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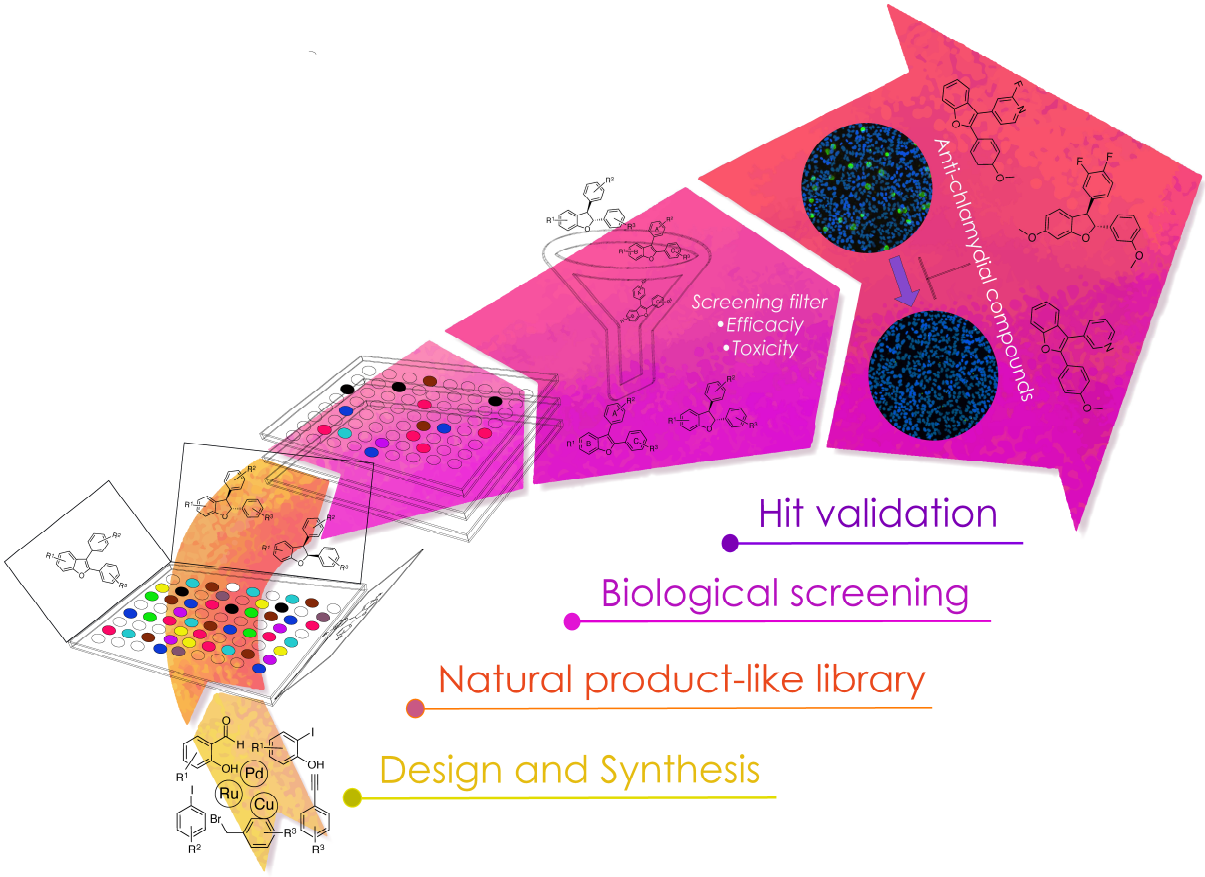

Natural product inspired library synthesis - Identification of 2,3-diaryl benzofuran and 2,3-dihydrobenzofuran based inhibitors of *Chlamydia trachomatis*

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Abstract

A natural product inspired library was synthesized based on 2,3-diarylbenzofuran and 2,3-diaryl-2,3-dihydrobenzofuran scaffolds. The library of forty-eight compounds was prepared by utilizing Pd-catalyzed one-pot multicomponent reactions and ruthenium-catalyzed intramolecular carbenoid C-H insertions. The compounds were evaluated for antibacterial activity in a panel of test systems including phenotypic, biochemical and image-based

screening assays. We identified several potent inhibitors that block intracellular replication of pathogenic *Chlamydia trachomatis* with $IC_{50} \leq 3$ µM. These new *C. trachomatis* inhibitors can serve as starting points for the development of specific treatments that reduces the global burden of *C. trachomatis* infections.

1. Introduction

Natural products have been a valuable source to identify candidates for drug discovery and development. [1-3] Our interest in biologically active natural products originates from the identification of the plant derived resveratrol tetramer, (-)-hopeaphenol (Figure 1) as an antibacterial agent that blocks the type III secretion system in *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis* without affecting bacterial growth. [4, 5] In addition, we found that (-)-hopeaphenol reduced cell entry of the obligate intracellular sexually transmitted pathogen *Chlamydia trachomatis*. [4] (-)-Hopeaphenol, the first characterized resveratrol oligomer, is based on a 2,3-*trans*-diaryl-2,3-dihydrobenzo[b]furan scaffold. Benzofuran and 2,3-disubstituted benzofuran moieties are widespread in nature and present in a large number of natural products that display numerous biological activities, including antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory and anticancer activities (Figure 1). [6-10] For example, 2-substiuted-3H-benzofurans and 3-benzoyl-2-aryl benzofurans were reported as antibacterial against *Mycobacterium tuberculosis* H37Rv and methicillin-resistant *S. aureus* (MRSA) respectively. [8]

In the present study, we designed, synthesized, and characterized a natural-product-like screening library based on 2,3-diarylbenzo[b]furan and 2,3-diaryl-2,3-dihydrobenzo[b]furan scaffolds to explore these scaffolds as a source for novel antibacterial compounds. This compound collection was interrogated in a number of phenotypic and biochemical screening assays leading to identification of potent inhibitors that block intracellular replication of the

Gram-negative pathogen *C. trachomatis.* To the best of our knowledge there no benzofurans besides (-)-hopeaphenol that have previously been reported to display antibacterial activity against *C. trachomatis*.

C. trachomatis is a clinically important pathogen that causes over 100 million cases of sexually transmitted disease annually, [11-13] which may give rise to infertility. [14] Furthermore, trachoma, an ocular infection caused by *C. trachomatis*, is responsible for preventable blindness. [15] More specific treatment of *Chlamydia* infections would be beneficial compared to the broad-spectrum antibiotics used today, which have a long-lasting effect on our commensal microbiota and contribute to the development of antibiotic resistance. These new anti-chlamydial compounds constitute starting points for the development of specific treatments that reduces the global burden of *C. trachomatis* infections.

2. Results and discussion

2.1 Synthesis

2.1.1 An overview of benzofurans synthesis

While 2,3-disubstituted benzofurans can be readily prepared, [16-22] only few reports describe efficient methods for the synthesis of 2,3-diaryl-2,3-dihydrobenzofurans and they mostly focus on the total synthesis of their parental natural products. [23-26] Wai-Hung *et al.* applied ruthenium porphyrins and *in situ* generated diazo compounds to synthesize *cis*-2,3-disubstituted-2,3-dihydro-benzofurans *via* carbenoid C-H insertion. [23] However, only one example of a non-substituted 2,3-diaryl-2,3-dihydrobenzofuran was disclosed. In a recent report Soldi *et al.* [24] elegantly carried out enantioselective synthesis of resveratrol-based

natural products containing the 2,3-dihydrobenzofuran scaffold by applying carbenoid C-H insertion with chiral dirhodium catalyst. [24, 27] This approach was also applied for the total synthesis of (-)-*E*-δ-viniferin. [26]

We have previously completed the total syntheses of (±)-ampelopsin B and (±)-ε-viniferin [28] (Figure 1), and more recently we reported the total syntheses of other polyphenolic nature products including viniferifuran, a resveratrol-picetannol hybrid and anigopreissin A. [29] To extend the scope of our studies on benzofuran-based natural products, we recently designed and synthesized a library based on 3-carboxy 2-aryl benzofuran and 3-carboxy 2-aryl *trans* 2,3-dihydrobenzofuran scaffolds. [30]

2.1.2 Synthesis of 2,3-diarylbenzo[b]furans series

In this study, we thought to synthesize a set of 2,3-diarylbenzo[*b*]furans as precursors to access *cis*- and *trans*-2,3-diaryl-2,3-dihydrobenzofuran *via* catalytic hydrogenation followed by epimerization based on our earlier results (Scheme 1). [31] The synthesis of 2,3-diarylbenzo[*b*]furan was achieved by utilizing efficient one-pot multicomponent reactions reported by Cacchi [32], Flynn [16, 33], and Larock. [18] It starts with Sonogashira coupling between an aryl alkyne and 2-iodophenol. Subsequently, one-pot heteroannulative coupling with aryl iodides affords the 2,3-diarylbenzo[*b*]furan as depicted in Scheme 2. The library was then designed by selecting nine commercially available aryl iodides (**A1**–**A9**), three 2-iodophenols (**B1**–**B3**), and two aryl-alkynes (**C1** and **C2**) to probe the chemistry outlined in scheme 1 (Figure 2). The two selected aryl-alkynes, 1-ethynyl-4-methoxybenzene (**C1**) and 1-ethynyl-4-benzyloxybenzene (**C2**), carry *para* protected hydroxyl group that is mechanistically required to assist the final epimerization under acidic condition as depicted in Scheme 1. [28, 31] Synthesizing all combinations would have yielded fifty-four molecules.

