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New Peptides with Metal Binding Abilities and Their Use as Drug Carriers

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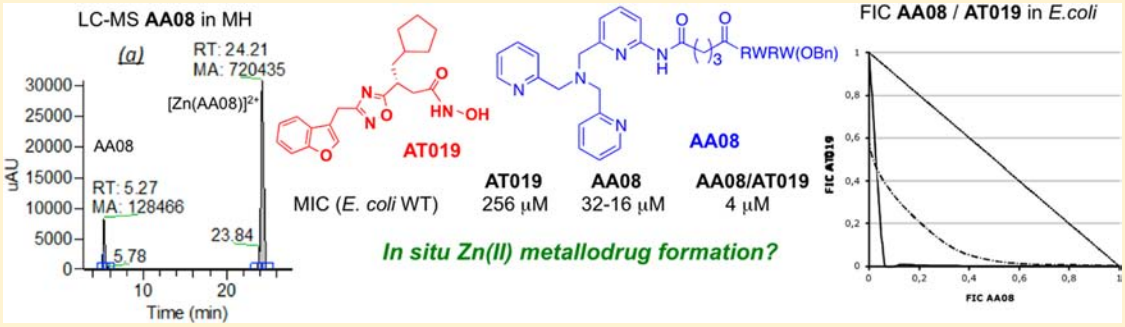
Supporting Information



ABSTRACT: Many new designed molecules that target efficiently *in vitro* bacterial metalloproteases were completely inactive *in cellulo* against Gram negative bacteria. Their activities were limited by the severe restriction of the penetration/diffusion rate through the outer membrane barrier. To bypass this limitation, we have assayed the strategy of metallodrugs, to improve the delivery of hydroxamic acid inhibitors to peptide deformylase. In this metal-chaperone, to facilitate bacterial uptake, the ancillary ligand tris(2-pyridylmethyl)amine (TPA) or di(picolyl)amine (DPA) was functionalized by a tetrapeptide analogue of antimicrobial peptide, RWRW(OBn) (**AA08** with TPA) and/or an efflux pump modulator PAβN (**AA09** with TPA and **AA27** with DPA). We prepared Co(III), Zn(II), and Cu(II) metallodrugs. Using a fluorescent hydroxamic acid, we showed that, in contrast to Cu(II) metallodrugs, Co(III) metallodrugs were stable in the Mueller Hinton (MH) broth during the time required for bacterial assays. The antibacterial activities were determined against *E. coli* strain wild-type (AG100) and *E. coli* strain deleted from *acr*AB efflux pump (AG100A). While none of the PDFinhs used in this study (**SMP289** with an indole scaffold, **AT015** and **AT019** built on a 1,2,4-oxadiazole scaffold) displayed activity higher than 128 μM, all the metallodrugs were active with MICs around 8 μM both against AG100 and AG100A. However, compared to the activities of equimolar combinations of PDFinhs and the free chelating peptides (**AA08**, **AA09**, or **AA27**), they showed similar activities. A synergistic association between **AT019** and **AA08** or **AA09** was determined using the fractional inhibitory concentration with AG100 and AG100A. Combinations of peptides lacking the chelating group with PDFinhs were inefficient. LC-MS analyses showed that the chelating peptides bind Zn(II) cation when incubated in MH broth. These results support the *in situ* formation of a zinc metallodrug, but we failed to detect it by LC-MS in MH. Nevertheless, this chelating peptides metalated with zinc act as permeabilizers which are more efficient than PAβN to facilitate the uptake of PDFinhs by Gram(−) bacteria.

INTRODUCTION

Facilitating intracellular accumulation of molecules into bacterial cells is of substantial interest because of the continuous emergence and dissemination of Multi Drug Resistant (MDR) bacterial pathogens.[1] A rapid overview of the literature shows that many newly designed molecules with high *in vitro* potency only show little *in cellulo* and further *in vivo* activities.[2] One reason for these discrepancies in Gram negative bacteria relies on the severe restriction of penetration/diffusion rate through the outer membrane (OM) barrier: in addition to inner/cytoplasmic membrane, OM drastically reduces the intracellular concentration of active molecules and protects the targeted cells.[3] Besides the low permeability of the OM, resistance also results from the overexpression of efflux systems that extrude the antibacterial agent from the cell before it can reach its target.[4] These last years, we have developed nonpeptide hydroxamic acids as new inhibitors of Peptide Deformylase (PDF), which is recognized as an attractive target for the design and synthesis of new antibacterials.[5,6] These nonpeptide compounds with an indole[7,8] or an oxadiazole scaffold,[9] while highly efficient *in vitro* toward PDF with IC_{50} in the nanomolar range, displayed

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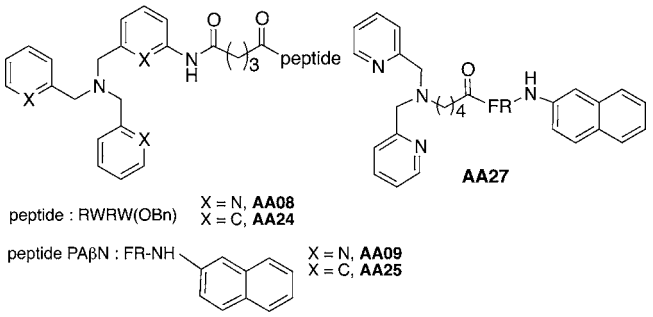
only modest activity against Gram positive bacteria but were totally inactive against Gram negative bacteria such as *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, or *Enterobacter aerogenes*.[9,10] Since these inhibitors target a metalloprotease, we propose to try and increase their antibacterial activities by using metallodrugs[11−14] and, namely, the metal-chaperone strategy, previously described by Hambley et al.[15,16] to improve the delivery of marimastat to matrix metalloprotease in tumor cells.[17] In these metallodrugs there are two partners, the drug and an ancillary ligand bound to a metal center such as Co(III). Recently, some Co complexes were found to possess both antiviral and antibacterial activities.[18] Our strategy to favor cell penetration of these peptide deformylase inhibitors (PDFinhs) is to graft a permeabilizer or an efflux pump modulator on the ancillary ligand. This allows us to address the inhibitor inside the cells without any modification susceptible to PDF affinity. As permeabilizer we first chose antimicrobial peptide (AMP) analogues.[19] AMPs are amphipathic cationic peptides that interact with the negatively charged phospholipids of the bacterial cell membrane, inducing an alteration of the membrane structure.[20] They are supposed to increase the permeability of the OM by formation of pores,[21] and thus, they can favor the penetration of antibiotics inside the cell. Otherwise, we also used PAβN (Phenylalanine arginine-β-naphthylamide), a peptidomimetic compound, with a broad spectrum efflux pump modulator that is routinely used as adjuvant to potentiate the effect of antibiotics, when the resistance is due to efflux pumps.[22] Recently, it has also been reported to permeabilize membranes.[23]

