Semisynthetic Nanoreactor for Reversible Single-Molecule Covalent Chemistry

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Supporting Information

ABSTRACT: Protein engineering has been used to remodel pores for applications in biotechnology. For example, the heptameric α-hemolysin pore (αHL) has been engineered to form a nanoreactor to study covalent chemistry at the single-molecule level. Previous work has been confined largely to the chemistry of cysteine side chains or, in one instance, to an irreversible reaction of an unnatural amino acid side chain bearing a terminal alkyne. Here, we present four different αHL pores obtained by coupling either two or three fragments by native chemical ligation (NCL). The synthetic αHL monomers were folded and incorporated into heptameric pores. The functionality of the pores was validated by hemolysis assays and by single-channel current recording. By using NCL to introduce a ketone amino acid, the nanoreactor approach was extended to an investigation of reversible covalent chemistry on an unnatural side chain at the single-molecule level.

KEYWORDS: nanoreactor, single-molecule chemistry, membrane protein, native chemical ligation, unnatural amino acid, protein semisynthesis

α-Hemolysin (αHL) is a pore-forming toxin secreted by Staphylococcus aureus. The pore contains seven subunits, and each subunit comprises 293 amino acids. Use of the heptameric αHL protein pore as a nanoreactor has proved profitable in studies of covalent chemistry at the single-molecule level. For example, the nanoreactor approach is advantageous because large, potentially interfering, fluorescent probes are not required. When a molecule undergoes a chemical reaction on the inner wall of the transmembrane β barrel of the αHL pore, the current carried by ions flowing through the pore is perturbed. Hence, individual reaction steps, including those that are not rate-limiting and therefore not detectable at the ensemble level, are visualized in the microsecond time domain, and the kinetics of each step can be determined. Recently, complex reaction networks and the motion of individual molecular walkers have been examined by this means. However, the chemistry carried out within engineered αHL nanoreactors has until recently been confined to the reactions of thiolates and derivatives of the side chains of cysteine residues. Lately, we expanded the range of chemistry that can be approached by introducing unnatural amino acid side chains into the αHL polypeptide by using native chemical ligation (NCL). By this means, an irreversible reaction of a side chain bearing a terminal alkyne was examined. In the present work, we advance the unnatural amino acid approach by introducing a ketone side chain which allows for observation of reversible chemistry. We also describe truncated pores made by the NCL approach.

RESULTS

General Approach to Two-Fragment Ligation. Two-fragment ligations involved the reaction of an N-terminal fragment (NTF) containing a C-terminal “thioester with a C-terminal fragment (CTF) bearing an N-terminal cysteine (N-Cys). The NTF coding sequence was fused in-frame with DNA encoding a Mycobacterium xenopi DNA gyrase A (Mxe GyrA) intein–chitin binding domain (CBD), that is, NTF–intein–CBD, in the pTXB3 plasmid (New England Biolabs). After expression of the protein in Escherichia coli, the NTF–thioester was cleaved from the intein–CBD with sodium 2-mercaptoethanesulfonate (MESNa), while the rest of the chimera remained bound to chitin beads. A CTF with an N-Cys can be generated by cleavage of a precursor fusion protein with a site-specific protease. However, this method may not work
Preparation of αHL pores. (A) αHL monomers were synthesized by native chemical ligation from two fragments (NTF and CTF) expressed in E. coli. Folding was performed by reducing the concentration of the denaturant (8 M urea) present during the purification of the synthetic monomers (SM). (B–D) Characterization of the synthetic αHL monomers by LC-MS. (B) SMΔ5: [M + H]+ = 34 983 (observed mass, obs), 34 981 (calculated mass, calcld). (C) SMΔ6–113F: [M + H]+ = 33 908 (obs), 33 907 (calcld). (D) SMΔ5–113F: [M + H]+ = 33 924 (obs), 33 923 (calcld). (E) Hemolysis assays (see Supporting Information, Experimental procedures). The decrease in light scattering over time was recorded in a microplate reader at 595 nm. WT αHL monomer (row 1) lysed rRBCs, whereas TBMΔ6 (row 3) did not due to its truncated β barrel. Similarly, the full-length synthetic αHL monomer SMΔ5 (5.9 μg mL−1, in well 1) lysed rRBCs, whereas SMΔ6–113M (7.4 μg mL−1) and SMΔ6–113F (7.8 μg mL−1) did not. WT and TBMΔ6 monomers were produced by IVTT. (F) SDS-PAGE gel analysis of WT and synthetic αHL (SMΔ5). Lane 1: molecular markers. Lane 2: radiolabeled αHL monomer (mon) produced by IVTT. Lane 3: radiolabeled WT pores (hep) produced in the presence of DPhPC liposomes (7 mg mL−1). Lane 4: (SMΔ5)pores assembled with purified SMΔ5 in the presence of DPhPC liposomes under the same conditions comigrate with the WT pore. An autoradiogram is superimposed on the Coomassie Blue-stained gel. (G) Heteroheptameric pores. WT αHL (radiolabeled protein) and SM were mixed in various ratios in the presence of rRBCm to yield heteromeric WT,SMα(n = 0–7) pores. The heptameric pores with different numbers of SM were separated by SDS-PAGE based on the different electrophoretic mobilities produced by D8 tails at the C-terminus of SMΔ5. (H) Homohexameric pores formed with SMΔ6–113M (left) and SMΔ6–113F (right). Homomeric pores were prepared in the presence of DPhPC liposomes (10 mg mL−1).