To evaluate the feasibility of the chemistry, a representative subset of twenty-eight molecules was selected.

The microwave-assisted one-pot three-component reaction (Scheme 2) reported by Markina *et al.* [18] allowed the synthesis of compounds **1–8** (Table 1) with the non-substituted 2-iodophenol **B1** in 35–91% yields. Unfortunately, this reaction resulted in low yield or no product when using 2-iodo-5-methoxyphenol **B2**, or 4-fluoro-2-iodophenol **B3**. This is due to that the initial Sonogashira coupling with both 4-methoxy phenyl acetylene **C2** and 4-benzyloxy phenyl acetylene **C3** resulted in premature cyclization to give 2-substituted benzo[*b*]furans (**P-II**, compound **9**) as product instead of the intermediate 2-(1-alkynyl) phenol (**P-I**) leading to the required target 2,3-diarylbenzofuran (**P-III**) (Scheme 3a). [16, 18] We, therefore, turned to a method reported by Chaplin and Flynn [16] (Scheme 2) in which two equivalents of Grignard reagent (CH_3MgCl) was used to mask the phenol and to eliminate the possibility of the premature cyclization. This method allowed the synthesis of the remaining benzofuran series, compounds **10–16** in 45–91% yields (Table 1). To expand our library further, variation of functional groups was achieved by demethyaltion of the phenolic groups with BBr_3 (Scheme 4) to yield the corresponding phenolic compounds **17–25** in 62–99% yields (Table 1).

2.1.3 Synthesis of cis- and trans-2,3-diaryl-2,3-dihydrobenzofurans series

We then used our knowledge from the total synthesis of (±)-ampelopsin B and (±)-ε-viniferin [28] to synthesize the corresponding *cis*-dihydrobenzofurans *via* catalytic hydrogenation. We were able to achieve 30–60% conversion of several benzofurans into the corresponding *cis*-dihydrobenzofurans using high-pressure hydrogenation (5 bar) and $Pd(OH)_2$ as a catalyst in iPrOH. However, it was not possible to obtain full conversion of the starting material while avoiding benzyl ether cleavage as a consecutive reaction (Scheme 3b). Various

heterogeneous and homogenous catalysts as well as reaction conditions and solvents were screened (data not shown). Nevertheless, we could not identify a reliable and efficient hydrogenation method that can be employed for a divergent synthesis to complete the library. These results are congruent with our findings during the attempts to synthesize 3-carboxy 2-aryl *cis*-2,3-dihydrobenzofurans. [30] Thus, due to these limitations the strategy to obtain dihydrobenzofurans outlined in Scheme 1 was abandoned.

We instead rerouted the synthetic strategy to synthesize dihydrobenzofurans *via* intramolecular carbenoid C-H insertion using salicylaldehydes, aryl iodides and benzyl halides as depicted in Scheme 5. We selected aryl iodides **A5**–**A11**, three salicylaldehydes **D1**–**D3** corresponding to iodophenols **B1**–**B3**, and two different benzyl bromides **E1** and **E2** as shown in Figure 2. From all possible forty-two combinations a representative subset of fourteen compounds in the form of *cis* and *trans* isomers was selected.

We exploited the ruthenium porphyrin catalyst [23] to synthesize *cis-trans* diastereomeric mixtures that can be readily separated (Scheme 5). The 2-hydroxydiarylketones (**P-IV**) are key intermediates in this pathway and they are typically prepared by addition of organolithium or Grignard reagent to aryl aldehyde followed by oxidation. [24, 26] However, while this procedure is straightforward, it often requires protection and deprotection of various base sensitive functionalities. In this work, we instead applied direct catalytic arylation of aldehydic C-H using palladium chloride to obtain **P-IV** as pluripotent intermediates. [34] The salicylaldehydes were coupled to the aryl iodides using palladium chloride to afford the corresponding 2-hydroxylarylketones in 40–93% yields (Scheme 5). Subsequently these were *O*-alkylated with two different benzyl bromides to afford intermediates **P-V** in essentially quantitative yields (Scheme 5). Aryl tosylhydrazones (**P-VI**)

were prepared from the corresponding ketone in 70–99% yields by condensation with *p*-toluenesulfonylhydrazide, which was then used as precursors for the carbenoid C-H insertion. Eventually, two-step one-pot ruthenium carbenoid intramolecular C-H insertion afforded a racemic *cis*-*trans* mixture of the dihydrobenzofurans (**P-VII**) *via in situ* generation of diazo compounds. In this study, we used commercially available ruthenium porphyrin (RuII(TPP)CO), which yielded a racemic *cis*-*trans* mixture in a ratio of 1:1–1:3 and 27–70% yields over two steps (Table 2). This diastereomeric mixture was subsequently resolved by HPLC and the stereochemistry was verified through the *J* coupling constants (see supporting information Figure S6). For compounds **28**, **37**, **44** and **48** only the *trans* isomer was isolated in > 95% purity, while the *cis* isomer was only > 85% pure in a mix with the *trans* isomer.

We have thus explored the chemistry of 2,3-diarylbenzofurans and demonstrated a facile construction of both 2,3-diarylbenzofurans as well as *cis*- and *trans*-2,3-diaryl-2,3-dihydrobenzofurans libraries. These methods can be further expanded to construct different libraries of natural products-like compounds based on benzofuran and 1*H*-indole scaffolds.

2.2 Biological evaluation

2.2.1 Biological screening

The 2,3-diarylbenzofurans and 2,3-diaryl-2,3-dihydrobenzofurans (Table 1 and 2) were then evaluated in a number of biological assays. Biochemical screening was performed to identify inhibitors of the ADP-ribose transferase activity of the *P. aeruginosa* toxin ExoS, [35] the *Y. pseudotuberculosis* phosphatase YopH, [36] and the acetaldehydedehydrogenase activity of AdhE from *Escherichia coli*. [37, 38] Phenotypic screening was executed with a *Y. pseudotuberculosis* luciferase reporter-gene assay for identification of type III secretion system inhibitors, [39] a lipase reporter assay for the twin arginine translocation (tat) system

in *P. aeruginosa*, [40, 41] and image-based screening for compounds that block intracellular replication of *C. trachomatis* in HeLa cells. [42] Active compounds were only identified in the *C. trachomatis* screen indicating that the compound library does not contain frequent hitters.

2.2.2 Identification of anti-chlamydial compounds

The *C. trachomatis* primary screen was performed in two independent experiments at 20 and 50 μM with Z' factor of 0.6 and 0.8 respectively (see supporting information Figure S1). A hit selection cut-off was set to two standard deviations based on inhibition of *C. trachomatis* replication but also considering toxicity of the compounds based on cell counts (see supporting information Figure S2 A). Seven compounds were found to be toxic to HeLa cells at both concentrations with < 70% cell viability at 50 μM and were therefore not considered for continued experimental investigation (see supporting information Figure S2 B).

2.2.3 Cytotoxicity profile of the identified anti-chlamydial

A toxic effect on the HeLa cells results in a reduction of *Chlamydia* inclusions and thus obscures deduction of structure-activity relationships. The resulting twenty-six primary hits were then subjected to extended cytotoxicity testing using the XTT reduction assay. [43] This led to the exclusion of additional six compounds due to their dose-dependent toxicity (see supporting information Table S1). The remaining non-toxic twenty compounds were subjected to full dose-response analysis at eleven concentrations and subsequent determination of IC_{50} values (i.e. 50% inhibition of control *Chlamydia* inclusion) (Table 3, supporting information Figure S5). From the 2,3-diarylbenzofuran series 11 compounds showed anti-chlamydial activity with a reproducible IC_{50} values between 2–27 μM (Table 3) and hill-slope [44, 45] values of 1–2. Representative immunofluorescence images for dose-response analysis of compounds **1**, **3** and **44** are shown in Figure 3.

