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Design and Synthesis of 2-Arylbenzimidazoles and Evaluation of Their Inhibitory Effect against *Chlamydia pneumoniae*

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Chlamydia pneumoniae is an intracellular bacterium that responds poorly to antibiotic treatment. Insufficient antibiotic usage leads to chronic infection, which is linked to disease processes of asthma, atherosclerosis, and Alzheimer's disease. The *Chlamydia* research lacks genetic tools exploited by other antimicrobial research, and thus other approaches to drug discovery must be applied. A set of 2-arylbenzimidazoles was designed based on our earlier findings, and 33 derivatives were synthesized. Derivatives were assayed against *C. pneumoniae* strain CWL-029 in an acute infection model using TR-FIA method at a concentration of 10 μM, and the effects of the derivatives on the host cell viability were evaluated at the same concentration. Fourteen compounds showed at least 80% inhibition, with only minor changes in host cell viability. Nine most potential compounds were evaluated using immunofluorescence microscopy on two different strains of *C. pneumoniae* CWL-029 and CV-6. The *N*-[3-(1*H*-benzimidazol-2-yl)phenyl]-3-methylbenzamide (**42**) had minimal inhibitory concentration (MIC) of 10 μM against CWL-029 and 6.3 μM against the clinical strain CV-6. This study shows the high antichlamydial potential of 2-arylbenzimidazoles, which also seem to have good characteristics for lead compounds.

Introduction

Acute *Chlamydia pneumoniae* infection causes usually mild upper respiratory track symptoms and prolonged coughing, but it is also estimated to cause 10% of community-acquired pneumonia and 5% of bronchitis and sinusitis in the adult population. This intracellular bacterium contributes widely to human diseases, but no reliable treatments are available as the effect of primary treatment antibiotics is controversial.[1,2] Almost every one of us is estimated to get infected at least once in a lifetime, as the seroprevalence of the bacteria is 60−70%.[3] This percentage is huge considered in the light of recent evidence suggesting that *C. pneumoniae* is a fairly new zoonotically acquired human pathogen.[4] Acute infection can be treated with existing antibiotics when used in timely manner and in sufficient intracellular concentrations. However, infection often transforms to persistent form, which is difficult to eradicate for the human immune system or by administering more antibiotics.[5,6]

C. pneumoniae causes inflammation of tissues, and with chronic infection of *C. pneumoniae*, the inflammation process is constant as chronic infection produces equal amounts of immunopathogenic antigen chlamydial heat shock protein 60 (hsp60) compared to acute infection.[7] It has been shown that this bacterium can infect multiple tissue and cell types and can be cultivated from most of them.[1,8,9] Chronic *C. pneumoniae* infection has been linked to asthma,[10−12] lung cancer,[13] atherosclerosis,[14,15] and late onset Alzheimer's disease,[16−18] all of which have inflammatory aspects in disease processes.

The ever growing evidence of the connection between the *C. pneumoniae* infection and the development of atherosclerosis is reviewed in detail by Watson and Alp[19] and Stassen et al.[20] In animal models of atherosclerosis, *C. pneumoniae* promotes formation of atherosclerotic plaques[21] and increases maximal intimal thickness of thoracic aortas.[22] Similar thickening of arteria and smooth muscle cell proliferation was seen after *C. pneumoniae* infection in an ex vivo setting.[23] The main mechanism for *C. pneumoniae* to trigger the formation of atherosclerosis is to cause constant inflammation and promote foam cell formation from macrophages,[24] which is suggested to be triggered through toll-like receptor 2 (TLR-2).[25]

Antibiotic treatments have given mixed results in treatment of atherosclerosis in general and in *C. pneumoniae*-induced human atherosclerosis in particular. In most animal models of *C. pneumoniae*-induced atherosclerosis, repeated infection is needed to induce atherosclerosis and early antibiotic treatment prevents this effect.[19] In some animal models of *C. pneumoniae* infection, the inhibitory effect of antibiotics depended on the timing and the dose of the treatment.[26,27] This might also be the case in human studies, as prolonged antibiotic treatments have not significantly reduced cardiac events or relieved symptoms.[28,29] Current antibiotics may fail

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preventing cardiac events because they fail to erase the pathogen from human macrophages in vivo, although they show good effect on the same strains cultured in vitro from these patients.[1] The human studies are also prone to fail if *C. pneumoniae* has already established chronic infection, as the chronic infection is highly refractory to antibiotic treatments but still capable of sustaining infection and inflammation by expressing chlamydial heat shock protein.[30]

The chlamydiae research was boosted by the complete genome sequences in 1998 and 1999.[31,32] The results based on the genomic approaches have increased our knowledge on the genus *Chlamydia*, but many features of the bacterium remain an enigma. As we still lack tools for genetic manipulation of chlamydiae, some of the general approaches for antimicrobial research cannot be included to the research tools. In chlamydiae research, the problems need to be solved from different points-of-view: the type three secretion system (TTSS[a]) apparatus is known to be present in *C. pneumoniae* at all stages, and TTSS is a part of the infectious elementary body (EB) as well as the inclusion membrane that separates the chlamydiae from host cell.[33] Compounds that inhibit TTSS secretion of *Yersinia* were also effective against *C. pneumoniae* in vitro.[34] Also a new fluoroketolide antibiotic was evaluated against *Chlamydia*, but the effect did not differ from other known antibiotics.[35] Only few intracellularly active substances are available to treat chlamydial infection. Development of resistance is thus of special concern and may warrant new treatment strategies when spreading.[36] Despite the new knowledge gained in past 10 years, only a few potential candidates for further drug development against *C. pneumoniae* have been reported. In this light, the treatment of *C. pneumoniae* infection is of utmost importance and new specific antichlamydial compounds are urgently needed.

In our previous study, a set of benzimidazole derivatives was found to be effective against the acute *C. pneumoniae* infection in vitro.[37] Benzimidazole derivatives are known to possess a wide variety of biological activities, in particular, antibacterial and antiviral activities.[38] Recently benzimidazole ureas have been shown to be potent antibacterial agents targeting DNA gyrase and topoisomerase IV.[39] More specifically, 2-arylbenzimidazole derivatives have shown effects as heparanase inhibitors,[40] as inhibitors of hepatitis C virus,[41] as suppressors of key markers of allergy,[42] inhibitors of sonic hedgehog (shh) signaling pathway,[43] antibacterial,[44,45] and antiprotozoal compounds.[46] In addition, benzimidazole structure as a privileged scaffold is a reasonable starting point to drug discovery.[47] Thus benzimidazole derivatives possess good lead compound characteristics for antimicrobial agents.

