ACS Macro Letters

Letter

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Micellar Paclitaxel-Initiated RAFT Polymer Conjugates with Acid-Sensitive Behavior

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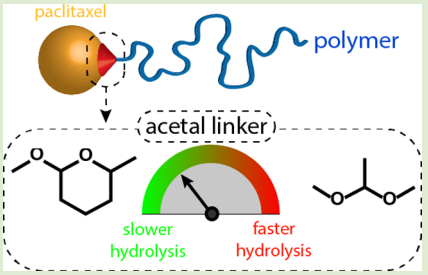
Supporting Information

ABSTRACT: Acid-sensitive paclitaxel (PTX)−polymer conjugates were designed by applying a grafting-from-drug RAFT approach. PTX was linked through either a cyclic or a linear, acid-sensitive acetal moiety. Relative to direct esterification of PTX, which occurred regioselectively at the C_2' OH-group, direct acetalization was observed at either the C_2' or the C_7 OH-group of PTX. This yielded two regioisomers of acetal-based PTX-functionalized RAFT chain transfer agents (CTAs). Subsequent polymerization with *N*,*N*-dimethylacrylamide (DMA) resulted in amphiphilic highly defined, acetal-based PTX−polymer conjugates with nearly identical features in terms of polymer definition and micellar self-assembly behavior, but with distinct PTX release kinetics and absence of burst release. This was further reflected by their *in vitro* biological performance, giving insights into the difference of the release mechanism between ester- and acetal-based PTX−polymer conjugates.



Taxanes (i.e., paclitaxel (PTX) and docetaxel (DTX)) are highly valuable anticancer drugs due to their broad-spectrum activity.[1,2] However, the widespread use of these drugs in clinic remains elusive. During discovery and early development, it was found that both drugs exhibited very low water solubility. In order to commercialize these drugs, formulation into a 1:1 ethanol:surfactant cosolvent mixture was required. Cremophor EL was used for the first commercial formulation of PTX (i.e., Taxol), while polysorbate 80 was used in the DTX formulation Taxotere. However, severe hypersensitivity reactions are attributed to these surfactants.[3−7] This urged the development of formulations based on more benign excipients, leading to the FDA approval of Abraxane (an albumin-stabilized PTX formulation) in 2005 and Genexol-PM (PTX, stabilized by poly(ethylene glycol)-*b*-poly(D,L-lactic acid); PEG-*b*-pDLLA) in South Korea two years later. The biocompatibility of both carriers significantly increased the therapeutic index, hence higher doses could be administered leading to a more effective chemotherapy.[8]

