the requirements related to safety and environmental protection. In addition, enzymes enable the synthesis of compounds without metal contamination, which is especially appreciated by the pharmaceutical industries. Among other uses, hydrolases are most often used as biocatalysts in organic synthesis.

Figure 1. Biologically active antimicrobial α-aminophosphonate derivatives.
Our work is more focused on discovering new unnatural catalytic activities of hydrolases. This phenomenon was defined as enzymatic promiscuity. Recently, a number of unnatural reactions catalyzed by hydrolases have been reported, such as the aza-Henry reaction [64], Michael additions [65,66], 1,2-addition of thiols to imines [67], and Morita–Baylis–Hillman reaction [68–70]. Although some chemical strategies work towards the synthesis of α-aminophosphonates, the biocatalytic preparation of target α-aminophosphonates remains unexploited. It was shown that some selected α-aminophosphonates could be obtained from aniline derivatives by the Kabachnik–Fields reaction using *Candida antarctica* lipase B (CAL-B) as a catalyst [71–73].

As a continuation of our research on seeking new catalytic activities of hydrolases [74–80,94,95], we focused our efforts on elaborating a sustainable metal-free method towards desired α-aminophosphonates 1–16 (Figure 2).

![Figure 2. α-Aminophosphonates 1–16 obtained via an enzyme-catalyzed Kabachnik–Fields reaction. Yields in brackets provided for isolated products 1–16.](image)

Regarding the promiscuous activity of lipases, [71] the model Kabachnik–Fields reaction of benzyl amine (1 mmol), benzaldehyde (1 mmol), and dimethyl phosphate (1 mmol) was conducted in neat at 25 °C (Scheme 1 and Table 1, entry 1).

\[
R_1\text{CHO} + R_2\text{NH}_2 + \text{HP}^\text{OR}_3^\text{OR}_4 \rightarrow \text{catalyst} \quad \text{solvent} \quad \rightarrow R_2\text{NH} \quad \text{OR}_3^\text{OR}_4
\]

\[R_1,R_2,R_3,R_4: \text{alkyl, aryl}\]

**Scheme 1.** Enzyme-catalyzed synthesis of α-aminophosphonates 1–16.
Table 1. Model Kabachnik–Fields reaction catalyzed by enzymes. [a] Optimization studies.

| Entry | Catalyst | T (°C) | Solvent | Yield [%] f |
|-------|----------|--------|---------|------------|
| 1     | None     | 25     | neat    | <5         |
| 2     | Porcine pancreas lipase (PpL) | 25 | neat | 73         |
| 3     | Porcine pancreas lipase (PpL) | 25 | Toluene | 64         |
| 4     | Porcine pancreas lipase (PpL) | 25 | EtOAc | 18         |
| 5     | Porcine pancreas lipase (PpL) | 25 | THF | 52         |
| 6     | Porcine pancreas lipase (PpL) | 25 | 2-Me THF | 55         |
| 7     | Porcine pancreas lipase (PpL) | 25 | TBME | 88         |
| 8     | Porcine pancreas lipase (PpL) | 30 | TBME | 91         |
| 9     | Porcine pancreas lipase (PpL) | 40 | TBME | 87         |
| 10    | Porcine pancreas lipase (PpL) b | 30 | TBME | 93         |
| 11    | Wheat germ lipase | 20 | neat | 29         |
| 12    | Pseudomonas cepacia lipase (Pfl) | 20 | neat | 44         |
| 13    | Candida cylindracea lipase (Ccl) | 20 | neat | 57         |
| 14    | Candida rugosa lipase (CrL) | 20 | neat | 35         |
| 15    | Novozym 435 | 20 | neat | 67         |
| 16    | Bovine serum albumin (BSA) | 30 | TBME | 9          |
| 17    | Bovine liver acetone powder (BLAP) c | 20 | neat | 43         |
| 18    | Denatured PpL d | 30 | TBME | <1         |
| 19    | CuI e | 25 | neat | 39         |
| 20    | CuO2 e | 25 | neat | 24         |
| 21    | Cu(OAc)2 e | 25 | neat | 33         |
| 22    | PhB(OH)2 e | 25 | neat | 14         |

