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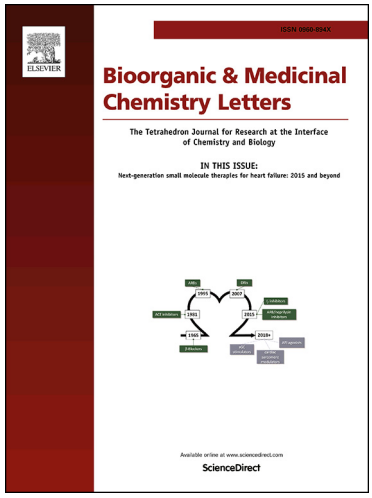

Synthesis and biological activity investigation of azole and quinone hybridized phosphonates

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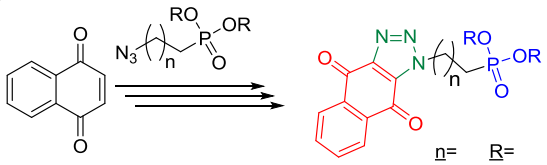

n = 1, 2, 3, 5, 7, and 9
R = Et or K

n=	R=	MIC against *F. graminearum* (μg/mL)
7	Et	1-2
9	Et	0.5

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Synthesis and biological activity investigation of azole and quinone hybridized phosphonates

Yagya Prasad Subedi[a], Madher N. Alfindee[a], Jaya P. Shrestha[a], Greg Becker[a], Michelle Grilley[b], Jon Y. Takemoto[b], Cheng-Wei Tom Chang[a]*

[a]*Department of Chemistry and Biochemistry, Utah State University, 0300 Old Main Hill, Logan, UT 84322-0300, USA*
[b]*Department of Biology, Utah State University, 5305 Old Main Hill, Logan, UT 84322-5305, USA*

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ABSTRACT

Phosphonates, azoles and quinones are pharmacophores found in bioactive compounds. A series of phosphonates conjugated to azoles and quinones with variable carbon chain lengths were synthesized in 3-4 steps with good yield. Antifungal assay of these compounds showed that ethyl protected phosphates have excellent inhibitory activity against phytopathogenic fungus *Fusarium graminearum*, and the free-base phosphates have good activity against human pathogenic fungi *Aspergillus flavus* and *Candida albicans*. Structure- activity relationship (SAR) studies showed activity increases with longer carbon chain length between phosphonate and anthraquinone analogs consisting of azole and quinone moieties. These newly synthesized compounds also have mild antibacterial activities to Gram positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA). Cytotoxicity analysis of these compounds against HeLa cells reveals that the phosphoric acid analogs are less toxic compared to ethyl protected phosphonates. Three leads compounds have been identified with prominent antifungal activity and low cytotoxicity.

Compounds containing quinone moieties are in great interest because of their wide biological activity[1]. Mitomycin C[2], streptonigrin[3], and anthracyclines[4] are the quinone based anticancer drugs used in the clinical practice (Figure 1). Along with anticancer properties, compounds containing the quinone motif are also antifungal and anti-bacterial.[5-8] Azole compounds have also attracted great attention due to their broad-spectrum biological activities, especially as antifungal agents like fluconazole, metconazole and itraconazole (Figure 2).[9, 10]

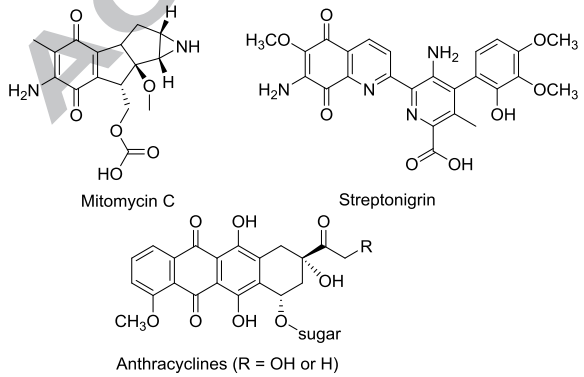


Figure 1. Structure of quinone-based anticancer drugs.