2.2.4 Structure-activity relationships

In terms of structure-activity relationships, compounds **1**, **3**, **12**, and **16,** the top potent inhibitors with IC_{50} below 5 µM all contain a pyridyl moiety as a substituent on position 3 of the benzofuran. Moreover, compounds **3**, **12** and **16**, the most potent analogues with IC_{50} of 2.0, 2.7 and 4.2 µM respectively, all shared the 2-flouro-4-pyridyl and the 4-methoxyphenyl substituents on position 3 and 2 of the benzofuran respectively, while their potency appear to be less affected by substitution on the fused ring. This trend in activity is valid also for the less potent analogues **7**, **11**, and **15**, that carry a 4-methoxyphenyl substituent on position 3. The 2,3-diarylbenzofurans with free phenolic groups were generally cytotoxic in the primary screening (see supporting information Figure S2 B) and even the few compounds that passed the primary screening cut-offs (i.e. **17**, **18**, **19**, **23**, and **25**) were later eliminated in the extended toxicity evaluation using the XTT assay (see supporting information Table S1). This data indicate the need for protected phenols in order to reduce the toxicity. When comparing compounds **1** and **3** with their benzyl-protected analogues, **6** and **4** respectively, it is clear that the large benzyl group reduces potency possibly due to steric factors and/or increased lipophilicity.

In the 2,3-diaryl-2,3-dihydrobenzofurans series the eight non-toxic hit compounds displayed weak *C. trachomatis* inhibition with IC_{50} typically above 20 µM (Table 3). In contrast to the 2,3-diarylbenzofuran series, all compounds that belong to the 2,3-diaryl-2,3-dihydrobenzofuran series featured steep dose-response curves and hill-slope values typically exceeding 3.5 [44, 45] (see supporting information Figure S5). This group of inhibitors is thus less attractive for further investigations. One intriguing exception is the non-toxic compound **44**, which showed a reproducible activity with IC_{50} of 0.9 µM. It cannot be

excluded that this compound has a different mode of action than the potent 2,3-diarylbenzofurans i.e. **1**, **3**, **12** and **16**.

2.2.5 Comparable activity with clinically-used antibiotics

We then proceeded to benchmark the most potent compounds (**1**, **3**, **12**, **16** and **44**) by comparing them to ofloxacin and azithromycin, two antibiotics used in the clinic to treat C. trachomatis infections. [46] Ofloxacin is a fluoroquinolone that inhibits bacterial DNA gyrase, [47] while azithromycin is a macrolide antibiotic that inhibits bacterial proteins synthesis by binding the ribosome 50S subunit. [48] The compounds demonstrated comparable IC50 values to the antibiotics (Table 4) and the slope of the IC50 curves was less steep for the compounds compared to the antibiotics (Figure 4). The most potent compound, 44, was however as potent as antibiotics, and thus constitute a promising starting point for further development.

2.2.6 Efficacy against Chlamydia strains serovar A and serovar D

As a final step, compound **1**, **3**, **12**, **16** and **44** were evaluated against two other different *Chlamydia* strains, serovar D and serovar A. Serovar D is one of the most prevalent *C. trachomatis* genital tract isolate worldwide, [49, 50] while serovar L2, which was used for the primary evaluation, is less common and more virulent causing the locally disseminated lymphogranuloma venerum infections. Both serovars are associated with variety of genital infections, but also neonatal infections. [14, 50, 51] In contrast, serovar A associates with ocular trachoma, which is a leading cause of infectious blindness worldwide. [14, 15, 51] We found that the compounds are equally active against all strains with IC_{50} values in the range of 0.079–5 µM (Table 4 and Figure 4).

2.2.7 Distinct mode of action compared to (-)-hopeaphenol

Giving that this novel anti-chlamydial class are based on the scaffold of the natural product (-)-hopeaphenol, we compared their inhibition profile with that of (-)-hopeaphenol. (-)-Hopeaphenol displays anti-chlamydial activity but only if the bacteria are pretreated with 13–100 µM of the compound 1 h before the infection of eukaryotic cells. [4] The lack of effect when adding bacteria and compound simultaneously to the host cells is most likely due to poor membrane permeability of (-)-hopeaphenol. Furthermore, it was established that (-)-hopeaphenol irreversibly inhibits the T3S in *Y. pseudotuberculosis*. [4] In contrast, the 2,3-diarylbenzofuran class does not block the T3S in *Y. pseudotuberculosis* and efficiently inhibits the intracellular replication of *Chlamydia* without the need for pretreatment. Altogether, this data suggest that the mechanism behind the anti-chlamydial activity of the benzofurans is distinct from the mode of action of (-)-hopeaphenol.

2.2.8 In-silico assessment of pharmacological properties

At this point of early drug discovery process, it was then important to incorporate an assessment of the pharmacological properties for those anti-chlamydial candidates. Membrane permeability is one of the most important factors in drug absorption, but also it is a crucial factor when it comes to the development of antibacterial. [52] Bypassing bacterial membranes has always been a challenging aspect particularly in Gram-negative organisms where the drug needs to penetrate outer membrane, inner membrane, and peptidoglycan layer. [52] In case of *C. trachomatis* additional permeability barriers are formed since *Chlamydia* is an obligate intracellular pathogen and a drug act intracellularly has to cross the host cell plasma membrane and inclusion membrane. We therefore applied *in silico* calculation of physicochemical properties including cell permeability and lipophilicity for the top potent inhibitors **1**, **3**, **12**, **16**, and **44** using QikProp program (Schrödinger, LLC, New York, NY, 2017) (see supporting information Table S2). We used two different *in silico* models, Caco-2 [53] and MDCK [54], to predict permeability through the intestinal epithelial

barriers. It appears that all compounds showed enhanced calculated Caco-2 and MDCK membrane permeability as well as drug likeness properties (supporting information Table S2). Clearly, the most potent inhibitor **44** appeared to have the highest permeability rate with > 9900 nm/sec. Taken together this result support our hypothesis regarding the need for these antibacterial compounds to cross the host cell membrane and maybe the bacterial inclusion membrane if this class of inhibitors target proteins or other components residing inside the inclusion body of *C. trachomatis*.

3. Conclusions

In conclusion, we have synthesized natural product-inspired compound libraries based on 2,3-diarylbenzofuran and 2,3-diaryl-2,3-dihydrobenzofuran. A phenotypic screening of this library against *Chlamydia* infection allowed us to identify non-toxic and potent small-molecules that inhibit strains representing all clinical presentations of *C. trachomatis*, serovar L2, D and A infection with IC_{50} values in the range of 0.079–3 μM and thus similar to ofloxacin and azithromycin. These small molecules are structurally distinct, yet more potent or in the same range of potency as known anti-chlamydial compounds e.g. salicylacylhydrazides, [55-58] 8-hydroxyquinoline based inhibitors, [59] *N*-acylated derivatives of sulfamethoxazole and sulfafurazole, [42] salicylacylhydrazide sulfonamide hybrids, [60] the *Chlamydia* protease inhibitor-peptide Boc-Val-Pro-ValP(OPh2) (JO146), [61, 62] the isoflavone biochanin A, [63] dibenzocyclooctadiene lignans, [64] thiadiazinons [65] and 2-pyridones. [66] These novel anti-chlamydial compounds can thus serve as starting points for a continued medicinal chemistry programs, lead optimization, and the development of a novel anti-*chlamydial* drug. Importantly, *Chlamydia* inhibitors, such as compounds **1**, **3**, **12**, **16** and **44** can also serve as chemical probes since genetic systems for *Chlamydia* spp. are inefficient and laborious.

