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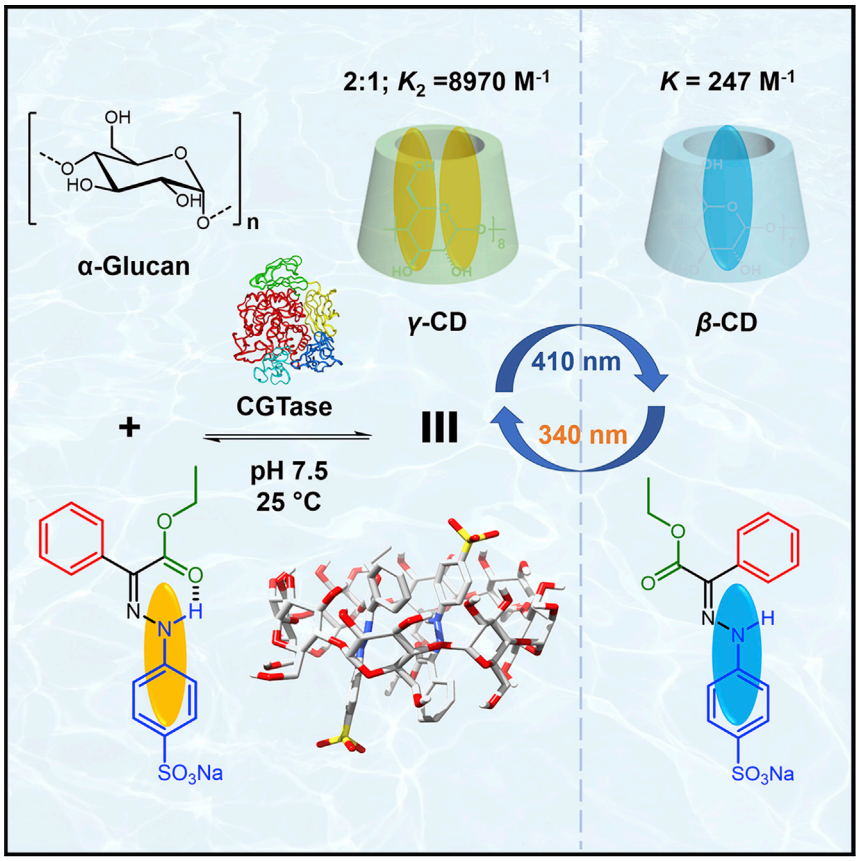
Dynamic enzymatic synthesis of γ-cyclodextrin using a photoremovable hydrazone template



A photoremovable hydrazone template is used to control the dynamic enzymatic synthesis of cyclodextrins (CDs). Because of the higher binding affinity of γ-CD (compared with α-CD and β-CD) for the *Z* form of the template, it is produced preferentially, leading to a 6-fold increase in yield. The low affinity of the *E* isomer of the template for γ-CD allows for its photoremoval, thus enabling isolation of the product using an alternative strategy to the energy-consuming distillation processes used in industry.

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Highlights

Water-soluble hydrazone photoswitch templates the enzymatic synthesis of γ-cyclodextrin

The hydrazone template improves the selectivity and yield of γ-CD production

The *Z* hydrazone isomer binds strongly to γ-CD while the *E* isomer has low affinity

The photoremovable template is a proof-of-principle strategy for efficient γ-CD isolation

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Dynamic enzymatic synthesis of γ-cyclodextrin using a photoremovable hydrazone template

Sirun Yang,[1] Dennis Larsen,[2] Maria Pellegrini,[1] Sebastian Meier,[2] Dale F. Mierke,[1,*] Sophie R. Beeren,[2,*] and Ivan Aprahamian[1,3,*]

SUMMARY

The high water solubility of γ-cyclodextrin (γ-CD) lowers its synthetic yield and complicates its post-synthetic recovery, thus increasing production costs. Here, we report the use of a photoremovable hydrazone template as a proof-of-concept strategy for increasing the efficiency of the enzymatic synthesis of γ-CD and lowering the associated production costs. Our results show that while both the *Z* and *E* isomers of the hydrazone switch form low affinity (K = 250–725 M^{-1}) complexes with β-CD (1:1), only the *Z* isomer of the switch can be included in γ-CD (2:1; K_2 = 8,970 M^{-1}). We used this property to preferably synthesize γ-CD and increase its yield by 6-fold and then took advantage of the photoremovable nature of the template to isolate the product. Considering the very limited number of photoswitches that can bind to γ-CDs, we anticipate that this newly discovered host-guest couple will open the way for designing γ-CD-based adaptive materials.