In this study, we report the design, synthesis, and antichlamydial activity of a set of new 2-arylbenzimidazoles. This set was synthesized on the basis of the structure−activity relationship (SAR) data available from our previous study.[37] In this previous study, in silico screening in a *Bacillus subtilis* RNA methyltransferase homology model revealed possible activity of certain 2-arylbenzimidazoles, and the antichlamydial activities were confirmed with in vitro studies. In the present study, a new set of compounds was assayed against *C. pneumoniae* reference strain CWL-029 with two different methods using the acute infection model, and the results were verified using a clinical vascular isolate CV-6, which is known to cause chronic infection in coronary arteries.[48] The set of 33 benzimidazoles was also studied for any effects on host cell viability by measuring the amount of intracellular adenosine-5′-triphosphate (ATP).

[a] Abbreviations: ATCC, American Type Culture Collection; ATP, adenosine-5′-triphosphate; *t*-Boc, *N*-*tert*-butoxycarbonyl; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DMAP, 4-(dimethylamino)pyridine; DMSO, dimethyl sulfoxide; EB, elementary body; HL, human line; IF, immunofluorescence; IFU, inclusion forming unit; MCC, minimum chlamydiocidal concentration; MIC, minimum inhibitory concentration; MOI, multiplicity of infection; MW, microwave; PBS, phosphate buffered saline; SAR, structure−activity relationship; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TR-FIA, time-resolved fluorometric immunoassay; *p*-TSA, *para*-toluenesulfonic acid; TTSS, type three secretion system.

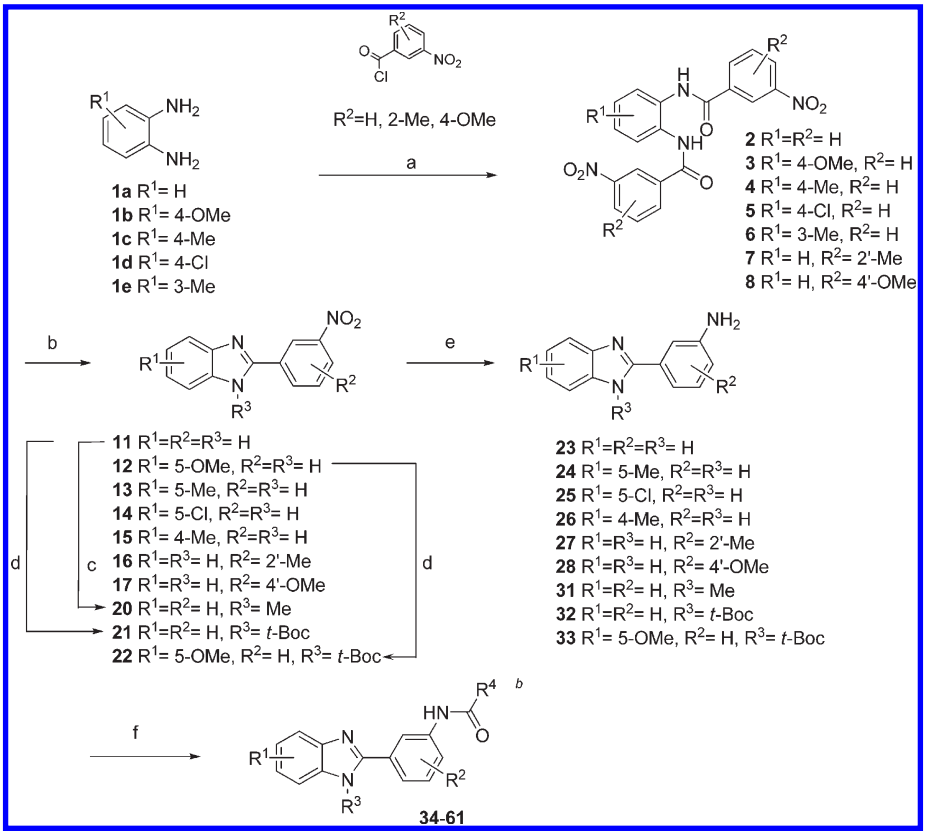
Results and Discussion

Chemistry. The preparation of the substituted 2-arylbenzimidazoles is outlined in Scheme 1. Oxidative methods[49−51] were first attempted for the one-step synthesis of 2-arylbenzimidazole, but they gave low yields probably due to the presence of deactivating *meta*-nitro group in the starting material. Therefore, the synthesis of the 2-arylbenzimidazole core was performed in two steps. First, 1,2-diaminobenzenes **1a**−**e** were diacylated with the corresponding acyl chlorides in the presence of 4-(dimethylamino)pyridine (DMAP) in pyridine to produce the bisamides **2**−**8**. This reaction was accomplished conveniently under microwave (MW) irradiation, which shortened reaction time to 15 min from the previously required 4−18 h at room temperature. The *para*-nitro-substituted **9** and 2-[(3-nitrobenzoyl)amino]phenyl 3-nitrobenzoate **10** were synthesized with the same procedure. In case the required 1,2-diaminobenzene was not commercially available, it was prepared by catalytic hydrogenation of the corresponding nitro compound in the presence of palladium catalyst. The subsequent condensation of the bisamides **2**−**8** was accomplished with *para*-toluenesulfonic acid (*p*-TSA) in refluxing *p*-xylene[52] to yield 2-arylbenzimidazoles **11**−**17**. The *para*-nitro-substituted compound **18** and the related 1,3-benzoxazole **19** were prepared with the same procedure from **9** and **10**. N_1-Methylated 2-arylbenzimidazole **20** was prepared from the unsubstituted benzimidazole **11** by alkylating with iodomethane in the presence of K_2CO_3 in *N*,*N*-dimethylformamide (DMF). Nitro group of nitroaryl benzimidazole was hydrogenated in the presence of palladium catalyst (**23**−**24**, **26**−**28**, **32**−**33**). Alternatively reduction was carried out with $SnCl_2 \cdot 2H_2O$ (**25**, **29**−**31**). Finally, the *N*-acylated products **34**−**48**, **51**−**52**, **55**−**59**, and **61**−**64** were prepared from **23**−**33** using the corresponding acyl chlorides in the presence of triethylamine (TEA) in tetrahydrofuran (THF). Due to the fact that final acylation step of the target compounds produced numerous byproducts and proceeded in low yield, the imidazole NH was protected as an *N*-*tert*-butoxycarbonyl (*t*-Boc) carbamate at room temperature ($(t\text{-Boc})_2O$, Cs_2CO_3, acetonitrile (MeCN)). Acylation and the subsequent deprotection with trifluoroacetic acid (TFA) in dichloromethane (DCM) yielded the benzimidazoles **49**−**50**, **53**−**54**, and **60**.

Synthesis of the target compounds **67**−**68** is outlined in Scheme 2. Their common starting material 2-(nitroaryl)-imidazole **65** was formed in the reaction between the corresponding nitrile and aminoacetaldehyde diethyl acetal,[53] followed by catalytic hydrogenation and acylation to give the final products.

