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Quinone-Methide Species, A Gateway to Functional Molecular Systems: From Self-Immolative Dendrimers to Long-Wavelength Fluorescent Dyes

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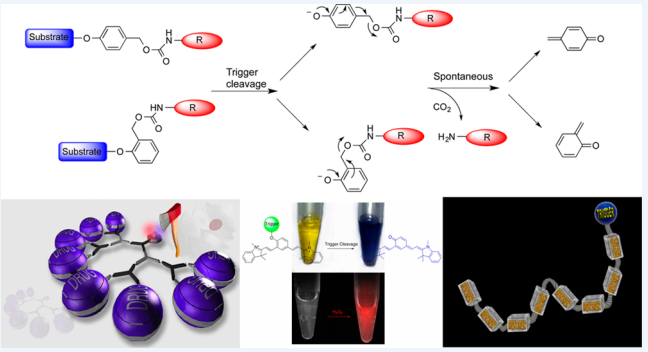
CONSPECTUS: Over the last 30 years, the quinone-methide elimination has served as a valuable tool for achieving various important molecular functions. Molecular adaptors based on quinone-methide or aza-quinone-methide reactivity have been designed, synthesized, and used in diagnostic probes, molecular amplifiers, drug delivery systems, and self-immolative dendritic/polymeric molecular systems. These unique adaptors function as stable spacers between an enzyme- or reagent-responsive group and a reporter moiety and can undergo 1,4-, 1,6-, or 1,8-type elimination reactions upon cleavage of the triggering group. Such reactivity results in the release of the reporter group through formation of a quinone-methide species. This type of elimination was applied to design distinct molecular adaptors capable of multiple quinone-methide eliminations.



Using this chemistry, we have developed unique molecular structures that are known today as self-immolative dendrimers. These dendrimers disassemble upon a single triggering event in a domino-like manner from the focal point to their periphery with the consequent release of multiple end-groups. Such molecular structures are used in self-immolative dendritic prodrugs and in diagnostic probes to obtain a significant amplification effect. To further enhance amplification, we have developed the dendritic chain reaction, which uses simple molecules to achieve functionality of high-generation virtual self-immolative dendrimers. In addition, we harnessed the quinone-methide elimination reactivity to design polymers that disassemble from head-to-tail initiated by an analyte-responsive event. Following this example, other chemical reactivities were demonstrated by scientists to design such polymeric molecules.

In a manner analogous to the quinone-methide elimination, electron rearrangement can lead to formation of conjugated quinone-methide-type dyes with long-wavelength emission of fluorescence. We have recently applied an intramolecular charge transfer to form a unique kind of quinone-methide type derivative based on a donor−two-acceptors molecular structure. This intramolecular charge transfer produces a new fluorochrome with an extended conjugation of π-electron system that is used for the design of long-wavelength fluorogenic probes with a turn-ON option. The rapidly expanding use of quinone-methide species, reflected in the increased number of examples reported in the literature, indicates the importance of this tool in chemistry. These species provide a useful gateway to functional molecular structures with distinct reactivities and spectroscopic characteristics.

INTRODUCTION

The term quinone-methide is used to describe a quinone analogue in which one of the carbonyl oxygens is replaced by a methylene group. If the second carbonyl-oxygen is replaced by a nitrogen the molecule is termed an aza-quinone-methide. In 1981, Katzenellenbogen's group demonstrated that 4-amino-benzyl-alcohol can be used to link a specific substrate to a target molecule via stable chemical linkages.[1] Enzymatic cleavage of the substrate results in formation of an aniline derivative, which undergoes rapid 1,6-elimination to release the target molecule. Such an elimination reaction generates a reactive aza-quinone-methide species that is usually trapped by an available nucleophile (e.g., a water molecule) to regenerate 4-amino-benzyl-alcohol. This example inspired many research groups to design adaptors that can be placed between an enzyme- or reagent-responsive group and a drug or a reporter moiety.[2] Scientists call these adaptors "self-immolative linkers" since they structurally "'sacrifice'" themselves in order to implement their designated function. These linkers are widely used in prodrug systems and in diagnostic probes.

The elimination reaction leading to formation of a quinone-methide species can take place in substituted phenol or aniline compounds in two possible directions as illustrated in Figure 1. Removal of the substrate from compound **1** or **2** leads to formation of phenolate **1a** or **2a**, respectively. While phenolate **1a** undergoes 1,6-elimination to release a reporter group and *p*-quinone-methide **1b**; phenolate **2a** undergoes 1,4-elimination to release the reporter and *o*-quinone-methide **2b**. The released end-group can be a chromogenic reporter, in the case of a

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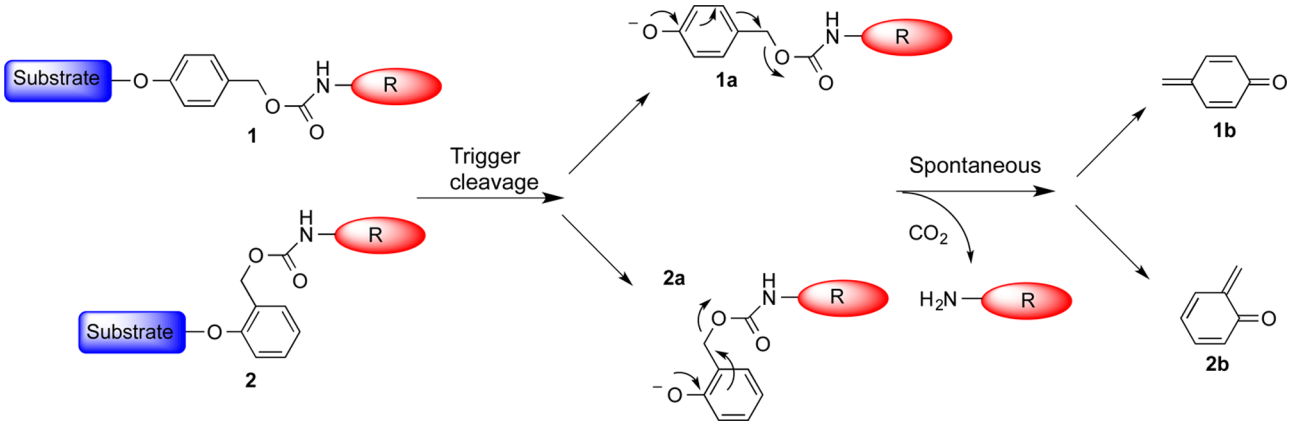

Figure 1. *p*-Quinone-methide vs *o*-quinone-methide elimination mechanism.

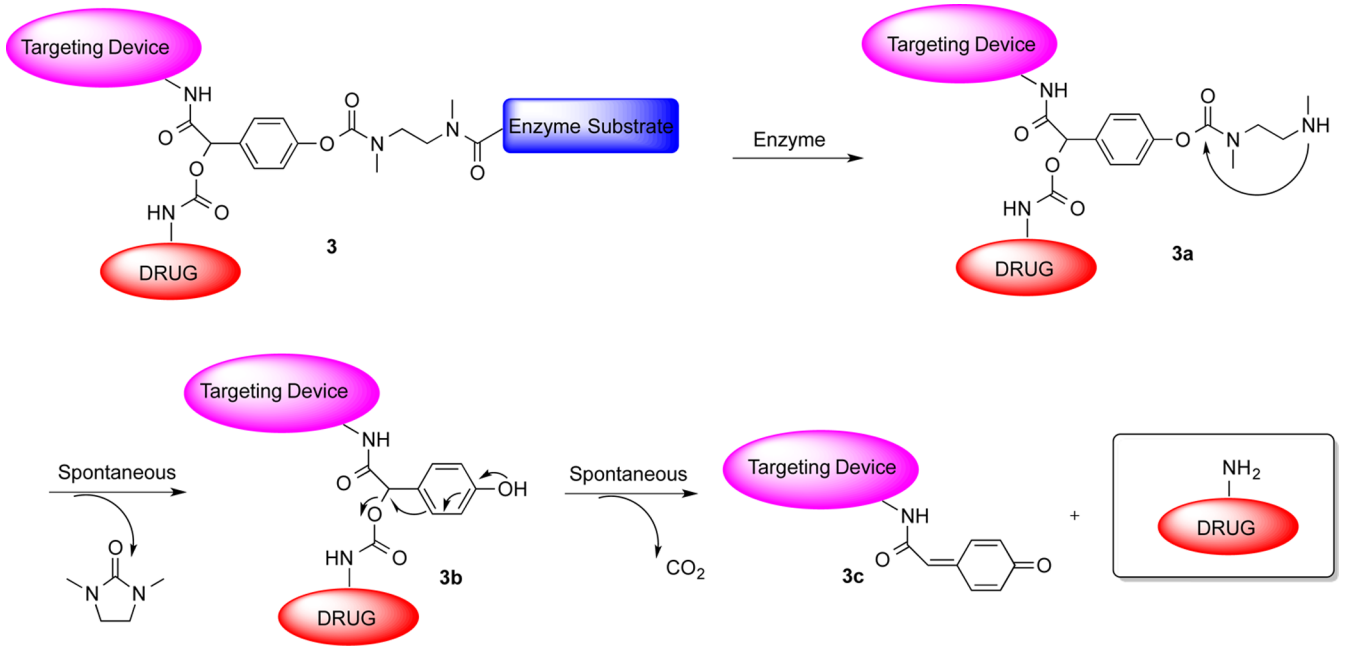

Figure 2. Activation pathway of a chemical adaptor based on a quinone-methide elimination reactivity.

diagnostic probe, or a drug molecule, in the case of a prodrug system.

CHEMICAL ADAPTORS BASED ON QUINONE-METHIDE ELIMINATION

Scientists are often required to link a specific chemical or biological reactivity to a target molecule that enables control of the activity. Therefore, a number of functional molecules have been designed and synthesized specifically for that purpose.[3,4] We have demonstrated how 4-hydroxy-mandelic acid can be used as a molecular adaptor to link between reactivity to a target molecule (Figure 2).[5] Adaptor molecule **3** acts as a platform to combine a tumor targeting device, a prodrug, and a prodrug activation trigger. The three functional groups of the 4-hydroxy-mandelic acid adaptor are each modified: The carboxylic acid functionality is linked to a targeting moiety, which is responsible for guiding the prodrug to the tumor site; the benzylic alcohol is attached to an active drug and, thereby, masks it to yield a prodrug; and the hydroxyl-phenol is attached to an enzyme substrate. When the corresponding enzyme cleaves the substrate, a spontaneous reaction is triggered that releases the active drug from the targeting moiety. As a result, prodrug activation is expected to occur preferentially at the tumor site.