INTRODUCTION

The hydrophobic cavity and water solubility of cyclodextrins (CDs) have made them some of the most ubiquitous host molecules used in supramolecular chemistry.[1–3] These characteristics, combined with the biocompatibility of CDs,[4–7] explain why they are regularly used in the food and pharmaceutical industries,[8–10] cosmetic products,[3] and pollutant remediation,[11] among other applications.[12–14] While CDs come in many sizes, the α-, β-, and γ-CDs, which differ in the number of glucose units forming the walls of the cavity (6, 7, and 8, respectively), are the most synthetically accessible. The lower water solubility of β-CD makes it more readily accessible at a commercially relevant scale, making it the most commonly used CD in industry.[1–3] This property is a double-edged sword, though, as the lower water solubility also limits the range of applications β-CDs can be used in. γ-CD, on the other hand, is more water soluble and has a larger cavity size compared with α- and β-CDs, allowing it to encapsulate larger guests and be used in more diverse applications, especially in the food and pharmaceutical industries.[15,16] For example, unlike β-CD, γ-CD can completely encapsulate bulky guests, such as vitamins, polyunsaturated fatty acids, pharmaceuticals, and sensitive dyes, thus providing better stabilization against autoxidation during storage. The better water solubility of γ-CD mitigates irritation and toxicity caused by drugs by lowering their dosage.[15] The high water solubility makes γ-CD the most difficult CD to synthesize and isolate on an industrial scale, thus limiting its broad application. To address this issue, organic templates are used during the enzymatic production of γ-CD, resulting in the precipitation of the host-guest complex, thus increasing its yield. However, the templates used in this process are not water soluble and have high boiling points, requiring the use of steam distillation for the separation of the host from the guest.

The bigger picture

Cyclodextrins (CDs) are extensively used as hosts for (bio) active molecules in the food, cosmetic, and pharmaceutical industries. Out of the three commonly used CDs, α-, β-, and γ-CD, the latter is the largest and most water soluble and thus can best encapsulate and solubilize bulky guests, such as vitamins and sensitive dyes. The encapsulation of drugs with γ-CD can provide stabilization against autoxidation during storage and allow for lower drug doses, thus mitigating irritation and toxicity. The high water solubility of γ-CD, however, complicates its isolation during industrial production, necessitating the use of an energy-consuming and cost-increasing distillation process. We show how a hydrazone photoswitch template can be used in the enzymatic production of CDs to specifically favor the formation of γ-CD. The template can then be removed from inside the γ-CD by photoirradiation, enabling product isolation processes that are more cost effective and environmentally friendly.

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The energy consumption of the distillation process greatly increases the cost of γ-CD production.[15] While recent efforts to improve selectivity for γ-CD using engineered enzymes and better templates have improved γ-CD synthesis yields,[17,18] further improvements in these strategies, and the development of alternative ones that address both production and isolation of γ-CD, are required to reach the level of production necessary to meet the market demand.

Light, which is being adopted by industry as a synthetic tool because of the benefits of photoredox catalysis,[19–21] can be used as an alternate strategy to dissociate the γ-CD host from its organic guest. Azobenzene,[22] which fits in the cavity of α- and β-CDs[23,24] in its *trans* form but not the *cis* form, is a candidate for such an application, considering that it has been extensively used in supramolecular chemistry for drug delivery,[25–27] self-assembly,[28–31] and sol-gel transitions,[32,33] among other applications.[34,35] The association of azobenzenes with the larger host γ-CD has garnered less attention, as the binding is usually not optimal. In one example, [33] a 2:1 complex was reported (K_a = 2,910 M^{-1}) without discussing the photoswitching process, whereas in another example, it was the *cis* isomer, which is obtained only in 50% at the photostationary state (PSS), that formed a complex (1:1; K_a = 1,660 M^{-1}) while the *trans* isomer was excluded.[36] We have recently described how the reversible enzymatic synthesis of CDs can be controlled[37,38] by adding photoreversible templates (guests).[39] However, the use of azobenzene as the photoswitchable guest only resulted in the optical control over the ratios of the α- and β-CDs, and no appreciable increase in the yield of γ-CD was observed. Addressing the synthetic and isolation challenges facing the production of γ-CD requires a paradigm shift in approach, which will require the development of new photochromic guests.[40]

Recently,[41–45] we reported a family of bistable and negatively photochromic hydrazones[46] that reversibly photoisomerize in organic solutions, the solid state, and aqueous media. Our interest in expanding the scope of photoswitches that can work in water, and act as guests to water-soluble macrocyclic hosts such as CDs, led us to the development of the first fully water-soluble hydrazone switch **1** (Figures 1 and S10). Here, we describe the photoswitching of **1** in pure water, and its complexation properties with β- and γ-CDs. While both isomers of **1** form inclusion complexes with β-CD (1:1), only **1**-*Z* binds to γ-CD (2:1), allowing for optical control over the complexation/decomplexation process. We took advantage of this process and used it in the light-controlled templated enzymatic synthesis of γ-CD, which has never been demonstrated before. Moreover, we used the fact that γ-CD does not bind to the *E* isomer of the switch to photo-remove the template from the host, and thus isolate the synthesized γ-CD. This new photoswitchable host/guest couple not only presents a proof-of-concept strategy for improving the yields of γ-CD production, but it also opens the way for designing new γ-CD-based adaptive self-assembled materials.

