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Tuning Isonitrile/Tetrazine Chemistry for Accelerated Deprotection and Formation of Stable Conjugates

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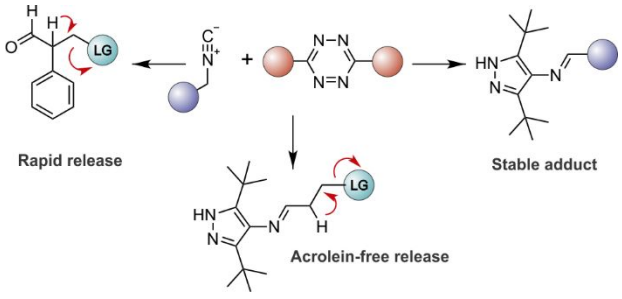


Abstract

The isocyano group is a valuable functionality for bioorthogonal reactions because it rapidly reacts with tetrazines to either form stable conjugates or release payloads from 3-isocyanopropyl groups. Here we provide mechanistic insights into the dissociative steps that follow the initial cycloaddition and analyze how structural modifications affect these processes. Three main outcomes of this study have important implications for designing such groups for bioorthogonal applications. First, anion-stabilizing substituents at C-2 of the 3-isocyanopropyl group promote β-elimination and accelerate deprotection. Second, tetrazines with bulky substituents form stable imine conjugates even with primary isonitriles that are otherwise rapidly hydrolyzed. Third, the elimination step is independent from hydrolysis to the aldehyde and instead can occur directly from the imine intermediate. These findings will allow tuning the structures of tetrazine and isonitrile reactants for application in bioorthogonal ligation and release chemistry.

Introduction

Several release reactions that work under physiological conditions have been developed[1-14] establishing a dissociative bioorthogonal chemistry toolbox.[15, 16] We are especially intrigued by the isocyano group that, despite its exceptional structural compactness and unique reactivity,[17, 18] remains remarkably underused as a bioorthogonal functionality. Isocyanides react rapidly with tetrazines (rates of up to 4.0 m^{-1} s^{-1} in PBS/DMSO (90:10) at 37 °C) in a (4+1) cycloaddition to form 4-aminopyrazoles (**Fig. 1**).[19, 20] This reaction is applicable to both labeling and release uses. Leeper *et al.* showed that tuning the structure of the isocyano reactant allows forming stable conjugates with tetrazines.[21-23] Our group recently developed biocompatible protecting groups based on this reaction. 3-isocyanopropyl (ICPr) and 3-isocyanopropyl-1-carbamoyl (ICPrc) groups react with tetrazines to spontaneously liberate phenols and amines under physiological conditions.[3] Our unexpected discovery that steric attractions in the transition state of the (4+1) cycloaddition accelerate the transformation further expanded the usefulness of this bioorthogonal reaction.[24] However, mechanistic questions remain and optimization of reactants will be needed to access the full potential of the chemistry.

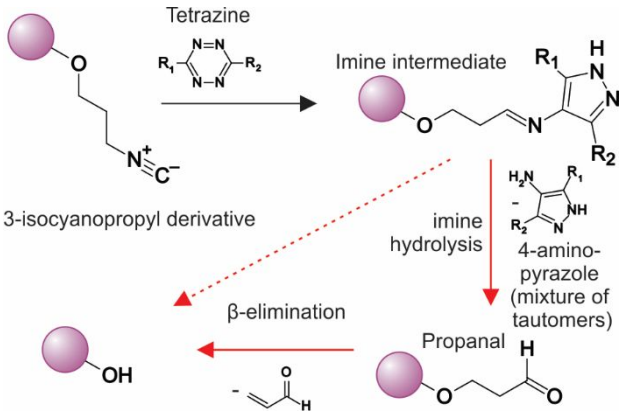


Figure 1: Mechanism of tetrazine-mediated removal of 3-isocyanopropyl groups. Red lines: steps analyzed in this study; dotted line: hypothetical transformation.

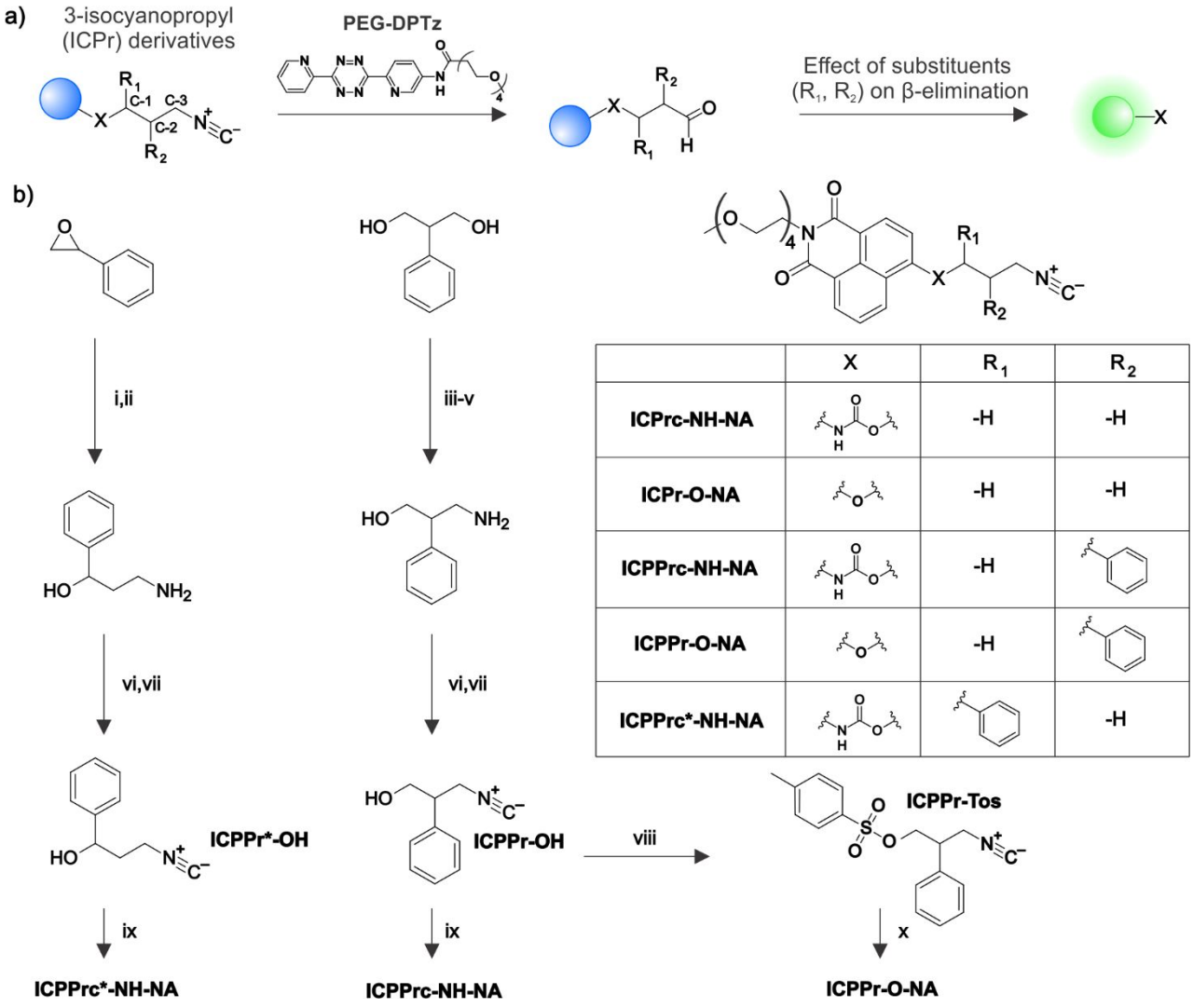
Here we analyze the dissociative steps of the transformations following the cycloaddition (**Fig. 1**). The tetrazine-triggered release of leaving groups from ICPr structures occurs in a cascade of steps. Release of N_2 and the formation of a five-membered ring with an exocyclic C=N bond (not shown in figure) follows the initial (4+1)-cycloaddition.[19] For most isonitriles the intermediate tautomerizes to an imine of 4-aminopyrazole (imine intermediate in **Fig. 1**). We previously hypothesized that hydrolysis of this imine intermediate into an aldehyde and 4-aminopyrazole elicits the release of leaving groups by a spontaneous β-elimination reaction.[25, 26] However, the later steps in this postulated mechanism await rigorous examination. Furthermore, structure-reactivity analysis might allow improving critical aspects of the reaction. First, serum albumin is required for rapid payload release from ICPr groups.[3] Chemical changes favoring elimination would be helpful to ensure rapid and condition-independent release. Second, identifying tetrazines

that form stable adducts with simple primary isonitriles would allow exploiting the minimal size of the isocyano group in bioconjugation chemistry. Previously, the formation of stable adducts required tertiary isonitriles or the presence of nearby carbonyls.[23] Third, achieving release independent of imine hydrolysis would avoid the formation of reactive acrolein side-products.

