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Communication

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Next Step toward Optimization of GRP Receptor Avidities: Determination of the Minimal Distance between $BBN_{(7-14)}$ Units in Peptide Homodimers

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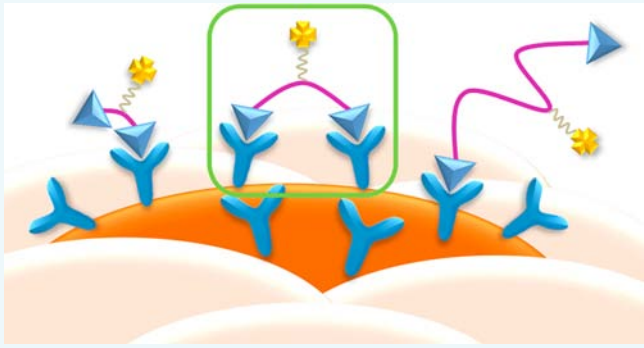
Supporting Information

ABSTRACT: As the gastrin releasing peptide receptor (GRPR) is overexpressed on several tumor types, it represents a promising target for the specific in vivo imaging of these tumors using positron emission tomography (PET). We were able to show that PESIN-based peptide multimers can result in substantially higher GRPR avidities, highly advantageous in vivo pharmacokinetics and tumor imaging properties compared to the respective monomers. However, the minimal distance between the peptidic binders, resulting in the lowest possible system entropy while enabling a concomitant GRPR binding and thus optimized receptor avidities, has not been determined so far. Thus, we aimed here to identify the minimal distance between two GRPR-binding peptides in order to provide the basis for the development of highly avid GRPR-specific PET imaging agents. We therefore synthesized dimers of the GRPR-binding bombesin analogue $BBN_{(7-14)}$ on a dendritic scaffold, exhibiting different distances between both peptide binders. The homodimers were further modified with the chelator NODAGA, radiolabeled with ^{68}Ga, and evaluated in vitro regarding their GRPR avidity. We found that the most potent of the newly developed radioligands exhibits GRPR avidity twice as high as the most potent reference compound known so far, and that a minimal distance of 62 bond lengths between both peptidic binders within the homodimer can result in concomitant peptide binding and optimal GRPR avidities. These findings answer the question as to what molecular design should be chosen when aiming at the development of highly avid homobivalent peptidic ligands addressing the GRPR.



The gastrin-releasing peptide receptor (GRPR), belonging to the bombesin receptor family, is overexpressed on a variety of tumors such as prostate, breast, and colon carcinomas, small cell lung cancer, gastrinomas, as well as head and neck tumors[1,2] and is thus a promising target structure for diagnostic imaging of these cancer types with positron emission tomography (PET).

For the development of suitable PET tracers applicable in imaging of the GRPR, many different agonistic and antagonistic analogues of the endogenous peptidic ligand bombesin were developed in order to obtain radiolabeled, highly GRPR-affine substances with increased stability compared to the natural lead.[3] However, some challenges still remain for many of these analogues, including limited in vivo stabilities, resulting in short plasma half-lives, fast excretion, high background, or low tumor uptakes. Nevertheless, promising candidates were also developed recently that could provide a basis for the development of bombesin analogues able to overcome these limitations.[4,5]

A strategy that has been followed by us and others to improve the pharmacokinetic properties of bombesin derivatives and to address the mentioned challenges is the multimerization of GRPR-affine peptides[6−9] as this approach is in principle able to result in radioligands exhibiting improved in vitro and in vivo properties compared to the respective monomers.[9−11]

As we were able to show before, the multimerization of bombesin analogues is able not only to increase the stability and receptor avidity of the peptide-based radiotracer, but also to substantially improve its in vivo pharmacokinetics and tumor visualization ability in PET imaging.[9] It was also found that, using this strategy, it is of crucial importance to determine the best-suited peptide multiplicity as well as to determine a suitable distance between the peptidic ligands in order to obtain