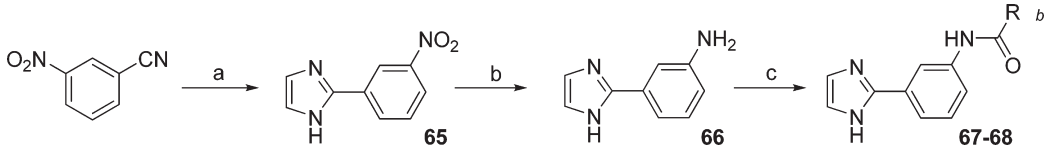
The A-ring substitution of the benzimidazole core gave rise to the 1,3-tautomers of the compounds, and they could be seen in some NMR spectra of the 2-arylbenzimidazoles in d_6-dimethyl sulfoxide (DMSO-d_6). Equivalence and fast exchange of the 4,7-protons and 5,6-protons accounted for the broad signals of the aromatic protons in the ^{1}H NMR

Scheme 1[a,b]



[a] Reagents and conditions: (a) DMAP, pyridine, 60 °C, MW, 15 min (yield 91−99%); (b) *p*-TSA, *p*-xylene, 140 °C, 18 h (yield 31−94%); (c) CH_3I, K_2CO_3, DMF, 19 h (yield 94%); (d) Cs_2CO_3, $(t\text{-Boc})_2O$, MeCN, 1 h (yield 82−94%); (e) $SnCl_2 \cdot 2H_2O$, EtOH, 0.5 h, reflux (yield 41−93%) or H_2, 10% Pd/C, EtOH/EtOAc, 5 h (yield 71−100%); (f) RCOCl, TEA (yield 14−71%) or RCOCl, TEA and TFA, DCM (yield 6−36%).
[b] R^4: see Tables 2−4.

Scheme 2[a,b]



[a] Reagents and conditions: (a) (i) 30% NaOMe, MeOH, 17 h, room temperature, (ii) $H_2NCH_2CH(OEt)_2$, 50 °C, 1 h, (iii) 6 M HCl, reflux 5 h; (b) H_2, 10% Pd/C, EtOH; (c) RCOCl, TEA, THF (yield 22−36%).
[b] R: see Table 4.

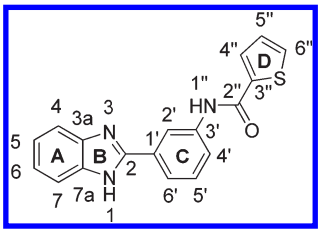
spectra and also for broad and even missing signals of the same aromatic carbon atoms in the ^{13}C NMR spectra.[54,55] The assignment of the ^1H and ^{13}C NMR signals of the representative 2-arylbenzimidazole (**34**) is based on the heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra and shown in Table 1.

TR-FIA Results and SAR. Thirty-three benzimidazole derivatives (Tables 2−4) were synthesized to investigate the SAR of the inhibitory effect of these compounds against *C. pneumoniae* strain CWL-029. All compounds were assayed for *C. pneumoniae* inhibition at a concentration of 10 μM using TR-FIA method[56] and for label binding properties and autofluorescence to exclude false positive signals. In this study, no false negative inhibition was found (data not shown). The biological activity results obtained from the new 2-arylbenzimidazoles were compared to the corresponding activity of **34**, known to inhibit the growth of *C. pneumoniae* in low micromolar concentration.[37] The minimal inhibitory concentration (MIC) for rifampicin was determined to be 0.012 μM (0.010 μg/mL) with the TR-FIA method, and this concentration was used as a control in the TR-FIA experiments.

The fused 2-arylbenzimidazole structure was found to be essential to activity because the corresponding 2-arylimidazole heterocycles **67** and **68** showed no activity. In addition, the *N*-methylated benzimidazole **61** was inactive and the related benzoxazole **63** showed only moderate activity. The A-ring-substituted compounds were all active. Methyl and halogen moieties in the benzimidazoles **55**−**57** were slightly better than methoxy **54**. Especially the methyl substitution at the 5-position of compound **55** turned out to be slightly better for antichlamydial activity than the corresponding 4-position in the compound **57.**

Modification of the C-ring substitution (compounds **58**−**59**) did not improve the antichlamydial activity, and

Table 1. Assignment of ^{1}H and ^{13}C NMR Signals of Compound **34**



	$\delta^{1}H_{ppm}$	$\delta^{13}C_{ppm}$		$\delta^{1}H_{ppm}$	$\delta^{13}C_{ppm}$
1	12.94		1′		130.6
2		151.1	2′	8.64	118.7
3			3′		139.3
3a		135.1	4′	7.90−7.87	121.5
4	7.67	118.9	5′	7.56−7.51	129.3
5	7.27−7.19	122.6	6′	7.90−7.87	121.5
6	7.27−7.19	121.7	1″	10.44	
7	7.56−7.51	111.4	2″		160.0
7a		143.8	3″		139.9
			4″	8.12	129.3
			5″	7.27−7.19	128.2
			6″	7.90−7.78	132.1

the further modifications were directed to the D-ring. An amide bond linking the C- and D-rings seemed to be beneficial in the *meta* position, and the D-ring was essential for the antichlamydial activity because the amide bond in the *para* position in compound **64** and a methyl group in place of the D-ring (compound **51**) led to completely inactive compounds. On the basis of our original finding[37] that thiophene ring was present in the most active compounds, other five-membered heterocyclic derivatives were synthesized. Among the furan and thiophene series of compounds, 2-positioned thiophene **34** was more potent than the corresponding 3-thiophene **35**, and 3-furan **38** was more potent than 2-furan **37**. The compound (**39**) with the *N*-methylated pyrrole as the D-ring moiety showed significant inhibition activity. This finding was in line with activities of the *meta* and *ortho*-substituted **42** and **48**, respectively.

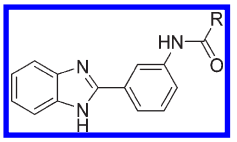
According to our previous screening study, *para*-substitution of the D-ring caused lack of activity.[37] In the present study, substituting the *meta*-position of the D-ring with various small substituents such as methyl (**42**), trifluoromethyl (**43**), chloro (**44**), and methoxy (**46**) proved to yield potent antichlamydial compounds, although activity differences to unsubstituted compound **41** were small. In the case of the *meta*-fluoro substituent in the D-ring (**45**), the observed antichlamydial effect was moderate. Compound **47** with the methoxycarbonyl functionality showed significant activity but was not selected to further testing because of its possible metabolic degradation via hydrolysis. The three best *meta* substituents, methyl, trifluoromethyl, and methoxy, were also tested in the *ortho* position in the 2-arylbenzimidazoles **48**−**50**. The substitution in compound **50** resulted in a significant decrease of antichlamydial activity. Taken together, *meta*-substitution in the D-ring seemed to result in good antichlamydial activity. The cycloalkane derivative, the cyclohexanoyl amide **53**, was highly active at a concentration of 10 μM, being among the most potent compounds determined by the screening method, but further testing showed superiority of the other derivatives (vide infra). In total, 14 of the synthesized compounds showed at least 80% inhibition activity at a concentration of 10 μM.

