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Synthesis and Evaluation of Anticancer Benzoxazoles and Benzimidazoles Related to UK-1

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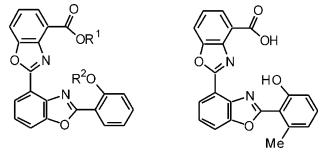
Abstract—UK-1 is a structurally unique bis(benzoxazole) natural product isolated from a strain of *Streptomyces*. UK-1 has been reported to possess anticancer activity but no activity against bacteria, yeast, or fungi. Previous work has also demonstrated the ability of UK-1 to bind a variety of di- and tri-valent metal ions, particularly Mg^{2+} ions, and to form complexes with double-stranded DNA in the presence of Mg^{2+} ions. Here we report the activity of UK-1 against a wide range of human cancer cell lines. UK-1 displays a wide spectrum of potent anticancer activity against leukemia, lymphoma, and certain solid tumor-derived cell lines, with IC_{50} values as low as 20 nM, but is inactive against *Staphylococcus aureus*, a methicillin-resistant strain of *S. aureus*, or *Pseudomonas aeruginosa*. A series of analogues of the bis(benzoxazole) natural product UK-1 in which the carbomethoxy-substituted benzoxazole ring of the natural product was modified were prepared and evaluated for their anticancer and antibacterial properties. An analogue of UK-1 in which the carbomethoxy-substituted benzoxazole ring was replaced with a carbomethoxy-substituted benzimidazole ring was inactive against human cancer cell lines and the two strains of *S. aureus*. In contrast, a simplified analogue in which the carbomethoxy-substituted benzoxazole ring was replaced with a carbomethoxy group was almost as active as UK-1 against the four cancer cell lines examined but lacked activity against *S. aureus*. Metal ion binding studies of these analogues demonstrate that they both bind Zn^{2+} and Ca^{2+} ions about as well as UK-1. The non-cytotoxic benzimidazole UK-1 analogue binds Mg^{2+} ions 50-fold weaker than UK-1, whereas the simple benzoxazole analogue binds Mg^{2+} ions nearly as well as UK-1. These results support a role of Mg^{2+} ion binding in the selective cytotoxicity of UK-1 and provide a minimal pharmacophore for the selective cytotoxic activity of the natural product.

Introduction

The bis(benzoxazole) natural products are a structurally unique class of *Streptomyces* secondary metabolites that have recently been reported in the literature.[1–3] In the course of a screening program for new bioactive compounds, Taniguchi and co-workers isolated UK-1 from the acetone extracts of *Streptomyces* sp. 517–02.[1] Subsequently, Tsuji and co-workers isolated AJI9561 from *Streptomyces* sp. AJ956.[2] Both UK-1 and AJI9561 were reported to possess growth inhibitory activity against the murine cancer cell line P388, with IC_{50} values in the 0.3–1.6 μM range.[1,2] Despite its cancer cell cytotoxic properties, UK-1 does not inhibit the growth of Gram-positive or Gram-negative bacteria, yeast, or fungi at concentrations as high as 250 μM.[1] However, the semi-synthetic derivatives methyl UK-1 (MUK-1) and demethyl UK-1 (DMUK-1) both have activity against Gram-positive and Gram-negative bacteria.[3,4] MUK-1 is also active against yeast and filamentous fungi.[3]



UK-1 (R^1 = Me, R^2 = H) **AJI9561**
MUK-1 (R^1 = Me, R^2 = Me)
DMUK-1 (R^1 = H, R^2 = H)

The selective cytotoxicity of UK-1 towards cancer cells versus bacteria and fungi indicates that UK-1 may have

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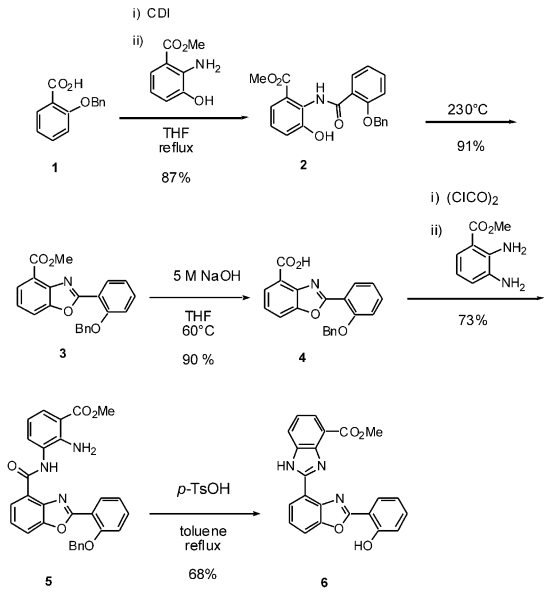
a unique mechanism of anticancer action. A recently reported synthetic route for the preparation of UK-1 has aided studies related to the mechanism of action of UK-1.[5] The 2-(2′-hydroxyphenyl)benzoxazole moiety present in UK-1 is also present in a number of synthetic metal ion chelators[6,7] and is analogous to the 2-(2′-hydroxyphenyl)oxazole moiety present in a class of microbial siderophores.[8] The metal ion binding ability of synthetically prepared UK-1 has been investigated.[9] These studies indicate that UK-1 is capable of binding a variety of biologically important metal ions, particularly Mg^{2+} ions. Like the Mg^{2+}-binding aureolic acid group of antitumor antibiotics[10,11] and synthetic antitumor quinobenzoxazines,[12,13] UK-1 binds DNA in a metal ion-dependent fashion. One consequence of this interaction with DNA is the inhibition of topoisomerase II.[9] A more recent investigation of the metal-mediated DNA binding of UK-1 by ESI-MS demonstrated that UK-1 forms complexes of the type [ds + UK-1 + M^{2+}] with a variety of metal ions including Ni^{2+}, Co^{2+}, and Zn^{2+}.[14] These results distinguish UK-1′s metal mediated DNA binding from that observed for the aureolic acids, which bind as [ds + 2×ligand + M^{2+}] complexes,[14,15] and the quinobenzoxazines, which bind as [ds + 2×ligand + 2×M^{2+}] complexes.[12,13]

In order to understand the structural basis for the selective cytotoxicity of UK-1, a number of structural analogues of this natural product have been prepared. A comparison of the anticancer activity of these compounds with their ability to inhibit the growth of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* demonstrates that a structurally simplified analogue of UK-1 retains the natural product's selective activity against cancer cells. It was also found that structurally conservative changes to UK-1 that diminish Mg^{2+}-binding ability result in a dramatic decrease in cancer cell cytotoxicity. Taken together, these results establish both a minimum structural pharmacophore as well as a functional role for Mg^{2+}-binding in the selective cytotoxicity of UK-1.