Preparation of Polypeptides for Two-Fragment Ligation. We targeted three αHL polypeptides for semisynthesis by two-fragment coupling (Figure 1A): a full-length αHL monomer and two different truncated barrel mutants (TBMΔ6) (Figure S1A–C). TBMΔ6 forms an αHL heptamer in which amino acids have been removed in pairwise fashion from both of the two β strands contributed by each subunit, resulting in a β barrel shortened by 6 amino acids. TBMΔ6 forms conductive pores despite the short length of the barrel, presumably by inducing the formation of toroidal lipid pores that span the bilayer. Two TBMΔ6α pores were produced by expression in E. coli at residue 113 (Met, Phe) were chosen, as it has been shown that the mutation Met-113→Phe significantly alters the binding kinetics of cyclodextrin adaptors to the pore. 19 Three different NTFs (NTF126 [Ala1–Gly126], NTF113M [Ala1–Met113], and NTF113F [Ala1–Phe113]) each bearing an “thioester at the C-terminus were obtained by thiolyis of the corresponding intein–CBD fusion proteins after expression in E. coli. The pTXB3–NTF126 and pTXB3–NTF113F plasmids were prepared by cloning PCR-amplified DNA, encoding residues 1–126 and 1–113 of αHL, upstream of the intein–CBD codons (Figure S2A,B). pTXB3–NTF113F was prepared by mutagenesis of pTXB3–NTF113M by homologous recombination (Figure S2C). Fusion proteins were produced in E. coli (BL21(DE3), N.E.B) and solubilized from inclusion bodies under denaturing conditions (8 M urea). The NTF-thioesters were obtained by on-column thiolyis of the fusion proteins bound to chitin columns with MESNa (Figure S3). The purified NTF2-thioesters were characterized by LC-MS (Figure S4, NTF126: [M + H]+ = 14 150 (obs), 14 150 Da (calcld); NTF113M: [M + H]+ = 12 838 (obs), 12 838 Da (calcld); NTF113F: [M + H]+ = 12 853 (obs), 12 854 Da (calcld)). The pT7-SC1-NTF126 plasmid was prepared by replacing plasmid DNA encoding residues 1–127 with the codons for Met-Cys as previously reported. 11 To produce CTF127-