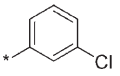
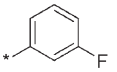
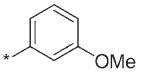
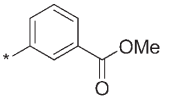
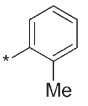
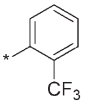
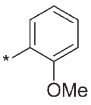
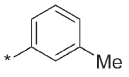
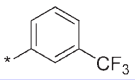
IF Measurement of Strain CWL-029: SAR. Nine of the most potential compounds were selected for further studies and divided into three subgroups (A−C) based on the similarities in the chemical structures and tested at smaller concentrations with traditional immunofluorescence (IF) labeling (Figure 1). The 2- and 3-thiophene derivatives **34** and **35** (group A) showed high activity against the bacterium at a 10 μM concentration and prevented *C. pneumoniae* growth almost completely with the highest concentration used. The same type of effect was seen in A-ring-substituted compounds **55**, **56**, and **60** in group C, where almost complete eradication was achieved at a 40 μM concentration. D-ring-modified benzimidazole derivatives, group B, were the only compounds eradicating *C. pneumoniae* strain CWL-029 from the cell cultures at used concentrations. From D-ring-modified derivatives, *meta*-substituted compounds **42** and **43** inhibited *C. pneumoniae* with lower concentration than *ortho*-substituted **48** or cyclohexyl derivative **53**. Derivatives **42** and **43** were the most effective antichlamydial compounds, having IC_{50} values of 2 and 3 μM, respectively, and both of them yielded over 80% inhibition at a 5 μM concentration. With all studied benzimidazoles, a clear trend emerged where increasing concentration of compounds produced significantly smaller but still detectable inclusions and only a few compounds reached complete inhibition of *C. pneumoniae* growth. Overall, IF inhibition of the benzimidazole derivatives were partly lower than TR-FIA inhibition and smaller inclusion size offers explanation for this phenomenon as the TR-FIA method measures the overall amount of *C. pneumoniae* formed whereas IF method measures the number of inclusions.

IF Measurement of Strain CV-6: First and Second Passage. The compounds with most potential and the reference antibiotic erythromycin were tested in an acute infection model using the cardiovascular strain of *C. pneumoniae* CV-6, which is a human coronary artery-derived clinical isolate known to cause chronic infections (Table 5).[48] The results verified the effect of the compounds against *C. pneumoniae* in general, but small differences in sensitivities were observed. We found the CV-6 strain to be more sensitive than the CWL-029 strain to most of the compounds. Differences between the antichlamydial efficiencies of the compounds became more obvious as the minimal chlamydiocidal concentration (MCC) ranged from 3.2 to >196 μM (Table 5). The most active compounds against CV-6 and CWL-029 were **42** and **43**. Overall, the results with the CV-6 strain were in line with the results from the CWL-029 experiments. Compounds **42**, **48**, **53**, **55**, and **60** were able to inhibit the growth of the CV-6 strain completely at the reported MIC within the first passage, and growth was also prevented at the same concentrations in the subcultures in antibiotic-free medium indicating a chlamydiocidal effect. However, compound **43**, which was highly effective at the first passage with a reported MIC value of 3.2 μM, displayed an MCC of 50 μM. Thus, the effect of **42** is chlamydiocidal and the effect of **43** is chlamydiostatic at low concentrations, although both are able to inhibit *C. pneumoniae* growth. Due to the chlamydiocidal effect of **42**, it is considered to be a better lead compound than **43** and will be used as the lead in future studies to enhance potency without increasing toxicity and to improve physicochemical properties.

Host Cell Viability. All of the plates during the study were controlled under microscope for abnormalities. In microscopic analysis, visible changes in cell monolayer and cell death was seen only at the concentration of 40 μM and only with compounds **43**, **56**, and **60**, otherwise the cell

Table 2. Chemical Structures of the Benzimidazoles **34**−**53**, Their Inhibition of *C. pneumoniae* Growth at a Concentration of 10 μM, and Their Effect on the Viability of the Host Cells at 10 μM



Compd	R	Inhibition % ± SEM	Viability %	Compd	R	Inhibition % ± SEM	Viability %
34		89 ± 1.8	98	**44**		94 ± 2.1	106
35		68 ± 4.2	98	**45**		58 ± 5.9	101
36		70 ± 8.3	107	**46**		98 ± 7.8	97
37		45 ± 2.1	98	**47**		99 ± 2.0	99
38		78 ± 9.3	101	**48**		99 ± 2.6	90
39		80 ± 2.6	104	**49**		96 ± 2.6	80
40		39 ± 4.7	98	**50**		0	107
41		99 ± 0.6	98	**51**	Me	0	100
42		98 ± 0.7	99	**52**	*t*-Bu	0	98
43		99 ± 0.5	69	**53**		100 ± 8.0	94

monolayers were comparable to the controls. The effect of all 2-arylbenzimidazoles on the host cell viability was studied after 72 h of incubation with a 10 μM concentration. The host cell viability percentage was assessed by the amount of intracellular ATP present in 2-arylbenzimidazole-treated cells compared to cells treated with DMSO-adjusted medium. The ATP concentration is considered to be a delicate indicator of cell viability.[57] Thirty-one compounds were determined to be nontoxic for HL cells, as the viability of the HL cells remained above the threshold of 80% set prior to the experiment (Tables 2−4). The selected nine compounds were evaluated for effects on host cell viability at four concentrations ranging from 0.625 to 40 μM (Figure 1). None of the nine compounds had an effect on host cell viability at a 2.5 μM concentration, and at a 40 μM concentration, four compounds did not cause over 20% decrease of the viability. Thus the compounds proved to be well tolerated by host cells, and from the results it was concluded that the effect on host cell viability cannot explain the antichlamydial effect of the compounds.

Other Antimicrobial Activities. None of the benzimidazoles showed any inhibition of the growth of the Gram-negative bacteria *Escherichia coli* at a concentration of 50 μM. In the case of Gram-positive *Staphylococcus aureus*, compound **43** showed 85% inhibition of resazurin metabolism, whereas the other compounds had no effect.

As the target of 2-arylbenzimidazoles in *Chlamydia* is still an enigma, there are multiple possible explanations why 2-arylbenzimidazoles were not effective against free living bacteria. At least four reasons are possible: (1) the 2-arylbenzimidazoles modify host cell processes, which are nonessential to host cells but essential to *C. pneumoniae* survival in host cells and thus have little effect on free living bacteria, (2) the compounds are modified by host cells to target bacterial functions, (3) the free living bacteria may have mechanisms to compensate the effects of 2-arylbenzimidazoles, (4) the

compounds affect directly the essential components or pathways of *C. pneumoniae* such as the TTSS.