The activation pathway of prodrug **3** is initiated by enzymatic cleavage of the substrate to form amine-intermediate **3a**, which undergoes a cyclization reaction to release phenol **3b**. The latter undergoes 1,6-elimination to release an active drug molecule and quinone-methide species **3c**. Proof of concept was demonstrated using etoposide as the drug, an HPMA-copolymer as the targeting device, and catalytic antibody 38C2 as the triggering enzyme. The system is generic and allows use of a variety of drugs, targeting devices, and enzymes by introducing the corresponding substrate as a trigger for drug release in the chemical adaptor. The potential of such adaptor molecules to provide chemical solutions for a variety of applications has well promoted their wide use.[6]

SELF-IMMOLATIVE DENDRIMERS

About 10 years ago, we demonstrated[7] how the *p*-quinone-methide and the *o*-quinone-methide eliminations could occur

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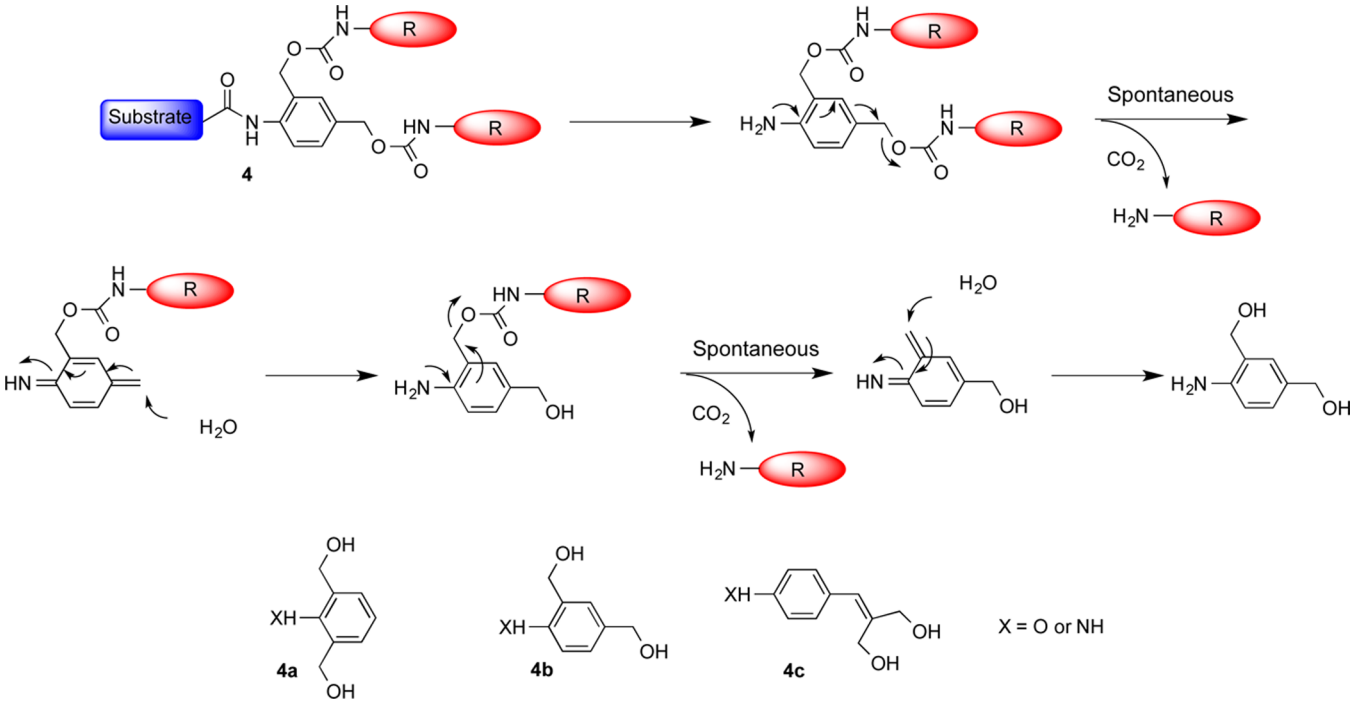

Figure 3. *p*-Quinone-methide and *o*-quinone-methide elimination sequences triggered by substrate cleavage.

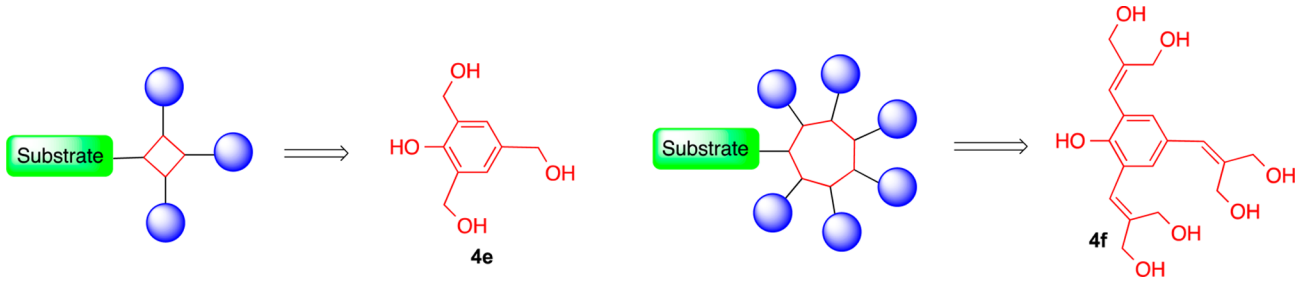

Figure 4. Molecular structure of phenols able to perform consecutive three and six quinone-methide type eliminations to release multiple end-groups.

consecutively on the same aromatic phenol[8] or aniline[9] derivative if proper leaving groups were introduced at *para* and *ortho* positions. This double elimination mechanism is presented in Figure 3. Removal of the substrate from compound **4** results in formation of an aniline derivative that undergoes *p*-quinone-methide elimination to release the first reporter group; this is followed by *o*-quinone-methide elimination to release the second reporter. Interestingly, this double elimination option was enabled at the same time by two additional independent research groups.[10,11] Several phenol or aniline derivatives have been evaluated. Some have leaving groups at the *para* and *ortho* benzylic positions (**4a** and **4b**), but the vinylogous benzylic position (**4c**) has also been used as a site for attachment of a leaving group. In molecular structures like **4c**, the disassembly mechanism occurs through double 1,8-eliminations through an extended quinone-methide species.

It was also realized that additional substituents on the *ortho*, *para*, or vinylogous benzylic positions of a phenol could produce molecules that are able to implement multiple types of quinone-methide eliminations. We have demonstrated this concept with molecules able to achieve three[12] (compound **4e**) and six[13] (compound **4f**) quinone-methide eliminations and to release their end-groups upon a single cleavage event of the triggering substrate (Figure 4).

Our group[7] and two others (de Groot[10] and Mcgrath[11]) have used such molecules as AB_2 building blocks to compose distinct kinds of dendrimers that can disassemble upon a single triggering event in a domino-like manner from the focal point to their periphery to release multiple end-groups. We have termed these molecules as self-immolative dendrimers.[7]

In a typical representative example, we demonstrated the synthesis of zeroth-, first-, and second-generation self-immolative dendritic molecules **5**, **5a**, and **5b** (Figure 5) based on an AB_2 aniline building block.[14] The dendrons were constructed with an enzymatic substrate that is cleaved by the enzyme PGA (penicillin-G-amidase), and the reporter groups were 5-amino-2-nitrobenzoic acid. As illustrated in Figure 6 for second-generation dendron **5b**, the disassembly is initiated after removal of the substrate by PGA. Aniline intermediate **5c** is then fragmented into two smaller aniline intermediates **5d** through double aza-quinone-methide eliminations. These intermediates are then further disassembled by additional double aza-quinone-methide eliminations to release four reporter units.

Since the free reporter 5-amino-2-nitrobenzoic acid has an absorbance at wavelength of 405 nm, the disassembly of the self-immolative dendrons is readily monitored by visible spectroscopy. As expected, the second-generation dendron **5b** generates a 2-fold stronger spectroscopic signal than does the

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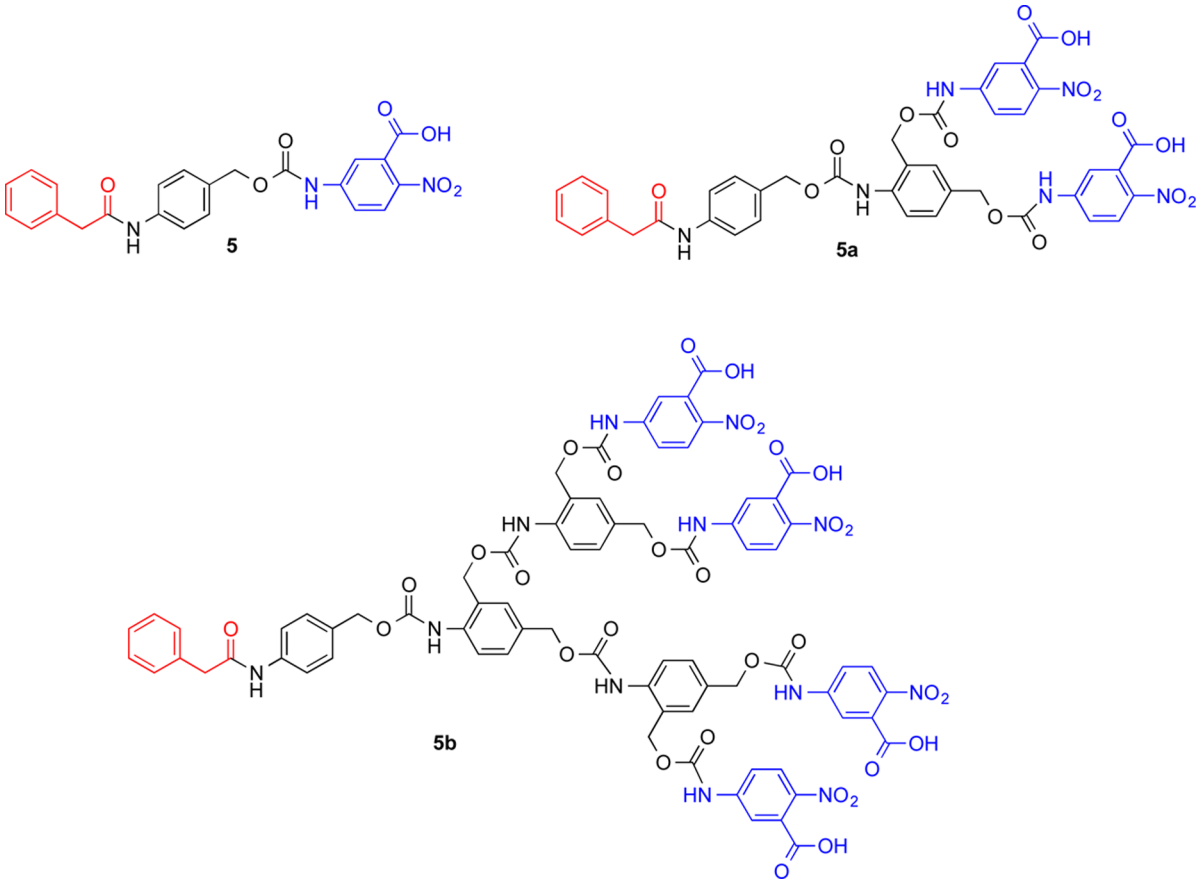

Figure 5. Chemical structures of zeroth-generation (**5**), first-generation (**5a**), and second-generation (**5b**) self-immolative dendrons. The substrate for PGA is shown in red, and the 5-amino-2-nitrobenzoic acid reporter groups are shown in blue.