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SC1-CTF<sub>Δ114-DH</sub> was prepared by two successive homologous recombination<sup>21</sup> from pT7-TBMΔ6, which encodes TBMΔ6 (Figure S5). The codons for residues 1–113 were removed (retaining the initiator Met), and codons for a D<sub>2</sub>H<sub>6</sub> (DH) tag were added in the first and second rounds, respectively. The two different C-terminal fragments (CTF<sub>Δ127</sub> [Cys<sup>127</sup>-Asn<sup>293</sup>]-D<sub>2</sub>H<sub>6</sub> and CTF<sub>Δ114</sub> [Cys<sup>114</sup>-Asn<sup>293</sup>, ΔPhe<sup>120</sup>-Thr<sup>125</sup>, ΔGly<sup>133</sup>, Ala<sup>138</sup>]-D<sub>2</sub>H<sub>6</sub>) were overexpressed in <i>E. coli</i>. Like the NTFs, the CTFs were obtained from inclusion bodies and, in this case, purified under denaturing conditions (6 M Gu-HCl) by FPLC (AKTA purifier, GE Healthcare Life Sciences) at room temperature by use of the His<sub>6</sub> tag at the C-terminus (Figure S6). The N-terminal Met was found to be absent, and the thiazolidine produced by condensation of the N-Cys with pyruvic acid was removed with 0.4 M HONH<sub>2</sub>·HCl for 4 h at room temperature. The purified CTFs were characterized by LC-MS (Figure S7, CTF<sub>Δ127</sub>: [M + H]<sup>+</sup> = 20 974 (obs), 20 974 Da (calcd); CTF<sub>Δ114</sub>: [M + H]<sup>+</sup> = 21 214 (obs), 21 212 Da (calcd)).

### α-Hemolysin Polypeptides by Two-Fragment Ligation

We ligated NTFs and CTFs (Figure S1) in NCL buffer [200 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6.9), 6 M Gu-HCl, 200 mM 4-mercaptoophenylacetic acid (MPAA), and 50 mM tris(2-carboxyethyl)phosphine (TCEP)] to make three different αHL constructs. In each case, an NTF and a CTF were mixed and the buffer was exchanged by dilution—concentration cycles with a centrifugal filter. NTF<sub>Δ27</sub> (∼0.5 mM) and CTF<sub>Δ127</sub> (∼0.5 mM) were coupled to produce the full-length αHL synthetic monomer (SM<sub>Δ</sub>). NTF<sub>Δ113M</sub> (∼0.6 mM) and NTF<sub>Δ113F</sub> (∼0.7 mM) were separately coupled with CTF<sub>Δ114</sub> (∼0.8 mM) to yield two different truncated monomer SMs (<sub>Δ</sub>6–113M and <sub>Δ</sub>6–113F). The rate of ligation is highly dependent on the steric properties of the C-terminal amino acid residue of an NTF<sup>22,23</sup> and the concentration of reactants.

The ligation reactions were carried out for 8–12 h at a final concentration of ∼1 mM as previous work<sup>22</sup> had suggested that ligations of NTFs containing C-terminal Ala, Val, Ile, Met, and Phe are completed within 9 h at a final peptide concentration of 1–3 mM. The two-fragment couplings gave SM<sub>Δ</sub>6–113M and SM<sub>Δ</sub>6–113F (Figures S8 and S9) in 48, 46, and 50% yields, respectively.

### Purification, Folding, and Functional Properties of α-Hemolysin Polypeptides

We purified the SMs (SM<sub>Δ</sub>6–113M<sub>α</sub> and SM<sub>Δ</sub>6–113F<sub>α</sub>) by gel filtration in 8 M urea (Figure S8) and characterized them by LC-MS (Figure 1B–D and Figure S8, SM<sub>Δ</sub>6–113M<sub>α</sub>: [M + H]<sup>+</sup> = 34 983 (obs), 34 981 Da (calcd); SM<sub>Δ</sub>6–113F<sub>α</sub>: [M + H]<sup>+</sup> = 33 908 (obs), 33 907 Da (calcd); SM<sub>Δ</sub>6–113M<sub>β</sub>: [M + H]<sup>+</sup> = 33 924 (obs), 33 923 Da (calcd)). We then folded the purified SMs by diluting the 8 M urea in the purification buffer to ∼60 mM and concentrating the proteins using a centrifugal filter (MWCO 3k). The folded monomers were examined for hemolytic activity toward rabbit red blood cells (rRBCs) (Figure 1E). We observed similarities between the synthetic monomers and the WT or truncated αHL monomers produced either in <i>E. coli</i> or by in vitro transcription and translation (IVTT). As expected, only SM<sub>β</sub> exhibited hemolytic activity toward rRBCs.<sup>19</sup> The specific hemolytic activity of SM<sub>β</sub> was HC<sub>50</sub> = 92 ng mL<sup>−1</sup>, which is in the same range as that of WT αHL (HC<sub>50</sub> = 31 ng mL<sup>−1</sup>). To visualize the formation of αHL heptamers, we incubated SM<sub>β</sub> at 37 °C in the presence of liposomes (10 mg mL<sup>−1</sup>, diphytanoylphosphatidylcholine, DPhPC), which produced a new band upon sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) corresponding to the size (∼240 kDa) of the heptamer (Figure 1F, left). We also incubated SM<sub>β</sub> in the presence of rabbit red blood cell membranes (rRBCMs) in different ratios with radiolabeled WT αHL produced by IVTT. The SM<sub>β</sub> oligomerized to form heteroheptamers with...
Figure 3. Preparation of αHL pores containing an unnatural amino acid. (A) An αHL monomer was synthesized from three fragments with a central segment (CSP) bearing an unnatural amino acid with a side chain bearing a ketone group (inset: Un, Fmoc-N^δ-(3-oxobutanyl)lysine, Fmoc-Ket-OH). (B) Characterization of the synthetic αHL monomer (SM_{agon}) by LC-MS. [M + H]^+ = 35109 (obs), 35107 (calc). (C) Hemolysis assay for SM_{ket}. The full-length synthetic αHL monomer [SM_{agon} (0.31 mg mL^{-1})] lysed rRBCs. rRBCs alone (row 6 in Figure 2E) are displayed for comparison. (D) Homo- (left) and hetero- (right) heptameric pores formed with SM_{ket}. The pores were prepared by the same methods described in Figure 1F,G.