The presented studies revealed that simple modifications of the 3-isocyanopropyl structure accelerated release without decreasing the reactivity to tetrazines. Additionally, we discovered that varying tetrazine substituents made it possible to tune the hydrolysis rate of the imine intermediate and stabilize the conjugate. Finally, a mechanistic analysis unraveled that the release step could occur independent of imine hydrolysis. The combined findings will guide the development of isonitrile and tetrazine reagents for diverse uses in chemistry and in the life sciences.

Results and Discussion

Accelerating β-elimination from tetrazine-activated 3-isocyanopropyl groups



	X	R_1	R_2
ICPrc-NH-NA		-H	-H
ICPr-O-NA		-H	-H
ICPPrc-NH-NA		-H	
ICPPr-O-NA		-H	
ICPPrc*-NH-NA			-H

Figure 2. Structures of probes to evaluate the effect of chemical modifications on the β-elimination step from tetrazine-activated 3-isocyanopropyl groups. a) General scheme of tetrazine-mediated deprotection reaction. b)

Structure and synthesis of fluorogenic naphthalimide used to study the release of amines and phenols. Reaction conditions: i: NaCN, MeOH, 62%; ii: BMS (borane-dimethyl sulfide), dry THF, 20%;[27] iii: TBDMSCl, NaH, dry THF, 59%; iv: Phthalimide, PPh_3, DEAD, dry THF, 50%; v: Hydrazine, EtOH, 30%;[28] vi: ethyl formate, RT; vii: Burgess reagent (yields for steps vi and vii: 55% for ICPPr-OH and 35% for ICPPr*-OH); viii: TsCl, TEA, dry THF, 59%; ix: N-(methyl-PEG4)-4-amino-1,8-naphthalimide,triphosgene, DIEA, dry toluene; x: N-(methyl-PEG4)-4-hydroxy-1,8-naphthalimide, K_2CO_3, acetone, 24%.

3-isocyanopropyl groups react with tetrazines to release amines (from carbamates) and phenols under physiological conditions.[3] Serum albumin catalyzes the β-elimination, and the release in serum occurs in minutes.[26] The rate-accelerating effect of albumin on β-elimination from oxopropyl groups, presumably caused by a general-base effect from the lysine residues in subdomain IIA of serum albumin, is well established. [25, 26, 29] However, the liberation step requires hours to go to completion in PBS buffer.[3] For certain applications, rapid release in the absence of albumin will be critical.

We tested whether modifications at the C-1 and C-2 positions of the 3-isocyanopropyl scaffold can facilitate the release of amines and phenols (**Fig. 2**). Of particular interest were substituents predicted to promote β-elimination by decreasing the proton affinity at C-2.[30] We designed 3-isocyano-2-phenylprop-1-yl (ICPPr) moieties as rapid-release protecting groups responsive to tetrazines (**Fig. 2b**). The design was inspired by the well-established pK_a decrease of carbonyl α-protons upon addition of phenyl substituents at the site of deprotonation (for example: pK_a(phenylacetone) = 19.8; pK_a(acetone) = 26.5).[31] The tetrazine-induced elimination from 3-isocyano-1-phenylprop-1-yl (ICPPr*) was assessed for comparison. The β-elimination from 3-ketoalkyl groups has been previously studied[25] and was not further investigated. As reporter probes, we prepared ICPr-modified naphthalimide dyes (**Fig. 2b**) because they undergo a characteristic bathochromic shift upon deprotection of amine/hydroxy groups on C-4.[32]

Reagents to access ICPPr-protected molecules were synthesized from 3-amino-2-phenylpropan-1-ol (**Fig. 2b**), which was prepared in a three-step literature procedure from 2-phenylpropane-1,3-diol.[28] Formylation of 3-amino-2-phenylpropan-1-ol (ethyl formate, room temperature) and dehydration with Burgess reagent,[3] afforded 3-isocyano-2-phenylpropan-1-ol (ICPPr-OH; 55% yield, two steps). Reacting ICPPr-OH with in situ generated 4-isocyanato-naphthalimide[3] provided reporter probe ICPPrc-NH-NA (**Fig. 2b**; ICPPrc: 3-isocyano-2-phenylpropylcarbamoyl) for testing the tetrazine-mediated deprotection of amines. The naphthalimide dye contained a PEG4-modification on the imide to enhance water solubility. The corresponding 3-isocyano-1-phenylpropylcarbamoyl (ICPPrc*) derivative of 4-amino naphthalimide (**Fig. 2b**) was synthesized analogously by reacting the dye-isocyanate with 3-isocyano-1-phenylpropan-1-ol, which was generated by formylation and dehydration (35% yield, two steps) of 3-amino-1-phenylpropan-1-ol[27] (**Fig. 2b**). To synthesize ICPPr-modified phenols, ICPPr-OH was converted into the tosyl compound ICPPr-Tos (TosCl, triethylamine, 59 % yield). Alkylation of 4-hydroxy naphthalimide with ICPPr-Tos (K_2CO_3, acetone) yielded ICPPr-O-NA in 24 % yield. All conjugates were stable for >72 h in diluted human serum (PBS:serum, 1:1 (v/v), T = 37 °C; **Fig. S1** in the SI).

The elimination of amines and phenols from ICPPrc-NH-NA and ICPPr-O-NA following the reaction with PEG4-3,6-di-2-pyridyl-1,2,4,5-tetrazine (PEG-DPTz)[3] was monitored spectrophotometrically by measuring the emergence of the characteristic absorbance band of 4-

amino/4-hydroxy naphthalimide (λ_{abs}(ICPPrc-NH-NA) = 435 nm, c = 0.1 mM; λ_{abs}(ICPPrc-O-NA) = 445 nm, c = 0.2 mM) and PEG-DPTz (c = 1 mM or 2 mM) in PBS:DMSO (4:1, v/v; **Fig. 3**). Elimination rates were calculated using a sequential first-order rate law (Equation 1 in the Supporting Information) using extrapolated bimolecular rate constants (**Fig. 3d**). As designed, the phenyl-group at the C-2 position significantly accelerated the release step (**Fig. 3b**). The rate constant of dye release from ICPPrc-NH-NA was 13.4-fold higher (k_{elim} = (9.56 ± 0.56) × 10^{-4} s^{-1}, $t_{1/2}$ = 0.20 h) than that for the ICPr-derivative ICPrc-NH-NA (k_{elim} = (7.13 ± 0.18) × 10^{-5} s^{-1}, $t_{1/2}$ = 2.70 h). A significant acceleration (9.1-fold) of the elimination step by the C-2 phenyl group was also observed for the phenolic dye (k_{elim}(ICPPr-O-NA) = (1.47 ± 0.01) × 10^{-3} s^{-1}, $t_{1/2}$ = 0.13 h; k_{elim}(ICPr-O-NA) = (1.61 ± 0.08) × 10^{-4} s^{-1}, $t_{1/2}$ = 1.20 h). Release yields were near-quantitative for both derivatives (98 ± 3 % for ICPPrc-NH-NA and 96 ± 3 % for ICPPr-O-NA; ICPPrc-NH-NA and ICPPr-O-NA (c = 1 mM) and DPTz (c = 3 mM), in PBS: DMSO (1:3, v/v), T = 37 °C, t = 3 h; **Fig. 3c**). Under these conditions, the release step occurs on the same timescale as the cycloaddition step, and it therefore is conceivable that the cycloaddition is the rate-limiting step and that the actual release step is faster. Control experiments with increased tetrazine concentrations to accelerate the bimolecular step provided comparable values for k_{elim} (not shown) establishing that the measured rates correctly reflect the kinetics of the elimination step.