RESULTS AND DISCUSSION

Photoisomerization of the water-soluble hydrazone

The target hydrazone (**1**; Scheme S1) was synthesized in a straightforward manner by reacting ethyl benzoylformate with 4-hydrazinobenzenesulfonic acid under reflux in water (78% yield). Both the *E* and *Z* isomers were separated using reversed-phase chromatography and characterized using NMR spectroscopy (Figures S1–S9) and high-resolution mass spectrometry.

The switching of **1** (Figure 1) in water was first studied using UV-vis spectroscopy. The absorption spectrum of **1**-*Z* (equilibrated in the dark) shows a band with a maximum

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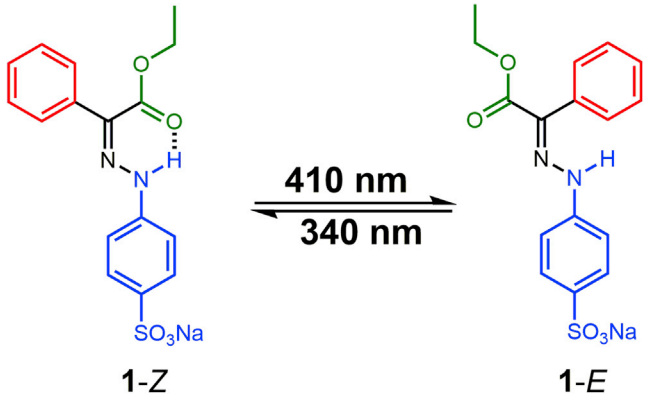

Figure 1. The photoswitching of hydrazone 1
The *Z*/*E* photoisomerization of hydrazone **1**

(λ_{max}) at 357 nm. Irradiating the solution with 410 nm light results in a shift of the λ_{max} to 338 nm (Figure S10A). The photoisomerization efficiency of **1** was studied using ^{1}H NMR spectroscopy (Figure S11). Upon 410 nm light irradiation, a sample of **1**-*Z* yields a PSS_{410} consisting of 92% of the *E* isomer. The quantum yield ($\Phi_{Z\rightarrow E}$) of the process was determined to be 2.6% ± 0.2% (Figure S12). Irradiation of the obtained sample with 340 nm light yields a PSS_{340} consisting of 65% of the *Z* isomer with $\Phi_{E\rightarrow Z}$ of 5.3% ± 0.3% (Figure S13). The switching process can be repeated ten times without any sign of degradation (Figure S10B). The study of the thermal half-life was not carried out because ester hydrolysis occurs at elevated temperatures in water. Nonetheless, the system is bistable as no signs of thermal isomerization of **1**-*E* are detected by ^{1}H NMR spectroscopy when stored in D_2O, in the dark, and at ambient temperature for at least 4 months (Figure S14).

Host-guest interaction between the hydrazone and cyclodextrins

Next, we studied the host-guest interaction of **1** with β-CD. NMR titrations were carried out by adding the CD into a D_2O solution of **1**-*Z*, resulting in slight upfield shifts for the CD proton signals, while mixed shifts were observed for the phenyl protons of **1**-*Z* (Tables S1 and S2). The presence of only one set of signals in the ^{1}H NMR spectra (Figure 2B) indicates that **1**-*Z* and β-CD are in a rapid dynamic inclusion/exclusion exchange equilibrium on the NMR timescale. The result of a Job's continuous variation analysis (Figure S18) indicated that the host and guest form a 1:1 complex, which was confirmed by ESI-MS measurements (Figure S19). Rotating frame nuclear Overhauser effect spectroscopy (ROESY) was used to elucidate the structure of the complex and rotating frame nuclear Overhauser effect (ROE) signals (Figure S35) were observed between the phenyl protons of **1**-*Z* and protons H_3' and H_5' (Figure 2A) inside the β-CD cavity. These interactions suggest that **1**-*Z* is encapsulated inside the β-CD through the stator phenyl group (Figure 1, shown in blue).