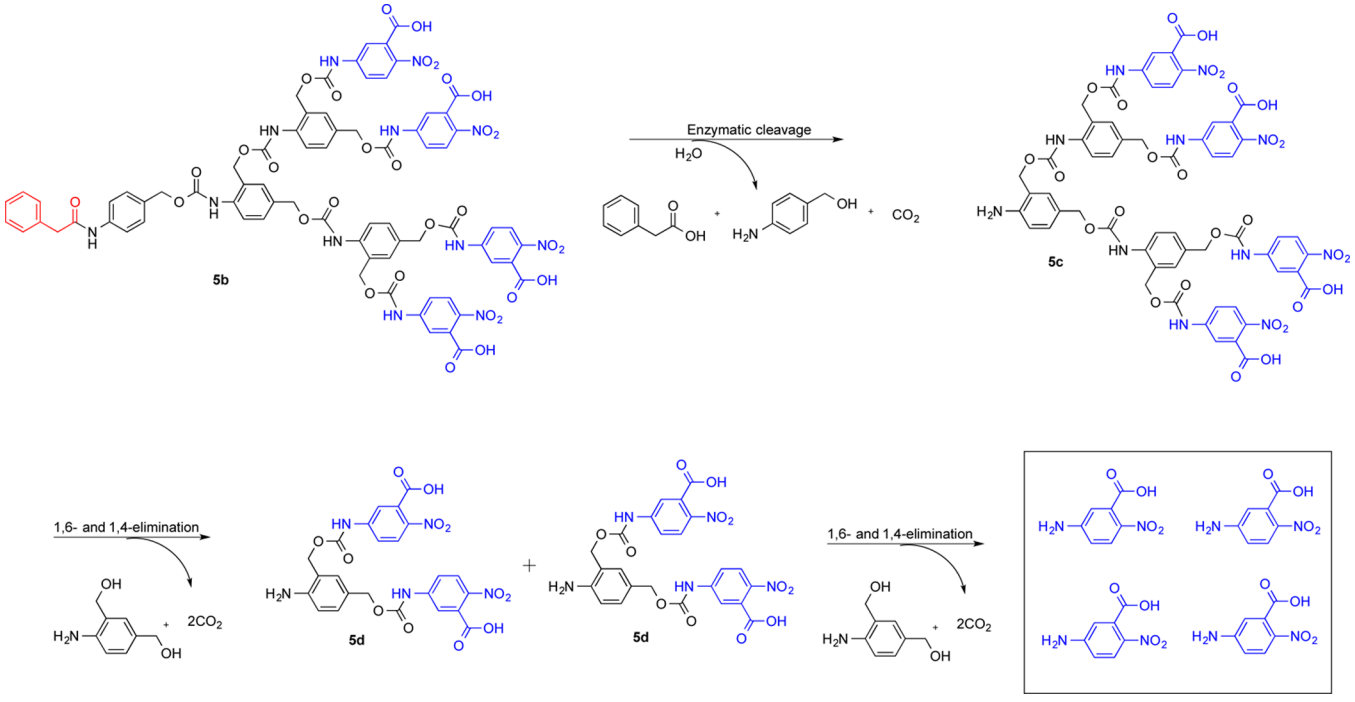

Figure 6. Disassembly pathway of second-generation self-immolative dendron.

first-generation dendron **5a**. The disassembly of these dendrons occurs from their focal point toward the periphery. It is also possible to construct dendrons that disassemble from the periphery toward the focal point.[15,16]

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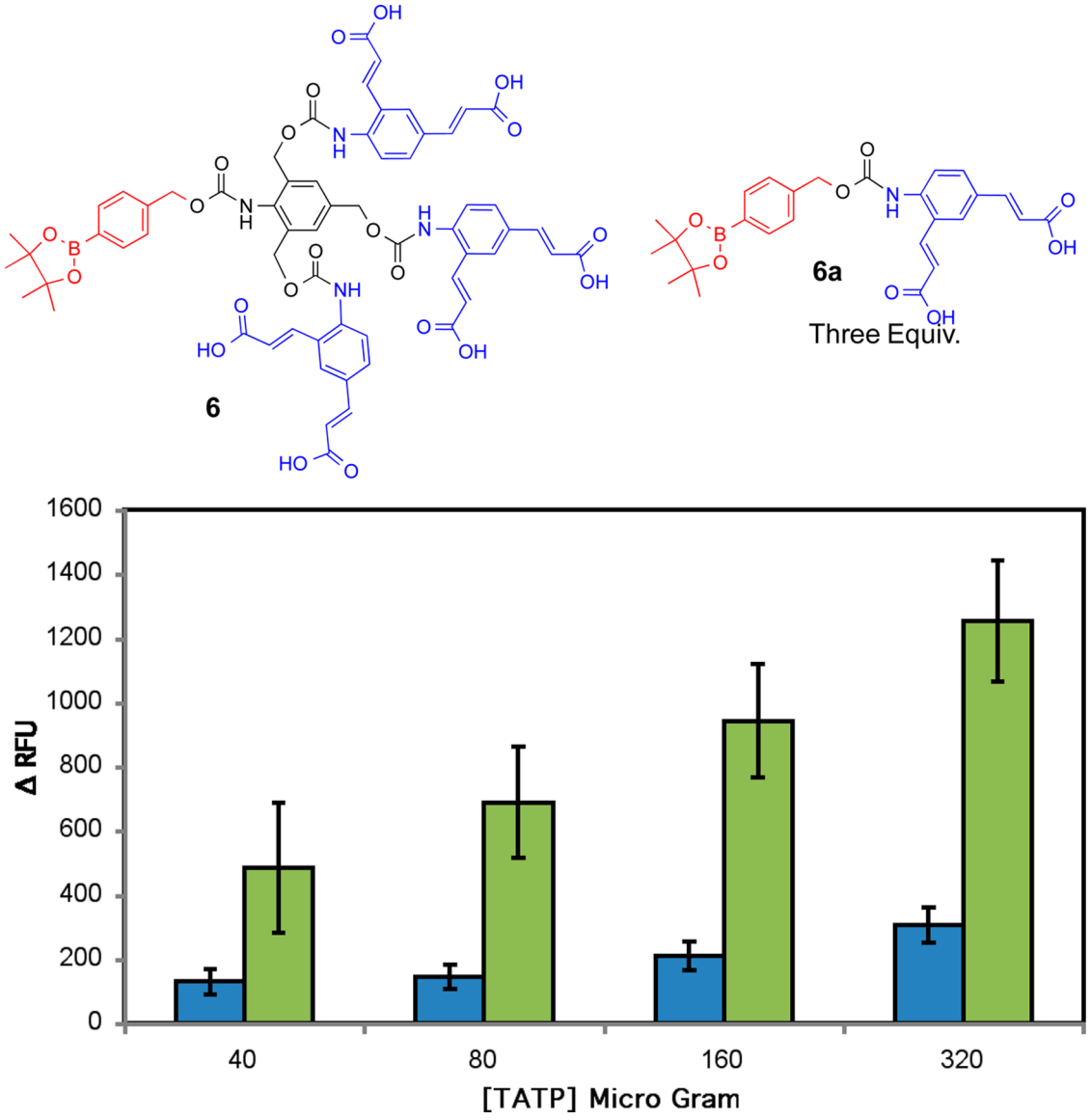

Figure 7. Fluorescence response (ex 270 nm, em 560 nm) of probes **6** (green) and **6a** (blue) upon incubation with various amounts of TATP. The probe was present at 150 mM, and solution contained 150 mM $NaHCO_3$ (pH 8.3).

SELF-IMMOLATIVE DENDRITIC PRODRUGS

Incorporation of drug molecules as end-units in a self-immolative dendron and a specific group as a triggering substrate produces a drug delivery carrier, capable of releasing payloads of drug upon a single activation event.[17,18] We have demonstrated this approach by evaluation of several self-immolative dendritic prodrugs.[19] In one example, we synthesized homo- and heterodimeric prodrugs of doxorubicin and camptothecin. Release is triggered by catalytic antibody 38C2, which functions as a model enzyme.[20] The drugs were efficiently released upon addition of catalytic antibody 38C2. The bioactivation of the dendritic prodrugs was evaluated in growth inhibition assays using the Molt-3 leukemia cell line, and the prodrugs showed a mild to significant increase in toxicity in comparison with the classical monomeric prodrugs. A remarkable increase in toxicity was observed upon bioactivation of the heterodimeric prodrug of doxorubicin and camptothecin. This study showed that introduction of more than one drug on the dendritic platform can result in synergetic effects, and suggested that precise drug combinations could be tailored for specific types of cancer.

