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A Bifunctional Monomer for On-Resin Synthesis of Polyfunctional PNAs and Tailored Induced-Fit Switching Probes

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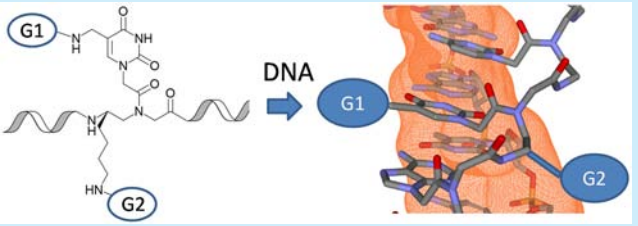
Supporting Information

ABSTRACT: A synthetic strategy for the production of polyfunctional PNAs bearing substituent groups both on the nucleobase and on the backbone C5 carbon of the same monomer is described; this is based on the use of a tris-orthogonally protected monomer and subsequent solid-phase selective functionalization. This strategy can be used for synthesizing PNAs that are not readily accessible by use of preformed modified monomers. As an example, a PNA-based probe that undergoes a switch in its fluorescence emission upon hybridization with a target oligonucleotide, induced by tailor-made movement of two pyrene substituent groups, was synthesized.



The basic structure of the peptide nucleic acid (PNA),[1] due to its excellent ability to hybridize with complementary target sequences and its high chemical and biological stability, can serve as a robust model scaffold for the synthesis of novel compounds, with applications in diagnostics, gene therapy, and assembly of nanostructured materials.[2] Chemical modification of PNA has emerged as a good strategy for overcoming innate drawbacks of the original structure[3] or for introducing additional groups with specific functions,[4] allowing for the development of increasingly efficient tools.[5]

PNAs with modified backbones have been reported, and modification either at C2 (α) or at C5 (γ) carbons of the aminoethylglycine unit has been largely exploited. Appropriate choice of stereochemistry allows for obtaining better performing PNAs with an increase in affinity and in sequence selectivity.[6] Of these, substitution at the C5 position shows some advantages because it provides a strategy to obtain preorganization,[7] to link the PNA to reporter or functional groups,[8] or to tether the probe to surfaces.[9] Nucleobase-modified PNAs have also been described,[10] and modifications with functional and reporter groups are especially used to improve the properties of PNAs as diagnostic molecular probes.[11]

Multifunctional systems in which different modifications are inserted into a single PNA strand to confer multiple properties can also be designed and synthesized. The main approach proposed for the synthesis of a polyfunctional PNA has been the preparation of modified monomers already bearing the desired functional group, followed by their insertion at the appropriate position along the strand.[2a] The advantage of this strategy is the use of standard protocols and fully automated peptide synthesis. However, this strategy is not convenient for generating molecular diversity and libraries. Additionally, it is possible that bulky substituents decrease the coupling yields in proximity of the modified monomers. As an alternative straightforward option, one might preform orthogonally protected monomers as "hidden reactive spots" that can be selectively deprotected during the solid-phase synthesis (SPS) of the PNA strand. This allows the reactive groups that are intended for the chemical introduction of the selected functional moieties and pro-reactive groups to be unleashed.[8,12] Recently, we reported a similar approach consisting of the introduction of an orthogonal function into a PNA thymine nucleobase.[13] Herein, we report the development of a SPS protocol based on a doubly modified PNA monomer that enables the selective introduction of functional residues both at the backbone and at the nucleobase side of the same monomer. This design has the advantage to (i) introduce a variety of moieties on the PNA which are difficult to introduce on the monomeric units; (ii) avoid solubility and reactivity problems for heavily substituted monomers during the SPS; and (iii) easily introduce two functional groups which can be positioned very close to each other and eventually interact.

