Evaluation of the Oxime Resin Based Segment Synthesis-Condensation Approach Using RNase T_1 as a Model Synthetic Target[1]

Tomikazu Sasaki,*,† Mark A. Findeis,*,‡ and Emil Thomas Kaiser[2]

Laboratory of Bioorganic Chemistry and Biochemistry, The Rockefeller University, 1230 York Avenue, New York, New York 10021

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The *p*-nitrobenzophenone oxime resin has been used as a support for the synthesis of a series of protected peptides based on the sequence of the guanyloribonuclease RNase T_1. As a further test of the utility of oxime resin, these protected peptides have been assembled by a convergent strategy using a combination of solid-phase and solution-phase coupling steps. An analogue in which 11 out of 17 residues in an α-helical region of the enzyme have been changed was also synthesized by changing three of the protected peptides in the synthesis of the native sequence enzyme. Following deprotection with the low-high HF method, the resulting synthetic enzymes had very low specific activity (<0.1%). The appropriate use of oxime resin in peptide synthesis is discussed.

For more than a decade our laboratory has pursued efforts to prepare models of biologically active peptides and polypeptides. Earlier and continuing studies have focused on structural elements such as α-helices and β-strands and have, to a first approximation, been able to neglect tertiary structure.[3] More recently, we have begun to pursue the question of whether the principles developed in the above studies can be extended to folded proteins. Namely, can we replace a naturally occurring secondary structural element with a redesigned element such that the protein can still fold properly and be active? To be able to pursue these questions, we have been developing synthetic methods to allow us to use a flexible chemical strategy for the preparation of compounds to test our hypotheses. DeGrado and Kaiser reported the development of the *p*-nitrobenzophenone polystyrene oxime resin for the synthesis of protected peptide segments in 1980, and the preparation of this resin has since been improved.[4-6] Since that time the oxime resin has been applied to the synthesis of apolipoprotein A-1 model peptides,[7] an *Antennapedia* homeo domain,[8] a hemeprotein model,[9] and a portion of the Cro repressor.[3a] While the earlier work established the general applicability of the oxime resin, we wanted to probe the usefulness of this resin and further develop protocols for its application to peptide synthesis by attempting the synthesis of a small globular protein, specifically the enzyme ribonuclease T_1 (RNase T_1).[10]

Our use of the oxime resin represents an effort to reach an efficient compromise between classical solution-phase peptide synthesis and solid-phase methods. Solution-phase synthetic methods have been successively applied to the synthesis of enzymes,[15] but while every intermediate can be isolated and well characterized, this approach is also time consuming. Solid-phase methods, first developed by Merrifield,[16] are notable for their speed of assembly of long peptide sequences,[17] but microheterogeneity can be difficult to assess in the products, and at times a point is reached in a target sequence beyond which the synthesis cannot be made to proceed.[18] With the oxime resin we can rapidly prepare protected peptide segments to carry on to subsequent solid-phase or solution-phase chemistry.

Results and Discussion

Our initial goal was to use the oxime resin to synthesize a series of small protected peptides corresponding to the entire sequence of RNase T_1. These syntheses proceeded straightforwardly to afford the desired protected peptide

* To whom correspondence should be addressed.

† Present address: Department of Chemistry, BG-10, University of Washington, Seattle, WA 98195.

‡ Present address: Department of Medicine, Harvard Medical School and New England Deaconess Hospital, 50 Binney St., Boston, MA 02115.

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(2) Deceased, July 18, 1988. This paper is dedicated to the memory of Tom Kaiser. T.S. and M.A.F. express their gratitude for the opportunity to work in Professor Kaiser's laboratory.

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(10) RNase T_1 is an attractive target for a synthetic study[11] and subsequent structural modeling. This enzyme is well characterized with a firmly established amino acid sequence,[12a] and a high-resolution crystal structure has been determined.[13] Folding studies have shown that the two disulfides in RNase T_1 can be reduced and the denatured enzyme then refolded and reoxidized to regain essentially full activity.[14] The enzyme is very stable, has a high specific activity, and can be assayed by several methods.[39,40]

(11) The sequence of RNase T_1 was recently revised.[12a] Two attempts[15c,d,17b] have been made to chemically synthesize RNase T_1 according to a wrong sequence.[12b,c]

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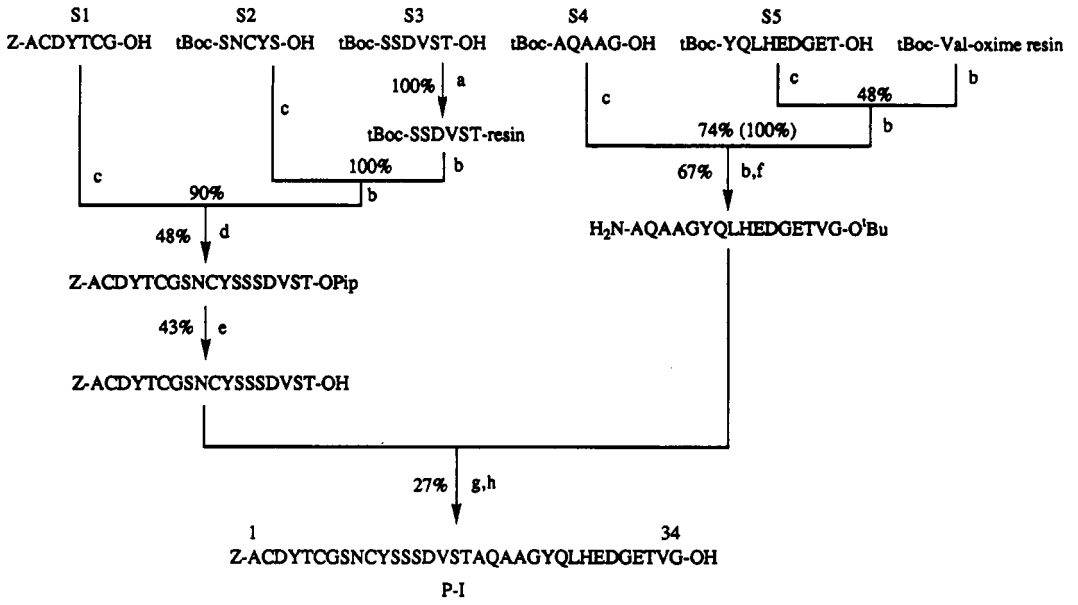
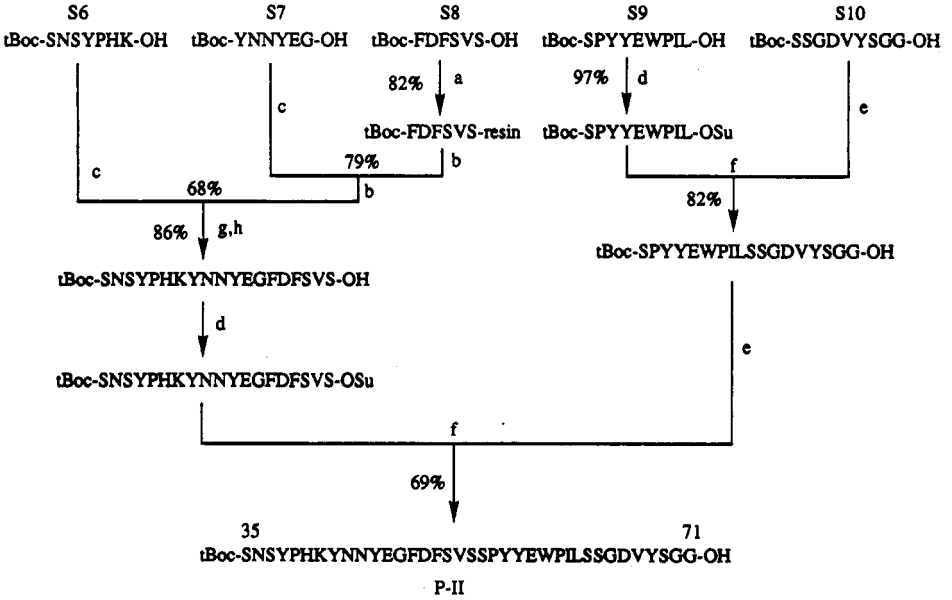
Scheme I[a]



[a] Key: (a) oxime resin, EACNOx, DCC, CH_2Cl_2; (b) 25% TFA/CH_2Cl_2; (c) DCC/HOBt, CH_2Cl_2/DMF; (d) HOPip, DMF/DMSO; (e) $Na_2S_2O_4$, TFA/DMF/DMSO; (f) Gly-O-*t*-Bu·HCl, DIEA, AcOH, DMF/DMSO; (g) DCC/HOBt, DMF/DMSO; (h) TFA, 0 °C.