Photoisomerization with 410 nm light slightly shifts all the CD proton signals back to low field (Figure 2B), especially proton H_5'. Similar chemical shifts were obtained when mixing pure **1**-*E* with β-CD (Figure S17), indicating that the shifts observed upon photoisomerization do not originate from the small amount of leftover **1**-*Z* at PSS_{410}. These experiments indicate that both isomers of **1** bind to β-CD. To get a better appreciation of the binding affinities of the two isomers with the CD, isothermal titration calorimetry (ITC) was used (Table 1).[47] The binding constant between **1**-*Z* and β-CD was calculated to be 725 ± 1 M^{-1} (Figure S21A) based on three

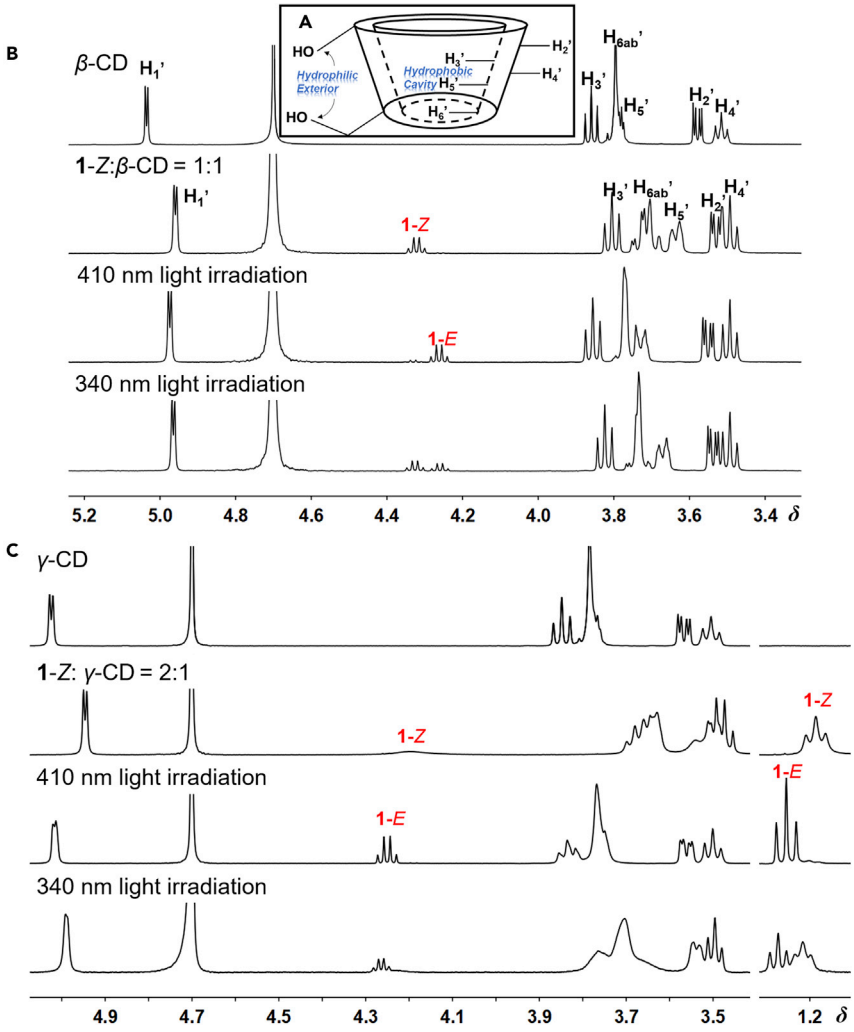

Figure 2. Host-guest interaction of 1 with β- and γ-CDs

(A) A Schematic representation of the cyclodextrins.

(B and C) Inclusion and switching of **1** in (B) β-CD and (C) γ-CD as monitored by ^{1}H NMR spectroscopy (D_2O at 298 K). The spectra mainly focus on the CD signals as these show the clearest shifts.

consecutive measurements, while the binding constant between **1**-*E* and β-CD was calculated to be 250 ± 25 M^{-1} under the same conditions (Figure S21B). Similar values were obtained from ^{1}H NMR titrations,[48–50] 835 ± 15 and 205 ± 5 M^{-1} for **1**-*Z* and **1**-*E*, respectively (Figures S23 and S25).

We also studied the interaction of **1** with γ-CD. The proton signals of γ-CD shifted upfield and were broadened upon mixing **1**-*Z* with γ-CD, indicating an interaction between the two (Figure 2C). The results of a Job's plot analysis showed the formation of a 2:1 complex between **1**-*Z* and γ-CD (Figure S26), and this stoichiometry was further confirmed by ESI-MS (Figure S27). ITC measurements (Table 1) yielded a binding constant of 320 ± 40 M^{-1} (K_1) and 8,970 ± 480 M^{-1} (K_2) between **1**-*Z* and γ-CD using the sequential binding model (Figure S31A). Similar values were obtained from ^{1}H NMR titrations (825 ± 285 and 8,205 ± 2,080 M^{-1}; Figure S29), while ROESY measurements (Figure S40) indicated that a head-to-tail orientation of the hydrazone inside the cavity of the γ-CD could be the most likely structure of the complex.