Results and Discussion

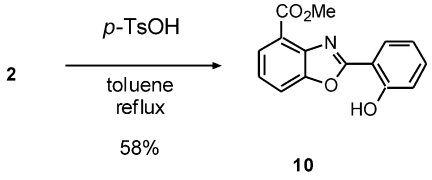
A number of analogues of UK-1 were prepared in which the carbomethoxy-substituted benzoxazole ring was either modified or deleted. The synthesis of these UK-1 analogues was carried out in analogy with the previously published total synthesis of the natural product,[5] with some improvements. An analogue of UK-1 in which the carbomethoxy-substituted benzoxazole ring is replaced with a substituted benzimidazole ring was prepared as shown in Scheme 1. Carbonyl diimidazole-coupling of 2-(benzyloxy)benzoic acid with methyl anthranilic acid affords the amide **2** in 87% yield. Pyrolysis of amide **2** at 230 °C under vacuum affords the benzoxazole **3** in 91% yield. Hydrolysis of the ester functionality proceeded to give the benzoxazole acid **4**, which was coupled with methyl 2,3-diaminobenzoate to afford the amide **5**. Final cyclodehydration and deprotection were carried out by heating **5** in the presence of *p*-toluenesulfonic acid (*p*-TsOH) to give the aza-analogue of UK-1 **6** in 35% overall yield from 2-benzyloxybenzoic acid.



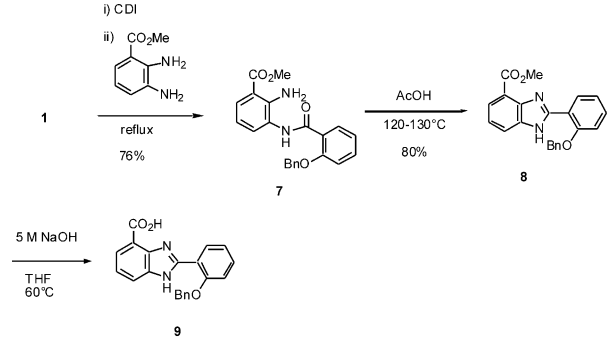
Scheme 1. Synthesis of an aza-analogue of UK-1.

The simple benzimidazole analogue **9** was prepared as shown in Scheme 2. Condensation of 2-benzyloxybenzoic acid with methyl 2,3-diaminobenzoate afforded the amide **7**, which was cyclized by heating in acetic acid to give the benzimidazole ester **8**. Hydrolysis of **8** afforded benzimidazole carboxylate **9** in 49% overall yield.

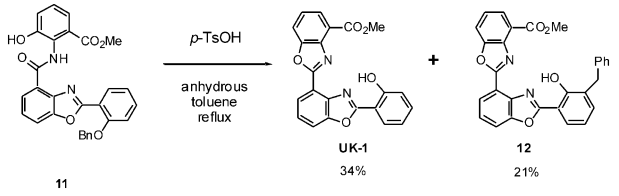
The substituted benzoxazole **10** was prepared directly from the amide **2** (Scheme 1) by *p*-TsOH-mediated cyclization and deprotection, as shown in eq 1 (below).



When this same *p*-TsOH-mediated cyclization/deprotection reaction was performed on a large scale in the synthesis of UK-1 from the precursor amide **11** (Scheme 3), a low yield of UK-1 was obtained, along with a side product that was difficult to separate from the crude product. Careful chromatography enabled the separations of



Scheme 2. Synthesis of a simplified benzimidazole UK-1 analogue.



Scheme 3. Formation of a benzylated analogue of UK-1.

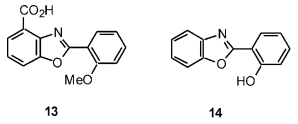
a pure sample of the side product, which was identified by ^{1}H and ^{13}C NMR, NOESY, COSY, and HRMS as the previously unreported benzylated UK-1 analogue **12**. Compound **12** apparently arises from interception of a benzyl cation formed by acid-mediated cleavage of the benzyl ester. In previous preparations of UK-1, this cation was presumably intercepted by adventitious water in the reaction mixture, but under more anhydrous conditions and larger scale, the cation is apparently able to participate in Friedel–Crafts alkylation chemistry, leading to the benzyl adduct **12**. The formation of this side product can be avoided; carrying out the transformation of **11** to UK-1 in the presence of 2–4 equivalents of water prevented the formation of **12**, and allowed for the isolation of UK-1 in high yields, as reported earlier.[5]

The cytotoxicity of UK-1 against P388, B16, and HeLa cell lines has been previously reported.[1] In order to determine the spectrum of cell lines whose growth is inhibited by UK-1, a range of human cancer cell lines were investigated. The results, shown in Table 1, demonstrate that UK-1 displays a wide spectrum of potent anticancer activity against leukemia, lymphoma, and certain solid tumor-derived cell lines. In particular, certain neuroblastoma cell lines (IMR-32 and NGP) were very sensitive to UK-1, with IC_{50} values <100 nM. Other cell lines, such as colon (HT-29) were much less sensitive. Despite the potent anticancer activity of UK-1, there was no indication of any antibacterial effect against *S. aureus*, methicillin-resistant *S. aureus*, or *Pseudomonas aeruginosa* at concentrations up to 50 μg/mL of UK-1, in accord to previous reports of selective cancer cell cytotoxicity of this natural product.[1]

