Peptide Synthesis

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Fragment Condensation of C-Terminal Pseudoproline Peptides without Racemization on the Solid Phase**

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Dedicated to Professor Horst Kessler on the occasion of his 70th birthday

Recent advances in the chemical synthesis of proteins have facilitated the study of modifications inaccessible by recombinant methods.[1] The most powerful methods[2] exploit the selective coupling between unprotected or partially protected peptides and peptide thioesters. Despite many improvements in peptide ligation methods the synthesis of the required fragments is mainly performed in a stepwise manner on a solid phase using Boc or Fmoc chemistry, which limits these peptides to about 50 amino acids because of the accumulation of side products. For glycopeptides, in particular, additional difficulties restrict the stepwise approach to shorter peptides. Here we report a convergent fragment-condensation method, which uses segments having a C-terminal pseudoproline.[3] These segments prevent racemization and can be used to overcome the size limitations in the stepwise synthesis of peptides and glycopeptides.

The semisynthesis of bovine ribonuclease C (RNase C) required glycopeptide thioester RNase 26–39, which was prepared on a dual-linker resin using an acetylated N-glycan. However, the stepwise elongation of RNase glycopeptide 26–39 by only a few amino acids resulted in truncated sequences because of acetyl-group migration as well as incomplete deprotections and couplings.[4] In contrast, elongation of glycopeptides with an unprotected carbohydrate may lead to additional O-acylation in each step.[5] We thus considered a convergent fragment condensation[6] on the solid phase for the elongation of glycopeptides with an unprotected sugar to circumvent the above-mentioned side reactions. A serious drawback of the fragment condensation is the racemization of the activated peptides at the C terminus, especially under microwave conditions,[7] which restricts this approach to fragments with a C-terminal glycine or proline[6] or *O*-acylisopeptides.[8] We were inspired by the special properties of commercially available pseudoproline dipeptides, which couple without racemization and significantly improve solubility.[9] Thus, protected fragments with a C-terminal pseudoproline should also couple without racemization. This would provide access to additional safe fragment-coupling sites at serine and threonine, which occur frequently in proteins. The synthesis of the demanding RNase 1–39 glycopeptide thioester was envisioned through three fragment condensations on the solid phase (Scheme 1).

Surprisingly, since Mutter et al. first proposed pseudoproline-based fragment couplings[9] only three brief notes have mentioned this topic.[10] For an expeditious synthesis of the RNase 23–32 fragment **B** (Scheme 1) we attached the pseudoproline dipeptide Fmoc-Lys(Boc)-Ser($\Psi^{Me,Me}$pro)-OH (**1a**) to the trityl resin **2**. After standard elongations (piperidine/NMP; TBTU) fragment **B** was obtained, albeit in an unexpectedly low yield.

Fmoc quantification combined with a quantitative ninhydrin assay showed that the loss of peptide occurred before elongation to the tripeptide. HPLC–MS analysis of the cleavage solution indicated the formation of diketopiperazine **3** (Scheme 2), which was isolated and confirmed by NMR spectroscopy (see the Supporting Information). Despite the resistance of trityl esters to diketopiperazine formation, the *cis*-configured[11] pseudoproline ester **2b** was readily cleaved from the resin by intramolecular cyclization.

Fmoc removal and peptide retention on the resin under varied conditions was quantified by determination of the free amino groups. This required liberation of the dipeptide from the resin since the ninhydrin test thermally induces the formation of diketopiperazine (Scheme S1 in the Supporting Information).

Fmoc removal and subsequent cyclization were dependent on the linker and on the cleavage conditions. The pseudoproline dipeptide was completely cleaved from the trityl linker after 15 min of incubation with 20% piperidine/NMP (Scheme S2 in the Supporting Information). Using the 2-Cl-Trt linker[12] under the same conditions delayed both diketopiperazine formation and deprotection (separately confirmed by LC–MS). Higher concentrations of piperidine (50%) accelerated both reactions, resulting in a narrow time window. When either DBU or 1-methylpyrrolidine[13] were used, mainly diketopiperazine formation was slowed and the best results were obtained with DBU/HOBt.[14]

Fragment **B** was resynthesized on 2-Cl-Trt resin **4** (Scheme 3). After deprotection of **4a** with DBU/piperidine/DMF (2:2:96) the third amino acid was coupled. The following deprotection with 50% piperidine was prolonged in order to fully cleave residual pseudoproline dipeptide as diketopiperazine (see Scheme S2 in the Supporting Information). Methionine residues were replaced with norleucine in order to avoid sulfoxide formation.[4] After peptide elonga-