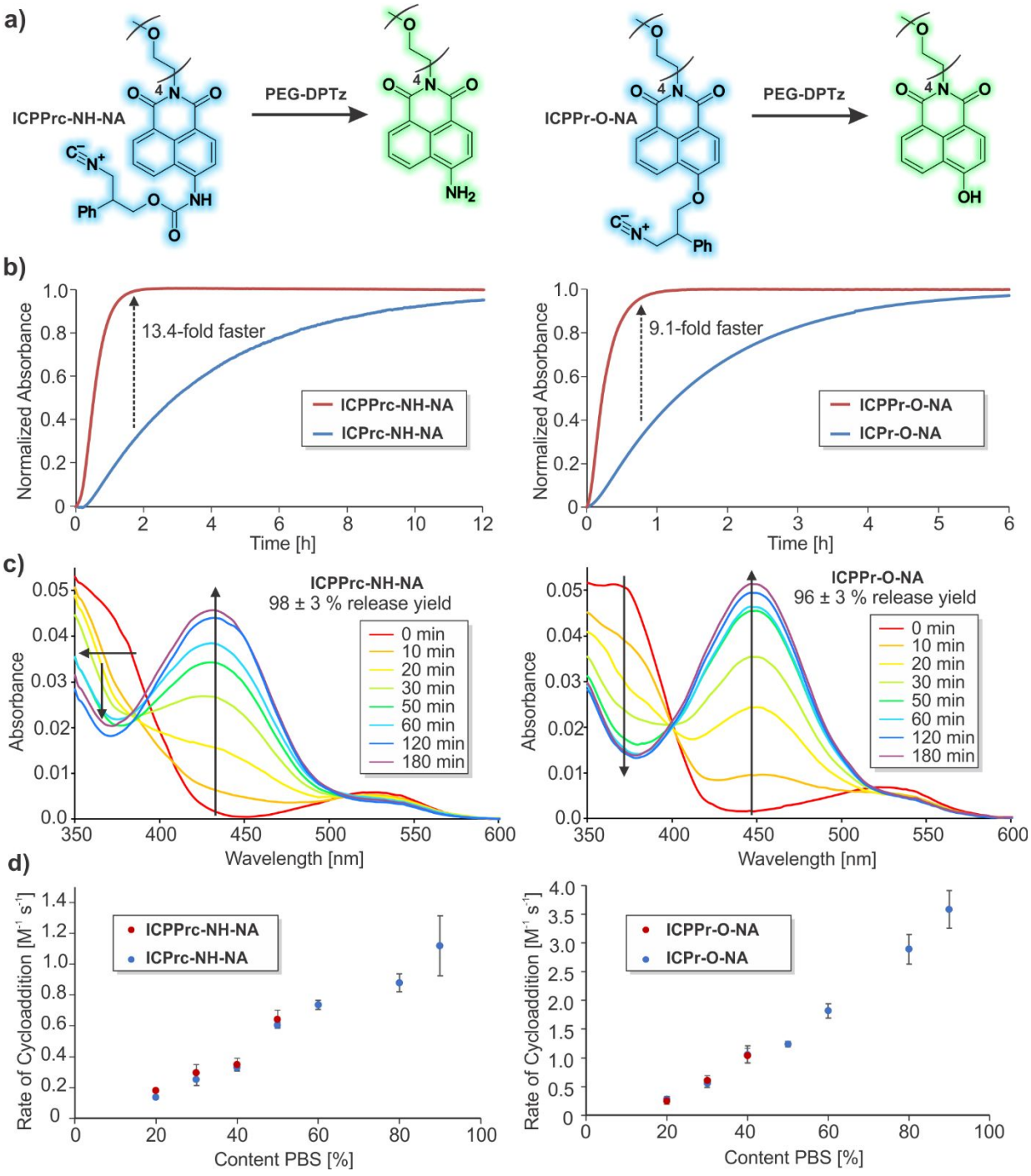


Figure 3. Effect of phenyl groups at C-2 of 3-isocyanopropyl groups on the tetrazine-induced release of naphthalimide dyes. a) Reaction schemes. b) Kinetics of the emergence of free naphthalimide dyes determined photospectrometrically. The phenyl groups accelerated the release of 4-amino/4-hydroxy napthalimides from ICPPr-derivatives relative to ICPr-derivatives. c) Quantification of amine and phenol release yields from ICPPr-modified reporter probes reacting with PEG-DPTz. d) Dependence of the cycloaddition rate of 3-isocyanopropyl derivatives with PEG-DPTz on the water content. Phenyl groups at the C-2 position had no statistically significant effect on the bimolecular reaction step. (Experimental conditions: PBS [pH 7.4]:DMSO 4:1 (unless stated otherwise, v/v, T = 37°C).

The accelerated elimination was achieved without affecting the rate of the bimolecular reaction of the isocyano group with tetrazines. The measured bimolecular rate constants were quasi-identical for ICPPr- and ICPr-derivatives with carbamate and phenol leaving groups at several solvent compositions (**Fig. 3d**; **Table S1** in the Supporting Information). This result established that it is possible to modulate the rates of the cycloaddition and the elimination steps independently. Interestingly, the reaction rates exhibited a quasi-linear dependence on the water content in the tested range (from 20% to 90% content PBS, v/v; T = 37 ˚C, **Fig. 3d**).

The effect of introducing a phenyl group at C-1 on the elimination and the bimolecular steps was modest (**Fig. 4**). The rate of elimination measured for ICPPrc*-NH-NA with PEG-DPTz of k_{elim} = (4.93 ± 0.03) × 10^{-5} s^{-1} ($t_{1/2}$ = 3.91 h), was 31 % slower than for the unmodified carbamate dye ICPrc-NH-NA (**Fig. 4b**). This result aligned with our expectations because the effect of the C-1 substituent on the acidity of the carbonyl α-proton is expected to be modest. The release yield of the naphthalimide from ICPPrc*-NH-NA is near-quantitative (97 ± 5 %; **Fig. 4c**) and the bimolecular rate (at 30% PBS in DMSO) was near-identical to that of ICPrc-NH-NA (**Table S1** in the Supporting Information). Previous experiments revealed that serum albumin catalyzes the elimination step from ICPr-groups.[3] The C-1 phenyl group did not obstruct the release-accelerating effect of serum on carbamate elimination and in diluted serum the dye release occurred in minutes (**Fig. 4b**; ICPPrc*-NH-NA, ICPrc-NH-NA (c = 0.1 mM) and PEG-DPTz (c = 1 mM) in PBS:DMSO (4:1, v/v) and Serum : PBS (1:1, v/v)). The outcome of these experiments has significant implications for designing probes and drug-release reagents. It establishes the C-1 position as a possible branching point for introducing handles for bioconjugation without interfering with the cycloaddition and release steps.

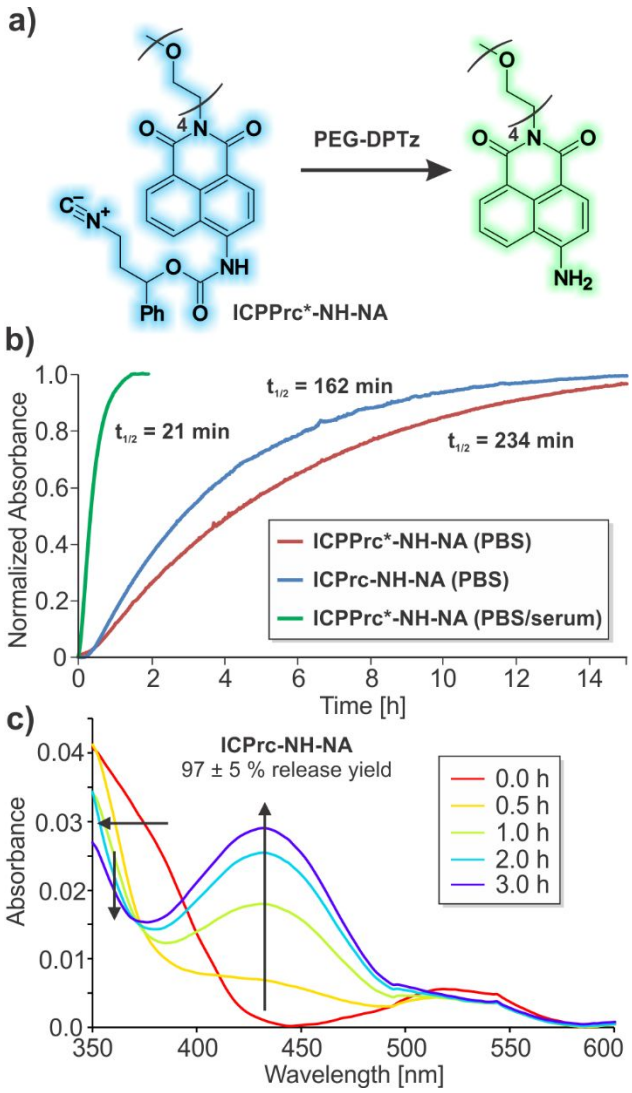


Figure 4. Effect of phenyl groups at C-1 of 3-isocyanopropyl groups on the tetrazine-induced release of carbamate groups. a) Reaction schemes. b) Kinetics of the emergence of free naphthalimide dye determined photospectrometrically. The phenyl group only modestly slowed the elimination step without interfering with the catalytic effect of human serum albumin. c) Quantification of amine release from ICPPrc*-NH-NA reacting with DPTz.