Herein, based on these data, we synthesized and characterized several cobalt metal-chaperones with some of our hydroxamic PDFinhs and tris(2-pyridylmethyl)amine (TPA) ancillary ligand functionalized with a short antimicrobial peptide analogue or with PAβN. To study the effect of the chelating group and of the metal cation, we also prepared similar copper and zinc metallodrugs based on di(picolyl)amine (DPA). The antibacterial activities of these metallodrugs were evaluated against *E. coli* parental strain (AG100) and its derivative (AG100A) deleted from *acr*AB efflux component. To assess the stability of our metallodrugs in the Mueller Hinton culture medium, we prepared equivalent cobalt and copper derivatives with a fluorescent hydroxamic acid. To evaluate the drug carrier ability of these metal-chaperones, their activities were compared with those of the free PDFinhs and of binary systems formed with an equimolar mixture of the PDFinhs and the functionalized ancillary ligands, also named chelating peptides. The results led us to check the inherent ability of these chelating peptides to bind a metal cation in the culture medium and presumably to form *in situ* a metallodrug with the PDFinhs.

RESULTS AND DISCUSSION

Chelating and Nonchelating Peptides. Several chelating peptides were prepared. A first series contains a tris(pyridylmethyl)amine (TPA) metal binding group linked via a glutaryl monoamide spacer to a small tetrapeptide analogue of antimicrobial peptide, RWRW(OBn), with a benzyl ester in C-terminal position for **AA08** and PAβN an efflux pump modulator, for **AA09**. We previously described the synthesis of **AA08**.[24] **AA09** was obtained by coupling PAβN to TPANHCO$(CH_2)_3$COOLi in DMF with HOBt and HBTU as activators in the presence of DIEA as base (Scheme 1; Supporting Information). To determine the contribution of the chelating group to the antibacterial activity of binary systems associating a free chelating peptide and a hydroxamic acid inhibitor, we also prepared, following the same procedure, derivatives **AA24** and **AA25** related to **AA08** and **AA09**, respectively, in which the TPA moiety has been replaced by a tris(benzyl)amine (TBA) with no chelating ability. Then, to study the effect of the chelating group we also synthesized **AA27**, a PAβN peptide linked to di(picolyl)amine (DPA) via a valerate spacer linked to the amine nitrogen of DPA. All the peptides, depicted in Scheme 1, were characterized by ^{1}H NMR and mass spectrometry.

Scheme 1. Peptides Synthesized in This Work



All the syntheses as well as characterizations are detailed in the Supporting Information.

Hydroxamic Acids. **SMP289**, **AT015**, and **AT019** shown in Scheme 2 are inhibitors of *E. coli* PDF loaded with Ni(II). IC_{50} value of **SMP289** is 312 nM[8] and those of **AT015** and **AT019** range around 10 nM. Syntheses and complete biological activities of the whole AT series will be detailed in an another paper.

Synthesis and Characterization of Metallodrugs. Synthesis of Co(III) complexes was achieved by dioxygen oxidation of the corresponding Co(II) species formed upon treatment, in DMF or MeOH, of [Co(II)(AA08)$(Cl)_2$] or [Co(II)(AA09)$(Cl)_2$] with the hydroxamic acid **SMP289**, **AT015**, or **AT019** in the presence of diisopropylethylamine (DIEA). The resulting metallodrugs, shown in Scheme 3, **AA21** [Co(AA08)(SMP289−2H)]$^{3+}$, **AA13** [Co(AA08)(AT015−2H)]$^{3+}$, **AA20** [Co(AA08)(AT019−2H)]$^{3+}$, **AA16** [Co(AA09)(SMP289−2H)]$^{2+}$, **AA14** [Co(AA09)(AT015−2H)]$^{2+}$, and **AA22** [Co(AA09)(AT019−2H)]$^{2+}$ were characterized by mass spectrometry and elemental analysis. In the formula, charges of the complexes were calculated assuming that the arginines in the peptides **AA08** and **AA09** were protonated. We recorded cyclic voltammetry in DMF of two metallodrugs, **AA21** and **AA22**. They displayed an irreversible reduction wave around −700 mV in agreement with the binding of the hydroxamic acid under its hydroximate form. Interestingly, a similar value was obtained for the Co(III)/Co(II) reduction peak of a Co(III) metallodrug model, in which one of the pyridine of the TPA moiety was substituted in *ortho* position by a pivaloylamide, [Co(III)((6-Piva)TPA)-$(CH_3CO{=}NO)$], mimicking a peptide chain.[25]

Syntheses of zinc and copper metallodrugs were performed upon mixing the free chelating peptides **AA09** or **AA27** with $ZnClO_4$ or $CuClO_4$, then with the hydroxamic acids in the presence of DIEA, affording hydroxamate metallodrugs, **AA26** [Zn(AA09)(SMP289-H)]$^{2+}$, **AA39** [Zn(AA09)(AT019-H)]$^{2+}$, **AA28** [Zn(AA27)(AT019-H)]$^{2+}$, and **AA29** [Cu(AA27)-

