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Degradable Conjugates from Oxanorbornadiene Reagents

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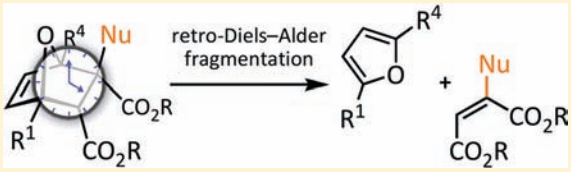
Supporting Information

ABSTRACT: Oxanorbornadienedicarboxylate (OND) reagents were explored for purposes of binding and releasing drugs from serum albumins as representative macromolecular carriers. Being highly reactive Michael acceptors, ONDs form adducts with thiols and amines, which then undergo retro-Diels–Alder fragmentation. A study of more than 30 model adducts revealed a number of modifications that can be used to influence adduct stability. For the most reactive OND linkers, the labeling of the single available bovine serum albumin (BSA) cysteine residue was complete within minutes at a mid-micromolar concentration of reactants. While a selectivity of greater than 1000-fold for thiol over amine was observed with model amino acids, the labeling of protein amines with ONDs is fast enough to be practical, as demonstrated by the reaction with thiol-depleted BSA. The OND–amine adducts were found to be up to 15 times more stable than OND–thiol adducts, and to be sensitive to acid by virtue of a stereochemically dependent acceleration of cycloreversion. The release rate of fluorescent cargo from serum albumins was tuned by selecting the coupling partners: the available half-lives ranged from 40 min to 7 days at 37 °C. Such versatility of release profiles from protein carriers, controlled by the nature of the OND linkage, is a useful addition to the drug delivery toolbox.



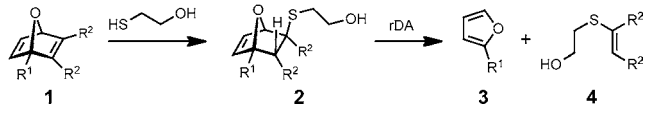
INTRODUCTION

Serum albumin (SA), the most abundant plasma protein in mammals,[1] is widely recognized as a long-circulating, nontoxic, nonimmunogenic, and biodegradable carrier for the delivery of lipophilic cargo, including endogenous metabolites and therapeutic drugs.[2] Among the most successful examples of this approach are human SA nanoparticles carrying adsorbed taxol[3] (Abraxane), approved for the treatment of breast cancer in 2005.

In most vertebrates, SA displays a single free cysteine residue that accounts for 90% of extracellular thiol content[1] (approximately 0.4–0.5 mM thiol in human plasma). The abundance of SA makes it possible to directly inject thiol-reactive electrophiles into the bloodstream, resulting in preferential covalent binding to SA with little or no off-target binding.[4] The observation of the intended drug activity then often requires breakdown of the albumin–drug linkage. Acid-sensitive hydrazones and protease-cleavable peptide sequences[5] have most commonly been used for this purpose. We present here a new approach to protein-modifying linkers and prodrugs, comprising electrophiles capable of tunable cleavage after reaction with thiols[6] and amines.[7]

We have recently described the conjugate addition reactivity of 7-oxanorbornadienedicarboxylate ester (OND) reagents (**1**) with thiols (Scheme 1).[8] The observed second-order rate constants of OND electrophiles are on par with those of standard maleimides (approximately 10–100 M^{-1} s^{-1} with glutathione, pH 7), while their aqueous stability generally exceeds that of the maleimides.[8,9] OND–thiol adducts **2** were found to undergo retro-Diels–Alder (rDA) fragmentation, giving furans **3** and thiomaleates **4**, whereas the rDA cleavage of the starting OND fragment is completely absent under the same conditions.[9] Thus, thiol addition constitutes a trigger for cleavage if the OND moiety bridges the carrier and the cargo. In our initial report, we noted a wide range of stabilities of different versions of **2** with changes in substitution pattern. We describe here analogous amine- and phosphine-triggered rDA cleavage and the results of a further investigation of the factors that contribute to cleavage rates of thiol and amine adducts, with the purpose of creating albumin-modifying agents with a useful range of release rates. While rDA cleavage has been employed in the context of drug release,[10] its use with convenient electrophilic linkages has not been previously reported.