Table 1. Cytotoxicity of UK-1 against cancer cell lines

Cell line	IC_{50}[a] (μM)
MCF-7	1.6
HT-29	65
HL60	0.32
PC-3	0.4
MDA-231	0.5
BT-20	0.17
DU145	0.2
SKBR3	0.1
A549	1.9
SK-N-AS	24
SK-N-D7	0.17
SK-N-F1	7
SK-N-MC	1.1
SK-N-SH	7
CHP-212	12
IMR-32	0.06
NGP	0.02

[a]Determined using the alamarBlue assay. See Experimental for details.



The cancer cell cytotoxicity and antibacterial activity of the UK-1 analogues were examined in order to determine the structural basis for the unique cancer cell selective cytotoxicity of the natural product. The previously reported benzoxazole **13**[5] and commercially available 2-(2′-hydroxyphenyl)benzoxazole (**14**) were also included in this study. Table 2 summarizes the results of cytotoxicity testing against MCF-7, HT-29, HL60, and PC-3 cell lines along with the antibacterial assays against *S. aureus* and a methicillin-resistant strain of *S. aureus*. UK-1 shows a wide range of cytotoxic activity against the four cancer cells lines, due to the relative resistance of the HT-29 cells to the action of UK-1. The benzylated UK-1 analogue **12** demonstrates a diminished cytotoxic effect against the cancer cell lines relative to UK-1; however, the spectrum of activity of this analogue is quite distinct. Compound **12** is more active than UK-1 against HT-29 cells (IC_{50} 5.2 μM vs 65 μM for UK-1), about 10-fold less potent than UK-1 against MCF-7 and PC-3 cells (IC_{50} 13 and 4.5 μM vs 1.6 μM and 0.4 μM for UK-1, respectively), and 20-fold less active than UK-1 against HL60 cells ($IC_{50}=27$ μM vs 0.32 μM for UK-1). Unfortunately, the limited availability of compound **12** prevented the determination of its antibacterial properties. It remains to be seen if substitution on the phenolic ring of UK-1 affects cancer cell versus bacterial cell selectivity of UK-1.

Interestingly, the aza-analogue of UK-1, compound **6** lacks any anticancer activity against three of the four cell lines examined, with IC_{50} values >100 μM against MCF-7, HT-29, and PC-3 cells (Table 2). Compound **6** did demonstrate very modest activity against HL60 cells ($IC_{50}=70$ μM). This analogue lacks any antibacterial activity against the two strains of *S. aureus* examined. The majority of the other analogues of UK-1 examined also lacked appreciable anticancer activity, although two analogues, benzoxazole acid **4** and benzimidazole acid **9**, displayed some antibacterial activity against the *S. aureus* strains. The activity of these acids against *S. aureus*, although modest, is interesting in light of the reported antibacterial activity of DMUK-1 but not the methyl ester UK-1 (see above); compound **3**, the methyl ester of **4**, also lacks antibacterial activity. The 2-(2′-hydroxyphenyl)benzoxazole (**14**) is neither cytotoxic to cancer cells nor antibacterial (Table 2), in accord with previous reports of the lack of antibacterial activity (>100 μM) for this compound.[16]

Within the series of truncated UK-1 analogues examined, only one analogue, compound **10**, demonstrated cancer cell cytotoxicity (Table 2). Compound **10** also displayed the selective activity against cancer cells versus bacterial cells that is characteristic of the natural product. The anticancer activity of compound **10** is somewhat less than that of UK-1 against HL60 and PC-3 cells ($IC_{50}=5.7$ μM vs 0.4 μM for UK-1, and 0.88 μM vs 0.32 μM for UK-1, respectively), but equal to or

Table 2. Antibacterial and Anticancer Activity of UK-1 and Analogues

Compd	Antibacterial IC_{50}[a]		Cytotoxicity IC_{50} range[b] (μM)
	S. aureus (μM)	Meth-resist *S. aureus*	
UK-1	NA[c]	NA[c]	0.32–65
12	nd[d]	nd[d]	4.5–27
6	NA	NA	7.0–>100
3	NA	NA	>100
4	43	29	>100
9	102	102	>100
10	NA	NA	0.88–9.1
13	NA	NA	>100
14	NA	NA	>100
Ciprofloxacin	0.45	0.45	nd
Mitomycin C	nd	nd	0.08–0.27

[a]Sample concentration that affords 50% growth of the test organism.
[b]Range of IC_{50} values obtained by alamarBlue cytotoxicity assays against MCF-7, HL-60, HT-29, and PC-3 cells after 72 h at 37 °C.
[c]No activity observed at concentrations up to 50 μg/mL.
[d]Not determined.

better than UK-1 against MCF-7 and HT-29 cells (IC_{50} = 1.5 μM vs 1.6 μM for UK-1, and 9.1 μM vs 65 μM for UK-1, respectively). Compound **10** was not active against *S. aureus* (Table 2) or against *P. aeruginosa, Cryptococcus neoformans,* or *Candida albicans* (IC_{50} > 50 μg/mL, data not shown).