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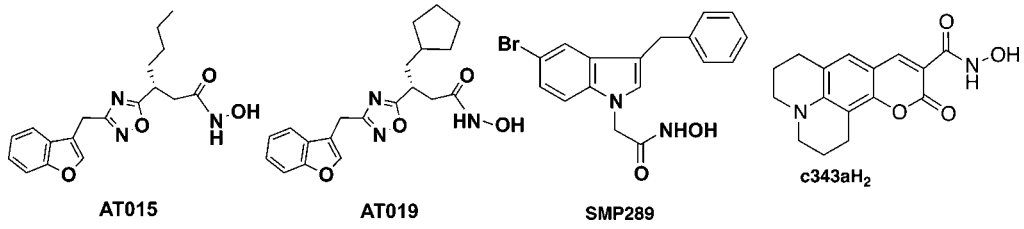
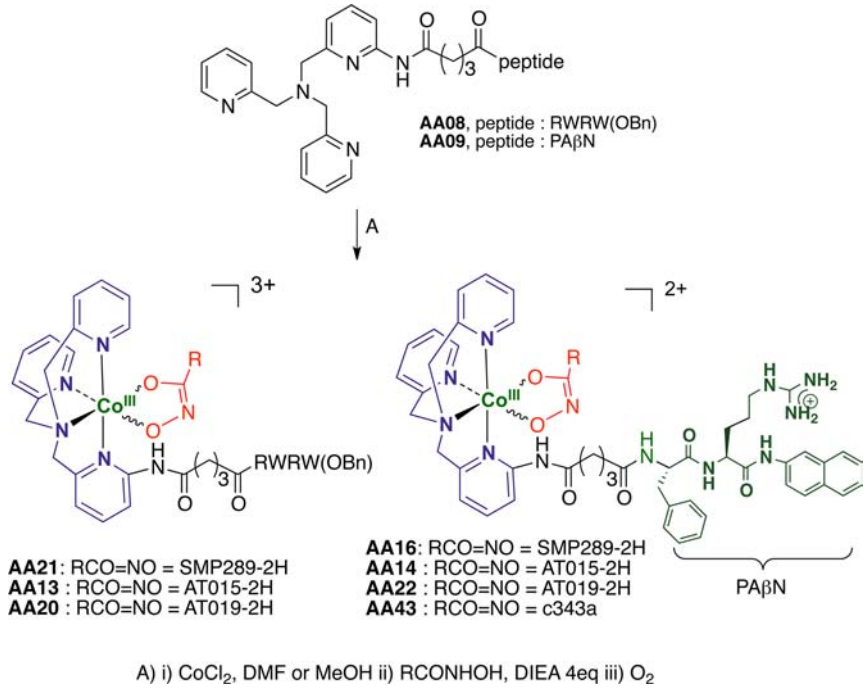
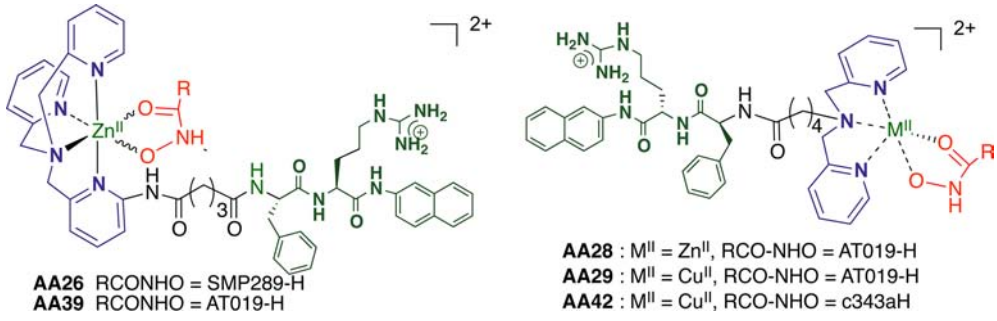
Scheme 2. Hydroxamic Acids Used in This Work



Scheme 3. Synthesis of Co(III) Metallodrugs



Scheme 4. Copper(II) and Zinc(II) Metallodrugs



(AT019-H)]$^{2+}$ (Scheme 4). They were also characterized by mass spectrometry and elemental analysis.

Stability of Metallodrugs in Mueller Hinton Culture Medium. To assess the stability of Co(III) and Cu(II) metallodrugs in the culture conditions, we prepared equivalent Co(III) and Cu(II) complexes with the fluorescent coumarin hydroxamic acid **c343aH$_2$** (Scheme 2) previously described by Hambley et al.[26] The resulting complexes, **AA43** [Co(III)-(AA09)(c343a)]$^{2+}$ and **AA42** [Cu(II)(AA27)(c343aH)]$^{2+}$, were incubated at 5 μM and 37 °C in the Mueller Hinton (MH) medium used for growth conditions. The fluorescence of **c343aH$_2$** was strongly quenched on coordination to Co(III) or Cu(II). The intensity of the emission at 490 nm upon excitation at 435 nm was very low when diluting the cobalt species in the culture medium, and it increased slightly over time, revealing a slow dissociation of **c343aH$_2$** (Figure 1). After 24 h, it reached 30% of the intensity of the free coumarin hydroxamic acid expected after complete release, as obtained upon addition of EDTA in excess, showing that **AA43** was stable during the antibacterial assays. In contrast, the copper metallodrug was not very stable for the biological assays. The coumarin ligand of the copper complex **AA42** was instantly partly dissociated at about 60% upon dilution in the Mueller Hinton medium, then the ratio, copper complex/free ligand remained stable (Figure S1 Supporting Information).

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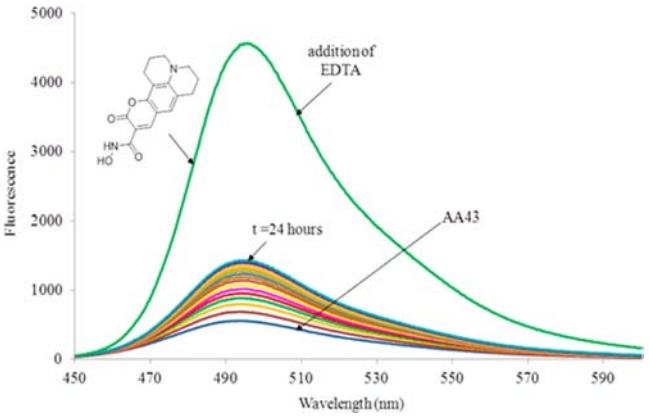

Figure 1. Stability of AA43, $[Co(III)(AA09)(c343a)]^{2+}$, in Mueller Hinton broth as a function of time. **AA43** (5 μM) was incubated up to 24 h in MH at 37 °C. Release of **c343aH$_2$** was monitored by fluorescence (λ_{exc}: 435 nm, λ_{em}: 490 nm)