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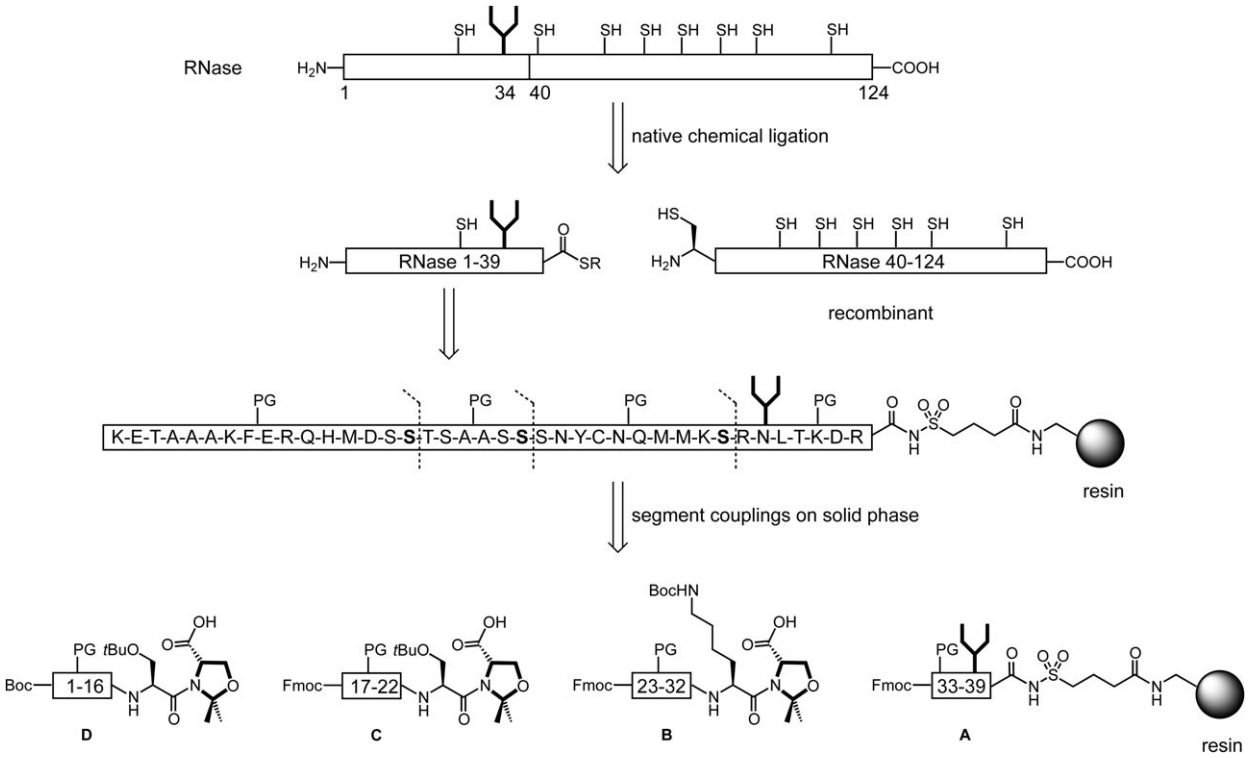
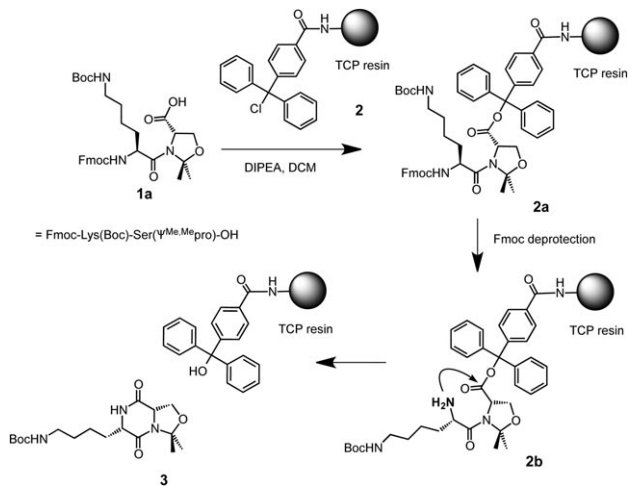


Scheme 1. Retrosynthesis of RNase 1–39 glycopeptide thioester via fragments with a C-terminal pseudoproline. Fmoc = 9-fluorenylmethoxycarbonyl, Boc = *tert*-butyloxycarbonyl, PG = protecting group.