4. Experimental section

4.1 General chemistry

All reactions were carried out under inert atmosphere (N_2 gas). Chemicals and reagents were purchased from Aldrich, Alfa Aesar, AK Scientific, Matrix Scientific or Apollo Scientific. 5,10,15,20-tetraphenyl-21H,23H-porphine ruthenium(II) carbonyl (RuII(TPP)CO) was purchased from Aldrich. Organic solvents were dried using the dry solvent system (Glass Contour Solvent Systems, SG Water USA) except CH_3CN, CH_3OH, $PhCH_3$ and DMSO, which were dried over activated molecular sieves 3Å. Microwave reactions were performed in Biotage Initiator. Flash chromatography was performed on Biotage Isolera One using appropriate SNAP Cartridge KP-Sil or SNAP Ultra HP-Sphere 25 µm, and UV absorbance at 254 nm. TLC was performed on Silica gel 60 F_{254} (Merck) with detection by UV light unless staining solution is mentioned. Preparative HPLC separation were performed on Gilson System HPLC, using a VP 250/21 NUCLEODUR C_{18} column HTEC 5 μm, with a flow rate 18 mL/min, detection at 214 nm and the eluent system: A: aq. 0.005% HCOOH, and B: 0.005% HCOOH in CH_3CN unless otherwise mentioned. The NMR spectra were recorded at 298 K on Bruker-DRX 400 MHz and 600 MHz using the residual peak of the solvent DMSO-d_6 (δ_H 2.50 ppm) or $CDCl_3$ (δ_H 7.26 ppm) as internal standard for ^{1}H, and DMSO-d_6 (δc 39.50 ppm) and $CDCl_3$ (δ_c 77.16 ppm) as internal standard for ^{13}C. HRMS was performed using a Bruker microtof II mass spectrometer with electrospray ionization (ES^+); sodium formate was used for calibration. LC-MS were recorded by detecting positive/negative ion (ES^+/ES^-) with an electrospray Water Micromass ZG 2000 instrument using XTerra MS C_{18} (5 μm, 19x50 mm column) and H_2O/CH_3CN (0.2% HCOOH) as the eluent system, or with Agilent 1290 infinity–6150 Quadrupole using YMC Triart C_{18} (1.9 μm, 20x50 mm column) and H_2O/CH_3CN (0.1% HCOOH) as the eluent system. Melting points were measured on

Electrothermal 1A9000 series Digital Melting Point Apparatus. All target compounds were >95% pure according to HPLC UV-trace, ^{1}H and ^{13}C NMR.

4.2 Synthesis of 2,3-diarylbenzofuran series

4.2.1 General procedures for the synthesis of 2,3-diarylbenzofuran

Method A [18] (Exemplified by compound 1) (Scheme 2 A):

3-(2-(4-methoxyphenyl)benzofuran-3-yl)pyridine, **1**: 2-Iodophenol (1.14 mmol, 250 mg) and $Pd(Ph_3P)_2Cl$ (5 mol%, 40 mg) were placed in a 20 mL microwave vial, and purged with N_2. Dry THF (0.8 mL) was added and the mixture was stirred until the iodophenol was completely dissolved. Then dry triethylamine (2 mL) and a solution of CuI (3 mol%, 5.4 mg) in triethylamine (1 mL) were added and the mixture was stirred for 10 min. 4-Ethynylanisole (1.2 equiv., 180.3 mg) in THF (0.5 mL) was then added under N_2, and the mixture was stirred in the microwave reactor for 30 min at 40 °C. The corresponding 3-iodopyridine (1.2 equiv., 279.6 mg) in dry CH_3CN (3.7 mL) was added under N_2, and the reaction mixture was heated in the microwave reactor at 100 °C for 30 min. The reaction was monitored with TLC and LC-MS. After cooling, the solvents were evaporated and the product was purified on silica followed by preparative HPLC (25→100% B over 25 min).*Method B[16] (Examplified by compound 12) (Scheme 2 B):*

2-fluoro-4-(6-methoxy-2-(4-methoxyphenyl)benzofuran-3-yl)pyridine, **12**: To a solution of the 2-Iodo-5-methoxyphenol (1 mmol, 252 mg) and the 4-Ethynylanisole (1.2 equiv., 166 mg) in dry THF (1.6 mL, 0.6 M of the Iodophenol), at 0 °C under N_2, was added CH_3MgBr (THF solution, 2.3 M, 2 equiv., 0.792 mL) dropwise. The mixture was allowed to warm to room temperature, and $Pd(Ph_3P)_2Cl$ (3 mol%, 21.3 mg) was added before the mixture was heated to 65 °C for 2 h under N_2. The reaction mixture was then cooled to room temperature

and the THF was removed under reduced pressure. DMSO (2 mL) was added to the residue followed by addition of 2-Fluoro-4-iodopyridine (1.2 equiv., 269.7 mg) and the mixture was heated to 80 °C for 4 h under N_2. The mixture was cooled to room temperature and diluted with EtOAc, washed with H_2O and brine. The organic phase was dried over Na_2SO_4, concentrated and purified on silica followed by preparative HPLC (25→100% B over 25 min).

4.2.2 General procedure for the deprotection of the permethylated compounds

Method C (Scheme 4):

To a solution of permethylated compound (0.53 mmol, 170 mg) in dry DCM (5 mL, 0.1 M) at -78 °C was added dropwise BBr_3 (0.136 mL, 1.5 equiv./OCH_3). The mixture was stirred and allowed to reach room temperature overnight. The reaction was monitored with TLC (DCM/CH_3OH 10%)/Heptane 7:3 and LC-MS, and quenched by addition of ice-cold saturated aqueous solution of $NaHCO_3$ dropwise. The mixture was diluted with EtOAc and washed with H_2O and brine. The organic phase was dried over Na_2SO_4, concentrated under vacuum and purified on silica using (DCM/CH_3OH 10%)/Heptane 7:3 to afford the corresponding phenol in quantitative yield. The compounds were also purified on preparative HPLC (25→100% B over 25 min).

4.3 Synthesis of cis- and trans-2,3-diaryl-2,3-dihydrobenzofuran series (Exemplified by compound 44) (Scheme 5)

4.3.1 General procedure for the synthesis of 2'-hydroxybenzophenone

Method D:[34]

(3,4-difluorophenyl)(2-hydroxy-4-methoxyphenyl)methanone: A vial was charged with 2-Hydroxy-4-methoxybenzaldehyde (1.97 mmol, 300 mg), $PdCl_2$ (5 mol%, 17.5 mg), 1,2-

Difluoro-4-iodobenzene (2 equiv., 946.7 mg), Na_2CO_3 (2 equiv., 418.1 mg), LiCl (0.4 equiv., 16.7 mg), and DMF (19.7 mL, 0.1 M of the aldehyde), purged with N_2 and stirred at 110 °C 4–10 h. The reaction was monitored with LC-MS and TLC (TLC conditions: Aliquot was diluted with CH_3OH, eluted with EtOAc/heptane 1:3, and stained with 2,4-dinitrophenylhydrazine solution). The reaction mixture was filtered over a pad of Celite, diluted with EtOAc, washed 3 times with water, and the aqueous layers was acidified and extracted twice with EtOAc. The combined organic layers was dried over Na_2SO_4, concentrated and purified on silica using EtOAc/Heptane 1:20→1:9 step gradient) to afford 2'-hydroxybenzophenone in 69.3% yield. (NMR data is given in the supporting information)*4.3.2 General procedure for the synthesis of 2'-benzyloxybenzophenone*

Method E:

(3,4-difluorophenyl)(4-methoxy-2-((3-methoxybenzyl)oxy)phenyl) methanone: To a solution of (3,4-difluorophenyl)(2-hydroxy-4-methoxyphenyl)methanone (0.56 mmol) in acetone (0.25 M, 2.4 mL) anhydrous K_2CO_3 (2 equiv., 157 mg) and KI (1 equiv., 94.3 mg) were added followed by 3-Methoxybenzyl bromide (2 equiv., 0.159 mL), and the mixture was refluxed and monitored with TLC (EtOAc/Heptane 1:3) till completion. After cooling to room temperature, the mixture was filtered, concentrated and purified on silica using EtOAc/Heptane (1:20→1:3 step gradient) to afford 2'-benzyloxybenzophenone in 96.7% yield. (NMR data is given in the supporting information)*4.3.3 General procedure for the synthesis of tosylhydrazones*

Method F:

(*E/Z*)-*N'*-((3,4-difluorophenyl)(4-methoxy-2-((3-methoxybenzyl) oxy)phenyl)methylene)-4-methylbenzenesulfonohydrazide: To a rapidly stirred solution of p-toluenesulphonohydrazide (2.08 mmol, 387.6 mg) in dry CH_3OH (0.6 mL, 3 M) at 60 °C was added dropwise a solution

of 2'-benzyloxybenzophenone (0.52 mmol, 200 mg) in dry CH_3OH (1 mL, 0.5 M). The reaction was stirred at reflux and monitored with TLC (EtOAc/Heptane 1:2) till completion. The product was precipitated as crystalline, which was filtered off and washed with ice-cold CH_3OH. The remaining reaction mixture and the filtrate was concentrated and purified on silica using (EtOAc/Heptane 1:2) to afford tosylhydrazone in 78.6% yield. (NMR data is given in the supporting information)

4.3.4 General procedure for the one-pot, two-step synthesis of 2,3-dihydrobenzofuran

Method G: [23]

3-(3,4-difluorophenyl)-6-methoxy-2-(3-methoxyphenyl)-2,3-dihydrobenzofuran, **44**: To a solution of tosylhydrazone (0.4 mmol, 226 mg) in dry THF (2 mL, 0.2 M) LiHMDS (1.05 equiv., 1 M in THF, 0.450 mL) was added at -78 °C. The mixture was stirred and allowed to warm to room temperature and stirred for additional 30 min before the solvent was evaporated under reduced pressure. To the residue was added tetrabutylammonium bromide (10 mol%, 13.2 mg), 4 Å crushed molecular sieve (0.2 g/mmol, 80 mg), dry toluene (4 mL, 0.1 M of hydrazone) and RuII(TPP)CO (1 mol%, 6 mg), and the mixture was stirred at 110 °C for 24 h under N_2. The reaction mixture was then filtered over a pad of Celite, concentrated and purified on silica using EtOAc/Heptane (99:1→95:5, linear gradient). The diastereoisomers (i.e. *cis* and *trans*) were separated with HPLC (25→100% B over 25 min).