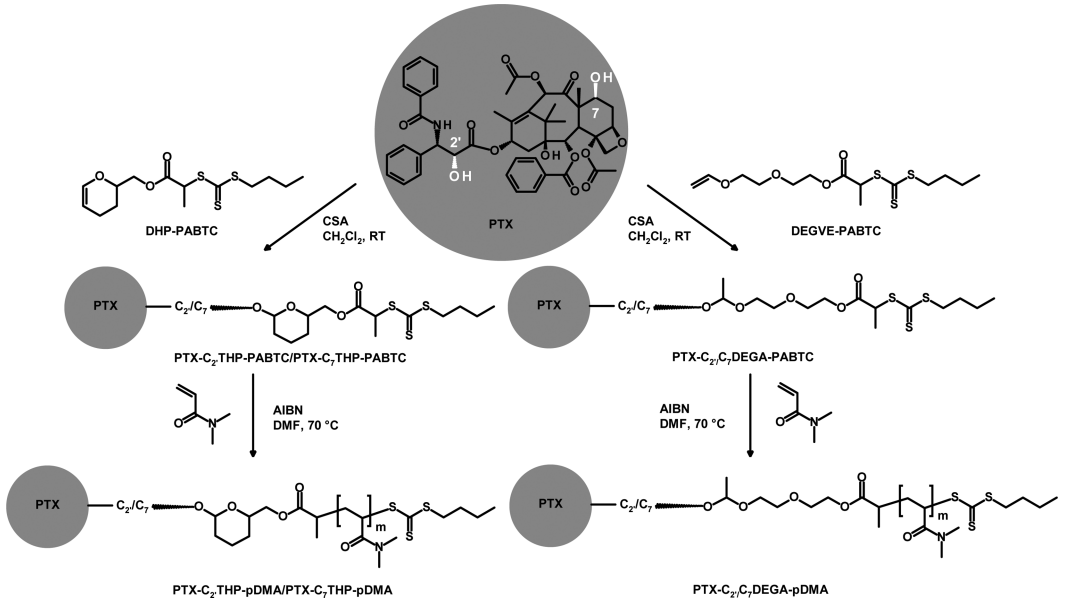
However, as the aforementioned formulations are based on physical drug entrapment, intravenous administration still holds the risk of systemic release and distribution of drug and hence does not prevent the intrinsic side effects of taxanes (i.e., neutropenia, neuropathy) from occurring. Besides physical drug entrapment by strong hydrophobic interaction, chemical conjugation is one of the most versatile strategies for more selective drug release.[9,10] Typically, the drug is chemically conjugated to the (polymeric) carrier vehicle through a stimuli-responsive linker. The latter can then be cleaved either by a specific enzyme (e.g., by cathepsin B lysosomal enzymes)[11] or by a subtle change in the chemical environment, as exploited by pH-sensitive (e.g., endosomal hydrolysis of acetals/ketals/hydrazone moieties) and redox-sensitive (e.g., cleavage of disulfides in response to hypoxic tumor microenvironment) carrier systems.[12−14] Polymer−drug conjugates with the highest progress in clinical trials (e.g., Opaxio; PTX conjugated to 48 kDa poly(L-glutamic acid)) are typically prepared by post-functionalization of the polymer backbone with the drug.[15] However, such a strategy has inherent reproducibility challenges and requires tedious purification to remove excess unconjugated drug which could cause burst release upon intravenous administration.

A recently uprising trend in polymer−drug conjugate design is direct polymerization from a drug molecule.[16,17] This technique is termed the "grafting-from-drug" or "drug-initiated" approach.[18] The promise of this method has also been

Received: December 28, 2016
Accepted: March 1, 2017
Published: March 3, 2017

DOI: 10.1021/acsmacrolett.6b00977
ACS Macro Lett. 2017, 6, 272−276

Scheme 1. Reaction Scheme for the Acid-Catalyzed Acetalization of Paclitaxel (PTX) with a Dihydropyran (DHP) or a Di(ethylene glycol) Vinyl Ether (DEGVE) Derivative of PABTC, Yielding a PTX RAFT CTA, Functionalized through a Cyclic or Linear Acetal Bond (i.e., THP or DEGA), Respectively[a]



[a]Note that acetalization occurs either at the C2′ or at the C7 OH-group of PTX. While the regioisomers of the THP-based PTX RAFT CTA could be separated, the regioisomeric mixture of the DEGA-based PTX RAFT CTA was used as such for polymerization of *N*,*N*-dimethylacrylamide. Key abbreviations: THP, tetrahydropyran-based cyclic acetal; DEGA, di(ethylene glycol)-based linear acetal; C2′/C7, regioisomeric mixture, modified either through the C2′ or the C7 OH-group of PTX.

demonstrated for taxane−polymer conjugates. For example, direct ring-opening polymerization (ROP) of lactic acid (LA) conjugated to PTX and DTX resulted in defined, hydrophobic PTX/DTX−PLA conjugates which could subsequently be formulated into nanoparticles.[16,17] Recently, our group has developed a grafting-from-drug reversible addition−fragmentation chain transfer (RAFT) polymerization approach for acquiring defined, amphiphilic PTX−polymer prodrug conjugates with high drug loading and aqueous compatibility.[19] The latter was obtained by functionalizing a RAFT chain transfer agent (CTA) with PTX by direct esterification. This ester bond was effectively cleaved *in vitro* as the IC_{50} matched the ones of Abraxane and Genexol-PM. These results motivated us to further explore this technique to develop acid-sensitive, acetal-based PTX−polymer conjugates that would hold the potential to more selectively release drug in response to the acidic milieu in tumor tissue or upon endocytosis and storage in intracellular vesicles. The literature reports that cyclic and linear acetal moieties can exert significantly different, acidic hydrolysis rates (i.e., up to several orders of magnitude), wherein linear acetals are known to degrade faster than their cyclic counterparts.[20] Hence, two acetal moieties were considered, one based on a cyclic, tetrahydropyran-based acetal (in this paper abbreviated as THP) and the other based on a linear, di(ethylene glycol)-based acetal (in this paper abbreviated as DEGA).