The antimicrobial selectivity was assayed as it is beneficial to find a highly selective antichlamydial lead compound as it can help avoiding some of the main issues of antimicrobial drugs. First, if the compound can be administered via oral route, it should not affect the normal gut flora and thus the most common adverse effects of antibiotics can be avoided. Second, if the effect is selective to *C. pneumoniae* or to intracellular bacteria in general, it is more unlikely that resistance would occur. The likelihood of finding a fast and complete clinical antichlamydial therapy is small, and thus compounds that can be used for prolonged times without effect to the normal microflora are wanted. With possible prolonged treatments, the importance of other possible adverse effects must be carefully addressed in further drug development. However, the time period of any antimicrobial treatment is normally fairly short (up to six months) compared to treatments of many other diseases that can be addressed to patients for a lifetime.

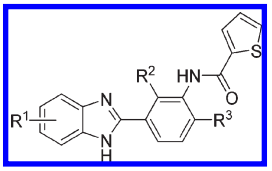
Conclusions

Antimicrobial drug development is at its turning point as high throughput screens have not produced reliable results, and this is why new approaches to antimicrobial drug discovery are needed.[58−60] The chlamydia research does not have tools to modify the bacterium genetically, and thus other methods of drug development than design of targeted inhibitors must be applied. On the basis of our previous in silico and in vitro studies, we synthesized a set of 2-arylbenzimidazoles and evaluated the SAR of the compounds against the *C. pneumoniae* strain CWL-029, the effects on host cell viability, and verified antichlamydial activity with a clinical *C. pneumoniae* isolate CV-6. We have also carried on further SAR studies based on the conformation of 2-arylbenzimidazoles.[61]

The benzimidazole derivatives seem to be highly selective in inhibiting intracellular *C. pneumoniae* compared to free living bacteria. The compound **42**, *N*-[3-(1*H*-benzimidazol-2-yl)phenyl]-3-methylbenzamide, completely abolished the *C. pneumoniae* infection of the strain CWL-029 at a 10 μM concentration measured by either TR-FIA or IF methods, and the clinical isolate strain CV-6 at a concentration of 6.3 μM was clearly more efficient in inhibiting *C. pneumoniae* than compound **34** published in our previous study on 2-arylbenzimidazoles.[37] The same 6.3 μM concentration of **42** also prevented the CV-6 strain from infecting new host cells at the second passage of *C. pneumoniae*, proving that the effect was chlamydiocidal. Overall, the *meta*-substituted **42** was the most prominent candidate for further lead optimization and drug development, as it was highly effective against *C. pneumoniae* and did not decrease host cell viability.

In this study, we showed how structure-based approach can be applied even without an unambiguous target. In the case of *Chlamydia*, it is often extremely difficult to determine what the target is and a highly efficient and predictably behaving compound is needed to proceed to more complex biological evaluations, which could reveal the target. Further proteomics studies have already been started to address the potential mechanism(s) of action.

Table 3. Chemical Structures of the Benzimidazoles **54**−**59**, Their Inhibition of *C. pneumoniae* Growth at a Concentration of 10 μM, and Their Effect on the Viability of the Host Cells at 10 μM

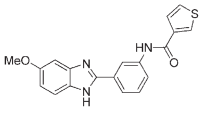
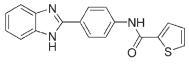
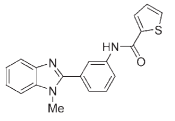
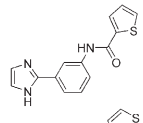
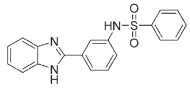
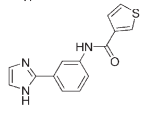
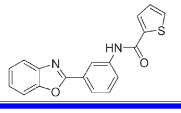


compd	R^1	R^2	R^3	inhibition % ± SEM	viability %
54	5-OMe			74 ± 6.3	98
55	5-Me			99 ± 0.9	95
56	5-Cl			89 ± 3.9	92
57	4-Me			89 ± 3.2	102
58		Me		59 ± 4.9	87
59			OMe	52 ± 7.9	101

Experimental Section

Materials and Methods. Cultivation of Cells and Infection Protocol. HL cell line (human line)[62] was cultivated and all cell-based experiments were done in a medium consisting of RPMI 1640 w/o L-glutamine (Biowhittaker, Lonza, Basel, Switzerland) supplemented with 7.5% fetal bovine serum (FBS)

Table 4. Chemical Structures of the Products **60**−**64** and **67**−**68**, Their Inhibition of *C. pneumoniae* Growth at a Concentration of 10 μM, and Their Effect on the Viability of the Host Cells at 10 μM

Compd	Structure	Inhibition % ± SEM	Viability %	Compd	Structure	Inhibition % ± SEM	Viability %
60		73 ± 12.6	95	**64**		0	96
61		0	102	**67**		0	115
62		0	77	**68**		0	133
63		61 ± 5.8	94				