Inspired by the success of quinone and azole compounds, we have pursued the synthesis of cationic anthraquinone analogs (CAAs), a hybrid of naphthoquinone and 1,2,3-triazole (Figure 3).[11-15] These compounds can be prepared in 2-3 steps and have a wide range of biological activities from anticancer to antibacterial and antifungal activity. For example, when attached with a linear alkyl chain, the CAAs, **1** are mainly antibacterial. With the attachment of aryl groups, compound **2** becomes primarily anticancer and antibacterial. The dimeric CAAs, **3** behave mainly as antifungal and antibacterial agents. These compounds all have a redox active naphthoquinone core, which plays the pivotal role in the exerted biological activities.

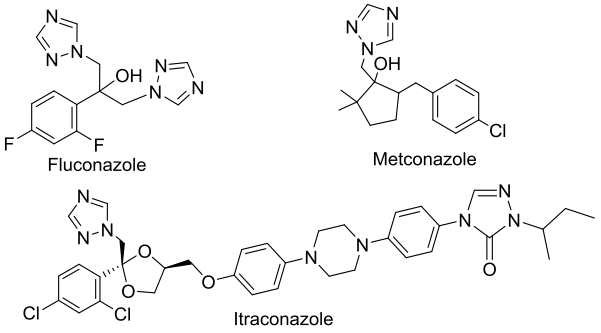


Figure 2. A caption is positioned left-justified below the figure or scheme.

Organophosphorus compounds are known to exert diverse biological effects[16] including antibacterial[17], herbicidal[18], and anti-inflammatory[19] activities. To increase the biological availability and stability, phosphonates are commonly employed

as analogs of organophosphorus compounds with a C-P bond. Organophosphorus compounds are also used as anticancer[20], and antiviral[21] prodrugs. Organophosphorus compounds with O-P bonds can be easily hydrolyzed in physiological conditions[22], whereas phosphonates with C-P bonds are stable under these conditions.[23] Similar to organophosphorus compounds, phosphonates are also used as antifungal, anticancer, and antiviral prodrugs, such as fosfluconazole, estramustine phosphate, fludarabine phosphonate, and fosamprenavir (Figure 4).[24] Conjugation of these phosphonate groups improves the solubilities of the parent drugs. Phosphonates conjugated to other biologically important moieties have shown antifungal and antibacterial activities (Figure 5).[25,26] Motivated by these applications of phosphonates, we began to systematically investigate the biological implications of conjugating phosphonates and anthraquinone analogs.

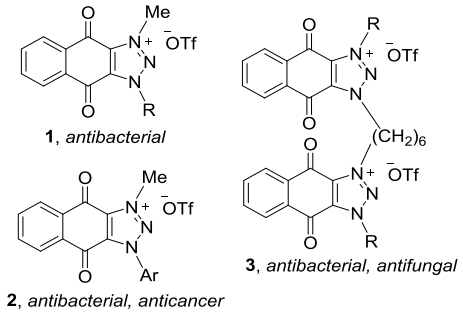


Figure 3. Structure and activity of CAAs.

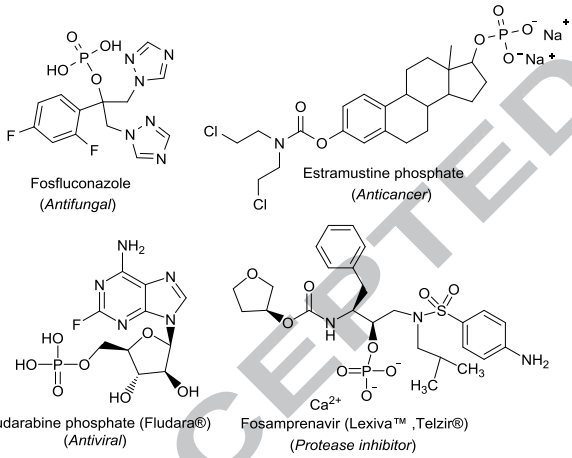


Figure 4. Structure of bioactive organophosphorus compounds.

