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Aminopyrrolic Synthetic Receptors for Monosaccharides: A Class of Carbohydrate-Binding Agents Endowed with Antibiotic Activity versus Pathogenic Yeasts

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Abstract: The biological activity of a set of structurally related aminopyrrolic synthetic receptors for monosaccharides has been tested versus yeast and yeast-like microorganisms and compared to their binding affinity toward mannosides. Antibiotic activity comparable to that of well-known polyene (amphotericin B) or azole (ketoconazole) drugs has been found for some members of the family, along with a general correlation with binding abilities. A systematic structure–activity–affinity investigation shed light on the structural and functional requirements necessary for antibiotic activity and identified the tripodal compound **1** as the most potent compound of the set. Together with toxicity tests and inhibitor localization experiments performed through fluorescence microscopy, these studies led to the characterization of a new class of carbohydrate binding agents possessing antibiotic activity, in which pyrrolic groups precisely structured on a tripodal architecture appear to be responsible for permeability through the cell wall of pathogens, as well as for antibiotic activity inside the cytoplasm.

Keywords: antibiotics · carbohydrates · microbiology · molecular recognition · synthetic receptors

Introduction

Carbohydrates are abundant on the surface of living eukaryotic cells, including microorganisms, in which they are involved in a number of functions, from adhesion to infection, from signal transmission to immune-system modulation.[1] Surface glycans are generally linked to proteins or lipids and form different structures that are cell-type specific, giving rise to a carbohydrate coating the diversity of which is essential for discriminating the "self" from the "nonself" in the interaction with other cells. Typically, the antigenic surface of viruses and pathogenic microorganisms is covered by a coating of glycolipids and glycoproteins, which trigger the immune response based on self/nonself carbohydrate discrimination.[2] On the other hand, in the case of viruses such as HIV, the immune response is escaped because the carbohydrates on the pathogen are themselves synthesized by the host and therefore not recognized as nonself.[3] All these phenomena rely on molecular recognition of carbohydrates as a key step of the process, which explains the attention that has been focused on this subject in the last decade.[4]

In this context, targeting the glycans of viral glycoproteins with carbohydrate-binding agents (CBAs) is recently emerging as a promising strategy for antiviral therapies and vaccines.[3a,5] Because mannose is frequently encountered as the terminal sugar of glycans and is densely expressed in the glycan shield of the viral envelope of several viral infections of high health risk (HIV, HCV, etc.), terminal oligomannosides have become attractive therapeutic targets.[6] For example, the pradimicin A and the benanomicin A antibiotics, which have been found to bind to the terminal mannosides exposed on fungal membrane,[7] have antiviral activity against HIV-1, with EC_{50} values in the micromolar range.[8] Microvirin, a cyanobacterial monovalent lectin, exerts a potent inhibitory activity toward HIV-1 infection (IC_{50} =

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2–12 nM) by selectively recognizing the Manα(1-2)Man motif of the high-mannose-type oligosaccharides of the gp120 viral protein.[9] It is thus clear that having available effective and selective receptors for mannosides would be of paramount importance for the development of CBAs.

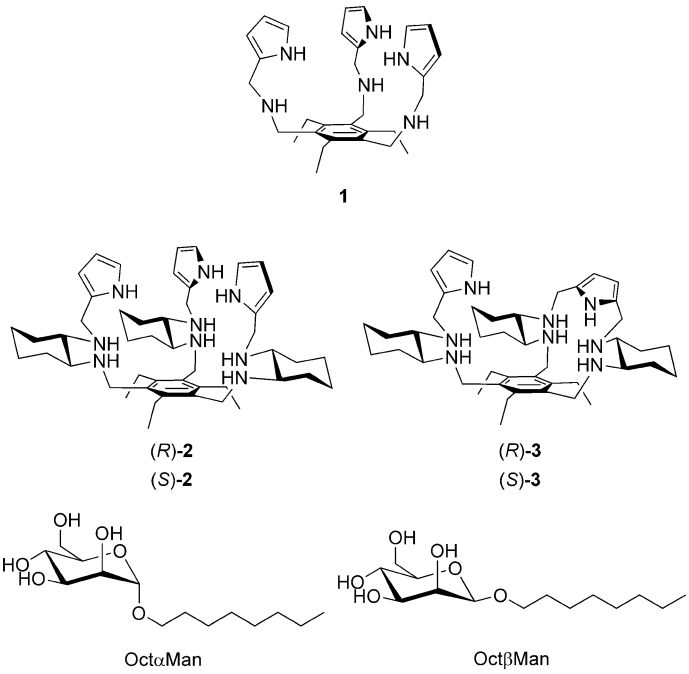
In the course of our studies on molecular recognition of carbohydrates, we developed a family of aminopyrrolic tripodal structures that proved to be effective receptors for monosaccharides in polar organic solvents, showing various levels of selectivity toward the biologically relevant monosaccharides.[10] Because of their binding ability, these molecules may interact with cell-surface glycans and consequently interfere with the living processes of the cell. Intrigued by this possibility, we were prompted to carry out an investigation on the biological activity of this class of synthetic receptors; in particular, we were interested in finding out whether any inhibitory activity could be associated to their binding properties.

