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Assembly/Disassembly of Drug Conjugates Using Imide Ligation

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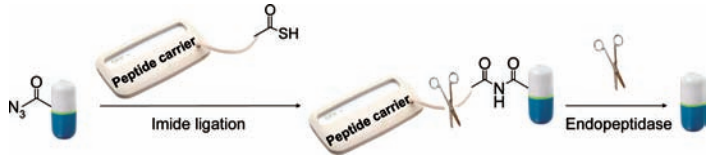
ABSTRACT



A strategy is described that allows the easy assembly and controlled disassembly of drug conjugates. Imide ligation, that is, the reaction of a peptide thioacid with an azidoformate, is used for conjugate assembly. The imide bond participates also with an endopeptidase-triggered cyclization-based disassembly mechanism.

In recent years, tremendous efforts have been focused on the design of novel ways to target drugs to specific cells[1] or organs.[2] Very often, polypeptide conjugates are used for targeting drugs to specific biomarkers.[3] Formation of a covalent bond between the peptide and the drug usually results in a biologically inactive conjugate. Release of the active substance at the delivery site can be triggered by a photochemical,[4] chemical, or biochemical event.[3b] In particular, the design of self-immolative linkers, whose fragmentation is triggered by a specific enzyme, has recently attracted utmost attention.[3b,5] However, preparation of such carrier-linked prodrugs requires multistep procedures and complex protection schemes.

We disclose here a novel strategy allowing the facile assembly/disassembly of peptide−drug conjugates **3** (Scheme 1). The first step involves an imide bond formation between a peptide carrier and a drug by reaction of thioacid **1** with azidoformate **2**. This imide bond plays a key role in the disassembly process leading to drug release. Indeed, enzyme-catalyzed cleavage of the peptide bond between amino acids $n-1$ and n unmasks the intermediate **5** amine group, which cyclizes on the imide to liberate drug **7**. Besides the efficiency of the assembly/disassembly process, the strategy depicted in Scheme 1 shows several other advantages: (1) the half-life of intermediate **5** can be potentially tuned by varying the last amino acid n; (2) peptide thioacids are easy to synthesize using standard solid-phase methods[6] or by hydrothiolysis of thioesters;[7] (3) the cleavage occurs after penultimate amino acid $n-1$, thus offering the possibility to optimize the last residue according to P-1′ endopeptidase specificity.

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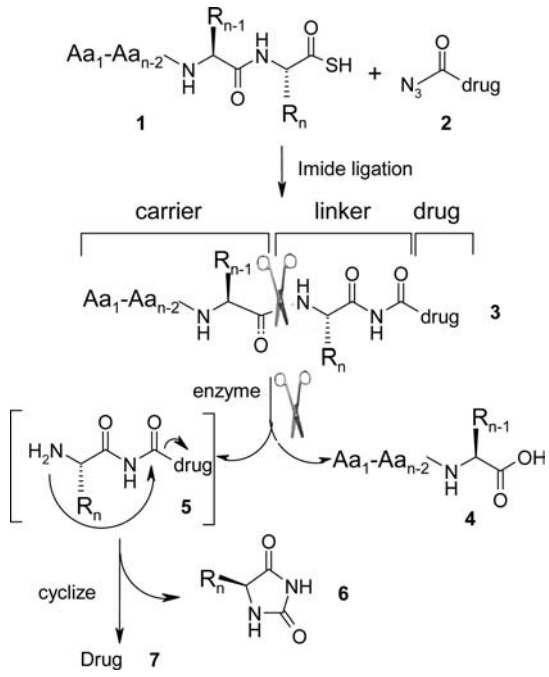
Scheme 1. Assembly of Conjugate **3** Using Imide Ligation: Disassembly Is Triggered by an Endopeptidase