Scheme II[a]



[a] Key: (a) oxime resin, EACNOx, DCC, CH_2Cl_2; (b) 25% TFA/CH_2Cl_2; (c) DCC/HOBt, DMF/DMSO; (d) EDC/HOSu, DMF (e) TFA; (f) DIEA, DMF/DMSO; (g) HOPip, DMF; (h) Zn, 5% TFA/DMF.

segments, which were then used to assemble protected intermediate peptides of 34, 37, and 33 residues in length. These intermediates, designated P-I, P-II, and P-III, respectively, corresponded to the amino-terminal [RNase T_1 (1–34)], middle [RNase T_1 (35–71)], and carboxyl-terminal [RNase T_1 (72–104)] portions of the RNase T_1 sequence. Junctions between these segments were chosen to have carboxyl termini glycyl residues on P-I and P-II to avoid racemization during couplings between major segments. Initially, we planned to prepare the major intermediates exclusively by solid-phase assembly of four or five smaller peptides on the oxime resin. When this was attempted, we found that two or three segments could be reattached to the resin in sequence with good to excellent yield but that subsequent couplings proceeded much more poorly. Further, when amino acid analysis indicated that the desired peptide had been completed by multiple couplings, cleavage and extraction of the protected peptide proved to be very difficult.[19] This difficulty prompted us to adopt

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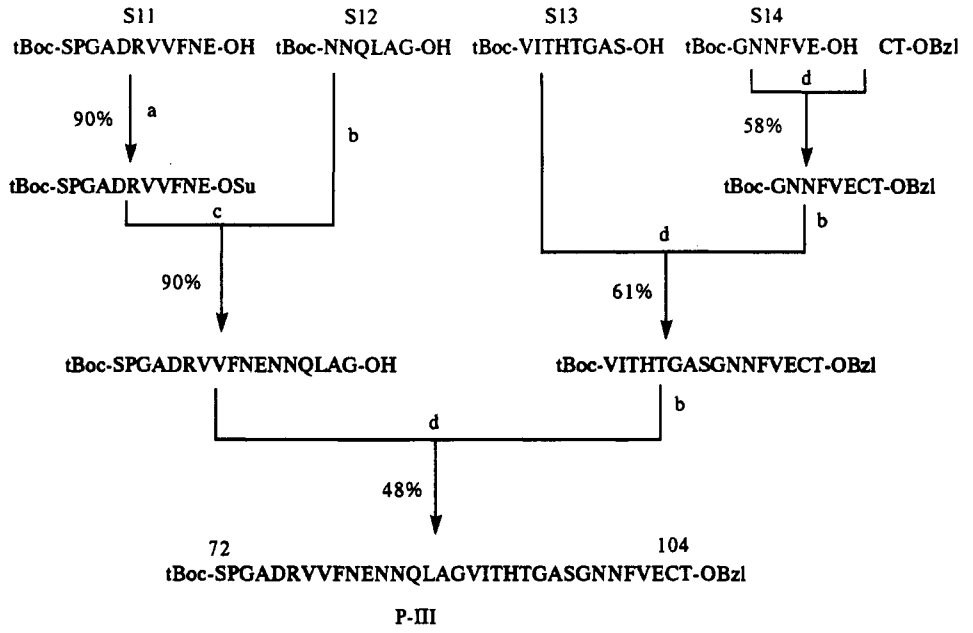
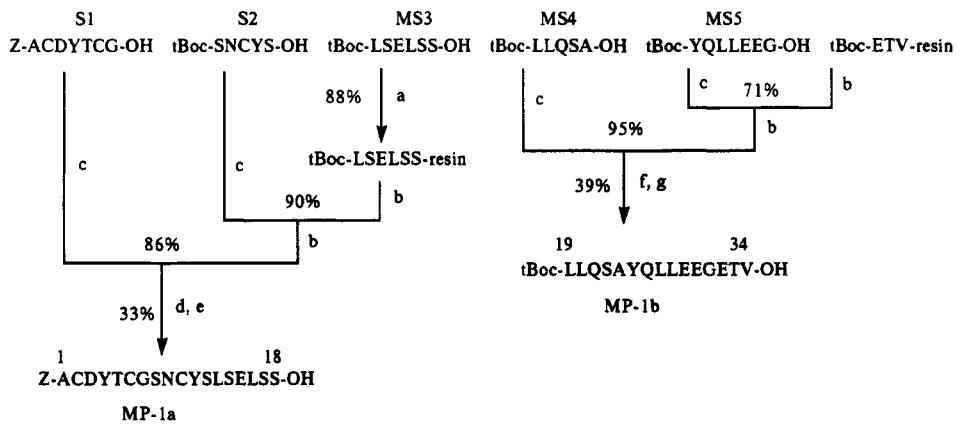
(18) While extensive refinement of solid-phase methods appears to have solved many problems encountered during the syntheses of various sequences,[16c] observations of "difficult" sequences, unfortunately, do not always make their way into the published literature, thus limiting the available knowledge regarding sequences that are unyielding to solid-phase methods.

Scheme III[a]



[a] Key: (a) EDC/HOSu, DMF; (b) TFA; (c) DIEA, DMF/DMSO; (d) DCC/HOBt, DMF/DMSO.

Scheme IV[a]



[a] Key: (a) oxime resin, EACNOx, DCC, CH_2Cl_2; (b) 25% TFA/CH_2Cl_2; (c) DCC/HOBt, DMF/DMSO; (d) HOPip, DMF (e) Zn, 5% TFA/DMF; (f) $GlyOCH_2CH_2Si(CH_3)_3$·HCl, DIEA, AcOH, DMF; (g) $(Bu)_4NF$, DMF/DMSO.

the following more convergent general strategy. We used the oxime resin as a support on which to assemble two or three segments to prepare protected peptides of approximately 15–20 residues in length. These protected peptides were then cleaved from the oxime resin for subsequent coupling in solution to obtain the intermediates P-I (Scheme I), P-II (Scheme II), and P-III (Scheme III).

As an initial effort at redesigning secondary structural elements in proteins, we also planned to synthesize a peptide in which the major α-helical region of RNase T_1 was substantially altered. In the modified sequence, changes have been made to accentuate the amphiphilicity of the 17-residue 4.5-turn α-helix and increase its net hydrophobicity by two residues (Figure 1).[13] According to several empirical rules[20] and our experience in earlier

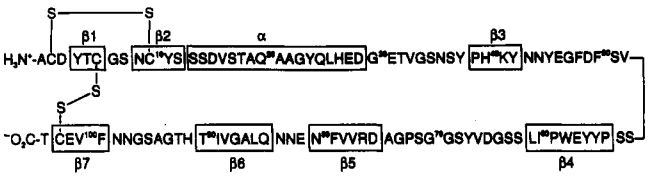


Figure 1. Sequence of the 104 residues of RNase T_1 indicating the two disulfides between positions 2 and 10 and positions 6 and 103. Secondary structural elements determined by X-ray crystallography,[13a] 7-β-strands and the 17-residue α-helix, are indicated by the boxed regions. In a redesign of the α-helical region, the sequence at positions 13–29 was changed to: LSELSSLLQSAYQLLEE. Single-letter notation is used for amino acids: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

modeling studies of peptide hormones,[21] leucine and glutamic acid/glutamine/serine residues have been used to construct the hydrophobic and hydrophilic faces of the

(19) In our experience and that of our co-workers this appears to be a fairly general problem. Depending on the solubility of a given protected peptide sequence, solid-phase segment condensations could proceed in better or worse yields.

(20) (a) Chou, P. Y.; Fasman, G. D. *Biophys. J.* **1979**, *26*, 367–384. (b) Chou, P. Y.; Fasman, G. D. *Adv. Enzymol.* **1978**, *47*, 45–148. (c) Eisenberg, D.; Scwartz, E.; Komaromy, M.; Wall, R. *J. Mol. Biol.* **1984**, *179*, 125–142. (d) Kyte, J.; Doolittle, R. F. *Ibid.* **1982**, *157*, 105–132. (e) Levitt, M. *Biochemistry* **1978**, *17*, 4277–4283.

(21) Taylor, J. W.; Kaiser, E. T. *Pharmacol. Rev.* **1986**, *38*, 291–319.