Scheme 1. Reaction of OND Electrophiles with BME as a Representative Thiol



RESULTS

Synthesis and Stability of OND–Thiol Adducts. Oxanorbornadienes **1** were readily obtained from inexpensive furans and dialkyl acetylenedicarboxylates in a single high-yielding step. A wide range of substituents was tolerated in this process, including sulfonamides, amides, ureas, alcohols, and thioethers, but not thioureas. Attempted Diels–Alder reactions

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of furfurylamine, as a free base or protected as various salts (trifluoroacetate, tosylate), invariably gave complex mixtures resulting from conjugate addition to dimethyl acetylenedicarboxylate. If the required acetylene precursor was inaccessible, the desired diester could be prepared by alkylation of saponified OND diacid salt (Supporting Information, Scheme S1). Despite their strongly electrophilic nature, dialkyl OND esters were found to be very stable in aqueous solutions, with the exception of dipropargyl esters that undergo intramolecular [2 + 2 + 2] cycloaddition.[9]

Adducts **2** were obtained by addition of β-mercaptoethanol (BME) to **1** in acetonitrile using catalytic amounts of tertiary amine base (Table 1). In all cases, the 3-*exo-syn* adduct was isolated as the sole product in near-quantitative yields, consistent with the general preference for attack on the less hindered *exo*-face of [2.2.1]-bicycles. The observed coupling between H^2 and bridgehead H^1 (J = 4.2 Hz) in **2a** corroborated the regiochemical assignment of substituted adducts **2b**–**2v**, in which the thiolic proton at C-2 resonates as a singlet. The bridgehead methyl substituent in **2b** was sufficient to direct the nucleophile addition to the distal maleate carbon (C3), while unsymmetric 1,4-disubstituted adducts **6b** and **6c** were obtained as mixtures of C2- and C3-isomers (Table 2). This suggests that the regioselectivity of thiol addition is governed by the steric bulk of the bridgehead substituents.

Table 1. Half-Lives of BME Adducts 2 (Scheme 1)

	R^1	R^2	half-life, days[a]
2a	H	CO_2Me	0.48 ± 0.03
2b	Me	CO_2Me	2.3 ± 0.1
2c	$CH_2NHDansyl$	CO_2Me	23.3 ± 0.8 (26.2 ± 0.5)[b]
2d	$CH_2N(Me)Dansyl$	CO_2Me	4.7 ± 0.4 (4.4 ± 0.2)[b]
2e	CH_2NHMs	CO_2Me	20.5 ± 0.5
2f	CH_2NHAc	CO_2Me	18.8 ± 0.5
2g	CH_2NHAc	CO_2Et	17.8 ± 0.7 (12.6 ± 0.4)[c]
2h	CH_2NHAc	CO_2Bu^t	11.3 ± 1.2
2i	CH_2NHAc	CO_2CH_2CCH	5.6 ± 0.1
2j	$CH_2NHCOC_6H_4pNO_2$	CO_2Et	19.8 ± 0.3
2k	$CH_2NHCOC_6H_4pNO_2$	CO_2Me	20.6 ± 0.8
2l	$CH_2NHCOC_6H_5$	CO_2Me	16.8 ± 0.5
2m	$CH_2NHCOC_6H_4pOMe$	CO_2Me	16.2 ± 0.5
2n	$CH_2NHCOC_6H_4pNMe_2$	CO_2Me	14.6 ± 0.3
2o	$CH_2NHCONHC_6H_5$	CO_2Me	7.7 ± 0.6
2p	CH_2NHBoc	CO_2Me	16.6 ± 1.1
2q	CH_2OH	CO_2Me	7.2 ± 0.5
2r	$CH_2NHDansyl$	CF_3, CO_2Et	241 ± 12
2s	$CH_2NHDansyl$	CF_3, CF_3	192 ± 10
2t	$CH_2NHDansyl$	$CO_2CH_2CH_2CCH$	8.9 ± 0.1
2u	$CH_2NHDansyl$	$CO_2(CH_2)_2O$-CH_2CCH	12.1 ± 0.2
2v	$CH_2N(Me)Dansyl$	$CO_2(CH_2)_2O$-CH_2CCH	7.0 ± 0.1

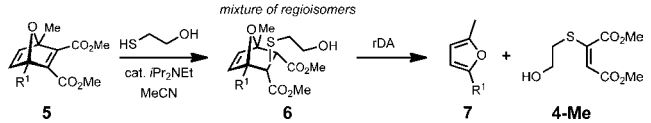
[a]Measured in $CDCl_3$ by NMR. [b]Measured in pH 7 phosphate buffer by HPLC of aliquots. [c]Measured in 9:1 buffer/DMSO by following appearance of thiomaleate absorbance.

The decomposition of oxanorbornene adducts **2** was followed by ^1H NMR in $CDCl_3$ at room temperature. No deviations from expected concentration-independent first-order decay were observed even at high conversions (up to 100%), suggesting that the reaction is irreversible. Adduct **2a**, prepared from the simplest OND, was found to have a half-life of approximately 12 h. Introduction of one bridgehead methyl group (**2b**) increased the half-life to 2.3 days. In the rest of the dimethyl ester series, replacing 2-methylfuran with furfurylamine derivatives, including sulfonamides (**2c**–**e**, **2t**–**v**), amides (**2f**–**2n**), urea (**2o**), carbamate (**2p**), and furfuryl alcohol (**2q**), resulted in an increase in thiol adduct stability. Fluorinated compounds **2r** and **2s** were very stable. Within this series, the steric bulk of the ester group made a modest difference (**2f**–**h**; **2j** and **2k**). However, the electronic properties of the ester groups (R^2) were quite influential: the more electron-poor the ester group as indicated by the pK_a of the corresponding alcohol, the less stable the OND adduct (**2i** vs **2g**, **2c** vs **2t**–**2v**; see also Table S2). In three cases, measurement of the cycloreversion rate in buffer (Figure S1) gave similar results to those obtained in chloroform (**2c**, **2d**, **2g**).