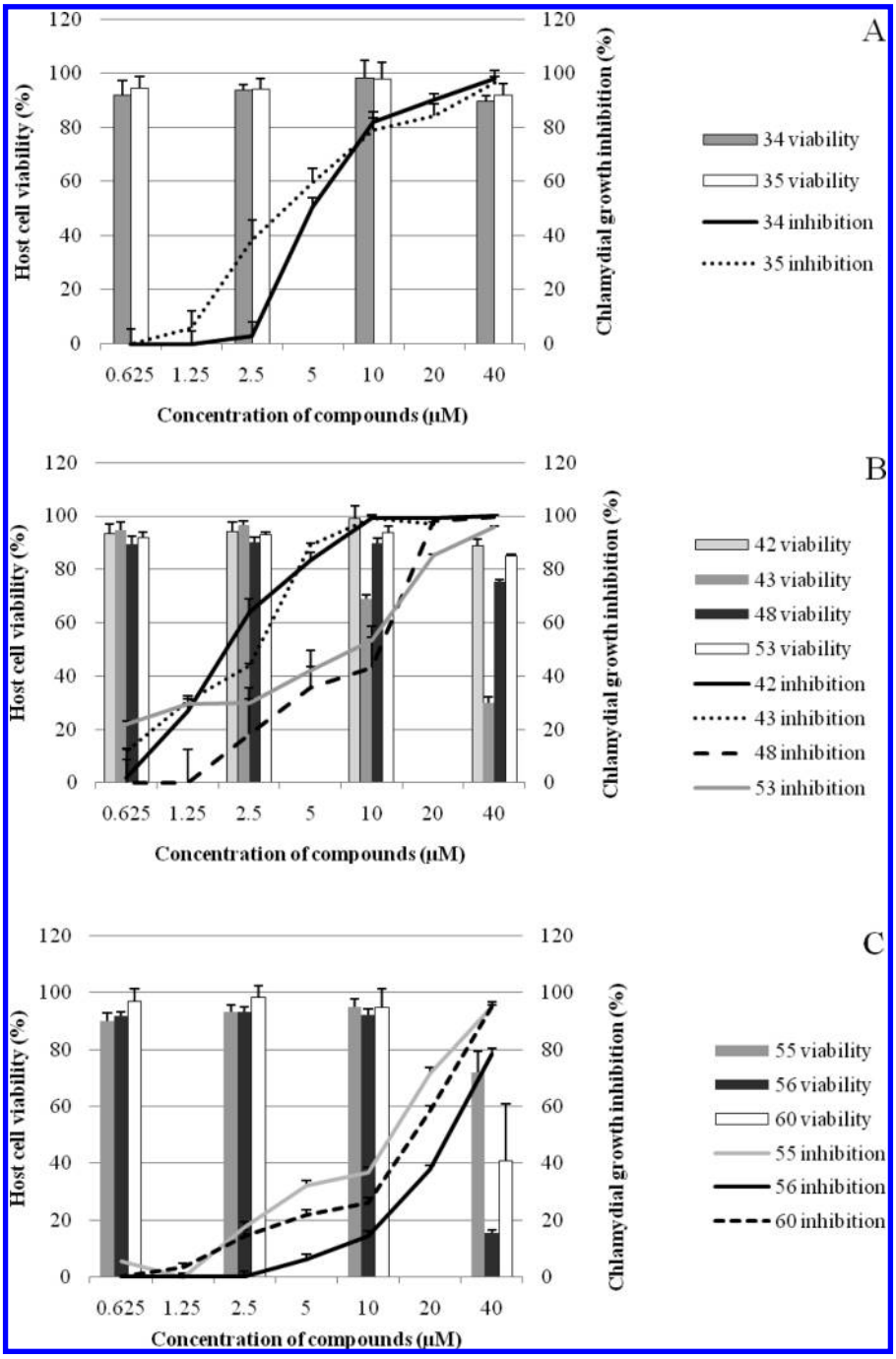


Figure 1. Differences in chemical structure of benzimidazoles (groups A−C) reflected well the antichlamydial effect (lines) and the effect on the host cell viability (bars). For chlamydial growth inhibition, $n = 12$ except for 40 μM, $n = 8$, error bars + SEM. The viability was compared to DMSO-adjusted control. For host cell viability $n = 3$, error bars + SD.

(Biowhittaker, Lonza, Basel, Switzerland), 2 mM L-glutamine (Biowhittaker, Lonza, Basel, Switzerland), and 20 μg/mL gentamicin (Fluka, Buchs, Switzerland). Cells were grown in cell culture flasks (Greiner bio-one, Frickenhausen, Germany) at 37 °C, 5% CO_2, and 95% humidity and dispensed either to 24-well plates (Cellstar, Greiner bio-one, Frickenhausen, Germany) with coverslips (Menzel-Gläser, Braunschweig, Germany) 4×10^5 cells/well or to 96-well plate with clear wells and white matrix (Wallac Isoplate 1450−516, PerkinElmer, Waltham, MA, USA) 6×10^4 cells/well. Twenty-four h after plating, the cells were infected with 0.2 multiplicity of infection (MOI) on 24-well and 96-well plates with *C. pneumoniae* sequenced American Type Culture Collection (ATCC) reference strain CWL-029 in medium supplemented with 0.5 μg/mL cycloheximide (Sigma-Aldrich, St. Louis, MO, USA). Plates were centrifuged at 550 for 1 h at room temperature (Heraeus Multifuge 3s, Thermo Fischer Scientific, Vantaa, Finland) and left to stand for 1 h in the incubator.

Following the infection, the infection medium was removed from the wells and samples were added in 0.5 μg/mL cycloheximide-supplemented medium, 1 mL/well on a 24-well plate, and 200 μL/well on a 96-well plate. The compounds were added as three parallel replicates on the 24-well plate and as six parallel replicates on the 96-well plate. On each plate, there were noninfected and infected controls as well as infected controls treated with the antibiotic rifampicin 0.012 μM (BioChemika >97.0% HPLC; Fluka, Buchs, Switzerland). Plates were incubated at 37 °C, 5% CO_2, and 95% humidity for 72 h, after which wells were washed with 1 mL/well or 300 μL/well of

Table 5. The Overall Pattern of Efficacy against *C. pneumoniae* Remained the Same with the CV-6 Strain Compared to the CWL-029 Strain ($n = 2$ except with **42** and **48** $n = 4$)

	MIC range		MCC range	
compd	μM	(μg/mL)	μM	(μg/mL)
34	196	(64)	>196	(>64)
35	>196	(>64)	>196	(>64)
42	3.2−6.3	(1−2)	3.2−6.3	(1−2)
43	3.2	(1)	50.4	(16)
48	6.1−48.9	(2−16)	24.5	(8)
53	24.5	(8)	24.5	(8)
55	12.6	(4)	12.6	(4)
56	196	(64)	>196	(>64)
60	12.6	(4)	12.6	(4)
erythromycin (control)	0.17	(0.125)	0.17	(0.125)

6.7 mM PBS (pH 7.4) solution (Biowhittaker, Lonza, Basel, Switzerland) and fixed with 1 mL/well or 300 μL/well of methanol (Mallinckrodt Baker, Phillipsburg, NJ, USA) for 10 min. The coverslips were removed from the 24-well plates and the methanol from the 96-well plates, and fixed cell layers were left to dry for at least 24 h.

TR-FIA Labeling. Assay buffer (PerkinElmer, Waltham, MA, USA) was supplemented with europium-conjugated chlamydial antibody (100 ng/mL), and 100 μL of this assay buffer was added to the wells of the 96-well plates. Plates were incubated for 30 min at 37 °C and washed six times with wash solution (PerkinElmer, Waltham, MA, USA) using a plate washer (BW 50, Biohit, Helsinki, Finland). Enhancement solution was added to the wells and plates shaken at low intensity for 5 min on a plate shaker (Delfia Plateshaker, PerkinElmer Finland, Turku, Finland), and signals were measured by a multilabel plate reader Victor^2 (PerkinElmer Finland, Turku, Finland). The TR-FIA method is described in detail elsewhere.[56]

Autofluorescence of Compounds and Affinity of Europium-conjugated Chlamydial Antibody to Compounds. The 96-well plate was handled according to the infection protocol, but no infection was induced. The compounds were added as in the antichlamydial TR-FIA assay. The plate was analyzed by Victor^2 plate reader after 72 h of incubation for autofluorescence by the compounds. The procedure was repeated but with labeling the plate as in TR-FIA and time-resolved fluorescence was measured with Victor^2 to detect unspecific binding of the label.