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Table 1. Summary of the inclusion behavior of 1 in CDs (25 °C in water)

Host/guest	Stoichiometry	ITC binding constants/M^{-1}
β-CD/**1**-*Z*	1: 1	725 ± 1
β-CD/**1**-*E*	1: 1	250 ± 25
γ-CD/**1**-*Z*	1: 1	320 ± 40 (K_1)
	1: 2	8,970 ± 480 (K_2)
γ-CD/**1**-*E*	binding affinity is too low to be measured	

To obtain further information about the nature of the complex between **1**-*Z* with γ-CD, structure refinement was carried out using molecular dynamics (MD) simulations. The initial structure was generated by a metric matrix distance geometry program,[51] which utilizes the distances generated from the geometry of the molecules (holonomeric matrix) and the ROEs. Because of the high symmetry of the γ-CD, the ROE distances were treated as an ensemble (i.e., penalty was applied only if all possible combinations of the specific protons were in violation). The resulting structure was solvated with TIP4 water, energy minimized, first with the complex held rigidly, and then 100 ps of MD run at 300 K using NAMD (nanoscale molecular dynamics).[52] The structure obtained from the simulations agrees with the one predicted by the ROESY measurements (Figure S40), i.e., two hydrazones are encapsulated in a head-to-tail manner inside the γ-CD (Figure 3).

Photoisomerization of **1**-*Z* upon 410 nm light irradiation shifts the proton signals of γ-CD back to where they were originally without the addition of the hydrazone, indicating minimal interaction between γ-CD and the *E* isomer (Figure 2C). A similar result is obtained when pure **1**-*E* is added to γ-CD (Figure S30). Moreover, no significant heat is released during ITC measurements when **1**-*E* is added to γ-CD (Figure S31B). These results indicate that photoisomerization results in the exclusion of the hydrazone from the cavity. This process is reversible as irradiation with 340 nm light results in the formation of the *Z* isomer and upfield shift of the ^{1}H NMR γ-CD signals.

Hydrazone-templated enzymatic synthesis of cyclodextrins

Considering the strong and selective molecular recognition of **1**-*Z* by γ-CD, we sought to use **1** as a photoresponsive template for the cyclodextrin glucanotransferase (CGTase)-mediated enzymatic synthesis of γ-CD.[53] CGTase can render the glycosidic linkages in α-1,4-glucans labile by catalyzing reversible transglycosylation and slow hydrolysis to generate dynamic mixtures of interconverting CDs and linear α-1,4-glucans. We have previously shown that CDs are formed as dynamic, but kinetically trapped, products in this system, while glucose is the ultimate thermodynamic product of CGTase activity on α-1,4-glucans.[37] Templates that bind to specific CDs can further stabilize these products, leading to both higher CD yields and selective synthesis of CDs.[37–39] We began by treating a solution of maltohexaose (**G6**) (6 mg/mL) in sodium phosphate buffer (50 mM, pH 7.5) with CGTase (0.6 mg/mL) in the presence of either **1**-*E* (10 mM) or **1**-*Z* (10 mM) in the dark. The reaction was monitored using HPLC coupled with an evaporative light scattering detector (ELSD).[54] In the presence of **1**-*E*, a modest increase in the β-CD composition was observed in comparison with the control experiment without template (Figure 4A). This is consistent with the relatively low association constant observed for the binding of **1**-*E* to β-CD (Table 1). With **1**-*Z*, on the other hand, a remarkable increase in the production of γ-CD was observed. Initially, β-CD was formed, but within 4 h, a stable equilibrium CD distribution was obtained with 60% γ-CD, 30% β-CD, and 10% α-CD. By comparison, γ-CD makes up only 8% of the CD distribution when a stable equilibrium is reached in the control experiment without template (Figure 4B). The observed distribution is consistent with the measured strong 2:1 interaction of **1**-*Z* with γ-CD (Table 1).