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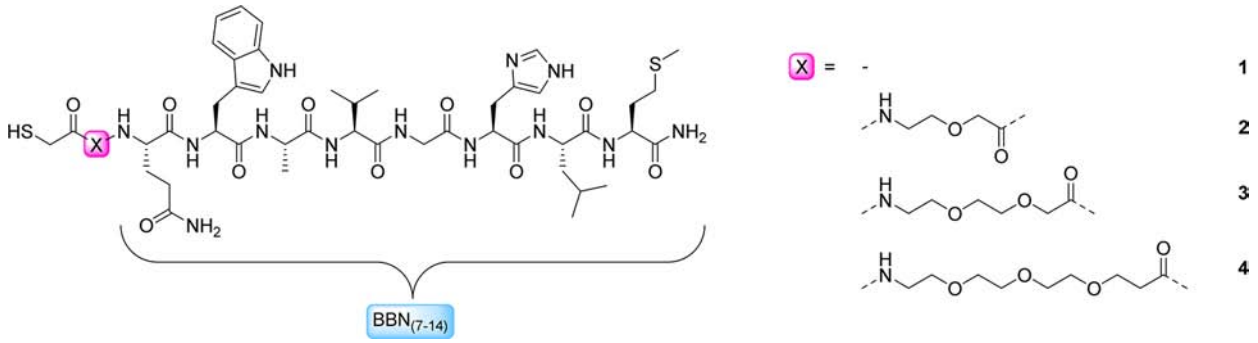
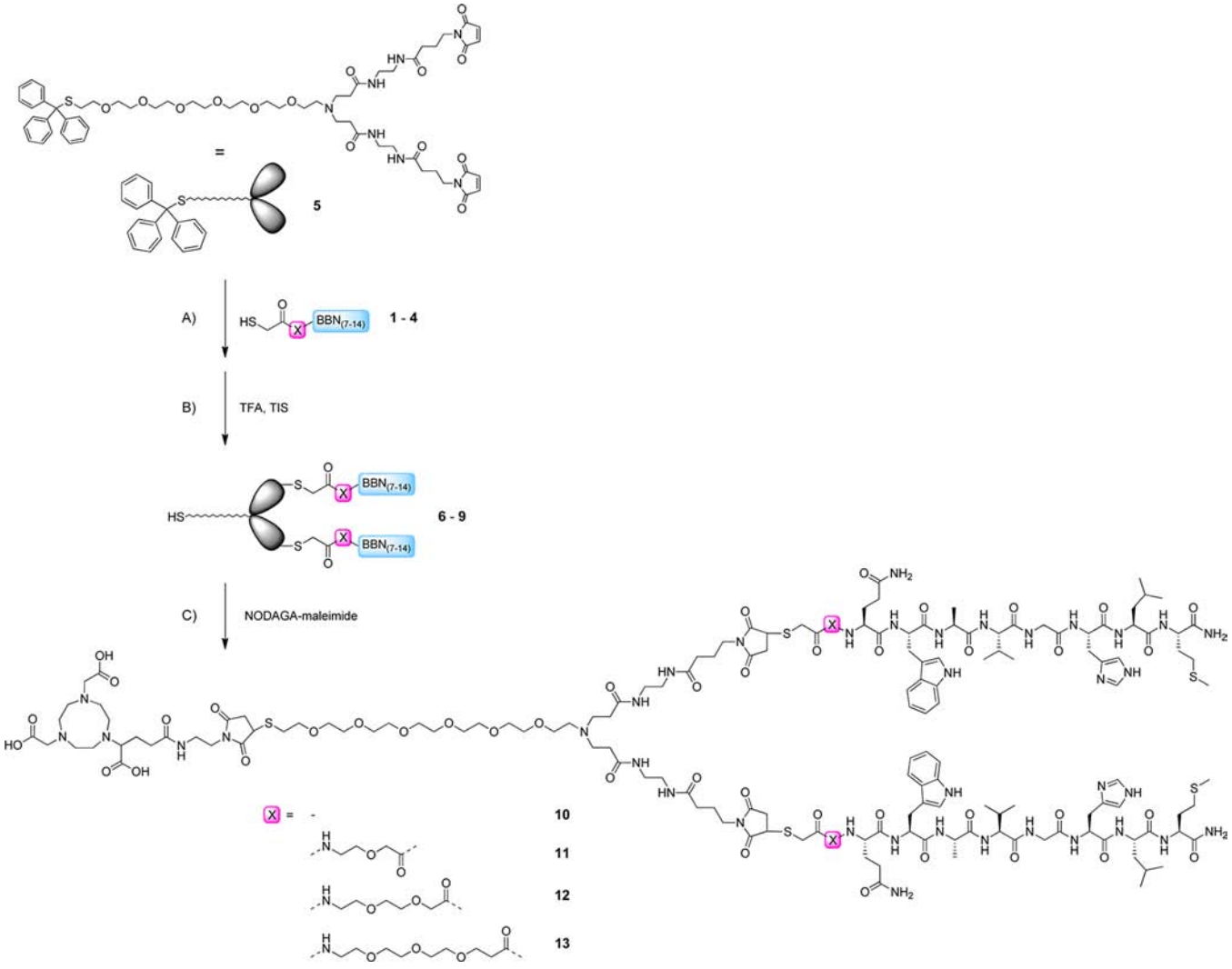


