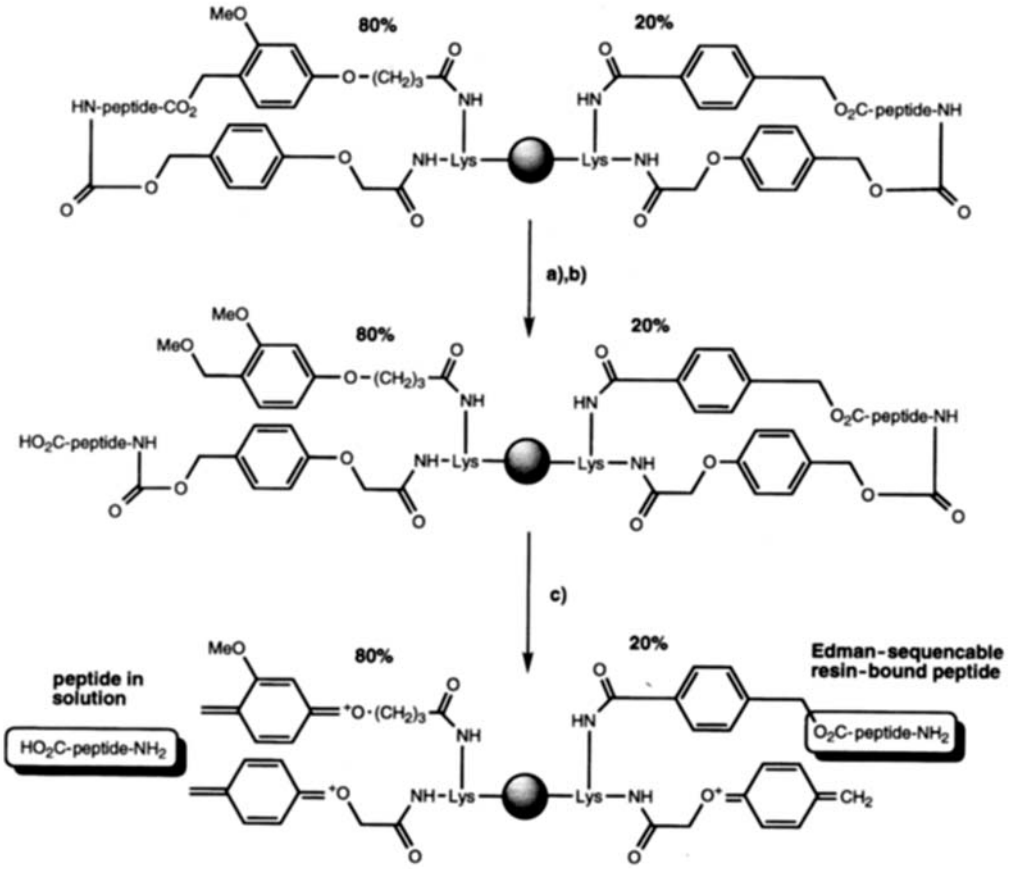

Scheme 2. Coupling two differently labile linkers to the resin prior to library synthesis and inversion, to afford an Edman-sequencable coding strand attached to the resin after peptide cleavage. a) 1% TFA/CH_2Cl_2; b) MeOH, c) 100% TFA.

halomethyl and acyloxymethyl ketones at the C-termini of peptides. This technique thus offers "another end to peptide synthesis".

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[15] Compounds **7a**–**7c** and **8**–**10** all gave a single major peak by reversed-phase HPLC (>80% purity). Retention factors [(elution time)/(solvent front)] were 4.69, 7.20, 7.90, 3.77, 7.12, and 7.79, respectively (4.5 × 250 mm octadecasiliyl column, solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile, gradient elution of 100% A to 100% B over 40 minutes, monitored at 220 nm). ESMS information was entirely consistent with the formation of the expected compounds: *m/z* (%), **7a** 324.2(100) $[MH^+]$, **7b** 437.4(100) $[MH^+]$, **7c** 494.3(100) $[MH^+]$, **8** 394.3(100) $[MH^+]$; **9** 526.3(100) $[MH^+]$; **10** 685.4 (100) $[MH^+]$.