Table I. Characterization of Small Protected Peptide Segments by Amino Acid Analysis and MS Spectra

peptide	sequence[a]	amino acid analysis	formula	calcd (ion)	found[b]
S1	Z-A-C-D-Y-T-C-G-OH	Asx, 1.07 (1); Thr, 0.99 (1); Gly, 0.99 (1) Ala, 1.00 (1); Tyr, 1.27 (1)	$C_{73}H_{79}N_7O_{14}S_2Cl_2$	1436.5 $(M + Na)^+$	1436.4
S2	*t*-Boc-S-N-C-Y-S-OH	Asx, 1.00 (1); Ser, 1.98 (2); Tyr, 0.87 (1)	$C_{56}H_{64}N_6O_{12}SCl_2$	1017.0 $(M - Boc + 2 H)^+$	1016.4
S3	*t*-Boc-S-S-D-V-S-T-OH	Asx, 1.00 (1); Thr, 0.96 (1); Ser, 2.63 (3) Val, 0.84 (1)	$C_{63}H_{76}N_6O_{15}$	1046.2 $(M - Boc + 2 H)^+$	1046.0
S4	*t*-Boc-A-Q-A-A-G-OH	Ala, 3.00 (3); Glx, 1.05 (1); Gly, 0.94 (1)	$C_{21}H_{36}N_6O_9$	417.4 $(M - Boc + 2 H)^+$	417.5
S5	*t*-Boc-Y-Q-L-H-E-D-G-E-T-OH	Asx, 1.00 (1); Thr, 0.84 (1); Glx, 3.11 (3); Gly, 0.93 (1); Leu, 1.01 (1); Tyr, 0.42 (1); His, 1.07 (1)	$C_{94}H_{110}N_{12}O_{22}Cl_2$	1731.8 $(M - Boc + 2 H)^+$	1731.4
S6	*t*-Boc-S-N-S-Y-P-H-K-OH	Asx, 1.09 (1); Ser, 1.24 (1); Pro, 0.83 (1); Tyr, 0.93 (1); His, 0.90 (1); Lys, 1.00 (1)	$C_{78}H_{90}N_{11}O_{17}Cl_3$	1561.4 $(M + H)^+$	1560.6
				1583.4 $(M + Na)^+$	1583.0
S7	*t*-Boc-Y-N-N-Y-E-G-OH	Asx, 1.99 (2); Glx, 1.15 (1); Gly, 1.00 (1); Tyr, 1.84 (2)	$C_{58}H_{64}N_8O_{15}Cl_4$	1289.6 $(M + Na)^+$	1289.5
				1311.3 $(M + 2 Na - H)^+$	1311.3
S8	*t*-Boc-F-D-F-S-V-S-OH	Asx, 0.99 (1); Ser, 1.71 (2); Val, 1.00 (1); Phe, 2.10 (2)	$C_{59}H_{70}N_6O_{13}$	1094.9 $(M + Na)^+$	1094.5
				1116.9 $(M + 2 Na - H)^+$	1116.7
S9	*t*-Boc-S-P-Y-Y-E-W-P-I-L-OH	Ser, 0.78 (1); Glx 1.00 (1); Pro, 2.20 (2); Ile, 0.94 (1); Leu, 1.07 (1); Tyr, 2.20 (2)	$C_{93}H_{106}N_{10}O_{18}Cl_4$	1817.0 $(M + Na)^+$	1817.7
				1839.0 $(M + 2 Na - H)^+$	1839.7
S10	*t*-Boc-S-S-G-D-V-Y-S-G-G-OH	Asx, 1.00 (1); Ser, 2.71 (3); Gly, 3.21 (3); Val, 0.62 (1); Tyr, 0.63 (1)	$C_{73}H_{85}N_9O_{18}Cl_2$	1470.9 $(M + Na)^+$	1471.2
				1492.9 $(M + 2 Na - H)^+$	1493.5
S11	*t*-Boc-S-P-G-A-D-R-V-V-F-N-E-OH	Asx, 4.26 (4); Ser, 0.61 (1); Glx, 1.98 (2); Pro, 1.08 (1); Gly, 2.00 (2); Ala, 2.07 (2); Val, 2.02 (2); Leu, 0.88 (1); Phe, 1.00 (1); Arg, 1.44 (1)	$C_{84}H_{111}N_{15}O_{22}S$	1737.8 $(M + Na)^+$	1560.6
				1759.8 $(M + 2 Na - H)^+$	1760.4
S12	*t*-Boc-N-N-Q-L-A-G-OH	Asx, 2.01 (2); Glx, 1.00 (1); Gly, 1.00 (1); Ala, 1.03 (1); Leu, 0.94 (1)	$C_{29}H_{49}N_9O_{12}$	738.8 $(M + Na)^+$	738.9
S13	*t*-Boc-V-I-T-H-T-G-A-S-OH	Ser + Thr, 1.16 (3); Gly, 1.00 (1); Ala, 1.06 (1); Val, 1.00 (1); Ile, 0.68 (1); His, 1.16 (1)	$C_{67}H_{90}N_{10}O_{15}$	1275.5 $(M + H)^+$	1275.4[c]
S14	*t*-Boc-G-N-N-F-V-E-OH	Asx, 2.13 (2); Glx, 1.06 (1); Gly, 1.00 (1); Val, 1.46 (1); Phe, 0.98 (1)	$C_{41}H_{56}N_8O_{13}$	869.9 $(M + H)^+$	869[c]
				891.9 $(M + Na)^+$	891[c]
MS3	*t*-Boc-L-S-E-L-S-S-OH	Ser, 2.11 (3); Glx, 1.00 (1); Leu, 1.94 (2)	$C_{59}H_{78}N_6O_{14}$	1118.3 $(M + Na)^+$	1118.1
				1140.3 $(M + 2 Na - H)^+$	1140.5
MS4	*t*-Boc-L-L-Q-S-A-OH	Ser, 0.72 (1); Glx, 1.05 (1); Ala, 1.00 (1); Leu, 2.01 (2)	$C_{35}H_{56}N_6O_{10}$	721.4 $(M + H)^+$	721[c]
MS5	*t*-Boc-Y-Q-L-L-E-E-G-OH	Glx, 3.01 (3); Gly, 1.00 (1); leu, 1.87 (2); Tyr, 0.99 (1)	$C_{64}H_{82}N_8O_{16}Cl_2$	1313.3 $(M + Na)^+$	1313.1
				1335.3 $(M + 2 Na - H)^+$	1335.5

[a] Single-letter abbreviations for the amino acids (see Figure 1). Amino acid side chain protecting groups: Arg, N^G-tosyl; Asp *O*-benzyl; Cys, 4-methylbenzyl; Glu, *O*-benzyl; His, N^π-(benzyloxy)methyl; Lys, [(2-chlorobenzyl)oxy]carbonyl; Ser, *O*-benzyl; Thr, *O*-benzyl; Trp, formyl Tyr, 2,6-dichlorobenzyl. [b] ^{252}Cf fission fragment time-of-flight mass spectra, unless otherwise noted. [c] FAB mass spectra.

designed amphiphilic α-helix, respectively. Alanine was chosen at residue 22 instead of a more bulky Leu residue to avoid an unfavorable steric interaction with the tryptophanyl residue at position 59. Two peptides corresponding to the peptide containing the redesigned helical region, mutant peptide I (MP-I), were synthesized in a manner analogous to that for the two precursors of P-I (Scheme IV).

To obtain the protected peptide sequence corresponding to RNase T_1, we coupled P-II and P-III in solution and then coupled P-I to the (P-II)–(P-III) segment. Finally, we deprotected the 104-residue peptide with the low–high HF method.[22] We had then planned to prepare the mutant-helix sequence by coupling the MP-I peptide to the 70-residue peptide (P-II)–(P-III). The mutant 34-residue MP-I proved to less soluble than the native sequence peptide, and we were unable to redissolve this material after isolation for use in the next coupling (see below). We worked around this problem by coupling the two precursors of MP-I to (P-II)–(P-III) sequentially. The intermediate in this approach, a protected 86-residue peptide, and the resulting mutant 104-residue protected peptide were both adequately soluble for further manipulation to complete the synthesis and for subsequent deprotection by the low–high HF method. Both synthetic enzymes were found to have extremely low specific activities.

Synthesis of Protected Peptide Segments on Oxime Resin. A conventional deprotection–coupling cycle with minor modifications was employed as reported previously[7] to prepare protected peptide segments as synthetic intermediates for the assembly of RNase T_1 and its structural mutant (Table I). The first amino acid was attached to the resin by a DCC[23]-mediated coupling in CH_2Cl_2[8,24]

(22) Tam, J. P.; Heath, W. F.; Merrifield, R. B. *J. Am. Chem. Soc.* **1983**, *105*, 6442–6445.

before starting synthesis. Boc groups were repeatedly deprotected with 25% TFA in CH_2Cl_2 for the synthesis of up to an 11-residue peptide (S11) without any difficulty. In addition to the symmetric anhydrides ordinarily used with the oxime resin, we also examined other coupling methods including the DCC/HOBt[25] method and BOP reagent[26] in this work. Although the DCC/HOBt method has been reported to be superior for the coupling of Gln and Asn residues in comparison to the corresponding symmetric anhydrides and we have used it routinely, it was found to be insufficiently effective for the coupling of the third amino acid residue to the oxime resin. Since the peptide–oxime resin ester bond is relatively labile toward nucleophilic attack, DCC/HOBt coupling appeared to be too slow to compete with the formation of diketopiperazines by intramolecular cyclization, which results in the cleavage of the first two residues from the resin.[27] On the other hand, use of the BOP reagent was found to be an efficient and economical coupling method.[28]

Cleavage of Protected Peptides from Resin. A useful property of the oxime resin in comparison with the conventional Merrifield resin is the ability to liberate a peptide from the resin with the protecting groups intact. Many mild nucleophiles[4,5,7] can be used for the cleavage of peptides from the oxime resin. *N*-Hydroxypiperidine (HOPip) was used extensively in this work, and the resulting OPip esters were converted to the corresponding acids by treatment with zinc dust in acetic acid as reported previously.[7] Preliminary experiments showed that the reductive cleavage of OPip esters was greatly accelerated in an acidic solvent, suggesting the possible involvement of a protonated OPip ester in the cleavage reaction. We found that acidified DMF (5% TFA) and DMF/DMSO (10% TFA) were effective as alternative solvents for the cleavage of acetic acid insoluble OPip esters. Zinc dust could be substituted by another reducing reagent such as sodium dithionite ($Na_2S_2O_4$) as well.[29]

Various amino acid esters were also examined as nucleophiles to cleave peptides from the resin in an effort to develop a general oxime methodology. Trimethylsilylethyl esters ordinarily used in peptide synthesis by Sieber[30] may prove to be very useful in the synthesis of larger peptides because of their unique reactivity toward fluoride ion. We found that N-terminal free amino peptide esters could be obtained directly by the treatment of Boc-deprotected peptides on the resin with amino acid esters. Since no appreciable amounts of cyclic peptides were isolated, intramolecular cyclization of the N-terminal free peptide segments on the resin appeared to be much slower than the nucleophilic cleavage by the amino acid esters under the conditions we employed.