Figure 1. Structures of the thiol-comprising $BBN_{(7-14)}$ derivatives **1**−**4** synthesized for subsequent homodimerization.

Scheme 1. Schematic Depiction of the Synthesis of the NODAGA-Derivatized $BBN_{(7-14)}$ Dimers 10−13[a]



[a]Conditions: (A) $BBN_{(7-14)}$-PEG_x-SH **1**−**4** (2.5 equiv), PB (phosphate buffer) (0.2 M, pH 5.0): MeCN 2:1, pH 6.9−7.2, RT, 5 min; (B) TFA:TIS 19:1, RT, 5 min, isolated yields of 19−30% over both steps (A) and (B); (C) NODAGA-maleimide (2.0 equiv), H_2O:MeCN 1:1 + 0.1% TFA, PB (0.2 M, pH 7.5), pH 6.9−7.2, RT, 5 min, isolated yields of 40−73%.

substances exhibiting a high avidity to the respective target receptor. By this approach, a strengthened binding to the GRPR compared to the respective monomer and—using a relatively short distance between both peptide binders—PET images of considerably improved quality with regard to tumor target detection and tumor-to-background ratios can be obtained. These positive effects can be attributed to a higher GRPR avidity but also to the favorable influence the peptide multimerization itself has on the in vivo pharmacokinetics of a radiotracer.[9,10] Regarding peptide multiplicity, a homodimer comprising two peptide copies is optimal for achieving high GRPR avidities and further peptide units cannot contribute to higher avidities, e.g., by increasing the probability of concomitant receptor binding by more than one peptide.

