A Novel Enzyme-Triggered Domino Reaction Producing Fluorescent Quinoxalin-2(1*H*)-one-based Heterocycles

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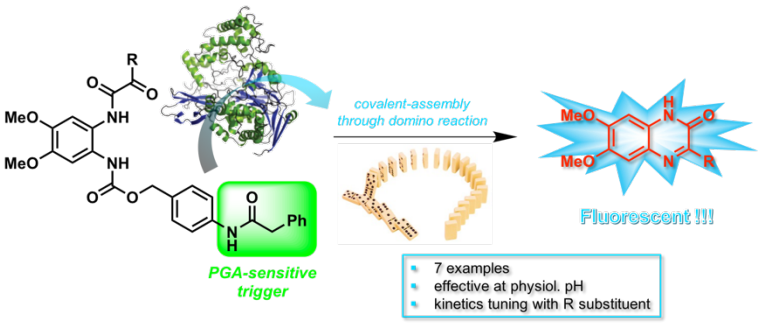


A simple and effective biocompatible domino reaction triggered by a model protease and leading to formation of strongly fluorescent quinoxalin-2(1*H*)-one *N*-heterocycles is described. Some positive attributes including versatility and ability to provide outstanding fluorescence "OFF-ON" responses were revealed by this work. They open the way for practical applications of this novel type of "covalent-assembly" based fluorescent probes in the fields of sensing and bioimaging.

Among the myriad of synthetic transformations and catalysts currently available for concise and efficient synthesis of organic molecules, bioinspired approaches based on the use of enzymes often intertwined with cascade/domino processes, occupy a special place and are receiving greater attention of late.[1] They have also contributed to the emergence of *in vivo* chemistry (often named as intracellular chemistry)[2] that can be defined as the use of living cells as reaction vessels and their constituents (*e.g.*, enzymes) possibly together with abiotic metal catalysts,[3] to achieve one or several reactions for internal construction of sophisticated nano-objects from exogenous synthetic precursors. In addition to possible applications devoted to the production of high-value chemicals, one of the most striking achievements in this emerging area is undoubtedly the biocompatible "click" reaction between an aromatic nitrile (*e.g.*, 6-amino-2-cyanobenzothiazole (CBT)) and D-cysteine leading to D-luciferin scaffold,[4] discovered by the Rao group in 2010 (Figure 1, top).[5] Its ability to be effective for the controlled assembly of nanoparticles in living cells together with its possible triggering by a biostimulus (pH, redox status and/or hydrolytic enzymes) has fostered the emergence of cutting-edge reactivity-based sensing approaches for *in vivo* molecular imaging, especially through the bioluminescence modality, for addressing challenges associated to light-based diagnosis.[6]

These remarkable achievements perfectly illustrate both utility and benefits of biocompatible/bioorthogonal transformations that are capable of generating a bright luminescence output signal upon the action of a biological trigger. In the field of activity-based fluorescent sensing, a new probe design principle namely the "covalent-assembly" approach, pioneered by the Swager group with the design of a "smart" semiconductive polymer for fluorogenic detection of fluoride ions,[7] and next rationalized by Anslyn and Yang to address relevant biological questions,[8,9] has emerged in the mid-2000s. This cutting-edge strategy is based on *in situ* formation of a fluorophore from a caged compound (not belonging to major classes of fluorescent dyes and, in theory at least, devoid of light emission ability) through a domino reaction triggered by the species to be detected and ideally designed to work properly in aqueous media (Figure 1, middle and bottom). Since this novel class of activatable fluorescent probes are supposed to dramatically improve signal-to-noise ratio (S/N) responses, thus providing optimal detection sensitivity, it is not really surprising that they have rapidly become popular tools for specific detection and imaging of enzyme activities (mainly, hydrolases and reductases). The vast majority of "covalent-assembly" based fluorescent probes devoted to enzyme biosensing involve *in situ* formation of blue-green emitting (2-imino)coumarins or related fluorophores through analyte-triggered lactonization or Pinner cyclization reactions[10,11,12] even if some of these published examples are also regarded as fluorogenic probes undergoing rigidification of their floppy pre-existing push-pull system upon the action of the bioanalyte.[8b] Our group, inspired by work of Yang et *al.*,[13] have recently contributed to this research field by expanding the scope to longer-wavelength fluorophores belonging to the popular class of xanthene dyes (*i.e.*, unsymmetrical pyronins with fluorescence features within the yellow-orange spectral region).[14] Some notable results were obtained with

several hydrolytic enzymes including penicillin G acylase (PGA) and leucine amino peptidase (LAP) and we demonstrated that a subtle equilibrium between reactivity and stability of caged precursors of pyronins can be readily obtained by means of effective and facile structural modifications.