Dendritic prodrugs that are activated through a single catalytic reaction by a specific enzyme offer amplification advantage in the inhibition of tumor growth,[21] especially if the targeted or secreted enzyme exists at relatively low levels in the malignant tissue. To demonstrate this effect, we prepared a self-immolative trimeric prodrug system with the anticancer drug camptothecin designed for activation by catalytic antibody 38C2.[22] As expected, this prodrug exhibited 3-fold amplification in growth inhibition of Molt-3 leukemia cells when compared with a classic monomeric prodrug. These results were also supported by studies of other groups.[23,24]

SELF-IMMOLATIVE DENDRITIC MOLECULAR PROBES

Self-immolative dendrimers can principally act as molecular amplifiers.[25] A single event at an analyte-responsive group leads to release of multiple end-units from the dendrimer periphery.[26] We demonstrated this amplification effect in a probe system designed to detect the explosive triacetone triperoide (TATP).[27] Self-immolative dendritic probe **6** is composed of three fluorogenic

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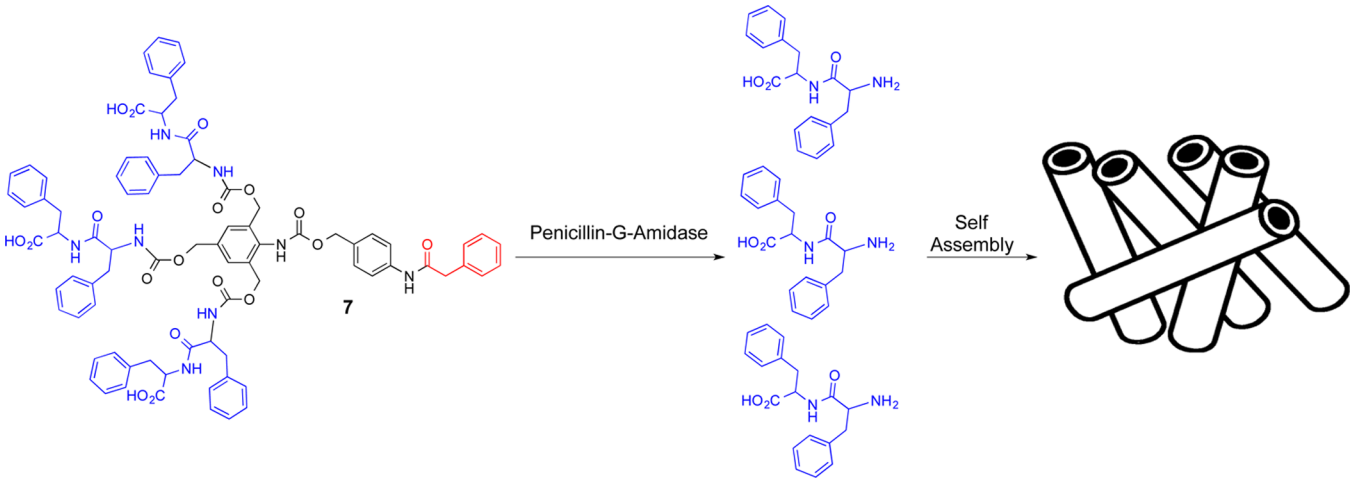

Figure 8. PGA-triggered controlled assembly of diphenylalanine peptide nanotubes from a self-immolative AB3 dendron.

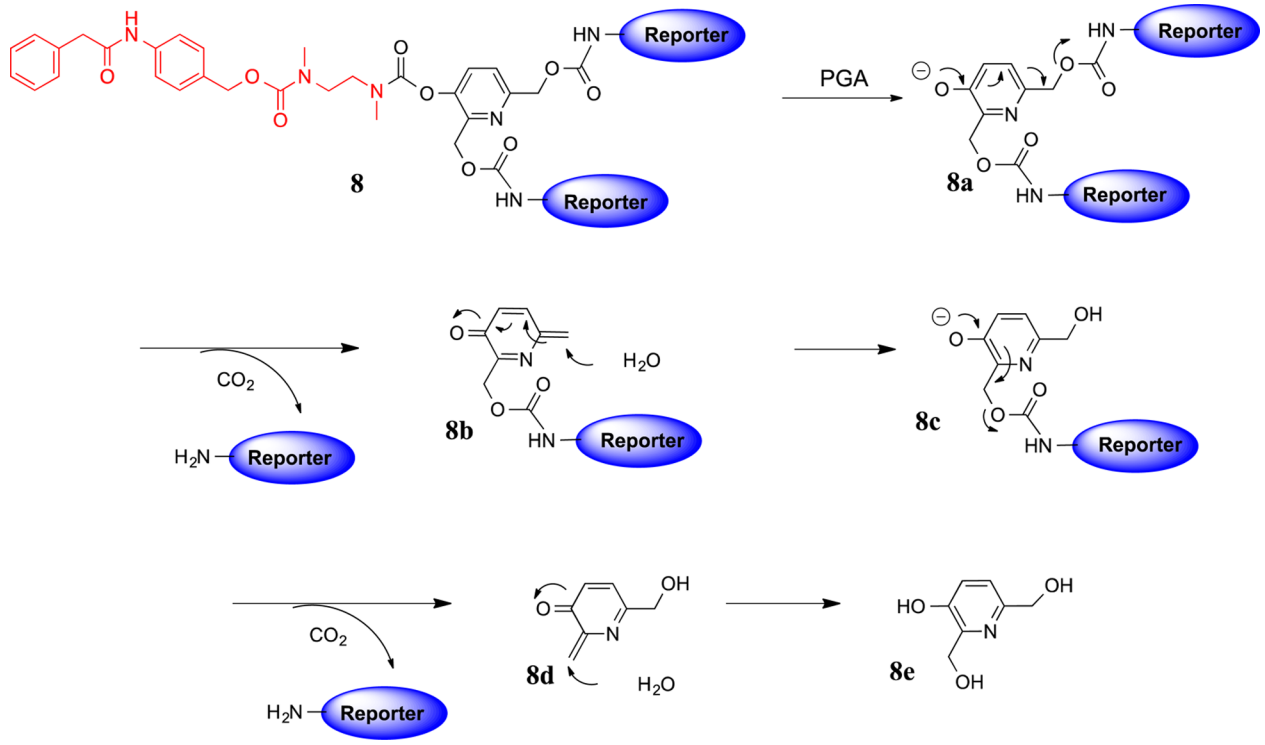

Figure 9. Disassembly pathway of self-immolative dendron **8** through double pyridinone-methide eliminations to release two reporter units.

reporter groups and a triggering substrate that can be activated by hydrogen peroxide (Figure 7). Exposure of the probe to TATP results in triple aza-quinone-methide eliminations to release the three reporters. A single molecule of hydrogen peroxide thus generates three molecules of free reporter, significantly amplifying the fluorescent signal in comparison to the nondendritic probe **6a**, which releases a single reporter upon exposure to TATP. Experimentally, the signal intensity produced by the dendritic probe is about three times higher than that of the nondendritic probe. With the self-immolative dendritic probe the explosive is detected in microgram amounts in aqueous buffer without any pretreatment.

The ability to release two different end-units simultaneously upon a single triggering event prompted us to develop a probe that allowed dual output detection. In this system, two different reporter units enabled detection by fluorescence and by UV−vis. The probe effectively detected the presence of the bacterial protease PGA; a single cleavage by the enzyme initiated the fragmentation of an AB_2 self-immolative dendritic platform to release two different reporter units. This was the first molecular probe with two different chromogenic reporter units activated by a specific stimulus.[28] Such AB_2 self-immolative dendritic platform has been used by us and other groups to design probes that enable detection by FRET,[29] fluorescence,[30] and chemiluminescence.[31]

SELF-IMMOLATION-TRIGGERED SUPERMOLECULAR ASSEMBLY

The ability to control the release of small molecules upon receipt of an external signal can be used to trigger processes such as the release of small-molecule building blocks. We designed a self-immolative dendritic system to serve as a transporter platform for

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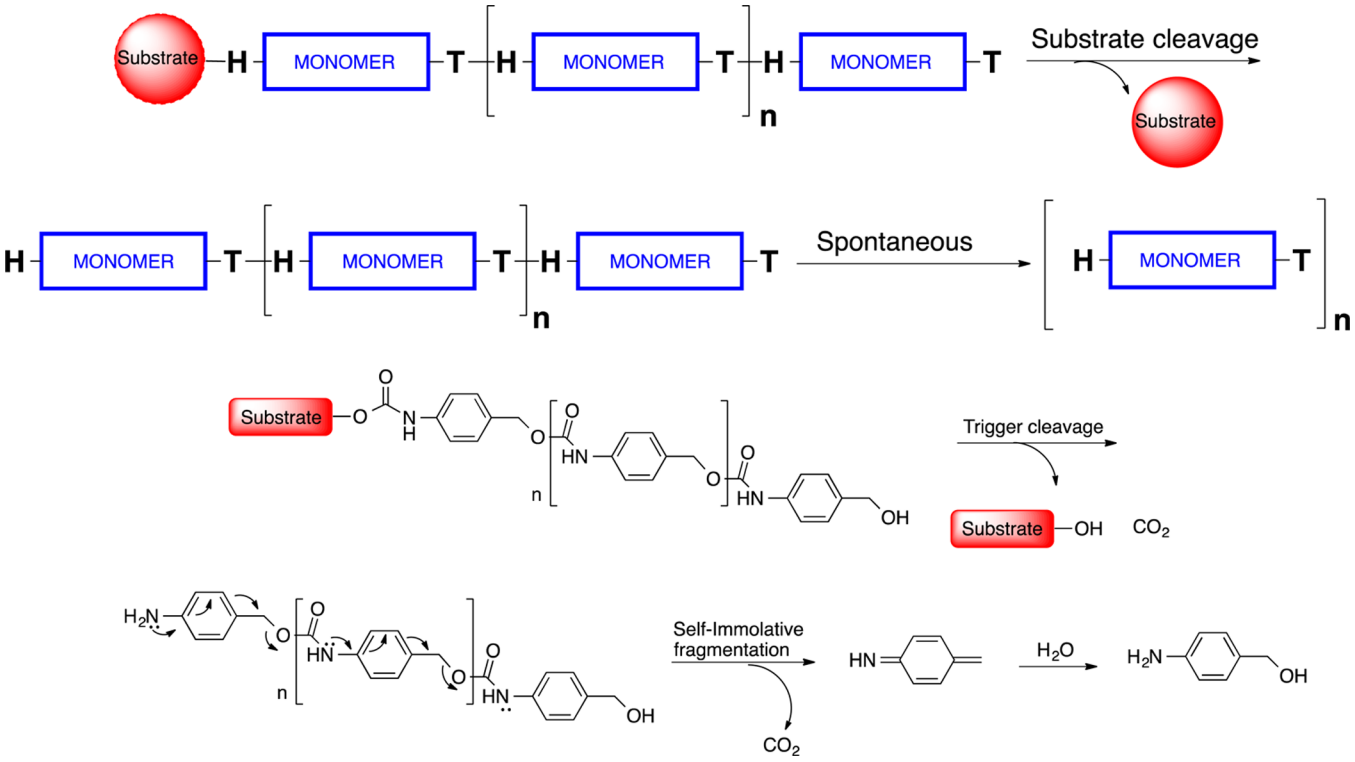

Figure 10. Graphical illustration of chemical design and disassembly mechanism of a self-immolative polymer.

the controlled assembly of the peptide nanostructures. AB_3 self-immolative dendron **7** was prepared with diphenylalanine end-units and a triggering substrate activated by PGA (Figure 8). Removal of the phenylacetamide group by PGA results in rapid release of the diphenylalanine end-units and apparent formation of peptide nanotubes. Because diphenylalanine cannot form tubular nanostructures while it is part of the AB_3 dendron, no organized structures were observed in the absence of the enzyme.[32]

This current example represents an important addition to the attempts to control bioorganic assembly at the nanoscale and could serve as an principal step toward the development of nanobiotechnological functions.