4.4 Analytical data for compound 1, 3 and 44

4.4.1 3-(2-(4-methoxyphenyl)benzofuran-3-yl)pyridine (1)

Synthesis: Methods A (300 mg, 88%, amorphous white solid, mp = 110–112 °C), Chromatography: (10% CH_3OH in DCM)/Heptane as the eluent (Step gradient 5→60%) followed by HPLC purification. ^{1}H NMR (600 MHz, DMSO-d_6, see SI for mentioned proton

number): δ_H 8.71-8.60 (m, 2H, H3a and H3c), 7.93 (dt, J = 8.0 Hz, 1.9 Hz, 1H, H3e), 7.69 (d, J = 8.3 Hz, 1H, H4), 7.56 (dd, J = 8.0 Hz, 4.8 Hz, 1H, H3d), 7.50 (d, J = 8.9 Hz, 2H, H2b and H2d), 7.47 (d, J = 7.7 Hz, 1H, H7), 7.39 (d, J = 7.7 Hz, 1H, H6), 7.30 (t, J = 7.7 Hz, 1H, H5), 7.00 (appear as d, J = 8.9 Hz, 2H, H2a and H2e), 3.78 (s, 3H, OCH_3) ppm. ^{13}C NMR (150 MHz, DMSO-d_6): δ_C 159.88, 153.24, 151.13, 149.84, 148.79, 137.01, 129.15, 128.37, 128.28, 124.94, 124.16, 123.53, 121.79, 119.23, 114.44, 112.30, 111.20, 55.25 ppm. HRMS *m/z* calcd for $C_{20}H_{15}NO_2$ 302.1176 [M+H$^+$]; observed 302.1215

4.4.2 2-fluoro-4-(2-(4-methoxyphenyl)benzofuran-3-yl)pyridine (3)

Synthesis: Methods A (58 mg, 67%, amorphous yellowish solid, mp = 113–114 °C), Chromatography: (1% CH_3OH in DCM)/Heptane as the eluent (Step gradient elution 1→20%) followed by HPLC purification. ^{1}H NMR (600 MHz, DMSO-d_6, see SI for the mentioned proton number): δ_H 8.35 (d, J = 5.2 Hz, 1H, H3b), 7.71 (d, J = 8.2 Hz, 1H, H3e), 7.59 (d, J = 7.6 Hz, 1H, H4), 7.55 (appear as d, J = 8.8 Hz, 2H, H2b and H2d), 7.48-7.39 (m, 2H, H3a and H7), 7.38-7.30 (m, 2H, H6 and H5), 7.04 (d, J = 8.7 Hz, 2H, H2a and H2e), 3.80 (s, 3H, OCH_3) ppm. ^{13}C NMR (150 M Hz, DMSO-d_6): δ_C 163.73 (d, $^1J_{CF}$ = 235.3 Hz), 160.27, 153.30, 152.11, 148.45 (d, $^3J_{CF}$ = 15.7 Hz), 146.38 (d, $^3J_{CF}$ = 8.8 Hz), 128.95, 128.06, 125.22, 123.77, 122.57 (d, $^4J_{CF}$ = 3.1 Hz), 121.20, 119.38, 119.38, 114.55, 112.38 (d, $^4J_{CF}$ = 3.1 Hz), 111.33, 109.48 (d, $^2J_{CF}$ = 38 Hz), 55.31 ppm. HRMS *m/z* calcd for $C_{21}H_{16}FNO_3$ 320.1074 [M+H$^+$]; observed 320.1081

4.4.3 3-(3,4-difluorophenyl)-6-methoxy-2-(3-methoxyphenyl)-2,3-dihydrobenzofuran (44)

Synthesis: Methods D–G (20% yield, sticky colorless oil). Diastereoisomers were separated by HPLC as described in general chemistry section. ^{1}H NMR (600 MHz, DMSO-d_6, see SI for mentioned proton number): δ_H 7.41 (dt, J = 10.8 Hz, 8.5 Hz, 1H, H3b), 7.33-7.25 (m, 2H, H3e and H2b), 7.08-7.02 (m, 1H, H3a), 6.94-6.90 (m, 1H, H2e), 6.90-6.86 (m, 2H, H2c and

H2a), 6.84 (d, *J* = 8.2 Hz, 1H, H4), 6.61 (d, *J* = 2.2 Hz, 1H, H7), 6.47 (dd, *J* = 8.2 Hz, 2.3 Hz, 1H, H5), 5.63 (d, *J* = 7.5 Hz, 1H, H2), 4.60 (d, *J* = 7.5 Hz, 1H, H3), 3.74 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3) ppm. ^{13}C NMR (150 MHz, DMSO-d_6): δ_C 160.61, 160.19, 159.33, 149.40 (dd, $^{1,2}J_{CF}$ = 246 Hz, 13 Hz), 148.52 (dd, $^{1,2}J_{CF}$ = 245 Hz, 13 Hz), 141.62, 139.74 (t, $^{3,3}J_{CF}$ = 4 Hz), 129.79, 125.13, 124.73 (m), 113.62, 111.47, 107.00, 95.99, 91.71, 55.36, 55.05, 54.58 ppm. HRMS *m/z* calcd for $C_{22}H_{18}F_2O_3$ 391.1116 [$M+Na^+$]; observed 391.1105

4.5 Biology

4.5.1 Cell culture and chlamydial infection

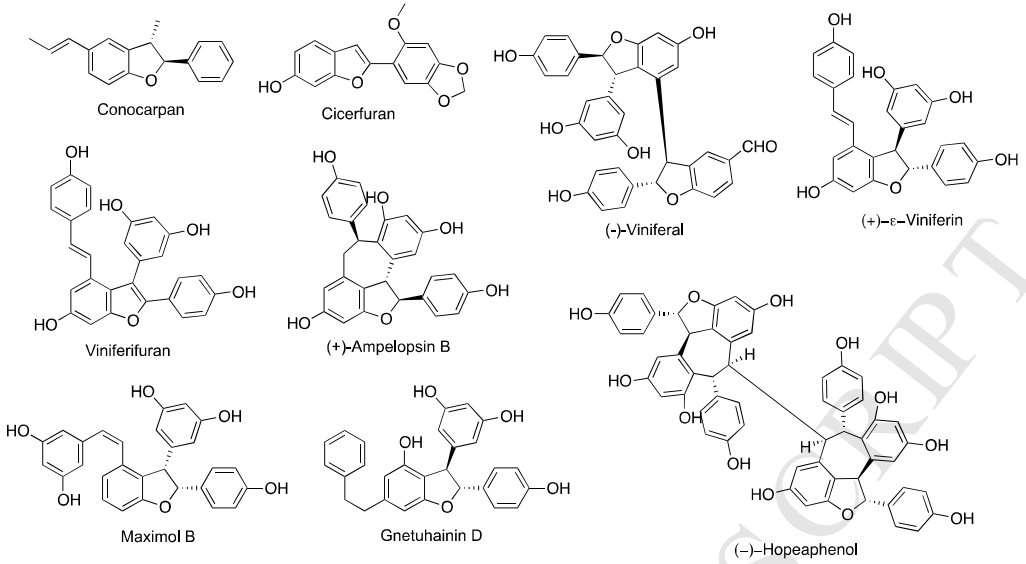
To determine compound IC_{50} values, HeLa299 cells (ATCC, Manassas, VA, USA) were inoculated into 96 well plates (Corning, New York, USA) with a density of 15,000 cells per well and cultured overnight at 37° C with 5% CO_2 in RPMI 640 (Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% Fetal Bovine Serum (FBS) and 2 mM L-glutamate. The cells were infected the following day with *C. trachomatis* serovar L2 454/Bu (ATCC, Manassas, VA, USA) at a multiplicity of infection (MOI) of approximately 0.3 in Hank's Balanced Salt Solution (HBSS) (Life Technologies, Carlsbad, CA, USA). At 1 h post infection, the inoculum was replaced with RPMI media supplemented as above and containing 0.5% DMSO and serial 1:1 dilutions of the tested compounds, starting at 50 µM and reaching 0.1953 µM. The infection was allowed to proceed for 44–48 h before fixation by aspiration of the media and adding methanol for 5 min. *Chlamydial* inclusions were stained with an in-house generated primary rabbit anti-*Chlamydia* antibody [42] and a secondary donkey anti-rabbit FITC-labeled antibody (Jackson ImmunoResearch, West Grove, PA, USA). The DNA of the cells and *Chlamydia* was stained by DAPI. The number of inclusions exceeding 130 square microns in size were counted by an ArrayScan VTI HCS automated reader (Thermo Scientific, Waltham, PA, USA). Inhibition of *Chlamydia* was

evaluated as the number of inclusions in compound treated infection compared to the numbers of inclusions in DMSO treated control infections (% of control). IC_{50} values are representative of at least three independent experiments. The data analysis was performed using nonlinear regression (curve fit) in GraphPad Prism v.5. For microscopy, HeLa299 cells were inoculated onto glass coverslips in 24 well plates. Cells were infected with *Chlamydia*, treated with compounds, fixed and stained as described, but using a rhodamine-labelled anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA). Infected cells were observed on a Nikon 90i Fluorescent Microscope using a 20x objective.