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Protease-Catalyzed Peptide Synthesis from N- to C-Terminus: An Advantageous Strategy**

Frank Bordusa, Dirk Ullmann, and Hans-Dieter Jakubke*

A step-by-step peptide synthesis from the N- to C-terminus cannot be achieved with chemical coupling methods as it risks racemization due to the repeated carboxyl activation procedures. Since the ribosomal synthesis of polypeptides occurs in the N → C direction, the development of an equivalent synthesis strategy should have more than academic interest—especially in light of the fact that even the dominating, stepwise Merrifield

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synthesis starting from the C-terminus cannot completely rule out partial epimerization.[1] Furthermore, it was recently demonstrated by capillary electrophoresis that 0.3–0.4% racemization resulted at every coupling step in the synthesis of a model peptide.[2] The ribosomal peptidyl transferase is only active within the ribosome, which rules out its in vitro application. Nevertheless, the reverse hydrolysis potential of proteases offers an alternative strategy.[3]

This communication introduces an enzymatic method that utilizes the cysteine protease clostripain to demonstrate the principle of a stepwise peptide synthesis in the nonconventional N → C direction with a simple model peptide as an example. To show the advantages of enzyme-catalyzed peptide synthesis, which result from the stereo- and regiospecificity of enzymes and allow mild ecological conditions without the use of side-chain protection, we chose the tetrapeptide H-Lys-Tyr-Arg-Ser-OH, which exclusively consists of trifunctional amino acids (Figure 1).

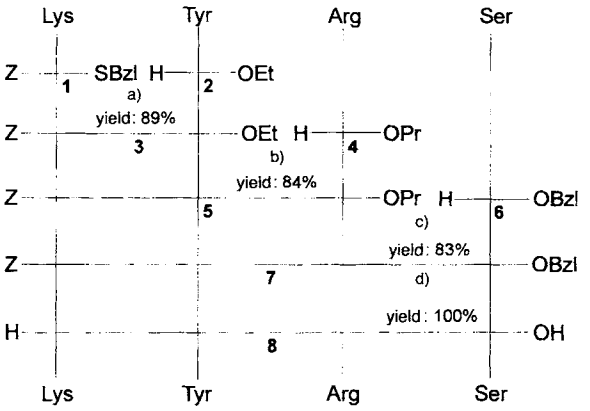

Figure 1. Scheme of the protease-catalyzed synthesis of the tetrapeptide **8**. a) and c) clostripain, 0.025 M borate buffer, pH 8.0, 25 °C, [**1**] = 8 mM, [**2**] = 24 mM, [**5**] = 10 mM, [**6**] = 50 mM; b) chymotrypsin, 0.025 mM borate buffer, pH 9.0, −25 °C, [**3**] = 10 mM, [**4**] = 70 mM; d) catalytic hydrogenation with 10% Pd.

Nucleophilic attack of the unprotected amino acid esters **2**, **4**, and **6** on the C-terminal ester group of the acyl donors **1**, **3**, and **5**, respectively, results in peptide esters **3** and **5**, which serve as acyl donors in the subsequent steps. The enzymes for the coupling steps were chosen based on their substrate specificity, which is known from previous studies of the S- and S′-subsite specificities.[4] The expected yields are mainly dictated by the S′-subsite specificity (nucleophile specificity) of the protease. Most of the studied proteases exhibit a lower nucleophile specificity for amino acid esters than amide and peptide derivatives. Consequently, such nucleophilic amino components result in low yields when used at room temperature.[5] A drastic improvement of the yield can be achieved by performing the enzymatic synthesis in frozen aqueous systems.[6] This was demonstrated for the serine protease chymotrypsin used in this study as well as the cysteine protease papain and the serine protease trypsin.[7] However, even under normal conditions clostripain exhibits an unusually high catalytic efficiency towards nucleophilic amino acid esters that is similar to its efficiency with amide derivatives. This enabled us to perform the clostripain-catalyzed steps a and c (Figure 1) in aqueous solution at room temperature. The chymotrypsin-catalyzed step b resulted in high yields when performed in frozen aqueous conditions at −25 °C. We were able to utilize clostripain as catalyst for two reaction steps due to its restrictive substrate specificity for Arg–Xaa bonds.[8] No hydrolysis occured for the Lys–Tyr bond, which is formed under kinetic control in the first step. This protease is particularly effective in the stepwise enzymatic synthesis of peptides due to its high efficiency for amino acid esters and its unusually broad nucleophilic specificity, which is not limited to proteinogenic L-amino acids.[9]