Antibacterial Activity against AG100 and AG100A *E. coli* Strains. Antibacterial activities were determined against isogenic *E. coli* strains: the wild-type AG100 and its *acr*AB-derivative AG100A. To take into account the difference in molecular weight, MICs were reported in micromolarity in order to compare the activities of all molecules. To ensure a facile conversion into μg mL^{-1}, molecular weights of all compounds are listed in Table S1 (Supporting Information). None of the three PDFinhs, **SMP289**, **AT015**, and **AT019**, showed significant activity against AG100 with MIC superior to 128 μM. As shown in Table 1, MICs of the three compounds decreased toward AG100A, an *E. coli* strain deleted from *acr*AB efflux pump. However, the major effect was observed against AG100 (*E. coli* wild-type) or AG100A by adding polymyxin B nonapeptide (PMBN) (see also refs 9 and 10), an adjuvant known to increase the membrane permeability of Gram negative bacteria (for **AT019**: MIC (AG100) 256 μM, + PMBN 2 μM; MIC (AG100A) 16 μM).[27] This indicates that the OM permeation rather than the efflux limits bacterial uptake of these compounds. The chelating peptides **AA08** and **AA09** displayed significant activity only against AG100A with MICs of 8 μM (4 μM with PMBN) (Table 2). The TPA moiety appears to play an important role in this increase of activity, since **AA09** is more active than its precursor PAβN, and both **AA08** and **AA09** are more active than the peptides with a tris-benzylamine in place of TPA, **AA24** and **AA25**, respectively. **AA27** with a di(picolyl)amine linked to PAβN via a spacer is only moderatly active against AG100A. This recalls the effect of peptides with ATCUN sites.[28,29]

Table 1. Minimal Inhibitory Concentrations against *E. coli* Strains, in μM, of PDFinhs Alone or in the Presence of the Permeabilizer PMBN or in Equimolar Combination with the Chelating Peptides or the Efflux Pump Modulator PAβN

	AG100	AG100A
SMP289	>128	64
SMP289 + PMBN[a]	16	8–4
SMP289/PAβN	32	16
SMP289/AA08	8	8–4
SMP289/AA24	64	32
SMP289/AA09	8	8–4
SMP289/AA25	128	32
AT015	>256	128
AT015 + PMBN	32	16
AT015/PAβN	64	16
AT015/AA08	8	8–4
AT015/AA24	64	32
AT015/AA09	8	4
AT015/AA25	>128	32
AT019	256	16
AT019 + PMBN	2	2
AT019/PAβN	32	4
AT019/AA08	4	2
AT019/AA24	32	16
AT019/AA09	4	2
AT019/AA25	128	16
AT019/AA27	16	4

[a]PMBN, a membrane permeabilizer was used at 1/5 of its MIC (51.2 μg mL^{-1}).

Table 2. Minimal Inhibitory Concentrations in μg mL^{-1} or in μM of Compounds against *E. coli* Strains[a]

	AG100	AG100A	
PMBN	256		In μg mL^{-1}
PAβN	>64 (>64)	64 (64)	in μM
AA08	32–16 (16–8)	8 (8–4)	
AA24	64 (32–16)	32 (16–8)	
AA09	64 (64–32)	8 (8–4)	
AA25	>128 (16)	128 (8)	
AA27	128 (128–64)	32 (32–16)	

[a]In brackets MIC values with PMBN at 1/5 of its MIC (51.2 μg mL^{-1}).

Presently the mode of action of these peptides is still unclear. However, using a fluorescent analogue of **AA08**, TPA-spacer-K*RWRW(OBn), with an additional dansylated lysine in the peptide sequence, we have imaged this peptide in single cell bacteria by deep ultraviolet fluorescence and we have shown that its accumulation inside the bacteria was dependent on both its concentration and incubation time within the cells.[24] Its localization was heterogeneous but more likely in the bacterial membranes as other antimicrobial peptides.[21] The TPA moiety of **AA08** or **AA09** could also bind a metal cation. Wether it was iron(II) or Cu(II), they could promote the formation of reactive oxygen species as reported for other metal binding groups.[30,31] This approach is under investigation.

For zinc and cobalt metallodrugs containing the putative PDFinhs **SMP289**, **AT015**, and **AT019** the antibacterial activities were enhanced when compared to those of the PDFinhs or of the chelating peptides alone, the best effect being observed with Co forms **AA14** $[Co(AA09)(AT015-2H)]^{2+}$, **AA20** $[Co(AA08)(AT019-2H)]^{3+}$, **AA22** $[Co(AA09)(AT019-2H)]^{2+}$, **AA28** $[Zn(AA27)(AT019\text{-}H)]^{2+}$, and **AA39** $[Zn(AA09)(AT019\text{-}H)]^{2+}$ (only against AG100A) (Table 3).

Compared to MICs obtained with equimolar combinations of PDFinhs and chelating peptides, **AA08** or **AA09** (Table 1), cobalt and zinc metallodrugs were less active except **AA14**, **AA20**, and **AA22** which showed similar activity to **AT015**/**AA09**, **AT019**/**AA08**, and **AT019**/**AA09**, respectively. Once again, in these combinations, the chelating group TPA or DPA (**AA27**) plays a key role since any combination of PDFinhs with **AA24** and **AA25** lacking the chelating group are inactive.

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Table 3. Minimal Inhibitory Concentrations in μM of Metallodrugs against *E. coli* Strains

		AG100	AG100A
	Cobalt Metallodrugs		
AA21	$[Co(AA08)(SMP289-2H)]^{3+}$	32	8
AA16	$[Co(AA09)(SMP289-2H)]^{2+}$	16	8
AA13	$[Co(AA08)(AT015-2H)]^{3+}$	64	16
AA14	$[Co(AA09)(AT015-2H)]^{2+}$	8	8
AA20	$[Co(AA08)AT019-2H)]^{3+}$	8	8–4
AA22	$[Co(AA09)(AT019-2H)]^{2+}$	8	8
	Zinc Metallodrugs		
AA26	$[Zn(AA09)(SMP289-H)]^{2+}$	16–8	8–4
AA39	$[Zn(AA09)(AT019-H)]^{2+}$	16–8	4
AA28	$[Zn(AA27)(AT019-H)]^{2+}$	8–4	8–4
	Copper Metallodrugs		
AA29	$[Cu(AA27)(AT019-H)]^{2+}$	16–8	8–4

AA27 was slightly less efficient than **AA08** or **AA09** to potentiate the antibacterial activity of **AT019**: **AT019**/**AA08** 4 μM, **AT019**/**AA09** 4 μM, and **AT019**/**AA27** 16 μM against AG100. However, **AA09** was strongly more efficient than PAβN (**AT019**/**PAβN** 32 μM) and this in any combination with the three PDFinhs. It is important to notice that in all the combinations, the chelating peptides were used well below their MICs (for **AA09** at MIC/8 or MIC/16, and for **AA08** at MIC/4 or MIC/8). At these concentrations they do not have substantial antibacterial activity, but more likely facilitate the penetration inside the bacteria of the PDFinhs and their delivery to their target.

