Journal of Research in Pharmacy

Research Article

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Aromatic Phosphinous Amides: A Promising New Generation of Antibiotics for Multidrug-Resistant Bacterial Infections

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Received: 18 April 2023 / Revised: 09 September 2023 / Accepted: 10 September 2023

ABSTRACT: There is a focus on using aromatic phosphinous amide as an antibacterial agent against serious multidrug resistant bacterial infections. N–(4–acetylphenyl)–P,P–diphenyl-phosphinous amide and its corresponding thioxo-phosphinous amide and selenoxo-phosphinous amide were synthesized and designated Ph1, Ph2, and Ph3, respectively. They were structurally identified by single crystal X-ray diffraction, multinuclear NMR spectroscopy, and elemental analysis. Because their antibacterial activities have never been examined, this study aimed to assess their antibacterial activities against both Gram-positive and Gram-negative bacteria as well as readily available standard bacterial strains. Two bacterial isolates were isolated from urine specimens collected from patients with urinary tract infections and designated CS1, and CS2. Phenotypic, biochemical, and molecular approaches were used to identify them. The antibiotic susceptibility/resistance pattern of these isolates was examined by the installed VITEK 2 system. Isolate CS1 is an aerobic Gram-positive, straight rod-shaped, spore forming, occurring singly or in a short chain, whereas isolate CS2 is an aerobic Gram-negative, coccobacilli-shaped, non-spore forming, occurring in pairs or in chains. Both isolates are positive for catalase and oxidase test. According to an examination of the 16S rRNA gene sequence, the isolates CS1 and CS2 have 98% of their similarities with Bacillus and Acinetobacter species, respectively. Isolates CS1 and CS2 are multidrug resistance species. Based on the results of the minimum inhibitory concentration, the standard bacterial strains and isolates CS1 and CS2 showed a wide range in the antibacterial capabilities of the studied phosphinous amides. In comparison to Ph2 and Ph3, Ph1 has the strongest antibacterial activity against all of the tested bacterial species. In conclusion, CS1 and CS2 isolates were identified as novel, multi-drug resistant members of the Bacillus and Acinetobacter genera, respectively. The Ph1 molecule represents a promising new generation of antibiotics with notable antibacterial efficacy against multidrug-resistant Gram-positive and Gram-negative microorganisms.

KEYWORDS: Phosphinous amide derivatives; Multidrug resistance bacteria; 16S rRNA gene sequencing technique; Structural analysis; Minimum inhibitory concentration.

1. INTRODUCTION

Multidrug resistant (MDR) gram-positive and gram-negative bacteria are difficult to eliminate from the environment, therefore their existence may represent an immediate hazard to public health, safety, or welfare. Previous research has shown that the emergence of novel MDR Gram-negative and Gram-positive bacteria has resulted in alarming levels of worldwide microbial infection. MDR infections have been related to developmental delays in newborns and children with immune system abnormalities, as well as those suffering from viral infections or other illnesses. This raises the risk of hospitalized patients getting multidrug-resistant bacterial infections, which can result in or cause considerable morbidity and mortality. Long hospital stays are also becoming one of the most serious issues in the global health system, resulting in high health and financial expenses for local governments in many nations and becoming a worldwide issue. Furthermore, MDR bacterial infections pose formidable therapeutic challenges due to limited efficacy of existing pharmaceutical agents. As a result, new antimicrobial drugs are still desperately needed to treat the

How to cite this article: Irshaid FI, Eshawakh SR, Hunaiti A, Al-Masri HT. Aromatic Phosphinous Amides: A Promising New Generation of Antibiotics for Multidrug-Resistant Bacterial Infections. J Res Pharm. 2024; 28(5): 1800-1811.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.852

newly emerging bacteria or MDR pathogens, as well as to prevent potentially fatal communicable infections [1-3].

In the hunt for new synthetic antibacterial drugs against serious or life-threatening MDR bacterial diseases, a number of novel synthetic and semi-synthetic antibacterial drugs with novel mechanisms have been developed and tested against a diverse range of MDR bacterial strains. These antibiotics surpassed natural antibiotics in terms of activity, therapeutic efficacy, side effects and toxicity. As a result, synthetic triazole, aminophosphane, and other derivatives continue to be essential for halting the spread of a number of infectious or communicable diseases [3-6].

Aminophosphanes, phosphanamine, phosphavanes or phosphinous amides are one of the promising candidates for new synthetic antibacterial agent. These compounds are notable for their carbon atoms arranged in aromatic rings, which are phosphonic analogs of tyrosine, tryptophan, and/or phenylalanine. They are interphosphoric organic compounds featuring a single P-N bond with each atom being saturated with protons or carbons. The alpha-aminophosphonic acids are similar in structure to amino acids and the transition state mimics peptide hydrolysis. These characteristics render them crucial antimetabolites, as they competitively bind with carboxylic analogs to occupy active enzyme sites and other cellular receptors [7-9].

In the pursuit of advancing phosphinous amides and their derivatives within pharmaceutical and biological chemistry, these compounds have exhibited diverse functionalities, serving as neuroactive and antibacterial agents, and finding extensive utility in both industrial and medical research settings. Notably, their intricate interactions with challenging metal chemistry ligands, as well as their catalytic potential, continue to spur ongoing research interest. Consequently, prior endeavors in the realm of organic phosphate compounds have predominantly concentrated on the development of novel and enhanced phosphorus ligands, aimed at facilitating the creation of innovative mineral complexes characterized by enhanced biological and catalytic attributes [7-10].