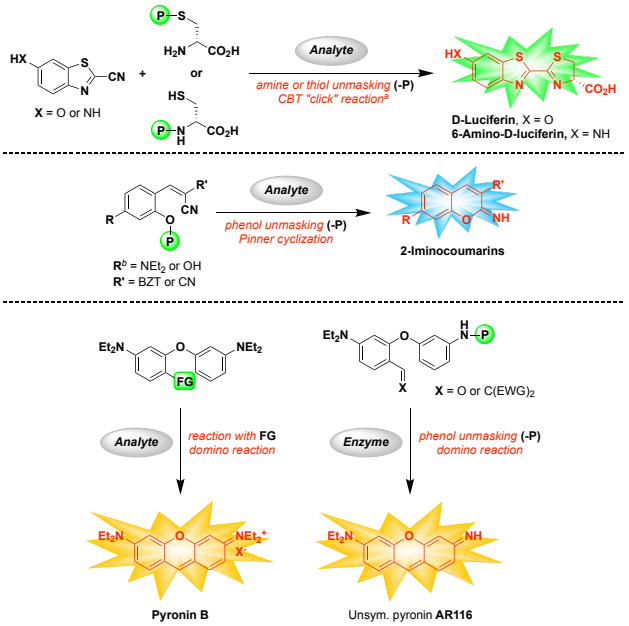


Figure 1. Background information about *in situ* synthesis of bioluminophores (top) and fluorophores (2-iminocoumarins (middle) and pyronins (bottom)) triggered by the (bio)analyte to be detected. (BZT = 2-benzothiazolyl, EWG = electron-withdrawing group, FG = functional group that reacts with the targeted analyte, P = recognition moiety of the targeted enzyme). *[a]For clarity, related polymerization reactions and intramolecular cyclizations leading to nano-aggregates were omitted. [b]Please note: fused julolidine, 4-dimethylaminophenyl and 1,4-diethylpiperazine fragments are also frequently used in the design of such "caged" precursors, to red-shift spectral features of in situ formed 2-iminocoumarin.*

Despite all these advances, "covalent-assembly" fluorogenic probes may sometimes have some drawbacks including (1) intrinsic red or far-red fluorescence due to aggregation-induced emission (AIE) phenomenon that prevents intensometric "OFF-ON" detection,[14b, 15] and (2) limited molecular diversity that may complicate optimization of their properties for a given application. Thus, there is a clear need to identify alternative fluorescent scaffolds that can be built by the targeted enzyme through a novel domino process. We have identified the quinoxalin-2(1*H*)-ones, a popular class of benzo-fused *N*-heterocycles with a wide spectrum of pharmacological activities.[16] They are frequently used as skeletons for the design of biologically active compounds and their spectral behavior close to those of 7-(dialkylamino)/7-hydroxycoumarins has recently promoted their use either as fluorophore alone or as optical reporter in "smart" fluorescent probes acting as chemodosimeters or chemosensors.[17] Furthermore, the very old condensation reaction historically used to prepare these *N*-heterocycles is easy to implement and involves the use of *ortho*-phenylenediamine and α-ketoacid (or α-ketoester derivative) as coupling partners.[18] Its versatility stems from the commercial or synthetic availability of a wide range of these starting materials bearing various substituents. We hypothesized that the cyclization/aromatization process which completes the *N*-heterocycle construction may occur spontaneously under physiological conditions. In the light of the mechanism generally accepted for this reaction, *i.e.*, the ketone moiety undergoing an intramolecular nucleophilic addition of the neighboring primary amino group followed by loss of a water molecule, it is reasonable to assume that a caged *ortho*-phenylenediamine derivative, whose the primary amino group would be acylated with an enzyme-specific trigger recognition moiety (the second one being acylated with the selected α-keto acid), should act as an effective "covalent-assembly" based fluorescent probe (Figure 2).