FIC Index (FICi) and Combination AT019 with AA08 and AA09. The dashed line (---) represents the additivity line.

The nature of the association between **AA** and **AT** molecules was studied using the Fractional Inhibitory Concentration (FIC) analysis with AG100 and AG100A *E. coli* strains. Combinations of active **AT019** + **AA08** or + **AA09** were performed, and the nature of the association was determined from the FICi average obtained for each combination (Table 4).

Table 4. FIC Index for Independent Duplicate Experiments[a]

	AG100		AG100A	
	FIC Index	NoI	FIC index	NoI
AT019/AA08	0.52 (0.06)	synergy	0.715 (0.045)	additivity
AT019/AA09	0.49 (0.00)	synergy	0.705 (0.03)	additivity

[a]The deviation is indicated in brackets: *in vitro* interactions between AT and AA compounds assessed on *E. coli* AG100 and AG100A strains. NoI, nature of interaction (ESCMID, 2000 Ref 38).

A synergistic association was observed between **AT019** and the two **AA** peptides with an average of FICi of about 0.5. In an efflux pump deficient context (AG100A), a higher index (around 0.7) was observed, reflecting an additive association.

A graphic representation was performed for each combination shown in Figure 2. In all cases, the isobole curve obtained was concave relative to the line of additivity, indicating an association more synergistic than additive, and more important in AG100 than in AG100A. Moreover, in AG100 a similar curve was obtained for the combinations with **AA08** or **AA09** (Figure 2) closer to the *x*-axis, showing an important synergistic effect of **AA08** and **AA09** on **AT019** inhibitor. In *acr*AB$^-$ context, a difference of profile was observed between the two

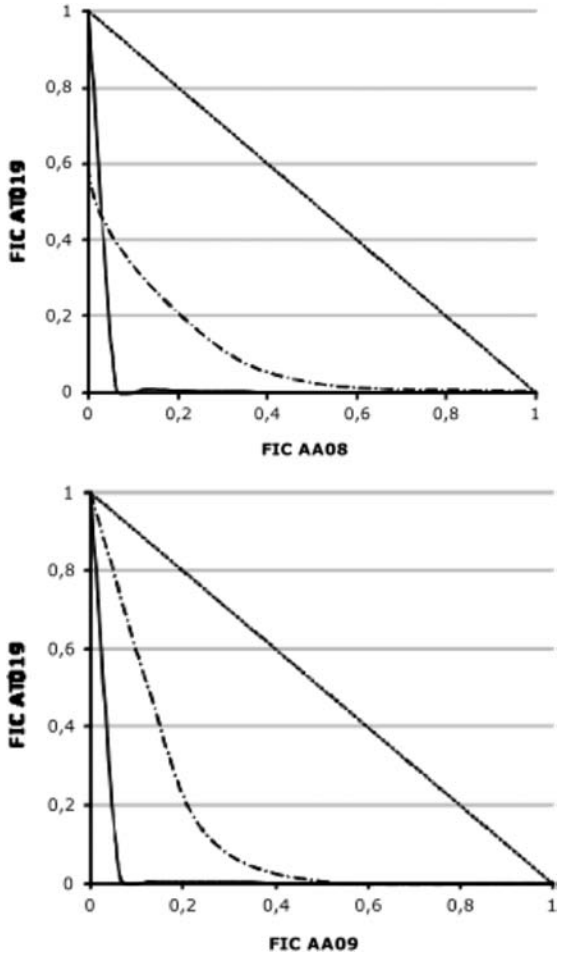

Figure 2. Representative isobolograms of *in vitro* interactions between AT019 and AA08 (top) or AA09 (bottom) against *E. coli* AG100 (WT) (—) and AG100A (acrAB$^-$) (—·—·—) strains.

combinations, with a decrease of **AA09** effect on **AT019** versus **AA08**.

When analyzing all these results, we suspected **AA08** and **AA09**, and to a lesser extent **AA27**, to be able to bind a metal cation in the culture medium, and when associated with PDFinhs in binary systems, to be able to form a metallodrug *in situ*.

Mass Analyses in the Mueller Hinton Medium. We used mass techniques to try and delineate the mode of action of our chelating peptides. These methods have been reported to contribute efficiently to the understanding of the mode of action of anticancer metallodrugs, *in vitro*, as well as *in vivo* and *in situ*.[32] The Mueller Hinton (MH) broth is rich in metal cations required for bacterial growth, and metal complexes could be formed *in situ* when diluting the chelating peptides in this culture medium. So, **AA08**, **AA09**, and **AA27** were incubated in MH broth at 25 μM and 37 °C for 2 h. Then the medium was extracted with methanol and the final solution was analyzed by LC-MS. In any case we identified both free ligands and zinc metalated forms. The HPLC peaks were assigned by comparison with authentic samples and by their ESI$^+$ mass spectra. As shown in Figure 3 for **AA08**, the HPLC chromatogram, run at 290 nm, displayed two peaks at 5.27 and 24.21 min corresponding to the free peptide (*m*/*z*: 410.39, $[M(AA08\ neutral\ form) + 4H + Cl]^{3+}/3$) and **Zn(AA08)** (*m*/*z*: 628.8, $[M(Zn(AA08)]^{2+}/2$; 419.7, $[M(Zn(AA08) + H]^{3+}/3$),