different stoichiometries (WT_{7–n}SM_{α}, n = 0–7) (Figure 1F, right). SM_{Δ6–113M} and SM_{Δ6–113F} were incubated with DPhPC liposomes (10 mg mL^{-1}) and oligomerized to form homomeric structures1 (Figure 1G).

Electrical Properties of Two-Fragment Pores and Binding of Cyclodextrins. To examine the electrical properties of the various heptameric αHL pores containing synthetic subunits [WT_{6}SM_{ff1}, (SM_{Δ6–113M})_{7}, and (SM_{Δ6–113F})_{7}], we determined the mean unitary conductance values under defined conditions and measured I–V curves (Figure S10a,b). The conductance values for the αHL pores containing synthetic monomers were similar to that of pores comprising WT αHL subunits produced by IVTT.

To confirm that the transmembrane β barrels of the semisynthetic pores were intact, we evaluated the binding kinetics at the single-molecule level of cyclodextrin molecular adapters (βCD and amβCD (heptakis(6-deoxy-6-amino)-β-cyclodextrin)) with the WT_{6}SM_{ff1} (SM_{Δ6–113M})_{7}, and (SM_{Δ6–113F})_{7}; pores.25,26 It was already known that the homohexamer formed from TBMΔ6/M113F binds amβCD very tightly.25,26 We determined the association and dissociation rate constants (k_{on} and k_{off}) of βCD for the three different protein pores. At least three measurements were made for each construct. βCD blocks the ionic current transiently when it is lodged within the lumen of the αHL pore. The dissociation constants (K_{D} = k_{off}/k_{on}; Figure S11) of βCD for WT_{6}SM_{β} (K_{β} = 14.5 ± 0.4 × 10^{-5} M, k_{on} = 10.0 ± 0.2 × 10^{6} M^{-1} s^{-1}, and k_{off} = 14.5 ± 0.2 × 10^{3} s^{-1}) and (SM_{Δ6–113M})_{7} (K_{β} = 6.5 ± 0.2 × 10^{-2} M, k_{on} = 2.4 ± 0.1 × 10^{5} M^{-1} s^{-1}, and k_{off} = 15.7 ± 0.3 × 10^{2} s^{-1}) were similar to the values obtained in our previous studies for WT_{7}25 and (TBMΔ6–113M)_{7}. The K_{β} (6.1 ± 1.3 × 10^{-3} M) of βCD for (SM_{Δ6–113F})_{7} (Figure S12A) was smaller by 3 orders of magnitude than the K_{β} for the (SM_{Δ6–113M})_{7} pore, which makes sense as it is known that βCD binds more tightly by 3–4 orders of magnitude to a pore formed by the full-length M113F subunit than it does to the WT pore.25 We also analyzed the binding of amβCD to the (SM_{Δ6–113M})_{7} pore (Figure S12B) and found that it remained bound to the pore “permanently” as previously reported for the same truncated pore produced by conventional means.1 The binding kinetics of βCD and amβCD suggest that the semisynthetic protein pores produced by two-fragment coupling, and thereby containing a Cys mutation (S114C), are very similar to the protein pores derived from WT αHL produced directly by IVTT from the corresponding genes.