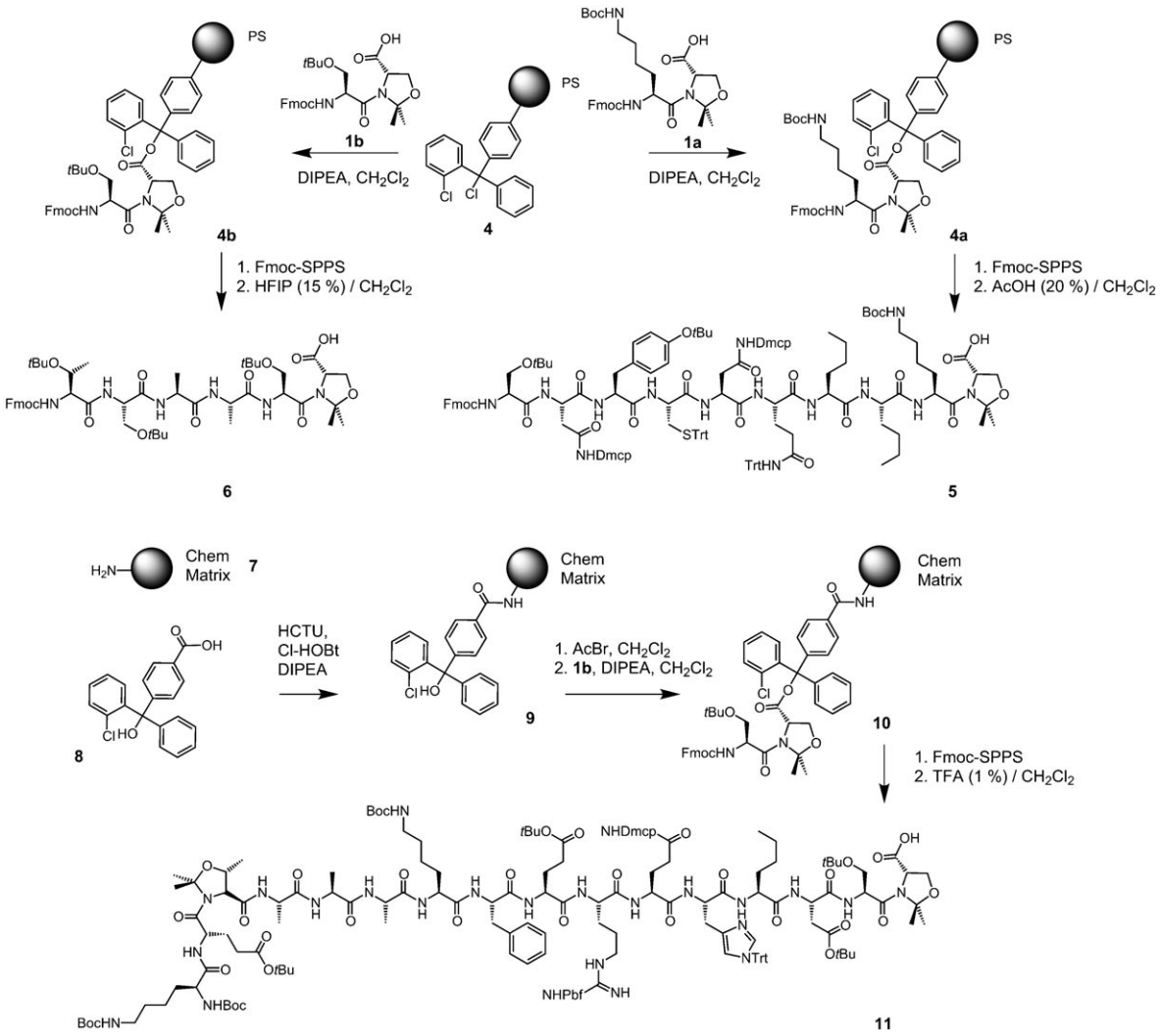
Scheme 2. Tertiary pseudoproline dipeptide esters (**2b**) are cleaved as diketopiperazines. DIPEA = diisopropyl ethyl amine.

tion, fragment **5** was cleaved from the resin using 20% $AcOH/CH_2Cl_2$ without affecting the C-terminal pseudoproline. Purification by gel filtration gave the fragment **5** in 23% yield.