Thiol addition to unsymmetrical 1,4-disubstituted ONDs **5** proceeded nonselectively, giving a mixture of 2-*exo-syn* and 3-*exo-syn*-adducts **6**, which were found to be relatively unstable toward decomposition to furan **7** and thiomaleate **4-Me** (Table 2). The 1,4-dimethyl-substituted adduct **6a** is extremely short-lived, decomposing by over 80% just 2 h after purification. Sulfonamide **6b** and amide **6c** were somewhat more stable.

Reactions of OND with Aromatic Thiols, Phosphines, and Tertiary Amines. Similar to maleimides,[11] OND electrophiles were found to be reactive toward other nucleophiles. The addition of an aromatic thiol to OND **1g** produced a mixture of *syn*- and *anti-exo* addition products that underwent rapid decomposition. Similarly, OND **1c** reacted rapidly with trimethylphosphine and tris(carboxylethyl)-phosphine (TCEP) to give adducts that instantaneously decomposed by rDA fragmentation (Scheme S2). In contrast, no reaction was observed after weeks of incubation of **1c** with triphenylphosphine at high concentration. As a representative

Table 2. Half-Lives of 1,4-Disubstituted Thiol Adducts 6



Adduct	R_1	regioisomer ratio	half-life[a]
6a	Me	–	<1 h
6b	$CH_2NHDansyl$	2:1	1.9 ± 0.1 h
6c	$CH_2NHCOC_6H_4pNO_2$	1.3:1	3.1 ± 0.2 h

[a]Measured in $CDCl_3$ by NMR.

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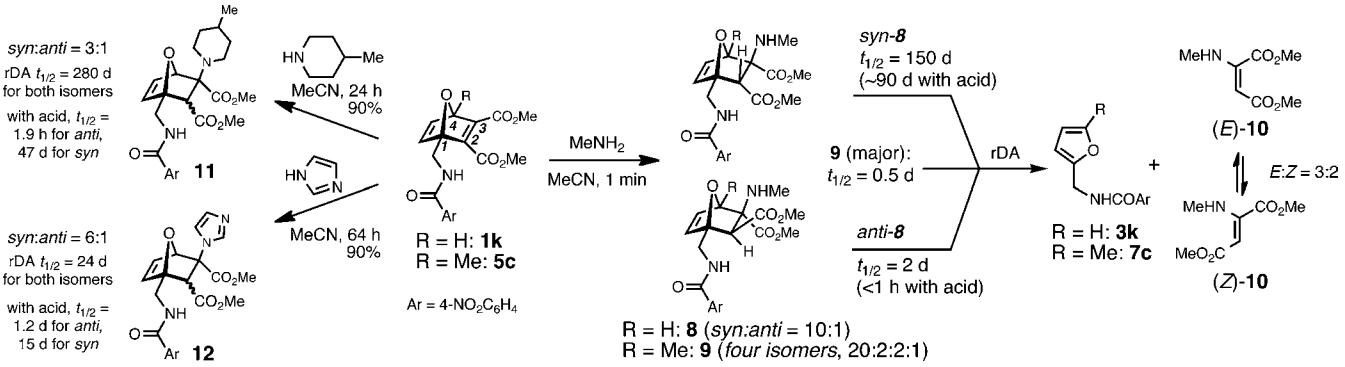
Scheme 2. Addition of Amines to OND Reagents and Cycloreversion of Adducts



case, OND **1k** was found also to be unreactive with triethylamine or 4-dimethylaminopyridine for at least one week in acetonitrile, highlighting the greater stability of ONDs to hard nucleophiles in organic solvent than to soft nucleophiles such as thiols and phosphines.

Synthesis and Stability of OND–Amine Adducts. The reactions of nontertiary amines with ONDs were studied in closer detail to model the potential Michael addition of proteinaceous nitrogen nucleophiles (Scheme 2, Table S1). Adducts **8** and **9** were obtained from reactions of ONDs **1k** and **5c** with 1.5 equiv of 40% aq. methylamine. In the former case, a 10:1 ratio of *syn* and *anti*-isomers of structure **8** was obtained with amine addition exclusively at C3. In the latter case, four isomers for **9** were observed by NMR (*syn* and *anti* structures from addition to C2 and C3), but one of these was dominant, presumably the C3-*syn* adduct.

