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Synthesis and Antimicrobial Activity of 2-Trifluoroacetylbenzoxazole Ligands and Their Metal Complexes

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Three 2-fluoroacetylbenzoxazole ligands **1a–c** and their new Zn(II) complexes **2a–c** have been synthesized. In addition, syntheses of new metal [Mg(II), Ni(II), Cu(II), Pd(II), and Ag(I)] complexes from **1a** have been also described. The molecular and crystal structures of six metal complexes **2b** and **2d–h** were determined by single-crystal X-ray diffraction analyses. Their antibacterial activities against six Gram-positive and six Gram-negative bacteria were evaluated by minimum inhibitory concentrations (MIC), which were compared with those of appropriate antibiotics and silver nitrate. The results indicate that some metal compounds have more antibacterial effects in comparison with free ligands and have preferred antibacterial activities that may have potential pharmaceutical applications. Noticeably, the Ag(I) complex **2h** exhibited low MIC value of 0.7 μM against *Pseudomonas aeruginosa*, which was even superior to the reference drug, Norfloxacin with that of 1.5 μM . Against *P. aeruginosa*, **2h** is bacteriostatic, exerts the cell surface damage observed by scanning electron microscopy (SEM) and is less likely to develop resistance. The new **2h** has been found to display effective antimicrobial activity against a series of bacteria.

Key words antibacterial activity; fluorinated benzoxazole; metal complex; trifluoroacetyl

Bacterial infection remains a serious threat to human lives due to rapid development of resistance to the existing antibiotics. In order to prevent this major medical problem, the discovery of new types of antimicrobial agents is a very important but challenging task.^{1,2} Transition metal complexes have received great attention because many drugs such as antibiotics and quinolone antibiotics possess better pharmaceutical properties when they are in the form of metal complexes.^{3–7}

Benzoxazole heterocycle system is an important pharmacophore and privileged structure in the medicinal chemistry.^{8–10} Its derivatives and particularly, 2-substituted benzoxazoles exert various biological activities such as antimicrobial, antiviral, antifungal, and anticancer.^{11–15} In previous studies, we have identified 2-trifluoroacetylbenzoxazole (**1a**) with *in vitro* antimicrobial activity against *Helicobacter pylori* and *Escherichia coli* (Ec) from our compound library of trifluoromethyl ketones obtained by our developed reactions.^{16,17}

The heteroaryl-substituted alkenol systems such as **1a** are known to chelate some metal ions such as aluminum¹⁸ and to form a stable six-membered metal complexes in a bidentate mode, where the complexes were reinforced by a positive inductive effect of the heterocyclic moiety and a negative inductive effect of the CF₃ groups.^{19–23}

In the light of these knowledge, three 2-fluoroacetylbenzoxazole ligands **1a–c** and their new Zn(II) complexes **2a–c** have been synthesized in order to check how the metal ion binding may influence their antibacterial activity (Chart 1). In addition, syntheses of new metal [Mg(II), Ni(II), Cu(II), Pd(II), and Ag(I)] complexes **2d–h** from **1a** have been also described (Table 1). The structures of six metal complexes **2b**

and **2d–h** were determined by single-crystal X-ray diffraction analyses. *In vitro* antimicrobial activities of the ligands **1a–c** and their metal complexes **2a–h** were investigated against six Gram-positive (G(+)) and six Gram-negative (G(–)) bacteria and the results were compared with those of silver nitrate and appropriate antibiotics such as Kanamycin, Gentamicin, Norfloxacin, and/or Linezolid. The most potent Ag(I) complex **2h** is bacteriostatic, disrupts membrane integrity observed by scanning electron microscopy (SEM) and is less likely to develop resistance development against *Pseudomonas aeruginosa* (Pa).

Results and Discussion

Chemistry First, three benzoxazole ligands **1a–c** and their new Zn(II) complexes **2a–c** have been prepared in order to investigate the relationship of the ligands and their Zn(II) complexes with their antibacterial activities.

Our synthetic protocol to obtain the ligand **1a** consists of a trifluoroacetylation of 2-methylbenzoxazole with trifluoroacetic anhydride (TFAA) in the presence of pyridine²⁴ (Chart 1). The Zn(II) complex **2a** was prepared by the reaction of **1a** with zinc dust in MeCN in 78% yield, consisting of **1a** with zinc(II) ion in 2 : 1 ratio.

Similarly, 2-chlorodifluoroacetylbenzoxazole (**1b**) was obtained in a good yield from a reaction of 2-methylbenzoxazole with (CF₂ClCO)₂O instead of TFAA. The **2b** was obtained from a reaction of **1b** with zinc dust in nitromethane in 98% yield.

On the other hand, the reaction of **1b** with zinc dust in the solvent of tetrahydrofuran (THF) instead nitromethane under-

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went the reduction of chlorodifluoromethyl (CF_2Cl -) group to difluoromethyl (CF_2H -) group and the formation of a new **2c** which consisted of a difluoroacetylbenzoxazole (**1c**) with zinc(II) ion in 2:1 ratio. The ligand **1c** was obtained from **2c** in a moderate yield by removing the Zn(II) using excess ethylenediaminetetraacetic acid ($\text{EDTA} \cdot 2\text{Na} \cdot 2\text{H}_2\text{O}$)²⁵⁾ (Chart 1).

Second, we have synthesized the magnesium(II), nickel(II), copper(II), palladium(II) and silver(I) complexes **2d–h** by the reaction of **1a** with the corresponding metal salts [MgBr_2 , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{Cu}(\text{OAc})_2 \cdot \text{H}_2\text{O}$, PdCl_2 , and Ag_2CO_3] (Table 1).