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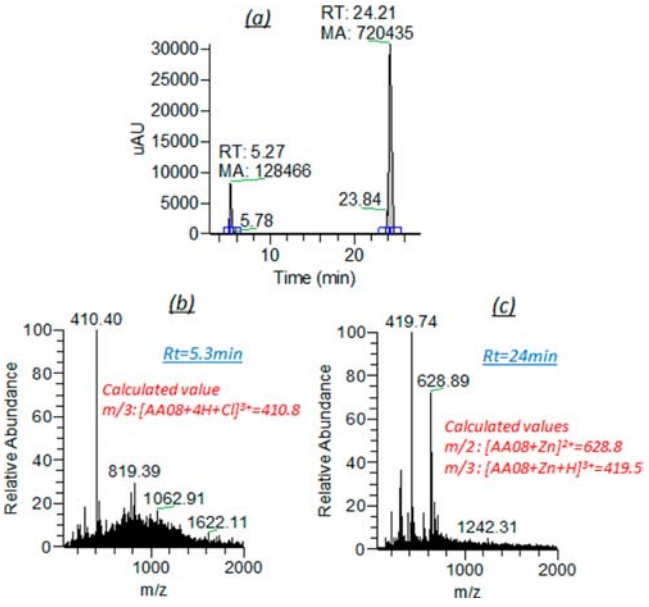

Figure 3. LC-MS chromatogram of the mixture obtained upon dissolution of AA08 at 25 μM, in Mueller Hinton broth after 2 h incubation at 37 °C. (a) MH broth was concentrated by evaporation, then extracted with MeOH. This solution was injected in HPLC eluted with $CH_3CN/MeOH/H_2O$, (7:2:1 v:v:v mixture), detection at λ 290 nm). (b) and (c): ESI^+ mass spectra of the peaks eluted at retention time of 5.3 and 24 min, respectively.

respectively. At 290 nm both compounds, diluted at the same concentration in methanol, have the same absorbance, so the ratio between free and Zn metalated form can be estimated, on the basis of the peak area, to 15/85. Regarding the detection of iron or copper complexes in the overall mass spectrum, it was only possible to identify some iron species (m/z: 624.76, $[M(Fe(AA08))]^{2+}/2$) (data not shown) in a low amount, about 1/6 of that of the zinc complex. Zinc, iron and copper eluted at the same retention time in HPLC. However, no copper derivative could be detected in the overall mass spectrum. This result is in agreement both with the ion content of MH broth and with the metal cation binding abilities of TPA ligand. The higher ion contents of MH are those of zinc and iron, while copper ion is in a very low amount.[33,34] The affinitiy of TPA is 2 orders of magnitude higher for Zn(II) than for Fe(II).[35] All these data show that **AA08** is mainly under its zinc metalated form when incubated in MH broth used for bacterial growth. Similar results are obtained with **AA09** (Figure S2, Supporting Information), but with a lower free/zinc form ratio of 42/58. However, **AA27** with a DPA chelating group is mainly under its free form, the zinc species only accounting for 7% (Figure S3, Supporting Information). Indeed, the binding abilities of DPA for metal cations are, on average, 3 orders of magnitude lower than those for TPA.[35]

The results of antibacterial activities associated with those of LC-MS experiments support the formation of a zinc metallodrug when we tested equimolar combinations of **AA08** or **AA09** and a PDFinh. Nevertheless, despite several attempts, when incubating **AA08** and **AT019** in MH broth, we failed to detect by LC-MS in ESI^+ mode the zinc metallodrug.

CONCLUSION

In this paper, we have described new strategies to improve the antibacterial activities of compounds targeting metalloproteases toward Gram negative bacteria, the activity of which was limited by the low permeability of the outer membrane or by the presence of efflux pumps. For this purpose, we developed chelating peptides, possessing PAβN and/or a small analogue of antimicrobial peptide grafted to a TPA or a DPA ligand, that can be used either in combination with the compounds in a 1:1 molar ratio, or as drug carrier in metallodrugs. Co and Zn metallodrugs are more active than any PDFinhs, but are as efficient as the binary combinations. Even though the chelating peptides have a low antibacterial activity, in the combinations they are used at concentrations well below their MICs. The presence of a metal binding group was essential for the activity of binary systems, and LC-MS analyses showed that they were not in their free form but rather under a zinc metalated form. The efficiency of binary systems could be related to the formation of a zinc metallodrug between the chelating peptides and the PDFinhs. These peptides act as permeabilizer facilitating the bacterial uptake of the drugs, and/or as drug carrier. Furthermore, FIC experiments run in AG100, revealed an important synergistic association of **AA08** and **AA09** peptides on **AT019** inhibitor.

EXPERIMENTAL PROCEDURES

Chemicals. All solvents and chemicals were purchased from SDS and Aldrich, respectively. DMF, MeOH, and CH_3CN were dried using standard. ^1H NMR spectra were recorded on a Bruker ARX-250 spectrometer or on a Bruker Avance-500 spectrometer and chemical shifts were reported in ppm downfield from TMS. Electrospray ionization (ESI) and HRMS mass spectrometry analyses were obtained using Thermo Finnigan LCD Advantage spectrometer. Elemental analyses were carried out by microanalysis service at Gif sur Yvette CNRS. Cyclic voltammetry experiments were performed at room temperature on a PGZ100/HVB100 setup (radiometer analytical) with a three-electrode system which consists of a NaCl saturated calomel electrode (SCE), a platinum auxiliary electrode, and a glassy carbon working electrode. The samples were dissolved in deaerated DMF (0.01 M). nBu_4NClO_4 was used as supporting electrolyte (0.10 M). The potential sweep rate was 100 $mV.s^{-1}$, and ferrocene was used as internal standard ($E_{1/2}$ = 342 mV vs SCE).

General Procedure for the Synthesis of Co(III) Metallodrugs. A mixture of the auxiliary TPA ligand **AA08** or **AA09** (37.8 μmol) with $CoCl_2$ (37.8 μmol, 4.9 mg) in MeOH (for **AA09**) or DMF (for **AA08**) (1 mL) and diisopropylethylamine (DIEA) (0.151 mmol) was stirred for 15 min under argon, and then a solution of hydroxamic acid ligand (**AT015** or **AT019** or **SMP289** or **c343aH2** in DMF) (37.8 μmol) was added to the mixture under argon. After dioxygen oxidation under stirring for 48 h, the solvent was removed under reduced pressure, and the residue was purified over Sephadex LH-20 using MeOH as eluent. The last green fraction was concentrated under reduced pressure, and the residue was purified by precipitation from MeOH into cold diethyl ether to afford the desired metallodrug with yields ranging from 25% to 65%.