Substantial differences in stability were observed for these molecules. *Anti*-**8** disappeared within 7 days at room temperature, while *syn*-**8** had a half-life of approximately 150 days. Similar to the thiol adducts, 1,4-bridgehead disubstitution induced much greater rates of retro-Diels–Alder fragmentation, with adduct **9** showing a half-life of approximately 12 h. All adducts **8** and **9** gave the corresponding furans and an equilibrated[12] mixture of vinylogous amides **10** (3:2 *E*/*Z* ratio on average) in quantitative yield as judged by NMR monitoring of the ongoing reactions. Secondary amines 4-methylpiperidine and imidazole reacted much slower than methylamine, again giving the *syn*-adducts (**11** and **12**) as the major products. The *syn*- and *anti*-isomers of **11** and **12** were equally stable in both cases ($t_{1/2} \approx$ 280 and 24 days, respectively). Interestingly, protonation (by addition of 2 equiv of trifluoroacetic acid) of adducts **8** and **11** made them less stable than the parent amines, to a much greater degree for *anti*-**8**•**TFA** ($t_{1/2} <$ 1 h) and *anti*-**11**•**TFA** ($t_{1/2} \approx$ 1.9 h) than for *syn*-**8**•**TFA** ($t_{1/2} \approx$ 90 d) and *syn*-**11**•**TFA** ($t_{1/2} \approx$ 47 d). In contrast, the *anti*- and *syn*-isomers of **12**•**TFA** showed a much smaller decrease in stability relative to the free base ($t_{1/2} \approx$ 1.2 and 15 d, respectively).

Stability Trends. It is apparent that the stability of OND adducts is governed by several stereoelectronic factors. Four trends were identified from the above data.

1. *Thiol adducts (2, 6) undergo retro-Diels–Alder fragmentation up to 15 times faster than amine adducts (8, 9).* These observations suggest transition state stabilization by n→σ^* donation, sulfur being better at this than nitrogen (structure **13**, Figure 1).[13] Aromatic thiols (which degrade rapidly) are likely to favor this interaction, whereas trifluoromethyl groups in place of esters on the electrophile discourage it, making the corresponding thiol adducts up to 10-fold more stable than the analogous diester compounds (**2r**, **2s** vs **2c**). That the attacking heteroatom is an rDA trigger is illustrated by the observation that furan adducts of both dimethyl maleate and dimethyl fumarate are quite stable to rDA cycloreversion even at elevated temperature (at least up to 65 °C).[14]

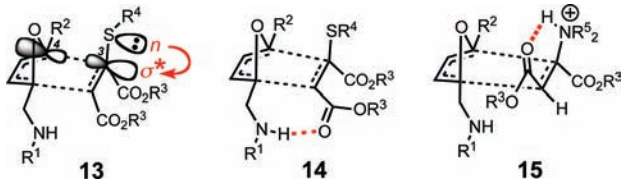


Figure 1. Orbital and hydrogen bonding interactions (in red) that influence the fragmentation rates of OND adducts.

2. Adducts with a single bridgehead substituent (**2**, **8**) are 100- to 300-fold more stable than the ones with 1,4-dialkyl substitution (**6**, **9**). The repulsion from a bridgehead methyl group in **6** and **9** may facilitate the aforementioned hyperconjugative n→σ^* interaction by increasing the C4–C3–X angle. Potential transition state stabilization by the second methyl cannot account for such a drop in stability, since the first methyl group makes the product more stable (stability order: **2b** > **2a** > **6a**).

3. The presence of a stabilizing hydrogen bond between a furfurylamine-derived NH donor and C-2 ester carbonyl acceptor provides up to 5-fold stabilization of the thiol adducts. Calculations suggest that this hydrogen bond is rather weak because of suboptimal geometry (Figure S3). Nonetheless, the rDA rates of thiol adducts **2** are correlated with the strength of the hydrogen bond donating ability of the furfurylamine-derived NH group at position 1 (Table 1). Within the benzamide series (**2k**–**2n**), the stability of thiol adducts decreases along with the decreasing acidity of *para*-benzamide substituent, as reflected in the chemical shift of the amide proton (Figure S2). In a direct test, alkylation of the NH-dansyl group produced a 5-fold destabilization of the corresponding dimethyl ester thiol adduct (**2c** vs **2d**). The much less pronounced 2-fold difference between substituted esters **2u** and **2v** suggests that this stabilization comes at a certain conformational cost (structure **14**, Figure 1).

4. *Cycloreversion is favored by H-bonding within the maleate fragment.* Among amine adducts, the imidazole **12** is the least stable, perhaps consistent with its ability to engage in π→σ^* donation analogous to that shown in **13**, or because of its relatively electron-deficient nature compared to methylamino or piperidino groups. However, amine adducts are very sensitive to protonation in a stereochemistry-dependent

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manner (Scheme 2). Thus, protonation increases the decomposition rate of *anti*-adducts to a far greater degree (20−3600-fold) than *syn*-adducts (1.6−6-fold). We attribute this to the formation of an intramolecular hydrogen bond in the *anti*-amine form that favors a coplanar alignment of ester and olefinic groups (**15**), stimulating cycloreversion. This effect is much less dramatic for **12●TFA**, which is protonated on the sp^2 imidazole nitrogen and therefore cannot engage in the proposed activating H-bonding interaction.