All the complexes **2a–h** are crystalline powders; as expected, the Zn(II) (**2a–c**) and Mg(II) (**2d**) are colorless complexes, but Ni(II) (**2e**) (dark green), Cu(II) (**2f**) (brown), Pd(II) (**2g**) (orange), and Ag(I) (**2h**) (yellow) complexes are colored. Complexes **2a–g** except the Ag(I) complex **2h** are stable and can be stored in a sealed flask for months without any change. The presence of fluorine atoms in the ligand periphery, ef-

fective steric shielding and bidentate chelating behavior toward the metal centers may impart these complexes **2a–g** as remarkable stability against air and moisture. However, the Ag(I) complex **2h** is slightly sensitive to light and darkens slowly under the light, especially when dissolved in the solution. Therefore, light stability of **2h** was studied in direct light under an air atmosphere at room temperature to mimic their exposure to light under every life conditions.²⁶⁾ Cotton pads were impregnated with 0.025 mol/L aqueous solutions of the compounds and exposed to air and light. The stability was monitored visually within 5 d. The photos of the samples (**2h**, **2a** and AgNO_3) are shown in Fig. S1. The **2h** started to be a little beige after 18 h and was completely blackened after 24 h. However, under the dark the **2h** is stable at room temperature for several months in the form of crystalline powder and their solution in dimethyl sulfoxide ($\text{DMSO}-d_6$) was remained unchanged at room temperature for 24 h. Indeed, the solutions of **2h** (3 mg) in $\text{DMSO}-d_6$ (0.5 mL) or $\text{D}_2\text{O}/\text{DMSO}-d_6$ (5:1) (0.5 mL) were standing at room temperature under the shading for 24 h and their NMR spectra remained unaltered. In the antibacterial assay and time–kill assay, the complexes were dissolved in DMSO and then diluted by Muller–Hinton broth. Therefore, the stability of **2h** (35 μM) in the buffer solution and DMSO were tested by monitoring their UV spectra at different intervals of time. On the other hand, 24 h of treatment is needed in biological activity studies. As shown in Fig. S2, only slight changes were observed in the UV spectra of the **2h** in DMSO and the buffer under the dark at 0 and 24 h.

The structures of new ligands **1b, c** and metal complexes **2a–h** were established through IR, UV, ^1H - and ^{13}C -NMR, and mass spectral studies.

The IR spectra of the ligands **1a–c** show a weak and broad band at 3100 cm^{-1} . This might be due to intramolecular hydrogen bonding between the enol hydroxyl hydrogen and nitrogen of the benzoxazole ring forming a six-membered ring.²⁷⁾ The absence of νOH in the complexes of Zn(II) (**2a–c**) and Cu(II) (**2f**) complexes suggested the deprotonation of the enolic–OH and its co-ordination through the O atom. Moreover, the $\nu\text{C}=\text{N}$ mode which occurred at 1644 cm^{-1} in the ligand **1a** was shifted in the complexes. This shifting of $\nu\text{C}=\text{N}$ towards lower frequency ($1580\text{--}1610\text{ cm}^{-1}$) suggested that the co-ordination of the ligands occurred through the deprotonated oxygen of the enol–OH group and nitrogen of the benzoxazole.²⁷⁾ However, the existence of water of hydration in the Mg(II) (**2d**), Pd(II) (**2g**), Ag(I) (**2h**) complexes or coordination in the Ni(II) (**2e**) complex causes difficulty in drawing conclusions from νOH band for the hydroxy groups of the free ligand **1a** which would overlap with those of water molecules. In the Ni(II) complex (**2e**), the absorption band at *ca.* 3100 cm^{-1} ($\nu(\text{O}–\text{H})$) disappeared, indicating the de-protonation of the enol after coordination Ni(II), while the emerging band at 3400 cm^{-1} was attributed to the O–H stretch of the coordinated water. Further conclusive evidence of the co-ordination of the ligands with the metals was confirmed by the appearance of weak low frequency bands at $480\text{--}510$ and $345\text{--}370\text{ cm}^{-1}$ due to metal–oxygen and metal–nitrogen stretching vibrations in the metal complexes and not observable in the spectra of the ligands **1a–c**.²⁸⁾

The Ni(II) (**2e**) and Pd(II) (**2g**) complexes would be paramagnetic and their ^1H -NMR spectra were appeared at wide range. The ^1H - and ^{13}C -NMR spectra of the ligands and dia-

Chart 1. Synthesis of Three Ligands **1a–c** and Their Zn(II) Complexes **2a–c**

Table 1. The Structures and Yields of Metal Complexes **2d–h**

2d : M=Mg(II)		2h	
2e : M=Ni(II)			
2f : M=Cu(II)			
2g : M=Pd(II)			
2	Metal salts (3.0 eq)	Time (h)	Yield (%)
d	MgBr_2	3	88
e	$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	6	82
f	$\text{Cu}(\text{OAc})_2 \cdot \text{H}_2\text{O}$	24	25
g	PdCl_2	3	23
h	Ag_2CO_3	3	30

magnetic Zn(II) (**2a–c**), Mg(II) (**2d**), Cu(II) (**2f**), Ag(I) (**2h**) complexes were obtained. The $^1\text{H-NMR}$ spectra of the Zn(II) complexes **2a–c**, assumed to have pseudotetrahedral coordination, affirming the simple spectrum expected for the ligand when all ligands are geometrically equivalent. The signal assigned to vinyl protons in $^1\text{H-NMR}$ spectra of three ligands **1a–c** and their Zn(II) complexes **2a–c** were appeared at a singlet between $\delta=5.86\text{--}6.07$ and $\delta=5.98\text{--}6.11$, respectively. The most notable changes were observed in the benzoxazole ring signals, particularly those assigned to the atoms of C2 ($\delta=163.9\text{--}164.8$ in the three ligands **1a–c**, $\delta=167.3\text{--}167.7$ in three Zn(II) complexes **2a–c**, 170.7 in the Mg(II) complex **2d**, 167.7 in the Ag(I) complex **2h** and C3a ($\delta=133.8\text{--}134.5$ in the three ligands **1a–c**, $\delta=136.8\text{--}137.0$ in three Zn(II) complexes **2a–c**, 140.4 in the Mg(II) complex **2d**, 141.7 in the Ag(I) complex **2h**).

The UV-Vis spectra data for compounds **1a–c** and **2a–h** in MeOH are summarized in Table 2. The major absorption bands of three ligands **1a–c** and all metal complexes **2a–h** are all around at 240 nm and $311\text{--}336\text{ nm}$, which were mainly unaffected (Fig. S2). Peaks at $311\text{--}336\text{ nm}$ can be attributed to the $\pi\text{--}\pi^*$ transitions due to the conjugated enol double bond. The molar absorption coefficient (ϵ) of metal complexes **2a–h** were much higher than those of the free ligands **1a–c**.