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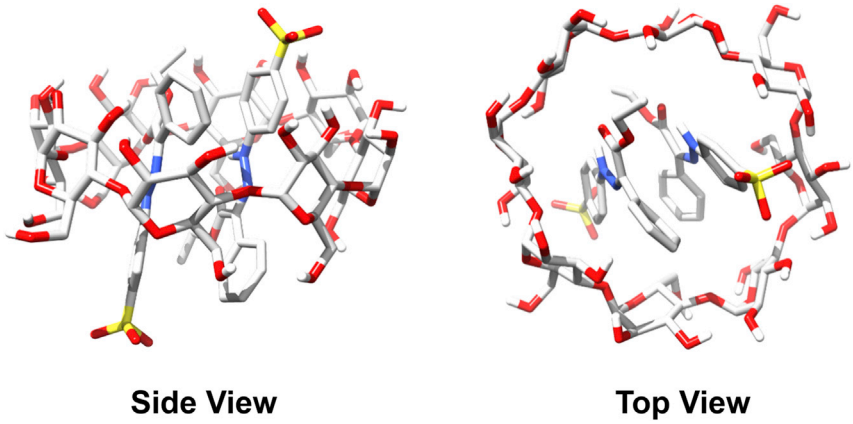

Figure 3. Proposed structure of (1-*Z*)$_2$@γ-CD based on MD simulations
The side and top views of the simulated 2:1 complex between **1**-*Z* and γ-CD are shown.

Irradiation of the reaction mixture at 415 nm switches **1**-*Z* to **1**-*E*, and the composition of the dynamic glucan mixture changed dramatically and quickly to reach a new CD distribution in ca. 30 min.[55] Now γ-CD is present in only very small quantities because of the poor affinity of γ-CD for **1**-*E*, while β-CD is the main component (Figure 4C), in agreement with the enzymatic reaction using **1**-*E* as the template (Figure 4B). Switching the irradiation wavelength to 340 nm results in a slow buildup of γ-CD again, consistent with the change of some **1**-*E* isomer back to the **1**-*Z* form. Applying irradiation with these two wavelengths (415 and 340 nm) in consecutive cycles shows that it is possible to reversibly change the composition in the dynamic CD system back and forth multiple times by controlling the relative *E*/*Z* ratio of photoswitch **1** (Figure 4C). The difference in CD distribution at the two PSSs becomes less pronounced in subsequent cycles and an overall decrease in the γ-CD composition is observed. This is a consequence of the significant buildup of short linear α-1,4-glucans during this long experiment (Figures S42 and S43). When the total concentration of CDs decreases, an increase in the concentration of smaller CDs at the expense of γ-CD is to be expected in this dynamic system.[38]

As a strategy for improving γ-CD production, our photocontrolled templated enzymatic synthesis should both increase the yield of γ-CD and facilitate the isolation of γ-CD by photorelease of the template. We thus set out to prepare γ-CD from **G6** and tracked the whole process using a combination of *in situ* NMR spectroscopy and HPLC. Optimized high-resolution ^{1}H-^{13}C HSQC NMR spectroscopy experiments were devised to resolve all the CD species in their respective binding regimes with **1**-*E* and **1**-*Z*, which enabled us to assign the H_1' signals for each of the CDs, both before and after irradiation. In the synthetic process, **G6** (6 mg/mL) was treated with CGTase in the presence of **1**-*Z* (10 mM) in the dark. β-CD accumulated first, and then slowly converted into γ-CD over approximately 3 h (Figures 5 and S44–S48). The γ-CD formed was bound to **1**-*Z*, as evidenced by an upfield shift of the anomeric (H_1') proton signal of γ-CD in the ^{1}H NMR spectrum (Figure 5), and from an ROE between γ-CD and **1**-*Z* (Figure S47). The ^{1}H NMR signal for H_1' in β-CD was also shifted slightly upfield as it forms a weak 1:1 complex with **1**-*Z*, which explains its initial accumulation in the reaction. After 3 h, the reaction was stopped by denaturing the enzyme (heating to 95 °C for 12 min). Upon irradiation of this now static mixture (415 nm), downfield shifts in the H_1' signals for both β-CD and γ-CD were observed, indicating that they are no longer bound to **1**-*Z* (Figure 5). The largest change in the chemical shift was observed for the anomeric signal of γ-CD, while the anomeric