Reactivity with Amino Acids. While we knew OND reagents to be highly selective for thiol-containing substrates such as cysteine and glutathione,[8] some knowledge of their reactivity with the large number of nonthiol nucleophilic residues found on proteins was required. We found that the relative and absolute reactivities of ONDs are similar to those of maleimides. Thus, **1t** reacted with a thiolate anion with an estimated second-order rate constant of 8540 M^{-1} s^{-1} (Table 3) vs a reported value of 14200 M^{-1} s^{-1} for an N-alkylmaleimide.[15] The Michael reaction of tri(glycine), a model for N-terminal protein amine,[16] with **1t** (0.2 M phosphate, pH 7.4, 25 °C) proceeded with k_2 = 0.011 ± 0.002 M^{-1} s^{-1}, again similar to the reported reaction of *N*-ethylmaleimide with N-terminal valine under the same conditions (k_{obs} = 0.006 M^{-1} s^{-1}).[17] *N*-Benzoylhistidine and 6-aminocaproate (model compounds for histidine and lysine) reacted much slower (Table 3). However, when corrected for the pre-equilibrium protonation state of each nucleophile (giving k_{Nu} and k_{rel}(Nu), Table 3), the model lysine amine was found to have an intrinsic reactivity with the OND electrophile 15 times greater than the N-terminal tripeptide. The substantial amine reactivity observed for BSA with OND reagents (see below) may therefore occur at a number of sites, depending on such factors as lysine acidity, intrinsic nucleophilicity, and steric accessibility.[18] As with maleimides, the position(s) of attachment to proteins must be assessed on a case-by-case basis.

Table 3. Reactivity of OND 1t with Model Peptidic Nucleophiles

Nucleophile (pK_a)	k_{obs}, M^{-1} s^{-1} [a]	k_{Nu}, $M^{-1}s^{-1}$ [b]	k_{rel} (Nu) [c]
N-acetylcysteine (9.52)[15]	64.3 ± 5.1	8540	143000
H-GlyGlyGly-OH (8.06)[19]	(10.7 ± 1.2) × 10^{-3}	0.0596	1
6-aminocaproate (10.80)[20]	(0.36 ± 0.03) × 10^{-3}	0.918	15.4
N-benzoylhistidine (7.08)[21]	(0.38 ± 0.04) × 10^{-3}	0.00057	0.0095

[a]Observed second-order rate constant in 0.2 M sodium phosphate, pH 7.4, 25 °C. [b]Reactivity of the nucleophilic form of an amino acid: k_{Nu} = k_{obs}●([NuH^+]/[Nu]), where Nu is the deprotonated (active) form of the nucleophile, the concentration of which was calculated from its reported pK_a. [c]Relative rate constants calculated from the preceding column.

Protein Labeling. The insights gained from the study of model compounds can be useful for designing a degradable material with a desired release profile. In order to demonstrate the potential of oxanorbornadiene reagents as modular cleavable linkers, we labeled the carrier protein bovine serum albumin (BSA) with four fluorogenic dansyl-OND reagents (Table 4). The substituents were chosen to provide a broad range of half-lives for release of cargo by rDA reaction, as inferred from the studies on small molecule adducts. BSA contains 61 amino groups (60 lysines and N-terminus), 17 histidine imidazoles, and one free cysteine residue. Although Cys-34 of BSA is not engaged in disulfide bonding within the protein, it is often blocked as a mixed disulfide with small thiols such as cysteine and can undergo aerobic oxidation to the corresponding sulfenic acid and other products.[22] Thus, the sulfhydryl content in commercial albumin samples usually varies from 0 to 70 mol % relative to protein concentration. In our case, a BSA sample with a thiol content of 11% (as determined by a modified Ellman assay[23]) was reduced by a brief treatment with dithiothreitol (DTT)[24] and purified by size-exclusion filtration. The resulting reduced BSA (rBSA) exhibited a thiol content of up to 95%.

The reaction profiles of BSA and rBSA with OND reagents were biphasic, showing an initial burst of fluorescence followed by a much slower nearly linear rise (Figure 2, Table 4). The magnitude of the initial reaction was found to correspond to the protein thiol content measured by the Ellman assay and thus is assigned to the addition of protein thiol (Cys34) to OND. The second phase of the BSA−OND reaction is proposed to be due to a much slower addition to amine nucleophiles.[16] Initial second-order rate constants of OND−amine reactions were obtained from the fluorogenic reactions with unreduced BSA (Figure 2, dotted lines; Table 4). The values for k_{amines} represent the sum of initial rate constants of all BSA nitrogen nucleophiles with each OND electrophile. For simplicity, the starting amine concentration was set equal to the protein concentration in the extraction of rate constants from the data.