4.5.2 XTT reduction assay

HeLa299 cells were inoculated into 96 well plates with a density of 7,000 cells per well and cultured overnight at 37 °C with 5% CO_2 in RPMI 640 supplemented with 10% FBS and 2 mM L-glutamate. The following day, the RPMI was replaced with DMEM without Phenol Red (Life Technologies, Carlsbad, CA, USA) containing 0.5% DMSO with or without test compounds at 25 and 50 µM concentrations, with at least three replicate wells for each compound concentration, and incubated at 37 °C with 5% CO_2 for 48 hours. Wells containing no cells were used as blank controls. Following this incubation with compounds, the XTT Cell Proliferation Assay kit was used according to the manufacturer's instructions (ATCC, Manassas, VA, USA). Briefly, activation reagent and XTT reagent were thawed at 37 °C, then mixed in a 1:50 ratio with enough volume to add 50 µL to each well. The plates were placed in the 37 °C incubator for 2.5 hours. The absorbance of each well was then read at 475 nm and 660 nm with a Tecan Infinite m200 plate reader (Tecan, Männedorf, Switzerland). To analyze the data, the 660 nm reading was subtracted from the 475 nm reading to help eliminate non-specific readings from the assay results. Average blank control well readings were subtracted from those containing cells. Final readings from wells containing compound were compared to DMSO-only control wells and expressed as a percent (% of control).

A)



B)

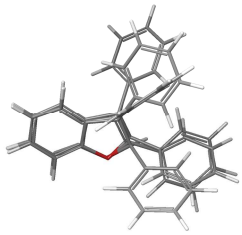


Figure 1 A) Structures of natural oligomeric resveratrol based on benzofuran and 2,3-diaryl-benzofuran scaffolds including (-)-hopeaphenol.[67, 68] **B)** An overlay of energy-minimized conformers of *cis*-2,3-diaryl-2,3-dihydrobenzofuran, *trans*-2,3-diaryl-2,3-dihydrobenzofuran and 2,3-diarylbenzofuran (created in Schrödinger Maestro version 10.5.014).

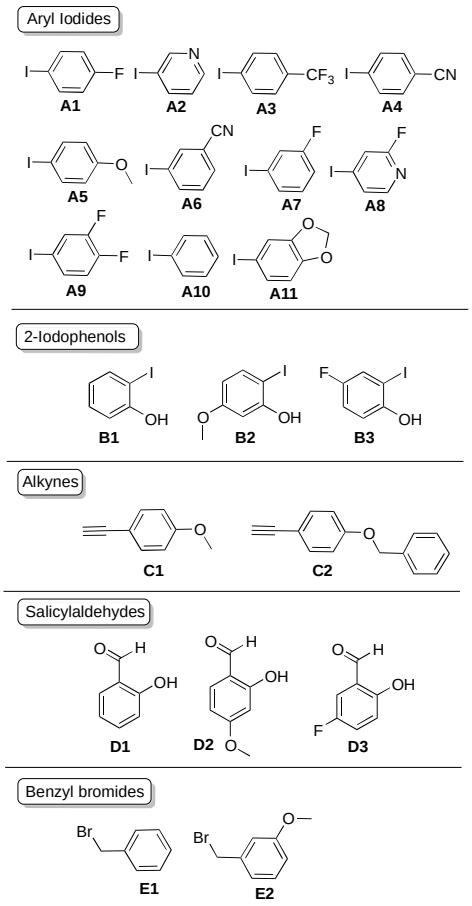


Figure 2. Building blocks selected for the design and the synthesis of the compound library.

A)

ID	3.12 µM	6.25 µM	12.5 µM	25 µM	50 µM
1					
3					

Infected control
0.5% DMSO

Uninfected control
0.5% DMSO

B)

ID	1.56 µM	3.13 µM	6.25 µM	12.5 µM

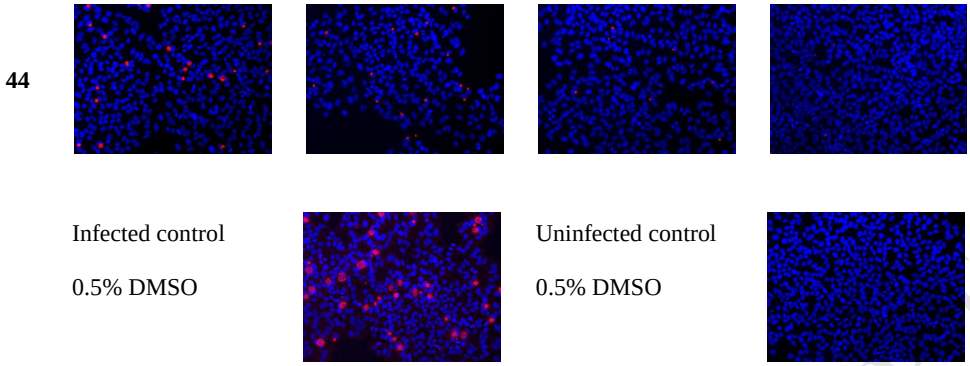


Figure 3. Immunofluorescence images for dose-response analysis of **A)** compounds **1** and **3** and **B)** compound **44** against *C. trachomatis* L2-454/Bu infected HeLa cells fixed with methanol at 48 h post infection and stained using antibodies specific for *Chlamydia* inclusions in red and DAPI for DNA (host cell nuclei) in blue. Epifluorescence images were obtained on a Nikon 90i Fluorescent Microscope (20× objective).