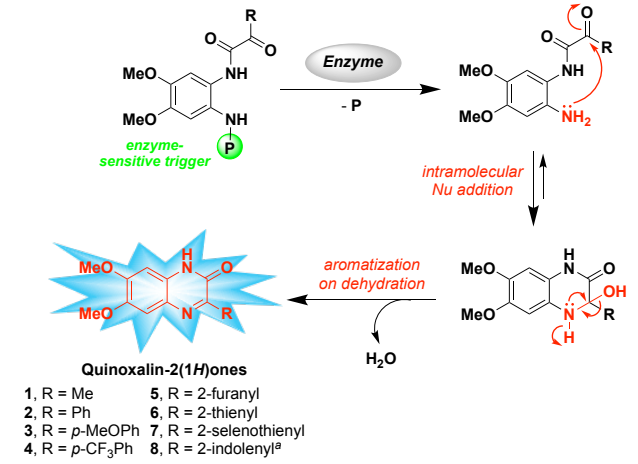


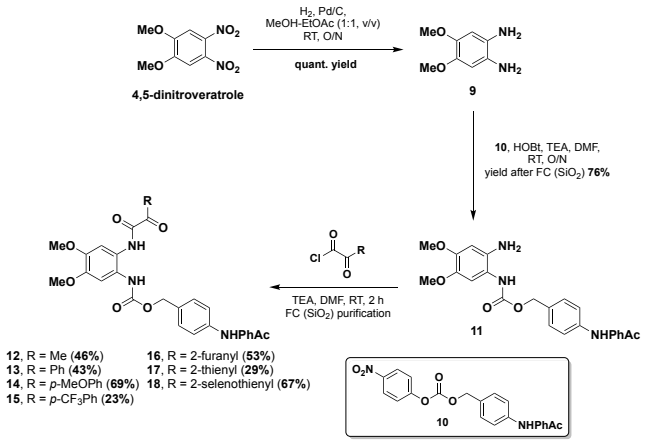
Figure 2. Enzyme sensing strategy explored in this work, based on the use of "covalent-assembly" fluorogenic probes whose activation leads to *in situ* formation of quinoxalin-2(1*H*)-ones. *[a]Due to its poor stability, we failed to synthesize the corresponding "covalent-assembly" fluorescent probe.*

We report here the practical implementation of this unusual fluorogenic reaction-based process through the synthesis of seven distinct *N*-caged *ortho*-phenylenediamine derivatives and using PGA as a model protease, a commercially available and widely used biocatalyst in the enzymatic synthesis of β-lactam antibiotics through the hydrolysis of phenylacetamide moieties.[19] The ability of this enzyme to trigger *in situ* formation of fluorescent quinoxalin-2(1*H*)-ones from these "covalent-assembly" type probes was studied in detail through *in vitro* fluorescence assays and HPLC-fluorescence/-MS analyses. All data generated have been used to rationalize the reaction mechanism and the influence of alkyl/aryl substituent of α-ketoamide moiety on the overall efficiency of this novel domino process.

The planned synthetic strategy was based on the sequential *N*-acylation of 4,5-dimethoxy-1,2-phenylenediamine **9** with *para*-nitrophenyl carbonate derivative of *para*-(phenylacetamido)benzyl alcohol (PhAc-PABA) **10**[20] and α-ketoacid chloride to install the recognition unit of PGA and electrophilic moiety respectively (Scheme 1). The substitution pattern of 2-aminoaniline **9** was chosen because the quinoxalin-2(1*H*)-ones obtained from this starting synthon are known to be the most fluorescent candidates form this class of benzo-fused *N*-heterocycles. Since **9** was found to be partially unstable over prolonged storage (degradation was observed after less than 2 months at -20 °C and under argon atmosphere), it was prepared immediately prior to use by hydrogenation over Pd/C catalyst of commercial, stable 4,5-dinitroveratrole.[21] The phenylacetamide unit known to be recognized and cleaved by PGA, was connected to 1,2-phenylenediamine scaffold through a self-immolative linker (*i.e.*, *para*-hydroxybenzyl) to lower steric hindrance and hence increase accessibility of the probe to the enzyme's active site.[22] Indeed, our preliminary investigations have shown that direct *N*-acylation of **9** with phenylacetyl chloride led to unreactive "covalent-assembly" fluorogenic probes, not recognized by PGA (data not shown). Molecular diversity was readily