[a] Reaction conditions: benzaldehyde (1 mmol), benzylamine (1 mmol), dimethyl phosphite (1 mmol), and enzyme (50 mg) in a solvent (2 mL) for 24 h, 200 rpm; b PpL (80 mg); c domestically prepared; d thermally deactivated at 100 °C for 24 h; e 10 mol%; f yield of the isolated product 1 after chromatography on silica gel.

As shown in Table 1, lipase from a porcine pancreas (PpL) was found as the best catalyst among the tested lipases for this addition reaction (Table 1, entry 2). The α-aminophosphonate 1 obtained a good yield (73%) after 24 h in neat at 25 °C. The yield did not increase substantially after 24 h. In the absence of enzyme only traces of the target product 1 was formed (Table 1, entry 1). In addition, four different nonenzymatic catalysts were reported in the literature as sustainable promoters of the Kabachnik–Fields reaction [81–83,94,95]: copper(I) iodide, copper(I) oxide, copper(II) acetate, and phenylboronic acid were tested under similar reaction conditions, leading to the target product 1 with up to a 39% yield (Table 1, entries 19–22). It is well recognized that the type of solvent used has a great impact on enzyme stability and activity [84]. Product 1 was provided with the highest yield of 88% in TBME (Table 1, entry 7); therefore, this solvent was applied in the following optimization.

Furthermore, the model reaction was carried out at elevated temperatures; however, the yield of product 1 was reduced at temperatures above 30 °C (Table 1, entries 8 and 9). Next, we studied if the amount of enzyme used had any impact on the reaction yield, and we found out that the yield of target compound 1 increased slightly by raising the amount of PpL from 50 mg to 80 mg. Thus, the 80 mg of PpL was the optimal amount for the further investigations [71,94,95].

Finally, we used the elaborated enzymatic protocol with various aromatic and aliphatic amines, aldehydes, and symmetrical as well as unsymmetrical H-phosphites [41] (Figure 2). The enzymatic Kabachnik–Fields reaction with aliphatic aldehydes and amines as well as 2-phenylethylamine provided products 4, 5, and 9–11 with lower yield ranges from 51% to 71% (Figure 2). A similar reduction in the reaction yield was observed for sterically bulky electron-rich aldehyde and amine with methoxy groups located at the phenyl ring, which resulted in product 8 with a 69% yield. Finally, the application of unsymmetrical H-phosphonates provided P-chiral products 14–16 as a mixture of diasteroisomers (1:1) with yields up to 76%. The structures of all obtained compounds 1–16 are presented in the experimental section (Supplementary Materials Figures S4–S65).
Additional experiments were performed to gather insights on the reaction pathway. Under developed conditions, N-(4-methoxylbenzylidene)benzylamine was used together with dimethyl H-phosphite in the presence of PpL as a catalyst, which resulted in an excellent yield of 95% of the target α-aminophosphonate 1. This observation constitutes the initial formation of an imine in the presence of lipase (Scheme 2).

![Scheme 2. Plausible mechanism of the porcine pancreas lipase-catalyzed Kabachnik – Fields reaction.](image)

**3.2. Cytotoxic Studies of the Library of α-Hydroxy Phosphonate Derivatives**

It is worth noting that the introduction of a fluorine atom into the structure of all 16 tested compounds did not have a significant effect on the activity of 2 and 12, which is often observed for various types of compounds exhibiting antibacterial activity [12] (Figures 3–6 and 8).