Searching for the appropriate target to test biological activity, we resorted to the use of cells of yeast and yeast-like microorganisms. Yeasts are among the most widely studied eukaryotic microorganisms and are extremely important in cell biology because they are similar to human cells in many respects. In particular, expression and processing of glycoproteins is largely analogous to that occurring in mammalian cells. Many of the secretory processes are indeed conserved between yeasts and mammals, and for this reason yeasts are considered a good model system to study glycoside processing.[11] In addition, some yeasts which are opportunistic pathogens and cause infections in humans, present a close carbohydrate similarity between the mannan portion exposed on the cell surface and the oligomannose glycans of the HIV gp120 glycoprotein of the viral envelope.[3a] As a matter of fact, the species *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* were for many years the most frequently isolated pathogens from clinical specimens.[12] Furthermore, the emergence of AIDS and the advances in biomedical procedures (e.g., organ transplants, aggressive chemotherapies, intravascular devices, etc.) increased the risk of infections in immunocompromised hosts.[13] Likewise, some yeast-like organisms are also associated with human and animal diseases, such as a few species of the genus *Prototheca* (*P. zopfii* and *P. whickeramii*).[13c,14]

We report, herein, the results of an investigation on the binding properties, antibiotic activity, and cytotoxicity of a family of structurally related aminopyrrolic carbohydrate receptors, together with a detailed analysis of the structure–binding–activity relationship.

Results and Discussion

Preliminary screening: In a preliminary screening, the affinities of the most representative members of the aminopyrrolic family of receptors, that is, the structures showing the most interesting binding properties toward the glycosides of the biologically relevant monosaccharides, in particular toward mannosides, were measured and compared to the growth inhibition of some yeast and yeast-like pathogenic target strains. Association constants were measured by ^{1}H NMR spectroscopic titrations according to a previously described protocol (see the Supporting Information for details), which consists of the simultaneous fit of the complexation-induced shifts of all the available signals from both the receptor and the glycoside to the appropriate association model by nonlinear regression analysis. Minimum inhibitory concentrations (MICs) were determined in RPMI 1640 culture medium (Sigma–Aldrich) by using 96-well microtiter plates. After incubation at 35 °C for 48 h under aerobic conditions, growth in the presence of two-fold increasing concentrations of each aminopyrrolic receptor was compared visually with the growth observed in the absence of antimicrobial agent. The compounds selected for testing, including the progenitor of the family **1** and the most effective receptors **2** and **3**, are depicted below (the *R* and *S* descriptors



are used to designate the receptors of *all-R* and *all-S* absolute configuration of the six stereocenters, respectively). The corresponding affinities toward octyl α- and β-mannosides (OctαMan, OctβMan), measured in a polar solvent (acetonitrile) and expressed as BC_{50}^{0} values,[15] and the MIC values toward four different microorganisms (*C. tropicalis*, *P. norvegensis*, *P. wickerhamii*, and *P. zopfii*) are reported in Tables 1 and 2, respectively, and in Figure S1 in the Supporting Information. The MIC values for two well-known antibiotic drugs, namely, Amphotericin B (AmB) and Ketoconazole (Keto), measured toward the investigated cell lines, are also reported in Table 2 for direct comparison.

Two main features were clearly emerging from this screening: 1) with the exception of (*R*)-**3**, the activity of which is uninteresting, all of the other compounds tested

Table 1. Binding affinities (BC_{50}^{0} [mM]) of synthetic receptors **1**–**3** for octyl mannosides in CD_3CN.[a]

	OctαMan	OctβMan
1	15.60	7.40
(*R*)-**2**	0.13	0.87
(*S*)-**2**	0.44	0.57
(*R*)-**3**	0.30	1.22
(*S*)-**3**	0.29	0.08

[a] Formation constants (log β) were measured by ^{1}H NMR spectroscopy (400 MHz) from titration experiments at $T=298$ K. Intrinsic median binding concentration (BC_{50}^{0}) values were calculated from the (log β) values by using the "BC_{50} Calculator" Program (see reference [15]).

markedly inhibited the growth of all yeast and yeast-like target species; the MIC values obtained, in particular those for compound **1**, are comparable to those measured for AmB and lower than those obtained for Keto; and 2) although in general binding to mannosides is associated to inhibition activity, which is noteworthy considering the widely different media in which these properties were measured, affinity and activity do not follow the same trend. Indeed, compound **1** shows the highest activities associated with the lowest affinities, whereas the poorly inhibiting (*R*)-**3** shows high affinities for the mannosides (see Figure S1 in the Supporting Information).

Structure–activity studies: On the basis of the results from the preliminary screening and of the interesting antibiotic activity observed toward pathogenic microorganisms, a detailed structure–activity investigation, developed from the structure of **1** as a lead compound, was carried out with the main goal of ascertaining the structural and functional requirements essential for antibiotic activity. Structure modifications on the parent tripodal aminopyrrolic architecture involved: 1) the variation of number and substitution pattern on the aromatic platform of the aminopyrrolic binding arms, 2) the introduction of additional functional groups on the binding arms, 3) the variation of the size of the aromatic platform, and 4) the dissection of the architecture into the constituent structural elements. The new family of compounds prepared through these modifications is depicted below, the MIC values determined toward 14 different strains of yeast and yeast-like microorganisms are reported in Table 3, the corresponding affinities measured toward octyl mannosides are reported in Table 4, and a graphical comparison of the activity–affinity values is depicted in Figure 1.