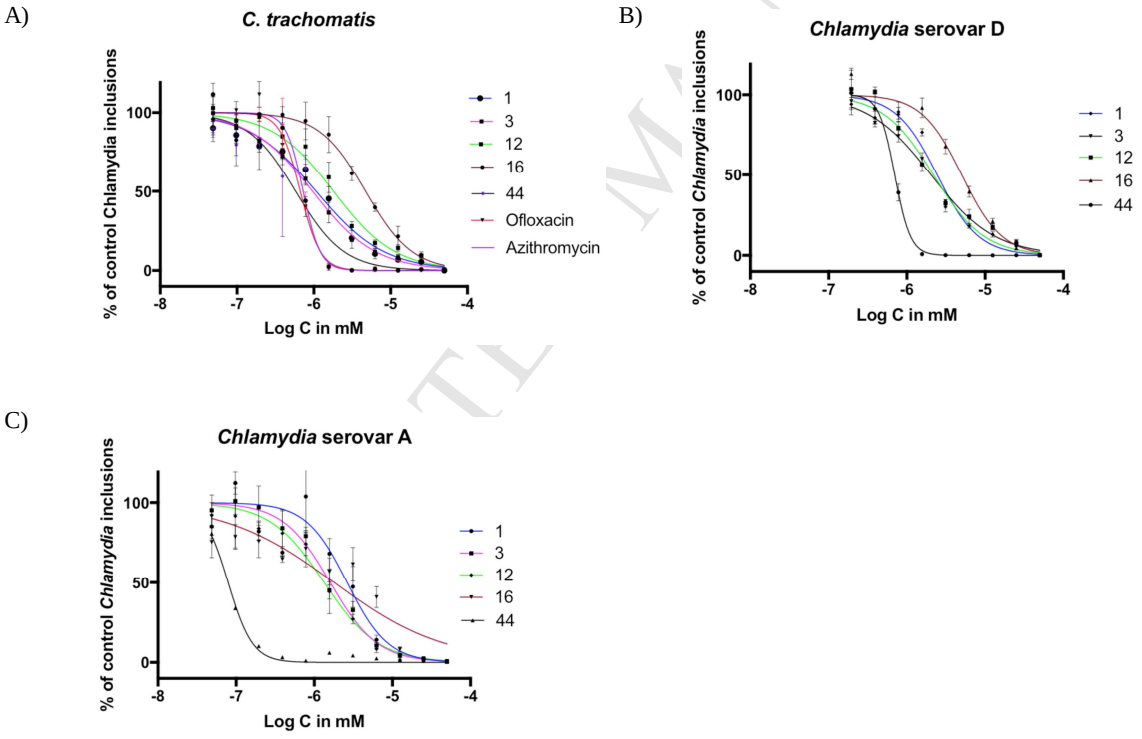
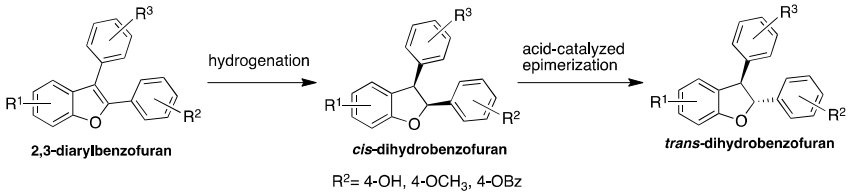
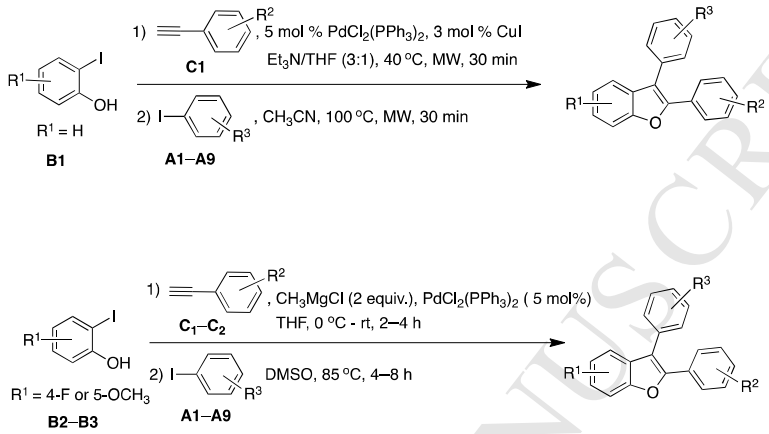


Figure 4. Dose-response curves of most potent compounds against **A)** *C. trachomatis* compared to antibiotics ofloxacin and azithromycin. **B)** *Chlamydia* serovar D. **C)** *Chlamydia* serovar A.

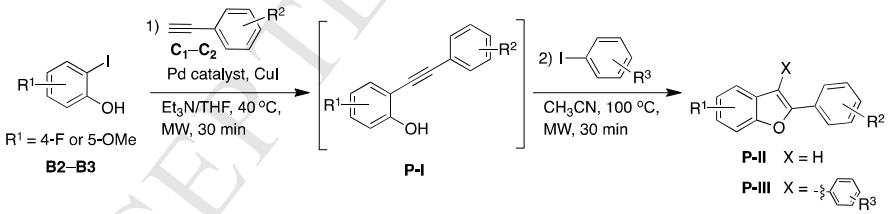


Scheme 1. Preliminary synthetic strategy for the generation of the 2,3-diarylbenzofurans library.

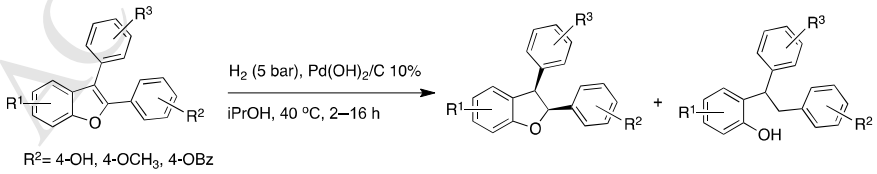


Scheme 2. Three-component one-pot reactions for the synthesis of 2,3-diarylbenzofurans as reported by Markina *et al.*[18] (top) and Chaplin and Flynn*[16]* (bottom).

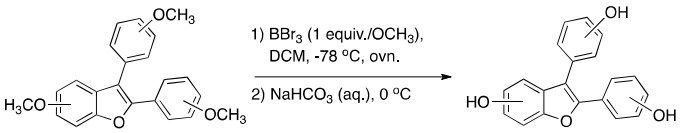
a)



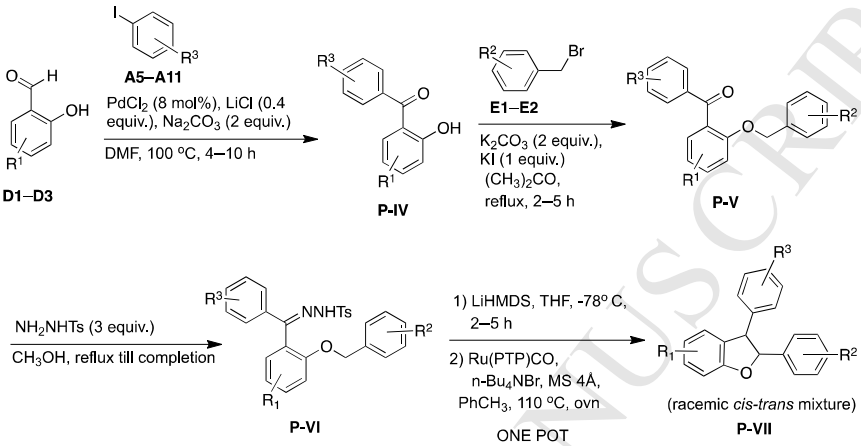
b)



Scheme 3. Synthetic challenges faced during the syntheses of 2,3-diarylbenzofuran and 2,3-dihydrobenzofuran. **a)** Synthesis of 2,3-diarylbenzofurans *via* 2-(1-alkynyl) phenol **P-I** showing the premature cyclized product **P-II**. **b)** Hydrogenation reaction showing the inevitable benzyl ether cleavage and ring opening.



Scheme 4. Demethylation of protected phenolic compound *via* BBr_3.



Scheme 5.Synthetic sequence for the synthesis of 2,3-diaryl-2,3-dihydrobenzofurans.

Table 1. Synthesized and characterized compounds in the 2,3-diarylbenzofurans series.

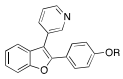
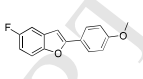
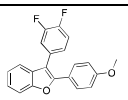
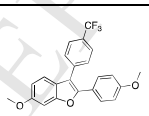
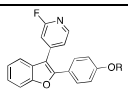
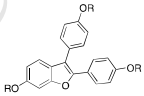
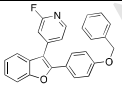
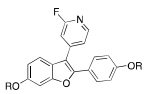
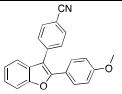
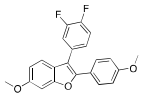
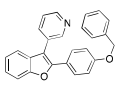
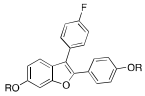
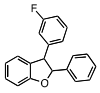
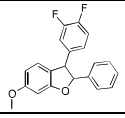
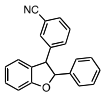
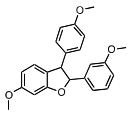
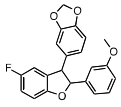
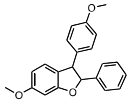
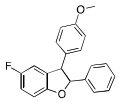
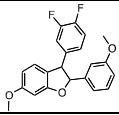
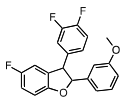
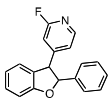
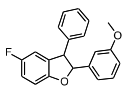
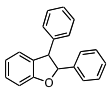
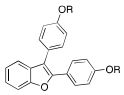
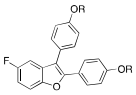
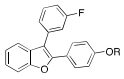
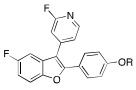
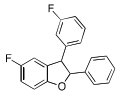
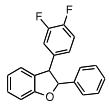
ID	Structure	Yield	ID	Structure	Yield
1 (R=CH_3) **17** (R=H)		88% 88%	**9**		32%
2		60%	**10**		73%
3 (R=CH_3) **18** (R=H)		67% 99%	**11** (R=CH_3) **21** (R=H)		62% 92%
4		61%	**12** (R=CH_3) **22** (R=H)		78% 85%
5		76%	**13**		45%
6		35%	**14** (R=CH_3) **23** (R=H)		67% 62%

Table 2. Synthesized and characterized compounds in the 2,3-diaryl-2,3-dihydrobenzofurans series.