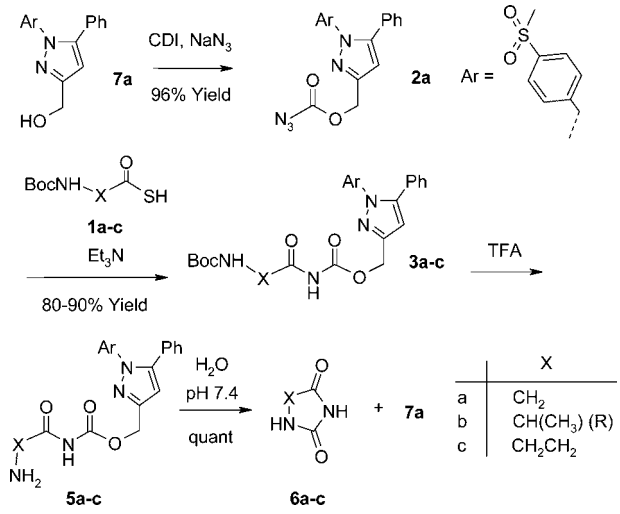


The reaction of thioacids with azides has been studied by several groups.[8] The reaction with electron-rich azides such as alkyl azides leads to the corresponding amides[8g,i,j] and can be promoted by Ru(III).[8h] The reaction is particularly effective with electron-poor azides such as sulfonyl azides[8b−g,k,l] and has been used by Liskamp et al.[8f,k] and others[8b,l] for peptide or protein conjugate synthesis. This chemistry is of great interest for linking molecules to peptide carriers, but the *N*-acyl sulfonamide bond formed in this reaction is stable toward nucleophilic attack and thus cannot be easily cleaved in a subsequent step as needed for our strategy (Scheme 1). Activation of the *N*-acyl sulfonamide toward nucleophiles can be achieved by alkylation[9] but requires an additional step and a protected peptide chain.[10] Alternately, we envisaged the chemoselective formation of an imide bond by reaction of peptide thioacids **1** with azidocarbonyl derivatives **2**. Indeed, besides the potential interest of this reaction for peptide−drug conjugate assembly, the known reactivity of imides toward nucleophiles[11] suggested that the imide group could react with an internal amine, unmasked in a preceding step by a specific biochemical event (Scheme 1).

Examination of the literature revealed that a unique example of a carbonyl carbamate bond formation by reaction of thiobenzoic or thioacetic acid with azido benzylformate was reported by the group of Williams.[8g] The feasibility of reacting chemoselectively azidoformates **2** with polyfunctional thioacids such as peptide thioacids **1** is to be established.

In this proof-of-concept study, cyclooxygenase (COX) inhibitor **7a** (Scheme 2) was used as the drug model (see Supporting Information). COXs are involved in a number of diseases.[12] In particular, COX-2 is overexpressed in prostate cancer[13] and considered as a molecular target in this disease.[14] **7a** features an alcohol group, which was used for attaching the azidocarbonyl moiety.

Scheme 2. Imide Ligation and Rearrangement Using Model Thioacids **1a−c**



In a preliminary approach, we have examined the reaction between model amino thioacids **1a−c** and azidocarbonyl derivative **2a**. Reaction in *N*,*N*-dimethylformamide in the presence of triethylamine successfully afforded imides **3a−c** in excellent yield, which were subsequently used to explore the cyclization-based disassembly step (Scheme 2). To this end, the amino group of **3a**,**c** was deprotected in TFA to provide

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5a,**c** quantitatively. Then, **5a**,**c** were dissolved in aqueous buffer at pH 2.5 or 7.4 and analyzed by RP-HPLC (Figure 1). Both

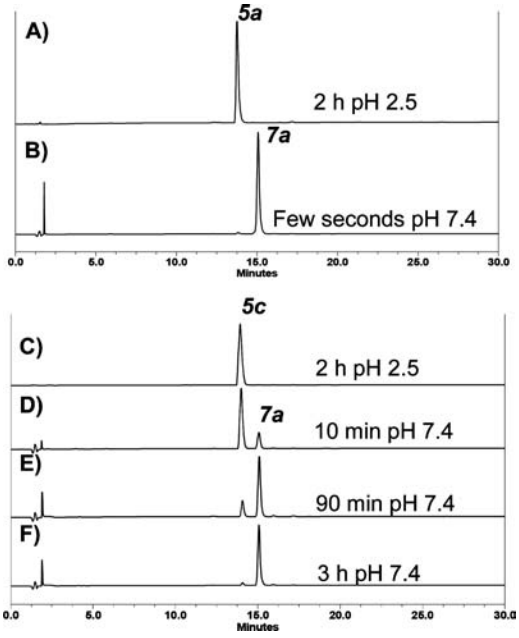
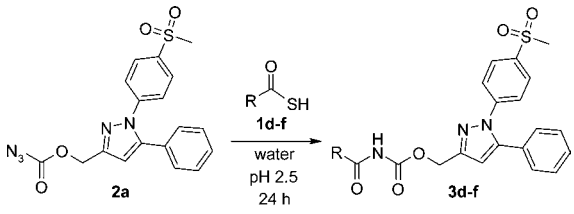