To further expand the scope of leaving groups, we tested the release of an aromatic thiol. An ICPPr-derivative of 2-naphthalenethiol was synthesized by nucleophilic substitution on ICPPr-Tos to afford ICPPr-S-NA. The release percentage was determined indirectly with Ellman's reagent and measured photospectrometrically. DPTz induced the release of the free thiol (65 ± 1 %; **Fig. S2** in the Supporting Information). Although the observed release yields were high, they were not near-quantitative as observed for phenols and amines (**Fig. 3**). Possibly, part of the released 2-nitro-5-thiobenzoic acid is alkylated by a downstream product (2-phenylacrolein or derivatives) of the ICPPr-release reaction, leading us to underestimate the actual release yield. Nonetheless, the

presented data clearly indicates that ICPPr-moieties can release diverse functional groups upon reaction with tetrazines.

In summary, the data that phenyl (as a carbanion-stabilizing substituent at the C2 position) accelerates payload release from ICPr-derivatives without negatively impacting the cycloaddition rates and release yields. This observation established the groundwork for developing tetrazine-responsive protecting groups with accelerated elimination rate.

Hydrolytic Stability of the Imine Intermediate

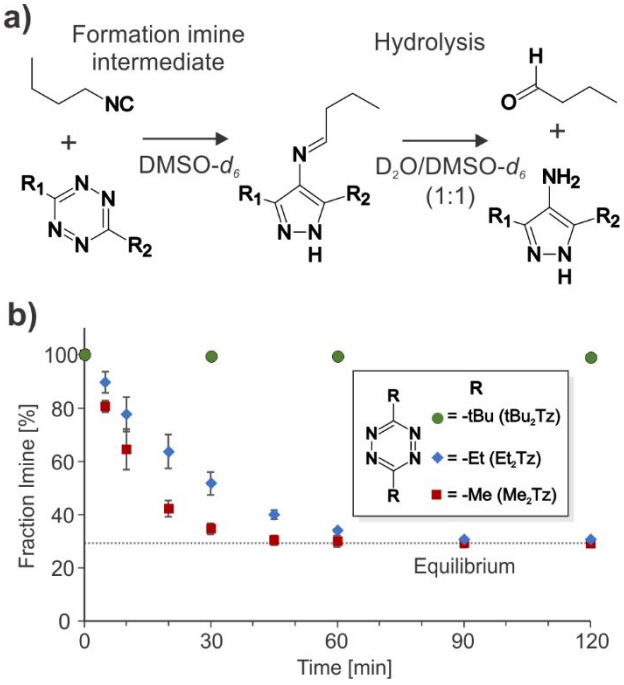


Figure 5. Hydrolysis of imine adduct formed by the reaction of isocyanides and tetrazines. a) Reaction sequence used to evaluate the stability of the adduct in aqueous phase. b) Time dependence of conversion of imine intermediate into butyraldehyde and 4-aminopyrazole; ^{1}H NMR spectra are shown in **Figs. S6, S7, S13** in the Supporting Information. (Conditions: DMSO-d_6:D_2O (1:1 v/v), T = 37 °C)

The tetrazine-mediated conversion of isonitriles to carbonyls involves the hydrolysis of an imine intermediate (**Fig. 1**).[19, 23] For bioorthogonal chemistry applications, it is important to control the rate of imine hydrolysis. For labeling applications, the adduct needs to be stable. Leeper *et al.* used tertiary isonitriles and methyl 3-isocyanopropionate to overcome the lability of the isonitrile-tetrazine adduct.[23] Slow imine hydrolysis on the other hand could impede ICPr-deprotection reactions. We therefore investigated the dependence of imine hydrolysis on the tetrazine substituents.

For these studies, we generated adducts of n-butyl isocyanide (n-BuNC) with tetrazines in DMSO-d_6 and monitored hydrolysis by ^{1}H NMR following dilution with D_2O (**Fig. 5a**). First, we assessed the effect of substituents with different electronic properties on the persistence of the imine intermediate. In agreement with results by Leeper *et al.*[23] even the traces of water present in the DMSO-d_6 solvent used for the NMR studies were sufficient to partially hydrolyze the imine formed with DPTz to the aldehyde and 4-aminopyrazole (**Fig. S3** in the Supporting Information), and a similar result was obtained for 3,6-di-2-pyrimidyl-1,2,4,5-tetrazine (**Fig. S4** in the Supporting Information). Therefore, adducts of simple alkyl isocyanides and these tetrazines hydrolyze rapidly. The corresponding imine adduct of n-BuNC and 3,6-diphenyl-1,2,4,5-tetrazine persisted in DMSO-d_6 and hydrolyzed upon addition of D_2O over a period of 2 h (**Fig. S5** in the Supporting Information). These results show that electron-withdrawing tetrazine substituents favor rapid hydrolysis of the imine adduct.

To assess steric effects of tetrazine substituents, we monitored imine hydrolysis for four symmetric dialkyl-tetrazines with methyl (Me_2Tz), ethyl (Et_2Tz), isopropyl (iPr_2Tz), and tert-butyl (tBu_2Tz) groups (**Fig. 5b**). The imine adducts of n-BuNC and Me_2Tz and Et_2Tz gradually hydrolyzed and reached an equilibrium within an hour under the given conditions (DMSO-d_6:D_2O (1:1 v/v), T = 37 °C, **Figs. S6, S7** in the Supporting Information). The rate of the dissociation of the adduct is $(8.77 \pm 0.99) \times 10^{-4}$ s^{-1} for Me_2Tz and $(4.73 \pm 0.90) \times 10^{-4}$ s^{-1} for Et_2Tz (**Fig. S8**, **Table S3** in the Supporting Information). The formation of an equilibrium was confirmed by temperature cycling experiments in which the ratio of imine to aldehyde products reproducibly adopted temperature-dependent values (Me_2Tz, T = 20 °C: $K_d = (3.56 \pm 0.25) \times 10^{-3}$ M; T = 37 °C: $K_d = (5.62 \pm 0.99) \times 10^{-3}$ M; T = 50 °C: $K_d = (1.61 \pm 0.24) \times 10^{-2}$ M; **Figs. S9-S10** in the Supporting Information). At the micromolar concentrations typical for chemical biology experiments, the dissociation would be expected to go to completion given these equilibrium constants. Reaction of n-BuNC with iPr_2Tz resulted in two imine intermediates that showed distinctly different hydrolysis rates (**Figs. S11, S12** in the Supporting Information). The adduct of tBu_2Tz and nBuNC is remarkably stable (**Fig. 5b**). After 2 h in the presence of 50 % D_2O, the imine signal was unchanged. Gradual imine hydrolysis occurred reaching 18 ± 3 % after 72 h (**Fig. S13** in the Supporting Information). The stability of the adduct of a primary isonitrile with tBu_2Tz was corroborated in 80% PBS:DMSO by HPLC analysis (**Fig. S14** in the Supporting Information) using a water-soluble isonitrile (CH_3O-PEG2-NC, structure shown in the Supporting Information).