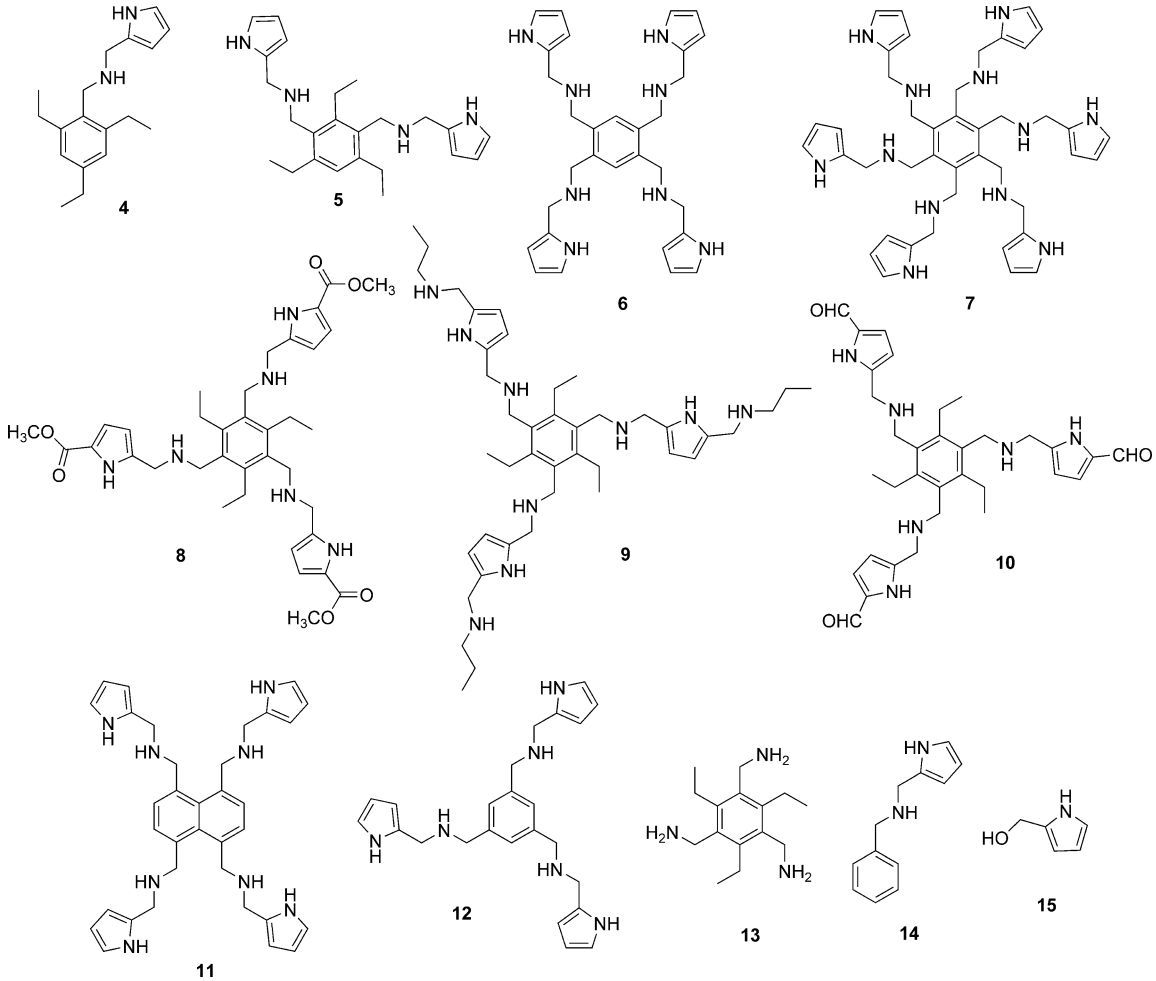


Table 2. Antibiotic activity (MIC [$\mu g\,mL^{-1}$]) of compounds **1**–**3**, AmB, and Keto versus strains of yeast and yeast-like microorganisms.[a]

	C. tropicalis 3982[c]	*P. norvegensis*[b] 6163[c]	*P. wickerhamii* 8879[c]	*P. zopfii* 8880[c]
1	4	2	2	8
(*R*)-**2**	8	4	4	4
(*S*)-**2**	8	4	4	4
(*R*)-**3**	64	64	64	128
(*S*)-**3**	16	16	16	16
AmB	2	1	2	1
Keto	32	8	16	32

[a] MIC values were determined after 48 h growth in RPMI 1640 medium. The experiments were performed as duplicates. [b] Formerly known as *Candida zeylanoides* (see reference [13]). [c] DBVPG collection accession number.

The main conclusion that can be drawn from the analysis of these data is that none of the modifications carried out improved the antibiotic activity of the parent structure. This quite unexpected outcome clearly shows that compound **1** is a structure already optimized toward the set of strains tested. Although sequentially removing the binding arms, as in **4** and **5**, predictably increases MIC values, introducing additional binding arms on the platform, as in **6** and **7**, does not increase activities. Likewise, additional functional groups on the binding arms, such as in **8**–**10**, introduced on the parent structure **1** to exert electronic effects and affecting H-bonding ability, had a marked effect on binding (e.g., in **9**) but did not produce increased antibiotic activity toward any strain. Replacing the benzene platform with a napthalene moiety in **11**[16] led to higher MIC values, even though the activity drop was less pronounced than that observed for the homologous structure **6**. Altogether, the systematic structural variations studied induced loss of activity in all cases, with the sole exception of **5** toward strain 6141, the activity of which showed a significant increase.

