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Development of a Synthetic Receptor for the Food Toxin Beauvericin: A Tale of Carbazole and Steroids

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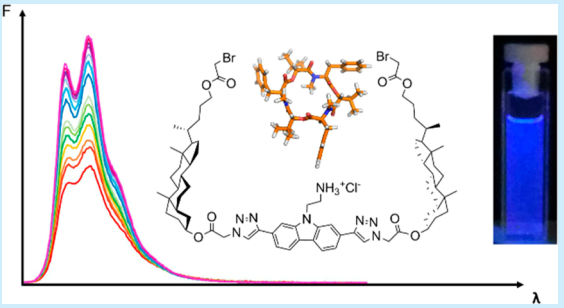
Supporting Information

ABSTRACT: The synthesis of the first synthetic receptor showing high affinity for the toxic ionophoric cyclodepsipeptide beauvericin is described. Binding results in a pronounced increase in fluorescence intensity of the receptor, while this increase is not observed for a very similar ionophore such as valinomycin. Experiments that shed light on the nature of this selectivity are discussed.



Beauvericin (BEA) is a toxic cyclodepsipeptide produced by the insect pathogenic fungus *Beauveria bassiana*[1] or plant pathogenic *Fussarium spp.* (See Figure 1.) The latter infects crops such as wheat and maize, which explains the high concentrations (up to 520 mg/kg) of beauvericin that can be found in infected kernels.[2] This toxin is able to bind K^+ and NH_4^+ cations and can transport these ions across lipid bilayers. By using membrane potential-sensitive fluorescent dyes, Tonshin et al. showed that low micromolar concentrations of beauvericin are sufficient to cause depolarization of human neural (Paju) cells.[3] Overnight incubation of human leukemia cells with 3 μM beauvericin resulted in an 80% decrease in survival rate, illustrating the toxic effect of this ionophore.[4] Moreover, when mice were treated intraperitoneally with 5 mg BEA/kg body weight for three consecutive days, bioaccumulation in the liver and lipophilic tissue was observed,[5] probably because of the hydrophobic nature of this toxin. It is this combination of prevalence in food with toxic properties and tendency to bioaccumulate that motivates the need to develop efficient and rapid detection systems for beauvericin.

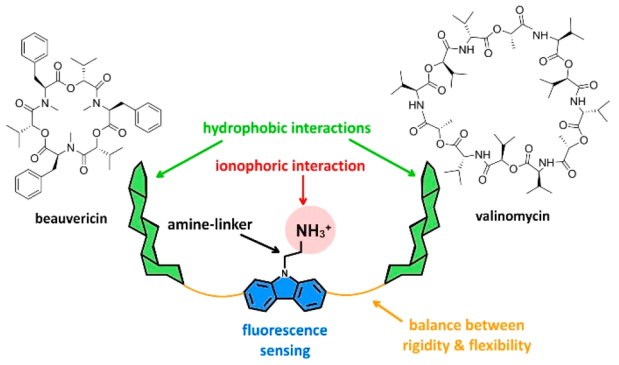


Figure 1. Chemical structure of beauvericin (left) and valinomycin (right), along with the design principles of the artificial receptor (middle).

Current methods rely on LC-MS(/MS),[6] and even though this technique provides low limits of detection/quantification, important drawbacks remain that limit the straightforward testing of large numbers of food samples for the presence of the toxin.