In summary, the data demonstrated that tetrazine substituents modulate the hydrolysis rate of imine intermediates. Adducts of isonitriles and tBu_2Tz are stable for days, which allows obviating the need of structurally engineering the isonitriles for bioconjugation. This possibility is especially appealing because we have shown that bis-tert-butyl tetrazines allow for triple-orthogonal protein labeling schemes.[24]

Mechanistic analysis of the elimination step

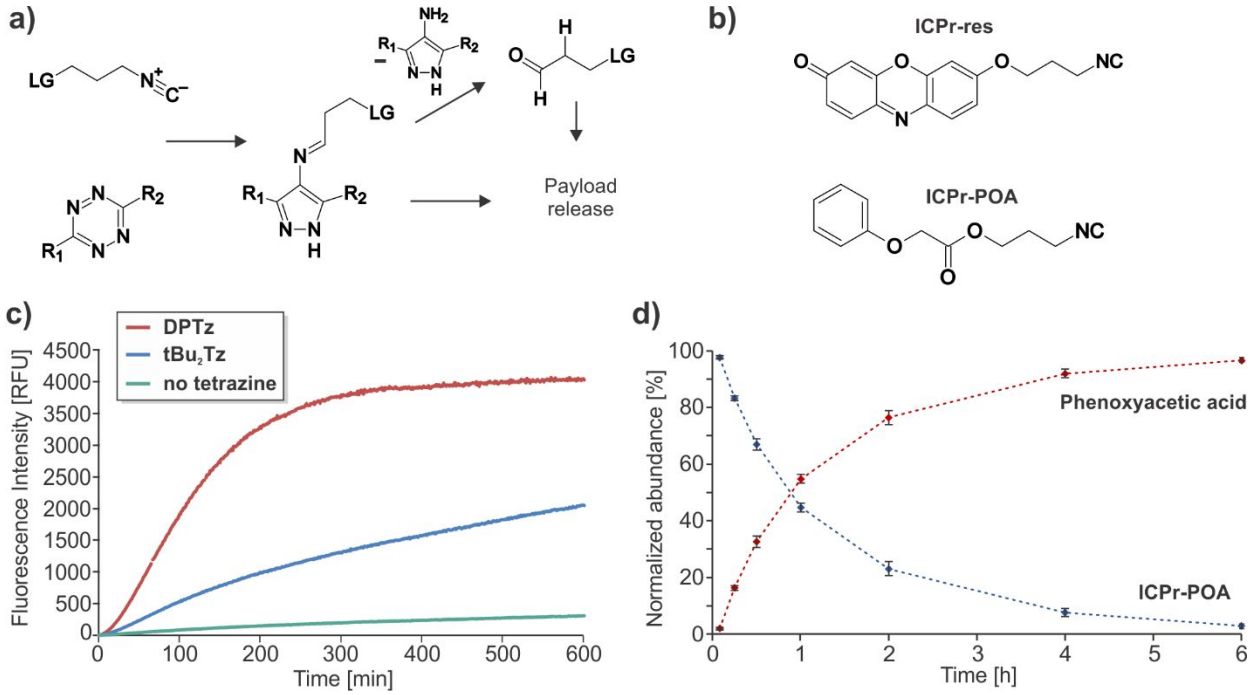


Figure 6. Mechanism of ICPr-removal by tetrazines. a) Possible reaction pathways. Hydrolysis of the imine intermediate may generate a propanal that spontaneously eliminates the leaving group. Alternatively, release may occur directly from the imine (or iminium) intermediate. b) Structures of probe molecules. c) Fluorescence monitoring of resorufin release from ICPr-res precursor after reaction with DPTz (red trace) and tBu_2Tz (blue trace) (ICPr-res (5 μM), tetrazine (100 μM) in PBS pH 7.4 : DMSO (4:1) at 37 ˚C). c) Time-dependent ^1H NMR analysis of tBu_2Tz-induced release of phenoxyacetic acid from ICPr-protected precursor (ICPr-POA (5 mM), tBu_2Tz (10 mM) in DMSO-d_6: D_2O (4:1) at room temperature).

The finding that tetrazine substituents influence the hydrolysis of the imine intermediate raises questions concerning the β-elimination step. The design of ICPr-chemistry assumed rapid hydrolysis of the imine intermediate to form a 3-propanal species followed by spontaneous β-elimination (**Fig. 6a**).[3] According to this mechanism, tetrazines forming long-lived imines would release payloads slowly. It is alternatively possible that release occurs directly from the imine (or iminium) intermediate, and β-elimination from imines has been postulated.[33]

To test this possibility, we examined the liberation of a resorufin fluorophore from an ICPr-caged precursor[3] (ICPr-res, **Fig. 6b**). ICPr-res (5 μM) and tetrazine (100 μM) were incubated in PBS pH 7.4 : DMSO (4:1 v/v) at 37 ˚C and emergence of the resorufin fluorescence was measured as a function of time (λ_{ex} = 590 nm; λ_{em} = 610 nm; **Fig. 6c**). A rapid fluorescence increase was observed with DPTz as expected. Importantly, emergence of a distinct resorufin signal was apparent for the reaction with tBu_2Tz even though the hydrolysis studies showed that the corresponding imine is stable in the given timeframe under these conditions (**Fig. 5**). This outcome provides evidence that resorufin release occurs from the imine/iminium intermediate. The slower uncaging of resorufin with tBu_2Tz relative to DPTz agrees with the different reaction rates of the cycloaddition step.[24]

Only low quantities of resorufin were generated in control experiments without tetrazine (**Fig. 6c**) confirming that resorufin release is tetrazine-mediated.

NMR experiments were performed to verify the hydrolysis-independent removal of ICPr-groups (**Fig. 6d**). We synthesized an ICPr-derivative of phenoxyacetic acid (ICPr-POA; **Fig. 6b**) having a characteristic ^{1}H NMR signal with a chemical shift at 4.74 ppm. ICPr-POA was synthesized in three steps from 3-aminopropanol. Formylation of 3-aminopropanol (ethyl formate, 45 ˚C) was followed by esterification with phenoxyacetic acid (EDC, DMAP, room temperature) and dehydration to the isocyanide (tosyl chloride, room temperature), which yielded ICPr-POA (30% yield, three steps). Time-dependent changes of the ^{1}H NMR spectra were recorded for samples of ICPr-POA (5 mM) and excess tBu_2Tz (10 mM) in DMSO-d_6:D_2O (4:1) at 25 ˚C (**Figs. S15-S16** in the Supporting Information). The signals linked to the ICPr-POA starting material progressively disappeared, giving rise to an intermediate whose ^{1}H NMR spectra agreed with that of the imine structure predicted by the mechanism. The intermediate gradually disappeared and a peak at 4.61 ppm emerged that coincided with that of a phenoxyacetic acid standard in this NMR-solvent system. Aldehyde signals were undetectable at any time of the measurement; instead distinct olefin ^{1}H NMR signals associated with a side product, plausibly the α,β-unsaturated imine, emerged in parallel with the release of phenoxyacetic acid, but disappeared as the reaction continued. The experimental results did not allow to make qualified conclusions on the formed side product. Broadening of the tert-butyl group at later time points suggested the formation of a complex mixture of aminopyrazole derivatives of possibly macromolecular nature.

The combined outcomes provide compelling evidence that elimination of leaving groups can occur directly from the imine/iminium intermediate. ICPr-chemistry is therefore hydrolysis-independent and may occur without generating acrolein side products.

Conclusions

This study provides important information on structure-activity relationships and mechanism of the tetrazine-induced removal of ICPr-groups. Three key conclusions result from the series of experiments. First, modifications that promote deprotonation at the C-2 position accelerate the elimination of payloads. Second, tetrazines with bulky substituents (-tBu) form stable adducts with primary isonitriles. Third, payload release can occur independently from imine hydrolysis. Each of these outcomes has important implications for practical translations.

The accelerating effect of the C-2 phenyl group on the β-elimination step indicates that it is possible to design ICPr-derivatives that, independent from albumin, release payloads rapidly. This outcome is important for many applications where the reaction has to occur without the presence of serum albumin. The ICPPr design establishes the basic principle for rate enhancement and provides guidance for future design; one can easily envision other modifications of the ICPr-group that would further accelerate tetrazine-mediated deprotection. Boosting the elimination step may additionally open up the possibility of applying ICPr-chemistry to the bioorthogonal unmasking of functional groups that are more difficult to eliminate than phenols and carbamates.