Our research centers on the synthesis of a novel group of antibiotics derived from aromatic phosphinous amides. Three specific aromatic phosphinous amides, namely N–(4–acetylphenyl)–P,P–diphenyl-phosphinous amide and its thioxo-phosphinous amide (monosulfide) and selenoxo-phosphinous amide (monoselenide) derivatives, have been synthesized in our laboratory [8]. Notably, the antibacterial properties of these aromatic phosphinous amides remain unexplored. Given the limited clinical benefits of recently approved antibiotic classes, most of which are derivatives of existing antibiotics and are susceptible to multidrug-resistant (MDR) bacterial strains, our study aims to address two main objectives. Firstly, we seek to isolate common bacterial pathogens from urine specimens of patients with suspected urinary tract infections (UTIs) and identify them at the genus or species level, while also determining their antimicrobial susceptibility profiles. Secondly, we aim to evaluate the antimicrobial efficacy of the newly synthesized aromatic phosphinous amides and their thioxo-phosphinous amide and selenoxo-phosphinous amide derivatives against both newly isolated bacterial species and established standard bacterial strains. This research addresses the pressing need for novel antibiotics in the face of MDR bacterial threats.

2. RESULTS

2.1. Isolation and identification of bacterial isolates from urine samples:

In this study, two clinical samples from two different people with urinary tract infections were cultured in Mueller-Hinton agar medium (MHAM). Two bacterial isolates were chosen for further investigation based on differences in colonial morphology (shape, size, elevation, surface, opacity, and edge). These bacterial isolates were identified as CS1 and CS2 for further characterization and identification. The cells of the CS1 isolate are Gram-positive, straight rod-shaped, spore generating, and range in diameter and length from 0.5-1.0 m to 3-6 μ (Table 1). CS1 cells can be found alone or in short chains. CS2 isolate cells are Gram-negative, coccobacilli-shaped, non-spore forming, and measure 0.8-1.7 by 1.3-2.2 μm.

Table 1. Gram staining, cell morphology and microscopic characteristics of bacterial isolates CS1 and CS2 obtained from urine specimens from adult patients with urine tract infections.

Isolate code	Source	Gram staining	Shape	Size (μm)	Sporulation	Cell arrangement
CS1	Urine	Positive	Rod	0.5-1.0 by 3.0-6.0	Spore forming	Single or short chain
CS2	Urine	Negative	Coccobacilli	0.8–1.7 by 1.3–2.2	Non-spore forming	Pairs or some in chains

2.2. Biochemical characterization of bacterial isolates by API 20E system:

Pure colonies from MHAM plates after 3 days of cultivation were used for biochemical assays. First, pure colonies from these two isolates were examined for catalase and oxidase activity. The API 20E Test System was then used to identify bacteria. Table 2 elucidates the biochemical characteristics of two clinical isolates, CS1 and CS2, isolated from urine samples of patients with UTI. These assays revealed that isolates CS1 and CS2 were catalase (CAT) and oxidase (OX) positive. AP120E tests indicated that cells from the CS1 isolate exhibited positive reactions for ADH, VP, GEL, GLU, MAN, SAC, MEL, and AMY. In contrast, the CS2 isolate demonstrated positivity for ONPG, ADH, CIT, TDA, GLU, MAN, RHA, SAC, MEL, AMY, and ARA. These analyses signify discernible distinctions in the biochemical and morphological attributes between the two isolates.

2.3. Bacterial identification in urine via 16S rRNA gene sequencing:

Table 3 displays the closest phylogenetic relatives of isolates CS1 and CS2 as determined by their 16S rRNA sequence similarity analysis. Due to the inability of the API 20E method to classify CS1 and CS2 isolates at the species level, 16S rDNA sequencing using the Sanger method with fluorescent dye-labels was employed. The obtained sequences underwent alignment and comparative analysis against the GenBank database. A BLAST search revealed that the strain CS1 was 98% identical to Bacillus spp. (Bacillus cereus, Bacillus tropicus, Bacillus luti, Bacillus albus, Bacillus toyonensis, and Bacillus nitratireducens). CS2 is 98% identical to Acinetobacter spp. (Acinetobacter sp., Acinetobacter lwoffii, Acinetobacter sp. PL15a_S20, and Acinetobacter sp.GC2). The 16S rDNA sequences for bacterial isolates CS1 and CS2 were deposited in the NCBI Gene Bankit nucleotide sequence database as accession numbers OP420469 and OP420470, respectively.

Table 2. Biochemical characteristics of the bacterial isolates CS1 and CS2 cultivated from urine specimens from adult patients with urinary tract infection.