Purification of Small Protected Peptide Segments. As discussed above, establishing the purity of peptide segments is a key step in our overall synthetic strategy. All small peptide segments were purified by preparative reversed-phase HPLC to ensure the complete removal of any potential side products such as deletion peptides. In order to keep protected peptides soluble in HPLC solvents and to increase the confidence in the purity ascertained by analytical HPLC, we had to limit the size of peptide segments to 6–8 residues per segment in most cases.[31] Proline-containing segments were found to be much more soluble, presumably due to the disruption of β-pleated-sheet formation.[32] We used acetonitrile/water acidified by 0.1% acetic acid as an HPLC solvent to minimize deprotection of Boc groups during the purification process.[33] For the purification of His(Bom) containing segments, 2-propanol–acetonitrile (1:1)/water with 0.5% acetic acid was found to be effective to reduce peak broadening. Some segments could be purified by a careful recrystallization instead of by time-consuming preparative HPLC.

Peptide segments were purified to homogeneity (>99%) by reversed-phase HPLC and characterized by amino acid analysis, MS, NMR, and sometimes UV. Both ^{252}Cf fission fragment MS and FABMS were used to confirm the purity of protected peptides in combination with amino acid analysis. Table I summarizes these data for each small peptide segment synthesized on oxime resin. ^1H NMR of the small protected peptides showed no indication of secondary structure formation in DMSO-d_6,[34] suggesting that the peptide segments would be easily coupled without steric hindrance of the reacting site.

Segment Condensation. Oxime resin allows a successive assembly of intermediate peptide segments on a

(23) Abbreviations: BOP, [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexaflurophosphate; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EACNOx, ethyl 2-(hydroxyimino)-2-cyanoacetate; EDC·HCl, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; His(Bom), N^{π}-[(Benzyloxy)methyl]-L-histidine; HOBt, 1-hydroxybenzotriazole; HOPip (OPip), *N*-hydroxypiperidine (*N*-hydroxypiperidyl); HOSu (OSu), *N*-hydroxysuccinimide (*N*-hydroxysuccinimidyl); HPLC, high-performance liquid chromatography; NMM, *N*-methylmorpholine; NMP, *N*-methylpyrrolidinone; TFA, trifluoroacetic acid. Dinucleotide substrates for enzyme assays: ApA, adenylyl(3′–5′)adenosine; ApG, adenylyl(3′–5′)guanosine; CpA, cytidylyl(3′–5′)adnosine; GpA, guanylyl(3′–5′)adenosine; UpA, uridylyl(3′–5′)-adenosine.

(24) Certain amino acid acid derivatives such as Boc-Arg(Tos) are practically insoluble in CH_2Cl_2. Although several coupling reagents including DCC, DCC/HOBt, and BOP reagent have been tried to attach these amino acids to the oxime resin in DMF as solvent, coupling yields were, in general, found to be very low. Mihara, H. Personal communication.

(25) Mojsov, S.; Michell, A. R.; Merrifield, R. B *J. Org. Chem.* **1980**, *45*, 555–559.

(26) (a) Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C. *Tetrahedron Lett.* **1975**, 1219–1222. (b) Felix, A. M.; Wang, C.-T.; Heimer, E. P.; Fournier, A. *Int. J. Peptide Protein Res.* **1988**, *31*, 231–238.

(27) Care must be taken in the attachment of the first three residues of a peptide to the oxime resin. If cleavage occurs and newly exposed oxime moieties are not acetylated, deletion peptide impurities may be introduced into the product.

(28) While BOP is costly, it allows the use of fewer equivalents of protected amino acid for a coupling step in comparison with symmetric anhydrides. In the case of an expensive amino acid derivative, the cost advantage can be significant. We note that a byproduct of BOP is hexamethylphosphoramide, which is highly toxic and a cancer suspect agent, and appropriate caution should be exercised in its handling.

(29) With 5% TFA in DMF, reductions of HOPip peptide esters proceed cleanly in 5–10 min using Zn or $Na_2S_2O_4$. Adding DMSO slows the reduction considerably. In DMF/DMSO (1:1) with 5% TFA, complete reduction requires 1 h or longer; with 10% TFA reduction is complete in about 30 min. The use of DMSO as a cosolvent is also complicated by gradual reduction of DMSO to dimethyl sulfide, which creates an unpleasant odor and may require the addition of greater excesses of reducing agent. The above observations were made on a small sample of peptide S10, by monitoring the progress of reduction by HPLC. Sasaki, T.; Findeis, M. A. Unpublished results.

(30) Sieber, P. *Helv. Chim. Acta* **1977**, *60*, 2711–2716.

(31) We found that protected peptides of 10 or more residues in length were typically not eluted from C4 HPLC columns with our chromatographic conditions, and if DMF was included in the solvent mixture, such peptides were eluted with poor resolution.

(32) (a) Narita, M.; Fukunaga, T.; Wakabayashi, A.; Ishikawa, K.; Nakano, H. *Int. J. Peptide Protein Res.* **1984**, *23*, 306–314. (b) Narita, M.; Ishikawa, K.; Chen, J.-Y.; Kim, Y. *Ibid.* **1984**, *24*, 580–587.

(33) TFA (0.1%) is commonly used in HPLC of peptides. With protected peptides care must be taken in the use of TFA to avoid concentrating it to the point where *t*-Boc or other protecting groups are removed before it is desired to do so.

(34) ^1H NMR spectra of each small segment appeared to be consistent with a linear combination of the reference spectra of constituent amino acids.

solid phase, thus offering flexibility in a synthetic strategy. Purified peptide segments were reattached to oxime resin by the DCC/EACNOx method in CH_2Cl_2, reported previously as an efficient and racemization-resistant method.[7] Although a peptide segment has to be soluble in CH_2Cl_2 to be successfully reattached to the resin, most large segments are not so soluble. We tried as an alternative, therefore, to coupling peptide segments to amino acyl oxime resin in DMF. Thus, a segment Boc-Ser(Bzl)-Ser(Bzl)-Gly-Val-Tyr(Cl_2Bzl)-Ser(Bzl)-Gly-COOH was coupled to glycine oxime resin in DMF to prepare S10-oxime resin. MS analysis of the cleaved peptide, however, showed the existence of a number of impurities including addition peptides and deletion peptides that could be attributed to instability of the neutralized glycine residue on the resin. Interestingly, the S5 segment was coupled to valine oxime resin without any difficulty, suggesting that the bulky side chain of the valyl residue might protect it against cleavage from the resin.

Once the first segment was attached to the resin, subsequent segment condensations were carried out by using the conventional DCC/HOBt method,[35] which has been shown to be effective and racemization resistant. The coupling yield of each step was estimated by quantitative amino acid analysis, using unique amino acids as a reference. The peptide resin was acetylated after each coupling reaction to limit the buildup of impurities due to incomplete coupling.

A major difficulty in carrying out segment condensations on the resin was found when a large peptide segment (20–40 residues in length) was coupled to the resin. We observed a sharp drop in coupling yield as the segment size increased above approximately 15 residues (MW = 2000–3000). This result could perhaps be explained as a result of the partition effect of peptide segments between a solution phase and the resin matrix. An effective concentration of larger segments in the polymer matrix would be decreased significantly since the exclusion limit (or effective pore size) of 1% cross-linked polystyrene has been reported to be MW 3000–10 000 depending on the conditions.[36]

Moreover, the hydrophobic nature of polystyrene resin would facilitate the formation of a hydrogen-bonded network of resin-bound peptides to prevent the further permeation of larger peptides into the polymer matrix. This apparent partition problem also presented a difficulty in extracting large peptide segments from the resin after several sequential segment condensations. We had to repeat a lengthy extraction several times (typically more than five times 1 day of extraction) to isolate peptides in a satisfactory yield. Although a polyamide-based oxime resin has been developed[6a] to provide a "peptide-compatible" environment in a polymer matrix, more work needs to be done to solve the above problems. In this work, therefore, we used the oxime resin to synthesize medium-size peptides while carrying out the segment condensations of larger peptides in solution.