The formation of stable adducts by the reaction between n-BuNC and tBu_2Tz suggests that one can use this chemistry to label a much broader range of isonitriles than previously thought possible. This result is significant because it obviates the need for engineering the isonitrile moiety (e.g. tertiary isonitriles[23]) and allows fully exploiting the isocyano functionality as the smallest bioorthogonal reactive group. Such chemistry could find widespread use. For example, carbohydrates with handles that are even smaller than those previously used for modifying sugars that can be metabolically incorporated into glycans[21, 22] could be used. Moreover, the structural compactness of the isocyano group is ideal for incorporation into proteins by codon expansion techniques and would minimally disrupt the structures of the biomacromolecules. The chemistry could also be useful for the discovery of isocyano-containing natural products. Recently, isonitrile-tetrazine chemistry was used in tandem with $NaBH_3CN$ reduction of the imine to isolate such molecules.[34] With sterically encumbered tetrazines, the reduction step could become unnecessary. The possibility to precisely tune the half-life of isonitrile-tetrazine adducts may open new possibilities in the biosciences.

An important outcome was the discovery that elimination of the payloads can occur from the imine intermediate. The fate of the imine of the 4-aminopyrazole following the reaction could not be determined from the NMR results and are likely dependent on the exact reaction conditions. Nevertheless, this result has significant implications. First, this result suggests that hydrolysis of the imine is not rate-limiting for release. Second, it means that release can occur independent of the structure of the tetrazine (i.e. even for tetrazines that form imine adducts that are inert to hydrolysis). Third, the outcome is important for drug delivery applications. For example, Royzen and Mejia Oneto *et al.* recently reported that implanted tetrazine-modified hydrogels combined with reactive prodrugs enabled the site-specific delivery of doxorubicin[35] and antibiotics.[36] Delayed drug release relative to the dissociation from the hydrogel would lead to diffusion of the prodrug away from the site of interest and diminish the spatial resolution of such an approach. Drug release from the imine intermediate overcomes this problem. Importantly, it can also avoid the formation of the acrolein side-product that was previously considered a primary disadvantage of ICPr-chemistry. Although it is possible that the formed side product may be intrinsically toxic, as it is retained on the hydrogel, or any other drug carrier, its detrimental effect will likely be greatly diminished. The therapeutic prospect of this chemistry will be explored in future studies.

In summary, the presented data provides detailed insights on the dissociative steps of tetrazine-induced ICPr-deprotection chemistry. The obtained knowledge will be valuable for designing applications based on this reaction in chemical biology and drug delivery.

Experimental Section

Materials and Instrumentation

All chemical reagents and solvents were obtained from commercial sources (Sigma-Aldrich, Alfa-Aesar, Combi-Blocks, Acros-Organic, TCI) and used without further purification. Thin-layer chromatography (TLC) analysis was carried out to monitor the process of reactions. Purification of compounds was performed by column chromatography with silica gel 300-400 mesh. ^1H NMR

and ^{13}C NMR spectra were recorded on a Varian Mercury-400 or Varian Inova-500 spectrometer as indicated with chemical shifts expressed as ppm (in $CDCl_3$, MeOD-d_4 or DMSO-d_6) using Me_4Si (TMS) as internal standard.

Exact Mass Spectra were measured by the University of California, Riverside Chemistry Mass Spectrometry Facility. All analytical preparative HPLCs were performed on a Dionex Ultimate3000 equipped with autosampler, diode array detector and robotic fraction collector (Dionex Thermo Scientific, USA) using a Gemini C18 column (5 μM, 250×10 mm, Phenomenex, USA) or Luna C18 column (5 μM, 150×2.0 mm, Phenomenex, USA). UV-VIS photospectrometic kinetic measurements were performed on a microplate reader SpectramaX M5 (Molecular Device, USA) in 96-well plates or 1 mL quartz cuvette.

Synthetic Procedures

3-((tert-butyldimethylsilyl)oxy)-2-phenylpropan-1-ol. A solution of 2-phenylpropane-1,3-diol (5 g, 33 mmol) in THF (25 mL) was added dropwise to a stirred and ice cooled suspension of NaH (1.5 g, 36 mmol) in THF (15 mL). The ice bath was removed and reaction mixture was allowed to warm to room temperature and kept at this temperature for 12 h. The reaction was quenched with ice, diluted with EtOAc (300 mL) and washed with brine (2×150 mL). The separated organic layer was dried with anhyd. Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (100% hexane) to afford the desired compound as a yellow solid in a yield of 5 g (59%; R_f = 0.8 in hexane : EtOAc = 100:1, v/v). ^1H NMR (400 MHz, $CDCl_3$) δ 7.33-7.30 (m, 2H), 7.24 (brs, 1H), 7.22-7.20 (m, 2H), 4.13-4.05 (m, 2H), 3.93-3.87 (m, 2H), 3.12-3.05 (m, 1H), 1.26 (t, J = 7.2 Hz, 2H), 0.90 (s, 9H), 0.06 (s, 6H). The ^1H NMR data agreed with the reported ^1H NMR spectrum of this compound.[28]

2-(3-((tert-butyldimethylsilyl)oxy)-2-phenylpropyl)isoindoline-1,3-dione.
Triphenylphosphine (6.3 g, 24 mmol) and phthalimide (2.8 g, 19.2 mmol) were added sequentially to a stirred and ice cooled solution of 3-((tert-butyldimethylsilyl)oxy)-2-phenylpropan-1-ol (4.4 g, 16 mmol) in THF (60 mL). To this mixture diethyl azodicarboxylate (3.34g, 19.2 mmol) in THF (15 mL) was added dropwise. The ice bath was removed and reaction mixture was allowed to warm to room temperature and kept at this temperature for 12 h. The reaction was quenched with ice, diluted with Et_2O (200 mL) and washed with sat. aq. $NaHCO_3$ (2×150 mL) and brine (2×150 mL). The separated organic layer was dried with anhyd. Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (hexane : EtOAc = 10:1, v/v) to afford the desired compound as a yellow oil in a yield of 3.2 g (50%; R_f = 0.6 in hexane : EtOAc = 5:1, v/v). ^1H NMR (400 MHz, $CDCl_3$) δ 7.78-7.76 (m, 2H), 7.66-7.64 (m, 2H), 7.25-7.18 (m, 5H), 4.11-4.00 (m, 2H), 3.85-3.81 (m, 2H), 3.49-3.44 (m, 1H), 1.26 (t, J = 7.2 Hz, 2H), 0.81 (s, 9H), 0.09 (s, 6H). The ^1H NMR data agreed with the reported ^1H NMR spectrum of this compound.[28]

3-Amino-2-phenylpropan-1-ol. Hydrazine hydrate (2 g, 40 mmol) was added to a stirred and ice cooled solution of compound *2-(3-((tert-butyldimethylsilyl)oxy)-2-phenylpropyl)isoindoline-1,3-dione* (1.6 g, 4 mmol) in EtOH (40 mL) and the reaction mixture was reflux for 2 h. The reaction

was quenched with ice, diluted with Et_2O (200 mL) and washed with sat. aq. $NaHCO_3$ (2×150 mL). The aqueous layer was extracted with Et_2O (100 mL) and the combined organic layers were washed with 1 M aq. HCl solution. The resulting acidic solution was then treated with 10 M aq. NaOH until reaching pH 11 and washed with EtOAc (200 mL). The combined organic layers were dried with anhyd. Na_2SO_4, filtered, and concentrated under reduced pressure to give the crude compound as yellow oil in a yield of 185 mg (30%; R_f = 0.1 in EtOAc : MeOH = 5:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36-7.30 (m, 3H), 7.24-7.22 (m, 2H), 4.10-4.04 (m, 2H), 3.99 (t, *J* = 7.6 Hz, 1H), 3.54-3.49 (m, 1H), 3.32-3.26 (m, 1H). HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd. for $C_9H_{14}NO$ 152.1070; Found 152.1095. The ^{1}H NMR data agreed with the reported spectrum of this compound.[28]

3-isocyanide-2-phenylpropan-1-ol (ICPPr-OH). Ethyl formate (850 mg, 12 mmol) was added in portions to a stirred ice cooled solution of 3-amino-2-phenylpropan-1-ol (185 mg, 1.2 mmol). The ice bath was removed and the reaction mixture heated to 50 °C for 2 h. Excess ethyl formate was removed by rotary evaporation to afford the desired product as a colorless oil in a near-quantitative yield (>95%). This compound decomposed upon storage and was immediately used in the next step. Burgess reagent (350 mg, 1.5 mmol) was added in portions over 10 min to a solution of the aforementioned intermediate in dry CH_2Cl_2 (2.5 mL). The solution was stirred at room temperature under nitrogen atmosphere for 2 h. The mixture was diluted with CH_2Cl_2 (50 mL), washed with brine (50 mL), dried with anhyd. Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (Hexane : EA = 3:1, v/v) to afford the desired compound as a yellow solid in a yield of 106 mg (55%; R_f = 0.3 in EtOAc : hexane = 1:3, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39-7.31 (m, 3H), 7.24-7.22 (m, 2H), 3.92-3.77 (m, 2H), 3.73-3.68 (m, 2H), 3.12 (brs, 1H). ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$) δ156.8, 156.7, 137.8, 128.9, 127.8, 127.7, 63.1, 46.9, 43.5, 43.5, 43.4. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd. for $C_{10}H_{12}NO$ 162.0913; Found 162.0929.