The above results raised the question as to whether the whole structure was required for the growth inhibition activity expressed by **1**. Dissection of the parent architecture into the constituent elements gave a clear answer to the question: compounds **13**–**15**, prepared to clarify the individual contributions of single functional groups, showed no inhibitory activity toward the target strains, clearly demonstrating that structuring all the constituent components into the tripodal architecture is essential for inhibiting the growth of yeast and yeast-like microorganisms. In this context, it is noteworthy that **12**, the congener of **1** in which the three ethyl groups have been removed, displays a dramatic loss of activity with respect to **1**, even though the removed substituents were not expected to have an effect on the activity.

Concerning the binding ability toward mannosides in relation to the inhibition properties, it is evident that for the set of compounds investigated a direct correlation between MIC and BC_{50}^{0} values does not hold, as anticipated from the preliminary screening, nor may be expected, when considering that affinities were measured in acetonitrile, whereas antibiotic activities are obtained from cultures in aqueous media. Nevertheless, a broad correspondence exists, in that compounds showing no affinity for mannosides also showed no inhibitory activity, whereas, with few exceptions, compounds exhibiting various levels of affinity are also endowed with antibiotic activity. This feature can be better appreciated from the graphical representation of data depicted in Figure 1, in which activities and affinities are compared face-to-face. The evidence that the two properties do not follow the same trend suggests that the origin of the antibiotic activity does not reside in the ability to bind to manno-

Table 4. Binding affinities (BC_{50}^{0} [mM]) of compounds **1** and **4**–**15** for octyl mannosides in CD_3CN.[a]

	1	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
OctαMan	15.6	n.d.	98.0	12.0	8.20	20.0	4.00	17.0	n.d.	22.0	n.d.	n.d.	n.d.
OctβMan	7.40	n.d.	44.5	5.90	8.00	10.4	0.65	4.80	n.d.	20.6	n.d.	n.d.	n.d.

[a] Formation constants (log β) were measured by ^{1}H NMR spectroscopy (400 MHz) from titration experiments at $T = 298$ K. BC_{50}^{0} values were calculated from the (log β) values by using the "BC_{50} Calculator" Program (see reference [15]). n.d. = non-detectable.

Table 3. Antibiotic activity (MIC [$\mu g\,mL^{-1}$]) of compounds **1** and **4**–**15** against different strains of yeast and yeast-like microorganisms.[a]

DBVPG[b]	Species	**1**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
6133	*Candida albicans*	64	128	512	>1024	512	256	256	>1024	256	>1024	>1024	>1024	>1024
6157	*Candida albicans*	16	128	512	1024	256	256	64	512	128	>1024	>1024	>1024	>1024
3828	*Candida glabrata*	8	256	128	1024	256	64	128	>1024	128	>1024	>1024	>1024	>1024
6140	*Meyerozyma guiliiermondii*	8	128	64	1024	64	64	64	512	64	512	>1024	>1024	>1024
6150	*Candida parapsilosis*	8	256	512	>1024	512	64	>1024	>1024	1024	>1024	>1024	>1024	>1024
3982	*Candida tropicalis*	4	128	64	512	32	32	8	128	64	256	512	>1024	>1024
6163	*Pichia norvegensis*	2	64	16	64	8	8	8	32	8	64	512	>1024	>1024
6142	*Clavispora lusitaniae*	32	128	256	>1024	256	128	128	512	512	>1024	>1024	>1024	>1024
6053	*Yarrowia lipolytica*	8	128	512	512	62	128	32	256	128	1024	1024	>1024	>1024
6782	*Pichia kudriavzevii*	16	128	64	512	64	64	128	128	128	512	>1024	>1024	>1024
6141	*Kluyveromyces marxianus*	8	16	4	256	32	8	8	128	32	256	1024	>1024	>1024
8879	*Prototheca wickeramii*	2	16	64	256	32	128	256	256	64	256	>1024	>1024	>1024
8880	*Prototheca zopfii*	8	32	128	256	64	128	256	1024	128	256	>1024	>1024	>1024
8830	*Prototheca zopfii*	8	64	128	256	32	64	256	1024	128	512	>1024	>1024	>1024

[a] MIC values were determined after 48 h growth in RPMI 1640 medium. The experiments were performed as duplicates. [b] DBVPG collection accession number.