Table 2. UV λ_{max} (nm) and ϵ Values of **1a–c** and **2a–h** in MeOH

Compd.	λ_{max} (nm)	ϵ ($\text{M}^{-1} \text{ cm}^{-1}$)
1a	326.5	5620
1b	333	10561
1c	320.5	1782
2a	312	47418
2b	316	46323
2c	317	33061
2d	311	40609
2e	317	38663
2f	315.5	38921
2g	335.5	— ^{a)}
2h	311.5	36049

^{a)} The ϵ value of **2g** was not determined due to the low solubility.

The molecular and crystal structures of six metal complexes **2b** and **2d–h** were determined by single-crystal X-ray diffraction analysis and are shown in Fig. 1. Their crystallographic data, including selected bond lengths [$\text{\AA}$] and bond angles [$^\circ$], were presented in Tables S1–8. Metal–ligand bond lengths and angles for **2b** and **2d–h** are in the range of values reported for other metal N–O systems.^{29–39)}

In the Zn(II) complex **2b**, the geometry at the Zn(II) center can be best described as a distorted tetrahedron in which two benzoxazole-2-chlorodifluoroacetate (**1b**) ligands are placed in a similar disposition about the zinc center.^{29–31)} One coordination plane (N–O–Zn) in **2b** is approximately perpendicular to the other with a dihedral angle of 89.2° . The molecular structure of **2b** was incorporated with hexane molecule. In the Mg(II) complex **2d**, the central Mg(II) atom was coordinated by two N atoms and two O atoms from two **1a** bidentate ligands and the axial sites were occupied by two coordinated acetonitrile molecules, forming a distorted N_4O_2 octahedral geometry.³²⁾ The nitrogen atoms of the benzoxazole ring situated mutually in the *trans* position. The Ni(II) complex **2e** exhibits a distorted octahedral geometry, in which two **1a** ligands are located in the equatorial plane, forming two stable six-membered rings with metal ion, and the axial sites are occupied by two coordinated water molecules.^{33,34)} In the Cu(II) complex **2f**, the Cu(II) atom was coordinated by two N atoms and two O atoms from two **1a**, forming a strictly square planar geometry (dihedral angle between two N–Cu–O planes: 0°).^{35–37)} In the Pd(II) complex **2g**, the geometry at Pd(II) center was coordinated as nearly perfect square planar which are similar to that of the Cu(II) atom in **2f**.³⁸⁾ The crystal structure of Ag(I) complex **2h** revealed to consist a 1:1 metal–ligand molar ratio and the Ag(I) ion exhibits a distorted trigonal pyramidal geometry by two **1a** and the another Ag(I) atom, forming a 4-membered parallelogram Ag_2O_2 ring.³⁹⁾ Thus, the complex **2h** crystallizes in a dimeric form with two $[\text{Ag}_2\text{O}_2]$ units held together by an Ag–Ag contact of $3.1340(4)\text{ \AA}$ which is longer than the Ag–Ag one in metallic silver ($2.88\text{ \AA}$), but much shorter than the sum of the van der Waals radii of two Ag(0) atom ($3.44\text{ \AA}$), implying the existence of $d^{10}\text{--}d^{10}$ interactions between two closed-shell Ag(I) ions. Such argentophilic

Fig. 1. Molecular Structures of **2b** and **2d–2h** Complexes Obtained by X-Ray Diffraction Analysis

(Color figure can be accessed in the online version.)

interactions have been observed in some multinuclear Ag(I) complexes.^{40,41} The crystal of **2h** formed a zigzag structure along the crystallographic *a* axis, with a pitch of 4.4377(3) Å (Fig. 2).

In Vitro Antibacterial Evaluation The synthesized compounds **1a–c** and **2a–h** were evaluated for their antimicrobial activities *in vitro* six G(+) bacteria of *Bacillus subtilis* (Bs), *Enterococcus faecalis* (Ef), *Staphylococcus aureus* (Sas), Methicillin-resistant *Staphylococcus aureus* (Sar), *Streptococcus pneumoniae* (Spn), and *Streptococcus pyogenes* (Spy) and six G(–) bacteria of *Acinetobacter baumannii* (Ab), *Escherichia coli* (Ec), *Klebsiella pneumoniae* (Kp), *Pseudomonas aeruginosa* (Pa), *Serratia marcescens* (Sm), and *Vibrio parahaemolyticus* (Vp). The minimum inhibitory concentrations (MIC, μM) were determined by means of standard serial dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS)⁴² and are presented in Table 3. Currently available antimicrobial drugs Kanamycin, Gentamicin, Linezolid, or Norfloxacin were used as the positive

Fig. 2. The Zig-Zag Structure of Ag(I) Complex **2h**

(Color figure can be accessed in the online version.)

control. In addition, AgNO_3 was also evaluated for its antimicrobial activities in order to compare with those of the Ag(I) complex **2h**. The MIC value of **1a** against Ec was already reported to be $8\mu\text{g/mL}$ ($34\mu\text{M}$).¹⁷ In order to assess the effect of three ligands and their metal complexes on the antibacterial activity, the three ligands **1a–c** and their Zn(II) complexes **2a–c** were first evaluated for *in vitro* antibacterial activity against six G(+) and six G(–) bacteria. The data revealed that the complexes **2a** and **b** showed more potent antibacterial activities against not only G(–) bacteria but also G(+) bacteria than the ligands **1a** and **b**, respectively. However, **2c** and **1c** showed higher MIC values against both G(–) and G(+) bacteria than **2a**, **b** or **1a**, **b**, respectively.