3-isocyano-2-phenylpropan-1-tosylpropane (ICPPr-Tos). To a stirred solution of compound ICPPr-OH (65 mg, 0.4 mmol) in dry THF (1 mL) was added dropwise over 30 minutes a solution of tosyl chloride (114 mg, 0.6 mmol) and triethylamine (60 mg, 0.6 mmol) in dry THF (1 mL). The solution was stirred at 0 °C under a nitrogen atmosphere for 4 hours. The reaction was diluted with DCM (20 mL) and washed with brine (2×30 mL). The organic layer was dried over anhyd. Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (DCM) to afford the desired compound as an oil in a yield of 75 mg (59%; R_f = 0.3 in DCM : hexane = 10:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 8.0 Hz, 2H), 7.35-7.32 (m, 5H), 7.16 (d, *J* = 8.0 Hz, 2H), 4.28-4.25 (m, 2H), 3.73 (t, *J* = 4.4 Hz, 2H), 3.29 (t, *J* = 6.4 Hz, 1H), 2.45 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$) δ 145.2, 135.6, 132.2, 130.0, 129.0, 128.4, 127.9, 127.6, 69.2, 43.9, 21.6, 14.1. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd. for $C_{17}H_{18}NO_3S$ 316.1002; Found 316.1005.

N-(methyl-PEG4)-4-(3-isocyanopropyl-2-phenylcarbamoyl)amino-1,8-naphthalimide (ICPPrc-NH-NA). To a mixture of N-(methyl-PEG4)-4-amino-1,8-naphthalimide[3] (48 mg, 0.12 mmol) and DIEA (44 mg, 0.36 mmol) in dry toluene (2.5 mL) was added a solution of triphosgene (39 mg, 0.13 mmol) in dry toluene (1 mL) dropwise. The solution was heated to reflux for 3 h.

After cooling to room temperature, the mixture was added a solution of ICPPr-OH (39 mg, 0.24 mmol) in dry CH_2Cl_2 (3 mL) and the reaction was stirred at room temperature for 12 h. The mixture was concentrated under reduced pressure and the residue was purified by column chromatography (DCM : MeOH = 20:1, v/v) to afford the desired compound as yellow oil in a yield of 21 mg (32%, ; R_f = 0.25 in DCM : MeOH = 20:1, v/v). ^{1}H NMR (400 MHz, CD_3OD) δ 8.54 (d, *J* = 7.6 Hz, 1H), 8.48 (t, *J* = 7.2 Hz, 2H), 8.14 (d, *J* = 7.6 Hz, 1H), 7.80-7.76 (m, 1H), 7.41-7.33 (m, 5H), 4.56-4.53 (m, 2H), 4.37 (t, *J* = 6.0 Hz, 2H), 4.03-3.91 (m, 2H), 3.79 (t, *J* = 6.0 Hz, 2H), 3.66-3.64 (m, 2H), 3.58-3.56 (m, 2H), 3.51-3.46 (m, 5H), 3.43-3.41 (m, 4H), 3.29 (s, 3H). ^{13}C{^{1}H} NMR (100 MHz, CD_3OD) δ 163.8, 163.3, 154.0, 140.9, 138.2, 132.0, 131.3, 129.73, 129.0, 128.8, 128.7, 128.5, 128.5, 128.0, 126.7, 124.3, 122.5, 118.7, 118.4, 117.5, 71.6, 70.2, 70.1, 70.0, 69.9, 67.3, 65.7, 58.4, 44.0. HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd. for $C_{32}H_{35}N_3O_8Na$ 612.2316; Found 612.2365.

N-(methyl-PEG4)-4-(3-isocyanopropyl-2-Phenol-1-oxy)-1,8-naphthalimide (ICPPr-O-NA). To a mixture of N-(methyl-PEG4)-4-hydroxy-1,8-naphthalimide[2] (41 mg, 0.1 mmol) in acetone (0.5 mL) was added anhydrous K_2CO_3 (22 mg, 0.16 mmol) and the mixture was allowed to stir at room temperature for 15 minutes. ICPPr-Tos (60 mg, 0.2 mmol) dissolved in acetone (0.5 mL) was added dropwise to the solution and the reaction mixture was allowed to stir at 50 ˚C for 12 h. The solution was cooled to room temperature, solid residues were removed by filtration, and the organic layer was concentrated under reduced pressure. Residues were purified by column chromatography (DCM : MeOH = 30:1, v/v) to afford the desired compound as an orange oil in a yield of 12 mg. (24%, ; R_f = 0.3 in DCM : MeOH = 20:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (d, *J* = 7.2 Hz, 1H), 8.52 (d, *J* = 8.0 Hz, 1H), 8.47 (d, *J* = 8.4 Hz, 1H), 7.72 (t, *J* = 7.6 Hz, 1H), 7.45-7.37 (m, 5H), 7.06 (d, *J* = 8.0 Hz, 1H), 4.58 (d, *J* = 6.4 Hz, 2H), 4.43-4.40 (m, 2H), 4.04-4.00 (m, 2H), 3.80 (d, *J* = 6.4 Hz, 3H), 3.70-3.67 (m, 4H), 3.62-3.56 (m, 8H), 3.52-3.49 (m, 2H), 3.35 (s, 3H), 3.15-3.09 (m, 1H). ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$) δ 164.3, 163.7, 159.1, 145.2, 136.7, 135.6, 133.2, 131.7, 130.0, 129.4, 129.2, 129.0, 128.4, 128.4, 128.2, 127.9, 127.6, 127.6, 126.2, 123.3, 122.5, 115.7, 106.0, 71.8, 70.5, 70.4, 70.0, 69.2, 69.0, 67.9, 44.4, 43.9, 21.6. HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd. for $C_{31}H_{34}N_2O_7Na$ 569.2258; Found 569.2295.

2-Cyano-1-phenylethanol. To a solution of styrene oxide (4 mL, 35 mmol) in MeOH (100 mL) was added a solution of sodium cyanide (2 g, 100 mmol) in water (20 mL). The reaction mixture was stirred for 12 h. The mixture was then quenched with water (80 mL) and a 2 aq. N HCl solution (100 mL). CAUTION: evolution of hydrogen cyanide gas! The reaction mixture was diluted with DCM (400 mL) and washed with brine (2×200 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (hexane : EA = 6:1, v/v) to afford the desired compound as an oil in a yield of 3 g. (62%, R_f = 0.2 in hexane : EA = 6:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41-7.36 (m, 5H), 5.05 (t, *J* = 6.4 Hz, 1H), 2.78 (d, *J* = 6.4 Hz, 2H). The ^{1}H NMR data agreed with the reported ^{1}H NMR spectrum of this compound. [37]

3-Amino-1-phenylpropan-1-ol. A solution of borane-dimethyl sulfide complex (2.2 mL, 23 mmol) in dry THF (10 mL) was added dropwise to a solution of 2-cyano-1-phenylethanol (3 g, 21 mmol) in dry THF (10 mL) at 0 °C under a N_2 atmosphere. The mixture was refluxed for 8 h. The

reaction was quenched with ice water, diluted with EtOAc (200 mL) and washed with brine (200 mL). The combined organic layers were dried over anhyd. Na_2SO_4, filtered, concentrated under reduced pressure. The residue was purified by column chromatography (EA : MeOH = 1:1, v/v) to afford a colorless oil in a yield of 600 mg (20%, R_f = 0.15 in EA : MeOH = 1:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39-7.23 (m, 5H), 4.97 (d, *J* = 8.4 Hz, 1H), 2.78 (brs, 2H), 1.89-1.74 (m, 2H). HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd. for $C_9H_{14}NO$ 152.1070; Found 152.1062. The ^{1}H NMR data agreed with the reported ^{1}H NMR spectrum of this compound.[27]

3-isocyanide-1-phenylpropan-1-ol (ICPPr*-OH). ICPPr*-OH was prepared following the same procedure as for ICPPr-OH. ICPPr*-OH was obtained as a colorless oil in a yield of 220 mg (35%, R_f = 0.35 in EA : hexane = 1:3, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38-7.31 (m, 5H), 4.90-4.86 (m, 1H), 3.67-3.59 (m, 1H), 3.47-3.39 (m, 1H), 2.08-2.03 (m, 1H). $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$) δ 156.0, 156.0, 155.9, 143.1, 128.7, 128.3, 128.1, 125.6, 70.6, 38.6, 38.5, 38.4, 37.9, 29.7. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd. for $C_{10}H_{12}NO$ 162.0913; Found 162.0905.