Name of biochemical test	Isolates	
	CS1	CS2
Ortho-nitrophenyl-β,D-galactoside	-	+
Arginine dihydrolase	+	+
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Citrate utilization	-	+
H_2S production	-	-
Urease	-	-
Tryptophan deaminase	-	+
Indole production	-	-
Voges–Proskauer	+	-
Gelatinase	+	-
Glucose	+	+
Mannitol	+	+
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	+
Saccharose	+	+
Melibiose	+	+
Amygdalin	+	+
Arabinose	-	+
Oxidase	+	+
Catalase	+	+

2.4. The antibiotic susceptibility profiles of the bacterial isolates CS1 and CS2:

The antibiotic susceptibility profiles of the CS1 and CS2 isolates recovered in this study using the VITEK® 2 microbial ID/AST testing systems were determined and presented in detail in Table 4. Results of susceptibility tests were classified as susceptible (S), intermediate (I) or resistant (R) according to criteria recommended by Clinical and Laboratory Standards Institute (CLSI guidelines) [13]. The results of this experiment revealed that CS1 isolate was found to be resistant benzylpenicillin (penicillin G), clindamycin,

erythromycin and quinupristin /dalfopristin. CS2 was also resistant to ampicillin, ampicillin/sulbactam, benzylpenicillin, clindamycin, erythromycin and imipenem. Bacterial isolate was classified to be MDR bacteria when it was resistant to at least two antibiotic compounds. Based on this classification, these two clinical isolated can be considered MDR bacteria.

Table 3. The closest relatives of bacterial isolates CS1 and CS2 based on identity in 16S rRNA gene sequencing data.

Isolate code	Total length (bp)	Coverage %	Close relative	Identity %	Gene bank accession no.
CS1[a]	1441	98	*Bacillus cereus*	98	MH114968.1
	1441	98	*Bacillus tropicus*	98	NR_157736.1
	1441	98	*Bacillus luti*	98	NR_157730.1
	1441	98	*Bacillus albus*	98	NR_157729.1
	1441	98	*Bacillus toyonensis*	98	KY085984.1
	1441	98	*Bacillus nitratireducens*	98	NR_157732.1
CS2[a]	1472	100	*Acinetobacter sp.*	98	EU567047.2
	1472	99	*Acinetobacter lwoffii*	98	JX966447.1
	1472	99	*Acinetobacter sp. PL15a_S20*	98	NR_157730.1
	1472	99	*Acinetobacter sp.GC2*	98	KJ867435.1

[a] The bacterial isolates CS1 and CS2 were cultivated from the urine specimen obtained from patients with urinary infection. The length of the resulting DNA sequences of isolates CS1 and CS2 were 1441 and 1472 base pairs (bp), respectively.

Table 4: The antibiotic susceptibility profiles of the bacterial isolates CS1 and CS2 from urine specimens from adult patients with urinary tract infection.

Antibacterial agent	Bacterial isolates	
	CS1	CS2
Ampicillin	S	R
Ampicillin/Sulbactam	S	R
Benzylpenicillin	R	R
Clindamycin	R	R
Erythromycin	R	R
Imipenem	S	R
Linezolid	S	S
Nitrofurantoin	S	S
Quinupristin/Dalfopristin	R	S
Teicoplenin	S	S
Vancomycin	S	S

S: Sensitive, R: Resistance, CS1: Clinical isolate 1, CS2: Clinical isolate 2.

2.5. Antibacterial activity of the three newly synthesized phosphinous amide derivatives:

Antibacterial activities of the three tested aromatic phosphinous amide derivatives (Ph1, Ph2, and Ph3) and colistin against the two clinical isolates and the three-laboratory control strain were shown in table 5. Phosphinous amide derivatives with highest antibacterial activity was Ph1 with MIC values ranging from 31-63 µg/ml against all tested bacterial species. This was followed by Ph2 compound with MIC values of 250 µg/ml against *Bacillus subtilis* and *E. coli* and 500 µg/mL against *Staphylococcus epidermidis*. On the other hand, Ph3 compound exhibited a poor antibacterial activity with MIC values of 500 to 1000 µg/ml for all test bacterial species. Furthermore, MIC values of colistin were found to be in the range of 13-32 µg/ml for all examined bacterial species.

Furthermore, antibacterial activity of these phosphinous amide derivatives were compared to colistin. Colistin (polymyxin E) is an antibiotic that is widely used to treat infections caused by MDR bacteria. The results revealed that MIC values of colistin antibiotic were in the range of 20-40 µg/ml for all test clinical isolates as seen in Table 6. Based on these data, MIC values of Ph1 compound were about two-folds higher than that for colistin. Whereas, MIC values of Ph2 compound were at least 11-fold higher than that recorded for colistin. On the other hand, Ph3 compound exhibited a poor antibacterial activity with MIC values of at least 900 µg/ml against all test clinical isolates and at least 23-fold higher than that reported for colistin.

Table 5. The minimum inhibitory concentrations (MIC) of synthetic phosphinous amide compounds and colistin towards Gram-positive and Gram-negative multidrug resistance bacterial species and control bacterial strains.

Isolate name	MIC (µg/ml)			
	Ph1	Ph2	Ph3	Colistin
Escherichia coli	30	250	1000	20
Bacillus subtilis	60	250	900	20
Staphylococcus epidermidis	60	500	1000	20
Bacillus spp. FICS1	60	450	950	40
Acinetobacter spp. FICS	60	500	1000	40

Ph1: N–(4–acetylphenyl)–*P*,*P*–diphenyl-phosphinous amide, Ph2: the corresponding thioxo-phosphinous amide, and Ph3: the corresponding selenoxo-phosphinous amide.