Among all the tested metal complexes **2a–h**, the bioactivity against the tested G(+) bacteria for various complexes decreased in the sequence $\text{Ag} > \text{Zn} > \text{Cu}$, $\text{Ni} > \text{1a} > \text{Mg} > \text{Pd}$. On the other hand, the bioactivity against the tested G(–) bacteria for various complexes decreased in the sequence $\text{Ag} > \text{Zn} > \text{1a} > \text{Cu}$, $\text{Ni} > \text{Mg}$, Pd . The Ag(I) complex **2h** showed broad antibacterial spectrum and potent antibacterial activities in comparison with the other metal complexes. In addition, all the MIC values estimated for the **2h** are considerably lower than AgNO_3 . Particularly, the **2h** was found to exhibit the strongest inhibitory activity against G(–) bacteria, such as Pa, Ab, Ec, Kp, Sm, and Vp, with MIC values of 0.7, 3, 7, 3, 7, and $3\mu\text{M}$, respectively. It is noted that the **2h** was the only one to display outstanding effect against Pa (MIC = $0.7\mu\text{M}$) and was found to be considerably stronger than the inhibitory concentration of the benchmark drug, Norfloxacin with MIC = $1.5\mu\text{M}$. On the other hand, Zn(II) complexes **2a** and **b** exhibited the strongest inhibitory activities against Bs, Sas, and Spn with MIC values of 2– $4\mu\text{M}$.

Generally, G(+) bacteria are more susceptible to the antibacterial effects of the ligands **1a–c** and the metal complexes **2a–g** than G(–) bacteria. On the other hand, G(–) bacteria are more susceptible to the Ag(I) complex **2h** and AgNO_3 than

Table 3. Antibacterial Activity of Compounds **1a–1c** and **2a–2h** (MIC in μM)^{a)}

Compd.	MIC (μM)											
	G(+) bacteria						G(–) bacteria					
	Bs	Ef	Sas	Sar	Spn	Spy	Ab	Ec	Kp	Pa	Sm	Vp
1a	9	34	17	17	17	≥ 280	69	34^b	69	≥ 280	140	8
1b	4	32	16	8	8	26	32	32	65	≥ 522	130	8
1c	19	75	75	75	37	75	75	37	75	303	75	18
2a	2	15	7	7	4	30	30	15	15	≥ 122	30	3
2b	2	28	2	14	4	15	28	28	28	≥ 115	57	3
2c	8	131	65	32	16	≥ 131	65	65	≥ 131	≥ 131	≥ 131	16
2d	8	67	17	33	33	≥ 133	≥ 133	67	≥ 133	≥ 133	≥ 133	8
2e	4	31	15	15	7	63	62	31	62	≥ 124	≥ 124	3
2f	4	30	15	15	4	31	61	30	61	≥ 123	≥ 123	3
2g	57	≥ 114	≥ 114	≥ 114	≥ 114	≥ 114	≥ 113	≥ 113	≥ 113	≥ 113	≥ 113	56
2h	4	7	7	7	28	56	3	7	3	0.7	7	3
Kanamycin	ND	ND	ND	ND	ND	ND	8	4	8	≥ 132	66	4
Gentamicin	0.5	67	2	1	8	16	4	1	2	16	2	4
Linezolid	ND	ND	ND	3	ND	ND	ND	ND	ND	ND	ND	ND
Norfloxacin	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.5	ND	ND
AgNO_3	11	47	94	94	189	94	11	11	11	23	23	11

a) Bs: *B. subtilis* ATCC6633, Ef: *E. faecalis* IID622, Sas: *S. aureus* 209P (MSSA), Sar: *S. aureus* N315 (MRSA), Spn: *S. pneumoniae* IID555, Spy: *S. pyogenes* 244, Ab: *A. baumannii* ATCC17978, Ec: *E. coli* TG1, Kp: *K. pneumoniae* IID5209, Pa: *P. aeruginosa* O1, Sm: *S. marcescens*, Vp: *V. parahaemolyticus* RIMD221051. b) The data was previously reported in Ref. 17.

Fig. 3. (A) Time-Kill Curve of **2h** and AgNO_3 against *P. aeruginosa*; (B) Growth Profile of *P. aeruginosa* in Muller-Hinton Broth, Treated with 0 (○) and 1 (●), 2 (△), 4 (▲), 8 (□)×MIC of **2h** and 4×MIC (■) of AgNO_3

Fig. 4. The Scanning Electron Microscopy (SEM) Photography of *P. aeruginosa*

Indicated photographs are an electron micrograph of untreated cells (a) and cells treated with 2×(b), 5×(c), 10×(d) MIC of the Ag(I) complex **2h** for 1 h at 37°C. The arrows represent the observed small morphological changes. The scale bar indicates 2 μm. Independent experiments were carried out three times and a similar result was provided.

G(+) bacteria, presumably due to their thinner wall, which may allow more rapid absorption or adhesion of the silver into the cell.^{43–45} The cell wall of G(–) bacteria contains lipopolysaccharide (LPS) as the outer leaflet of the outer membrane bilayer. However, the G(+) bacteria are surrounded by a thick peptidoglycan layer, which explains the elevated activity of the metal complex over G(–) bacteria than G(+) bacteria species.

Time-kill studies were performed with 10^7 colony-forming units (CFU/mL) of Pa at baseline (Fig. 3A). Pa was incubated in the presence of the Ag(I) complex **2h** at 0, 1, 2, 4 and 8 times respective MIC (MIC=0.7 μM) and the number of surviving bacteria were determined by colony counting. The data are judged relative to the conventional definition of bactericidal activity, that is, a 3 log₁₀ CFU/mL or greater reduction in the initial inoculum by 24 h.^{42,46}

In growth curve studies (Fig. 3B), no influence was observed on the growth of Pa in liquid medium supplemented with up to 2 times the MIC of **2h**. However, Pa was submitted to 8 times the MIC of **2h**, the growth was suppressed until 12 h and then the regrowth occurred. This regrowth was attributed to adaptation,^{47,48} because the doubling rate of the cell collected after 24 h in the experiment of 8 times MIC of **2h** was the same as that for the growth curve when the same experiment was repeated at 8 times MIC of **2h**. This bacterial regrowth was not observed for AgNO_3 . We conclude that **2h** is bacteriostatic against Pa (<3 log₁₀ CFU/mL of drop). On the other hand, AgNO_3 exhibited bactericidal activity against Pa with MIC of 23 μM, demonstrating killing of approximately 3 to 4 log units. It is also reported that Ag(I)-coated carbon nanotubes are bactericidal against Pa.⁴⁹