A rational design of a doubly modified monomer that can be easily modified during SPS relies on carefully choosing orthogonal protective groups. These must be independently and selectively deprotected in order to avoid the formation of side products and must be compatible with the linker group connecting the PNA with the solid support. We focused on a Boc-based peptide synthesis strategy so that the Fmoc group could be used as an orthogonal protective function of a lysine side chain linked to the γ position of the PNA monomer. This enables the use of the commercially available Boc-L-Lys(Fmoc)−OH as starting material. Furthermore, modifications on this position were shown to lead to an enhancement of DNA binding strength, as preorganization is induced.[7a,14] The azide group of 5-azidomethyluracil was chosen as a second orthogonal function

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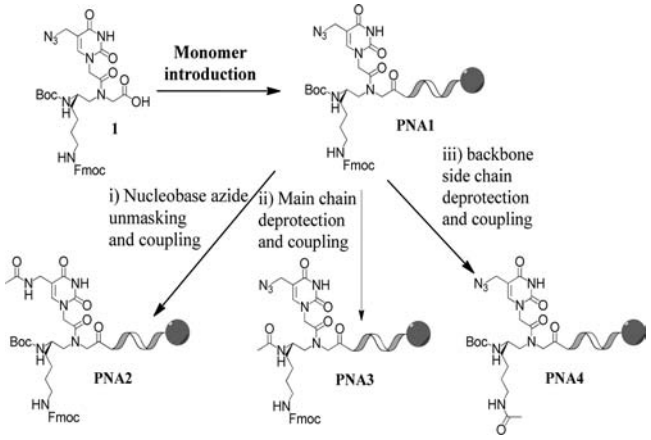
because of its efficient "unmasking" (i.e., transformation into an amine) under mild Staudinger conditions. The azide group may also be useful to carry out 1,3-dipolar cycloadditions under Huisgen's conditions.[13,15] Monomer **1** was thus synthesized as described in the Supporting Information. Briefly, the carboxylic group of Boc-L-Lys(Fmoc)−OH was reduced to aldehyde using Weinreb amide reduction strategy; the product was then reacted with glycine methyl ester under reductive amination conditions. The backbone thus obtained was linked to 2-(5-azidomethylur-acil-1-yl)acetic acid[13] using EDC/DhBtOH coupling. Selective deprotection of the methyl ester under basic conditions gave the monomer **1**.

Monomer **1** was inserted into a growing PNA strand using standard SPS protocols with HBTU/DIPEA as activating mixture, giving the modified **PNA1**. The **PNA1**-carrying resin was then split into three aliquots in order to assess the effective orthogonality of the different deprotection conditions (Scheme 1). Acetylation was carried out (i) after the preliminary reduction

Scheme 1. Summary of the Three Possible Reactive Pathways for Selective PNA Functionalization

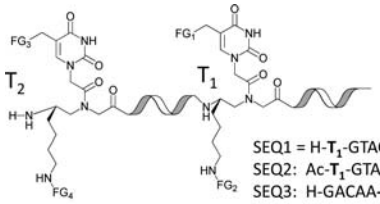


of the azide function (0.33 M trimethylphosphine in a 1:1 THF/H_2O mixture, 10 min, 2 cycles; Scheme 1, path (i)) to yield **PNA2**; (ii) after deprotection of the terminal Boc-protected amino function using standard Boc-based SPS protocol (5% *m*-cresol in TFA, 4 min, 2 cycles; Scheme 1, path (ii)), thus giving **PNA3**; and (iii) after the Fmoc group on the lysine side chain was removed (20% piperidine in DMF, 8 min, 2 cycles; Scheme 1, path (iii)), resulting in **PNA4**.

Using Boc chemistry, the azide function should be converted into derivatives on the solid support because cleavage of azide-containing PNA generates a series of degradations products (e.g., with reduced azide), with a characteristic pattern of mass changes (Figures S7−S9).[16] In all cases, absence of starting material and of polyfunctionalized side products due to unwanted deprotections was confirmed by ESI-MS analysis after cleavage from the solid support.

A series of modified PNAs was then synthesized using one or two distant units of the doubly modified monomer **1** (Table 1). These PNAs feature two or four groups available for further substitution. **PNA5** was first synthesized, with a sequence complementary to that of a segment of miR-221, which is a micro-RNA particularly relevant in gene regulation and overexpressed in several types of cancers.[17] To further prove the mutual orthogonality of azide and Fmoc groups, independently from the order of reactions and the possibility to efficiently introduce

Table 1. General Scheme of PNA Synthesized, Bearing One or Two Doubly Functionalized PNA Monomers with Different Modifications and Sequences[a]