3. DISCUSSION

A previous report noted a rising prevalence of multidrug-resistant (MDR) pathogenic bacteria in patients with various infections, including urinary tract infections (UTI), over recent decades [1]. In our study, we focused on isolating and identifying MDR bacteria from urine samples obtained from adult patients with suspected UTI. Two distinct isolates, CS1 and CS2, were selected based on their physical characteristics. CS1 is an aerobic Gram-positive rod-shaped bacterium, while CS2 is an aerobic Gram-negative coccobacillus. Both isolates exhibited catalase and oxidase positivity. Biochemical analysis using the API 20E system revealed noticeable differences in biochemical properties between CS1 and CS2. These analyses conducted reveal noticeable differences in both morphology and biochemical properties between the two isolates, CS1 and CS2. As a result, each isolate has a distinctive and special profile, highlighting their obvious difference. The biochemical testing results for isolates CS1 and CS2 were, however, vague and indeterminate, making suitable taxonomic categorization difficult.

Morphological and biochemical tests failed to classify two unidentified bacterial isolates at the genus or species level, necessitating genetic analysis via 16S rRNA sequencing. Through a BLAST search, the 16S rRNA gene sequences were aligned with prokaryotic type strain sequences to determine similarity. The sequence identity between isolate CS1 and *Bacillus* spp. was 98%, while isolate CS2 showed a 98% identity with *Acinetobacter* spp. based on current sequencing data. Our findings suggest that 16S rRNA sequence analysis could be effective in evaluating whether or not an unknown bacterial isolate belongs to a given taxon. This method was widely used to detect strains and bacteria that were poorly labeled, seldom isolated, or exhibited phenotypically abnormalitie [5, 6, 12-17]. According to CLSI recommendations for establishing taxonomic links between prokaryotic strains and bacterial categorization through DNA target sequencing, If the 16S rRNA sequences are 99% identical or more, the strains are almost certainly the same species, whereas 97 to 99% similarity was used to identify an organism at the genus level [13-14]. It is crucial to remember that the 16S rRNA gene sequence data cannot offer a conclusive solution since it cannot tell apart recently diverged species. As a result, the species of the CS1 and CS2 isolates is unknown. The CS1 and CS2 isolates, however, might represent novel *Bacillus* and *Acinetobacter* species, according to CLSI recommendations. Taking into consideration these findings, the isolate CS1 was designated as *Bacillus sp.*FI-CS1 and the isolate CS2 was designated as *Acinetobacter* sp. FI-CS2.

According to previous and present research, many *Bacillus* species exhibit a diverse range of physiologic features that allow them to thrive in a variety of natural environments. These *Bacillus* species have also been proven to be MDR, capable of causing a variety of severe ailments such as respiratory tract infections, urinary tract infections, and others, some of which can be deadly to humans or animals. These analyses further confirmed that these *Bacillus* species are aerobic, Gram-positive, endospore-forming, and rod-shaped, providing more convincing support for our findings [15-19]. According to our findings, the morphological, biochemical, and molecular characteristics of isolate CS1 were comparable to previously known *Bacillus* species such as *Bacillus cereus* and *Bacillus tropicus*, suggesting that this isolate might be a unique *Bacillus* species.

The Gram-negative saprophytic bacteria *Acinetobacter* was revealed to be completely aerobic, non-pigmented, non-fermenting, and non-motile. This genus has enclosed cocco-bacilli rods, which are tiny and commonly occur in pairs or long chains of various length. *Acinetobacter* species are not difficult to culture in normal laboratory conditions. Catalase-positive, oxidase-negative, indole-negative, and citrate-positive species were discovered, with G + C concentrations ranging from 39 to 47% [20-23]. With the exception of being oxidase positive, the morphological and biochemical traits of isolate CS2 appear to be comparable with previous results of Acinetobacter morphological and biochemical properties. These *Acinetobacter* genus

traits, together with 16S rRNA gene sequence analysis, offered an extra layer of support to the CS2 isolate's identification.

To successfully minimize urinary tract infections in patients while limiting antibiotic resistance, it is critical to first establish the susceptibility properties of the bacteria causing UTI before giving medicinal supplies. According to antibiotic susceptibility test findings, the clinical isolate CS1 was resistant to at least four commercially available antibiotics, including benzylpenicillin, clindamycin, erythromycin, and quinupristin/dalfopristin. Several *Bacillus cereus* group strains have been reported to be resistant to routinely used antibiotics, including penicillin, ampicillin, ciprofloxacin, cloxacillin, erythromycin, tetracycline, and streptomycin. [24, 25]. Fiedler et al. (2019) used genome sequencing to determine the incidence of antibiotic resistance in multiple *Bacillus cereus* strains and revealed a gene responsible for conferring resistance [25]. The CS2 appears to be resistant to at least six commonly used antibiotics, including ampicillin, ampicillin/sulbactam, benzylpenicillin, clindamycin, erythromycin, and imipenem. Previous research on newly emergent *Acinetobacter* species found resistance to all routinely used antibiotics [1, 3, 26-28]. As a consequence, our findings are consistent with previous research from different parts of the world. Furthermore, the scientists attributed antibiotic misuse and/or the availability of antimicrobials without a prescription for the global rise in MDR *Acinetobacter* species. These findings may help to explain the high prevalence of antibiotic resistance in our isolates, which we believe is important for our investigation. Antibiotic resistance in our isolate, on the other hand, might be due to the existence of unexplored genes with unknown functions and processes.