THE PYRIDINONE-METHIDE ELIMINATION

Pyridine is a water-soluble compound, whereas benzene is almost insoluble in aqueous media. Thus, use of self-immolative linkers based on a pyridinone-methide reactivity should increase water solubility and enable application under physiological conditions. We have shown that the pyridinone-methide elimination can take place in a pyridine-based linker in a manner analogous to that from a benzene-based system.[33] Dendron **8** was composed with two reporter units attached to a pyridine core and phenylacetamide group as a triggering substrate (Figure 9). The dendron releases phenolate **8a** upon reaction with the enzyme PGA. Next, it undergoes a first elimination to release one reporter and *p*-pyridinone-methide **8b**, which further reacts with a water molecule to generate phenolate **8c**. The latter undergoes a second elimination to release the other reporter and *o*-pyridinone-methide **8d**.

Increased aqueous solubility was observed with compounds based on pyridine relative to those based on benzene. The pyridinone-methide elimination was demonstrated as an alternative suitable for design of self-immolative linkers that function in an aqueous environment.

SELF-IMMOLATIVE POLYMERS

A major challenge in synthesis of self-immolative dendrimers is that multiple steps are required. Furthermore, steric hindrance limits the number of the tail groups that a dendrimer can carry. To overcome such difficulties, we have developed novel self-immolative polymeric molecules.[34] Like self-immolative dendrimers, these self-immolative polymers starts to disassemble upon a triggering event into their building blocks, in a domino-like manner.[35] The polymer is prepared by polymerization of an appropriate monomer. Capping of the polymer terminal headgroup with a specific protecting group generates a polymeric molecule with a triggering substrate (Figure 10). Selective cleavage of the trigger initiates sequential fragmentation into the building blocks from the head (H) to the tail (T). Our design for a self-immolative polymer is based on a polycarbamate backbone of 4-amino-benzylalcohol with a protecting group on the terminal amine that acts as a triggering substrate. Cleavage of the protecting group generates an amine molecule that then undergoes sequential 1,6-elimination and decarboxylation reactions to form carbon dioxide and aza-quinone-methide (Figure 10). We have demonstrated the unique disassembly pathway of these polymers with an example of a polymer made of aniline building blocks that are fluorescent. The fluorescence of the aniline derivative is turned OFF when the molecule is part of the polycarbamate backbone. Once the triggering substrate is removed, the polymer disassembles to generate the fluorescent aniline molecule. Thus, the polymer disassembly can be usefully monitored by a fluorometer.

Insertion of additional hydroxymethyl substituents at the *ortho*-position of each aniline monomer generated self-immolative comb-polymers with side-releasable groups.[36] A water-soluble prototype of a self-immolative comb-polymer was prepared and activated under physiological conditions by a protease. The self-immolative comb-polymer underwent complete

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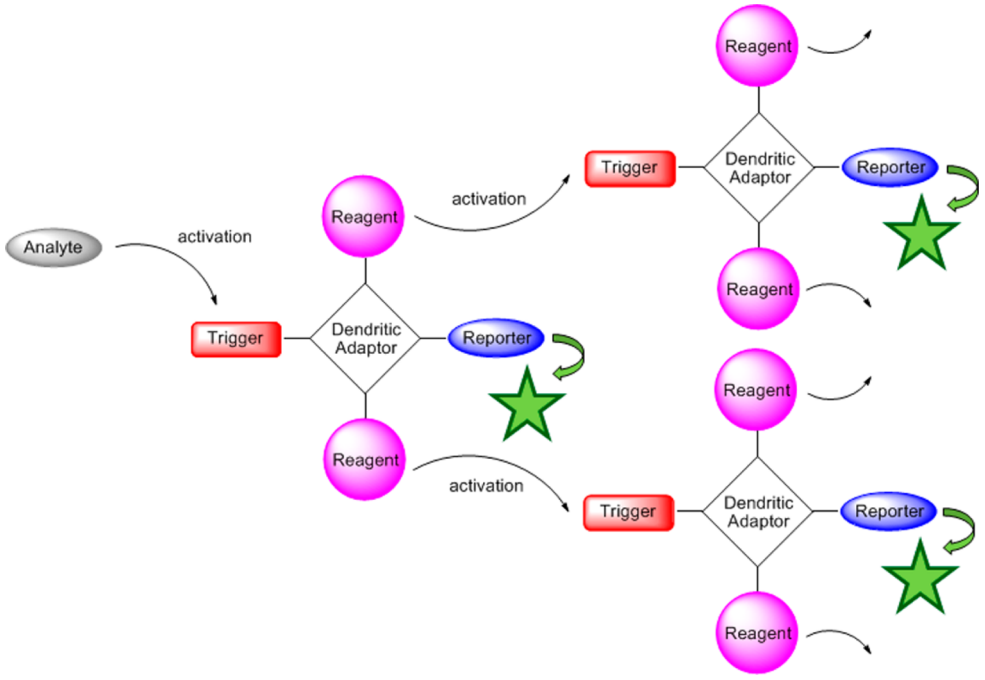

Figure 11. Graphical illustration of the DCR technique.

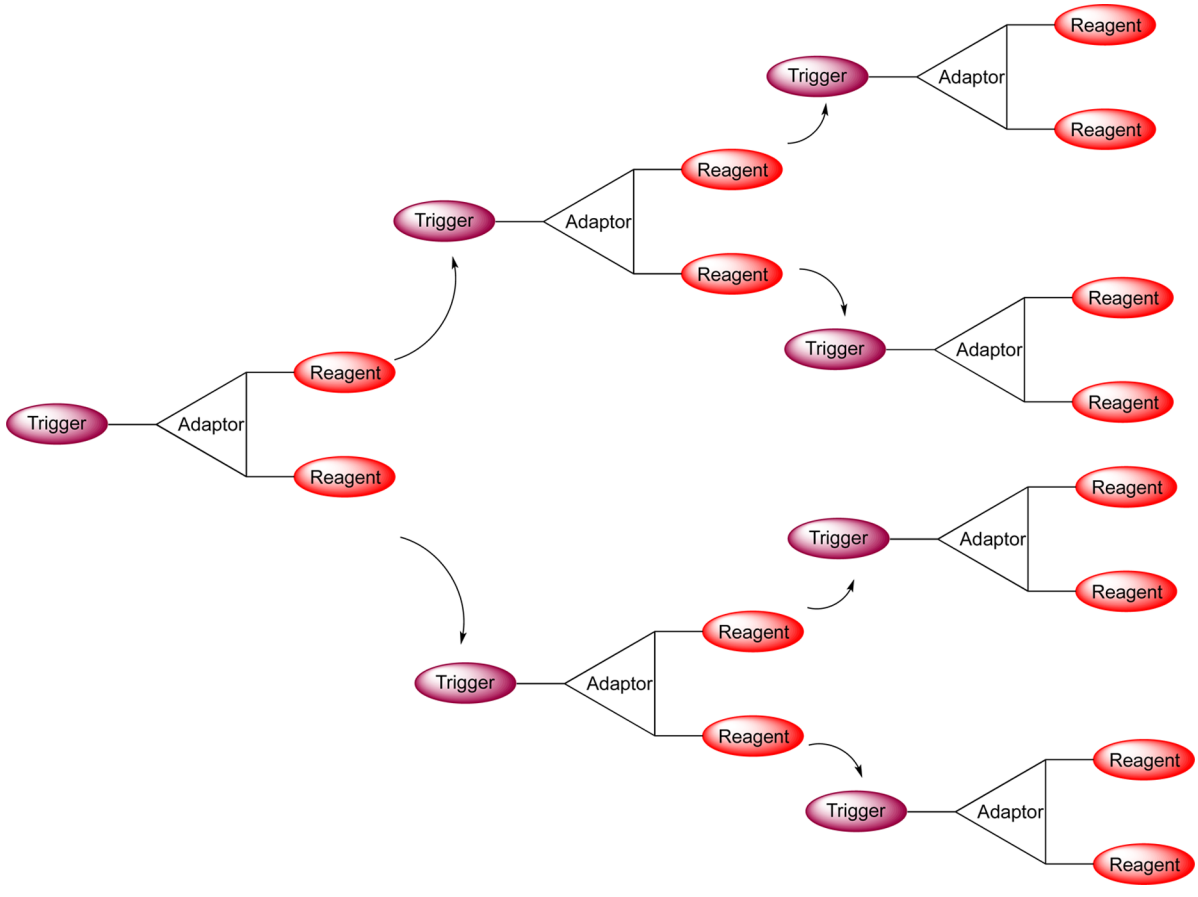

Figure 12. Disassembly pattern of virtual high-generation self-immolative dendron.

disassembly to release its multiple reporter groups. Use of drug molecules instead of reporter units would generate a polymeric drug delivery system that can selectively release a high payload of drug upon stimulus by a specific enzyme.

In addition, we have demonstrated how these polymers can be applied as activity-based labeling probes for proteins with catalytic activity.[37] A real-time detection of the protein labeling can be monitored due to a change in the fluorescence emission