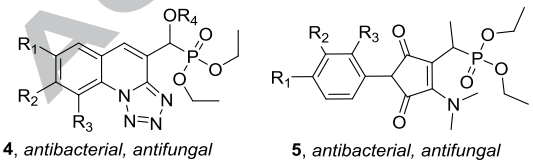
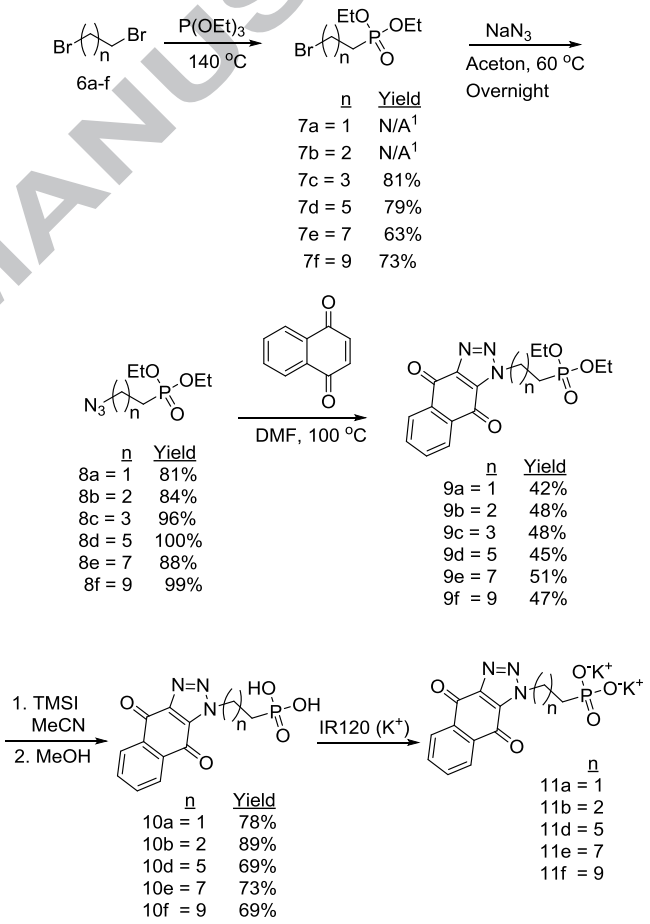


Figure 5. Phosphonate conjugated biologically compound with antibacterial and antifungal activities.

The synthetic design in this work was to combine the redox active naphthoquinone core, predecessor of the CAA core, and phosphonate with various linkers. The synthesis began with an Arbuzov reaction using dibromoalkanes and triethylphosphite (Scheme 1). Reacting the resulting bromoalkyl phosphonates with sodium azide provided compound **8a-8f** ready for a [2+3] cycloaddition with 1,4-naphthoquinone. During this cycloaddition, the cyclization was followed by an oxidation *in situ* in the presence of excess naphthoquinone, and yielded compounds **9a-f**.[12] Following the cycloaddition/oxidation process, the ethyl groups of the phosphonate were removed using trimethylsilyl iodide (TMSI). Deprotection of ethyl groups of **9c** led to the formation of the desired product mixed with inseparable impurities. Therefore, no corresponding adducts in the forms of phosphoric acid or potassium phosphonate were tested. The resulting phosphoric acids, **10a**, **10b**, and **10d-10f,** were purified and fully characterized. However, to improve solubility, these phosphoric acids were converted to potassium salts using IR120 ion exchange resin, and assayed as potassium salts, **11a**, **11b**, and **11d-11f**. Compounds **9a-9f**, ethyl ester of phosphonates, can be viewed as prodrugs of compounds **11a**, **11b**, and **11d-11f**. (potassium phosphonates), and both were evaluated for their biological activities. In short, there are two main structural differences among the synthesized compounds: 1) chain-length connecting the triazole and phosphonate; and 2) the forms of phosphonates (ethyl ester vs. potassium phosphonates).

Scheme 1.



N/A[1]: Not applicable, compound was purchased from commercial source.