In the second phase, we employed three recently synthesized aromatic phosphinous amides, denoted as Ph1 and its derivatives, Ph2 and Ph3. These compounds were synthesized through the reaction of phosphorus ligands (Ph1) with mineral complexes that contained either sulfur or selenium. This reaction facilitated the efficient incorporation of sulfur or selenium atoms into the P-N linkage of the Ph1 molecule [7-11]. We postulated that these modifications would render them a prolific reservoir of innovative bioactive compounds, with the potential to emerge as potent antibacterial agents. We assessed the antibacterial effectiveness of these compounds for the first time by investigating their activity against newly isolated MDR bacterial strains, namely *Bacillus* sp. FI-CS1 and *Acinetobacter* sp. FI-CS2, as well as established laboratory control strains. In this experiment, it was found that the Ph1 compound exhibited the highest antibacterial activity, with minimum inhibitory concentration (MIC) values ranging from 40 to 60 g/mL against both newly isolated strains (*Bacillus* sp. FI-CS1 and *Acinetobacter* sp. FI-CS2) and laboratory control strains. In contrast, Ph2's MIC values against all investigated bacterial species varied from 250 to 500 g/mL. Furthermore, Ph3 molecule demonstrated exceedingly weak antibacterial action against all tested bacterial species. Previous research demonstrated that several alpha-aminophosphonic acid derivatives have bacteriostatic action against Gram-positive and Gram-negative bacterial species [7-11, 29-31]. These prior research' conclusions are completely consistent with our findings. Furthermore, for the majority of the tested bacterial species, colistin as a normal antibiotic was still more effective than the Ph1 compounds. Taken together, these findings are encouraging and can be used to justify developing a broad-spectrum antibiotic based on the structure of the Ph1 molecule.

Our findings showed that the antibacterial activity of Ph2 and Ph3 compounds was much lower than that of Ph1, which exhibits robust antibacterial action. Indeed, differences in antibacterial activity across these substances can be explained simply by structural differences. A deeper examination of the structure of Ph1 chemical found that it lacks sulfur and/or selenium when compared to its comparable Ph2 and Ph3 derivatives, which do include sulfur and selenium, respectively. The addition of sulfur and selenium atoms in Ph2 and Ph3 structures, respectively, can cause significant changes in the space group and atomic arrangement of the original Ph1 structure. Furthermore, these structural modifications influence both the physical and chemical characteristics of Ph1. According to several research investigations, structural alterations and/or substitutions frequently result in the production of a new chemical with unique features. As a result, many groups of researchers have produced multiple aromatic phosphinous amide derivatives [7-11, 29-31]. These studies demonstrated that several of the newly synthesized phosphinous amide derivatives have better biological features, including antibacterial activity. In contrast, the changes we made to the Ph1 molecule had a detrimental impact on its antibacterial properties. As a consequence, it is feasible to deduce that the addition of sulfur or selenium to Ph1 molecule appears to display more steric hindrance, and as a result, the antibacterial activities of these compounds have been severely reduced due to this steric impact.

Previous research revealed that metal-ligand bonding might increase the interaction and therapeutic activities of various aminophosphonic acids [7-10, 29-31]. As previously noted, it was discovered that adding sulfur or selenium atoms to Ph1 significantly lowered its antibacterial effects. Both sulfur and selenium are known to be present in proteins as components of alpha-amino acids such as cysteine, methionine,

selenocysteine, and selenomethionin. Because they are in the same category, selenium and sulfur have more in common. They contain the same amount of valence electrons and will react similarly. Sulfur, on the other hand, has a somewhat greater electronegativity than selenium. Furthermore, the atomic radius of sulfur is less than that of selenium [32]. Because the selenium atom is bigger than the sulfur atom, the steric hindrance is stronger in the Ph3 molecule than in the Ph2 compound. The observed differences between Ph2 and Ph3 chemicals explain why Ph2's MIC values against all tested bacteria species were much lower than those of Ph3.

As previously indicated, the antibacterial activity of Ph1 compound was greater than that of Ph2 or Ph3 molecule. Aside from the steric action, the Ph1 molecule has a free phosphate group. This considerable antibacterial action might be attributed to this free group. Similarly, Rapp et al. (2021) suggest that adding a phosphonate group to biological active chemicals might alter their physicochemical characteristics as well as biological activities such as antibiotic properties [33]. They also showed that even little changes in their physicochemical features can result in major alterations in their biochemical characteristics or activities. According to these data, the significant antibacterial activity of Ph1 molecule appears to be related to its physicochemical property. Furthermore, based on the structure of the Ph1 molecule, the results of this study are positive and promising, and may be utilized to design and create a new class of phosphinous amide compounds with broadened spectrum antibiotics.