The main limitation resides in the fact that LC-MS(/MS) does not allow parallel measurements of many samples simultaneously, nor can it be used on site (in the field or in food processing facilities) where fungal growth and concomitant toxin production occurs. Such limitations have been circumvented by the development of enzyme-linked immunosorbent assays (ELISA) and lateral flow devices,[7] which are commercially available for the more well-known mycotoxins, such as aflatoxins, deoxynivalenol, and many others. However, both methods require antibodies to recognize the mycotoxin under investigation. While mycotoxins such as aflatoxins, zearalenone, and deoxynivalenol contain functional groups that allow conjugation to carrier proteins (which is necessary to

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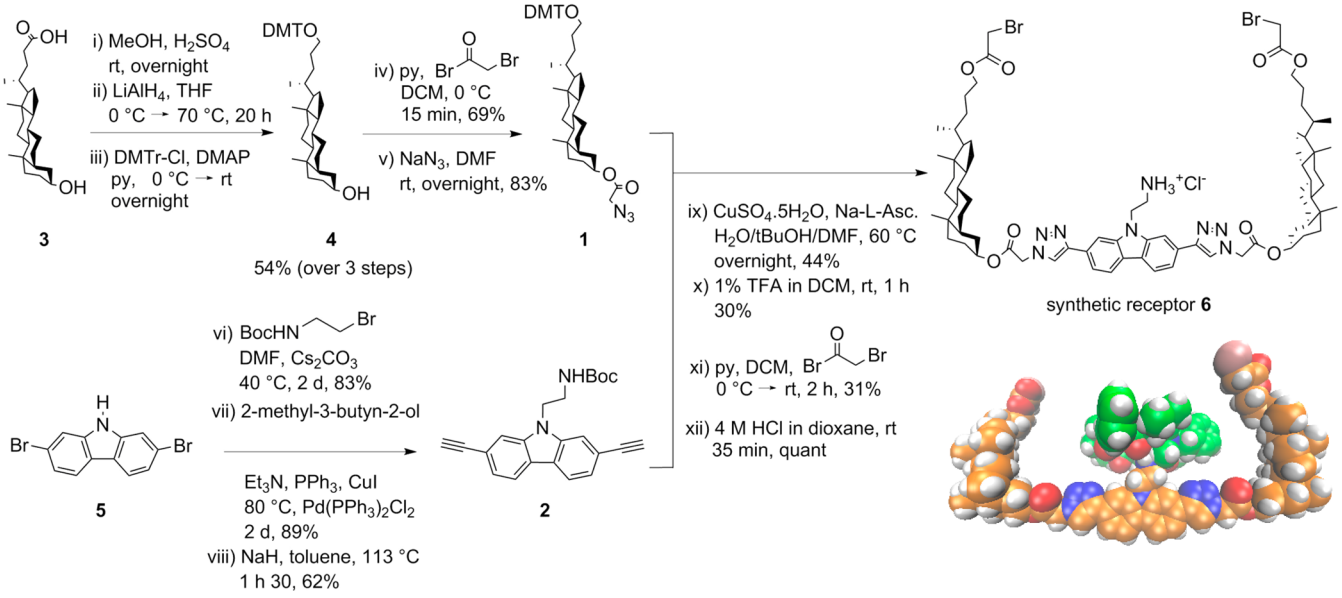
Scheme 1. Synthetic Scheme toward Artificial Receptor 6, with Visual Representation of BEA (Green) Binding to 6 (Orange), Using VMD



elicit an immune response), beauvericin does not. Given the difficulty to generate antibodies for beauvericin, and in view of the inherent limitations of antibodies including limited long-term stability, short shelf life, and cost, we became interested in the development of a synthetic receptor capable of selectively binding this toxin.

A wealth of artificial receptors has been developed for a broad range of ligands such as anions,[8] cations, saccharides[9] and small linear dipeptides and tripeptides,[10] to name just a few. In the context of peptide ligand binding, association constants in the range of 10^3-10^6 M^{-1} have been reported.[11−13] However, cyclic medium-sized depsipeptides such as beauvericin have remained unexplored as ligands for synthetic hosts. In terms of ligand scope, the work presented herein is a first step toward filling this gap.