The synthesized compounds were tested against both plant and human fungal pathogens, including *Aspergillus flavus*, *Fusarium graminearum* B4-5A, *Candida albicans* ATCC 64214 (azole resistance), *Candida albicans* MYA2876, *Cryptococcus neoformans* H99, and *Rhotorula pilimanae* (Table 1) as previously described.[27] Voriconazole and amphiphilic aminoglycoside **K20**[28] were used as positive controls. *F. graminearum* is a well-known plant pathogen causing head blight in wheat and other grains and destruction of food crops.[29] *C. albicans* and *A. flavus* are opportunistic pathogenic fungi.[30,31] *C. albicans* is the most common *Candida* species responsible for

candidiasis humans. *A. flavus* produces toxic mycotoxins in mammals[32] and is responsible for aspergillosis in animals and humans.[31] Minimum inhibitory concentrations (MICs) from antifungal assays showed that the ethyl protected analogs (**9a-9f)** have excellent inhibitory activity against *F. graminearum,* and activity increases with lengthening of the connector chain in between the phosphonate group and anthraquinone moiety. Compounds **9a-9d** were also active towards *C. neoformans* but the activity decreased with an increase in connector chain length. On the other hand, there was a significant drop in antifungal activity against *F. graminearum* for potassium phosphonate analogs, **11a-11f.** In addition, there was no significant effect when the linker chain length. Interestingly, among the potassium phosphonate analogs, **11e** is active against *A. flavus and C. albicans* with MICs ranging from 8-32 µg/mL. In short, there is no clear SAR against tested fungi except for *F. graminearum*. It is speculated that the variation in antifungal susceptibility is due to differences in the membrane lipid composition among various fungal species. Similar results have been observed for the recently described amphiphilic antifungals.[28]

Table 1. Minimum inhibitory concentration of phosphonate compounds against fungi[a].

Compound	*Aspergillus flavus*	*Fusarium graminearum B4-5A*	*Candida albicans 64124*	*Candida albicans MYA2876*	*Cryptococcus neoformans H99*	*Rhodotorula pilimanae*
9a	64 - 128	**16**	>128	>128	**16**	64 - 128
9b	64 - 128	**16**	>128	>128	**16**	64 - 128
9c	>128	**8**	>128	>128	32	128
9d	>128	**4**	>128	>128	32	64 - 128
9e	>128	**1-2**	>128	>128	>128	>128
9f	ND[b]	**0.5**	>128	>128	>128	>128
11a	>128	32	>128	>128	64	128
11b	16 - 32	32	32	32	32	32
11d	64	64	32 - 64	32 - 64	64	128
11e	**8 - 16**	32	**16 - 32**	**8 - 16**	32	>128
11f	ND	32	128	128	32	64
K20	> 256	**32**	256	64	**4**	**4**
Voriconazole	1	32	>256	0.125	0.125	8

[a] Unit: µg/mL, [b] ND: Not determined

For the evaluation of antibacterial activity, the synthesized compounds were examined against Gram negative (G-) bacteria, *E. coli* (25922), and Gram-positive (G+) bacteria, *S. aureus* (ATCC 25923), *S. aureus* (ATCC 33591, MRSA), *S. aureus* (ATCC 43300, MRSA) (Table 2). Among the tested compounds, the MICs against *E. coli* were more than 128 µg/mL for ethyl esters and 256 µg/mL for potassium phosphonate analogs. Most of the tested compounds have moderate activity towards G+ bacterial with MIC ranges from 16 – 128 µg/mL. Among these compounds, **9e** is the most active against *S. aureus* (ATCC 43300) (MRSA) with MIC value 16-32 µg/mL. However, these newly synthesized compounds show relatively lower antibacterial activity than their antifungal activity.

Table 2. MICs of phosphonate compounds against bacteria[a].

Compound	*E. coli* (ATCC 25922)	*S. aureus* (ATCC 25923)	*S. aureus* (ATCC 33591)	*S. aureus* (ATCC 43300)
9a	>128	64	64	64
9b	>128	64	64	64-128
9c	>128	128	128	64-128
9d	>128	32-64	64	32-64
9e	>128	64	32	16-32
9f	>128	>128	64	16- 64
11a	>256	32	128	128
11b	>256	32	128	64-128
11d	>256	32-64	64	64
11e	>256	64	256	>256
11f	>256	32-64	>256	>256