3.1. Limitations

Concerning the study's limitations, the known antibacterial mechanism associated with phosphinous amide chemicals is unknown. Bacillus colistinus generates colistin, a cyclic cationic polypeptide molecule that kills bacteria by entering and disrupting the bacterial cell membrane through lipopolysaccharide interactions [34]. This may help to explain the Ph1 molecule's antibacterial characteristics. As a result, membrane disruption is one of the Ph1 molecule's potential antibacterial modes of action. It is not impossible that the Ph1 molecule's antibacterial activity is due to its interaction with molecular targets on the surface of the bacteria's cell membrane. Furthermore, this action might be attributed to its capacity to connect with a genuine enzyme and function as enzyme inhibitors in peptide metabolism. Numerous investigations have shown that aminophosphonic acid compounds are effective inhibitors of a variety of receptors, including gamma-aminobutyric acid receptors and cysteine proteases like papain, cathepsin B, and cathepsin K [7, 8, 31]. Covalent interaction of these compounds' phosphate groups with the side chains of important amino acid residues located inside the active regions of the target enzymes may have a significant role in lowering their activity, resulting in persistent inhibition. Other potential mechanism must not be disregarded. Despite their advantages, aminophosphonates can affect the environment. As a result, before using aminophosphatonates as biological agents, their pharmacological and pharmacokinetic characteristics, as well as their environmental toxicity, must be studied.

4. CONCLUSION

For the first time, we describe the isolation of two clinically relevant pathogenic bacterial species, CS1 and CS2, from urine samples of adult patients with UTI. Our data imply that isolate CS1 is a Gram-positive rod-shaped bacterium, whereas isolate CS2 is a Gram-negative coccobacilli bacterium. Both isolates CS1 and CS2 are catalase and oxidase positive. Biochemical property variation was also discovered utilizing the AP1 20E technique for bacterial identification. The isolates CS1 and CS2 represent two distinct and independent *Bacillus* and *Acinetobacter* species, respectively, according to 16S rRNA gene sequencing. This might result in the emergence of a novel and/or distinct bacterial species or genus. The isolate CS1 tested resistant to benzylpenicillin, clindamycin, erythromycin, and quinupristin/dalfopristin in an antibiotic susceptibility test. The isolate CS2 was resistant to ampicillin, ampicillin/sulbactam, benzylpenicillin, clindamycin, erythromycin, and imipenem. These findings demonstrate that these two unique isolates differ phenotypically, genetically, and immunologically. These two isolates CS1 and CS2 appear to be connected to separate genera based on their morphological, biochemical, immunological, and genetic sequences.

This study also discovered that oxidation with elemental sulfur or selenium changed the Ph1 molecule, yielding the equivalent Ph2 and Ph3, respectively. Only Ph1 demonstrated substantial antibacterial activity against MDR Gram-positive and Gram-positive bacterial species when compared to the comparable Ph2 (monosulfide) and Ph3 (monoselenide) derivatives. This discovery shown that substituting Ph1 with a sulfur or selenium atom lowers antibacterial effectiveness. This fascinating discovery holds promise for the creation of new classes of potent broad-spectrum antibiotics based on the structure of the

Ph1 molecule. Furthermore, the antibacterial mechanism of this compound remains undisclosed. Further research is essential to evaluate its efficacy against diverse bacterial species.

5. MATERIALS AND METHODS

5.1. Patients and clinical sample collections:

This current study was conducted in the framework of a research project investigating the antibacterial activities of some newly synthesized phosphinous amide compounds against newly characterized multidrug resistant bacterial species. Urine samples were collected from two patients (adult woman and adult man) with urinary tract infection attending their first clinical appointment at microbiology laboratory of King Abdullah University Hospital, Irbid, Jordan. The clinical specimens were obtained prior to any prescription or use of antibiotics, for microbiological analyses and antibiotic susceptibility testing (AST) to insure optimal conditions for the recovery of pathogens. The technique was reviewed and authorized by the department's ethics committee for scientific research. It received ethical approval from the Institutional Review Board of the Faculty of Science. A letter of approval from University Hospital's clinical director was also obtained prior to the collection of the samples.

About 5 ml of fresh urine specimens were collected from two different patients with urinary tract infections in sterile urine containers and then placed in a sealed plastic bag. Then, the specimens were labeled with the patient's full name and date of birth. Next, the specimens were placed inside a sterile ice box, and immediately transported to our laboratory where analyses were carried out.

5.2. Cultivation and isolation of bacteria from urine samples:

The two urine specimens were first cultivated in Mueller-Hinton agar medium (MHAM) (HiMedia Laboratories Pvt. Mumbai, India). Standard loop technique was followed for bacterial culture [2]. To obtain pure colony from each specimen, the specimens were cultured on MHBM by using the spread plate technique and incubated aerobically at 35°C for 48 hours. After incubation, one suspected colony was selected from each urine specimens and repeatedly sub-cultured on a new MHAM plates. Then, plates were incubated as above to get a single pure colony. Pure colonies from teach isolates were obtained and stored for further biological analysis. This study also included three common bacterial strains known as laboratory control strains (Escherichia coli (ATCC 25922), Bacillus subtilis (ATCC 6633), and Staphylococcus epidermidis (ATCC 12228) provided by The American Type Culture Collection (ATCC) in Manassas, Virginia. Standard bacterial strains were cultivated in nutrient agar medium (NAM) using standard procedures. Plates were inoculated with standard bacterial strains and incubated at 35°C for 24 hours. Standard bacterial strains were cultivated in NAM using standard procedures. Plates were inoculated with standard bacterial strains and incubated at 35°C for 24 hours.