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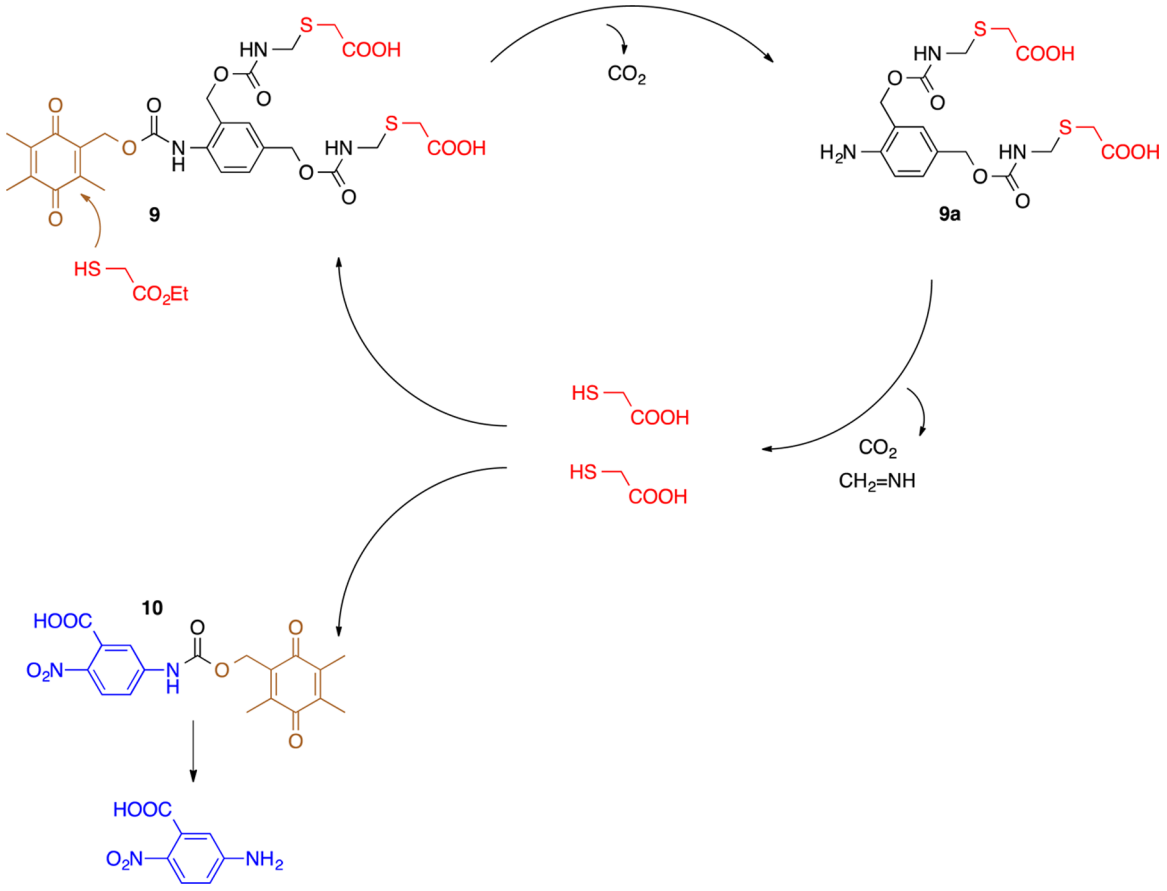

Figure 13. A two-component DCR system to detect sulfhydryl compounds; mercaptoacetic acid reagent units and 5-amino-2-nitrobenzoic acid reporter are indicated by red and blue color, respectively.

wavelength of the probe during the polymer disassembly process. The self-immolative polymer-based labeling approach conserves catalytic activity of the labeled protein, and we observed a significant preference toward labeling of the activating protein.

Several other research groups have adopted our self-immolative strategy for responsive polymer disassembly.[38,39] New ideas based on other chemical reactions were cleverly developed[40] to achieve head-to-tail disassembly of polymers.[41,42] These concepts have resulted in polymer-based capsules,[43] polymeric nanoparticles,[44] and biodegradable polymers[45] with self-immolative disassembly patterns.[46]

DENDRITIC CHAIN REACTION

The amplification effect achieved by self-immolative dendrimers or polymers is limited mainly by practical synthetic concerns. To overcome such limitations, we have developed a new amplification approach based on a distinct dendritic chain reaction (DCR).[47] The amplification principle of the DCR technique is illustrated in Figure 11. The approach is based on a simple AB_3 self-immolative dendron, which is equipped with an analyte-responsive group (trigger), one reporter, and two reagent end-groups. Removal of the trigger by the analyte will generate the release of one chromogenic reporter and two reagent units. The two free reagents will then acquire the same reactivity of the analyte of interest and activate two additional dendrons by removal of their substrates. These events will produce two more chromogenic reporters and four additional free reagent units. The process will proceed exponentially until all dendrons have been disassembled.

In general, the DCR amplification approach can be viewed as activation of a virtual high-generation self-immolative dendron (Figure 12). A simple self-immolative dendritic AB_2 unit is able to produce an exponential disassembly pattern that is equivalent to disassembly of a self-immolative dendron of an endless generation.[48]

Initially, we demonstrated our DCR approach with a probe system designed to detect hydrogen peroxide.[49] However, the modular design of the DCR probe enables synthesis of diagnostic probes for various analytes. In a second example, we developed a DCR probe system for the detection of ubiquitous sulfhydryls.[50] The probe is activated by a thiol analyte through a stoichiometric reaction to generate a chain reaction that exponentially amplifies a diagnostic signal. We have also prepared a DCR probe that amplifies a spectroscopic signal for the direct detection of fluoride.[51] The amplification occurs through reaction of fluoride with a responsive chromogenic probe. The probe activity is based on a dendritic chain reaction that generates a fluoride anion, which acts to catalyze the disassembly pathway of the dendritic probe. Such an autoinductive amplification mechanism may be applied for detection of other analytes by coupling activity of a modified probe with that of the fluoride amplifier.

A simplified version to achieve exponential amplification was also developed based on a two-component dendritic chain reaction.[52] In this version, a self-immolative dendron serves as an amplifier component and a chromogenic probe component is used to produce a diagnostic signal.[53] The two components are equipped with identical triggering substrates and therefore are able to react with same analyte of interest. A two-component

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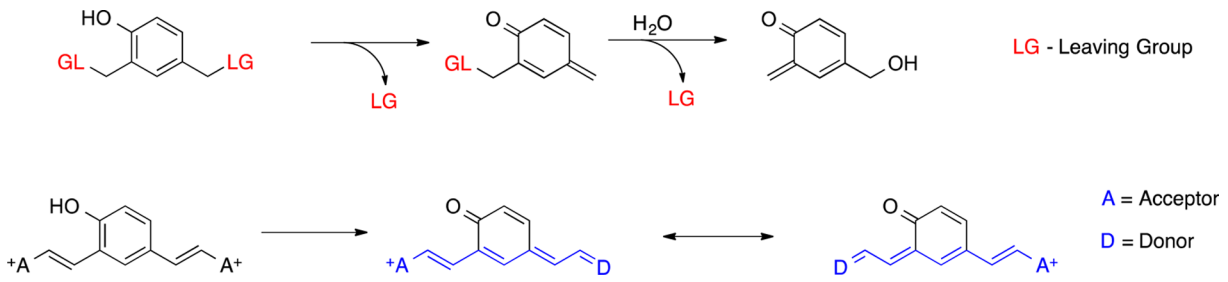

Figure 14. Comparison of a quinone-methide elimination (top) and an intramolecular charge transfer process between a donor and two acceptors (bottom).

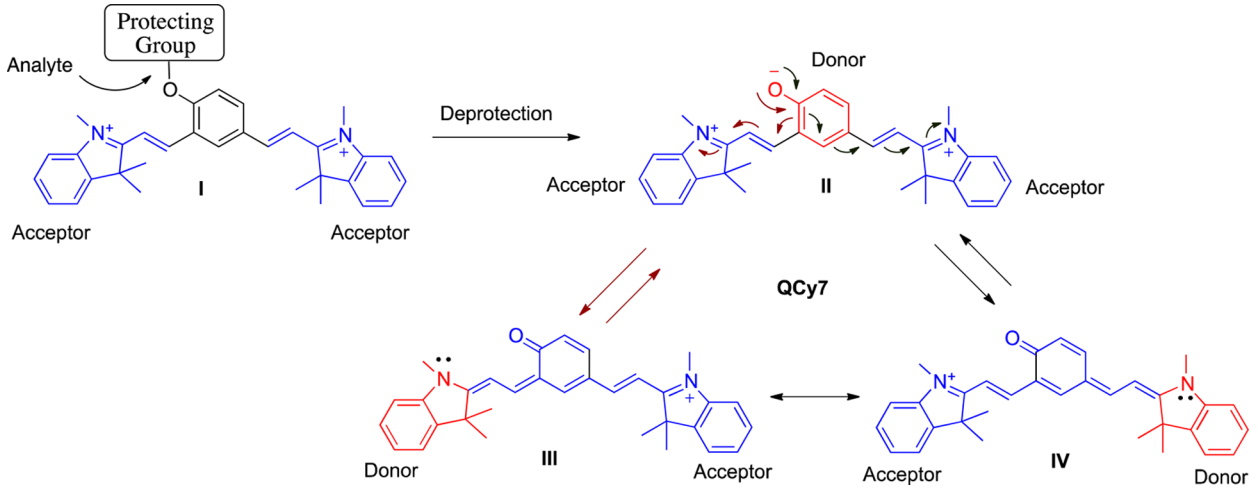

Figure 15. Activation of a QCy7 modular probe by a specific analyte to produce a fluorescent turn-ON response.

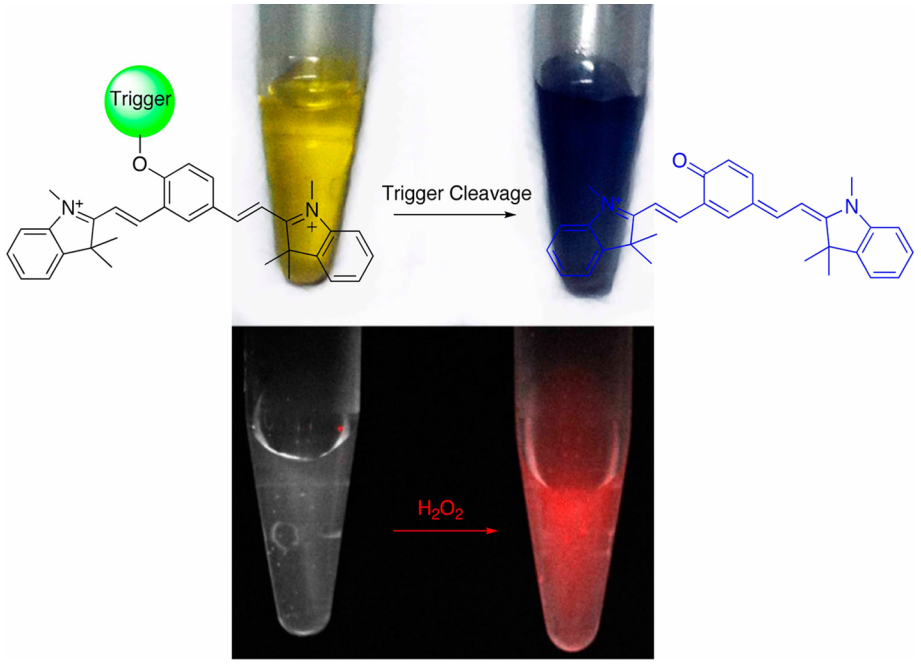

Figure 16. Images of a QCy7-based probe and free QCy7 solutions [50 μM] in PBS 7.4 as observed by the naked eye and by the NIR camera.