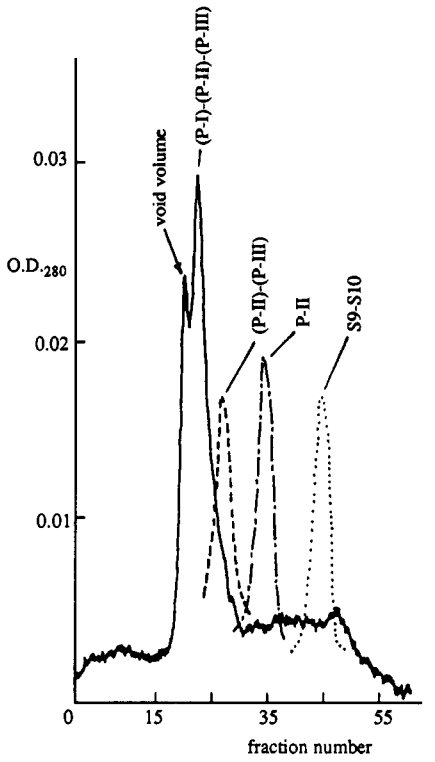


Figure 2. Gel filtration chromatographs of the fully protected RNase T_1 ((P-I)-(P-II)-(P-III)) and smaller peptide segments on Sephadex LH-60 with DMF as the eluting solvent. Molecular weights of the peptide segments: (P-I)-(P-II)-(P-III), 16 800; (P-II)-(P-III), 11 200; P-II, 6700; S9-S10, 3100.

Large protected peptides thus synthesized were easily purified by gel filtration chromatography. Among the column packing materials we examined, Sephadex LH-60 was found to be the most suitable gel filtration media for the purification of our protected peptide segments. Figure 2 shows the gel filtration elution profiles for the purification of protected peptide segments having different molecular weights. DMF was used exclusively as a solvent for gel filtration except for the P-III segment in which purification we replaced DMF by *N*-methylpyrrolidinone (NMP) to avoid the precipitation of the peptide in the column. We also found that only a minimum amount of DMSO (<50% v/v) might be added to the sample solution, when necessary, to ensure the complete dissolution of peptides without losing chromatographic resolution.

All protected peptides that we synthesized were eluted at the expected volume in accord with the literature.[37] The operational range for chromatography of protected peptides on Sephadex LH-60 appeared to be approximately MW 1000–20 000 in DMF. We were able to separate compounds whose molecular weights were different by as little as ~30% of the MW of a desired peptide. Large peptide segments thus synthesized were further purified, if necessary, by rechromatography on the Sephadex LH-60 column to obtain a symmetrical peak.

We encountered a serious solubility problem during the synthesis of the mutant segment (1–34), which was initially assembled on the oxime resin by successive segment condensations of five protected peptides. The resulting peptide resin was treated with glycine *tert*-butyl ester and extracted with several solvents. The desired 34-residue peptide was, unfortunately, practically insoluble in all solvents we tried except TFA and was impossible to purify.

(35) (a) We chose either glycine or racemization-resistant amino acids as a C-terminal amino acid of each peptide segment. The extent of racemization during segment condensations should, however, be strongly affected by various experimental conditions such as reaction time, temperature, and the chain length of peptide segments. See: Bennoiton, N. L.; Kuroda, K. *Int. J. Peptide Protein Res.* **1981**, *17*, 197–204. (b) Human parathyroid hormone, an 84-residue peptide, has successfully been synthesized via a segment condensation approach using carbodiimide/HOBt as a coupling reagent. See: Kimura, K.; Takai, M.; Masui, Y.; Morikawa, T.; Sakakibara, S. *Biopolymers* **1981**, *20*, 1823–1832.

(36) (a) Halasz, I.; Vogtel, P. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 24–28. (b) Heitz, W. *J. Chromatogr.* **1970**, *53*, 37–49. (c) *Bio-Rad Catalog* **1988**, p 50.

(37) Galpin, I. J.; Jackson, A. G.; Kenner, G. W.; Noble, P.; Ramage, R, *J. Chromatogr.* **1978**, *147*, 424–428.

We therefore chose to divide the segment into two medium-size segments (MP-Ia and MP-Ib, see Scheme IV), which individually turned out to be more soluble and could be coupled to the 70-residue peptide (P-II)–(P-III) sequentially to synthesize the mutant RNase T_1 (see the supplementary material). All other segments were adequately soluble in either DMF or DMF/DMSO mixtures. Since the segment synthesis–condensation strategy allows a variety of condensation patterns, many solubility problems could potentially be overcome by minor modifications of a synthetic route. Furthermore, an empirical rule that predicts the solubility of protected peptides has been proposed,[32b] which may be useful in designing a synthetic strategy.

Large protected peptide segments were charactrized by amino acid analysis and ^{252}Cf fission fragment mass spectrometry. The purity of large peptide segments was estimated to be more than 90% based on gel filtration chromatography and amino acid analysis. Although we failed to detect monocations of the fully protected RNaseT_1, a broad peak was observed at the expected position for dications (MW_{app} ~8500). Fully protected synthetic enzymes thus obtained were treated with anhydrous HF by using the low–high procedure[22] to remove all protecting groups.

Refolding, Characterization, and Assay of Synthetic RNase T_1 and Its Analogue. Crude synthetic enzyme was gel filtered to remove salts and residual scavengers from the deprotection. The synthetic enzyme was then taken up in the denaturation–reduction buffer of Pace and Creighton[38] followed by gel filtration to remove denaturant and dithiothreitol to allow refolding and oxidation. Enzymes were assayed in a yeast hydrolysis[39] and dinucleotide hydrolysis assays.[40] The observed specific activity was very low, 0.07% for the native-sequence synthetic enzyme and 0.004% for the redesigned enzyme.[41]

The low specific activity of the native-sequence enzyme indicates that our synthesis of RNase T_1 is severely inefficient. At this time we cannot account for the extent to which different potential problems affect this synthesis. We have used standard coupling procedures that should minimize racemization, but we have not examined the extent to which particular residues may have been racemized.[35] Lack of efficiency in deprotection and refolding may also be a source of losses in activity. These last problems would be encountered regardless of the manner in which the final protected polypeptide was prepared.

Conclusions

Oxime resin is clearly a useful support for the synthesis of small protected peptides. This resin can also be used for the straightforward solid-phase coupling of protected segments to prepare peptides of intermediate size. Just as clearly, however, the oxime approach suffers from the same problems that other synthetic approaches encounter as the size of intermediate and target peptides increases: namely, decreasing solubility and reactions rates. Sequential solid-phase synthesis of large peptides and small proteins (to ca. 100 residues) is currently well-enough established to be the best chemical method for preparing such materials.[17] Oxime resin based syntheses can be an appropriate alternative for syntheses of peptides of 40–60 residues in length when synthetic flexibility is desirable. Access to protected peptides is also of importance in the synthesis of peptide–organic molecules. Further, in those solid-phase syntheses that fail at an intermediate point, the coupling of a protected peptide segment that bridges the problematic sequence is a potential solution.

Experimental Section

General Procedures. Protected amino acids were purchased from Peninsula Laboratories or Bachem. Hydroxybenzotriazole monohydrate was from Pierce. Guanylyl(3′–5′)adenosine, adenylyl(3′–5′)adenosine, cytidylyl(3′–5′)adenosine, uridylyl(3′–5′)-adenosine, and adenylyl(3′–5′)guanosine were purchased as the ammonium salts from Sigma. Guanosine 2′(3′)5′-diphosphate agarose was from Sigma. Polystyrene oxime resin,[4-6] *N*-hydroxypiperidine,[44] and EACNOx[45] were prepared by published procedures. Gel permeation chromatography of protected peptides was performed with a Sephadex LH-60 column (2.5 × 80 cm) eluting with DMF or NMP after samples were applied in DMF or DMF/DMSO mixtures. Hydrolyses of protected peptides were performed in HCl/propionic aicd (1:1, v/v; Peninsula) at 130 °C for 2 h, and amino acid analyses were performed with a Dionex system equipped with a Pickering cation-exchange column. Linear regression analyses of Eadie–Hofstee plots were performed with a Hewlett-Packard HP-25 calculater programmed in the appropriate manner. Synthetic and commercial RNase T_1 was analyzed by reversed-phase HPLC using a Vydac C4 analytical column eluted with acetonitrile/water/0.1% TFA mixtures.

Protected Peptide Segments. Small protected peptide segments were prepared by methods essentially as described previously.[4,5,7] Most amino acids were coupled as the symmetric anhydrides. *t*-Boc-Asn, *t*-Boc-Gln, and *t*-Boc-His(Bom) were coupled by the DCC/HOBt procedure.[24] Peptides were cleaved from the oxime resin by *N*-hydroxypiperidine, reduced to the peptide free acids with zinc in 90% aqueous acetic acid, and purified by reversed-phase HPLC using a Vydac C4 preparative HPLC column eluting with acetonitrile/water/0/1% acetic acid mixtures. Peptides were characterized as noted in Table I.