Fragment RNase 17–22 (**6**) was synthesized analogously. A side product with an additional alanine moiety could be removed by flash chromatography to afford fragment **6** in 25% yield.

The 16-mer **D** was first assembled on 2-Cl-Trt polystyrene resin **4**. However, cleavage of the hydrophobic peptide under mild conditions was not efficient, and under more acidic conditions the terminal pseudoproline opened. Since a more hydrophilic 2-Cl-Trt resin was not commercially available, we modified ChemMatrix resin **7** with the 2-Cl-Trt linker **8**, which was activated as a bromide and coupled with **1b**.[15] The deprotection of resin **10** was further improved by washing with 0.5% HOBt after the DBU/HOBt-mediated Fmoc cleavage. The hydrophobic peptide was readily liberated from the hydrophilic resin and was obtained in 40% yield after gel filtration. Only after incorporation of the internal pseudoproline fragment did fragment **11** display good solubility in acetonitrile–water.[9]

The RNase 33–39 fragment **A** was synthesized on a double-linker PEGA resin as described previously.[4] Deacetylation of the GlcNAc moiety with dilute hydrazine hydrate[16] yielded glycopeptide resin **12**. Each of the three segment condensations with **5**, **6**, and **11** was carried out with 2 equivalents of pseudoproline peptide and PyBOP in NMP and reached completion within 1 day at room temperature or within 1 h under microwave irradiation[7,17] at 55 °C (Scheme 4). No epimerization was observed after the segment condensations. The purity of the glycopeptides after the first and the second segment condensation was very high (Figures S5 and S6 in the Supporting Information). Only after the last fragment coupling did the HPLC profile show some truncated sequences caused by impurities of fragment **11** (see Figure S7 in the Supporting Information). The RNase 1–39 glycopeptide **13** was alkylated at the safety-catch linker with TMS-diazomethane.[18] Subsequent thiolysis and deprotection gave the desired RNase 1–39 thioester **15** in 23% yield after HPLC. Analysis of the amino acid components of thioester **15**



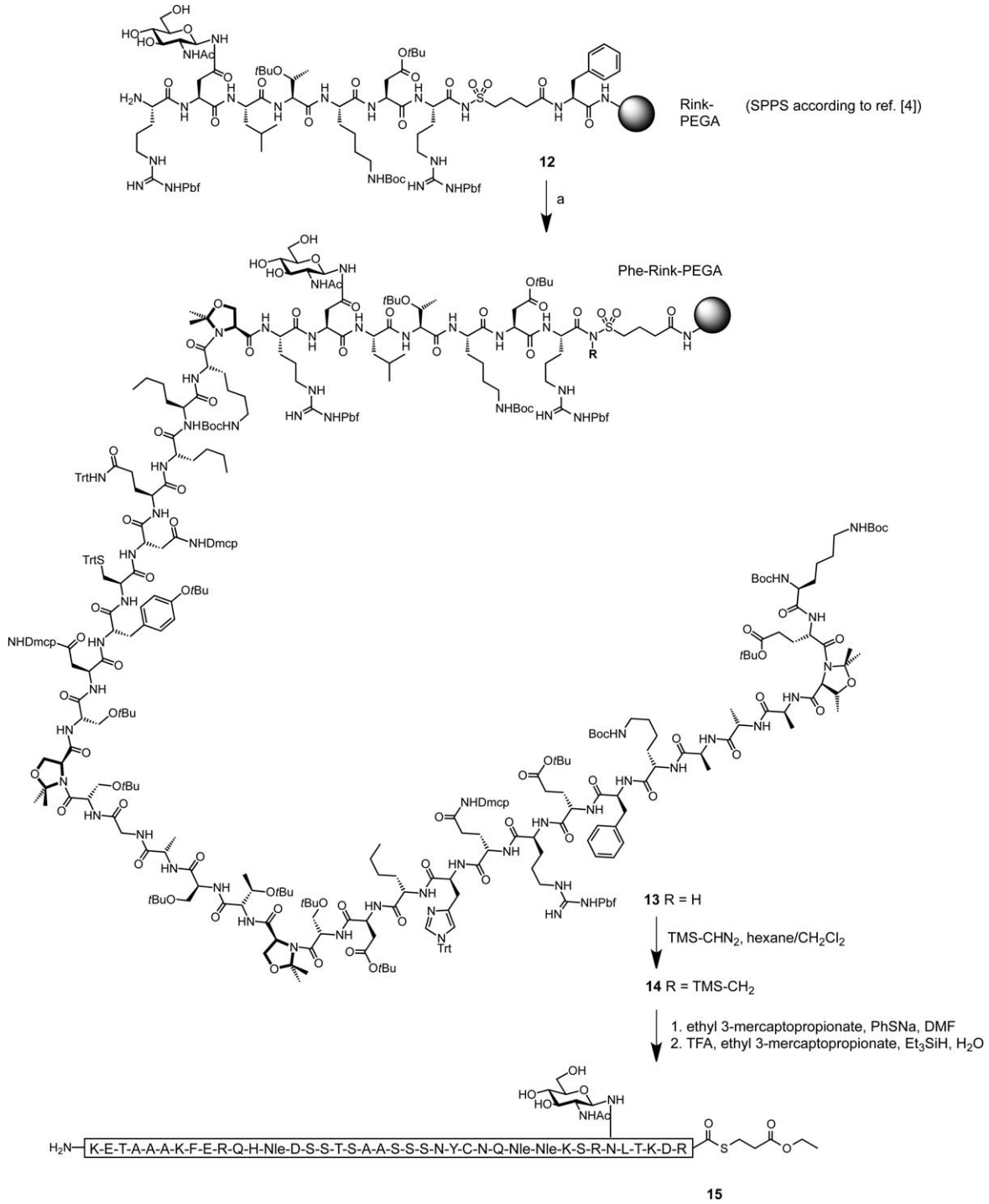
Scheme 3. Synthesis of peptide acids **5**, **6**, and **11**. DMF = *N*,*N*-dimethylformamide, DIC = *N*,*N*′-diisopropylcarbodiimide, HOBt = 1-hydroxybenzotriazole, NMP = *N*-methylpyrrolidone, HFIP = 1,1,1,3,3,3-hexafluoroisopropanol, Trt = trityl, Dmcp = dimethylcyclopropyl, HCTU = *N*-[(1*H*-6-chlorobenzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate-*N*-oxide, Pbf = 2,2,4,6,7-pentamethyl-2,3-dihydro-1-benzofuran-5-sulfonyl, TFA = CF_3COOH.