Considering that some Ag complexes can interfere with

the cell wall synthesis,^{50,51)} scanning electron microscopy (SEM) was used to visualize the outer surface change of Pa in the presence of the Ag(I) complex **2h**. Pa cell morphology changes were examined after 1 h treatment of exponential phase bacteria (O.D.₆₀₀ of about 1.4) with 2, 5, 10×MIC of **2h** (Fig. 4). When 2×MIC of **2h** was allowed to act, small morphological changes (marked by the arrow) were observed on the cell surface as compared with the control (Figs. 4a, b). Furthermore, obvious morphologic alternations on the cell surface, irregular, pitted, and shriveled, were observed in the cells treated with the concentration of more than 5×MIC of **2h** (Figs. 4c, d). The morphological alternations on the surface might result in the leakage of contents of the cells. These images suggest a probable mechanism of action whereby the Ag(I) complex **2h** interacts with the cell membrane, causing membrane permeabilization and degradation, with accompanying changes in cell morphology.

Next, in order to assess the ability of Pa to develop resistance to the Ag(I) complex **2h**, we exposed a standard strain of Pa to liquid medium supplemented with 4×MIC of **2h**. We passed the strain 4 times in the same concentration medium and passaged to stepwise increasing concentration of **2h**. The MIC of **2h** for each passage of Pa was measured, but it showed the same MIC as the standard strain. Furthermore, we cultured a standard strain of Pa (4×10^9 CFU cells) on an agar medium supplemented with 4 and 8×MIC of **2h** and isolated several candidate strains growing on the agar medium. The MIC of **2h** for these strains was measured, but it showed the same MIC as the standard strain. These assay indicates that Pa do not develop significant resistance against **2h**, as easily as the bacteria do against Norfloxacin.⁵²⁾

Indeed, bacterial resistance to silver has been rarely reported.^{53,54)} The reason why **2h** is less likely to develop resistance may be that the multiple actions of the Ag complex make it difficult for bacteria to simultaneously employ existing strategies for resistance. The probable mechanisms of action of Ag(I) ions are reported to include binding to DNA, interaction with the cell membrane, interference with the electron transport chain and reaction with the thiol group in vital enzymes.^{50,51)} However, we cannot exclude the conclusion that antibacterial activity is connected to silver ion release.⁵⁵⁾

Conclusion

The 2-trifluoroacetylbenzoxazole ligand (**1a**) coordinates with monovalent Ag(I) metal in 1:1 and divalent metals [Zn(II), Mg(II), Ni(II), Cu(II), and Pd(II)] in 1:2 metal–ligand ratios, as confirmed by IR, MS and X-ray. In the antimicrobial screening, it has been observed that the ligands were found to be biologically active and the antibacterial activity increased on complexation with Ag(I) or Zn(II). Such increased activity of the metal complexes can be explained on the basis of Overtone concept and Tweedy chelation theory.^{56,57)} According to the overtone concept of cell permeability, the lipid membrane that surrounds the cell favors the passage of only lipid-soluble materials; therefore, lipophilicity is an important factor which controls the antibacterial activity. On chelation, polarity of the metal ion is reduced to a greater extent due to the overlapping of ligand orbital and partial sharing of the positive charge of the metal ion with donor groups. Moreover, delocalization of the π -electrons over the whole chelate ring is increased and lipophilicity enhances the penetration of the complexes into

the lipid membranes and blocks the metal binding sites in the enzymes of microorganisms. Therefore, a significant increase in antimicrobial activity with Ag(I) complex can be explained on the basis of the above described Overtone concept and Tweedy chelation theory.

The Ag(I) complex **2h** displayed significant antibacterial activities. In particular, the inhibition against Pa was observed at low micromolar concentration, which was found to be considerably stronger than the inhibitory concentration of the benchmark drug, Norfloxacin with MIC=1.5 μ M. The **2h** is bacteriostatic and exerts the cell surface damage against Pa. Serial transfer of Pa on drug gradient plates produced no evidence of rapid resistance development. Compared to small-molecular antibiotics, the Ag(I) complex may be less likely to develop resistance partly because multiple actions of Ag(I) complex may not be easily overcome by strategies employed by bacteria to develop resistance simultaneously. These results can be utilized for developing a new class of novel antibiotics.⁷⁾ Furthermore, it guides us to synthesize some analogs of substituted benzoxazole ligands and their metal complexes and to study their biological activities.

Experimental

General Remarks All melting points were determined using a Yanagimoto hot-stage melting point apparatus and are uncorrected. ¹H-NMR and ¹³C-NMR spectra were measured on Bruker AVANCE500 (500 MHz for ¹H and 126 MHz for ¹³C) spectrometer. Both ¹H- and ¹³C-NMR spectral data are reported in parts per million (δ) relative to tetramethylsilane (Me₄Si). IR spectra were recorded on a JASCO FT/IR-4100 spectrometer. Low- and high-resolution (LR/HR)-MS were obtained with a JEOL JMS-GC mate II or a JEOL JMS-700 spectrometer with a direct inlet system at 70 eV. X-ray crystallographic data were recorded on a Rigaku VariMax SaturnCCD724/ α diffractometer using graphite monochromated Mo-K α radiation at the Advanced Research Support Center, Ehime University.

Materials and Methods Dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as solvent control. Kanamycin (Wako Pure Chemical Industries, Ltd.), Gentamicin (Wako Pure Chemical Industries, Ltd.), Linezolid (Wako Pure Chemical Industries, Ltd.), Norfloxacin (Wako Pure Chemical Industries, Ltd.) and AgNO₃ (Nacalai Tesque, Kyoto, Japan) were used as standard drugs. (Z)-1-(Benzo[d]oxazol-2-yl)-3,3,3-trifluoroprop-1-en-2-ol (**1a**) was prepared by the literature method.²⁴⁾ **1a**: mp 160–162°C (mp²⁴⁾ 165°C).