The observed rate constant of reaction of **1t** with BSA was measured as a function of pH from 6.0 to 8.0. Fitting those

Table 4. Reactivity of BSA with OND Electrophiles and Half-Lives of the Resulting Adducts

OND	k_{thiol} ($M^{-1}s^{-1}$) [a]	k_{amines} (M^{-1} s^{-1}) [b]	kinetic selectivity [c]	adducts per BSA [d]	adducts per rBSA [e]	BSA-thiol−OND adduct half-life (days) [f]	BSA-amine−OND adduct half-life (days) [f]
1t	44.9 ± 1.4	0.926 ± 0.089	48 ± 5	0.89 ± 0.11	1.57 ± 0.23	0.55 ± 0.08	6.7 ± 0.3
1u	27.7 ± 1.9	0.823 ± 0.087	34 ± 4	0.79 ± 0.11	1.42 ± 0.41	0.48 ± 0.06	6.9 ± 0.4
1v	15.4 ± 0.6	0.403 ± 0.048	38 ± 5	0.47 ± 0.11	1.18 ± 0.20	0.16 ± 0.01	4.5 ± 0.3
5b	10.9 ± 0.5	0.146 ± 0.026	75 ± 14	0.22 ± 0.12	0.48 ± 0.05 [g]	0.03 ± 0.01	0.41 ± 0.04

[a]Observed initial second-order rate constant for reaction of rBSA thiol with OND reagents. [b]Observed initial rate constant for secondary reaction with amines on BSA; starting amine concentration was set equal to protein concentration. [c]Rate ratio k_{thiol}/k_{amine}. [d]At 2 h with unreduced BSA. Calculated from the ratio of [dansyl] (determined by absorbance at 332 nm) and [protein] (determined by Bradford assay), normalized to initial [OND]. [e]At 2 h with reduced BSA (thiol content of 65.0 ± 1.0 μM by Ellman's assay). Calculated as for (d). [f]At 37 °C, calculated from first-order rate constants for the rDA fragmentation of rBSA-OND adducts by extraction of liberated dansyl-furan at various time points and monitoring remaining aqueous fluorescence. [g]This value is lower than expected because of simultaneous rDA cleavage during the analysis procedure.

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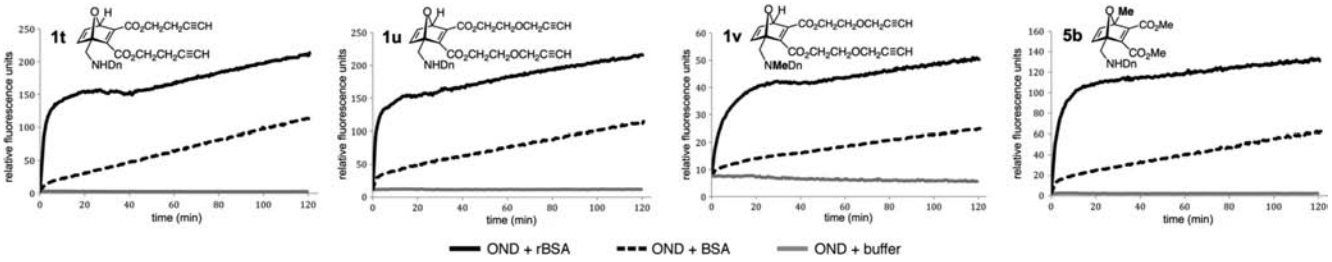
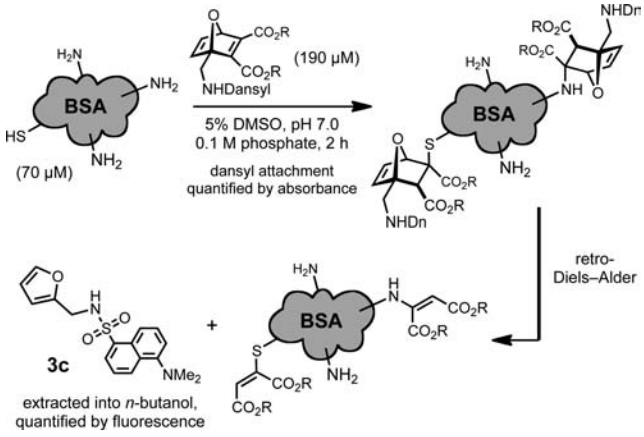


Figure 2. Labeling of BSA and rBSA (70 μM) with the indicated fluorogenic OND reagents (190 μM); pH 7.0, 23 °C. Concentration of active thiol of rBSA for reactions with **1t**, **1u**, **5b** = 65 μM; for reaction with **1v** = 40 μM.

rates to the calculated protonation state of a single amine nucleophile gave an apparent pK_a of 7.9 for this reaction site, a value well within the possible range of lysine side chain pK_a (Figures S4 and S5).[18] The precise locations of OND-amine labeling on BSA were not determined.