![Figure 3. Minimum inhibitory concentration (MIC) of the phosphonate derivatives in model bacterial strains.](image)

**Figure 3.** Minimum inhibitory concentration (MIC) of the phosphonate derivatives in model bacterial strains. The x-axis features compounds 1–16 used sequentially. The y-axis shows the MIC value in μg/mL⁻¹. Investigated strains of E. coli K12 as the control (blue), R2 strains (orange), R3 strains (grey), and R4 strains (yellow). The order in which the compounds were applied to the plate is shown in Supplementary Materials Figure S1.

![Figure 4. Minimum bactericidal concentration (MBC) of the phosphonate derivatives.](image)

**Figure 4.** Minimum bactericidal concentration (MBC) of the phosphonate derivatives. The x-axis features compounds 1–16 used sequentially. The y-axis shows the MIC value in μg/mL⁻¹. Investigated strains of E. coli K12 as control (blue), R2 strains (orange), R3 strains (grey), and R4 strains (yellow). The order in which the compounds were applied to the plate is shown in Supplementary Materials Figure S1.
Figure 4. Minimum bactericidal concentration (MBC) of the phosphonate derivatives. The x-axis features compounds 1–16 used sequentially. The y-axis shows the MIC value in µg/mL⁻¹. Investigated strains of E. coli K12 as control (blue), R2 strains (orange), R3 strains (grey), and R4 strains (yellow). The order in which the compounds were applied to the plate is shown in Supplementary Materials Figure S1.

Figure 5. The ratio of MBC/MIC of the phosphonate derivatives. The x-axis features compounds 1–16 used sequentially. The y-axis shows the MIC value in µg/mL⁻¹. Investigated strains of E. coli K12 as control (blue), R2 strains (orange), R3 strains (grey), and R4 strains (yellow). The order in which the compounds were applied to the plate is shown in Supplementary Materials Figure S1.

Table 2. Statistical analysis of all analyzed compounds by MIC, MBC, and MBC/MIC; <0.05 *, <0.01 **, <0.001 ***.

| No. of Samples | 13  | 15  | 16  | Type of Test |
|----------------|-----|-----|-----|-------------|
| K12            | *** | *** | *** | MIC         |
| R2             | *** | *** | *** | MIC         |
| R3             | *** | *** | *** | MIC         |
| R4             | *** | *** | *** | MIC         |
| K12            | **  | *   | **  | MBC         |
| R2             | **  | *   | **  | MBC         |
| R3             | **  | *   | **  | MBC         |
| R4             | **  | *   | **  | MBC         |
| K12            | *** | **  | **  | MBC/MIC     |
| R2             | *** | **  | **  | MBC/MIC     |
| R3             | *** | **  | **  | MBC/MIC     |
| R4             | *** | **  | **  | MBC/MIC     |

3.3. Analysis of R2–R4 E. coli Strains Modified with α-Aminophosphonate Derivatives

The obtained MIC values as well as our previous studies with various types of the analyzed compounds [85–95] indicate that α-aminophosphonate derivatives also show a strong toxic effect on the analyzed bacterial model strains. The three analyzed compounds were selected for further analysis by modifying their DNA. Modified bacterial DNA was digested with Fpg as described earlier [85–93]. All selected analyzed α-aminophosphonate derivatives (Figure 6), including different types of alkoxy groups, substituents located at the phenyl ring, and the length of the alkyl chain, can strongly change the topology of bacterial DNA. After digestion with Fpg, approximately 3.5% of oxidative damage was identified, which very strongly indicates oxidative damage in bacterial DNA, similar to the previous observations [85–93]. The different types of alkoxy groups, substituents located at the phenyl ring, and the length of the alkyl affected this outcome (Figure 6).
4. Conclusions

The performed studies prove that the analyzed and newly synthesized compounds can potentially be used as “substitutes” for the currently used antibiotics in hospital and clinical infections, (Figures 7 and 8 and Supplementary Materials Figure S3).