N-(methyl-PEG4)-4-(3-isocyanopropyl-3-phenylcarbamoyl)amino-1,8-naphthalimide (ICPPrc*-NH-NA). ICPPrc*-NH-NA was prepared following the same procedure as for ICPPrc-NH-NA. ICPPrc*-NH-NA was obtained as a yellow oil in a yield of 15 mg (35%, R_f = 0.35 in EA : hexane = 1:3, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (t, *J* = 8.0 Hz, 2H), 8.32-8.29 (m, 2H), 8.09 (brs, 1H), 7.73 (t, *J* = 8.0 Hz, 1H), 7.59-7.49 (m, 5H), 6.13-6.09 (m, 1H), 4.52-4.49 (m, 2H), 3.97 (t, *J* = 6.0 Hz, 2H), 3.87-3.85 (m, 2H), 3.78-3.68 (m, 6H), 3.63-3.59 (m, 5H), 3.55-3.53 (m, 2H), 3.42 (s, 3H), 2.63-2.37 (m, 2H). $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$) δ163.9, 163.4, 157.0, 152.2, 138.8, 138.3, 132.1, 131.0, 128.8, 128.8, 128.3, 126.3, 126.2, 122.7, 117.3, 116.8, 74.7, 71.6, 70.5, 70.3, 70.3, 70.2, 70.0, 67.9, 58.8, 38.9, 38.2, 35.5, 29.5. HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd. for $C_{32}H_{35}N_3O_8Na$ 612.2316; Found 612.2371.

(3-isocyano-2-phenylpropyl)(naphthalen-2-yl)sulfane. To a mixture of 2-naphthalenethiol (6 mg, 0.033 mmol) in DMF (0.1 mL) was added anhydrous K_2CO_3 (17 mg, 0.12 mmol) and the mixture was stirred at room temperature for 15 minutes. ICPPr-Tos (10 mg, 0.03 mmol) dissolved in DMF (0.1 mL) was added dropwise and the reaction mixture was stirred at room temperature for 12 h. Solid residues were removed by filtration, and the organic layer was concentrated under reduced pressure and the residue purified by column chromatography (Hexane : EA = 10:1, v/v) to afford the desired compound as an orange oil in a yield of 8 mg. (20%, ; R_f = 0.3 in Hexane : EA = 10:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82-7.76 (m, 4H), 7.50-7.31 (m, 6H), 7.22 (d, *J* = 7.2 Hz, 2H), 3.87-3.76 (m, 2H), 3.43-3.40 (m, 2H), 3.17 (brs, 1H). $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$) δ 139.1, 133.6, 132.2, 132.0, 128.9, 128.8, 128.0, 127.7, 127.5, 127.5, 127.1, 126.7, 126.0, 45.8, 44.0, 36.2, 31.9, 29.7, 22.7. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd. for $C_{20}H_{18}NS$ 304.1154; Found 304.1154.

3-formamidopropyl 2-phenoxyacetate. Ethyl formate (311 mg, 4.2 mmol) was added in portions to 3-aminopropanol (247.5 mg, 3.3 mmol) while stirring at 0 ℃. The solution was removed from the ice-bath and was heated at 50 °C for 2 h. Excess ethyl formate was removed by rotary evaporation to afford the desired product as a colorless oil in near-quantitative yield (>95%). This compound decomposed upon storage and was immediately used in the next step. To a solution of phenoxyacetic acid (456 mg, 3 mmol) in dry DCM (5 mL), a solution of the aforementioned

intermediate and DMAP (508 mg, 4.2 mmol) in dry DCM (5 mL) was added at 0 ℃ under a nitrogen atmosphere. The solution was stirred at 0 ℃ for 30 min, followed by the addition of EDC•HCl (1.15 g, 6 mmol) in portions. The solution was stirred at room temperature under nitrogen atmosphere overnight. The mixture was diluted with CH_2Cl_2 (50 mL), washed with water (30 mL), followed by brine (50 mL), concentrated under reduced pressure, and purified by column chromatography (Hexane : EA = 1:1, v/v) to afford the desired compound as a yellow oil in a yield of 426 mg (62% NMR yield [determined from ^{1}H integration and measured weight taking into account residual DMAP]; R_f = 0.15 in EtOAc : hexane = 1:1, v/v) as a mixture with DMAP. It was carried forward without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (s, 1H), 7.28 (t, *J* = 8.0 Hz, 2H), 6.98 (t, *J* = 8.0 Hz, 1H), 6.88 (d, *J* = 8.0 Hz, 2H), 6.34 (brs, 1H), 4.63 (s, 2H), 4.25 (t, *J* = 6.0 Hz, 2H), 3.29 (q, *J* = 6.3 Hz, 2H), 1.91-1.80 (m, 2H).

3-isocyanopropyl 2-phenoxyacetate. 3-formamidopropyl 2-phenoxyacetate (420 mg, 1.7 mmol) was dissolved in dry DCM (3 mL), and dry pyridine (2 mL) was added. A solution of tosyl chloride (TsCl, 646 mg, 3.4 mmol) in dry DCM (4 mL) was added to it dropwise at 0 ℃ under a nitrogen atmosphere while stirring. The solution was stirred at room temperature under nitrogen atmosphere overnight. The mixture was purified by column chromatography (Hexane : EA = 4:1, v/v) to afford the desired compound as a brown oil in a yield of 191 mg (49%; R_f = 0.3 in EtOAc : hexane = 4:1, v/v). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.27 (t, *J* = 7.6 Hz, 2H), 6.95 (t, *J* = 7.2, 1H), 6.88 (d, *J* = 8.0 Hz, 2H), 4.74 (s, 2H), 4.18 (t, *J* = 5.8 Hz, 2H), 3.50-3.41 (m, 2H), 1.94-1.84 (m, 2H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ 168.7, 157.5, 156.1, 129.5, 121.2, 114.4, 64.4, 61.1, 38.0, 27.7. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd. for $C_{12}H_{14}NO_3$ 220.0973; Found 220.0973.

1-isocyano-2-(2-(2-methoxyethoxy)ethoxy)ethane. Ethyl formate (67 mg, 0.9 mmol) was added in portions to 2-(2-(2-methoxyethoxy)ethoxy)ethanamine (98 mg, 0.6 mmol) while stirring at 0 ℃. The solution was removed from the ice-bath and was heated at 50 °C for 2 h. Excess ethyl formate was removed by rotary evaporation to afford the desired product as a colorless oil in a near-quantitative yield (>95%). This compound decomposed upon storage and was immediately used in the next step. The intermediate was dissolved in dry DCM (2 mL), and dry pyridine (1 mL) was added to it. A solution of TsCl (228 mg, 1.2 mmol) in dry DCM (2 mL) was added to it dropwise at 0 ℃ under a nitrogen atmosphere while stirring. The solution was stirred at room temperature under nitrogen atmosphere overnight. The mixture was purified by column chromatography (Hexane : EA = 4:1, v/v) to afford the desired compound as a light brown oil in a yield of 68 mg (63%; R_f = 0.25 in EtOAc : hexane = 4:1, v/v). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.72-3.61 (m, 8H), 3.58-3.51 (m, 4H), 3.36 (s, 3H). ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ 157.3, 72.0, 70.9, 70.7, 68.7, 59.1, 50.7, 41.8, 29.7. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd. for $C_8H_{16}NO_3$ 174.1130; Found 174.1123.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at XXXX

Supporting figures and tables

NMR spectra of products and intermediates

Acknowledgements

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