IF Measurement of Strain CWL-029. The dried coverslips were stained with Pathfinder Chlamydia Culture Confirmation System (Bio-Rad, Hercules, CA, USA) containing isothiocyanate-conjugated monoclonal antibody. The Pathfinder solution was distributed to a marked slide, and coverslips were placed on the slide cells facing down and incubated for 30 min at 37 °C in a humid environment. The excess label was removed by dipping the coverslips twice to 6.7 mM PBS (pH 7.4) and once to purified water (Milli-Q). Coverslips were mounted to objective glasses, and the chlamydial inclusions were counted under a fluorescence microscope (Nikon Eclipse TE300, Tokyo, Japan) in a magnification of 200×. Four eye-fields from every coverslips were counted, totaling 12 measurements per compound.

IF Measurement of Strain CV-6. MIC testing of *C. pneumoniae* was carried out in cell culture with HEp-2 cells (ATCC CCL-23) grown to confluency in 24-well plates with glass coverslips. After a centrifugation step (1700*g* for 45 min, 35 °C) with 10^3 inclusion forming units (IFU) per mL, the medium was replaced with minimal essential medium containing cycloheximide (1 μg/mL) and assayed compounds in serial 2-fold dilutions. Visualization of chlamydial inclusions was performed after incubation for 72 h at 35 °C and 5% CO_2, with a fluorescein-conjugated monoclonal anti-*Chlamydia* lipopolysaccharide antibody (Oxoid, Ely, UK). The MIC was defined as the lowest concentration at which no IFU were observed. The MCC was subsequently determined by removing the drug-containing medium, washing the wells with 6.7 mM PBS (pH 7.4), and adding fresh medium without antibiotic supplementation. The infected monolayer was then disrupted, passed onto fresh monolayers, and incubated and stained as above. The MCC was the lowest drug concentration that inhibited the production of IFU in the antibiotic-free passage. All titrations were carried out in duplicate. The compounds **42** and **48** were tested twice, as they were first tested separately and then retested among the other seven selected compounds.

Host Cell Viability Measurement. In the viability measurement, a commercial kit "CellTiter-Glo Luminescent Cell Viability Assay" (Promega, Madison, WI, USA) was used. The 96-well plate was seeded with HL cells as described above, but one column was left empty for blank measurement. Twenty-six hours after seeding the plate, the compounds were added to three wells each, according to the infection protocol, as neither infection nor centrifugation was needed. Cells were incubated for 72 h and handled according to the instructions of the kit with one exception. Approximately 200 μL of old medium was removed and 100 μL of fresh medium was applied just before adding "CellTiter-Glo" reagents. The plate was shaken on the plate shaker on low intensity for 2 min to induce complete cell lysis. After this, the signal was allowed to stabilize for 10 min and luminescence was measured by VarioSkan flash plate reader (Thermo Fischer Scientific, Vantaa, Finland). Viability percentage was calculated by comparing the ATP concentration of cells treated with DMSO-adjusted medium to the ATP concentration of cells treated with 2-arylbenzimidazoles.

Other Antimicrobial Activities. To determine general antimicrobial effects of the benzimidazoles, they were tested against *Staphylococcus aureus* ATCC-25923 (Gram-positive) and *Escherichia coli* ATCC-25922 (Gram-negative). Both microbes were first grown overnight in 5 mL of nutrient broth at 37 °C and repassaged by adding 0.1 mL of the bacterial suspension to 10 mL of nutrient broth and grown overnight at 37 °C in a shaker at the speed of 100 rpm. Of this suspension, 0.1 mL was suspended to 100 mL of nutrient broth and suspension was mixed thoroughly. Then 130 μL of formed bacterial suspension was added to the wells of 96-well plate (Wallac Isoplate 1450−516, PerkinElmer, Waltham, MA, USA) excluding negative growth and background wells, where plain nutrient broth was added. To all wells were added either 50 μL of 0.01% (w/v) resazurin (cat. no. R-2127, Sigma-Aldrich, St. Louis, MO, USA) solution in sterile water or plain sterile water, and 50 μL of assayed compounds or control compound ampicillin (cat. no. A-6140, Sigma-Aldrich, St. Louis, MO, USA) in nutrient broth or plain nutrient broth. The end concentration of benzimidazoles and ampicillin were 50 and 25 μM (10 μg/mL), respectively. Plates were incubated at 37 °C for 18 h, and the amount of resazurin was measured by photometric method with VarioSkan flash plate reader at the wavelength of 600 nm.

Synthesis. All reagents were commercially available and were acquired from Fluka (Buchs, Switzerland), Aldrich (Schnelldorf, Germany), Mallinckrodt Baker (Phillipsburg, NJ, USA), Riedel-de Haën (Seelze, Germany), and Alfa Aesar (Karlsruhe, Germany). THF was distilled over sodium/benzophenone ketyl. Anhydrous MeCN (Aldrich) was stored under an inert atmosphere of dry argon. All reactions in anhydrous solvents were performed under an argon atmosphere. The progress of chemical reaction was monitored by thin-layer chromatography on silica gel 60-F_{254} plates acquired from Merck (Darmstadt, Germany). The eluent consisted of EtOAc and *n*-hexane or CH_2Cl_2 and MeOH, and detection was conducted at 254 or 366 nm. MW-assisted synthesis was conducted by Biotage SP1MW instrument (Uppsala, Sweden). Flash SiO_2 column chromatography was performed with a Merck silica gel 60 (230−400 mesh), or products were purified by flash chromatography on a silica gel with a Biotage SP1 purification system (Uppsala, Sweden) using 25 + M or 12 + M cartridges (25 or 12 mL/min flow rate, detection 254 nm). Melting points were measured using an

IA9100 digital melting point apparatus (Electrothermal Engineering, Essex, UK) and are uncorrected. IR spectra were recorded on a Bruker Vertex 70 FT-IR spectrometer (Ettlingen, Germany) with KBr technique. The synthesized compounds were analyzed by NMR on a Varian Mercury 300 MHz spectrometer (Varian, Palo Alto, CA). ^{1}H and ^{13}C NMR were recorded as solutions in DMSO-d_6, $CDCl_3$, or CD_3OD. Deuterated solvents were purchased from Aldrich. Chemical shifts (δ) are given in parts per million (ppm) relative to the NMR solvent signals (DMSO-d_6 2.50 and 39.51 ppm, $CDCl_3$ 7.26 and 77.16 ppm, CD_3OD 3.31 and 49.00 for ^{1}H and ^{13}C NMR, respectively). LC-MS analyses were performed by the use of an HP1100 instrument with UV detector (λ 210 nm) and Esquire LC spectrometer (Bruker Daltonik, Bremen, Germany) with ESI ion source. Signal separation was carried out by use of a Waters XTerra MS RP18 column (4.6 mm × 30 mm, 2.5 μm). The eluent consisted of water (+0.1% HCO_2H) and acetonitrile (+0.1% HCO_2H) (gradient run 90:10 → 10:90). Purity of all tested compounds was >95%. High resolution mass spectra (HRMS) were measured on a JEOL MStation JMS-700 (Tokyo, Japan) instrument operating at 70 eV.