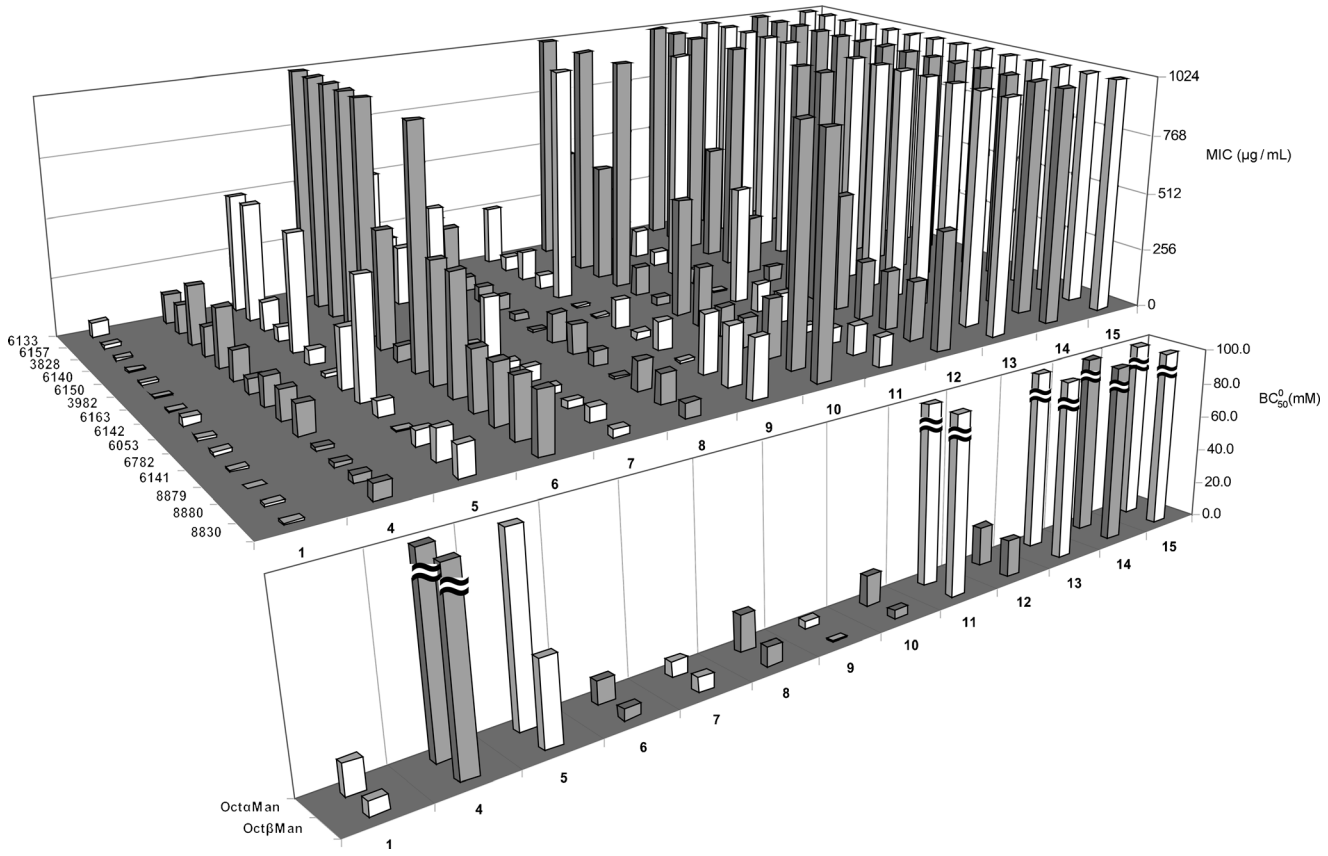


Figure 1. Graph of the antibiotic activity (MIC [$\mu g\,mL^{-1}$]) of compounds **1** and **4**–**15** versus 14 different strains of yeast and yeast-like microorganisms (top) and the corresponding affinities (BC_{50}^{0} [mM]) for octyl mannosides (bottom).

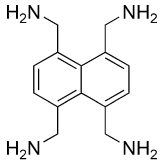
sides; on the other hand, binding seems to be a prerequisite to exert inhibition activity, which suggests that binding is involved in the process at some stage. This may be compatible with the hypothesis that the ability to bind to mannosides would facilitate the approach of the compounds to the yeast and yeast-like cells, triggering a process that would subsequently involve adhesion to the cell wall and penetration across the membrane into the cytoplasm, in which they might exert antibiotic activity by interacting with some specific target. This latter hypothesis is based on the very strict structural requirements associated to the inhibition activity expressed against yeast and yeast-like cells, which appear intolerant of even small variations, including the presence of apparently innocent components (such as the ethyl groups on the aromatic platform), thus suggesting a very precise fit of the molecule into a binding pocket.

Uptake and toxicity studies: To gain more information and support for this hypothesis, we investigated the fate of the aminopyrrolic inhibitors when incubated with the microorganisms. Fluorescence appeared to be the convenient property to follow for the localization of inhibitors in the cells. However, monitoring **1** by fluorescence was unfeasible, whereas **11** showed excitation and emission spectra that could be exploited for fluorescence microscopy. On the other hand, the lower activity of **11** with respect to **1** could be compensated for by using a higher concentration of **11** in cell cultures for fluorescence experiments, under the as-

Table 5. Viability [%] of human hepatic cell lines[a] after treating with increasing concentrations [μM] of inhibitor, and the corresponding lethal concentration (LC_{50} [μM]).[b] [Please note: headings have been centered on columns]

	Concentration [μM]										LC_{50} [μM]
	0	0.03	0.1	0.3	1	3	10	30	100	300	
1	100.0±3.4	100.1±2.9	104.3±4.4	107.4±1.1	104.7±2.9	25.1±3.5	18.3±2.8	17.4±3.9	17.3±3.3	8.3±0.1	2.6±0.8
11	100.0±2.7	98.1±1.4	102.4±2.9	105.9±3.5	105.8±2.0	101.0±4.0	94.0±3.9	42.6±1.4	25.2±0.4	21.3±3.8	27.2±2.1
16	100.0±2.6	105.2±4.9	109.9±6.8	106.7±3.6	103.1±4.3	96.9±4.8	93.5±3.6	90.0±6.2	65.3±3.9	41.8±6.5	189.4±3.2
AmB	100.0±4.6	106.7±3.7	97.7±2.2	98.5±3.4	99.0±4.4	91.0±1.8	34.7±4.3	16.5±4.4	14.8±2.1	8.2±0.2	8.2±1.0
Keto	100.0±2.8	100.7±1.8	103.6±3.7	103.7±2.4	97.2±0.7	72.5±5.6	44.9±5.4	24.3±6.4	15.6±5.1	8.0±3.2	9.0±1.3

[a] HuH-7 human hepatoma cell line. [b] Data are expressed as % of the basal value (0 μM). Viability was evaluated by MTT test. All treatments were performed after 48 h incubation with the analyzed compounds in DMEM/HamF12 culture media with 10% serum and 1% DMSO.