Based on previous work that implicated metal ion binding in the mechanism of action of UK-1, the ability of selected UK-1 analogues to bind a variety of metal ions was determined by the method of continuous variation (Table 3). Methanolic solutions of UK-1 or analogues and metal ion salts [$Ca(NO_3)_2$, $Mg(NO_3)_2$, $Zn(NO_3)_2$, or $Fe(NO_3)_3$] at various molar ratios and 20 μM combined total concentration were prepared and the absorbance at 418 nm was determined. Plots of A_{418nm} versus mole ratio of ligand demonstrated maxima at 0.5 mole ratio for all metal ions, indicating 1:1 stoichiometry for each of the metal ion complexes. The aza-analogue **6**, which lacks cancer cell cytotoxicity, binds Mg^{2+} and Fe^{3+} ions much less well than UK-1, while the Ca^{2+} and Zn^{2+} metal ion binding ability of this analogue is slightly higher than that of UK-1. The cancer cell cytotoxic analogue **10** retains much of the Zn^{2+} metal ion binding ability of the natural product, while the ability of **10** to bind Mg^{2+}, Ca^{2+}, and Fc^{2+} ion is slightly diminished relative to UK-1.

An appropriate 4-substituent on the 2-(2′-hydroxyphenyl)benzoxazole core is important for the efficient Mg^{2+} binding of ability of these compounds. The 4-unsubstituted 2-(2′-hydroxyphenyl)-benzoxazole (**14**) does not complex Mg^{2+} ions (data not shown), although **14** does form complexes with Zn^{2+}.[17,18] Introduction of 4-carbomethoxy substituent, as in **10**, or a 4-(benzoxazo-2-yl) substituent (as in UK-1) on to the 2-(2′-hydroxyphenyl)benzoxazole core imparts Mg^{2+} ion binding ability, but the 4-(benzimidazo-2-yl) substituent (as in **6**) does not. Taken together, these results support a role for Mg^{2+} ion binding in the cancer cell cytotoxicity of these compounds, in that the cytotoxic UK-1 and analogue **10** bind Mg^{2+} ions better than the non-cytotoxic analogues **6** and **14**. Moreover, the selective activity of compound **10** against cancer cells but not bacteria reflects the selective activity of UK-1. Compound **10** thus represents a minimum pharmacophore for the selective anticancer activity of UK-1.

Table 3. Association constants for metal ion binding by UK-1 and analogues

Metal ion	Compd		
	UK-1	**6**	**10**
Mg^{2+}	2.0×10^{6} M^{-1}	4.0×10^{4} M^{-1}	3.2×10^{5} M^{-1}
Ca^{2+}	4.0×10^{4} M^{-1}	1.0×10^{5} M^{-1}	6.3×10^{3} M^{-1}
Zn^{2+}	2.0×10^{6} M^{-1}	3.2×10^{6} M^{-1}	1.3×10^{6} M^{-1}
Fe^{3+}	2.5×10^{5} M^{-1}	1.0×10^{4} M^{-1}	7.9×10^{3} M^{-1}

Conclusion

The relatively simple yet novel bis(benzoxazole) natural products UK-1[1] and AJI9561[2] represent a new class of cytotoxic secondary metabolites. Interest in this class of bis(benzoxazoles) is fueled by the finding that UK-1 is selectively cytotoxic to cancer cells but not bacterial cells, yeast, or fungi.[1] This selective cytotoxicity may be mediated through the specific interaction of UK-1 with an as yet undefined cancer cell-associated target. While the putative target of UK-1 remains unknown, previous studies have demonstrated that this natural product forms complexes with a variety of physiologically relevant metal ions, particularly magnesium ions.[9] Furthermore, the metal ion binding of UK-1 increases the affinity of this compound for DNA in a manner reminiscent of the aureolic acid class of antitumor antibiotics.[9,14] Here it is shown that within a series of analogues, the structural basis for Mg^{2+} ion binding and selective cytotoxicity of UK-1 are the same; both Mg^{2+} ion binding and selective cytotoxicity require an appropriately 4-substituted 2-(2′-hydrophenyl)benzoxazole moiety. The elucidation of the structural basis for Mg^{2+} ion recognition by these 4-substituted 2-(2′-hydroxyphenyl)benzoxazoles requires further investigation. While it is unlikely that Mg^{2+} ion binding per se is the origin of the selective cytotoxicity of UK-1 and analogue **10**, the ability of UK-1 to form metal ion complexes that can bind to DNA[9,14] and inhibit DNA-processing enzymes[9] indicates that Mg^{2+} ion binding by UK-1 may lead to biologically relevant complexes with a specific target in cancer cells. Further work with UK-1 and analogues is required in order to determine if such targeting is involved in the selective cytotoxicity of UK-1 and if the promising spectrum of in vitro anticancer activity of UK-1 reported here is also reflected in vivo.

Experimental

General

All reagents and solvents were purchased from Aldrich Chemical Company and used without further purification,

unless noted. THF was distilled from sodium/benzophenone immediately prior to use. CH_2Cl_2 and DMF were distilled from CaH_2 immediately prior to use. Toluene was distilled from sodium metal. UV–Vis spectra for the continuous variation plots were determined on a Varian Cary 3E spectrophotometer. Melting points (open capillary) are uncorrected. Unless otherwise noted, ^1H and ^{13}C NMR spectra were determined in $CDCl_3$ on a spectrometer operating at 300 and 75.5 MHz, respectively. All mass spectra were obtained by chemical ionization using methane as the ionizing gas. Chromatography refers to flash chromatography on silica gel, and R_f values were determined using silica gel-GF TLC plates (Merck) using the solvent system indicated.