Coupling of S5 to Valine Oxime Resin. *t*-Boc valine oxime resin (1.05 g, 0.48 mmol/g) was deprotected in 25% TFA/CH_2Cl_2 (10 mL × 25 min), washed, and neutralized. Peptide S5 (291 mg, 156 μmol, dissolved in 50 μL DMF diluted with a further 0.5 mL of CH_2Cl_2) and EACNOx (44 mg, 310 μmol) were added to the resin in CH_2Cl_2 (7 mL) followed by the addition of DCC (0.5 M in CH_2Cl_2, 0.312 mL). After the mixture was shaken overnight, the resin was washed and dried. Quantitative amino acid analysis indicated that the coupling yield was 48% (75 μmol of S5 on the resin).

H_2N-S4-S5-Val-Gly-OtBu. The peptidyl resin from the previous step was acetylated with acetic anhydride (57 μL, 0.6 mmol) and NMM (66 μL, 0.6 mmol) for 1 h, then deprotected,

(38) Pace, C. N.; Creighton, T. E. *J. Mol. Biol.* **1986**, *188*, 477–486.
(39) Takahashi, K. *J. Biochem.* **1961**, *49*, 1–8.
(40) Ipata, P. L.; Felicioli, R. A.; Zucchelli, G. C. *Ital. J. Biochem.* **1969**, *18*, 114–122.
(41) The native-sequence enzyme was found to hydrolyze GpA as a substrate with a K_m of 86 μM. This value is close to the K_m of 66 μM measured with commercial RNase T_1. The substrate specificity of the synthetic native-sequence enzyme was evaluated in assays with other dinucleotides as substrates. Of ApA, ApG, UpA, and CpA, none were found to be substrates of the synthetic enzyme (<5% of the activity with GpA) as is the case with native RNase T_1. The specific activity of the synthetic enzyme, however, was very low. Calculation of specific activity gave a value for k_{cat} of 0.28 min^{-1}, which is 0.004% that of the native enzyme. In an effort to purify the enzyme further, affinity chromatography on GMP-agarose was performed.[42] This procedure increased the specific activity only by about a factor of 2–0.5 min^{-1}. Reversed-phase HPLC was also used in an attempt to obtain higher activity material. In comparison to native enzyme, the synthetic enzyme was eluted with a broader peak shape. Isolation of the fraction eluted at the position of native enzyme afforded a material with a specific activity of ~5 min^{-1}, an increase in specific activity by a factor of 18, but still only 0.07% of the value for native enzyme.[43] The mutant enzyme was found to be active against GpA. ApA was tried as a substrate and found not to be hydrolyzed to a significant extent by the mutant enzyme. Interestingly, the mutant enzyme bound GpA more tightly than native enzyme with a K_m of 5 μM. The mutant enzyme's specific activity was lower, however, with a k_{cat} of 0.019 min^{-1}.
(42) Jervis, L.; Pettit, N. M. *J. Chromatogr.* **1974**, *97*, 33–38.
(43) This value of k_{cat} was estimated from a single assay at a concentration of 50 μM GpA.
(44) (a) Sabel, W. *Chem. Ind.* **1966**, 1216–1217. (b) Handford, B. O.; Young, G. T.; Johnson, T. F. N. *J. Chem. Soc.* **1965**, 6814–6827.
(45) Conrad, M.; Schultz, A. *Ber. Dtsch. Chem. Ges.* **1909**, *42*, 735–737.

and washed. Peptide S4 (57.8 mg, 112 μmol, 1.5 equiv) was coupled with HOBt (34.3 mg, 224 μmol) and DCC (0.5 M, 224 μL) in DMF/CH_2Cl_2 (8 mL) overnight. Amino acid analysis indicated essentially complete coupling to the S5-Val resin but with some loss of total peptide. The calculated amount of product present was 117 mg. The peptide resin was deprotected, washed, and then treated several times with glycine *O-tert*-butyl ester hydrochloride (42 mg, 0.25 mmol), acetic acid (14 μL, 0.24 mmol), and DIEA (0.43 mL, 0.25 mmol) in DMF/DMSO (1:1, 6 mL). Solvent was removed from the crude product in vacuo, then applied to a Sephadex LH-60 column, and eluted with DMF, and the major high molecular weight peak was collected. Amino acid analysis: Asx, 1.10 (1); Thr, 0.98 (1); Glx, 4.00 (4); Gly, 2.98 (3); Ala, 2.64 (3); Val, 1.05 (1); Leu, 1.09 (1); Tyr, 0.68 (1); His, 1.08 (1). MS (^{252}Cf fission fragment), *m/e* 2342.8 (calcd for $C_{116}H_{149}N_{20}O_{28}Cl_2$ $(M + H)^+$, 2342.5); 2364.8 (calcd for $(M + Na)^+$ ion, 2364.5).

S3-Oxime Resin. Peptide S3 (172 mg, 0.15 mmol) was coupled to oxime resin (0.6 g) with EACNOx (42.6 mg, 0.30 mmol) and DCC (0.5 M, 0.30 mL) overnight in CH_2Cl_2 (6 mL). The resin was washed and dried. By weight gain of the resin, all of the peptide coupled. Excess oxime was acetylated with acetic anhydride (43 μL, 0.45 mmol) and NMM (50 μL, 0.45 mmol) in CH_2Cl_2 (6 mL) for 1 h.

S2-S3-Oxime Resin. Peptide resin from the preceding step was deprotected and washed. Peptide S2 (198 mg, 177 μmol, 1.5 equiv) was coupled with HOBt (54.2 mg, 354 μmol) and DCC (0.5 M, 354 μL) in DMF overnight and then washed and dried, yielding 890 mg of peptide resin. Amino acid analysis indicated complete coupling of S2 to S3 resin with 0.10 mmol of peptide on the resin.

Coupling of S1 to S2-S3-Oxime Resin. Peptide resin from the preceding step was deprotected and washed and coupled to peptide S1 (162 mg, 115 μmol) with HOBt (35 mg, 320 μmol) and DCC (0.5 M, 230 μmol) in DMF/CH_2Cl_2 (1:2, 6 mL). The yield of peptide resin was 1.00 g. By amino acid analysis the coupling yield was >90%, with the resin containing 0.33 g of S1-S2-S3-OH. The peptide resin was treated several times with *N*-hydroxypiperidine (32 mg, 0.32 mmol) in DMF/DMSO (2:1, 6 mL) to obtain peptide S1-S2-S3 as the *N*-hydroxypiperidyl ester, which was purified by Sephadex LH-60 chromatography. Yield 157 mg (48%). MS (^{252}Cf fission fragment), *m/e* 3545.1 (calcd for $C_{186}H_{208}N_{20}O_{35}S_3Cl_4Na$ $(M + Na)^+$, 3544.8).

Reduction of S1-S2-S3-OPip Ester. The OPip ester (33 mg, 9.4 μmol) was dissolved in 1.5 mL of DMSO, diluted with 1.5 mL of DMF, and cooled to 0 °C on ice. TFA (0.3 mL) was added with stirring to 5% (v/v). Over the course of 80 min sodium dithionite ($Na_2S_2O_4$, 190 mg) was added in portions. After 1.5 h the peptide was precipitated with 10% citric acid, collected by filtration, washed with 2 mL of water, and dried in vacuo. The resulting gray solid (40 mg) was taken up in 1 mL of DMSO, diluted with 2 mL of DMF, and chromatographed on Sephadex LH-60. A single peak was obtained, which was collected and evaporated. Yield 14 mg (43%). Amino acid analysis: Asx, 3.05 (3); Thr, 2.05 (2); Ser, 3.27 (5); Gly, 0.88 (1); Ala, 1.00 (1); Val, 0.93 (1); Tyr, 1.73 (2). MS (^{252}Cf fission fragment), *m/e* 3461.7 (calcd for $C_{181}H_{199}N_{19}O_{35}S_3Cl_4Na$ $(M + Na)^+$, 3461.7).

Z-S1-S2-S3-S4-S5-Val-Gly-OH (P-I). Z-S1-S2-S3-H (40 mg, 11.6 μmol), H_2N-S4-S5-V-G-OtBu (27.2 mg, 11.6 μmol), and HOBt (1.8 mg, 11.8 μmol) were placed in a small flask and kept under high vacuum for 1 day. DMSO (1.5 mL) was added to dissolve the peptides after which DMF (1.5 mL) was added. DCC (2 M in DMF, 6 μL) and DIEA (4 μL) were added to the reaction mixture. After 1 day an additional amount of DCC (2 M, 6 μL) and DIEA (5 μL) were added followed by more DCC (2 M, 5 μL) after 1 day more. The reaction mixture was chromatographed 1 day later on Sephadex LH-60. The leading peak was collected and rechromatographed. Amino acid analysis: Asx, 5.10 (4); Thr, 2.72 (3); Ser, 4.48 (5); Glx, 4.00 (4); Gly, 3.86 (4); Ala, 4.00 (4); Val, 2.39 (2); Leu, 0.97 (1); Tyr, 2.24 (3); His, 0.87 (1). MS (^{252}Cf fission fragment) *m/e* 5411.4 (broad) (calcd for $C_{297}H_{345}N_{39}O_{62}Cl_6$ $(M + H)^+$, 5763.1). This material was treated with TFA at 0 °C for 30 min. The TFA was blown off with nitrogen; the residue was triturated with either and then dried in vacuo. Yield 17.8 mg (27%).