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sumption that the two inhibitors would follow the same pathway. To ascertain the existence of a correlation between the inhibition activity and the localization of the inhibitor in the cell, the tetraminonaphthalene derivative **16**, the homologue of **11** devoid of pyrrolic groups, was prepared as an inactive, fluorescent counterpart of **11**. The latter was used as the reference compound in blank experiments, in analogy to compound **13**, used as a reference for **1**, which completely lost its inhibition properties when the pyrrolic moieties had been removed.[17]

Pichia norvegensis DBVPG 6163, which was the strain most effectively inhibited by **11**, was thus incubated a) in the absence of any additive, b) in the presence of **11**, and c) in the presence of **16**. The cultures were then observed by fluorescence microscopy and the corresponding images are reported in Figure 2. The images in Figure 2b and c clearly show that both compounds have been absorbed by the yeast cells, which exhibited an unequivocally stronger fluorescence than the background, concentrated in distinct areas of the cell.

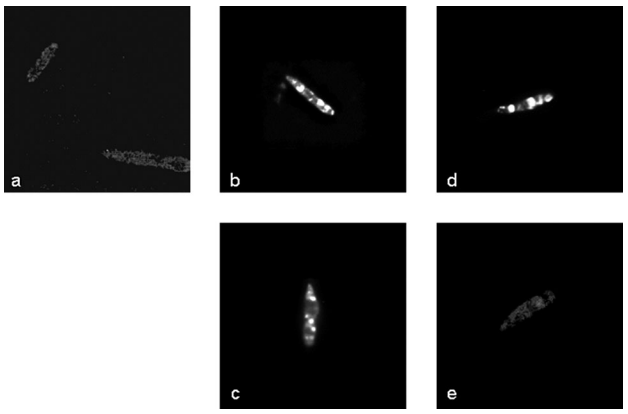

Figure 2. Fluorescence emitted by cells of *Pichia norvegensis* DBVPG 6163 after 48 h growth in RPMI 1640 medium a) in the absence of any additive; b) in the presence of **11** (128 $\mu g\,mL^{-1}$); c) in the presence of **16** (128 $\mu g\,mL^{-1}$); d) as in (b), after washing with 0.9% NaCl sterile solution; and e) as in (c), after washing with 0.9% NaCl sterile solution.

Apparently, **11** and **16** were taken up from the culture in much the same way; however, when cells were washed with 0.9% NaCl sterile solution, fluorescence persisted unaltered in cells incubated with **11** (Figure 2d), whereas it turned off to the background level in cells treated with **16** (Figure 2e), which demonstrated that the latter was actually just "deposited" on the cell surface, whereas the former had been internalized in the cytoplasm. This conclusion was confirmed by fluorescence scanning focused on different planes, which showed emission from the whole body of cells treated with **11**, but only from the cell surface for those treated with **16**.

A direct conclusion from these experiments is that the presence of pyrrolic groups seems to be responsible for the permeability of the active inhibitor **11** through the yeast cell wall; in the absence of pyrrolic groups, the aminic compound **16** does not penetrate into the cytoplasm, in addition to be biologically inactive and incapable of binding to mannosides. It is also evident that the inhibition activity is exerted inside the cytoplasm, rather than at the level of the cell surface. Whether pyrrolic moieties are also determinant for the activity of the inhibitor or just for its permeability cannot be assessed unambiguously from the present data; nevertheless, this evidence appears to support the existence of a chain of events in which binding to the oligomannosides of the cell wall may play a crucial role in the internalization process.

Toxicity is a critical property of compounds possessing antibiotic activity. In particular the well known antifungal drugs AmB and Keto are associated with the development of hepatotoxicity and nephrotoxicity.[18] To characterize the in vitro toxicity of the aminopyrrolic inhibitors, the viability of a human hepatic cell line (HuH-7 human hepatoma cell line) has been tested. HuH-7 cells were incubated in the presence of increasing concentrations of compounds **1**, **11**, and **16**, and the cell viability was monitored after 24 and 48 h incubation. Results are reported in Table S2 in the Supporting Information and Table 5, respectively, together with the corresponding LC_{50} values. Cell viabilities obtained by incubation with AmB and Keto and corresponding LC_{50} values are also reported for comparison. A graphical representation of toxicity data is reported in the graph in Figure 3.