Figure 1. Rearrangement of imides **5a** (A,B) or **5c** (C−F). RP-HPLC analysis of the reaction mixture (detection at 215 nm, C18 Nucleosil column).

compounds **5a** and **5c** were stable in water at pH 2.5 (**5a**, Figure 1A; **5c**, Figure 1C) due to protonation of the amine. Importantly, at pH 7.4 used hereinafter for endopeptidase-triggered disassembly of conjugates **3e**,**f** (Scheme 3, Figure 2), rearrangement

Scheme 3. Synthesis of Peptide Imides **3d**−**f**



R		isolated yield
H-ILKEPGHV	d	62%
H-ANIQR	e	50%
Ac-ISSGY	f	28%

of both imides **5a**,**c** induced release of **7a**. For **5a**, rearrangement was complete after a few seconds only (Figure 1B), whereas about 3 h was necessary for imide **5c** to rearrange (Figure 1D−F).

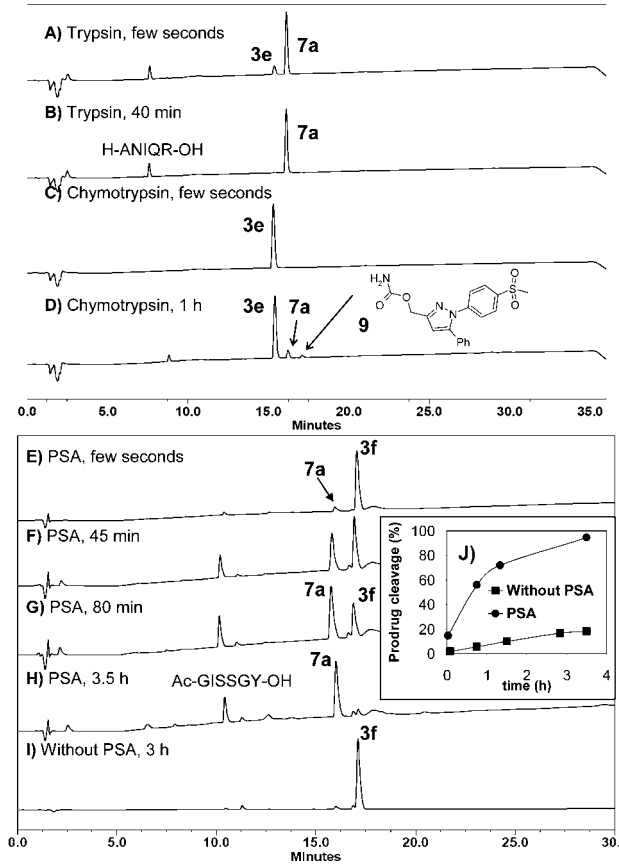


Figure 2. Unmasking of the drug using an endopeptidase (PBS pH 7.4, 37 °C). Conjugate **3e** (40 μM) was incubated with trypsin (A,B) (40 ng/mL) or chymotrypsin (C,D) (40 ng/mL). Conjugate **3f** (40 μM) was incubated with (E−H) (10 μg/mL) or without (I) PSA. RP-HPLC analysis of the reaction mixtures on a C18 Nucleosil column (215 nm).

Imide **5b** derived from alanine rearranged as rapidly as the glycine analogue **5a** (see Supporting Information). Careful LC-MS analysis of the reaction mixture showed the exclusive formation of hydantoin **6b** and **7a**. The potential hydrolysis products of imide **5b**, such as alanine or alanine amide, were not observed.[15] Moreover, thorough NMR analysis of the same reaction mixture showed the formation of hydantoin **6b**[16] and of **7a** in a 1/1 molar ratio. Taken together, these data support the cyclization mechanism depicted in Scheme 1. These results also show that the nature of the amino acid involved in the imide bond influences the rate of cyclization and thus of drug liberation.