achieved by selecting 7 different α-ketoacids including pyruvic acid or derivatives arising formally from benzylic oxidation of phenylacetic or heteroarylacetic acid (phenyl, *para*-methoxyphenyl, *para*-(trifluoromethyl)phenyl, 2-furyl, 2-thienyl or 2-selenothienyl as fragment). They are commercially available or easily prepared by SeO_2-mediated oxidation of the corresponding α-methylketone (for details, see the Supporting Information). Conversion into the corresponding acyl chlorides was achieved by treatment with an excess of dichloromethyl methyl ether (acting as both chlorinating agent and solvent) at 50 °C, except for 2-selenopheneglyoxylic acid found to be unstable under these conditions; $SOCl_2$ in dry DMF was used in this latter case. Practical implementation of the two distinct *N*-acylation reactions was performed as follows: first, 1,2-phenylenediamine **9** was reacted with activated carbonate **10** (stoichiometrically deficient relative to **9**, 0.36 equiv.) in dry DMF, in the presence of 1-hydroxybenzotriazole (HOBt, 0.36 equiv.) and TEA (1 equiv.). Thereafter, amidation of the remaining primary aniline with α-ketoacid chloride (1.2 equiv.) was conducted under nearly identical conditions (in dry DMF, in the presence of TEA). Purification by flash-column chromatography over silica gel provided the PGA-responsive fluorogenic probes in moderate to good yields (23-69%). All spectroscopic data (see the Supporting Information), especially multinuclear NMR and high- and low-resolution mass spectrometry), were in agreement with the structures assigned. Interestingly, selenophene-based probe **18** was further characterized by ^{77}Se NMR (proton decoupled experiment) and the single resonance peak at 675.3 ppm is a valuable evidence for the presence of a single species within the product sample (Figure S114).

Scheme 1. Synthesis of PGA-responsive "covalent-assembly" fluorogenic probes 12-18



We have also prepared the quinoxalin-2(1*H*)-one fluorophores **1-7** assumed to be formed upon the enzymatic activation of probes **12-18**. Indeed, their use as references in fluorescence assays and HPLC analyses is essential to clearly demonstrate the reaction-based sensing mechanism (see the Supporting Information for synthesis and characterization of fluorophores **1-7**). The synthesis of indole-based derivative **8** was also achieved but we failed to obtain the corresponding PGA-responsive probe owing to its poor stability. To have an indication of the magnitude of fluorogenic "OFF-ON" response arising from PGA activation of probes **12-18**, photophysical properties of fluorophores were also determined both in phosphate buffer (PB, 100 mM, pH 7.6) and DMSO (see Tables S1 and S2, and Figures S115-S130). Compounds **1-3** and **5** have been identified as the most brilliant dyes within the violet-blue-green spectral range typical for quinoxalin-2(1*H*)-ones (B ($\varepsilon \times \Phi_F$) values within the range 5000 - 9100 M^{-1} cm^{-1} for PB medium). It is worth noting that fluorescence quantum yield decreases from 0.37 for furane-based dye **5** to 0.20 for thiophene-based dye **6** to 0.06 for selenophene-based dye **7** which is consistent with heavy-atom effects promoting triplet formation relative to fluorescence.[23] Another interesting feature that will be subjected to additional investigations, concerns the indole-based dye **8** which was found to be almost non-fluorescent ($\Phi_F < 0.01$) in PB, probably as a consequence of photoinduced electron transfer (PeT) process.[24] Conversely, preliminary fluorescence fixed-cell imaging experiments have shown the emission ability of this quinoxalin-2(1*H*)-one in a real biological context and its superior capability for cellular staining compared to the phenyl-based dye **2** otherwise characterized by a dramatically higher Φ_F in PB (0.6) (Figures S66-S67). This campaign of photophysical studies was completed by spectral measurements that confirmed the lack of fluorescence emission in PB for all PGA-responsive "covalent-assembly" type probes **12-18**.