Two-Fragment Ligation Forms a Native Amide Bond. To verify the existence of a native amide bond formed between Gly126 and Cys127 in SM_{β}, we carried out thiolate chemistry on single WT_{6}SM_{β} pores by using the side chain of Cys-127 generated by NCL. In the absence of methyl-PEG-OPSS (MPO, 5.0 kDa), the open state of WT_{6}SM_{β} had a long duration (>30 min). The addition of MPO (0.1 mM) to the trans compartment at +100 mV generated an irreversible current drop (Figure 2A), due to the formation of a disulfide bond between MPO and the side chain of Cys-127. The pore remained blocked over a range of potentials (−100 to +100 mV), indicating that the current drop is not due to simple clogging of the pore. In the presence DTT (5 mM, both compartments), the open current level was restored (Figure 2B) because the PEG chain was cleaved from the pore.

Three-Fragment Ligation To Form a Ketone-Containing αHL Polypeptide. With NTF_{113M} and CTF_{127}, and a synthetic central peptide, we next carried out three-fragment coupling to construct a full-length αHL monomer containing a ketone group (Figure 3A). The ketone is a versatile functional group in organic chemistry and participates in a large number of reactions.27–30 However, reactions of a ketone have not been observed yet at the single-molecule level. The synthetic
methods used to obtain an unnatural amino acid containing a ketone group are not very efficient, and the techniques used to incorporate a ketone amino acid into the middle of a polypeptide chain are often arduous. We made an unnatural amino acid bearing a ketone (Fmoc-Ket-OH; Fmoc-N6-(3-oxobutanoyl)lysine), Figure 3A, inset) from Fmoc-Lys-OH and N-hydroxysuccinimidyl acetoacetate (NHA). Fmoc-Ket-OH was used for SPPS of a central segment of the polypeptide chain (CSP: Thz114 ThrLeuKetTyrGlyPheAsnGlyAsnVal-ThrGly126-Nbz), such that the Ket side chain would project into the transmembrane β barrel of an αHL pore. CSP was prepared with a C-terminal acylurea (Figures S13 and S14), which yields a peptide arylthioester with 4-mercaptophenylacetic acid (MPAA), accelerating the NCL reaction. We then proceeded to assemble a full-length αHL bearing the ketone group with two sequential NCL reactions (Figure S15). The final product (SMket) was purified (Figure S16) and characterized by LC-MS (Figure 3B and Figure S9D; [M + H]+ = 35,109 (obs), 35,107 Da (calcd)). A hemolysis assay showed that folded SMket (HC50 = 47 ng mL−1) had similar activity to the WT αHL monomer (Figure 3C). SMket also formed homo- and heteroheptameric pores in the presence of liposomes and rRBCm, respectively (Figure 3D).

We determined the mean unitary conductance values for individual WT6SMket1 pores in 1 M KCl and 50 mM Na acetate buffer over a range of applied potentials (−100 to +100 mV) (Figure S18A). The buffer was adjusted to pH 3.4 in anticipation of an acid-catalyzed addition reaction (imine formation) on the ketone side chain (see below). The conductance of WT6SMket1 (0.93 ± 0.10 nS, n = 9) at +100 mV was similar to that of the WT pore (1.07 ± 0.02 nS, n = 9) under the same conditions. We also determined the association and dissociation rate constants at pH 3.4 (k_on and k_off) for βCD binding from the values of the mean dwell times (τ_on and τ_off).
transoximination with HONH$_2$, the current increased from mean reaction time for oxime formation with HONH$_2$. The measured mean lifetimes the O-alkyloxime and the oxime were 52 ± 2 s (n = 79) and 51 ± 2 s (n = 78) (Figure S19), which yield rate constants for transoximation of $k_{\text{ao}} = 10^{-3}$ s$^{-1}$ and $k_{\text{ao}} = 2$ M$^{-1}$ s$^{-1}$, respectively, in 1 M KCl, Na acetate buffer (pH 3.4), at +75 mV, where $k_{\text{ao}} = k_{\text{on}} = 1/\tau_{\text{ox}}$. [MPHA] and $k_{\text{ao}} = k_{\text{off}} = 1/\tau_{\text{ox}}$. [NH$_2$OH].