[a] Unit: µg/mL

To investigate the cytotoxicity, the synthesized compounds **9a-9f** and **11a**, **11b**, and **11d-11f** were tested against human HeLa cells and cervical cancer cells. Among the tested compounds, potassium phosphonate analogs **11a**, **11b**, and **11d-11f** did not show any toxicity up to 10 µg/mL. These compounds exert toxicities with cell viabilities around 50% at 100 µg/mL. Similarly, ethyl-protected analogs with the longest connector chain **9e** and **9f** did not show toxicity up to 10 µg/mL and some toxicity at 100 µg/mL. The calculated IC_{50} values showed that the toxicity of the ethyl protected compound **9a-9f** decreases with increase in the connector chain length increase (Table 3). In general, potassium phosphonates were less toxic relative to the ethyl-protected analogs particularly with compounds **11a** and **11b** as compared to their ethyl protected counterparts. Conceivably, the negative charge on the compound, which make the compound difficult to go inside the cells may be the reason for reduced toxicity towards mammalian cells.[21]

Table 3. IC_{50} values of the compounds against HeLa cells[a].

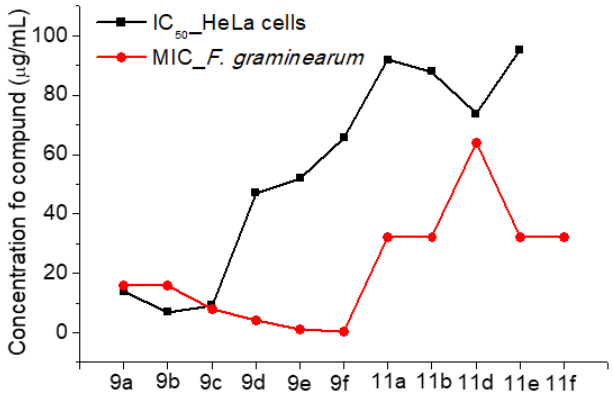
Compound	IC_{50} value	Compound	IC_{50} value
9a	13.83± 0.52	**11a**	91.80±5.08
9b	6.97±0.66	**11b**	87.98±8.37
9c	9.11±0.65	**11d**	73.64±0.94
9d	46.99±5.55	**11e**	95.31±4.63
9e	52.00±4.49	**11f**	>100
9f	65.66±1.30		

[a] Unit: µg/mL

Since the newly synthesized compounds were most active against *F. graminearum*, the trend of their antifungal activity and cytotoxicity was further analyzed. To provide a clear SAR, the cytotoxicity and MICs of the tested compounds against *F. graminearum* was plotted in the same chart (Figure 6). Based on the comparison of MICs and IC_{50}, it is obvious that the antifungal

activity of **9a-9f** increases with increases in the chain-length of linkers between anthraquinone moiety and phosphonate group while the cytotoxicity towards mammalian cells decreases. Specifically, there is no significant difference in the cytotoxicity of the compounds with linkers ranging from C2 to C4 (compound **9a-9c**) and significantly decreases for the compound with linkers ranging from C6 to C10 (compounds **9c-9f**). In contrast, there is no significant difference in the antifungal activity of the potassium phosphonate analogs (**11a, 11b**, and **11d-11f**) although all of these compounds display lower cytotoxicity. Combining these results, compound **9f** stands out as the best lead.

Figure 6. Comparison of the MIC towards *F. graminearum* vs IC_{50} against HeLa cells.



In summary, we have synthesized a library of novel bioactive compounds that contains significant pharmacophores, including naphthoquinone, triazole and phosphonate. Ethyl-protected compounds bearing a phosphonate diethyl ester have excellent antifungal activity towards the phytopathogenic filamentous fungus, *F. graminearum*. Potassium phosphonates (ethyl group-deprotected) are less effective towards filamentous fungi. However, compound **11e** with an 8C chain between the phosphonate group and azole group displayed antifungal activity towards *A. flavus* and *C. albicans*. All compounds were less active against bacteria. Considering the SAR of cytotoxicity and antifungal activity, compounds, **9f** is the most active compound with least cytotoxicity followed by **9d** and **9e**. These compounds can serve as the leads for further development.

Acknowledgments

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Highlights

- Novel bioactive compounds consist of quinone, azole and phosphonate groups.
- Leads show excellent activity against *Fusarium graminearum* and low cytotoxicity.
- Optimal structures revealed via SAR study.