Fluorogenic PGA assays and blank experiments (that confirmed hydrolytic stability of compounds **12-18**) were achieved through time-course measurements following a reliable protocol previously used by us.[11f, 14] In all cases, addition of recombinant PGA (from *Escherichia coli*) caused a rapid increase of blue fluorescence emission centered at a wavelength value within the range 455-490 nm, that reached a plateau in less than 10 min (Figure 3 and Figures S138-S151), except for the probe **15** bearing *para*-(trifluoromethyl)phenyl moiety as the ketone substituent. Electron-withdrawing effect of $-CF_3$ may negatively impact the last step of domino process namely dehydration, leading to quinoxalin-2(1*H*)-one aromatization (Figure 2); this rate-determining step should be favored by combined push electron effects of *N* atom and neighboring alkyl/aryl substituent toward carbon atom center undergoing the loss of hydroxyl group.

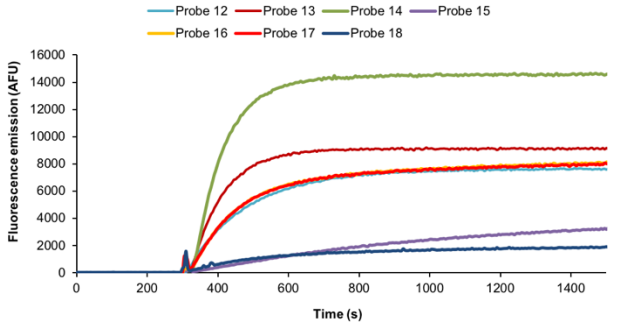


Figure 3. Time-dependent changes in the blue-green fluorescence intensity (Ex./Em. at the respective absorption/emission maxima, distinctive for each fluorophore, see the Supporting Information, slit = 2 nm) of fluorogenic probes **12-18** (concentration: 1.0 μM) in the presence of PGA (1 U) in PB (100 mM, pH 7.6) at 37 °C. Please note: PGA was added after 5 min of incubation of probe in PB alone.

Outstanding fluorescence "OFF-ON" responses were obtained even for the lowest fluorescent derivative bearing 2-selenothienyl moiety (Figures S139, S141, S143, S145, S147, S149 and S151). This highlights the main valuable feature of "covalent-assembly" approach in a striking manner. Furthermore, enzymatic activation and fluorogenic response arising from this was not impacted by the presence of biological interferents such as glutathione (Figures S152-S158). To confirm that the intense blue fluorescence signal detected was due to *in situ* formation of quinoxalin-2(1*H*)-one *N*-heterocycle, each

enzymatic reaction mixture was subjected to RP-HPLC-fluorescence analyses (Figure 4 for phenyl derivative **13** and Figures S159-S207). For each sample, a single peak was detected and unambiguously assigned to the expected fluorophore (comparison of retention times and co-injection with authentic samples of quinoxalin-2(1*H*)-ones **1-7** independently prepared, *vide supra*). To prove the validity of our assumption that PGA-initiated domino reaction to yield these benzo-fused *N*-heterocycles, the same enzymatic mixtures were next analyzed by RP-HPLC-MS both in "full-scan" and single ion monitoring (SIM) modes (Figure 5 for phenyl derivative **13** and Figures S208-S235). The disappearance of the probe **13** peak (t_R = 5.0 min) and unambiguous quantitative formation of quinoxalin-2(1*H*)-one **2** (t_R = 4.1 min) were clearly observed and both structure and integrity of fluorophore were supported by MS-ESI+/- and UV-vis data. Interestingly no trace of elusive intermediate aniline arising from PGA-mediated removal of PhAc moiety was detected, confirming the high reactivity of its primary amino group toward the adjacent carbonyl of α-ketoamide fragment. It should also be noted that both the existence and presence in enzymatic mixtures of the transient 2-hydroxy-1,2-dihydroquinoxalinone-type intermediate (*i.e.*, intermediate undergoing dehydration, Figure 2) cannot be confirmed due to its rapid conversion to final fluorophore under acidic conditions of mobile phases used for RP-HPLC-MS analyses. Nevertheless, all results presented here allowed us to confirm both viability and effectiveness of the sensing mechanism initially claimed and depicted in Figure 2. Furthermore, depending on the electronic effects of R substituent found in the probe's structure, the reactivity of α-ketoamide fragment and by extension the quinoxalinone-production rate, are impacted. This paves the way to a trivial molecular design strategy for fine-tuning reactivity of these quinoxalinone caged precursors.