Scheme 1 depicts the strategy applied for the syntheses of cyclic and linear acetal-based PTX−polymer conjugates. First, a RAFT CTA (i.e., 2-(butylthiocarbonothioylthio)propanoic acid (PABTC)) was modified, with either a dihydropyran (DHP) or a di(ethylene glycol) vinyl ether (DEGVE) moiety, yielding DHP-PABTC and DEGVE-PABTC respectively. Both structures were confirmed by nuclear magnetic resonance (NMR) spectroscopy and electron spray ionization-mass spectroscopy (ESI-MS) (Figures S1−S4). DHP-PABTC and DEGVE-PABTC were subsequently used for direct, acid-catalyzed acetalization of PTX through a cyclic or linear acetal, respectively. In contrast to the regioselective esterification of PTX at the C_2' hydroxyl(OH)-group we observed previously,[19] in the present study we found that acetalization occurred at two sites (i.e., either at the C_2' or at the C_7 OH-group).

This resulted in two regioisomers of PTX-THP-functionalized CTA, which could be separated by silica gel chromatography. Detailed NMR analysis (Figures S5−S14) indeed confirmed the absence of the C_2' OH-group (at 2.51 ppm in the ^{1}H NMR spectrum) and the presence of the C_7 OH-group (at 2.46 ppm) for the PTX-C_2'THP-PABTC regioisomer, while the opposite was observed for the PTX-C_7THP-PABTC regioisomer. As expected, both isomers produced the identical product ion on ESI-MS (Figure S15). Synthesis of PTX-DEGA-PABTC resulted in a reaction mixture from which one fraction could be purified by silica gel chromatography. However, NMR characterization (Figures S16−S20) confirmed the presence of the two regioisomeric species, as in the ^{13}C NMR spectrum, and the anomeric carbon of the acetal moiety was observed at 99.72 and 100.52 ppm for the PTX-C_2'DEGA-PABTC and PTX-C_7DEGA-PABTC isomer, respectively. Direct injection ESI-MS revealed ions related to the desired product (Figure S21). These ions were also detected by liquid chromatography diode array detection mass spectrometry (LC-DAD/MS), showing two fractions eluting closely after one another (Figure S22), further confirming that the obtained PTX-DEGA-PABTC is a regioisomeric mixture. Both isomers could not be separated on a preparative scale, but LC analysis (Figure S22) yielded an 82/18 ratio of the respective isomers.

Both isomers of PTX-THP-PABTC and the regioisomeric mixture of PTX-DEGA-PABTC were used for RAFT polymer-

DOI: 10.1021/acsmacrolett.6b00977
ACS Macro Lett. 2017, 6, 272−276

ization of *N*,*N*-dimethylacrylamide (DMA). pDMA is a hydrophilic polymer that exhibits excellent *in vivo* biocompatibility and significantly lower antibody-mediated accelerated blood clearance (ABC) compared to the widespread polyethylene glycol (PEG) and polyoxazolines (POx).[21] In analogy to our previous endeavors using ester-based PTX−polymer conjugates (abbreviated as PTX−pDMA), a degree of polymerization (DP) of 30 was aimed to balance between solubility and high drug loading. For both the cyclic (PTX-THP-pDMA) and the linear acetal-based conjugates (PTX-DEGA-pDMA), similar molecular weight (MW) and narrow dispersity were measured by size exclusion chromatography (SEC, Figure 1A)