Metallodrug AA13, $[Co(AA08)(AT015-2H)]^{3+}$. Green powder, 49% yield. MS (ESI^+, m/z, MeOH): 797.5 (100%, $[M-H]^{2+}/2$). El. anal. calcd. for $C_{82}H_{96}N_{20}CoO_{11}\cdot PF_6\cdot 2Cl\cdot 7H_2O$: C, 50.80; H, 5.72; N, 14.45. Found: C, 50.79; H, 5.45; N, 14.53. Cyclic voltammetry: E_{pc} = −710 mV vs ECS (DMF, nBu_4NClO_4 (0.1 M)).

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Metallodrug AA20, [Co(AA08)(AT019−2H)]$^{3+}$. Green powder, 53% yield. MS (ESI$^+$, *m/z*, MeOH): 810.3 (100%, [M-H]$^{2+}$/2). El. anal. calcd. for $C_{84}H_{98}N_{20}CoO_{11}$·1.2$PF_6$·1.8Cl·0.35$H_2O$·2.3DMF: C, 53.65; H, 5.69; N, 15.35. Found: C, 53.35; H, 5.53; N, 15.65.

Metallodrug AA21, [Co(AA08)(SMP289−2H)]$^{3+}$. Green powder, 50%. MS (ESI$^+$, *m/z*, MeOH): 805.5 (100%, [M-H]$^{2+}$/2). El. anal. calcd. for $C_{81}H_{89}N_{19}CoBrO_9$·0.9$PF_6$·2.1Cl·1$H_2O$·2.1DMF: C, 52.72; H, 5.41; N, 14.86. Found: C, 52.45; H, 5.48; N, 15.16.

Metallodrug AA14, [Co(AA09)(AT015−2H)]$^{2+}$. Green powder, 55%. MS (ESI$^+$, *m/z*, MeOH): 453.1 (100%, [M+1e-(AT015-H)]$^{2+}$/2), 624.5 (50%, [M+e]$^{2+}$/2). El. anal. calcd. for $C_{66}H_{73}N_{14}CoO_8$·2Cl·5H_2O: C, 56.21; H, 5.93; N, 13.90. Found: C, 56.48; H, 5.99; N, 13.80.

Metallodrug AA22, [Co(AA09)(AT019−2H)]$^{2+}$. Green powder, 55% yield. MS (ESI$^+$, *m/z*, MeOH): 637.1 (100%, [M]$^{2+}$/2), 1419.2 (25%, [M+PF_6]$^+$). El. anal. calcd. for $C_{68}H_{75}N_{14}CoO_8$·1Cl·1PF_6·5.5H_2O: C, 52.53; H, 5.58; N, 12.61. Found: C, 52.46; H, 5.76; N, 12.48.

Metallodrug AA16, [Co(AA09)(SMP289−2H)]$^{2+}$. Green powder, 52% yield. MS (ESI$^+$, *m/z*, MeOH): 632.5 (100%, [M]$^{2+}$/2), 1261.9 (25%, [M-H]$^+$). El. anal. calcd. for $C_{65}H_{67}N_{13}CoBrO_6$·2.5MeOH·2Cl.0·5$H_2O$: C, 56.89; H, 5.52; N, 12.78. Found: C, 56.99; H, 5.76; N, 12.53. Cyclic voltammetry: Epc = −700 mV vs ECS.

Metallodrug AA43, [Co(AA09)(c343a)]$^{2+}$. Yellow solid, 45% yield. HRMS (ESI$^+$, *m/z*, MeOH): Calcd for $C_{64}H_{68}N_{13}CoO_8$ [M]$^{2+}$/2, 602.7323; found 602.7316. El. anal. calcd. for $C_{64}H_{68}N_{13}CoO_8$·1Cl·1PF_6·2.3H_2O·1.3DMF: C, 53.54; H, 5.41; N, 13.15. Found: C, 53.14; H, 5.52; N, 13.52.

General Procedure for the Synthesis of Zn(II) and Cu(II) Metallodrugs. A mixture of the auxiliary DPA ligand **AA27** (37.8 μmol, 32.6 mg) with the metal salt $ZnClO_4$·6H_2O (37.8 μmol, 14.1 mg) or $CuClO_4$·6H_2O (37.8 μmol, 14.0 mg) and DIEA (0.113 mmol, 20 μL) in MeOH (1 mL) was stirred for 15 min under argon, and then a solution of hydroxamic acid (**AT019** or **SMP289** or **c343aH$_2$**) (37.8 μmol) was added to the mixture. The reaction was stirred at r.t. for 30 min. The solvent was evaporated under reduced pressure, and the residue was purified over Sephadex LH-20 using MeOH as eluent. The last fraction was concentrated under reduced pressure, and the residue was purified by precipitation from MeOH into cold diethyl ether to give the desired metallodrug with yields ranging from 25% to 65%.

Metallodrug AA28, [Zn(AA27)(AT019-H)]$^{2+}$. White powder, 65%. MS (ESI$^+$, *m/z*, MeOH): 580.1 (100%, [M]$^{2+}$/2), 1259.3 (40%, [M+ClO_4]$^+$). El. anal. calcd. for $C_{62}H_{72}N_{12}ZnO_7$·HPF_6·ClO_4·2.2H_2O: C, 51.47; H, 5.32; N, 11.62. Found: C, 51.46; H, 5.48; N, 11.62.

Metallodrug AA29, [Cu(AA27)(AT019-H)]$^{2+}$. Green solid, 25% yield. MS (ESI$^+$, *m/z*, MeOH): 579.6 (100%, [M]$^{2+}$), 1258.2 (60%, [M+ClO_4]$^+$), 1304.2 (40%, [M+PF_6]$^+$). El. anal. calcd. For $C_{62}H_{72}N_{12}CuO_7$·0.5PF_6·ClO_4·0.5Cl·8H_2O: C, 49.82; H, 5.93; N, 11.25. Found: C, 49.84; H, 6.22; N, 11.51. Cyclic voltammetry: $E_{1/2}$ = 115 mV vs ECS, ΔE = 620 mV.

Metallodrug AA42, [Cu(AA27)(c343aH)]$^{2+}$. Yellow solid, 25% yield. HRMS (ESI$^+$, *m/z*, MeOH): Calcd for $C_{58}H_{65}N_{11}CuO_7$ [M]$^{2+}$ 545.2182; found 545.2164. Cyclic voltammetry: $E_{1/2}$ = 108 mV vs ECS, ΔE = 602 mV.