For long-term storage, pure cultures from the two-suspected colonies were prepared in 50% glycerol solution. Briefly, after bacterial growth in MHBM, 500 µL of the overnight culture of each pure culture were added into 500 µL of 50% glycerol in a 2 mL screw top tube and gently mix. The resulting glycerol stock of each pure culture was stored in freezer at -20 ^{o}C for future studies [2]. All sample containers, solutions and all glassware used during these procedures or experiments were rinsed thoroughly with distilled water and then subjected to sterilization at 121°C for 15 minutes using an autoclave before being used again for any procedure or experiments

5.3. Characterization and identification of bacterial isolates:

For the purpose of characterization and identification of the two selected isolates, pure colonies of the two isolates were first inoculated in MHAM and incubated aerobically at 35°C for 72 hours. Characterization and identification of these isolates were first performed on the basis of their morphological characteristics as described previously [2]. Two isolates were first selected based on morphological characterization (color, shape, transparency and margin. Finally, two bacterial isolates were selected and subjected to further characterization and identifications. For these purposes, the two bacterial isolates were designated as CS1 and CS2. Gram staining techniques were used to identify these two bacterial isolates (CS1 and CS2). The staining procedure was conducted for the isolates as previously described [11]. Microscopic features were examined and recorded for the two isolates via Gram stain protocol.

The isolates CS1 and CS2 were also subjected to biochemical identification. The isolates were examined for the oxidase and catalase activities. The oxidase and catalase activities were carried out for the two isolates according to a previously described protocol [11]. API 20Esystem (standardized identification

system) was also used for identification of these two isolates (BioMérieux, Marcy-l'Etoile, France). This system was widely used for biochemical characterization and identification of bacteria in specimens from human infections. This system is a multiple test system allowing the determination of 20 different biochemical tests nearly simultaneously. The identification kit of API 20E system strips composed of 20 separate microtubes containing dehydrated substrates. The biochemical tests investigated with API 20E system are: O-nitrophenyl-b-D-galactopyranoside (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), H_2S production (H_2S), urease (URE), tryptophan deaminase (TDA), indole production (IND), Voges–Proskauer (VP), gelatinase (GEL), glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), saccharose (SAC), melibiose (MEL), amygdalin (AMY) and arabinose (ARA).

Briefly, biochemical identification for these two isolates was performed using API 20E strips system following the manufacturer's instructions. A fresh overnight culture of each pure isolate was inoculated into MHAM plates. Plates were incubated at 35°C for 72 hours. For preparation of the inoculums, a single colony from each plate was removed and suspended into an ampule of API NaCl 0.85 % Medium (5 ml) without additives. To obtain a homogeneous bacterial suspension, the suspension was mixed by pipette. The results were tabulated on the result sheet and recorded according to the reading table provided by the manufacturer. API 20E database included in APILAB Plus software was used to identify the isolated strains to the species level.

5.4. Identification of bacterial isolates in urine samples by 16S rRNA gene sequencing:

To accurately identify these isolates (CS1 and CS2) to the species level, DNA-based molecular technique was employed as previously described [12]. Briefly, fresh bacterial culture from each isolate was prepared by cultivation of each isolate in MHB medium overnight at 37 °C with shaking at 175 rpm. DNA from each pure isolate was first extracted and purified. Norgen's Bacterial Genomic DNA Isolation Kit (Thorold, ON, Canada L2V 4Y6) was used for the rapid isolation and purification preparation of genomic DNA from viable bacterial cells. Isolation and purification of DNA from these urine samples was performed in accordance with the manufactures' instructions (Norgen Biotek Corp, Canada). The purified DNA was stored at -20°C in refrigerator for future analysis.

The purified DNA obtained from each pure isolate was subjected to amplification by polymerase chain reaction (PCR). For each isolate,16S rRNA gene was amplified by PCR. After that, the resulting PCR product for each isolate was sequenced as described previously [11]. To identify the name of species for each isolate, the resulting DNA nucleotide sequence for each isolate was compared to those nucleotide sequence data obtained from GenBank database using Basic Local Alignment Search Tool (BLAST**)**. Database search and comparisons were carried out by National Center for Biotechnology Information's (NCBI) Web BLAST Service (http://ncbi.nlm.nih.gov).

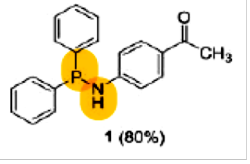
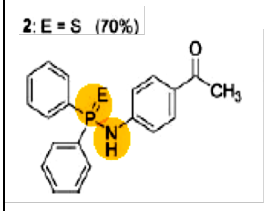
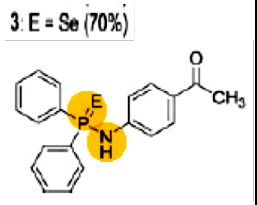
5.5. Antibiotic susceptibility testing (AST) of the selected bacterial isolates by VITEK 2 system:

One of the primary goals of this study was to analyze the Multidrug-Resistant (MDR) pattern exhibited by two bacterial isolates CS1 and CS2. To assess the susceptibility of these bacteria to antibiotics, we employed the VITEK® 2 microbial ID/AST testing systems developed by BioM'erieux, located in Marcy-l'Etoile, France. This system is widely recognized for its accuracy in identifying and determining the antibiotic susceptibility of microorganisms (http://localhost/jsp/ident/index.jsp). Antibiotic susceptibility testing was carried out in accordance with the CLSI 2015 standards, previously the National Committee for Clinical Laboratory Standards [13]. Briefly, fresh overnight culture on MHA plates from each isolates was prepared. All plates were inoculated with pure colony and incubated at 35°C for 24 hours. The inoculums of each isolate was placed into the VITEK®2 Cassette at the SMART CARRIER STATION™, where the VITEK® 2 Card and sample were linked virtually. The card used contains amikacin, ampicillin/sulbactam, aztreonam, ceflxime, ceftriaxone, cefepime, cefuroxime, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, levofloxacin, meropenem, piperacillin, piperacillin/tazobactam, tigecycline, and trimethoprim. Cards were automatically filled, sealed, and loaded into the VITEK 2 instrument for incubation and reading of each card. MICs of each antimicrobial drug measured by the VITEK 2 system were compared with each MIC determined by the CLSI procedure. A susceptibility category was given to each MIC value according to the current CLSI breakpoint criteria [13].