PNA	sequence	FG_1	FG_2	FG_3	FG_4
PNA1	SEQ1	N_3	Ac		
PNA2	SEQ1	Ac	Fmoc		
PNA3	SEQ2	N_3	Ac		
PNA4	SEQ1	N_3	Fmoc-AEEA		
PNA5	SEQ3	N_3	Fmoc		
PNA6	SEQ3	Fl-NH	Fmoc		
PNA7	SEQ3	N_3	Fmoc-AEEA		
PNA8	SEQ3	Fl-NH	Fmoc-AEEA		
PNA9	SEQ4	N_3	Fmoc		
PNA10	SEQ4	N_3	Fl		
PNA11	SEQ5	N_3	Fl	N3	Fmoc
PNA12	SEQ5	Py-NH	Fl	Py-NH	Fmoc
PNA13	SEQ5	Py-NH	Fl	Py-NH	Rh
PNA14	SEQ6	Py-NH	Py-NH		

[a]Fl, 5(6)-fluoresceincarbonyl; AEEA, 2-(2-aminoethoxy)ethoxyacetyl; Rh, B-Rhodaminyl; Py, 1-pyrenylacetyl.

different groups in the middle of the strand, the resin bearing **PNA5** was split in two different batches and subjected to different orders of functionalization of the lateral protective groups, that is, on the nucleobase at first (leading to **PNA6**) and next on the backbone or, vice versa, first on the backbone side chain (leading to **PNA7**) and then on the nucleobase, to obtain the same doubly functionalized **PNA8** (Figure S8). In both cases, we obtained complete conversions without the presence of side products and with similar purity of the final **PNA8** (see Supporting Information), which confirms and strengthens the previous experiments conducted on **PNA1** of complete reciprocal independence of the orthogonal reactions and the lack of interference by the elongated PNA chain. We further tested the generality of this strategy for producing multifunctional PNA, together with the possibility to functionalize two positions at the same time, by synthesizing a series of PNAs (**PNA9−13**, Table 1) with a different sequence, previously used by us in combination with pyrene-containing nucleobase-modified monomers.[18] After insertion of the first monomer **1** unit, deprotection of the Fmoc group (**PNA9**) and subsequent derivatization with fluorescein (**PNA10**) were carried out. Elongation of the PNA chain and insertion of a second unit of monomer **1** led to **PNA11**, and at this step, the two azide functions of this PNA were simultaneously reduced and then coupled with 1-pyreneacetic acid to yield the triply functionalized **PNA12**. This latter could be deprotected and further functionalized using rhodamine, giving **PNA13**. A key point of this approach is that, using the doubly modified PNA monomers and harnessing the flexibility offered by the on-resin modification, it is possible to obtain systems in which the mutual position of the two units on the same monomer can be changed upon interaction with DNA.

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In fact, in the flexible single-stranded PNA, the two moieties on the same monomer can interact with each other, whereas in a PNA:DNA duplex (as illustrated in Figure 1A, using a model

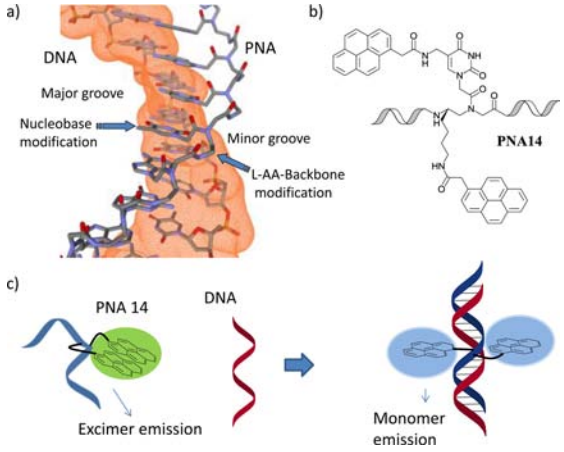

Figure 1. (a) Position of C5 (γ) and uracil substituents in the PNA:DNA duplex, directed toward the minor and major grooves, respectively, according to the structure reported in ref 19. (b) Structure of **PNA14**. (c) Scheme of induced-fit movement observed for **PNA14** upon interaction with complementary DNA (see Figure S26 for a static 3D model).