Our receptor design (see Figure 1) is based on the bridging of two steroid arms via a linker to create a hydrophobic cavity in an effort to accommodate the lipophilic surface of beauvericin. The use of steroidal bile acid derivatives was inspired by reports in the literature describing their use in artificial receptors,[14,15] as well as some of our previous work.[16,17] Incorporation of an easily protonated primary amine functionality in the linking unit between the steroid moieties should further promote binding, because of the ionophoric nature of the toxin. Initially, a construct was investigated in which the steroid arms were connected through a flexible linker and further decorated with peptide chains providing additional diversification points (see Figure S1 in the Supporting Information (SI)). However, next to potential scaleup being hampered by the required solid-phase peptide synthesis, determining the binding affinity for beauvericin has remained difficult without introducing adequate labels in either of the two partners. Therefore, in the current optimized design, shown in Figure 1, a carbazole moiety was introduced as linking fragment between the steroid arms to ensure an adequate level of rigidity, while, at the same time, equipping the receptor with fluorescent properties. Although carbazoles have been used in artificial receptors for caffeine[18] and iodine[19] among others, our design is the first example that combines both bile acid derivatives and this heterocycle within one receptor entity. In order to prevent the hydrophobic cavity from being too small to accommodate the toxin, the envisaged complex was visualized by taking the van der Waals volume of both binding partners into account, using visual molecular dynamics (VMD) (Scheme 1).[20] This allowed to determine the minimum length of the linkers connecting the carbazole with the bile acid moieties.

The convergent synthesis of the receptor (Scheme 1) involves two fragments: a steroid fragment **1** and a carbazole fragment **2**, which are joined together via a copper-catalyzed alkyne azide cycloaddition (CuAAC) to yield the bipodal receptor **6** after some manipulations. For the steroid fragment, we started from commercially available lithocholic acid **3**. Reduction of the carboxylic acid and selective dimethoxytrityl (DMT) protection of the primary alcohol in the presence of the secondary alcohol yielded DMT-protected steroid derivative **4**, with a yield of 54% over three steps. Next, acylation with bromoacetyl bromide, followed by a S_N2 reaction with NaN_3, resulted in the formation of the final steroid fragment **1**.

For the synthesis of the carbazole fragment, N-alkylation of 2,7-dibromocarbazole allowed the introduction of the required primary amine, needed for complexation within the beauvericin interior. This was followed by a Sonogashira reaction and alkyne-deprotection using NaH in toluene,[21] which completed the synthesis of compound **2**.

With these two building blocks, we could proceed to the construction of the bipodal receptor using the CuAAC reaction. While typically this "click" reaction is very efficient, no reaction occurred at room temperature using standard Fokin−Sharpless conditions. By heating the mixture at 60 °C overnight, the desired CuAAC product could be obtained. The 1,5-regioisomer that can potentially be formed as a result of a Huisgen rather than copper-catalyzed pathway was not observed. In principle, at this stage, deprotection of both DMT- and Boc-group would yield a functional receptor. However, the limited solubility of the resulting construct in DMF alone, severely limits its future application. Therefore,

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the free alcohols were acylated with bromoacetyl bromide to render the receptor more hydrophobic. Subsequently, following Boc-deprotection with 4 M HCl in dry dioxane, we were glad to observe that the final receptor **6** was indeed soluble in $CHCl_3$. Note that the introduced α-bromoacetyl moiety potentially allows for further derivatization (e.g., immobilization on solid support or labeling).

We then set out to determine the new receptor's affinity for beauvericin. It is known that fluorescence is highly dependent on the molecular environment. Therefore, we reasoned that binding of beauvericin in close proximity to the fluorescent carbazole, would influence the local environment, thus permitting the use of fluorescence titration for the determination of binding affinity. Gratifyingly, upon addition of beauvericin to receptor **6** a clear concentration-dependent increase in fluorescence is observed at ~373 and 389 nm (Figure 2), which corresponds to the typical blue carbazole emission. When this increase in fluorescence intensity is plotted versus the added ligand concentration, a binding curve can be obtained. As shown in the inset of Figure 2, this curve can be fitted to a 1:1 stoichiometry resulting in a mean association constant of 4.0×10^5 M^{-1} $\pm$ 2.1×10^4 (K_a $\pm$ standard deviation (SD)) (see also Figure S3 in the Supporting Information). When performing the titration at a lower receptor concentration (1 μM), a 2.6-fold higher association constant was obtained, which seems to point toward a mass balance limitation. As the limited fluorescence of carbazole does not allow the reliable determination of K_a at lower receptor concentrations, the value reported here for the binding affinity should be considered as the minimal value, which can be determined using fluorescence.