5.6. General procedure for the synthesis of phosphinous amide compounds and their structures:

The current study was part of a wider effort to test the antibacterial activities of freshly synthesized phosphinamide compounds against newly identified multidrug-resistant bacterial species. Our lab earlier synthesized N-(4-acetylphenyl)-P,P-diphenyl-phosphinous amide, as well as its related thioxo-phosphinous amide and selenoxo-phosphinous amide [8]. The three new aromatic phosphinous amides were designated as Ph1, Ph2, and Ph3. The chemicals were powdered and maintained at room temperature in test tubes. The physical and chemical features of the three new phosphine compounds, such as molecular weight and solubility, were were also measured. The features of the investigated chemical compounds are shown in Table 6.

Table 6. The chemical names, the given symbol for each compound, initial formula and molecular weight for the three novel phosphinous amide derivatives.

Compound names	Symbol	Initial formula	Structural formula	MW
N–(4–acetylphenyl)–*P*,*P*-diphenyl-phosphinous amide	Ph1	(*p*-CH_3CO)$C6H_4N(H)PPh_2$		319 g/mol
Monosulfide (thioxophosphino)	Ph2	(*p*-CH_3CO)$C_6H_4N(H)P(S)Ph_2$		351 g/mol
Monoselenide (selenoxophosphino)	Ph3	(*p*-CH_3CO)$C_6H_4N(H)P(Se)Ph_2$		387 g/mol

5.7. Antibacterial assessment of N–(4–acetylphenyl)–P, P–diphenyl-phosphinous amide and its derivatives:

Antibacterial activity of Ph1, Ph2, and Ph3 compounds against three bacterial species: Staphylococcus epidermis ATCC 12228, Escherichia coli ATCC 25922, and Bacillus cereus ATCC 11778, as well as newly discovered bacterial species from clinical cases, was examined. Furthermore, colistin (colistimethate sodium) is a popular commercial antibiotic used to treat a range of ailments, including infectious diarrhea and UTI. This antibiotic was used as both a reference medicine and a positive control for the study of the antibacterial activity of three freshly synthesized phosphinous amides. It was purchased from a local supplier (BioRad in Hercules, California, USA).

The antibacterial activities of the three compounds and the colistin antibiotic were evaluated on MHB culture medium using a CLSI-approved microdilution method [13]. A liquid culture of each unique bacterial species was generated by growing the clinically isolated bacterial strain in MHB medium and aerobically incubating it overnight at 35°C with shaking at 175 rpm. From the stock solution (20 mg/L), 100 µL aliquots of each of the three produced compounds (concentration 2 mg/L) were prepared. Previously, these chemicals were dissolved in sterile dimethyl sulphoxide. One hundred µL aliquots of the examined compounds and colistin antibiotic were administered in triplicate to wells in the first row of the 96-well microtiter plate, then serially diluted (1/2) down the vertical axis of the microplate from column 1 to column 8. In addition, as a control well, 100 µL of each growth control (culture broth containing DMSO but no antibacterial agents or antibiotics) were added to column 10. As a negative control wells, 100 µL of sterility control (MHB medium only) were added to column 11. Following that, each test and growth control well received 10 µL of a bacterial solution (OD value between 0.5 and 1.0). The plates were then incubated for 24 hours in aerobic conditions at 36 °C. Finally, the plates were incubated at 36 °C for four hours after each well

received 20 L of a resazurin solution (0.1 mg/mL). The MIC values were calculated as the lowest concentration of each synthetic drug that completely prevented the color change, signifying full suppression or no visible growth of the tested bacterial isolates. The results were given in micrograms per milliliter.

Acknowledgements: The authors are indebted to all members of the clinical microbiology laboratory, King Abdullah University Hospital, Irbid, Jordan for their kind assistance in collecting the specimens of the study. Authors also would like to express their gratitude to staff members of the Biology Department for their invaluable support and assistant in completing this study.
Author contributions: Concept – F.I.I., S. R. E.; Design – F.I.I., S. R. E., A. H., H. T. A.; Supervision – F.I.I.; Resource – S.R.E.; Materials – F.I.I, S. R. E., A. H., H. T. A.; Data Collection &/or Processing – F.I.I., S. R. E; Analysis &/or Interpretation - F.I.I., S. R. E., A. H., H. T. A.; Literature Search – F.I.I., S. R. E., A. H., H. T. A.; Writing –– F.I.I., S. R. E.; Critical Reviews – F.I.I., S. R. E., A. H., H. T. A.
Conflict of interest statement: The authors declared no conflict of interest.

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