derived from one of the available crystal structures[19]), it is predicted that a nucleobase-linked group would protrude into the major groove, while a substituent on the (C5) γ position, with the stereochemistry derived from L-amino acids, would be directed either outward or toward the minor groove, thus forcing the two groups to be separated. This model and the newly developed method were used to obtain **PNA14**, which bears two pyrene moieties on the same monomer in tweezers-like fashion. Unmodified **PNA15** (H-GCAGACAATGTAGCT-Gly-NH_2) was also synthesized for comparison. **PNA14** was designed to exploit the vicinity of the two pyrenes, which, combined with the high flexibility of the single-stranded PNA, allows the interaction of the aromatic moieties. The high stability of the PNA:DNA and PNA:RNA duplexes can then force the two residues to adopt the conformation of the duplex and thus to be parted (also see models in Figure S26), which allows to take advantage of the switching between the excimer and monomer fluorescent states of the pyrene couple induced by the interaction with DNA. Attempts to synthesize this PNA using a doubly pyrene-modified monomer failed due to its lower solubility and steric hindrance (data not shown). In this case, we focused on a sequence fully complementary to a longer tract of miR-221 because this is an interesting target for cancer diagnostics and for anti-miR therapy.[17] SPS of the PNA probe was carried out following the above-reported procedure, except for the subsequent release of both the amino groups before the coupling with 1-pyreneacetic acid (Supporting Information). **PNA14** exhibited the typical pyrene excimer band in solution (broad band at 464 nm, Figures 2 and S13–S15), suggesting that the two pyrene units are able to interact in aqueous solution; this can be attributed to the PNA flexibility, and it is driven by hydrophobic effect and $\pi-\pi$ stacking interactions. We then investigated the ability of **PNA14** to hybridize with the target sequence and to give a detectable fluorescent response. A DNA strand (**DNA-FM**: 5′-AGCTAC-ATTGTCTGC-3′) with a sequence homologous to that of miR-221 was used as the model target.

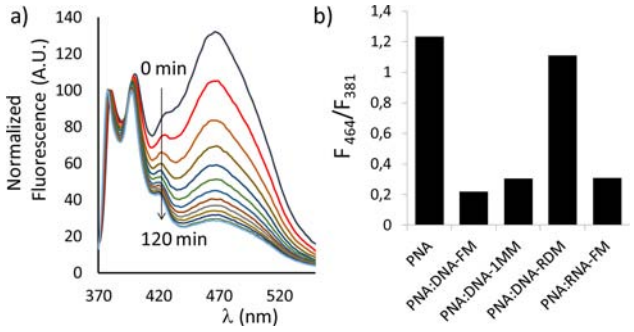

Figure 2. (a) Normalized fluorescence emission spectra of a 5 μM solution of **PNA14** hybridizing with the target DNA sequence (T = 25 °C, at different incubation times: top to bottom 0–120 min, time gaps of 10 min). (b) Excimer/monomer emission intensity ratio for **PNA14** (1 μM) at 50 °C (after annealing at 90 °C) in the presence of full match (FM), single mismatch (MM), and random (RDM) DNA sequences, and full match RNA. Measurements were in PBS with 0.7% acetonitrile at pH 7.0.

Figure 2a reports the trend of fluorescence emission spectra at different incubation times (here at 25 °C; see also Supporting Information for the same phenomenon investigated at different concentration and temperatures) upon addition of the complementary target DNA to the **PNA14** solution. The formation of a PNA/DNA duplex led to a drastic drop of the excimer band and a simultaneous increase of the monomer bands (Figure S14). The observed switching effect was due to the specific recognition of the DNA counterpart, as shown by the hybridization of the mismatched sequence **DNA-MM** (5′-AGCTAC**G**TTGTCTGC-3′), which also caused a decrease of the excimer/monomer emission ratio but with slower kinetics and with a less pronounced final effect (Figure S14). Melting experiments revealed a hysteresis profile (results not shown), confirming a slow kinetics of the association process. These kinetics meaurements were affected by temperature, strand concentrations, ionic strength, and percentages of acetonitrile (Figures S14–S17). We found that by being annealed at 90 °C with fluorescence measurements at 50 °C, the process was sufficiently fast to allow the comparison of final intensities without the bias of kinetic effects. **DNA-FM** and, to a slightly lesser extent, **DNA-MM** showed a net decrease of the excimer/monomer fluorescent intensity ratio (Figure 2b and Figure S17). A comparable decrease was observed for complementary **RNA-FM** (5′-AGCUACAUUGUCUGC-3′).