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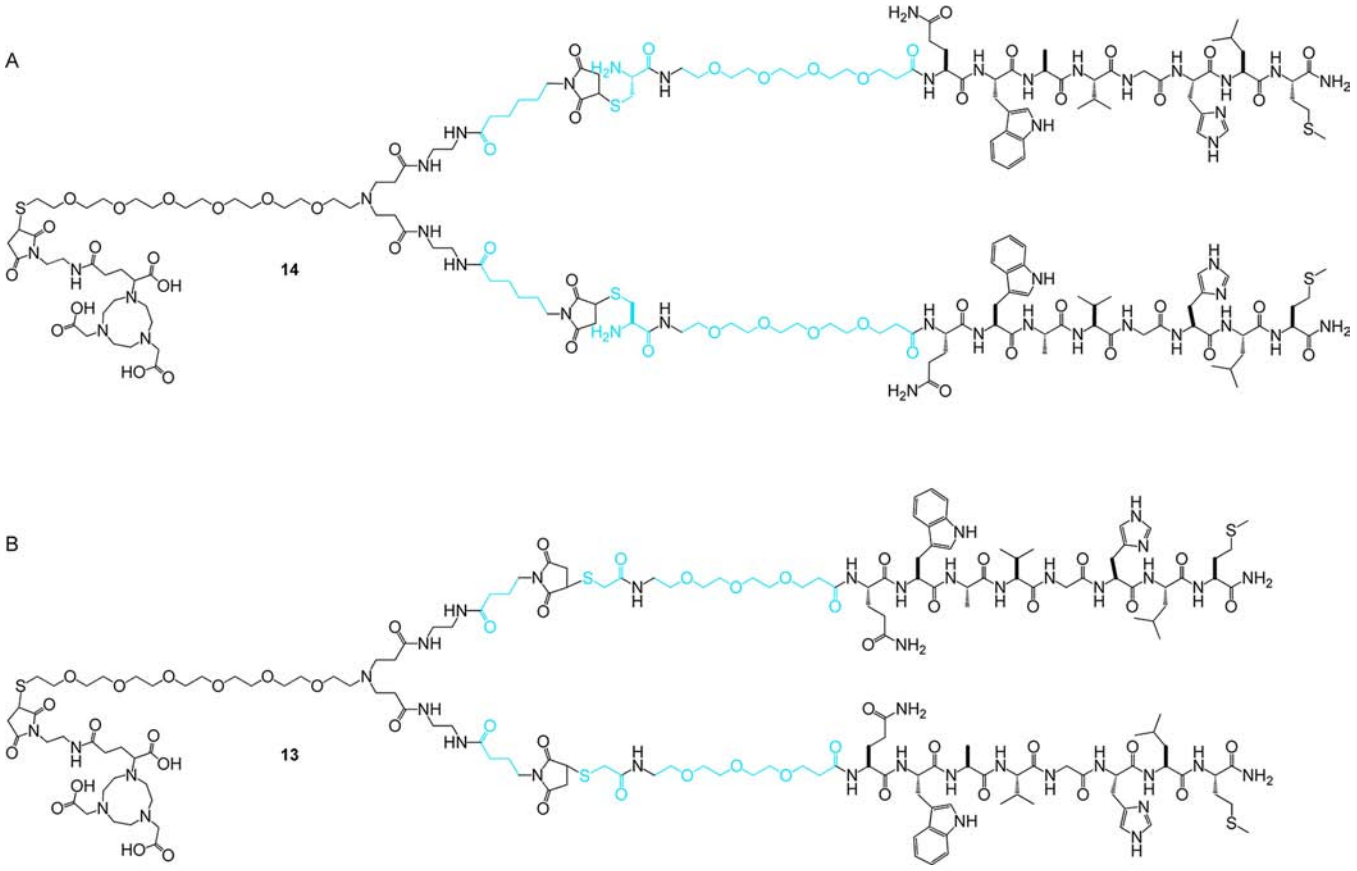

Figure 2. Comparative depiction of the structures of (A) the homodimeric reference substance **14**[9] and (B) the most potent newly developed substance **13**. Structure elements differing between both homodimer generations are depicted in color.

From the data available, it seems most likely that the minimal distance between both GRP receptor ligands within a peptide homodimer, resulting in optimal binding avidities by enabling concomitant peptide binding, has not been found so far, as this should range between 28 bond lengths (which were shown before to result in a slightly increased avidity of the dimer compared to the respective monomer)[6] and 74 bond lengths (resulting in a considerably increased avidity of the dimer compared to the monomer).[9]

Thus, we intended in this study to determine that particular minimal distance between two bombesin$_{(7-14)}$ (BBN$_{(7-14)}$) copies within a respective peptide homodimer which results in the lowest possible system entropy while enabling a concomitant peptide binding and thus provides optimal GRPR avidities of bombesin homodimers. These insights should be able to provide the basis for the development of highly potent bombesin homodimer-based PET imaging agents for the visualization of GRPR-expressing tumors.