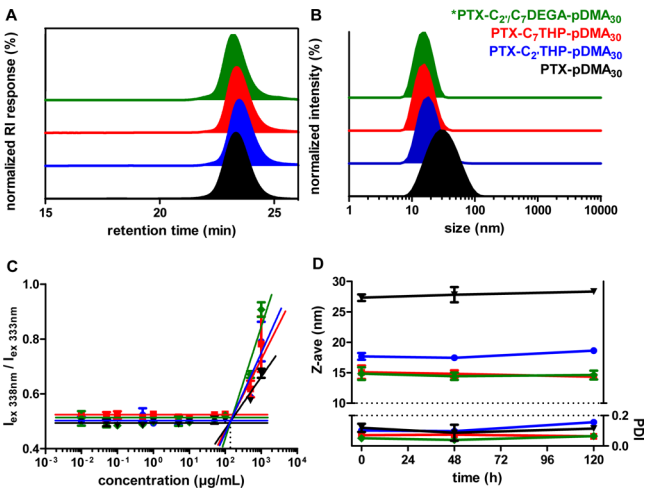


Figure 1. SEC elugrams (A), intensity size distribution (B), CAC (C), and colloidal stability (D) of PTX−polymer conjugates. B,D: n = 3, measured by DLS in PBS (30 mg/mL). C: n = 2, measured in PBS by pyrene assay. Data points and error bars in (C) and (D) represent mean value and standard deviation (SD), respectively. *Regioisomeric mixture of the PTX−polymer conjugate, modified through either the C_2' or the C_7 OH-group of PTX.

in addition to a high α- and ω-end group fidelity measured by NMR (Figures S23 and S24). These findings underline the value, even at high monomer conversion, of the grafting-from-drug RAFT approach for the synthesis of well-defined polymer−drug conjugates. The high similarity between our earlier reported ester-based and present acetal-based conjugates in terms of total MW, polymer chain length, and PTX loading capacity (Table 1) is highly favorable and allows an adequate, head-to-head comparison of both generations *in vitro*. As pDMA has good aqueous solubility, its conjugation with PTX yields an amphiphilic polymer due to the hydrophobicity of the PTX terminal end and therefore likely self-assembles in water into micellar structures.

We investigated the self-assembly behavior of the acetal-based conjugates in aqueous medium (i.e., phosphate-buffered saline (PBS)) and compared their behavior to our previously synthesized ester-based conjugates. All conjugates could easily be dissolved in PBS up to elevated concentrations (at least 30 mg/mL). Both PTX-THP-pDMA and PTX-DEGA-pDMA self-assembled into micellar nanoparticles with similar size as the ester-based conjugates, as observed by dynamic light scattering (DLS, Figure 1B and Table 2). No difference in particle size

Table 2. Supramolecular and Cytotoxicity Features of PTX−Polymer Conjugates

polymer	Z-Avg (nm)[a]	PDI[a]	CAC (μg/mL)[b]	IC_{50} (μM)[c]
PTX-$pDMA_{30}$	27.3 ± 0.5	0.119 ± 0.026	102 ± 12	37×10^{-3}
PTX-C_2'THP-$pDMA_{30}$	17.6 ± 0.5	0.102 ± 0.018	113 ± 2	95
PTX-C_7THP-$pDMA_{30}$	15.0 ± 1.1	0.071 ± 0.020	109 ± 6	95
[d]PTX-C_2'/C_7DEGA-$pDMA_{30}$	14.8 ± 1.0	0.051 ± 0.001	100 ± 1	51×10^{-1}

[a]Numeric values for Z-Average hydrodynamic diameter and PDI of PTX−polymer conjugates, measured in PBS (30 mg/mL) at 37 °C by DLS (n = 3). [b]CAC in PBS at 20 °C, measured by pyrene assay (n = 2). [c]Relative IC_{50}-values calculated by nonlinear regression analysis of MTT data presented in Figure 3. IC_{50}-values of Abraxane and Genexol-PM were 45×10^{-3} and 46×10^{-3} μM, respectively. [d]Regioisomeric mixture of PTX−polymer conjugate, modified through either the C_2' or the C_7 OH-group of PTX.

was observed between the PTX-C_2'THP-pDMA and PTX-C_7THP-pDMA isomers. The critical aggregation concentration (CAC) of the acetal-based conjugates was mutually similar and in good concordance with the first-generation conjugates (Figure 1C and Table 2). Finally, all conjugates showed high colloidal stability in PBS for several days at 37 °C (Figure 1D).