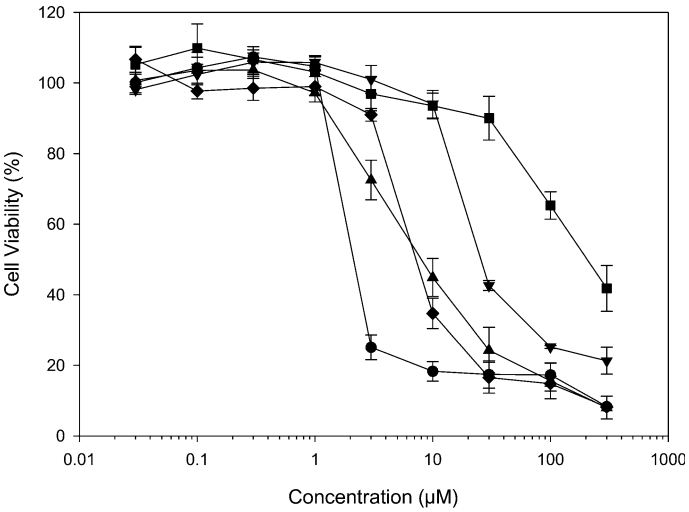

Figure 3. Viability [%] of HuH-7 hepatocytes after 48 h incubation with increasing concentration [μM] of **1** (●), **11** (▼), **16** (■), AmB (◆), and Keto (▲).

Although up to 1 μM of all the tested compounds are nontoxic, a steep decrease in cell viability is observed for **1** above 1 μM, for AmB above 3 μM, and for **11** above 10 μM;

for Keto, a quite shallow decrease is observed from 1 μM, whereas for **16** a shallow decrease above 30 μM provides evidence of an overall low toxicity. In terms of LC_{50}, AmB and Keto show similar values, whereas **1** exhibits a slightly smaller value. It is worth noting that the decreasing toxicities of compounds **1**, **11**, and **16**, with LC_{50} values spanning nearly two orders of magnitude, follow the same trend observed for their overall inhibition activity toward the yeast and yeast-like target strains, which suggests a possible common mode of action.

Localization experiments on compounds **11** and **16** in cells of human hepatocytes provided additional information clarifying some ambiguities found with pathogens. Fluorescence microscopy images obtained on HuH-7 cell lines from experiments analogous to those performed on *Pichia norvegensis* are shown in Figure 4.

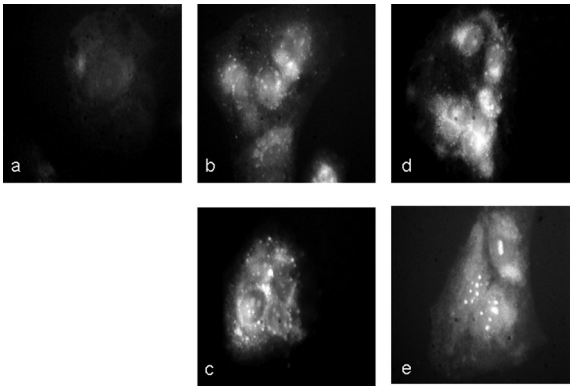

Figure 4. Fluorescence emitted by cells of HuH-7 after 15 min incubation a) in the absence of any additive; b) in the presence of **11** (128 $\mu g\,mL^{-1}$); c) in the presence of **16** (128 $\mu g\,mL^{-1}$); d) as in (b), after washing with Dulbecco's modified eagle medium (DMEM); and e) as in (c), after washing with DMEM.

It can be seen that, as for yeast cells, fluorescence substantially stronger than that of the background is emitted from the hepatocytes, when incubated with **11** and **16** (Figure 4b and c, respectively, in comparison to Figure 4a). After 15 min incubation, both inhibitors were internalized into the cytoplasm and appeared concentrated in small fluorescent spots, most likely because of their inclusion into small vesicles by a cell cytoplasmic process. This observation was confirmed by subsequent washing of cells with DMEM solution, which caused a somewhat attenuated fluorescence, but did not remove emission from either one of the two treated strains, showing that both compounds were largely internalized into the cytoplasm (Figure 4d and e). It appears that, in contrast to the yeast cell wall, the hepatocyte membrane is permeable to both aminic compounds.[19] The different permeability behavior of hepatocites compared to yeast cells may be ascribed to the glycoconjugate coating of the yeast cell wall. This may discriminate **11** from **16** by selectively allowing carbohydrate recognition-mediated entry into the yeast cell of the aminopyrrolic inhibitor but not of its structurally related aminic counterpart. Taking into account the different binding abilities of **11** and **16** toward mannosides, this evidence reinforces the hypothesis that permeability of the inhibitor mediated by carbohydrate recognition on the cell wall of yeasts is a crucial step of a cascade of events responsible for the antibiotic activity of these compounds. It should be noted, however, that the carbohydrate recognition-mediated entry into the yeast cell, characterizing this family of aminopyrrolic structures, may not be limited to their interaction with mannosides. Because monosaccharidic glycosides other than mannosides are also recognized, it cannot be excluded that interaction with different glycans of the cell wall may indeed be responsible for the permeability features of the pyrrolic inhibitors.

A further aspect sheding light on the above ambiguities is the activity–toxicity differences between **11** and **16**. Both are found inside the cytoplasm after incubating the hepatocytes in their presence; yet they show a markedly different toxicity (see Table 5 and Figure 3). Considering that the antibiotic activity of this family follows the toxicity trend, it may be expected that the lack of activity observed for **16** with pathogenic yeast and yeast-like microorganisms would be due to poor inhibiting activity towards microbial cell growth, rather than to poor permeability through the cell wall, due to the lack of pyrrolic groups. In turn this would confirm that pyrrolic groups on the aminic scaffold are not only responsible for the permeability of **11** (and of related aminopyrrolic compounds of the family), but also for the actual inhibition properties exerted in the cell cytoplasm.