A noncomplementary random sequence (**DNA-RDM**, 5′-GGTGAATGAGTAAC-3′) was used to make the sequence dependence of the process more evident. The results reported in Figure 2b show an almost negligible switch from the excimer to monomer emission state for the **DNA-RDM**, with this very minute effect likely ascribed to nonspecific interactions. This demonstrates that the disruption of the pyrene–pyrene stacking is mediated by the sequence-selective interaction of the PNA with the DNA, in accordance with the model depicted in Figure 1. Circular dichroism spectra (Figures S18–S24) revealed that the **PNA14**:**DNA-FM** duplex conformation is similar to that obtained for unmodified **PNA15** and is formed both at 25 °C and at 50 °C. The same was observed for **DNA-MM** and for **RNA-FM**.

Analysis of the melting temperatures of the **PNA14**:DNA hybrids (Table S1) indicated a T_m of 69.3 °C for the **DNA-FM**, 70.6 °C for **RNA-FM**, and 65.9 °C for the **DNA-MM**; thus both are still formed, though to a different extent, at 50 °C.

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Comparison with the T_m obtained for **PNA15** allows one to conclude that the pyrene units destabilize the complex with **DNA-FM** and do not affect that of **DNA-MM**, thus slightly decreasing the sequence selectivity.

In conclusion, the proposed strategy for the synthesis of polyfunctional PNAs, based on the use of a modified monomer equipped with three orthogonal protective groups, allowed us to perform the successive introduction of desired functional moieties directly on solid phase and to produce multifunctional PNAs. We believe this method may serve as a robust and efficient way to produce tools both for diagnostics and nanofabrication purposes. As an example, the novel pyrene-based **PNA14** switching probe able to undergo a detectable and programmed conformational change upon hybridization with DNA could be easily obtained.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02363.

Experimental procedures, characterizations, and additional hybridization studies (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497−1500. (b) Nielsen, P. E. *Acc. Chem. Res.* **1999**, *32*, 624−630.

(2) (a) Sacui, I.; Hsieh, W. C.; Manna, A.; Sahu, B.; Ly, D. H. *J. Am. Chem. Soc.* **2015**, *137*, 8603−8610. (b) Flory, J. D.; Shinde, S.; Lin, S.; Liu, Y.; Yan, H.; Ghirlanda, G.; Fromme, P. *J. Am. Chem. Soc.* **2013**, *135*, 6985−6993. (c) Anstaett, P.; Zheng, Y.; Thai, T.; Funston, A. M.; Bach, U.; Gasser, G. *Angew. Chem., Int. Ed.* **2013**, *52*, 4217−4220. (d) Shiraishi, T.; Nielsen, P. E. *Bioconjugate Chem.* **2012**, *23*, 196−202.

(3) (a) Hyrup, B.; Nielsen, P. E. *Bioorg. Med. Chem.* **1996**, *4*, 5−23. (b) Koppelhus, U.; Nielsen, P. E. *Adv. Drug Delivery Rev.* **2003**, *55*, 267−280. (c) Torres, A. G.; Fabani, M. M.; Vigorito, E.; Williams, D.; Al-Obaidi, N.; Wojciechowski, F.; Hudson, R. H. E; Seitz, O.; Gait, M. J. *Nucleic Acids Res.* **2012**, *40*, 2152−2167. (d) Zhou, P.; Wang, M.; Du, L.; Fisher, G. W.; Waggoner, A.; Ly, D. H. *J. Am. Chem. Soc.* **2003**, *125*, 6878−6879.

(4) (a) Corradini, R.; Sforza, S.; Tedeschi, T.; Totsingan, F.; Manicardi, A.; Marchelli, R. *Curr. Top. Med. Chem.* **2011**, *11*, 1535−1554. (b) Tedeschi, T.; Sforza, S.; Corradini, R.; Marchelli, R. *Tetrahedron Lett.* **2005**, *46*, 8395−8399. (c) Rozners, E. *J. Nucleic Acids* **2012**, *2012*, No. 518162.

(5) Zhao, C.; Hoppe, T.; Setty, M. K. H. G.; Murray, D.; Chun, T. W.; Hewlett, I.; Appella, D. H. *Nat. Commun.* **2014**, *5*, No. 5079.

(6) Sforza, S.; Tedeschi, T.; Corradini, R.; Marchelli, R. *Eur. J. Org. Chem.* **2007**, *2007*, 5879−5885.