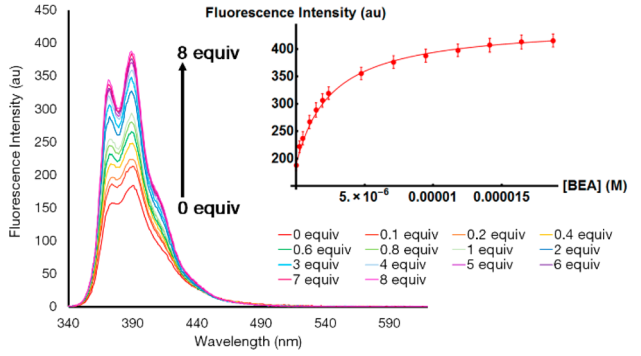

Figure 2. Fluorescence titration of 0 to 8 equiv beauvericin (BEA) to 2.5 μM receptor **6** in $CHCl_3$ using λ_{ex} = 329 nm. The inset shows the mean fluorescent intensity of **6** at 389 nm in function of added BEA concentration. The mean and standard error at each titration point were calculated based on three independent titrations. Data points were fitted to a 1:1 stoichiometry (red line).

Even though the Job plot has been the golden standard in terms of determining binding stoichiometry, multiple authors have raised concerns and criticized its use in supramolecular chemistry.[22,23] Therefore, it has been recommended to fit the data to different binding models and to assess which model is most suitable for explaining the experimental data.[22,23]

When trying to fit the above titration points to a 1:2 or 2:1 host–guest stoichiometry (see Figures S4 and S5 in the SI), no proper fit could be obtained for the individual titrations. The fact that a good mathematical fit can be found for a 1:1 binding model and no reasonable fit is achievable using either a 1:2 or 2:1 binding model confirms that the observed binding is best explained based on a 1:1 stoichiometry, which is further consistent with the proposed binding mode (recall the space-filling model in Scheme 1).

As expected, when the amine is still Boc-protected no change in fluorescence is observed (data not shown). More importantly when only the carbazole fragment is tested without the steroid arms, a much weaker association (factor 10^3 smaller) is observed (Figure S6 and S7 in the SI). This decrease in binding strength clearly emphasizes the role of the hydrophobic cavity generated by the steroid arms.

To assess the degree of selectivity, the capacity of receptor **6** to bind valinomycin (see Figure 1) was also tested. This bacterial toxin is produced by *Streptomyces spp.*[24] and, like beauvericin, is an ionophoric cyclodepsipeptide with selectivity for K^+ and NH_4^+ ions. The main difference between the two toxins resides in their molecular weight and size (783 Da for beauvericin and 1111 Da for valinomycin). Interestingly, even though both ionophores are very similar, when valinomycin was added to receptor **6**, a negligible increase in fluorescence occurred (see Figure 3, left).