The coupling steps were optimized in preliminary experiments. We studied the influence of changes in enzyme concentration, pH, type of ester, and, in particular, the most economical ratio between acyl donor and acceptor (shown in Figure 2 for step a). The yield given in Figure 1 is not identical with the

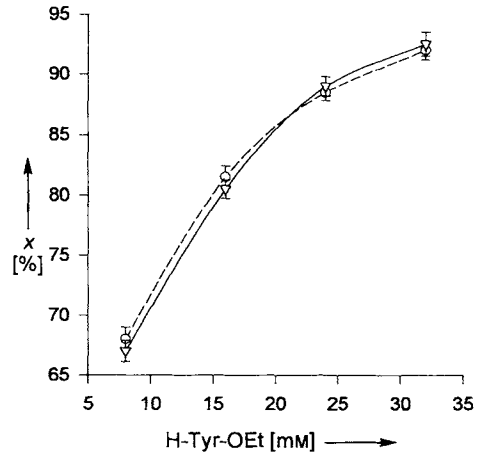

Figure 2. Influence of nucleophile concentration and pH on product yield for the clostripain-catalyzed synthesis of **3**. –▽– pH 8.0; –○– pH 9.0; [**1**] = 8 mM, 0.025 M borate buffer, 25 °C.

maximum yield but represents a compromise between the utilized excess of nucleophile and the resulting yield. Our goal was to achieve yields exceeding 80% for all synthesis steps. Our calculation is based on HPLC analyses as well as preparative yields of peptide products.[10] The error values given in Figure 2 result from the different chromophores that were considered by specific methods of analysis.[11] The analysis of all other reaction steps was straightforward for detection at $\lambda = 280$ nm. Subsequent to step c and final product purification, we removed protecting groups from **7** by catalytic hydrogenation, which occurred easily and completely. The final product **8** (total yield 62%) was characterized and identified with conventional techniques;[12] the chromatographic analysis is shown in Figure 3. The protected peptide ester **7** could be elongated in an elastase-catalyzed reaction in a frozen aqueous system, which should lead to a pentapeptide derivative that could be further extended in subsequent couplings. In principle this enzymatic strategy allows C-terminal modifications of peptides and even proteins

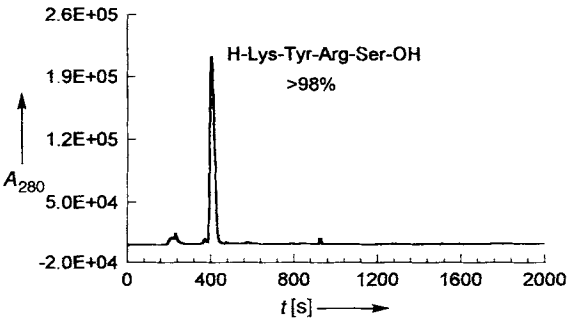

Figure 3. HPLC analysis of **8**; conditions are as given in ref. [10].

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containing chiral amino acids starting from appropriate ester derivatives that could not be achieved by conventional chemical methods.

Limitations for the universal application of enzymatic peptide synthesis result from the restrictive specificity of proteases and from the risk of proteolytic cleavage of the starting materials and the product. Utilization of substrate mimetics that were originally named "inverse substrates" in trypsin-catalyzed peptide syntheses[13] and of proteolytically inactive zymogens[3] in conjunction with the results shown here offer an enzymatic strategy with emerging practical relevance.