Methyl 2-{[2-(benzyloxy)benzoyl]amino}-3-hydroxybenzoate (2). Carbonyldiimidazole (CDI) (5.10 g, 31.5 mmol) was dissolved in 50 mL dry THF with stirring at room temperature under an argon atmosphere, and 2-(benzyloxy)benzoic acid (7.18 g, 31.5 mmol)[19] was then added carefully. After the evolution of CO_2 ceased (ca. 5 min) the reaction mixture was stirred for an additional 10 min and then methyl 2-amino-3-hydroxybenzoate (3.5 g, 21 mmol)[20] was added. After stirring for an addition 10 min at room temperature, the reaction mixture was heated under reflux for 18 h. The reaction mixture, brown in color, was concentrated and dissolved in a minimum volume of EtOAc. Silica gel (60–100 mesh) was added to make the slurry and solvent was evaporated to dryness. Column chromatography was performed using 20% EtOAc in hexane to give a light yellow solid (6.9 g, 87%): mp 104–105 °C; R_f 0.328 (20% EtOAc in hexanes); ^1H NMR δ 3.80 (s, 3H), 5.50 (s, 2H), 7.05 (d, 1H, $J=8.1$ Hz), 7.10 (t, 1H), 7.24 (m, 2H), 7.34 (m, 3H), 7.47 (m, 3H), 7.61 (d, 1H, $J=7.8$ Hz), 8.27 (d, 1H, $J=9.0$ Hz), 9.28 (brs, 1H, NH), 12.27 (s, 1H, OH); ^{13}C NMR δ 52.20, 70.80, 113.34, 120.94, 121.27, 121.58, 122.97, 125.70, 126.05, 126.93, 128.01, 128.21, 128.60, 132.69, 133.93, 136.22, 150.76, 157.01, 165.51, 167.57; CIMS m/z 378 (MH^+); HRMS m/z calcd for $C_{22}H_{20}NO_5$: 378.1341, found 378.1343.

Methyl 2-[2-(benzyloxy)phenyl]-1,3-benzoxazole-4-carboxylate (3). Compound **2** (6.9 g, 1.83 mol) was heated neat at 230 °C for 2 h with stirring in a long neck 50-mL round-bottomed flask under vacuum, which was applied slowly at small intervals (20 min) to remove the water vapor generated in the reaction. Upon cooling, a light orange solid mass was obtained, which was dissolved in a minimum volume of EtOAc. To this solution, hexane was added with stirring to precipitate the product benzoazole, which was filtered to give a white solid (6.0 g, 91%): mp 100–102 °C; R_f 0.468 (40% EtOAc in hexanes); ^1H NMR δ 3.99 (s, 3H), 5.28 (s, 2H), 7.07–7.13 (m, 2H), 7.27–7.33 (m, 1H), 7.35–7.42 (m, 3H), 7.48 (ddd, 1H, $J=7.8$, 1.7 Hz), 7.63 (br d, 1H), 7.73 (dd, 1H, $J=8.1$, 1.0 Hz), 8.02 (dd, 1H, $J=7.8$, 1.2, 1.0 Hz), 8.25 (br d, 1H); ^{13}C NMR δ 52.40, 70.56, 113.61, 114.67, 116.31, 121.03, 121.98, 124.12, 126.79 (2C), 127.65, 128.45, 131.92, 133.19, 136.66, 141.46, 151.24, 157.74, 163.60, 165.97; CIMS m/z 360 (MH^+); HRMS m/z calcd for $C_{22}H_{18}NO_4$: 360.1235, found 360.1228.

2-[2-(Benzyloxy)phenyl]-1,3-benzoxazole-4-carboxylic acid (4). Compound **3** (6.0 g, 1.67 mol) was dissolved in 80 mL of THF and 40 mL of 5 M NaOH was added. The reaction mixture was stirred at 60 °C with stirring for 2 h. The reaction mixture was cooled to room temperature, diluted with EtOAc and acidified with concentrated HCl. The organic layer was washed with brine two times and dried over anhydrous Na_2S0_4. The solvent was evaporated and the residue was dried under vacuum to afford a white solid that was recrystallized from EtOAc and hexanes (5.2 g, 90%): mp 103–104 °C; R_f 0.15 (40% EtOAc in hexanes); ^1H NMR δ 5.28 (s, 3H), 7.12–7.20 (m, 2H), 7.37–7.49 (m, 4H), 7.52–7.60 (m, 3H), 7.77 (dd, 1H, $J=7.9$, 0.9 Hz), 8.14 (dd, 1H, $J=7.6$, 1.2 Hz), 8.21 (dd, 1H, $J=7.8$, 1.5 Hz); ^{13}C NMR δ 70.98, 113.76, 114.61, 115.28, 120.07, 121.27, 125.48, 127.08, 127.31, 128.39, 128.90, 131.72, 134.44, 136.09, 141.13, 149.90, 158.27, 163.16, 165.00; CIMS m/z 346 (MH^+); HRMS m/z calcd for $C_{21}H_{16}NO_4$: 346.1079, found 346.1078.

Methyl 2-amino-3-({[2-(2-benzyloxyphenyl)-1,3-benzoxazol-4-yl]carbonyl}aminobenzoate (5). To a solution of compound **4** (1.035 g, 3 mmol) in 40 mL of dry CH_2Cl_2 was added 10 mL of freshly distilled oxalyl chloride. After stirring for 10 min, five drops of DMF was added to the reaction mixture. The reaction mixture was stirred for 2 h at room temperature and the solvent was removed by evaporation. The resultant yellow solid was dried under vacuum for 3 h. The yellow solid was then dissolved in 15 mL of dry CH_2Cl_2 and transferred via canula to a solution of methyl 2,3-diaminobenzoate (498 mg, 3 mmol)[21] dissolved in 20 mL of dry CH_2Cl_2 and 2 mL of pyridine. The reaction mixture was stirred at room temperature overnight and then diluted with CH_2Cl_2. The solution was washed with 1% HCl, satd aq $NaHCO_3$ and satd aq NaCl. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to give the residue which was recrystalized from EtOAc to afford light yellow solid (1.077 g, 73%): mp 178 °C; R_f 0.447 (40% EtOAc in hexanes); ^1H NMR δ 3.91 (s, 3H, CH_3), 5.30 (s, 2H, CH_2), 6.23 (brs, 2H, NH_2), 6.75 (t, 1H, $J=7.9$ Hz), 7.14–7.30 (m, 6H), 7.45–7.58 (m, 4H), 7.77 (dd, 1H, $J=8.1$, 0.7 Hz), 7.80 (dd, 1H, $J=8.1$, 1.5 Hz), 7.93 (dd, 1H, $J=7.6$, 1.2 Hz), 8.24 (dd, 1H, $J=8.0$, 1.7, 1.5 Hz), 8.28 (dd, 1H, $J=7.6$, 0.7 Hz), 10.64 (s, 1H, NH); ^{13}C NMR δ 51.59, 70.89, 111.92, 113.94, 113.97, 115.46, 115.77, 121.28, 123.82, 124.74, 125.02, 125.87, 1126.83, 127.81, 128.23, 128.41, 129.37, 131.55, 133.75, 136.12, 139.45, 139.45, 144.36, 150.33, 157.87, 162.77, 162.82, 168.64; CIMS m/z 494 (MH^+); HRMS m/z calcd for $C_{29}H_{24}N_3O_5$: 494.1715, found 494.1711.