Metallodrug AA39, [Zn(AA09)(AT019-H)]$^{2+}$. Colorless solid, 62% yield. MS (ESI$^+$, *m/z*, MeOH): 304.1 (100%, [M-(AT019-H)]$^{3+}$),1379.2 (55%, [M+ClO_4]$^+$). El. anal. calcd. for $C_{68}H_{76}N_{14}ZnO_8$·1.4PF_6·0.6ClO_4·4.2H_2O: C, 50.38; H, 5.25; N, 12.10. Found: C, 50.07; H, 5.19; N, 12.46.

Metallodrug AA26, [Zn(AA09)(SMP289-H)]$^{2+}$. Colorless solid, 64% yield. MS (ESI$^+$, *m/z*, MeOH): 635.4 (60%, [M]$^{2+}$), 1369.9 (25%, [M+ClO_4]$^+$), 1416.0 (20%, [M+PF_6]$^+$). El. anal. calcd. for $C_{65}H_{68}N_{13}ZnBrO_6$·1.8$PF_6$·0.2$ClO_4$·4$H_2O$: C, 48.03; H, 4.71; N, 11.20. Found: C, 47.76; H, 4.58; N, 11.50.

HPLC-MS Studies. These studies were performed on a Surveyor HPLC instrument coupled to a LCQ Advantage ion trap mass spectrometer (Thermo, Les Ulis, France), using an Aquapore Butyl C4 column (100 × 2.1 mm, 7 μm) from Applied Biosystems, and a gradient A + B starting from 30% B for 1 min then increasing linearly to 100% B in 6 min and then with 100% B up to 20 min (A = H_2O, B = CH_3CN/CH_3OH/H_2O 7:2:1 (v:v:v mixture)) at 300 μL/min. Mass spectra were obtained by electrospray ionization in positive ionization mode (ESI$^+$), detection under the following conditions: source parameters, sheath gas, 30 L/min; auxilary gas, 15 L/min; spray voltage, 4.5 kV; capillary temperature, 250 °C; capillary voltage, 30 V; and *m/z* range for MS recorded between 50 and 2000.

Microbiology. *Bacterial Growth.* Two *Escherichia coli* (*E. coli*) strains were used. Briefly, AG100 was an *E. coli* wild-type strain and AG100A its *acr*AB derivative. Strains were routinely grown at 37 °C in Mueller-Hinton (MH) broth, supplemented with kanamycine (50 μg.mL^{-1}) for AG100A.

Susceptibility Determination. Minimal inhibitory concentrations (MICs) were determined by broth dilution method as previously described.[36] Susceptibilities were determined in 96-wells microplates with an inoculum of 2 × 10^5 cfu in 200 μL of MH broth containing 2-fold serial dilutions of each compounds. Molecules were tested alone or in combination with other molecules in equimolar ratio. MICs were determined in the absence and in the presence of a membrane permeabilizer, polymyxin B nonapeptide (PMBN) used at 1/5 of its direct MIC previously determined. The MIC was determined as the lowest concentration of each compound at which no visible growth was observed after 18 h of incubation at 37 °C. Each test was performed in duplicate or triplicate. Results were expressed in μM.

Fractional Inhibitory Concentration (FIC) Analysis. The interaction between compounds was analyzed based on the fractional inhibitory concentration index (FICi).[37] A two-dimensional checkerboard with 2-fold dilutions of each compound was set up for the study. For the first clear well in each row of the microplate containing antimicrobial agent, the FIC was calculated as follows: FIC of compound A (FIC A) = MIC of compound A in combination with B/MIC of compound A alone, and FIC of compound B (FIC B) = MIC of compound B in combination with A/MIC of compound B alone. The FICi was calculated as the sum of the FIC of each compound. The nature of the interaction was classified as follows: synergy FICi ≤ 0.5; additivity 0.5 < FICi ≤ 1; indifference 1 < FICi ≤ 2; and antagonism FICi > 2.[38] For each combination, an isobologram of graphically displaying drug interaction was constructed: FIC A was showed in abscissa and FIC B in ordinate. The isobole form depends on the nature of the interaction. The different forms are presented in Figure 4.

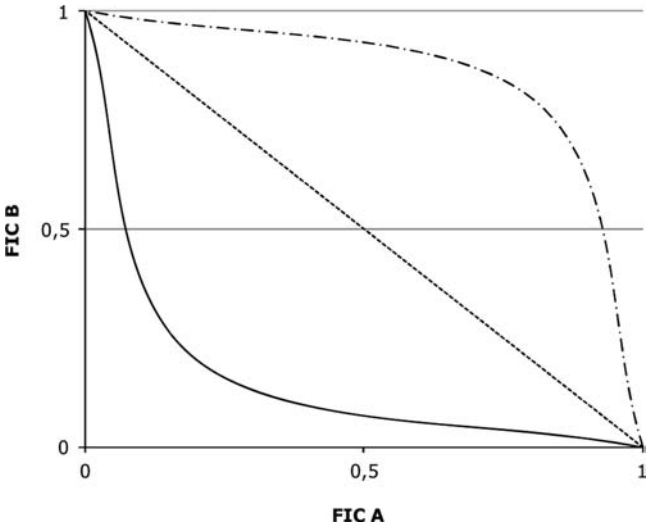

Figure 4. Different forms that the isobole can take. Representative isobolograms of *in vitro* interactions between two compounds A and B. The straight line represents a synergistic association (—), the dashed lines represent additive or indifferent association (---) and antagonistic association (—·—·—).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table of the molecular weights of all compounds tested. Synthesis and characterization of peptides **AA09**, **AA25**, and **AA27**. Synthesis and characterization of **c343H$_2$**. Characterization of **AT015** and **AT019** hydroxamic acids. Stability of **AA42** metallodrug [Cu(AA27)(c343aH)]$^{2+}$ in MH broth. LC-MS analyses in MH of **AA09** and **AA27**. ^{1}H NMR spectra of **AA09**, **AA25**, and **AA27**. Comparative ^{1}H NMR of **AA09** free ligand and Co(III) metallodrug **AA16**, [Co(AA09)(SMP289−2H)]$^{2+}$. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HOBt, *N*-hydroxybenzotriazole:; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate; DIEA, diisopropylethylamine

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