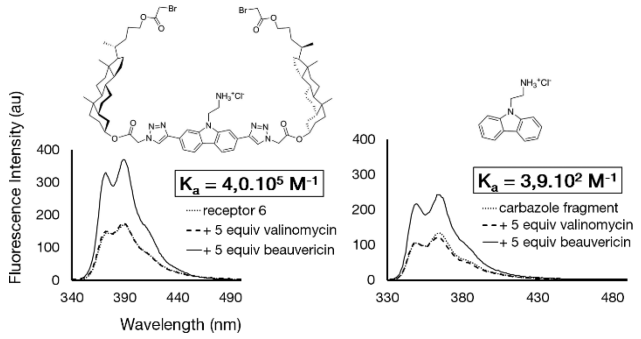

Figure 3. Fluorescence emission spectrum upon addition of 5 equiv beauvericin or valinomycin to 2.5 μM receptor **6** using λ_{ex} = 329 nm (left) or to 10 μM of the carbazole fragment using λ_{ex} = 290 nm (right) in $CHCl_3$.

Intrigued by this observation, we became interested in the structural reason behind this selectivity. We originally hypothesized that the cavity size was responsible for this effect. In order to assess this hypothesis, we synthesized a carbazole fragment that contains the amine linker but is devoid of any hydrophobic cavity, thereby expecting that both toxins would interact similarly. Surprisingly, even though beauvericin can perfectly interact with this carbazole fragment, albeit with lower affinity, valinomycin cannot (Figure 3, right). This seems to indicate that the length of the chain connecting the primary amine to the central carbazole moiety plays an important role in the selectivity. This observation might be explained by taking the size of the toxins into consideration. The short amine linker forces the toxin in close proximity of the carbazole fragment. We reason that valinomycin, being much larger than BEA, is experiencing steric hindrance due to the carbazole moiety, while this is not the case for the smaller beauvericin.

Finally, we investigated if the fluorescence sensing capabilities of receptor **6** toward BEA would still stand in more-complex solutions. To this end, 4 mL human urine was extracted with 3 mL $CHCl_3$. The organic phase was isolated and the fluorescence emission was measured. This was followed by the subsequent addition of 10 μM receptor **6**

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and 2 equiv BEA. As can be seen in Figure 4, the receptor is still able to sense the toxin, even in the chloroform extract of urine. A similar experiment was performed using urine that was spiked with BEA (see the SI).

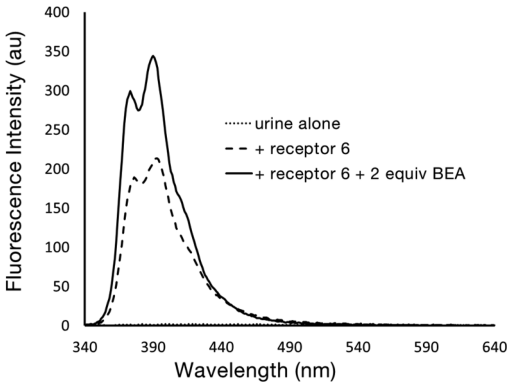

Figure 4. Fluorescence emission spectrum of a $CHCl_3$ extract from urine (dotted line) and after addition of 10 μM receptor **6** (dashed line) or both 10 μM receptor **6** and 2 equiv BEA (full line), using λ_{ex} = 329 nm.

To conclude, we have described the first synthetic receptor for the ionophore beauvericin with an association constant of 4.0×10^5 M^{-1} in $CHCl_3$. This receptor shows a pronounced increase in fluorescence intensity upon addition of the toxin, thus allowing easy detection of binding in solution without immobilization of either one of the binding partners. Interestingly, when a very similar ionophore such as valinomycin is added, no change in fluorescence is observed, providing evidence for the selectivity of our receptor. Preliminary experiments seem to indicate that not only the cavity size, but also the length of the amine linker, is responsible for this selective behavior. Artificial receptors have been described for short linear peptides consisting of a maximum of 3−4 natural amino acids (vide supra). The receptor developed here binds the 6-mer cyclic depsipeptide BEA with an affinity that is at least similar or slightly higher than those previously reported for receptors binding small peptides, making it the first synthetic receptor for beauvericin reported to date.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b02630.

Detailed synthetic procedures, spectroscopic characterization, fluorescence measurements, equations used for K_a determination and experiments with urine samples (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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