General Procedure A. To the solution of diamine or aminophenol compound (5.5 mmol, 1 equiv) in pyridine (20 mL) was added 3-nitrobenzoyl chloride (2.14 g, 11.6 mmol, 2.1 equiv), unless otherwise stated, and DMAP (34 mg, 0.31 mmol, 0.05 equiv). After MW irradiation (15 min, 60 °C) in a sealed tube (20 mL), the mixture was poured into 2 M HCl solution (30 mL) on an ice bath. The precipitate was collected and washed with 4 M HCl solution (2 × 20 mL), H_2O (20 mL), 1 M NaOH solution (20 mL), H_2O (20 mL), and Et_2O (20 mL) to give a crude product, which was used for the next step without purification.

3-Nitro-*N*-[2-[(3-nitrobenzoyl)amino]phenyl]benzamide (2). Synthesis according to the general procedure A using 1,2-diaminobenzene (0.59 g, 5.5 mmol) as a starting compound gave compound **2** (2.21 g, 99%) as an off-white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.37 (s, 2H), 8.78 (t, J 2.0 Hz, 2H), 8.44−8.38 (m, 4H), 7.82 (t, J 8.1 Hz, 2H), 7.71−7.68 (m, 2H), 7.36−7.31 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.6 (2C), 147.7 (2C), 136.1 (2C), 134.2 (2C), 131.3 (2C), 130.2 (2C), 126.3 (2C), 126.2 (2C), 125.9 (2C), 122.6 (2C). R_f = 0.53 (EtOAc: *n*-hexane = 1:1).

General Procedure B. Bisamide compound (5.00 mmol, 1 equiv) and *p*-TSA (1.52 g, 8.00 mmol, 1.6 equiv) were refluxed in *p*-xylene (40 mL) for 18 h. Reaction mixture was partitioned between EtOAc (100 mL) and 1 M NaOH solution (100 mL), and the aqueous layer was extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtrated, and evaporated in vacuo.

2-(3-Nitrophenyl)-1*H*-benzimidazole (11). Following the general procedure B, the product was synthesized from **2** (2.03 g, 5.00 mmol). Recrystallization from EtOH gave **11** as yellow crystals (0.86 g, 72%); mp 208 °C (lit. 206−207 °C[61]). ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.28 (s, H), 9.01, (t, J 1.8 Hz, 1H), 8.61 (d, J 7.8 Hz, 1H), 8.31 (dd, J 8.0, 1.7 Hz, 1H), 7.84 (t, J 8.1 Hz, 1H), 7.66−7.64 (m, 2H), 7.28−7.22 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 149.1, 148.3, 132.5, 131.7, 130.7, 124.2, 122.7, 120.8 (^{1}H NMR and ^{13}C NMR were in agreement with reported data[63]). LC-MS: $[M + H]^+$, m/z 340 (t_r = 4.5 min). R_f = 0.51 (EtOAc: *n*-hexane = 1:1).

General Procedure C. To the solution of the nitro compound in EtOH, EtOH/EtOAc, or EtOH/EtOAc/MeOH, 10% Pd/C was added in small portions and the solution was hydrogenated at room temperature. The reaction mixture was filtered through a small pad of Celite, and the filtrate was evaporated in vacuo to give the aniline.

3-(1*H*-Benzimidazol-2-yl)aniline (23). According to the general procedure C, compound **11** (2.39 g, 10.0 mmol) in EtOH (250 mL) was hydrogenated (Pd/C 0.02 g) for 5.5 h to give **23** as a beige crude product (1.96 g, 94%), which was carried to the next step without further purification. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.69 (s, 1H), 7.55 (s, br, 2H), 7.42 (t, J 1.8 Hz, 1H), 7.27 (dt, J 7.8, 1.5 Hz, 1H), 7.19−7.14 (m, 3H), 6.67 (ddd, J 8.0, 2.4, 1.0 Hz, 1H), 5.30 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 152.1, 149.1, 143.8, 134.9, 130.7, 129.3, 122.2, 121.4, 118.7, 115.5, 113.9, 111.9, 111.2 (^{1}H NMR and ^{13}C NMR were in agreement with reported data[43]). R_f = 0.47 (EtOAc: *n*-hexane = 1:1).

General Procedure E. To the solution of compound (**23**−**31**, 1.0 mmol, 1 equiv) in anhydrous THF was added the appropriate acyl chloride (1.1 mmol, 1.1 equiv) and TEA (210 μL, 1.5 mmol, 1.5 equiv). After the given time, the eluent was evaporated in vacuo and the residue was partitioned between water (100 mL) and EtOAc (50 mL). The aqueous layer was separated and extracted with EtOAc (50 mL), unless otherwise stated, and the combined organic phases were washed with saturated $NaHCO_3$ (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4, filtered, and the filtrate was evaporated in vacuo to give the product.

***N*-[3-(1*H*-Benzimidazol-2-yl)phenyl]thiophene-2-carboxamide (34).** Following the procedure E mixture of **23** (0.21 g, 1.0 mmol), TEA, and 2-thiophenecarbonyl chloride (120 μL, 1.1 mmol) in THF (10 mL) was stirred for 30 min. The residue was purified by column chromatography on silica gel (EtOAc: *n*-hexane = 1:1 → 3:2) to give **34** as a white powder (0.17 g, 55%); mp 134 °C (recrystallized from MeOH). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.94 (s, 1H), 10.44 (s, 1H), 8.64 (t, J 1.8 Hz, 1H), 8.12 (dd, J 1.2, 3.6 Hz, 1H), 7.90−7.87 (m, 3H), 7.67 (d, J 6.3 Hz, 1H), 7.56−7.51 (m, 2H), 7.27−7.19 (m, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.0, 151.1, 143.8, 139.9, 139.3, 135.1, 132.1, 130.6, 129.3, 129.3, 128.2, 122.6, 121.7, 121.5, 118.9, 118.7, 111.4. LC-MS: $[M + H]^+$, m/z 320 (t_r = 4.3 min). FT-IR (KBr, cm^{-1}): 3301, 2962, 1641, 1555, 1262, 742. R_f = 0.22 (EtOAc: *n*-hexane = 1:1). HRMS (EI): calcd for $C_{18}H_{13}N_3OS$, 319.0779; found, 319.0780.

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Supporting Information Available: Material describing the synthesis and characterization data of **1b**, **3**−**22**, and **24**−**68**, and the calculations of cLogP values for all final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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