ID	Structure[a]	Yield[b]	ID	Structure[a]	Yield[b]
26 (*cis*) **27** (*trans*)		22% 22%	**38** (*cis*) **39** (*trans*)		23% 46%
28 (*trans*)		40%	**40** (*cis*) **41** (*trans*)		17% 17%
29 (*cis*) **30** (*trans*)		14% 14%	**42** (*cis*) **43** (*trans*)		22% 22%
31 (*cis*) **32** (*trans*)		15% 35%	**44** (*trans*)		20%
33 (*cis*) **34** (*trans*)		13% 25%	**45**[c]		27%
35 (cis) **36** (*trans*)		16% 35%	**46** (*cis*) **47** (*trans*)		24% 46%

7 (R=CH_3)		58%	**15** (R=CH_3)		50%
19 (R=H)		96%	**24** (R=H)		99%
8 (R=CH_3)		91%	**16** (R=CH_3)		58%
20 (R=H)		89%	**25** (R=H)		92%

37 (*trans*)		28%	**48** (*trans*)		20%

[a] All *cis and trans* compounds are racemic. [b] Isolated yield over two-step, one-pot reaction and after column chromatography and/or HPLC separation of the diastereomeric mixture. [c] A single isomer was isolated as the other isomer was not stable, thus the relative stereochemistry could not be assigned unambiguously.

Table 3. Anti-chlamydial activity and toxicity profile of hit compounds.

ID	IC_{50} (μM)[a]	log IC_{50}[a]	HeLa cell viability % at 25 μM	HeLa cell viability % at 50 μM
1	2.4	-5.612	82 ± 5	103 ± 4
2	8.7	-5.059	90 ± 8	96 ± 8
3	2.0	-5.690	84 ± 3	97 ± 8
6	15.0	-4.824	76 ± 7	82 ± 9
7	11.6	-4.937	92 ± 8	94 ± 5
8	22.1	-4.655	79 ± 2	84 ± 3
11	12.8	-4.892	106 ± 15	100 ± 2
12	2.7	-5.564	68 ± 7	93 ± 10
13	27.0	-4.568	96 ± 11	101 ± 5
15	15.2	-4.819	82 ± 7	99 ± 2
16	4.2	-5.376	90 ± 19	103 ± 5
28	25.0	-4.602	90 ± 4	96 ± 5
30	25.8	-4.589	88 ± 2	103 ± 3
33	19.8	-4.702	80 ± 4	118 ± 4
37	24.9	-4.604	83 ± 12	84 ± 4
38	23.4	-4.631	84 ± 7	102 ± 1
41	10.6	-4.974	76 ± 6	90 ± 3
44	1.0	-6.001	93 ± 9	95 ± 1
46	25.4	-4.595	72 ± 10	88 ± 10
48	27.2	-4.566	75 ± 6	87 ± 8

[a] IC_{50} represents 50% inhibition of control *Chlamydia* inclusion.

Table 4. IC_{50} values for the most potent compounds, ofloxacin and azithromycin against *C. trachomatis*, *C.* serovar D and *C.* serovar A.

ID	IC_{50} (μM) *C. trachomatis* serovar L2 (IC_{50} 95% CI)[a]	IC_{50} (μM) *C. trachomatis* serovar D (IC_{50} 95% CI)[a]	IC_{50} (μM) *C. trachomatis* serovar A (IC_{50} 95% CI)
1	1.0 (0.925–1.25)	2.44 (2.14–2.80)	2.63 (1.96–3.46)
3	0.965 (0.876–1.06)	2.04 (1.85–2.25)	1.60 (1.39–1.84)
12	1.81 (1.45–2.25)	2.19 (1.87–2.58)	1.34 (1.1–1.63)
16	4.73 (3.92–5.71)	5.04 (4.46–5.70)	1.84 (1.15–2.75)
44	0.581 (0.415–0.795)	0.700 (0.673–0.727)	0.079 (0.074–0.084)
Ofloxacin	0.666 (0.577–0.764)	nd[b]	nd[b]
Azithromycin	0.727 (0.670–0.784)	nd[b]	nd[b]

[a] IC_{50} range calculated with 95% confidence interval (CI). [b] not determined

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Conflict of interest

All authors have given approval to the final version of the manuscript and declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

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Supporting Information

The supporting information is available on the publication website.
SI contents: Primary screening statistics and scattar plots, immunofluorescence images and hit selection critiera, cytotoxicity and dose-response curves for all hit compounds, ^{1}H NMR, ^{13}C NMR, HRMS or LC-MS and melting points for all final compounds and synthesized intermediates, and ^{1}H NMR and ^{13}C NMR spectra for the reported compounds and intermediates (file type: PDF).

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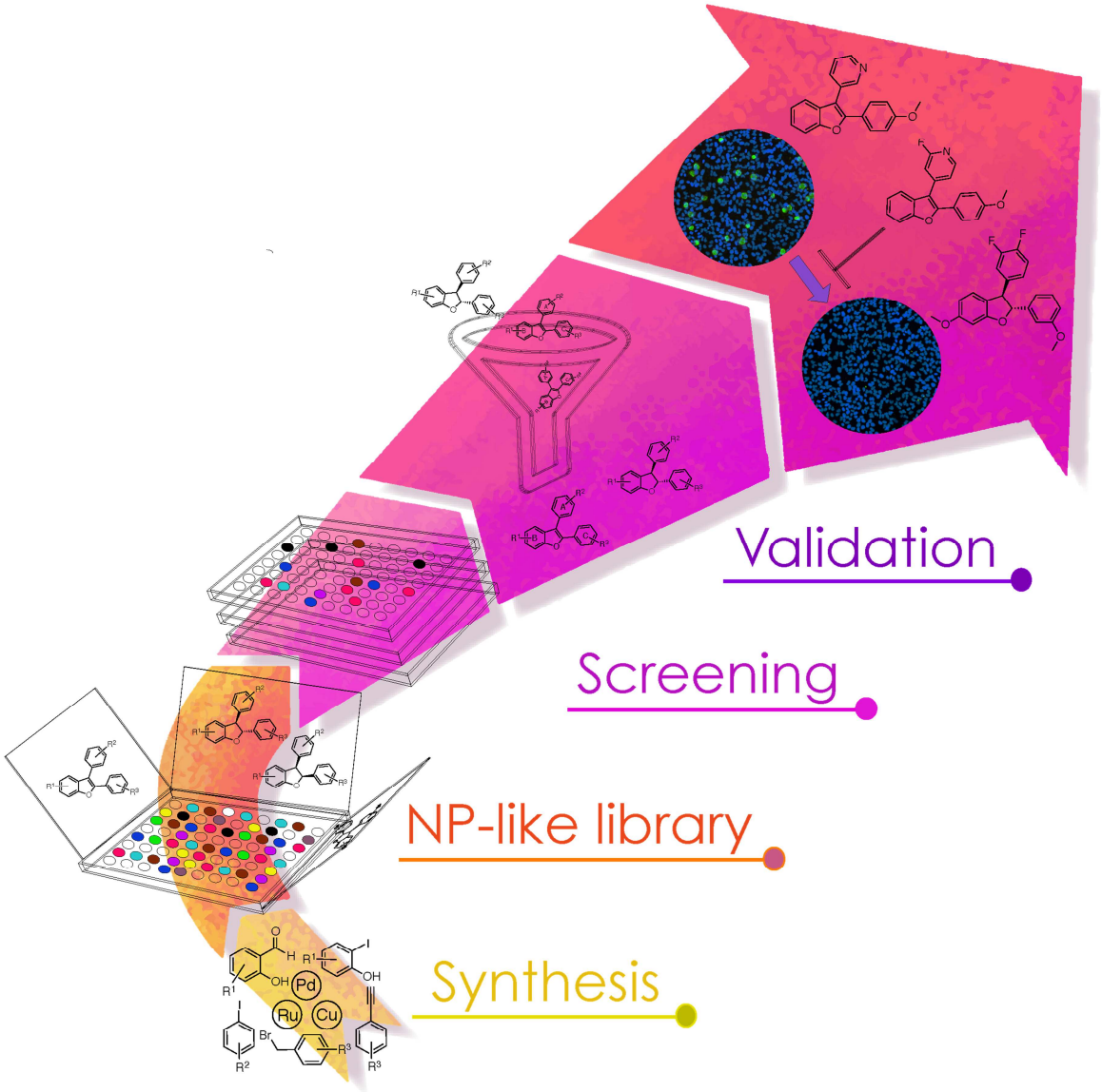
Graphical abstract



Highlights:

- Synthesis of natural product inspired library based on benzofuran scaffolds.
- Facile construction of *cis-/trans*-dihydrobenzofuran *via* Ru-catalyzed C-H insertion.
- Phenotypic screening for antimicrobial activity of natural product-like benzofurans.
- Identification of inhibitors of clinically relevant strains of *Chlamydia* with $IC_{50} \leq 3\ \mu M$.