Release from BSA. Tests of retro-Diels–Alder release from BSA were performed as shown in Scheme 3. The protein was

Scheme 3. BSA Labeling and the Retro-Diels–Alder Breakdown of the Labeled Protein



labeled with a moderate excess of fluorogenic OND reagents under conditions designed to promote the complete alkylation of available thiol and a modest level of reaction with protein amino groups. After removal of excess OND reagent by organic extraction, labeling yields were estimated by measuring the dye absorbance of aliquots of the aqueous layer under denaturing conditions. The release of attached dansylfurfurylamine **3c** from nondenatured BSA at 37 °C was then established by removal of aliquots and extraction with *n*-butanol, followed by fluorescence spectroscopy of the aqueous layer.

The resulting release profiles, likewise found to be curved (Figure 3), were assigned to two first-order processes as expected from the adduct half-lives summarized in Table 4. Thiol adduct half-lives were calculated from the first-order rate constants determined from the slope of the steeper linear portion of these graphs, corrected for the rates of amine adduct release that contribute slightly to the observed data. Amine adduct half-lives were independently measured from corresponding experiments using unreduced BSA (Figure S6). Although we have not measured the rate of degradation of amine adducts as a function of pH, the release is expected to be pH-dependent. The pK_a of adducts like **8** can be quite low due to pendant ester groups,[25] suggesting that both the fast-

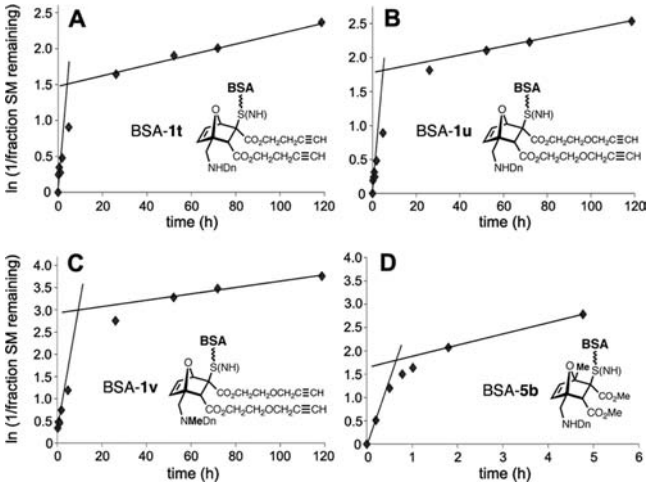


Figure 3. Kinetic profiles for retro-Diels–Alder fragmentation of rBSA-adducts of the indicated ONDs. Straight lines mark the linear approximations for the two first-order processes (thiol and amine adduct release) that are assumed to contribute to the overall reaction.

degrading protonated form and slow-degrading free base form are accessible at physiological conditions.

The results, summarized in the last two columns of Table 4, show that (a) the bridgehead disubstituted case **5b** provided the shortest-lived adducts, (b) sulfonamide alkylation led to a decrease in thiol adduct half-life (**1u** vs **1v**), and (c) BSA-amine adducts were generally found to be more stable than BSA-thiol adducts. Each of these findings correlated well with the observations made above for BME adducts.

We also examined the stability of a representative BSA-**5b** conjugate at 23, 30, and 37 °C, showing the thiol adduct to be 4.5 times more stable and the amine adduct(s) to be 11.6 times more stable, at room temperature vs physiological temperature (Table S5). Arrhenius activation energies of 20 ± 1 and 32 ± 3 kcal/mol were estimated for thiol- and amine-adduct cycloreversion, respectively. For practical purposes, the more stable of the BSA–OND adducts may be stored in solution at reduced temperature for extended periods of time without significant fragmentation.

OND reagents can also be used to selectively label serum albumins in complex mixtures. Reagent **1t** (75 μM) was incubated with newborn calf serum (25 ± 2 μM in thiol by Ellman–Wilson assay) at 37 °C. Electrophoretic analysis after 2–30 min of labeling revealed a major fluorescent band near 60 kDa corresponding to OND-labeled BSA (Figure 4, top). Heating the sample under conditions known to effect the rDA cleavage of OND–thiol adducts (80 °C, 5 min) resulted in only partial loss of fluorescence, showing that amine labeling occurred to a significant extent (as observed for pure BSA)