S9-OSu. Into an ice-cold solution of S9 (0.12 g, 0.067 mmol) and *N*-hydroxysuccimimide (15.4 mg, 0.134 mmol) in DMF (1.5 mL) was added EDC·HCl (25.6 mg, 0.134 mmol) with stirring. The mixture was stirred for 16 h at 4 °C. The solvent was removed under reduced pressure, and the residue was triturated with H_2O. The white precipitate was collected, washed with H_2O, and dried under vacuum. Yield 122 mg (97%).

S9-S10-OH. Peptide S10 (90 mg, 0.062 mmol) was treated with TFA (2 mL) to remove the Boc group. The peptide was triturated with ether after the solvent was removed with a stream of N_2, dried under vacuum, and redissolved in DMF/DMSO (1:1, 2 mL). The apparent pH (on wet pH paper) of the solution was adjusted to 8.5 by the addition of DIEA. S9-OSu (0.115 g, 0.061 mmol) was added into the solution, and the mixture was stirred for 16 h at room temperature. The desired product, S9-S10-OH, was purified by gel filtration chromatography on Sephadex LH-60 with DMF as an eluting solvent. Yield 0.157 g (82%). Amino acid analysis: Asx, 1.11 (1); Ser, 2.54 (4); Glx, 0.83 (1); Pro, 2.19 (2); Gly, 2.88 (3); Val, 1.00 (1); Ile, 0.86 (1); Leu, 0.96 (1); Tyr, 2.92 (3). MS (^{252}Cf fission fragment) *m/e* 3146.6 (calcd for $C_{161}H_{181}N_{19}O_{33}Cl_6Na$ $(M + Na)^+$ 3146.0).

S8-Oxime Resin. To a solution of the peptide S8 (0.39 g, 0.364 mmol) in CH_2Cl_2 (10 mL) were added EACNOx (0.11 g, 0.774 mmol) and oxime resin (1.0 g). The mixture was cooled on an ice–water bath, and DCC (0.5 M in CH_2Cl_2, 0.75 mL, 0.375 mmol) was added with stirring. The mixture was shaken for 1 day at room temperature. Resin was collected on a glass filter, washed with CH_2Cl_2 (20 mL), CH_2Cl_2/EtOH (1:1, 20 mL), and EtOH (20 mL), and dried under vacuum. Yield 1.375 g (82%, 0.22 mmol of Val/g).

S7-S8-Oxime Resin. The peptide resin S8-oxime resin (0.53 g, 0.117 mmol) was acetylated with acetic anhydride (72 μL, 0.71 mmol) and NMM (677 μL, 0.71 mmol) for 1 h, deprotected, neutralized, and coupled with the peptide S7 (0.15 g, 0.118 mmol) by DCC (0.5 M in CH_2Cl_2, 0.24 mL, 0.12 mmol) and HOBt·H_2O (40 mg, 0.3 mmol) in DMF/DMSO (1:3.5, 4.5 mL) for 27 h. Yield 0.616 g (79% based on the ratio of Glu to Val).

S6-S7-S8-Oxime Resin. The peptide resin S7-S8-oxime resin (0.616 g, 0.08 mmol) was acetylated, deprotected, neutralized, and coupled with the peptide S6 (0.20 g, 0.126 mmol) by DCC (0.5 M) in CH_2Cl_2/DMF (2.5:2, 4.5 mL) for 26 h. The resin was then acetylated again. Yield 0.691 g (68% based on the ratio of Lys to Glu). The resin was found to contain 0.221 g of the desired compound based on the amino acid analysis.

S6-S7-S8-OH. The peptide resin S6-S7-S8-oxime resin (0.691 g) was treated with HOPip (0.5 M in CH_2Cl_2, 2 mL, 1 mmol) in CH_2Cl_2 (5 mL) for 1 day. The resin was collected by filtration. The peptide was extracted with DMF/DMSO (1:1, 5 mL) twice. The filtrate and extracts were combined, concentrated to 5 mL, and applied to a Sephadex LH-60 column. The column was eluted with DMF, and the major peak at the expected elution volume was collected. The fractions were combined and concentrated to 10 mL. To the solution were added 10% TFA in DMF (10 mL) and Zn dust (0.5 g) with stirring. The mixture was vigorously stirred for 1 h at room temperature. Zn was removed by filtration through a Millex HV 0.45-μm filter, and the filtrate was concentrated to dryness. The residue was triturated with 5% aqueous citric acid (5 mL). The white precipitate was collected, washed with H_2O, and dried under vacuum. Yield 0.181 g (86%). Amino acid analysis: Asx, 4.11 (4); Ser, 2.54 (4); Glx, 0.99 (1); Pro, 0.80 (1); Gly, 1.01 (1); Val, 0.99 (1); Tyr, 2.84 (3); Phe, 2.00 (2); His, 0.70 (1); Lys, 0.99 (1). MS (^{252}Cf fission fragment) *m/e* 3686.7 (calcd for $C_{186}H_{205}N_{25}O_{39}Cl_7$ $(M + H)^+$ 3663.0); *m/e* 3686.7 (calcd for $C_{186}H_{204}N_{25}O_{39}Cl_7Na$ $(M + Na)^+$ 3685.0).

S6-S7-S8-S9-S10-OH (P-II). The peptide S6-S7-S8-OH (57 mg, 0.016 mmol) was converted to the corresponding *N*-hydroxysuccinimide ester by the same procedure described for S9-OSu. The peptide S9-S10-OH was dissolved in TFA (0.5 mL). The solution was kept standing for 1 h at 0 °C. The peptide was triturated with ether after the solvent was removed with a stream of N_2, washed with ether, and dried under vacuum. The peptide was redissolved in DMF/DMSO (1:1, 1.5 mL). After the apparent pH of the solution was adjusted to ca. 8.5 with DIEA, the *N*-hydroxysuccinimide ester of S6-S7-S8-OH was added and the mixture was stirred for 2 days at room temperature. The mixture was applied to a Sephadex LH-60 column that had been equilibrated with DMF. The column was eluted with DMF, and the major peak at the expected elution volume was collected. Yield

74 mg (69%). Amino acid analysis: Asx, 5.17 (5); Ser, 5.41 (8); Glx, 1.95 (2); Pro, 3.16 (3); Gly, 4.00 (4); Val, 2.10 (2); Ile, 1.00 (1); Leu, 1.08 (1); Tyr, 5.84 (6); Phe, 2.21 (2); His, 1.01 (1); Lys, 0.85 (1). MS (^{252}Cf fission fragmentt) m/e 6714.4 (calcd for $C_{342}H_{374}N_{44}O_{69}Cl_{13}Na_2$ (M + 2 Na − H)$^+$ 6711.9).

H_2N-Cys(MeBzl)Thr(Bzl)-OBzl·HCl (CT-OBzl·HCl). Boc-Thr(Bzl)OBzl was prepared from Boc-Thr(Bzl) and benzyl alcohol by the reported procedure.[46] Boc-Thr(Bzl)OBzl (0.90 g, 2.2 mmol) was treated with 4 N HCl in dioxane (10 mL, 40 mmol) for 1 h at room temperature. The solvent was removed under reduced pressure. The residue was redissolved in dioxane (10 mL), and the solvent was evaporated again to remove excess HCl. The resulting oily residue was dried under vacuum and then dissolved in DMF (10 mL). The solution was neutralized with DIEA (pH 8.0 on wet pH paper). Into the solution were added Boc-Cys(MeBzl) (0.71 g, 2.2 mmol), HOBt·H_2O (0.51 g, 3.3 mmol), and EDC·HCl (0.46 g, 2.4 mmol). The mixture was stirred for 2 h at 0 °C and then 20 h at room temperature. After the solvent was removed under reduced pressure, the residue was dissolved in EtOAc. The solution was washed with 10% aqueous citric acid, 5% $NaHCO_3$, and H_2O and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure. The residue was treated with 4 N HCl in dioxane as described above. The product was crystallized from EtOAc/ether. Yield 0.91 g (82%). MS (FAB) m/e 507 (calcd for $C_{29}H_{34}N_2O_4S$ (M + H)$^+$ 507.0).