We next explored the usefulness of imide ligation for the chemoselective and racemization-free modification of unprotected peptides (Scheme 3). Azidoformate **2a** can potentially react with α or ε-amino groups within peptides to give carbamate derivatives. One way to avoid this side reaction is to work at acidic pH, thereby allowing amine protection by protonation. In this context, peptide thioacid **1d** featuring a free N-terminal amino group and a Lys residue was reacted

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with compound **2a** in water at different acidic pH values. Interestingly, the reaction proceeded efficiently and chemoselectively at pH 2.5 within 24 h and permitted isolation of conjugate **3d** in good yield. These experimental conditions appeared optimal since the reaction rate and purity of the crude product diminished by increasing the reaction mixture pH (see Supporting Information). Importantly, the ligation proceeded without racemization of the C-terminal Ala residue (0.28% D-Ala by GC-MS).

The data presented here show that imide ligation is chemoselective, racemization-free, gives good yields using simple reaction conditions in water, and requires only readily available starting materials. Moreover, dinitrogen sulfide, formed as a byproduct of this reaction,[8a,d,g] is highly unstable and decomposes into molecular nitrogen and elemental sulfur,[17] which are nontoxic. Besides its utility for the facile assembly/disassembly of conjugates as shown here, we believe that imide ligation is a valuable chemical tool that will enrich the ligation repertoire available for assembling complex peptide scaffolds.[18]

The successful chemoselective and racemization-free assembly of conjugates **3** sets the stage for studying drug release using endopeptidases. To this end, conjugate **3e** featuring an Arg residue in the penultimate position was synthesized as described before and incubated with trypsin, which cleaves after Arg/Lys residues (Figure 2A,B). Chymotrypsin, which cuts after aromatic amino acid residues, was used in the control experiment (Figure 2C,D). Figure 2A shows that unmasking of **7a** was almost complete after a few seconds of trypsin digestion only. Unmasking of **7a** occurred in the mixture because rearrangement of intermediate **5b** did not occur in the eluent used for RP-HPLC analysis (pH 2). The early eluting peak corresponded, by LC-MS, to peptide H-ANIQR-OH, showing the Arg−Ala peptide bond cleavage by trypsin. Chymotrypsin was unable to induce release of **7a** using the same experimental conditions (Figure 2C). Longer incubation times led to release of a small proportion of **7a** and **9** due to slow hydrolysis or degradation of the imide bond in water (Figure 2D, $t_{1/2} \sim 6$ h).

To illustrate applicability of the strategy to an enzyme of medical interest, we have used a peptide substrate of prostate specific antigen (PSA),[19] an endopeptidase which is relatively specific to prostate tissue and overexpressed in prostate cancer cells. This enzyme has been used by several groups for vectorization of anticancer drugs because PSA is active only in the prostate cells' microenvironment.[20]

The sequence of peptide imide **3f** (Figure 2) was chosen according to the known substrate specificity of PSA.[21] Incubation of imide **3f** (40 μM) with PSA (10 μg/mL) in PBS buffer at 37 °C led to successful unmasking of **7a** ($t_{1/2} \sim 40$ min) and to concomitant formation of peptide Ac-GISSGY-OH, in a similar manner as in the experiment using trypsin and peptide **3e**. The rate of hydrolysis in the absence of PSA was 22 times lower ($t_{1/2} \sim 15$ h), in accord with the relative stability of acyclic imides in water at neutral pH.[15]

In summary, the data presented in this communication show that the reaction of peptide thioacids with azidoformates (i.e., imide ligation) allows the chemoselective and racemization-free assembly of the peptide−drug conjugates. Moreover, we show that the disassembly of the conjugate can be triggered specifically by an endopeptidase, which generates an internal amine able to cyclize on the imide through a five-membered ring intermediate. The amino acid residue engaged in the imide bond has been shown to influence the rate of cyclization and thus of liberation. The assembly/disassembly strategy or imide ligation chemistry reported in this study should find many interests in different fields of research and, in particular, in the design of biologically relevant conjugates.

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Supporting Information Available: Experimental procedures and characterization data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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