Methyl 2-[2-(2-hydroxyphenyl)-1,3-benzoxazol-4-yl]-1*H*-benz imidazole-4-carboxylate (6). Compound **5** (493 mg, 1 mmol) was dissolved in 20 mL of toluene and *p*-toluenesulfonic acid monohydrate (475 mg, 2.5 mmol) was added. The reaction mixture was refluxed for 2 h and the solvent was distilled off. The residue was treated with CH_2Cl_2 and the solution was washed with satd aq $NaHCO_3$ and satd aq NaCl. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated

to give a solid which was stirred with EtOAc and filtered. The residue was dried and purified by preparative TLC using 40% EtOAc in hexanes to afford a white solid (200 mg, 68%): mp 243 °C; R_f 0.170 (40% EtOAc in hexanes); ^{1}H NMR δ 4.20 (s, 3H, CH_3), 7.10 (t, 1H, J=8.1, 7.2 Hz), 7.24 (d, 1H, J=8.4 Hz), 7.41 (t, 1H, J=7.8, 7.5 Hz), 7.52–7.61 (m, 2H), 7.74 (d, 1H, J=7.5 Hz), 8.02 (d, 1H, J=7.8 Hz), 8.09–8.14 (m, 2H), 8.56 (d, 1H, J=7.2 Hz), 10.53 (s, 1H, NH), 11.95 (brs, 1H, OH); ^{13}C NMR δ 52.65, 109.97, 112.49, 113.88, 117.84, 119.20, 120.19, 122.61, 124.30, 125.71, 126.08, 127.87, 134.10, 134.45, 134.54, 137.65, 142.83, 149.23, 149.39, 158.54, 163.96, 166.64; CIMS m/z 386(MH^+); HRMS m/z calcd for $C_{22}H_{16}N_3O_4$: 386.1141, found 386.1150.

Methyl 2-amino-3-{[2-(benzyloxy)benzoyl]amino}benzoate (7). Carbonyldiimidazole (2.43 g, 15 mmol) was dissolved in 30 mL dry THF with stirring under argon at room temperature and 2-(benzyloxy)benzoic acid (3.42 g, 15 mmol) was then added carefully. Heavy evolution of CO_2 was observed for 3–4 min. After stirring for 10 min, methyl 2,3-diaminobenzoate (1.66 g, 10 mmol) was added and stirring continued further for 10 min. The reaction mixture was then heated under reflux for 5 days. The reaction mixture, dark brown in color, was concentrated and dissolved in a minimum volume of EtOAc. Silica gel (60–100 mesh) was added to make a slurry and the solvent was evaporated. The resulting solid was added to the top of a dry-packed chromatography column that was eluted with 20% EtOAc in hexane to give the product as a white solid (2.86 g, 76%): mp 111–113 °C; R_f 0.373 (40% EtOAc in hexanes); ^{1}H NMR δ 3.86 (s, 3H, CH_3), 5.25 (s, 2H, CH_2), 5.58 (s, 2H, NH_2), 6.66 (t, 1H, J=8.0 Hz), 7.13–7.18 (m, 2H), 7.43–7.58 (m, 7H), 7.75 (d, 1H, J=7.8 Hz), 8.27 (d, 1H, J=7.8 Hz), 9.20 (brs, 1H, NH); ^{13}C NMR δ 51.68, 71.72, 112.57, 112.59, 116.10, 121.75, 121.83, 124.76, 128.49, 128.77, 129.12 (2C), 130.09, 132.72, 133.32, 135.01, 144.51, 156.54, 163.98, 168.40; CIMS m/z 377 (MH^+); HRMS m/z calcd for $C_{22}H_{21}N_2O_4$: 377.1501, found 377.1499.

Methyl 2-[2-(benzyloxy)phenyl]-1*H*-benzimidazole-4-carboxylate (8). Compound **7** (2.86 g, 76 mmol) was heated in glacial acetic acid (20 mL) at 120 °C for 30 min with stirring in a 50 mL round bottom flask. The reaction mixture was allowed to cool and then poured into ice cold water and extracted with CH_2Cl_2. The combined organic layers were washed with satd aq $NaHCO_3$, brine and dried (Na_2SO_4). The solvent was removed by evaporation and the residue was purified by chromatography on silica gel (60–100 mesh) using 20 to 40% EtOAc in hexane to afford benzimidazole ester **8** as a white solid (2.3 g, 80%): mp 100–101 °C; R_f 0.412 (40% EtOAc in hexanes); ^{1}H NMR δ 3.67 (s, 3H, CH_3), 5.36 (s, 2H, CH_2), 7.17–7.23 (m, 2H), 7.33 (t, 1H, J=7.7 Hz), 7.40–7.49 (m, 4H), 7.56 (dd, 2H, J=7.6, 1.7 Hz), 7.90 (dd, 1H, J=7.6, 1.0 Hz), 8.03 (d, 1H, J=8.4 Hz), 8.62 (dd, 1H, J=7.8, 1.8 Hz), 11.56 (brs, 1H); ^{13}C NMR δ 51.69, 71.29, 112.95, 113.40, 117.93, 121.68, 121.90, 124.30, 124.52, 127.95, 128.51, 128.92, 130.51, 131.58, 134.16, 135.64, 143.74, 150.91, 156.33, 166.33; CIMS m/z 359 (MH^+); HRMS m/z calcd for $C_{22}H_{19}N_2O_3$: 359.1395, found 358.1393.