In summary, we managed to expand the scope of "covalent-assembly" fluorescent probe design principle to quinoxalin-2(1*H*)-ones. Indeed, the facile construction of such benzo-fused *N*-heterocycles from structurally simple caged precursors and through an effective cyclization/dehydration sequence triggered by a model protease was demonstrated. We have also shown that kinetics of this fluorogenic process is dramatically influenced by the substituent nature of α-ketoamide fragment (found in C-3 position of quinoxalin-2(1*H*)-one formed *in situ*); the latter also having a significant impact on fluorescence properties and hence on the quality of detection. This feature combined to the wide scope of this approach (*i.e.*, facile exchange of the recognition moiety introduced on the primary aniline of probe for targeting a wide range of distinct bioanalytes), will open new perspectives in the field of activity-based optical sensing/imaging and, more broadly, expand the molecular toolbox for *in vivo* chemsitry. Lastly, the presented enzyme-triggered cyclizaion/aromatization reaction could be a useful source of inspiration to medicinal/or chemists to devise novel advanced prodrug strategies.[25]

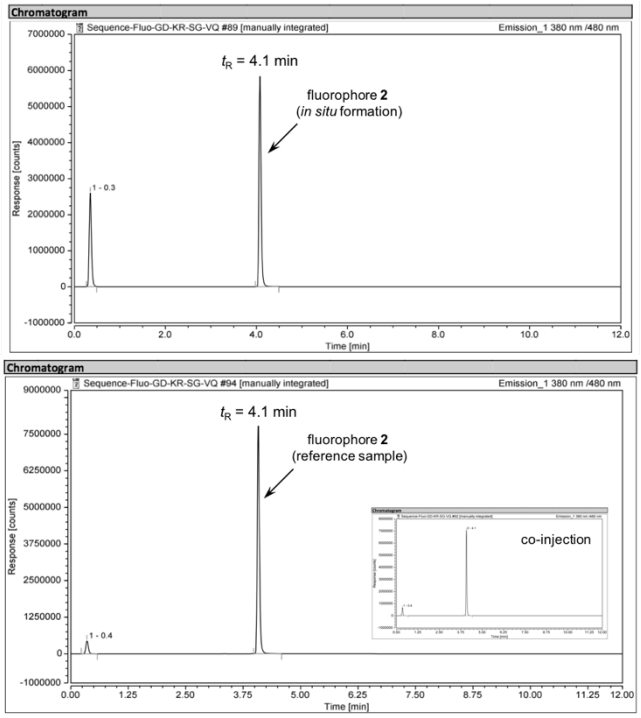


Figure 4. RP-HPLC elution profiles (fluorescence detection Ex./Em. 380/480 nm, see the Supplementary Information for elution conditions) of enzymatic reaction mixture of phenyl-based probe **13** with PGA (top) and authentic sample of fluorophore **2** (bottom) and co-injection of both (inset).

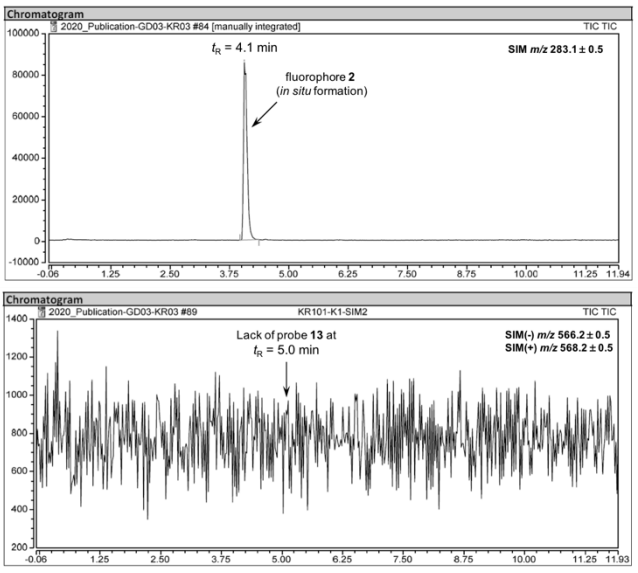


Figure 5. RP-HPLC elution profile (ESI-MS detection, SIM mode: ESI+ for detecting fluorophore (top) and ESI-/+ probe (bottom)) of enzymatic reaction mixture of phenyl-based probe **13** with PGA.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, spectroscopic and analytical data, IR, NMR, MS and UV-vis/fluorescence spectra, elution profiles for all RP-HPLC-fluorescence/-MS analyses (PDF). The Supporting Information is available free of charge at xxx.

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