Subsequently we investigated the hydrolysis behavior of the PTX−polymer conjugates at different pH values (i.e., pH 7.4 to mimic the conditions in the circulation and extracellular fluids, pH 5 to mimic the acidic milieu in intracellular vesicles, and pH 4 to test an even more acidic milieu) and in the presence of serum (i.e., PBS supplemented with 10% fetal bovine serum (FBS)). For this purpose, conjugates were incubated for fixed periods of time in the respective media, followed by ultraperformance liquid chromatography (UPLC) analysis. Only limited (i.e., <5%) PTX release was observed at pH 7.4 in 96 h for the ester-based conjugates, most probably through a base-catalyzed process, while the acetal-based conjugates remained fully stable within the time frame of the experiment at this pH value (Figure 2A). Additionally, the presence of

Table 1. Compositional Data of the Synthesized Polymers

polymer	$[DMA]_0$/ [CTA]	conversion DMA (%)[a]	DP^{conv},[b]	$DP^{end\ group}$,[c]	M_n (Da)[d]	Đ[d]	PTX loading capacity (%)[e]
PTX-$pDMA_{30}$	30	99	30	31	4356	1.07	21
PTX-C_2'THP-$pDMA_{30}$	30	94	28	30	3975	1.08	21
PTX-C_7THP-$pDMA_{30}$	30	95	28	30	4226	1.09	21
[f]PTX-C_2'/C_7DEGA-$pDMA_{30}$	30	97	29	32	4999	1.08	21

[a]Determined by ^{1}H NMR spectroscopy. [b]Determined by ^{1}H NMR spectroscopy based on monomer conversion. [c]Determined by ^{1}H NMR spectroscopy based on end group analysis. [d]Analyzed by SEC in DMAc, calibrated with PMMA standards. [e]Calculated based on conversion by ^{1}H NMR spectroscopy: $MW_{PTX}/MW_{PTX\text{-}polymer} \times 100\%$. [f]Regioisomeric mixture of the PTX−polymer conjugate, modified through either the C_2' or the C_7 OH-group of PTX.

DOI: 10.1021/acsmacrolett.6b00977
ACS Macro Lett. 2017, 6, 272−276

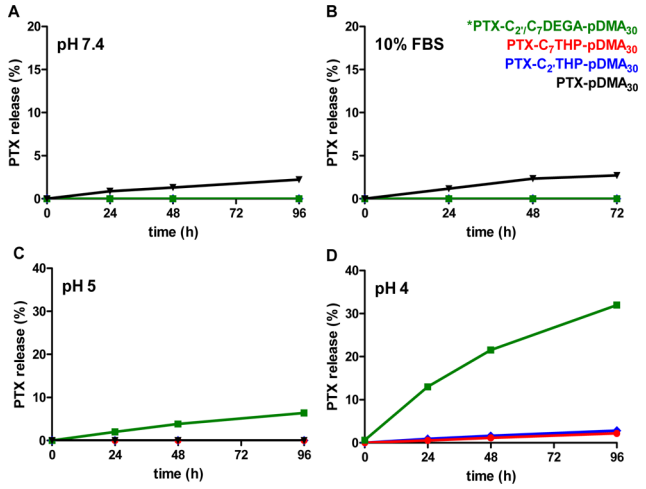


Figure 2. Drug release kinetics at 37 °C of PTX–polymer conjugates (10 mg/mL) in 100 mM citrate buffer (pH 4; A and pH 5; B), phosphate buffer (pH 7.4; C) and PBS supplemented with 10% FBS (D), determined by UPLC (n = 2). Data points represent mean value. *Regioisomeric mixture of PTX–polymer conjugate, modified through either the C_2' or the C_7 OH-group of PTX.

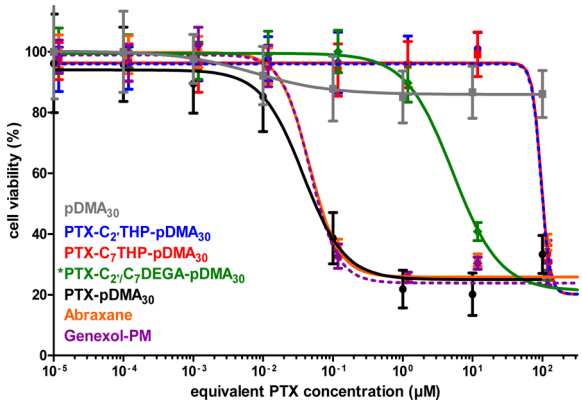


Figure 3. *In vitro* cytotoxicity of PTX–polymer conjugates versus commercial PTX nanoformulations Abraxane and Genexol-PM (n = 6), coincubated with SKOV-3 cells for 72 h. Data points and error bars represent mean value and SD, respectively. *Regioisomeric mixture of the PTX–polymer conjugate, modified through either the C_2' or the C_7 OH-group of PTX.