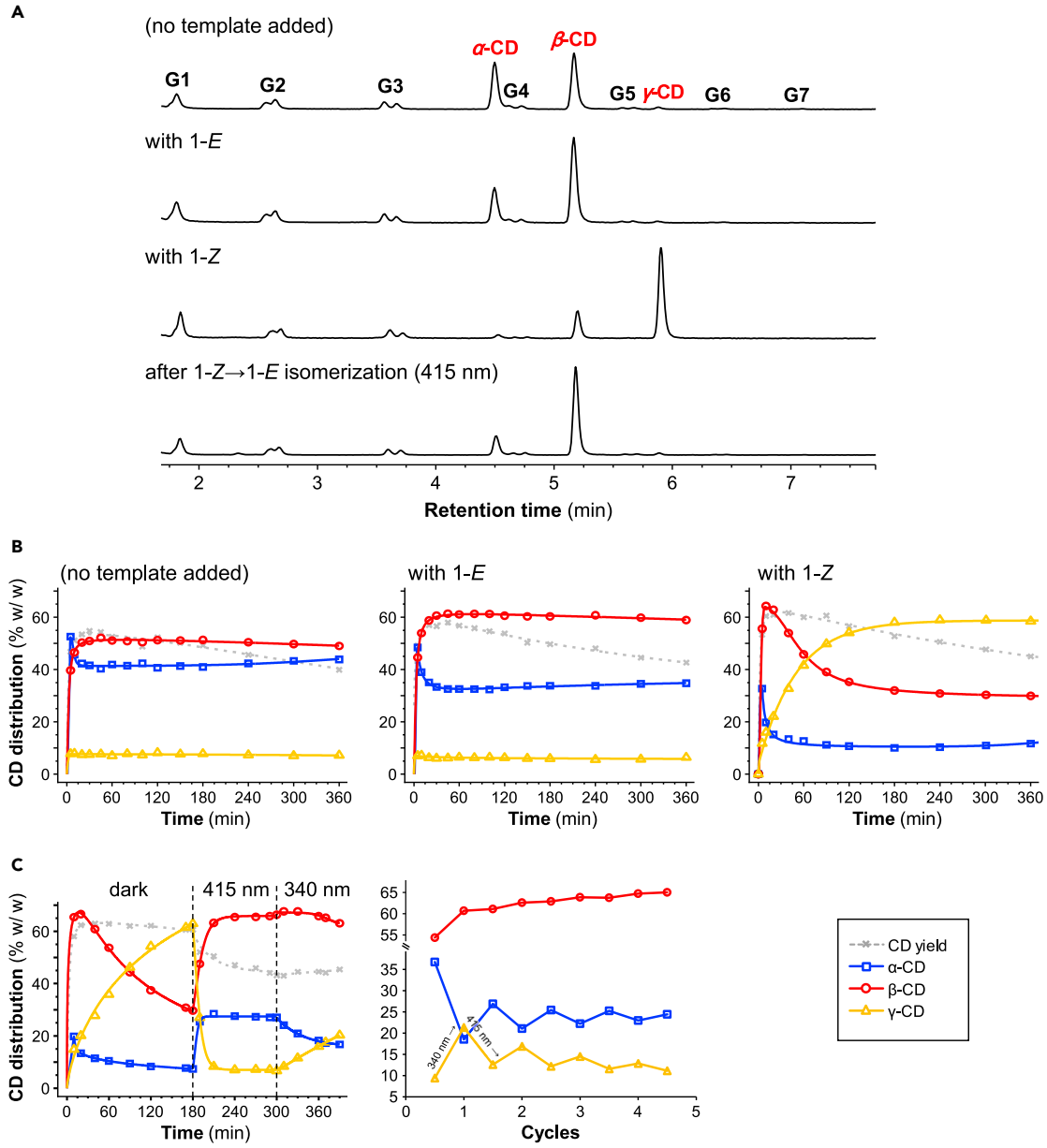

Figure 4. Cyclodextrin glucanotransferase (CGTase)-mediated dynamic synthesis of cyclodextrins templated with 1 and manipulated with light irradiation at different wavelengths

(A) Chromatograms (HPLC-ELSD) showing equilibrium product distributions generated when CGTase acts on maltohexaose (**G6**) (6 mg/mL) in sodium phosphate buffer (50 mM, pH 7.5) under various conditions: without template; with **1**-*E* in the dark; with **1**-*Z* in the dark; and with **1**-*Z* after irradiation at 415 nm.

(B) Change in composition of the templated and untemplated reactions over time. The CD composition is shown in % by weight of all CDs present, while the total amount of CDs (gray line) is shown in % by weight out of all the saccharides in the mixture. For the concentrations of each different saccharide see Figure S43.

(C) Change in the composition of **1**-templated reactions when irradiated sequentially and in cycles at 415 and 340 nm.

signal of β-CD changed less, presumably because of its affinity to **1**-*E* (Table 1). The now unbound γ-CD was isolated from this mixture on a milligram scale using preparative HPLC (Figure S49), showcasing how this photoremovable template approach can be used in both synthesizing and isolating γ-CD.