Synthesis of Thiol-Functionalized BBN$_{(7-14)}$ Analogues. As we intended to systematically determine the minimal distance between two BBN$_{(7-14)}$ copies in a peptide homodimer enabling concomitant peptide GRPR binding, anticipating it to range between 28[6] and 74[9] bond lengths, we first synthesized BBN$_{(7-14)}$ derivatives comprising no or hydrophilic ethylene glycol linkers of differing lengths. These ethylene glycol-based linkers were applied in order to obtain highly hydrophilic substances ensuring a predominantly renal clearance of the resulting radiotracers. The BBN$_{(7-14)}$ derivatives were further modified with a thiol functionality and subsequently dimerized on a dendritic core, resulting in distances between both peptide binders of 36, 48, 54, and 62 bond lengths in the finally obtained labeling precursors.

The linker- and thiol-modified peptide analogues were synthesized by standard Fmoc-based solid-phase peptide synthesis (SPPS) by successive conjugation of the respective amino acids and linkers to a rink amide resin using standard reaction conditions.[9] As we intended to apply the same molecular design as in the mentioned preceding study—yielding a structural lead with advantageous in vivo pharmacokinetics and PET tumor imaging properties—we functionalized the BBN$_{(7-14)}$ analogues with *S*-trityl-mercaptoacetic acid to obtain a *N*-terminal thiol functionality amenable to dendron conjugation via Michael addition. The respective peptide monomers **1**−**4** (Figure 1) used for subsequent homodimerization were obtained in overall isolated yields of 30% to 39%.

Synthesis of Chelator-Comprising Peptide Dimers 10−14 and ^{68}Ga-Radiolabeling. A small PAMAM (polyamidoamine) dendron—built on the basis of a *S*-trityl-protected amino-functionalized PEG$_5$ linker and derivatized with maleimidobutanoic acids[9] for peptide conjugation via Michael addition—was used as symmetrical scaffold structure for peptide homodimerization (**5**, Scheme 1). The applied PEG$_5$ linker enables the efficient introduction of a chelating agent—necessary for the labeling of the substances with radiometal nuclides such as ^{68}Ga used for PET imaging—in the last synthesis step despite the sterically demanding homodimeric peptide structures.

This maleimide-modified PAMAM dendron scaffold **5** was at first quantitatively reacted within minutes with the thiol-

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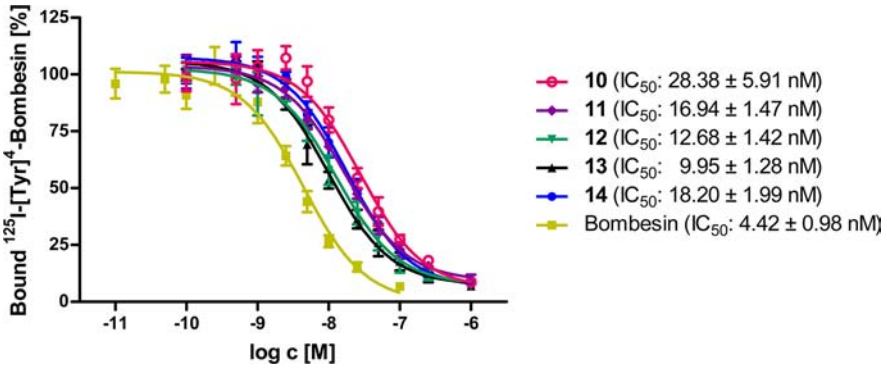

Figure 3. Binding curves and IC_{50} values for the $BBN_{(7-14)}$ homodimers **10**–**14** and bombesin obtained by competitive binding experiments on viable human prostate carcinoma PC-3 cells. Results were obtained from at least three independent experiments, each performed in triplicate.

modified peptides **1**–**4** (Scheme 1A) and afterward deprotected with neat trifluoroacetic acid (TFA) and triisopropylsilane (TIS) (Scheme 1B), resulting in the intermediate products **6**–**9** which were obtained in good isolated yields.