serum did not substantially accelerate the PTX release rate for the ester- nor for the acetal-based conjugates, as for the latter the zero baseline also persisted in the presence of FBS (Figure 2B). Next, the conjugates were incubated at endosomal pH (i.e., pH 5). While release was observed for the linear acetal-based conjugate (i.e., 6% in 96 h), no release of PTX was observed for the cyclic acetal- and ester-based conjugates within a similar time frame (Figure 2C). The latter confirms a higher stability of cyclic acetals compared to their linear counterparts and suggests that, after swift endosomal uptake of the conjugates which we previously confirmed by confocal microscopy,[19] the ester-based conjugates most likely did not release PTX through an acid-catalyzed process but predominantly through an enzymatic pathway instead. The acetal-based conjugates were also incubated at pH 4 to verify the influence of pH on acetal hydrolysis rate. Indeed, PTX-THP-pDMA showed higher, but still limited, release at pH 4, while a substantially higher PTX release was observed for the linear acetal-based conjugates (Figure 2D). This further suggests that the release of PTX from the acetal-based conjugates is primarily triggered chemically instead of enzymatically, more specifically by means of acid-catalyzed hydrolysis.

Finally, the biological performance of the acetal-based conjugates was evaluated *in vitro* on human ovarian SKOV-3 cells by MTT assay after 72 h of coincubation. Prior to these experiments we verified that none of the conjugates showed a tendency toward aggregate formation in the presence of serum (Figure S25). As depicted in Figure 3, pDMA did not exert any intrinsic cytotoxic effect. Both the acetal-based conjugates induced a decrease in cell viability up to the same extent as the ester-based conjugates, and two commercial PTX formulations based on physical entrapment, albeit significantly higher concentrations, are required. These data are in accordance with the *in vitro* release studies, which demonstrated that the extent of PTX release is incomplete at pH 5 after 72 h of incubation. Additionally, the higher release rate observed for PTX-DEGA-pDMA compared to PTX-THP-pDMA indeed results in a higher IC_{50} value of the latter (Table 2). Finally, the MTT results further propose a difference in release mechanism between ester- and acetal-based PTX–polymer conjugates (i.e., enzyme- and acid-catalyzed, respectively). Both mechanisms can be of interest in developing advanced polymer–drug conjugates with adequate, selective drug release and limited systemic burst release. For acquiring PTX–polymer conjugates with faster release kinetics at pH 5, highly pH-sensitive acetal/ketal moieties are to be considered. The acetal/ketal chemistry, developed by Fréchet and co-workers, could provide valuable insights for designing the latter purpose.[22,23]

In conclusion, the grafting-from-drug RAFT approach allowed the preparation of well-defined acetal-based PTX–polymer conjugates with nearly identical features in terms of polymer composition and amphiphilic properties but with distinct PTX release properties. The findings of this paper clearly highlight the broad chemical versatility and robustness of the polymerization technique and show that, due to the high definition of the obtained polymers, RAFT should be considered as a key player in future rational design of advanced polymer–drug conjugates. Whereas the *in vivo* behavior of these systems remains to be elucidated, ester-based systems show the advantage of a potentially higher activity on a short-term scale. However, the specific acid-triggered PTX release, exhibited by acetal-based systems, in combination with their resilience to enzymatic cleavage might be beneficial as well.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.6b00977.

Experimental details and extensive characterization details (PDF)

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DOI: 10.1021/acsmacrolett.6b00977
ACS Macro Lett. 2017, 6, 272–276

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.

■ ACKNOWLEDGMENTS

BL acknowledges the IWT Flanders for a PhD scholarship. LN acknowledges the Alexander von Humbold Foundation and the FWO Flanders for funding. BGDG acknowledges the FWO Flanders, BOF-UGent and the VLK for financial support.

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ACS Macro Lett. 2017, 6, 272−276