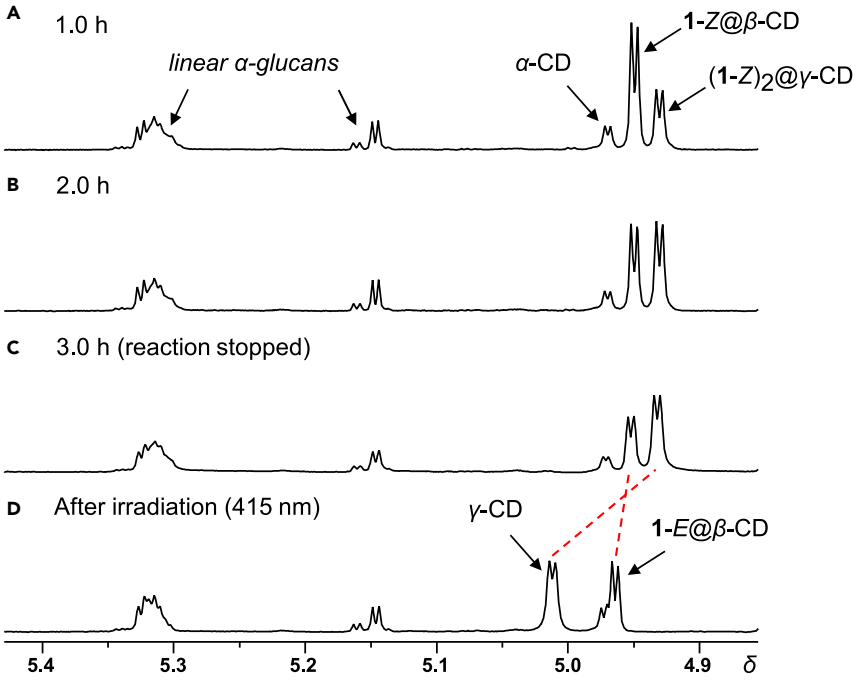

Figure 5. CGTase-catalyzed production of γ-CD monitored by ^{1}H NMR spectroscopy

(A–D) Anomeric region of the ^{1}H NMR spectra showing (A–C) the signals from the anomeric protons of the CDs and linear α-1,4-glucans during the **1**-*Z*-templated enzymatic synthesis of γ-CD, and (D) the photocontrolled release of the template after reaction quenching. See also Figures S1–S48.

In conclusion, the host-guest interaction between a hydrazone switch and β- and γ-CDs was studied in pure water. We found that both **1**-*Z* and **1**-*E* form 1:1 inclusion complex with β-CD, but with different binding affinities. More interestingly, **1**-*Z* forms a 2:1 inclusion complex with γ-CD while no inclusion with the *E* isomer is observed. We exploited this property for optical control over the templated enzymatic synthesis of γ-CD and showed how the template can increase the yield of γ-CD by 6-fold. Coupled with the fact that **1**-*E* has a low affinity for γ-CD, thus allowing for the isolation of the synthesized product by photoremoval of the template, this proof-of-concept example constitutes a new paradigm for increasing the efficiency of industrial production of γ-CD using photoreversible templated enzymatic synthesis. We speculate that this new photoswitchable host-guest couple between γ-CD and hydrazone **1** will also open the way for additional opportunities in host-guest chemistry and the formulation of new water-soluble adaptive materials.[12–14,34,35]

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Ivan Aprahamian (ivan.aprahamian@dartmouth.edu).

Materials availability

All materials generated in this study are available from the lead contact without restriction.

Data and code availability

The Ca(**1**-*Z*)$_2$ structure reported in this article has been deposited in the Cambridge Crystallographic Data Center under accession number CCDC: 1941587

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Full experimental procedures are provided in the supplemental information.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chempr.2021.05.013.

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AUTHOR CONTRIBUTIONS

S.Y. performed the synthesis and characterization of the photoswitch and host-guest complexes. M.P. and D.F.M. performed the MD simulations and related ROE measurements. D.L. performed dynamic library experiments. S.M. developed the NMR methods used for monitoring dynamic libraries. D.F.M., S.R.B., and I.A. designed the experiments and supervised the research. All authors contributed to the preparation and editing of the manuscript.

DECLARATION OF INTERESTS

I.A. is a member of the advisory board of *Chem*.

A provisional patent application related to this publication has been submitted.

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55. We are describing the full *E*/*Z* isomerization process here to demonstrate the efficacy of the templation and switching processes. In the presence of the enzyme, and as shown here, isomerization to the E form will result in a re-equilibration of the CD mixture in favor of the β-CD. To prevent this process from occurring in an industrial application, and to proceed with the isolation of the synthesized γ-CD (Figure S49), the enzyme needs to be deactivated before the *Z*→*E* photoisomerization process is initiated. Following this strategy, the re-equilibration process will be circumvented, allowing for the isolation of the γ-CD product after photoremoval of the hydrazone template (Figures 5 and S44).