These intermediate $BBN_{(7-14)}$ homodimers **6**–**9** were in the following efficiently reacted under mild conditions within minutes with NODAGA-maleimide (NODAGA = 1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid), giving the chelator-functionalized $BBN_{(7-14)}$ homodimeric labeling precursors **10**–**13** (Scheme 1C) in high isolated yields. NODAGA was chosen as chelating agent as it allows for the stable and efficient complexation of $^{68}Ga^{3+}$ at ambient temperature.[12]

In addition to these new $BBN_{(7-14)}$ homodimers **10**–**13**, exhibiting distances between both peptides of 36, 48, 54, and 62 bond lengths, the most potent bombesin analogue dimer of the preceding study (**14**, Figure 2A) was synthesized using the same protocols. It exhibits a distance of 74 bond lengths between both peptide units and served as a reference substance in the following in vitro competitive receptor binding assay.

In the following, the $BBN_{(7-14)}$ homodimers **10**–**14** were radiolabeled with ^{68}Ga using the fractioned elution method of $^{68}Ge/^{68}Ga$ generators,[13] omitting a pre-reaction purification of the generator eluate. The radiolabeling protocol used does not require a heating step for complexation of the radiometal allowing for a very efficient labeling reaction at ambient temperature within 10 min.[12] The ^{68}Ga-labeled products [^{68}**Ga**]**10**–[^{68}**Ga**]**14** could be obtained in high radiochemical yields and purities of 96–99% as well as nonoptimized specific activities of 11.2 to 64.6 GBq/μmol, starting from 162 to 829 MBq of $^{68}Ga^{3+}$.

In Vitro GRPR Avidity Evaluation of Peptide Homodimers 10–14 and Bombesin on GRPR-Expressing PC-3 Cells. The homodimers **10**–**14** and bombesin (which was taken as endogenous peptide reference) were evaluated as to their GRPR avidity applying a competitive binding assay on the human prostate carcinoma PC-3 cell line using ^{125}I-[Tyr^4]-bombesin as tracer. The PC-3 cell line expresses the GRPR in high density of up to $2.7 \pm 0.1 \times 10^6$ receptors per cell, and thus represents a suitable cell line for the determination of the minimal distance between both $BBN_{(7-14)}$ peptide units of a respective peptide homodimer.[14] By this approach, the effects of the distance between both peptide binders within the homodimer on the achievable GRPR avidities were comparatively assessed. In Figure 3, the results of the competitive binding assay are summarized.

From these results, a clear trend regarding the influence of the distance between both peptide parts of the homodimer on the resulting GRPR avidities can be observed: A distance of 62 bond lengths (**13**) seems to be the minimal distance in order to achieve a concomitant and thus strong GRP receptor binding as shorter distances between both peptides (**10**–**12**) resulted in weaker GRPR binding (Figure 3). The GRPR binding potential found for **13** furthermore is twice as high as that of the so far most potent homodimer **14**[9] and comes near that of the endogenous ligand bombesin, meaning that the developed homodimeric substance **13** has a high GRP receptor-targeting ability.

These results indicate that a minimal distance of 62 bond lengths is necessary to enable a concomitant binding of both peptidic ligands of $BBN_{(7-14)}$-based homodimers and thus to achieve an optimal target GRPR binding.

As we intended to ensure an in-principle sufficient stability of the ^{68}Ga-labeled homodimer [^{68}**Ga**]**13** for in vivo application, we studied its stability in human serum. Over the 2 h period of investigation, no fragmentation of the peptide homodimer was observed, indicating its high stability, being in principle suitable for in vivo use.

In summary, it was shown before that binding affinities/avidities can give a valid indication of the biological potency of peptide multimers and that multivalency itself is also able to contribute to improved in vivo pharmacokinetics as well as tumor accumulation properties of the radioligands.[9,10,15] Thus, the results presented here—answering the question as to which distance should minimally be maintained between two peptides in a GRPR-targeting peptide homodimer—can contribute to the development of highly potent homobivalent peptidic ligands addressing the GRPR.

The next step will be to assess which of the known GRPR agonists and antagonists gives the best results with regard to GRPR-targeting and in vivo tumor visualization when homodimerized using the molecular design presented here.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and compound characterization data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00362.

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Notes

The authors declare no competing financial interest.

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