**CONCLUSIONS**

The ability to introduce unnatural amino acids into the αHL pore has the potential to provide a large variety of reactive side chains for the investigation of single-molecule covalent chemistry. We have previously produced αHL polypeptides with unnatural alkyl and aryl amino acids by using in vitro chemically acylated tRNAs. However, this approach is demanding and often gives poor results when more than one amino acid is introduced. The coupling of polypeptide segments by NCL has been used extensively to produce proteins and is a favorable alternative means to incorporate unnatural amino acids. In the present work, we demonstrate a variety of synthetic protein pores using αHL polypeptides and use the synthetic pore containing a ketone as a single-molecule nanoreactor.

Oxime chemistry was examined in an aqueous environment at the single-molecule level with a ketone-containing αHL pore (WT$_{6}$SM$_{\text{ket1}}$), and the work described here is the first observation of reversible covalent chemistry using an unnatural amino acid side-chain in a nanoreactor. Oxime formation from a ketone proceeds via nucleophilic addition to form a tetrahedral intermediate, followed by the elimination of water. Transoximation also proceeds reversibly through a tetrahedral intermediate that subsequently breaks down to form a new oxime and a hydroxylamine. In our work, no intermediates were observed in both the O-alkyloxime formation by MPHA and the transoximation reaction. Presumably, the lifetimes of the tetrahedral intermediates are too short or the current changes too small to observe under our recording conditions. We obtained rate constants for oxime and O-alkyloxime formation within the pore ($k_{\text{ao}} = 10^{-3}$ s$^{-1}$ and $k_{\text{ao}} = 2$ M$^{-1}$ s$^{-1}$). Earlier determinations in bulk solution are in the range $\sim$1 to 10$^4$ M$^{-1}$ s$^{-1}$ and depend strongly on substituents, solvent, pH, and temperature. In our case, the partitioning of the polymer reactant, MPHA, into the pore must be considered.

We have observed oxime formation by using a semisynthetic pore containing an unnatural amino acid as a nanoreactor. The ketone-containing pore expands the range of covalent chemistry that can be studied by the nanoreactor approach to reversible reactions for which statistically significant data can be acquired rapidly without tedious repeats. Taken together with our recent demonstration of alkyne chemistry, the versatility of the nanoreactor approach is apparent, and we look forward to developing even more ambitious possibilities, such as single-molecule catalysis, which may require the placement of several different unnatural amino acids within a single polypeptide chain.

**METHODS**

**Native Chemical Ligation. Two-Fragment Coupling.** Fifty microliters of CTF (>0.5 mM), from which pyruvate had been removed, was mixed with 50 μL of NTF (>0.5 mM) in 0.4 mL of NCL.
buffer [200 mM NaH2PO4 (pH 6.9) containing 6 M Gu-HCl, 50 mM tris(2-carboxyethyl)phosphine (TCEP), and 200 mM 4-mercaptophenylacetic acid (MPAA)] and concentrated to 100 μL using a centrifugal filter (Amicon, MWCO 3k) at 14,000 g for 20 min. The buffer containing NCL buffer was replaced with NCL buffer by repeated (5 times) dilution and concentration with the same filter. The reaction was allowed to proceed overnight at room temperature.

Three-Fragment Coupling. CTF (0.5 mM), from which pyruvate was removed by passing the mixture through a size-exclusion column (Superdex 200 10/300 GL). The product was analyzed by LC-MS.

N-terminal Thz group was subsequently cleaved by treatment with 0.4 M HONH2 (Superdex 200 10/300 GL). The product was analyzed by LC-MS. The N-terminal Thz group was subsequently cleaved by treatment with 0.4 M HONH2 (Superdex 200 10/300 GL). The product was analyzed by LC-MS.

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Notes
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Three-Fragment Coupling. CTF (0.5 mM), from which pyruvate was removed by passing the mixture through a size-exclusion column (Superdex 200 10/300 GL). The product was analyzed by LC-MS.

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