dendritic chain reaction example for a sulfhydryl analyte is illustrated in Figure 13. Dendron **9** is composed of two mercaptoacetic acid units and a benzoquinone moiety as a trigger, which is cleaved upon reaction with any sulfhydryl. Probe **10** is composed of the 5-amino-2-nitrobenzoic acid reporter attached to the benzoquinone trigger. Cleavage of the trigger of dendron **9** by a thiol molecule will initially generate intermediate **9a**. The latter will undergo double eliminations to release two mercaptoacetic acid molecules that will then activate some AB_2 dendrons and some probe molecules. Since the used concentration of dendron **9** is at least twice that of probe **10**, the rate of the system disassembly should exponentially increase until all of the reporter

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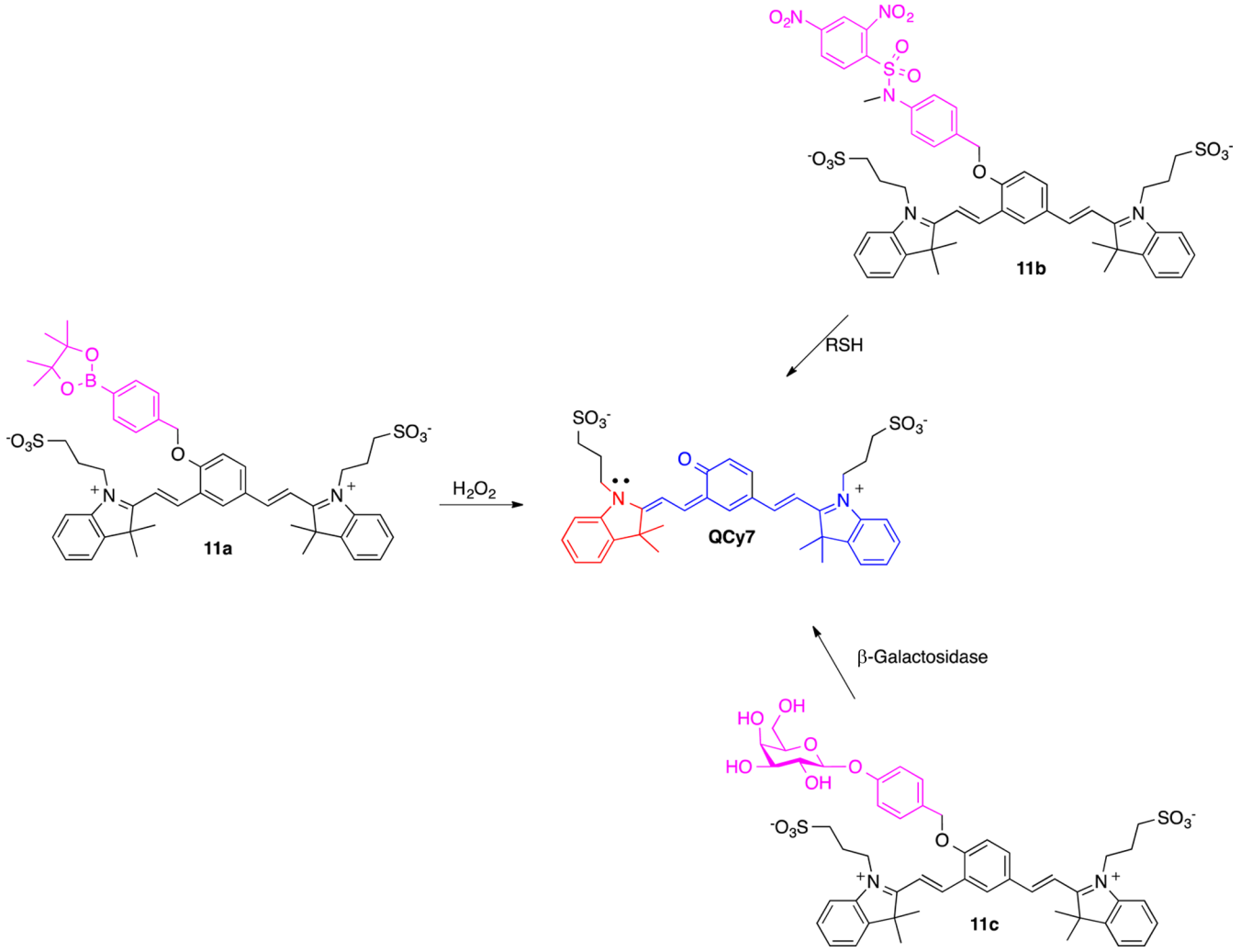

Figure 17. Chemical structures of three different QCy7-based probes for detection of hydrogen peroxide (**11a**), ubiquitous sulfhydryl (**11b**), and *β*-galactosidase (**11c**).

molecules have been released. The signal can be detected with a spectrophotometer by monitoring the yellow color of the released 5-amino-2-nitrobenzoic acid. Following this concept, other groups have also developed exponential chain reactions with diagnostic signal amplification for detection of analytes.[54]

FLUORESCENT PROBES: DONOR–TWO-ACCEPTORS DYE DESIGN

As described above, the quinone-methide elimination occurs if an appropriate leaving group is introduced in either *ortho* or *para* positions of a phenol derivative. However, if conjugated acceptors are incorporated instead of leaving groups, quinone-methide species are formed through an intramolecular charge transfer (ICT). A comparison between the quinone-methide elimination and an ICT process is presented in Figure 14.

Such an ICT process between a phenol donor and two acceptors results in formation of an elongated conjugated *π*-electron system. We have shown how this strategy can be applied for the design of long-wavelength fluorogenic dyes with a turn-ON option.[55] This strategy was translated into synthesis of a library of dyes with fluorescence emission in the near-infrared (NIR) region.[56,57] This optical range is particularly valuable for *in vivo* imaging applications, since live tissues have minimal absorbance and emission in NIR wavelengths and such photons have the capability to infiltrate deeper into tissues.

The donor–two-acceptors dye system is constituted of a protected phenol moiety (**I**) that acts as a latent donor conjugated with two indolium acceptors (Figure 15). The protecting group is typically a substrate that can undergo a cleavage reaction in the presence of the analyte of interest. Deprotection of the phenol leads to formation of a phenolate active donor **II** that is able to donate a pair of *π*-electrons to either one of the conjugated indolium acceptors (structures **III** and **IV**). This intramolecular charge transfer generates a resonance species with a *π*-electron pattern similar to that of a cyanine fluorochrome. Accordingly, we have termed this new fluorochrome quinone-cyanine-7 (QCy7). The donor capability of the phenolate species **II** can be masked by an analyte-responsive group to generate molecular probes for detection or imaging of specifice analytes.

The color-shift of the turn-ON response obtained by such a donor–two-acceptors dye system is shown in Figure 16. A QCy7-based probe was prepared with a triggering substrate that is responsive to hydrogen peroxide. In the right eppendorf, one can see the typical cyan color for solution of QCy7 obtained under physiological conditions. Due to the obtained 150 nm blue Stokes shift, the masked form of the dye has a yellow color in aqueous solution. When imaged using a NIR imaging camera, the

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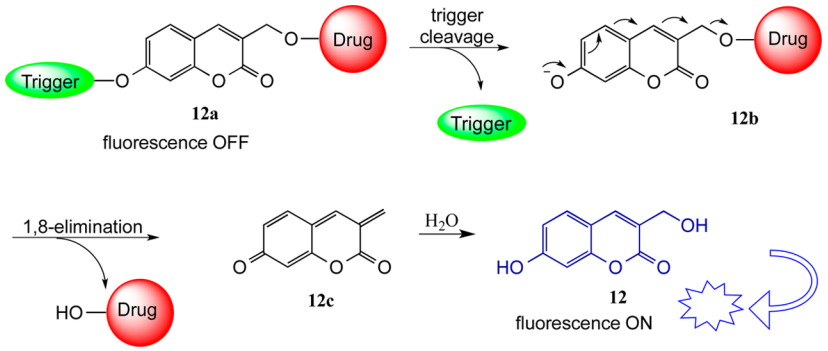

Figure 18. Theranostic prodrug design and activation based on a coumarin self-immolative linker.

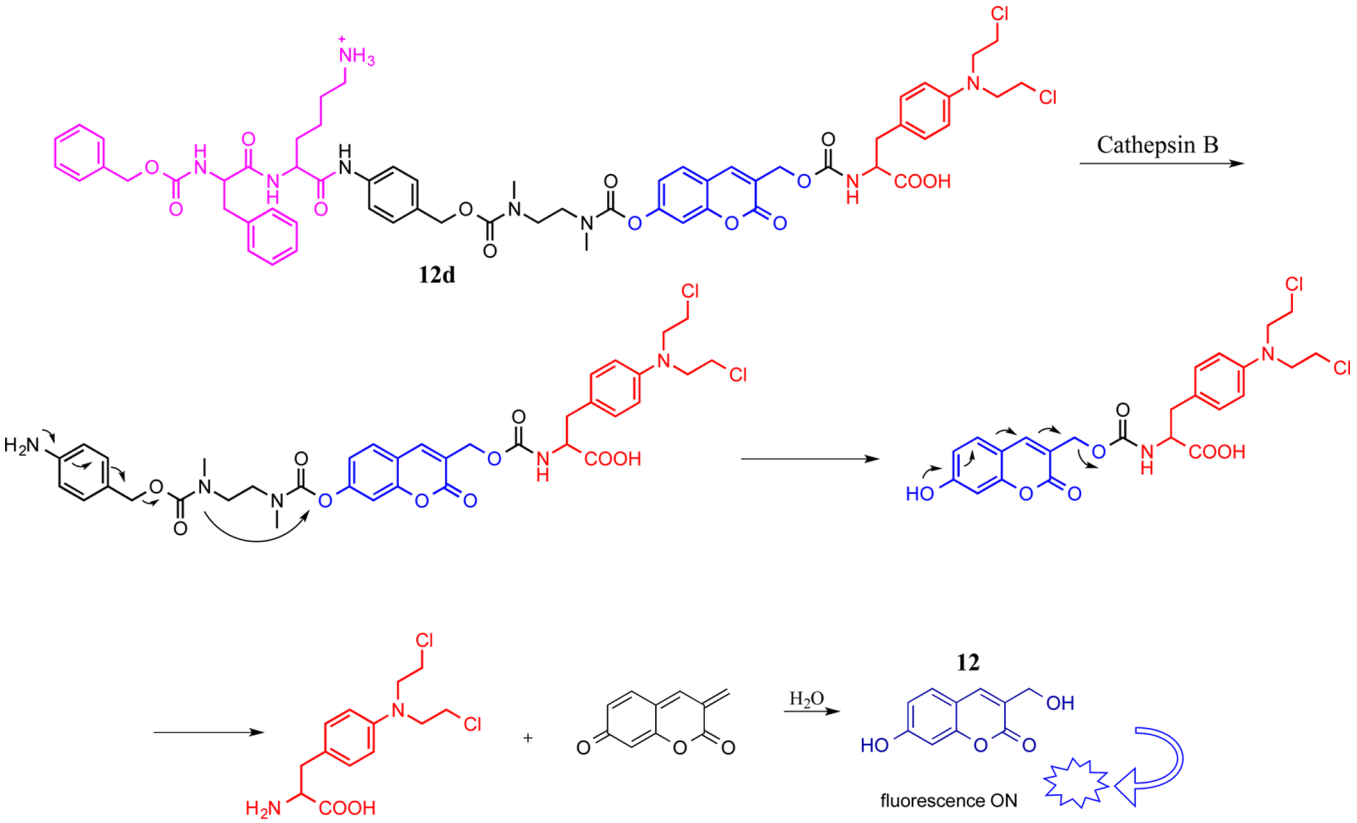

Figure 19. Melphalan theranostic prodrug activation-pathway by cathepsin B.

solution of the protected probe is clearly transparent, while the solution of free QCy7 exhibits a well-observed NIR fluorescence.