3.4. R2-R4 E. coli Strains with Tested α-Aminophosphonate Derivatives

The performed studies prove that the analyzed and newly synthesized compounds can potentially be used as “substitutes” for the currently used antibiotics in hospital and clinical infections, (Figures 7 and 8 and Supplementary Materials Figure S3).

**Figure 6.** Percentage of plasmid DNA recognized by the Fpg enzymes (y-axis) with model bacterial, K12, and R2–R4 strains (x-axis). All analyzed compounds numbered were statistically significant at <0.05 (see Table 2).

**Figure 7.** Percentage of bacterial DNA recognized by the Fpg enzymes in model bacterial strains after ciprofloxacin, bleomycin, and cloxacillin treatment. The compounds were statistically significant at \( p < 0.05 \).

**Figure 8.** Examples of MIC with model bacterial strains K12, R2, R3, and R4 for studying the antibiotics ciprofloxacin (cipro), bleomycin (bleo), and cloxacillin (clox). The x-axis features antibiotics used sequentially. The y-axis features the MIC value in \( \mu g/mL^{-1} \).
Large modifications of plasmid DNA were observed for the three analyzed compounds numbered 13, 15, and 16, showing high superselectivity.

4. Conclusions

Our developed protocol provides an efficient mild and metal-free synthesis of the target products with a high yield (51–93%). Among the studied derivatives, the compounds possessing alkoxy groups with halogen atoms or nitro groups in phosphate moieties 13, 15, and 16 turned out to be the most active compared to derivatives with the dimethyl groups (Figure 2). Finally, the reported α-aminophosphonate derivatives are more cytotoxic in the model bacterial cells than the following commonly used antibiotics: ciprofloxacin, bleomycin, and cloxacillin.

Supplementary Materials: The supporting information can be downloaded at the following: https://www.mdpi.com/article/10.3390/ma15113846/s1. Figure S1: examples of MIC and MBC on mi-
possessing alkoxy groups with halogen atoms or nitro groups in phosphate moieties (phenylethyl) phosphite. Figure S57: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S7: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S27: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S56: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S18: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S15: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S12: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S13: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S4: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S5: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S6: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S3: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S9: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S2: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S36: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S32: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S35: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S31: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S29: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S26: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S20: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S14: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S10: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S11: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S47: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S48: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S49: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S50: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S51: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S52: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S53: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S54: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S55: (100 MHz, CDCl$_3$) spectra of compound 1. Figure S56: (100 MHz, CDCl$_3$) spectra of compound 1.
phosphate. Figure S58: $^1$HNMR (400 MHz, CDCl$_3$) spectra of methyl (4-chlorophenylethyl) phosphate. Figure S59: $^{13}$CNMR (100 MHz, CDCl$_3$) spectra of methyl (4-chlorophenylethyl) phosphate. Figure S60: $^{31}$PNMR (162 MHz, CDCl$_3$) spectra of methyl (4-chlorophenylethyl) phosphate. Figure S61: $^1$HNMR (400 MHz, CDCl$_3$) spectra of methyl (4-nitrobenzyl) phosphate. Figure S62: $^{13}$CNMR (100 MHz, CDCl$_3$) spectra of methyl (4-nitrobenzyl) phosphate. Figure S63: $^{31}$PNMR (162 MHz, CDCl$_3$) spectra of methyl (4-nitrobenzyl) phosphate. Figure S64: $^1$HNMR (400 MHz, CDCl$_3$) spectra of N-(4-methoxybenzylidene)benzylamine. Figure S65: $^{13}$CNMR (100 MHz, CDCl$_3$) spectra of N-(4-methoxybenzylidene)benzylamine.

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**Abbreviations**

| Acronym | Definition |
|---------|------------|
| MIC     | minimum inhibitory concentration |
| MBC     | minimum bactericidal concentration |
| Oc      | open circle |
| CCC     | covalently closed circle |
| BER     | base excision repair |
| Fpg     | DNA-formamidopyrimidine glycosylase |

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