Procedures for the Preparation of Benzoxazoles and Their Zinc(II) Complexes

(Z)-1-(Benzo[d]oxazol-2-yl)-3-chloro-3,3-difluoroprop-1-en-2-ol (**1b**)

Chlorodifluoroacetic anhydride (23.4 mL, 138.6 mmol) was added dropwise to a solution of 2-methylbenzoxazole (5.4 mL, 46.2 mmol) and pyridine (18 mL, 230.7 mmol) in anhydrous toluene (60 mL) at 0°C. The mixture was stirred at room temperature for 24 h and diluted with 3% Na₂CO₃ (80 mL) and extracted with EtOAc (80 mL)×3, washed brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. Recrystallization gave the pure product (**1b**). Brown solid, 81% yield. mp 182°C. IR (KBr) 3191, 1642, 1619, 1576, 1298, 1198, 1141, 1094, 975, 955, 831, 748, 718, 500 cm⁻¹;

$^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 5.98 (s, 1H, CH), 7.26–7.39 (m, 2H, ArH), 7.50 (t, $J=9.2$ Hz, 2H, ArH); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 78.5, (t, $^3J_{\text{C-F}}=3.4$ Hz, CH), 120.8, 114.9, 121.7 (t, $^1J_{\text{C-F}}=295.4$ Hz, CF), 125.0, 125.7, 133.1, 147.4, 164.8, 169.4 (t, $^2J_{\text{C-F}}=29.3$ Hz, CO); LR-MS (electron ionization (EI) $^+$): $m/z=245$ (M^+ , 13.7), 160 (100); HR-MS (EI) Calcd for $\text{C}_{10}\text{H}_6\text{ClF}_2\text{NO}_2$ (M^+): 245.0055. Found: 245.0047.

Bis((Z)-1-(benzo[d]oxazol-2-yl)-3,3,3-trifluoroprop-1-en-2-ate)zinc(II) (**2a**)

A mixture of **1a** (245.5 mg, 1 mmol) and zinc dust (196.6 mg, 3 mmol) in CH_3CN (5 mL) was stirred for 24 h at 80°C . After being cooled to room temperature, the reaction mixture was filtered through a pad of Celite and the filtrate was concentrated *in vacuo*. Column chromatography of the crude product on silica gel eluting with hexane–ethyl acetate provided a pure product (**2a**). Colorless solid, 78% yield. mp 201°C . IR (KBr) 3096, 1594, 1562, 1461, 1431, 1328, 1232, 1204, 1137, 1121, 1021, 881, 754, 947, 718 cm^{-1} ; $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 6.11 (s, 1H, CH), 7.18–7.34 (m, 3H, ArH), 7.52 (dd, 1H, $J=7.6$ and 7.3 Hz, ArH); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$) δ : 81.1, 110.9, 115.7, 119.4 (q, $^1J_{\text{C-F}}=279.6$ Hz, CF), 125.5, 125.8, 136.7, 147.6, 166.9 (q, $^2J_{\text{C-F}}=33.6$ Hz, CO), 167.2; LR-MS (EI) $^+$: $m/z=520$ (M^+ , 3.7), 160 (100); HR-MS (EI) Calcd for $\text{C}_{20}\text{H}_{10}\text{F}_6\text{N}_2\text{O}_4\text{Zn}$ (M^+): 519.9836. Found: 519.9825.

Bis((Z)-1-(benzo[d]oxazol-2-yl)-3-chloro-3,3-difluoroprop-1-en-2-ate)zinc(II) (**2b**)

A mixture of **1b** (245.5 mg, 1 mmol) and zinc dust (196.6 mg, 3 mmol) in nitromethane was stirred for 24 h at 80°C . After being cooled, the reaction mixture was filtered through a pad of Celite and the filtrate was concentrated *in vacuo*. Column chromatography of the crude product on silica gel eluting with hexane–ethyl acetate provided pure product (**2b**). Colorless solid, 98% yield. mp 165°C . IR (KBr) 3136, 3106, 3077, 2922, 2852, 1578, 1549, 1459, 1424, 1310, 1205, 1112, 1024, 962, 931, 831, 771, 744, 727, 678, 635, 522 cm^{-1} ; $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 6.09 (s, 1H, CH), 7.22–7.34 (m, 3H, ArH), 7.52 (dd, $J=7.2$ and 6.9 Hz, 1H, ArH); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 79.4 (t, $^3J_{\text{C-F}}=3.6$ Hz, CH), 110.9, 115.9, 122.8 (t, $^1J_{\text{C-F}}=298.5$ Hz, CF), 125.5, 125.8, 136.8, 147.8, 167.4, 169.9 (t, $^2J_{\text{C-F}}=27.6$ Hz, CO); LR-MS (EI) $^+$: $m/z=552$ (M^+ , 16.0), 159 (100); HR-MS (EI) Calcd for $\text{C}_{20}\text{H}_{10}\text{Cl}_2\text{F}_4\text{N}_2\text{O}_4\text{Zn}$ (M^+): 551.9245. Found: 551.9230.

Bis((Z)-1-(benzo[d]oxazol-2-yl)-3,3-difluoroprop-1-en-2-ate)-zinc(II) (**2c**)

A mixture of **1b** (245.5 mg, 1 mmol) and zinc dust (196.6 mg, 3 mmol) in THF (5 mL) was stirred for 24 h at 80°C . After being cooled to room temperature, the reaction mixture was filtered through a pad of Celite and the filtrate was concentrated *in vacuo*. Column chromatography of the crude product on silica gel eluting with hexane–ethyl acetate provided a pure product (**2c**). Colorless solid, 97% yield. mp 183°C . IR (KBr) 1590, 1557, 1432, 1297, 1121, 765 cm^{-1} ; $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 5.98 (s, 1H, CH), 6.05 (t, $J=55$ Hz, CF_2H), 7.15 (d, $J=7.5$ Hz, 1H, ArH), 7.23–7.30 (m, 2H, ArH), 7.50 (d, $J=7$ Hz, 1H, ArH); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 80.3, 110.7, 111.5 (t, $^1J_{\text{C-F}}=245.3$ Hz, CF), 115.7, 125.0, 125.6, 137.1, 147.6, 167.7, 173.1 (t, $^2J_{\text{C-F}}=22.5$ Hz, CO); LR-MS (EI) $^+$: $m/z=484$ (M^+ , 100); HR-MS (EI) Calcd for $\text{C}_{20}\text{H}_{12}\text{F}_4\text{N}_2\text{O}_4\text{Zn}$ (M^+): 484.0024. Found: 484.0001.