(7) (a) Dragulescu-Andrasi, A.; Rapireddy, S.; Frezza, B. M.; Gayathri, C.; Gil, R. R.; Ly, D. H. *J. Am. Chem. Soc.* **2006**, *128*, 10258−10267. (b) Dragulescu-Andrasi, A.; Zhou, P.; He, G.; Ly, D. H. *Chem. Commun. (Cambridge, U. K.)* **2005**, *2*, 244−246. (c) Sahu, B.; Sacui, I.; Rapireddy, S.; Zanotti, K. J.; Bahal, R.; Armitage, B. A.; Ly, D. H. *J. Org. Chem.* **2011**, *76* (14), 5614−5627.

(8) Englund, E. A.; Appella, D. H. *Angew. Chem., Int. Ed.* **2007**, *46*, 1414−1418.

(9) De, A.; Souchelnytskyi, S.; Van Den Berg, A.; Carlen, E. T. *ACS Appl. Mater. Interfaces* **2013**, *5*, 4607−4612.

(10) (a) Hudson, R. H. E.; Viirre, R. D.; Liu, Y. H.; Wojciechowski, F.; Dambenieks, A. K. *Pure Appl. Chem.* **2004**, *76*, 1591−1598. (b) Wojciechowski, F.; Hudson, R. E. *Curr. Top. Med. Chem.* **2007**, *7*, 667−679.

(11) (a) Oquare, B. Y.; Taylor, J. S. *Bioconjugate Chem.* **2008**, *19*, 2196−2204. (b) St. Amant, A. H.; Hudson, R. H. E. *Org. Biomol. Chem.* **2012**, *10*, 876−881. (c) Wojciechowski, F.; Hudson, R. H. E. *Org. Lett.* **2009**, *11* (21), 4878−4881. (d) Müllar, S.; Strohmeier, J.; Diederichsen, U. *Org. Lett.* **2012**, *14*, 1382−1385.

(12) (a) de Koning, M. C.; Petersen, L.; Weterings, J.; Overhand, J. M.; van der Marel, G. A.; Filippov, D. V. *Tetrahedron* **2006**, *62*, 3248−3258. (c) Manicardi, A.; Gyssels, E.; Corradini, R.; Madder, A. *Chem. Commun.* **2016**, *52*, 6930−6933.

(13) Manicardi, A.; Accetta, A.; Tedeschi, T.; Sforza, S.; Marchelli, R.; Corradini, R. *Artif. DNA PNA XNA* **2012**, *3*, 53−62.

(14) (a) Totsingan, F.; Rossi, S.; Corradini, R.; Tedeschi, T.; Sforza, S.; Juris, A.; Scaravelli, E.; Marchelli, R. *Org. Biomol. Chem.* **2008**, *6*, 1232−1237. (b) Rapireddy, S.; He, G.; Roy, S.; Armitage, B. A.; Ly, D. H. *J. Am. Chem. Soc.* **2007**, *129*, 15596−15600.

(15) Schilling, C. I.; Jung, N.; Biskup, M.; Schepers, U.; Bräse, S. *Chem. Soc. Rev.* **2011**, *40* (9), 4840−4871. Wang, X.; Hudson, R. H. E. *ChemBioChem* **2015**, *16*, 2156−2161.

(16) Schneggenburger, P. E.; Worbs, B.; Diederichsen, U. *J. Pept. Sci.* **2010**, *16*, 10−14.

(17) (a) He, H.; Jazdzewski, K.; Li, W.; Liyanarachchi, S.; Nagy, R.; Volinia, S.; Calin, G. A.; Liu, C. G.; Franssila, K.; Suster, S.; Kloos, R. T.; Croce, C. M.; de la Chapelle, A. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102* (52), 19075−19080. (b) Esquela-Kerscher, A.; Slack, F. J. *Nat. Rev. Cancer* **2006**, *6*, 259−269. (c) Falkenberg, N.; Anastasov, N.; Rappl, K.; Braselmann, H.; Auer, G.; Walch, A.; Huber, M.; Höfig, I.; Schmitt, M.; Höfler, H.; Atkinson, M. J.; Aubele, M. *Br. J. Cancer* **2013**, *109*, 2714−2723.

(18) Manicardi, A.; Guidi, L.; Ghidini, A.; Corradini, R. *Beilstein J. Org. Chem.* **2014**, *10*, 1495−1503.

(19) Menchise, V.; De Simone, G.; Tedeschi, T.; Corradini, R.; Sforza, S.; Marchelli, R.; Capasso, D.; Saviano, M.; Pedone, C. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 12021−12026.

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