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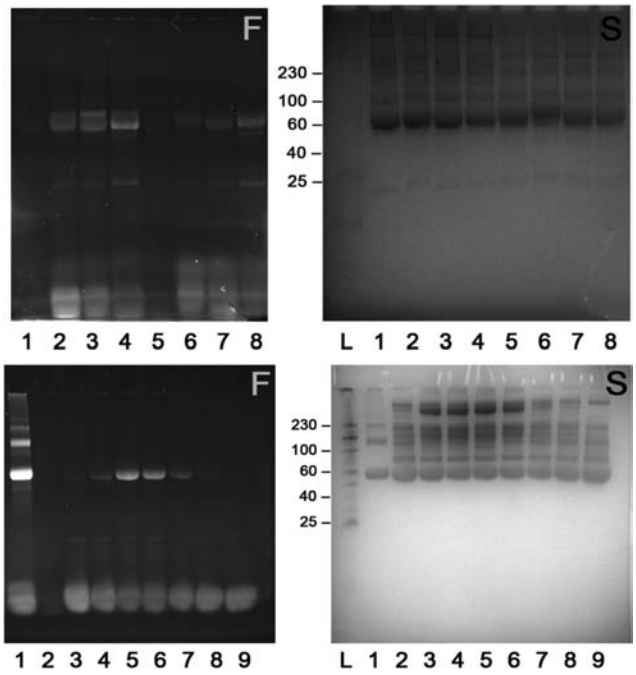

Figure 4. (Top) Gel electrophoresis (1 mM BME, 1% SDS) of newborn calf serum labeled with OND **1t**. Lane 1−4 = labeling with **1t** for 0 (no OND), 2, 6, and 30 min, quenched with BME; lanes 5−8: samples from lanes 1−4, heated for 5 min at 80 °C. (Bottom) Gel electrophoresis (1 mM BME, 1% SDS) of human plasma labeled with OND **5b**. Lane 1 = prelabeled BSA; lanes 2−5: human plasma labeled with **5b** for 0 (no OND), 2, 6, and 30 min; lanes 6−9: human plasma labeled for 30 min, quenched with BME, incubated for 1, 3, 6, and 24 h at 37 °C, and stored at 4 °C prior to analysis. F = fluorescence imaging, S = stained (Coomassie), L = molecular weight ladder. The fluorescence on the bottom of the gels is from small molecule fluorophores.

and that release from the amine sites was significantly slower. A similar reaction of OND **5b** (75 μM) with human plasma gave much cleaner labeling of serum albumin (Figure 4, bottom), consistent with its much greater thiol content (345 ± 20 μM in thiol). Rapid release of dansyl-furan **7b** was observed upon incubation of the resulting conjugate at 37 °C, consistent with the expected lability of the thiol adduct.

■ DISCUSSION

We chart here the various modes of reactivity of OND reagents with proteins under simulated physiological conditions, focusing on the example of serum albumin attachment and release. Although the kinetic selectivity for thiols is much greater than for amines, we find that amine reactivity can be significant. As demonstrated by successful labeling of thiol-depleted BSA, temporary and reversible blockage of thiols makes it possible to direct the OND labeling to nitrogen nucleophiles.

ONDs are unique among cleavable linkers because the release function is masked within a potent electrophile, and the cleavage conditions are exceptionally mild. Reagents that form mixed disulfides, such as Ellman's reagent and S-thiosulfonates,[26] also combine labeling and release functions, but the resulting disulfides fail to cleave under the nonreducing conditions of human plasma. To the best of our knowledge, no other reaction that forms a covalent bond has been reported in the context of reversible labeling of plasma components.

Several popular protein tagging techniques use strong noncovalent (biotin-streptavidin[27]) or metal−ligand (oligohistidine−metal[28]) interactions. In the latter case, the metal ion is essentially a reversible electrophilic label, cleavable with imidazole. Both tagging methods have been modified to incorporate optical signals.[29] Additional manipulations are required to dispose of such labels, such as reductive cleavage of an electrophilic biotin−diazonium tag,[30] or proteolytic removal of a His tag cleavage with a tag-specific peptidase.[31] Fluorogenic OND labels conveniently combine the electrophile, cleavage, and detection functions. It has been shown that thiomaleate **4** and related thiomaleimide[8] moieties that remain on the protein thiols after rDA cleavage of OND-modified substrates can be cleaved under reducing conditions,[32] thereby rendering the OND modification of protein thiols fully reversible as well.

The OND family of reagents described here allows for the convenient tuning of reaction rate, labeling site, and release rate for the marriage of cargo molecules with serum albumin carrier proteins.[33] By extension, we expect a wide variety of other scaffolds to be similarly addressable. The ability to control the rate of fragmentation by altering the substituents on the OND core and the nucleophile involved in carrier−OND adduct formation provides flexibility in the design of diverse degradable biomaterials.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures; synthesis of complex OND esters (Scheme S1), reaction of ONDs with aromatic thiols and phosphines (Scheme S2), amines (Table S1), UV monitoring the formation of **4** (Figure S1), dependence of stability of **2** on alcohol pK_a (Table S2), data for release rates vs benzamide substituents (Figure S2), calculated structure of hydrogen-bonded thiol adduct (Figure S3), influence of pH on BSA labeling rates (Figures S4, S5, Table S3), relative reactivity of nonthiol residues (Table S4), kinetic traces of rDA fragmentation of labeled BSA (Figures S6 and S7), and ^{1}H and ^{13}C NMR spectra of all new compounds. The material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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