(Z)-1-(Benzo[d]oxazol-2-yl)-3,3-difluoroprop-1-en-2-ol (**1c**)

A mixture of **2b** (242.8 mg, 0.5 mmol) and EDTA·2Na·2H₂O

(1.7 g, 5 mmol) in EtOH (5 mL) was stirred for 6 h at 80°C . After being cooled to room temperature, the reaction mixture was diluted with 3% NaOH (30 mL) and extracted with EtOAc (30 mL×3), washed brine, dried over Na_2SO_4 , filtered and concentrated under reduced pressure. Column chromatography of the crude product on silica gel eluting with hexane–ethyl acetate provided a pure product (**1c**). White solid, 66% yield. mp $133\text{--}135^\circ\text{C}$. IR (KBr) 3109, 1638, 1553, 1446, 1347, 1296, 1251, 1190, 1163, 1094, 1069, 1005, 977, 813, 740, 484 cm^{-1} ; $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 5.26 (brs, 1H, OH), 5.86 (s, 1H, CH), 6.36 (t, $J=54$ Hz, 1H, CF_2H), 7.32–7.41 (m, 2H, ArH), 7.60 (d, $J=6.9$ Hz, ArH); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 79.1 (t, $^3J_{\text{C-F}}=3.4$ Hz, CH), 111.0, 111.3 (t, $^1J_{\text{C-F}}=243.0$ Hz, CF_2H), 115.2, 124.9, 125.8, 133.8, 146.9, 163.9, 172.9 (t, $^2J_{\text{C-F}}=23.0$ Hz, CO); LR-MS (EI) $^+$: $m/z=211$ (M^+ , 49.4), 160 (100); HR-MS (EI) Calcd for $\text{C}_{10}\text{H}_7\text{F}_2\text{NO}_2$ (M^+): 211.0445. Found: 211.0450.

General Procedure for Synthesis of Metal Complexes (**2d–h**)

A mixture of **1a** (229 mg, 1 mmol), metal salts (3 mmol) in THF (5 mL) was stirred at room temperature. Reaction time was described in Table 1. The reaction mixture was filtered through a Celite and filtrate was concentrated under vacuum. The resulting product was recrystallized several times from CHCl_3 and hexane. Or chromatography (hexane/AcOEt) on silica gel gave the pure product.

Bis((Z)-1-(benzo[d]oxazol-2-yl)-3,3,3-trifluoroprop-1-en-2-ate)magnesium(II)·2(MeCN) (**2d**)

Purified by chromatography on silica gel. Colorless solid, 88% yield. mp $274\text{--}275^\circ\text{C}$. IR (KBr) 3173, 1603, 1552, 1429, 1317, 1232, 1188, 1127, 874, 778, 751 cm^{-1} ; $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ : 5.73 (s, 1H, CH), 7.20–7.24 (m, 2H, ArH), 7.53–7.55 (m, 1H, ArH), 7.87 (brs, 1H, ArH); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$) δ : 79.1, 110.2, 118.3, 120.6 (q, $^1J_{\text{C-F}}=284.4$ Hz, CF), 124.0, 124.6, 140.4, 148.2, 163.9 (q, $^2J_{\text{C-F}}=31.5$ Hz, CO), 166.9; LR-MS (EI) $^+$: $m/z=480$ (M^+ , 100); HR-MS (EI) Calcd for $\text{C}_{20}\text{H}_{10}\text{F}_6\text{MgN}_2\text{O}_4$ (M^+): 480.0395. Found: 480.0389.

Bis((Z)-1-(benzo[d]oxazol-2-yl)-3,3,3-trifluoroprop-1-en-2-ate)nickel(II)·2H₂O (**2e**)

Purified by chromatography on silica gel. Dark green solid, 82% yield. mp $172\text{--}173^\circ\text{C}$. IR (KBr) 3399, 1595, 1560, 1458, 1429, 1318, 1231, 1186, 1129, 876, 775, 749 cm^{-1} . The compound exhibit ferromagnetism and unmeasurable by $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$. LR-MS (EI) $^+$: $m/z=514$ (M^+ , 88.9), 160 (100); HR-MS (EI) Calcd for $\text{C}_{20}\text{H}_{10}\text{F}_6\text{N}_2\text{NiO}_4$ (M^+): 513.9898. Found: 513.9870.

Bis((Z)-1-(benzo[d]oxazol-2-yl)-3,3,3-trifluoroprop-1-en-2-ate)copper(II) (**2f**)

Purified by chromatography on silica gel. Brown solid, 25% yield. mp $214\text{--}215^\circ\text{C}$. IR (KBr) 3162, 1609, 1554, 1430, 1347, 1330, 1235, 1186, 1143, 1021, 933, 883, 782, 758 cm^{-1} ; $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 5.19 (brs, 1H), 6.44 (brs, 1H). $^{13}\text{C-NMR}$ signals could not be unambiguously assigned due to a set of broad signals for aromatic carbons. LR-MS (EI) $^+$: $m/z=519$ (M^+ , 93.7), 291 (100); HR-MS (EI) Calcd for $\text{C}_{20}\text{H}_{10}\text{CuF}_6\text{N}_2\text{O}_4$ (M^+): 518.9841. Found: 517.9837.

Bis((Z)-1-(benzo[d]oxazol-2-yl)-3,3,3-trifluoroprop-1-en-2-ate)palladium(II) (**2g**)

Washed by CHCl_3 and hexane. Orange solid, 23% yield. mp $200\text{--}204^\circ\text{C}$. IR (KBr) 3466, 1604, 1557, 1427, 1344, 1235, 1207, 1136, 1123, 895, 775, 753 cm^{-1} . $^1\text{H-NMR}$ and

^{13}C -NMR were unmeasurable. LR-MS (EI^+): $m/z=562$ (M^+ , 100); HR-MS (EI) Calcd for $\text{C}_{20}\text{H}_{10}\text{F}_6\text{N}_2\text{O}_4\text{Pd}$ (M^+): 561.9580. Found: 561.9580.