2-[2-(benzyloxy)phenyl]-1*H*-benzimidazole-4-carboxylic acid (9). Compound **8** (1.0 g, 2.78 mmol) was dissolved in 20 mL of THF and 8 mL of 5M NaOH was added. The reaction mixture was stirred at 60 °C with stirring for 3 h. The reaction mixture was allowed to cool to room temperature and acidified with concentrated HCl. The white solid that was obtained was filtered, washed with water, and dried under vacuum to afford the acid **9** (780 mg, 81%): mp 188 °C; R_f 0.125 (40% EtOAc in hexanes); ^{1}H NMR (DMSO-d_6) δ 5.32 (s, 2H, CH_2), 7.15 (t, 1H, J=7.5 Hz), 7.29–7.41 (m, 5H), 7.49 (t, 1H, J=8.1 Hz), 7.56 (d, 2H, J=7.4 Hz), 7.78 (d, 1H, J=7.7 Hz), 7.92 (d, 1H, J=7.5 Hz), 8.32 (dd, 1H, J=7.9, 1.8 Hz), 11.85 (brs, 1H); ^{13}C NMR (DMSO-d_6) δ 70.27, 107.28, 114.01, 117.73, 121.49, 121.66, 124.21, 123,68, 127.85, 128.12,128.64,130.00, 131.80, 134.68, 136.13, 141.02, 150.23, 155.89, 167.11; CIMS m/z 345 (MH^+); HRMS m/z calcd for $C_{22}H_{17}N_2O_3$: 345.1239, found 345.1233,

Methyl 2-(2-hydroxyphenyl)-benzoxazole-4-carboxylate (10). Compound **2** (103 mg, 0.3 mmol) was dissolved in 4 mL of toluene and *p*-toluenesulfonic acid monohydrate (142 mg, 0.75 mmol) was added. The reaction mixture was refluxed for 1.5 h and after cooling at room temperature was diluted with EtOAc. The organic layer was washed with satd aq $NaHCO_3$ and satd aq NaCl and dried over anhydrous Na_2SO_4 and the solvent was evaporated to afford the benzoxazole as a solid (45 mg, 58%): mp 134–135 °C; R_f 0.578 (30% EtOAc in hexanes); ^{1}H NMR δ 4.05 (s, 3H, CH_3), 7.01 (t, 1H), 7.13 (d, 1H, J=8.1 Hz), 7.4–7.5 (m, 2H), 7.78 (d, 1H, J=8.1 Hz), 8.01 (d, 1H, J=7.5 Hz), 8.06 (d, 1H, J=8.1 Hz); ^{13}C NMR δ 52.38, 109.91, 114.86, 117.63, 119.55, 121.24, 124.69, 127.15, 127.40, 134.19, 139.36, 149.70, 159.33, 164.26, 165.52; CIMS m/z 270 (MH^+); HRMS m/z calcd for $C_{15}H_{12}NO_4$: 270.0766, found 270.0766.

Methyl 3-hydroxy-2-{[2-(benzyloxyphenyl)-1,3-benzoxazol-4-yl] carbonyl}aminobenzoate (11). To a solution of compound **4** (3.45 g, 10 mmol) in 60 mL of dry CH_2Cl_2 was added 10 mL of freshly distilled oxalyl chloride. The reaction mixture was stirred for 10 min and 0.3 mL of dimethyl formamide was added to initiate the reaction. The reaction mixture was stirred for 2 h at room temperature and the solvent was removed by evaporation. The resultant yellow solid was dried under vacuum for 3 h. The yellow solid was then dissolved in 20 mL of dry CH_2Cl_2 and transferred via canula to a solution of methyl 2-amino-3-hydroxybenzoate (1.67 g, 10 mmol) dissolved in 40 mL of dry CH_2Cl_2 and 3 mL of pyridine. The reaction mixture was stirred at room temperature overnight and then diluted with dichlomethane. The solution was washed with 1% HCl, satd aq $NaHCO_3$ and satd aq NaCl. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to give a residue which was subjected to column chromatography using CH_2Cl_2 as eluant to afford a light yellow solid (3.8 g, 77%): mp 157–158 °C; R_f 0.329 (CH_2Cl_2); ^{1}H NMR δ 3.79 (s, 3H), 5.29 (s, 2H), 7.13–7.39 (m, 8H), 7.50–7.55 (m, 4H), 7.65 (dd, 1H, J=7.5, 1.5 Hz), 7.80

(brd, 1H, $J=8.1$ Hz), 8.31 (brd, 1H, $J=7.8$ Hz), 8.50 (dd, 1H, $J=7.5$, 1.8 Hz); ^{13}C NMR δ 52.23, 70.71, 113.76, 114.71, 115.76, 121.18, 122.51, 122.98, 123.62, 124.73, 125.62, 126.35, 126.50, 126.69, 127.68, 127.87, 132.31, 133.66, 136.28, 139.91, 150.87, 151.20, 157.89, 163.46, 164.40, 167.11 ; CIMS m/z 495 (MH^+); HRMS m/z calcd for $C_{29}H_{23}N_2O_6$: 495.1556, found 495.1553.