by GC–MS (see the Supporting Information) detected only 0.1 % of D-Ser, which indicates that the pseudoproline-based segment condensation effectively precludes racemization even under microwave conditions (55 °C). Without the pseudoproline, C-terminal serine is prone to considerable racemization during conventional segment condensation.[19]

The fragment couplings with C-terminal pseudoprolines were investigated on glycopeptides bearing an unprotected oligosaccharide. Glycopeptide **16** was synthesized as described previously (Scheme 5).[4] The segment couplings were carried out at room temperature since elongation with the shortest peptide **6** resulted in significant acylation of the sugar moiety (Figure S10 in the Supporting Information). The transient O-acylation was conveniently removed on the resin[16] with hydrazine hydrate prior to Fmoc cleavage. The unprotected nonasaccharide complicated selective N-alkylation of the safety-catch linker owing to the limited solubility of glycopeptide **17**. Despite incomplete linker activation and some sugar O-alkylation, the 39-mer thioester **19** bearing a nonasaccharide was obtained in 9 % yield after deprotection and HPLC purification.

Because of the difficulties in the activation of the safety-catch linker, an alternative synthesis of thioester **19** was carried out on 2-Cl-Trt ChemMatrix resin **9** (Schemes S5–S7 in the Supporting Information). Here the coupling of the glycosyl asparagine **24** and the segment condensations were nearly quantitative with only some O-acylation (Figures S16 and S18); these products were removed by hydrazinolysis (Figures S17 and S19). The protected glycopeptide RNase 1–39 was released from the resin **23** with dilute TFA and was subsequently thioesterified following an in situ procedure (Scheme S7).[20] After deprotection and purification the yield of isolated RNase 1–39 thioester **19** increased significantly (13 %).

In summary, a linear synthesis of peptide segments with a C-terminal pseudoproline was established on the solid phase. These segments permitted robust epimerization-free fragment condensations at serine and threonine residues, as



Scheme 4. Synthesis of RNase 1–39 thioester (**15**): a) coupling: peptide **5**, **6**, or **11**, PyBOP, DIPEA (2 equiv each) in NMP, μW (55 °C, 2×30 min); deprotection: 20% piperidine/NMP, PyBOP = benzotriazolyl-1-oxytripyrrolidinophosphonium hexafluorophosphate, TMS = trimethylsilyl.

demonstrated with the convergent solid-phase synthesis of challenging glycopeptide thioesters. This method should also facilitate the synthesis of peptides and glycopeptides not directly accessible by ligation strategies.