Silver(I) (Z)-1-(benzo[*d*]oxazol-2-yl)-3,3,3-trifluoroprop-1-en-2-ate (**2h**)

Recrystallized by CHCl_3 and hexane. Yellow solid, 30% yield. mp 209°C . IR (KBr) 3434, 1684, 1595, 1556, 1458, 1438, 1250, 1225, 1184, 1123, 1103, 863, 768, 749 cm^{-1} ; ^1H -NMR (500 MHz, $\text{DMSO}-d_6$) δ : 5.36 (s, 1H, CH), 7.19 (t, $J=7.5\text{ Hz}$, ArH), 7.25 (t, $J=7.6\text{ Hz}$, ArH), 7.52 (dd, $J=16.9$ and 16.9 Hz , ArH); ^{13}C -NMR (125 MHz, $\text{DMSO}-d_6$) δ : 74.2, 109.9, 116.9, 119.9 (q, $^1J_{\text{C-F}}=287.3\text{ Hz}$, CF), 123.4, 124.5, 141.7, 147.3, 166.3 (q, $^2J_{\text{C-F}}=29.0\text{ Hz}$, CO), 167.7.

The molecular ion peak was not observed by MS. The molecular weight of **2h** was estimated to be 672 as Ag_2L_2 and the MIC value was calculated based on the estimated molecular weight.

X-Ray Single-Crystal Structure Determination The single crystals of **2b**, **e**, **f** and **h** were prepared at room temperature by the vapor diffusion method using two solvents, AcOEt and hexane for **2b** and **f**, acetone and hexane for **2e**, and CHCl_3 and hexane for **2h**, respectively.⁵⁸ The single crystals of **2d** and **g** were recrystallized from acetonitrile or THF, respectively. Their crystal structures were determined by single-crystal X-ray diffraction. Data collection was carried out at 100–296 K on a Rigaku VariMax Saturn CCD724/ α diffractometer using graphite monochromated Mo- $K\alpha$ radiation at the Advanced Research Support Center, Ehime University. Basic information pertaining to crystal parameters and structure refinement is summarized in Table S1-7. Further crystallographic details for the structure reported in this paper may be obtained from the Cambridge Crystallographic Data Center, on quoting the depository numbers CCDC 1574811(**2b**), CCDC 1574812 (**2d**), CCDC 1574814 (**2e**), CCDC 1574815 (**2f**), CCDC 1574816 (**2g**), and CCDC 1574817 (**2h**).

Light Stability of the Ag(I) Complex 2h Light stability of the Ag(I) complex **2h** was studied in direct light under an air atmosphere at room temperature to mimic their exposure to light under every life conditions.²⁶ The positive controls were AgNO_3 and Zn(II) complex (**2a**). Cotton pads were impregnated with 0.025 M aqueous solutions of the compounds and exposed to air and light. The stability was monitored visually within 5 d. The photos of the samples are shown in Fig. S1. The complex **2h** became slightly darkened already after 5 h whereas **2a** remained unaltered until 24 h.

Evaluation of Antibacterial Activity *in Vitro*

Bacterial Strains

Gram-negative bacteria of *Acinetobacter baumannii* ATC C17978, *Escherichia coli* TGI, *Klebsiella pneumoniae* IID5209, *Pseudomonas aeruginosa* O1, *Serratia marcescens* (clinical isolate), and *Vibrio parahaemolyticus* RIMD221051 and Gram-positive bacteria of *Bacillus subtilis* ATCC6633, *Enterococcus faecalis* IID622, *Staphylococcus aureus* FDA209P, methicillin-resistant *Staphylococcus aureus* N315, *Streptococcus pneumoniae* IID555, and *Streptococcus pyogenes* 124 were used in this study.

Antimicrobial Activity

The MICs of all compounds (**1a–c** and **2a–h**) for all 12 strains were determined by the standard two-fold dilution method.⁴² Briefly, the bacterial cells (10^5 CFU/mL) were added into $100\mu\text{L}$ of the compounds dilutions in Muller-

Hinton broth, and then incubated at 37°C for 24 h. The microbial growth was examined by visual inspection. In this assay, all compounds were dissolved in DMSO. The final concentration of DMSO in broth did not affect the microbial growth.

Time–Kill Curve and Growth Curve

Pa cells, in the stationary growth phase, were diluted to $1.0\times 10^7\text{ CFU/mL}$ in Muller Hinton broth (Becton, Dickinson and Company, Sparks, MD, U.S.A.) and incubated at 37°C . The concentrations equivalent to 0, 1, 2, 4 and 8 times MIC of compounds **2h** and 4 times MIC of AgNO_3 were added to cell suspension after 2 h of incubation. In the time–kill curve, CFU/mL after 0, 2, 4, 6, 8, 12, and 24 h of incubation was determined by counting colonies of diluted cell suspension at each time point on Muller Hinton agar plates. On the other hand, in the growth curve, the optical density at 600 nm (O.D._{600}) of the cell suspension was measured over time.⁵⁹

Scanning Electron Microscopy (SEM) Detection

The morphology of Pa after incubation with $0\times$, $2\times$, $5\times$ and $10\times\text{MIC}$ (0, 1, 5 and $10\mu\text{g/mL}$) of Ag(I) complex **2h** was observed under SEM. Pa was aerobically cultured in LB medium at 37°C . The Ag(I) complex **2h** was added when the absorbance at 600 nm was about 1.5 and then aerobically cultured another 1 h at 37°C . One milliliter of Cells was harvested by centrifugation at $21500\times g$ for 30 s and then suspended the cells in 0.5 mL of PBS. The Cells were subsequently fixed by adding 0.5 mL of 5% (v/v) glutaraldehyde (Grade for electron microscope, Wako Pure Chemical Industries, Ltd.) in PBS and then incubated overnight at 4°C . The fixed cells were spotted onto ISOPORETM membrane filter (pore size: $0.2\mu\text{m}$, MILLIPORE), washed with PBS and dehydrated with a graded ethanol series on filter. After lyophilized and platinum coating, the samples were visualized by Hitachi S-3400N Scanning Electron Microscope (Hitachi High-Technologies, Tokyo, Japan).⁶⁰

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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