Conclusion

In this work, we have shown that a family of structurally related aminopyrrolic synthetic receptors for monosaccharides represents a new class of carbohydrate binding agents possessing antibiotic activity against yeast and yeast-like pathogens, some of which exhibit potencies comparable to that of well-known polyene (amphotericin B) or azole (ketoconazole) drugs. Summarizing the main conclusions emerging from this work:

1) The tripodal aminopyrrolic compound **1** is the optimal structure in terms of antibiotic activity within the family. None of the modifications carried out improved the antibiotic activity of the parent structure. On the other hand, structuring all the constituent elements into the tripodal architecture turned out to be essential for inhibiting the growth of yeast and yeast-like microorganisms.
2) Although a direct correlation between MICs and affinities for mannosides was not found, a broad correspondence exists, in that compounds showing no affinity for mannosides also lack any inhibitory activity, whereas compounds showing various levels of affinity are also endowed with antibiotic activity. This suggests that the origin of the antibiotic activity does not reside in the ability to bind mannosides, which are densely expressed on the cell wall of these pathogens, but that binding is in-

volved in the process at some stage, possibly facilitating the approach of the inhibitor and its permeation through the cell wall.

3) By following, through fluorescence microscopy, the fate of the inhibitors when incubated with the microorganisms it was found that aminopyrrolic compounds were internalized into the cytoplasm, whereas the corresponding structures devoid of pyrrolic groups did not pass through the cell wall. While supporting the previous conclusion, this evidence indicates that pyrrolic groups are required for inhibitor permeability.
4) The toxicity of this family of inhibitors parallels their antibiotic activity, in that the most active compounds are also the most toxic toward human hepatocytes. However, the toxicity of the most effective inhibitors is comparable to that of amphotericin B and ketoconazole.
5) Localization experiments through fluorescence microscopy on human hepatocytes showed that both the (inactive) amino- and the (active) aminopyrrolic compounds were internalized into the cytoplasm, which demonstrated that pyrrolic groups are responsible not only for the permeability, but also for the antibiotic activity and toxicity of the inhibitors. Furthermore, this evidence reinforces the hypothesis that inhibitor permeation mediated by carbohydrate recognition on the cell wall of yeasts is a crucial step of a cascade of events responsible for the antibiotic activity of these compounds.

As a closing remark, quoting from the recent ACS Webinar on the future of antimicrobial drug discovery: "Antimicrobial resistance is growing at an alarming rate and The Infectious Disease Society of America has issued the challenge to develop ten new antibiotics by 2020. How difficult will this be? What are the hurdles?". In this context, it appears that the aminopyrrolic inhibitors of yeast and yeast-like proliferation presented in this paper may constitute a new tool against pathogenic microorganisms and may have an impact on the development of new drugs circumventing antibiotic resistance.

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[13] Among these yeasts (labeled as emerging pathogens), the literature reports cases of cutaneous or systemic mycoses mainly caused by strains of *Candida glabrata*, *Meyerozyma guilliermondii* (teleomorphic state of *Candida guilliermondii*), *Pichia kudriavzevii* (teleomorphic state of *Candida krusei*), *Pichia norvegensis* (former *Candida zeylanoides*), and *Clavispora lusitaniae*, see: a) M. A. Lachance in *The Yeasts: A Taxonomic Study, Vol. 2*, 5th ed. (Eds.: C. P. Kurtzman, J. W. Fell, T. Boekhout), Elsevier Science, Amsterdam, **2011**, pp. 349–353; b) C. P. Kurtzman in *The Yeasts: A Taxonomic Study, Vol. 2*, 5th ed. (Eds.: C. P. Kurtzman, J. W. Fell, T. Boekhout), Elsevier Science, Amsterdam, **2011**, pp. 685–707; c) R. S. Pore in *The Yeasts: A Taxonomic Study, Vol. 3*, 5th ed. (Eds.: C. P. Kurtzman, J. W. Fell, T. Boekhout), Elsevier Science, Amsterdam, **2011**, pp. 2071–2080.

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[15] The BC_{50} (median binding concentration) parameter, which can be calculated from the measured association constants, is a generalized affinity descriptor univocally defining on a common scale the overall binding ability of receptors for ligands in chemical systems involving any number of complex species. The BC_{50} is defined as the total concentration of receptor necessary for binding 50% of the ligand and directly provides a quantitative measure of binding ability as, in analogy to the IC_{50} parameter, the lower the value, the higher the affinity. The BC^0_{50} (intrinsic median binding concentration) is the value assumed by the BC_{50} when the fraction of bound receptor is zero, that is, when forming the first complex molecule; this is a receptor specific constant value, independent of concentration, which coincides with the dissociation constant K_d when the 1:1 association is the only equilibrium present in solution. A detailed description of the treatment of the BC_{50} descriptor has been reported (see referen-

ce [10a], and references therein). To expedite the calculation of the BC_{50} parameter, a computer program has been developed for PC platforms ("BC_{50} Calculator") and is available for free upon request from the corresponding author. A detailed description of the equations used by the program for computing BC_{50} and BC^{0}_{50} values is reported in the above references.

[16] Replacement of the benzene platform with a tetrahydropyrene moiety was also tested, but the resulting MIC values were unreliable, likely because of the interference of the inhibitor with the culture medium, which turned dark blue on addition of the inhibitor.

[17] Compound **16** gave MIC values $>1024\,\mu g\,mL^{-1}$ for all the strains tested. No evidence of binding to mannosides could be detected.

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