Methyl 2′-(3-benzyl-2-hydroxyphenyl)-2,4′-bi-1,3-benzoxazole-4-carboxylate (12). A mixture of dry toluene (20 mL) and *p*-toluenesulfonic acid monohydrate (950 mg, 5 mmol) was refluxed under a reflux condenser. Compound **11** (988 mg, 2 mmol) was added and the reaction mixture was refluxed for an additional 2 h. After the solvent was removed by distillation, the residue was dissolved in CH_2Cl_2 and the solution was washed with satd aq $NaHCO_3$ and satd aq NaCl. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to give 432 mg of solid that was a mixture of UK-1 and compound **12** (TLC and ^{1}H NMR) in the ratio of 1.6:1.0. Careful column chromatography using 5 to 20% EtOAc in hexane afforded 10 mg pure compound **12** (1%) along with 300 mg UK-1 (13%) and a large mixed fraction (122mg). Compound **12**: mp 204–205 °C; R_f 0.357 (30% EtOAc in hexanes); ^{1}H NMR δ 4.09 (s, 3H, CH_3), 4.15 (s, 2H, CH_2), 6.96 (t, 1H, $J=7.6$ Hz, C_5-H), 7.20–7.14 [m, 1H, C_3-benzyl-H(*para*)], 7.26 [s, 2H, C_3-benzyl-H(*meta*)], 7.27 [s, 2H, C_3-benzyl-H(*ortho*)], 7.30 (dd, 1H, $J=7.5$, 1.2 Hz, C_4-H), 7.46 (t, 1H, $J=7.9$ Hz, C_6-H), 7.52 (t, 1H, $J=8.0$ Hz, $C_{6'}$-H), 7.77 (dd, 1H, $J=8.1$, 1.0 Hz, $C_{7'}$-H), 7.86 (dd, 1H, $J=8.1$, 1.0 Hz, C_7-H), 7.96 (dd, 1H, $J=7.9$, 1.6 Hz, C_6-H), 8.10 (dd, 1H, $J=7.7$, 1.0 Hz, C_5-H), 8.31 (dd, 1H, $J=7.8$, 0.9 Hz, $C_{5'}$-H); ^{13}C NMR δ 35.83, 52.63, 109.72, 113.82, 115.09, 117.61, 119.33, 122.57, 124.93, 125.11, 125.24, 125.51, 126.07, 127.57, 128.42, 128.81, 129.77, 135.26, 138.76, 140.38, 141.56, 150.01, 151.22, 157.65, 161.71, 165.05, 166.32 : CIMS m/z 477 (MH^+); HRMS m/z calcd for $C_{29}H_{21}N_2O_5$: 477.1450, found 477.1442.

Cytotoxicity assays

Cell culture cytotoxicity assays were performed using the alamarBlue method, as previously described.[22] Briefly, aliquots of 100 μL of cell suspension (1.0–2.5×10^3 cells) were placed in 96-well microtiter plates in an atmosphere of 5% CO_2 at 37 °C. After 24 h, 100 μL of culture medium and 2 μL of compound in vehicle (culture media with 40% pyridine) or vehicle alone were added, and the plates incubated an additional 72 h. The final pyridine concentration in all cases was 0.4%; at this pyridine concentration, there was no affect on the growth of cells compared to cells in culture media without added pyridine. Compounds, along with mitomycin C as positive control, were evaluated in duplicate at final concentrations ranging from 0.001 to 100 μM. After the culture media had been removed from each well, 200 μL of fresh media and 20 μL of 90% alamarBlue reagent were added, followed by an additional 6 h incubation. The fluorescent intensity was measured using a SpectrafluorPlus plate reader with excitation at 530 nm and emission at 590 nm. Results are reported as IC_{50} values, the average concentration required to produce a decrease of fluorescent intensity of 50% relative to vehicle-treated controls in two separate determinations.

Antimicrobial bioassay

S. aureus ATCC 29213 and methicillin-resistant *S. aureus* ATCC 43300 (MRS) were obtained from the American Type Culture Collection (Rockville, MD, USA) and stored on agar slants at 4 °C until needed. Susceptibility testing was performed using a modified version of the NCCLS methods.[23,24] Microorganisms were subcultured prior to the assay by suspending cells from the slant in Eugon broth (BBL, MD, USA) and incubating for 24 h at 37 °C. Inocula were prepared by diluting the subcultured organism in its incubation broth after comparison to the 0.5 McFarland Standard (a $BaSO_4$ suspension) to afford final inocula ranges of *S. aureus*: $1.0–5.0\times10^5$ and MRS: $0.2–6.0\times10^5$ CFU/mL. Test compounds were dissolved in DMSO, serially-diluted using normal saline, and transferred in duplicate to 96-well flat-bottomed microtiter plates. The microbial inocula were added to the samples to achieve a final volume of 200 μL and final compound concentrations starting with 50 μg/mL. Drug [Ciprofloxacin (ICN Biomedicals, OH, USA)] as well as growth and blank (media only) controls were added to each test plate. Plates were read at 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, VT, USA) prior to and after incubation at 37 °C for 24 h. Percent growth was calculated and plotted versus concentration to afford the IC_{50}, or sample concentration that affords 50% growth of the organism relative to controls.

Continuous variation plots

A 100 μM solution of UK-1 or analogue and 100 μM solutions of $Ca(NO_3)_2$, $Mg(NO_3)_2$, $Zn(NO_3)_2$, or $Fe(NO_3)_3$ were prepared in methanol. From these stock solutions, 15 samples (1 mL) of varying mole fraction of the metal ion were prepared at a constant, combined concentration of 20 μM for the ligand and the metal ion. The change in absorbance was monitored from 500 to 200 nm for each sample using as a reference a 1 mL solution containing the same concentration of ligand as the sample being measured but without the metal ion. The maximum absorbance change was typically around 418 nm but varied somewhat for each metal ion. The absorbance at 418 nm was plotted as a function of the mole fraction of the metal ion and the maximum absorbance change was determined. The absorbance of a sample containing the metal ion concentration corresponding to this maximum change and a 15-fold excess of metal ion at this ligand concentration, subtracted from a reference containing the same concentration of ligand but no metal ion was used to normalize the curve and obtain the conditional formation constant as described.[25]

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