The modular structure of the probe enables one to introduce various substrates as protecting groups. Substrates can be tailored to react with a specific analyte. Recently, we have shown how a modular synthetic procedure can be used for preparation of three different QCy7 probes. The probe were designed for detection and imaging of hydrogen peroxide, ubiquitous sulfhydryl, and β-galactosidase.[58] Probe **11a**, designed for detection of hydrogen peroxide, is equipped with a phenyl-boronic-ester protecting group as a substrate; probe **11b**, designed for detection of ubiquitous sulfhydryl, is equipped with a dinitro-benzene-sulfonyl protecting group; and probe **11c**, designed for detection of the enzyme β-galactosidase, is equipped with a β-galactose protecting group (Figure 17). Reaction of the specific analyte or enzyme with the appropriate probe results in a turn-ON response through the release of active QCy7 dye.

In addition, we evaluated the ability of QCy7-based probe **11a** to visualize endogenously produced hydrogen peroxide using a noninvasive imaging technique. The signal-to-noise ratio of the NIR fluorescence intensity observed by the hydrogen peroxide imaging probe in mice was about 100-fold higher compared with the control group. Such ratio should be adequate to obtain strong contrast images.[55]

THERANOSTIC PRODRUGS WITH FLUORESCENCE MODE-OF-ACTION

Efficacy of chemotherapy drugs significantly depends on the concentration of the agent in the cancerous tissue (therapeutic effect) relative to that in healthy tissues (side effects). The low concentrations of chemotherapeutics present in the body are difficult to follow in an individual patient in real time in order to personalize treatment. Thus, there is an urgent medical need for new more efficient methods of cancer treatment with fewer side effects and drugs that allow imaging of biodistribution in real

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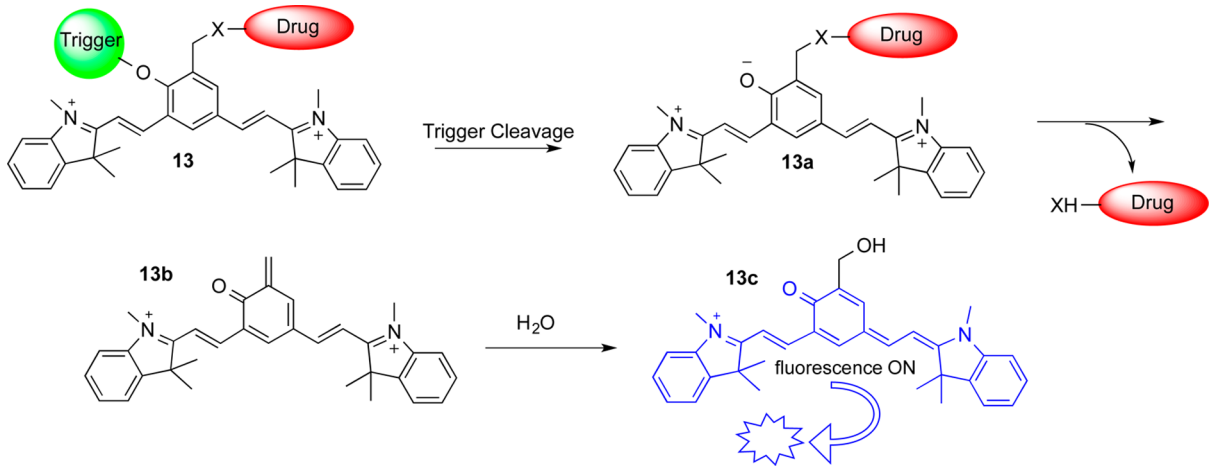

Figure 20. Activation pathway of a theranostic prodrug based on a QCy7 LF-linker with NIR fluorescence turn-ON response.

time. Monitoring of drug release would enable real time *in vivo* kinetic studies of the process. Therefore, drug delivery systems that instantaneously report on the release of their active drug could be of great benefit, especially if the reported signal could be detected by a noninvasive imaging technique.[59] Latent fluorophores are attractive candidates for this type of reporter. By coupling latent fluorophore activation to the drug-release event in a delivery system, real-time information about the release process can be obtained.

Several years ago, we developed a theranostic prodrug system that was based on a 7-hydroxycoumarin latent fluorophore-linker (compound **12**, Figure 18).[60] The phenolic alcohol of **12** is attached to a triggering substrate, and the hydroxymethyl substituent serves as a self-immolative linker for attachment of a drug molecule. The release of the drug is initiated by removal of the trigger from molecule **12a** resulting in formation of phenolate **12b**. A spontaneous 1,8-elimination reaction releases the drug through generation of coumarin-quinone-methide derivative **12c**. Addition of a water molecule to the reactive quinone-methide **12c** results in formation of the highly fluorescence coumarin derivative **12**.

To evaluate this concept, we synthesized prodrug **12d**, equipped with the dipeptide Phe-Lys (pink) as the triggering-substrate for cathepsin B and melphalan (red) as a chemotherapeutic drug (Figure 19). Cleavage of the amide bond at the C-terminus of the lysine initiates the disassembly cascade, resulting in the release of free melphalan and the formation of fluorescent coumarin derivative **12**. Direct correlation was observed between tumor cell growth inhibition activity and emitted fluorescence in MOLT-3 cells. The amount of drug release can be calculated by quantifying the emitted fluorescence and, therefore, should allow prediction of therapeutic effect.

In the above example, fluorescence emission is in the blue/green region, which is not practical for *in vivo* use. For clinical use, LF linkers with longer emitted wavelengths are necessary. We are currently trying to incorporate a QCy7 derivative as a latent fluorophore linker for a prodrug (Figure 20). Prodrug **13** is composed of triggering substrate attached through a QCy7 linker to a drug molecule. Removal of the triggering substrate should form intermediate **13a**, which can then undergo 1,4-elimnation to release the active drug through formation of quinone-methide derivative **13b**. Addition of a water molecule to quinone-methide **13b** will generate dye **13c** and result in a turn-ON response of the QCy7 fluorophore. It should be possible to monitor the activation of such theranostic prodrug *in vivo* since the emitted fluorescence of the QCy7 derivative is in the NIR region.

CONCLUDING REMARKS

In recent years, the quinone-methide elimination has proven to be a valuable tool for drug delivery, molecular probe design, signal amplification, stimulus supermolecular assembly, and self-immolative dendritic and polymeric molecular systems. In a manner analogous to this elimination, electron rearrangement can lead to formation of conjugated quinone-methide-type dye compounds with long-wavelength emission of fluorescence. The mounting number of publications on this topic clearly indicates the interest of the scientific community in the quinone-methide elimination and its analogous electron rearrangement. In this Account, we described the development of functional molecular systems related to the quinone-methide elimination. Since the discovery of molecules that enable multiple quinone-methide eliminations, we have developed unique molecular structures that are known today as self-immolative dendrimers.[41,61] These systems have been used to construct self-immolative dendritic prodrugs and diagnostic probes that self-amplify. To further improve this amplification effect, we have developed the dendritic chain reaction, which uses simple molecules to achieve functionality of a high-generation virtual self-immolative dendrimer. In addition, we have harnessed the quinone-methide elimination as tool to design distinct polymers that disassemble from head-to-tail upon an analyte-responsive event. Other chemical reactivities have been reported for construction of such polymeric molecules.

Inspired by the quinone-methide elimination mechanism, we have recently applied an intramolecular charge transfer to form unique quinone-methide type derivatives based on donor−two-acceptor chemical structures. This intramolecular charge transfer produces a fluorochrome with an extended π-conjugated system that could be used for the design of long-wavelength fluorogenic probes with a turn-ON option. One such probe was successfully used to image hydrogen peroxide *in vivo* in a mouse inflammation model. The donor−two-acceptor concept was translated to a library of new dyes with long-wavelength fluorescence emission. These dyes may also be applied as LF linkers of theranostic prodrugs that provide a turn-ON NIR fluorescence response upon activation.

The rapidly expanding use of quinone-methide species, reflected in ever increasing numbers of publications and

examples, highlights the importance of this tool in chemistry. These species provide a useful gateway to functional molecular structures with distinct reactivities and spectroscopic characteristics. It is quite obvious that quinone-methide reactivity will continue to "play" a valuable role in the design of functional molecular systems.

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Notes

The authors declare no competing financial interest.

Biographies

Samer Gnaim was born in 1991 in Baqa El-Garbia, Israel. He received his B.Sc. degree in Chemistry & Biology with distinction in 2013 from Tel Aviv University. He is now a Ph.D. student in the Organic Chemistry department in Tel Aviv University under the supervision of Prof. Doron Shabat. His research focuses on the development of a new approach for targeted drug delivery systems. He is the recipient of the "Israeli Council for Higher Education" scholarship for outstanding research students in 2013.

Doron Shabat studied chemistry at the Technion-Israel Institute of Technology between 1987 and 1990. After obtaining his B.Sc. degree, he continued toward his Ph.D. degree under the supervision of Prof. Ehud Keinan in the field of catalytic antibodies. Upon the completion of his Ph.D. thesis in 1997, he joined a group led by Profs. Richard A. Lerner and Carlos F. Barbas, III, at The Scripps Research Institute in La Jolla, California, as a postdoctoral fellow. There, he continued to work in the area of catalytic antibodies. In 2000, he returned to Israel to start his independent career in the School of Chemistry at Tel Aviv University as a senior lecturer. He was promoted to the rank of associate professor in 2005 and to full professor in 2008. His research is focused in bioorganic chemistry with particular interests in self-immolative molecular systems and long-wavelength fluorescent dyes for *in vivo* imaging. He is the recipient of the Juludan Prize for 2005, administered by the Technion-Israel Institute of Technology, and the Israel Chemical Society's Prize (2005) for Outstanding Young Chemists.

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