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[10] A 4.6 × 250 mm RP18 column Vydac 218TP54 (The Separations Group) was used. Preparative separations were performed with a similar column (22 × 290 mm) and run under isocratic (step b) and gradient (steps a and c) conditions. The products were obtained with a fraction collector, the solvent was removed under vacuum, and the products were lyophilized from aqueous solutions.
[11] Peptide yields were calculated based on the difference of the determined concentration of acid and the small amounts of remaining substrate ester as well as based on the tyrosine content. The indicated yields represent the mean of both determinations, and the error intervals represent their deviations.
[12] H-Lys-Tyr-Arg-Ser-OH·3TFA·4H_2O: elemental analysis calcd for $C_{30}H_{51}F_9N_8O_{17}$ (966.77): C 37.27 H 5.32 N 11.59; found: C 37.14 H 5.11 N 11.21; ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C, TMS): δ = 1.51–1.53 (m, 2H, δ-CH_2, Lys), 1.55–1.62 (m, 2H, γ-CH_2, Arg), 1.62–1.69 (m, 2H, β-CH_2, Lys), 1.70–1.77 (m, 2H, β-CH_2, Arg), 2.92–2.95 (d, 2H, β-CH_2, Tyr), 3.09–3.10 (m, 2H, ε-CH_2, Lys), 3.59–3.63 (t, 2H, β-CH_2, Ser), 4.10–4.12 (m, 1H, α-CH, Lys), 4.30–4.32 (q, 1H, α-CH, Arg), 4.47–4.49 (m, 1H, α-CH, Tyr), 6.64–6.67 (d, 2H, Tyr H_{arom} (3,5)), 7.04–7.07 (d, 2H, Tyr H_{arom} (2,6)), 7.70–7.73 (t, 2H, ε-NH_2, Lys), 7.98–8.00 (d, 1H, NH, Tyr), 8.15–8.20 (d, 1H, α-NH, Arg), 8.53 (s, 1H, OH, Ser), 9.21 (s, 1H, OH, Tyr); MS: m/z = 553 [M^+ + H].
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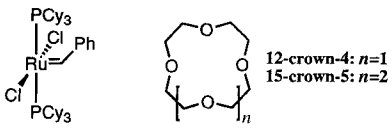
Template-Directed Ring-Closing Metathesis: Synthesis and Polymerization of Unsaturated Crown Ether Analogs**

Michael J. Marsella, Heather D. Maynard, and Robert H. Grubbs*

Ring-closing metathesis (RCM) has become an established protocol for converting dienes into corresponding cyclized adducts.[1] Recently the synthetic utility of RCM was expanded greatly by the development of catalyst **1** (Cy = cyclohexyl), which demonstrates high tolerance to a number of functional groups.[2] As with all ring-forming reactions, the RCM of medium-sized rings is determined by several factors including the kinetics of ring closing, ring strain, and competing metathesis-based polymerization. In some cases, particularly in eight-membered rings, it has proven beneficial to incorporate conformational constraints into the substrates to promote the desired RCM.[3–6] For such systems, it has been proposed that RCM is favored (in part) due to the close proximity of the olefins. To date structurally restricted conformations or hydrogen bonding have been the only reported methods for mediating olefin proximity.[3–6] Herein we present template-directed RCM, an approach that utilizes noncovalent interactions between a diene and an appropriate template to enhance the RCM of linear dienes devoid of other conformational constraints. In addition to the template-directed RCM of **2**, the activity of **1** towards the



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acyclic diene metathesis polymerization (ADMET) of **2**, the ring-opening metathesis polymerization (ROMP) of **3**, and the template-directed depolymerization of polymer **4** to regenerate **3** will be addressed.

Yields of crown ethers derived from linear polyethers can be significantly increased when an appropriate metal-ion template is used to preorganize the substrate and promote the desired macrocyclization.[7] We anticipated that preorganization of linear polyether possessing terminal olefins about a complementary metal ion should provide the conformational restrictions required to enhance RCM. Crown ethers **3** (n = 1, 2) were chosen as the target compounds with which to test this concept due to their similarities to the known ionophores [12]crown-4 and [15]crown-5. As depicted in Scheme 1, the synthesis of **3** by RCM of **2** is subject to competing reaction pathways: 1) formation of the desired RCM product, 2) ROMP of the RCM product, and 3) ADMET of **2** to yield **4**. Side reactions occurring by ROMP and ADMET can be suppressed if the RCM is run at relatively high dilution (about 0.02 M).[4] Therefore, the RCM of **2** at 0.02 M in the presence of the appropriate metal-ion template should favor the desired RCM product **3**.

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