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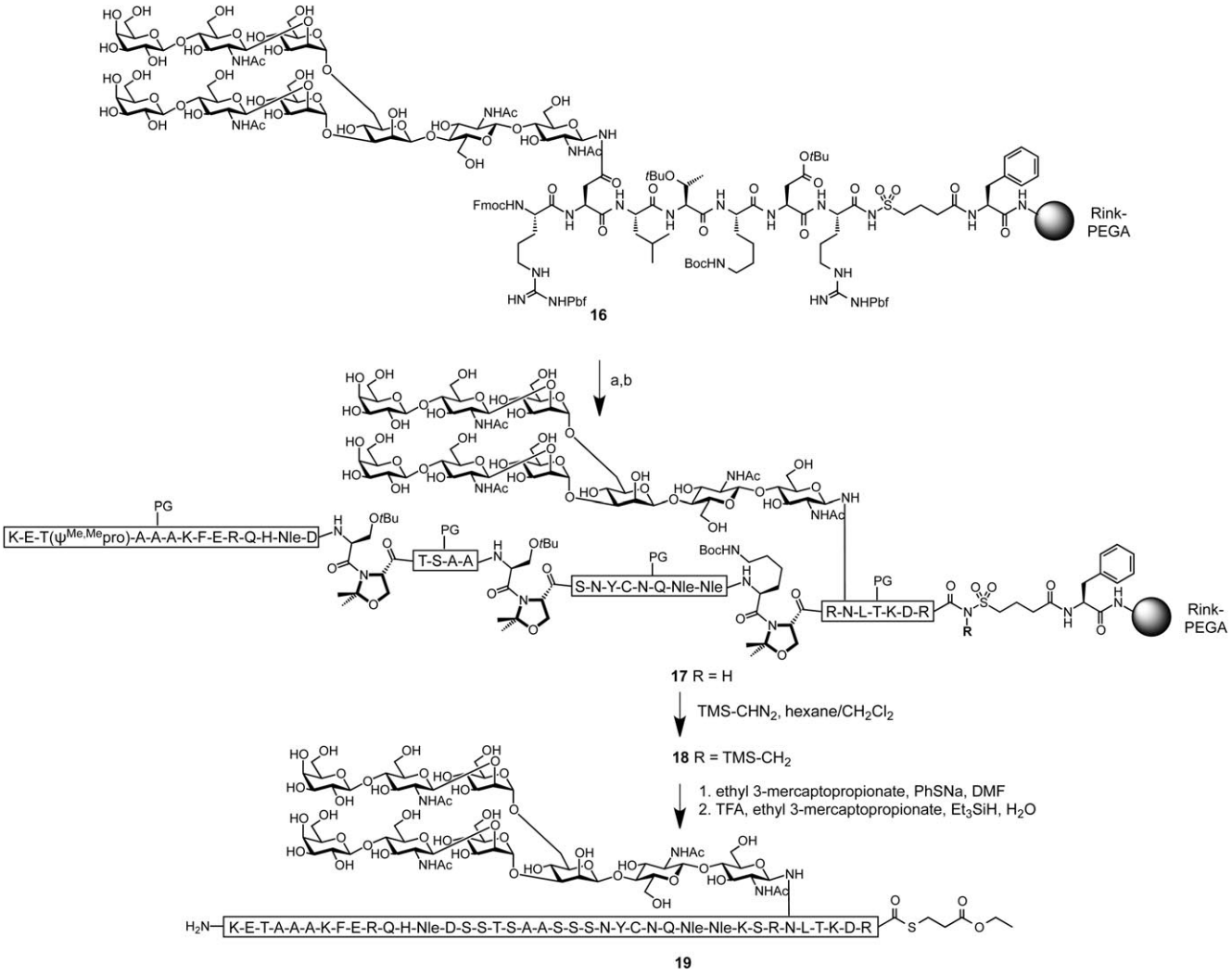

Scheme 5. Synthesis of RNase 1–39 thioester (**19**): a) deprotection: 20% piperidine/NMP; b) coupling: peptide **5**, **6**, or **11**, PyBOP, DIPEA (2 equiv each) in NMP, 24 h.

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