S14-CT-OBzl. Peptide S14 (52 mg, 0.06 mmol), Cys-(MeBzl)Thr(Bzl)OBzl·HCl (49 mg, 0.09 mmol), and HOBt·H_2O (14 mg, 0.09 mmol) were dissolved in DMF/DMSO (2:1, 1.5 mL). The apparent pH of the mixture was adjusted to ca. 8 (on wet pH paper) by the careful addition of DIEA (16 μL) with stirring. After the mixture was cooled on an ice–water bath for 5 min, EDC·HCl (17 mg, 0.09 mmol) was added. The mixture was stirred for 2 h at 4 °C and then for 1 day at room temperature. The product was precipitated by the slow addition of H_2O (1.5 mL). The precipitate was collected, washed with H_2O (1 mL) twice, and dried under vacuum. The crude product thus obtained (91 mg) was purified by reversed-phase HPLC. Yield 48 mg (58%). Amino acid analysis: Asx, 2.13 (2); Thr, 1.05 (1); Glx, 1.06 (1); Gly, 1.00 (1); Val, 1.46 (1); Phe, 0.98 (1). MS (^{252}Cf fission fragment) m/e 1380.4 (calcd for $C_{70}H_{88}N_{10}O_{16}SNa$ (M + Na)$^+$ 1380.6).

S13-S14-CT-OBzl. The peptide S14-CT-OBzl (50 mg, 0.037 mmol) was dissolved in TFA (0.5 mL). The solution was allowed to stand for 30 min at 0 °C, and then the solvent was evaporated under a gentle N_2 stream. The oily residue was triturated with ether and redissolved in 4 N HCl/dioxane (0.5 mL). The solvent was removed under reduced pressure. The residue was triturated with ether and dried under vacuum. The white residue was dissolved in DMF/DMSO (1:1, 1.5 mL), and peptide S13 (40 mg, 0.35 mmol) and HOBt·H_2O (11.3 mg, 0.074 mmol) were added. After the apparent pH was adjusted with DIEA to ca. 8.0, EDC·HCl (11 mg, 0.056 mmol) was added and the mixture was stirred for 1 day at room temperature. Precipitated product was collected as before, washed with DMF and H_2O, and dried under vacuum. Yield 54 mg (61%). Amino acid analysis: Asx, 2.30 (2); Thr, 2.73 (3); Ser, 0.59 (1); Glx, 1.08 (1); Gly, 2.00 (2); Ala, 0.96 (1); Val, 1.71 (2); Ile, 0.74 (1); Phe, 0.97 (1); His, 1.01 (1).

S11-OSu. To a solution of the peptide S11 (70 mg, 0.048 mmol) in dry DMF (1.5 mL) were added HOSu (10 mg, 0.087 mmol) and EDC·HCl (15.6 mg, 0.082 mmol). The mixture was stirred for 16 h at 4 °C and then evaporated to dryness under reduced pressure. The oily residue was triturated with cold water (5 mL) on an ice–water bath. The precipitate was collected, washed with cold water, and dried under vacuum. Yield 67 mg (90%).

S11-S12-OH. The peptide S12 (23 mg, 0.032 mmol) was dissolved in TFA (0.5 mL) at 0 °C. The solution was allowed to stand for 30 min at 0 °C and then evaporated to dryness with a stream of dry N_2. The oily residue was triturated with ether (1 mL). The precipitate was collected, washed with ether, dried under vacuum, and then redissolved in DMF/DMSO (1:1, 2 mL). To the solution was added DIEA (14 μL, 80 μmol) to neutralize the amino group (pH 8.5 on wet pH paper). A solution of S11-OSu (55 mg, 30 μmol) in dry DMF (1 mL) was added, and the mixture was stirred for 16 h at room temperature. The solvent was removed under reduced pressure, and the residue was triturated with 10% citric acid (10 mL). The white precipitate was collected, washed with H_2O, EtOAc, and CH_2Cl_2, and dried under vacuum. Yield 69 mg (90%). Amino acid analysis: Asx, 4.26 (4); Ser, 0.61 (1); Glx, 1.98 (2); Pro, 1.08 (1); Gly, 2.00 (2); Ala, 2.07 (2); Val, 2.02 (2); Leu, 0.88 (1); Phe, 1.00 (1); Arg, 1.44 (1).

S11-S12-S13-S14-CT-OBzl (P-III). The peptide S13-S14-CT-OBzl (50 mg, 0.02 mmol) was treated with TFA (0.5 mL) to remove the t-Boc group. The deprotected peptide, S11-S12-OH (47 mg, 0.02 mmol), and HOBt·H_2O (6.1 mg, 0.04 mmol) were dissolved in hot DMF/DMSO (1:1, 1.5 mL) with brief sonication. The apparent pH of the mixture was adjusted to ca. 8.0 by the addition of DIEA. After the mixture was cooled to room temperature, EDC·HCl (6.2 mg, 0.033 mmol) was added and the mixture was stirred for 3 days. Precipitated product was collected by suction filtration and redissolved in hot DMSO (3 mL). The solution was combined with the filtrate and applied to a Sephadex LH-60 column that had been equilibrated with N-methylpyrrolidinone (NMP). The column was eluted with NMP, and the major peak at the expected elution volume was collected. Yield 47 mg (48%). Amino acid analysis: Asx, 6.20 (6); Thr, 2.20 (3); Ser, 0.72 (2); Glx, 2.83 (3); Pro, 0.98 (1); Gly, 3.89 (4); Ala, 3.00 (3); Val, 3.79 (4); Ile, 0.88 (1); Leu, 1.00 (1); Phe, 1.89 (2); His, 0.95 (1); Arg, 1.12 (1). MS (^{252}Cf fission fragment, after treatment with TFA) m/e 4646.4 (calcd for $C_{230}H_{300}N_{44}O_5S_2Na_2$ (M + Na + O)$^+$ 4648.3).

(P-II)-(P-III). The peptide P-III (10 mg, 2.4 μmol) was treated with TFA (0.5 mL) for 1 h at 0 °C to remove the Boc group. The solvent was removed with a stream of N_2. The residue was triturated with ether, dried under vacuum for 1 h, and redissolved in hot DMF/DMSO (1:1, 0.5 mL) with brief sonication. After the solution was cooled to room temperature, the apparent pH was adjusted to ca. 8 by addition of DIEA. Into the mixture were added peptide P-II (16 mg, 2.4 μmol) and BOP reagent (2.1 mg, 4.8 μmol). The apparent pH of the mixture was readjusted to ca. 8 with DIEA after 2 h, 1 day, and 2 days. The mixture was stirred for a total of 3 days and applied to a Sephadex LH-60 column in DMF as an eluting solvent. The desired 70-residue protected peptide was eluted at the expected elution volume as a well-resolved peak. The peptide was further purified by a rechromatography on the same column. Yield 7.0 mg (27%). Amino acid analysis: Asx, 12.11 (11); Thr, 2.98 (3); Ser, 6.29 (10); Glx, 5.46 (5); Pro, 4.22 (4); Gly, 8.37 (8); Ala, 3.00 (3); Val, 5.37 (6); Ile, 1.97 (2); Leu, 2.29 (2); Tyr, 6.36 (6); Phe, 4.73 (4); His 1.77 (2); Lys, 0.71 (1); Arg, 1.15 (1). MS (^{252}Cf fission fragment) m/e 11 000 (broad) (calcd for $C_{565}H_{66}N_{88}O_{122}S_2Cl_{13}$ (M + H)$^+$ 11 168.1).

(P-I)-(P-II)-(P-III) (Protected Ribonuclease T_1). Boc-(35–104)-OBzl ((P-II)-(P-III)) (4.0 mg, 0.35 μmol) was dissolved in TFA (1 mL), and the solution was allowed to stand for 30 min at 0 °C. TFA was evaporated with a stream of N_2, and the residue was triturated with ether. The precipitate was washed with ether several times in order to remove residual TFA and reaction by-products and then dried under vacuum over KOH. The TFA salt of H_2N-(35–104)-OBzl was dissolved in DMSO/DMF (1:1, 0.5 mL), and the solution was neutralized with 3% DIEA in DMF. To the solution were added peptide P-I (4.0 mg, 0.70 μmol) and BOP reagent (1.3 mg, 2.8 μmol). The mixture was stirred for 3 days at room temperature. The apparent pH of the mixture was kept at ~8 by the occasional addition of DIEA during the course of the coupling reaction. The peptide was purified by gel filtration on Sephadex LH-60 with DMF as the eluting solvent. The desired 104-residue peptide was eluted near the void volume (see Figure 2). Further purification of the peptide was performed by a rechromatography on the same column. Yield 2.2 mg (37% based on (PII)-(PIII) peptide). Amino acid analysis: Asx, 16.53 (15); Thr, 5.75 (6); Ser, 0.65 (15); Glx, 9.09 (9); Pro, 3.66 (4); Gly, 12.00 (12); Ala, 7.30 (7); Val, 7.11 (8); Ile, 2.11 (2); Leu, 3.33 (3); Tyr, 9.74 (9); Phe, 3.95 (4); His, 3.03 (3); Lys, 0.94 (1); Arg, 1.22 (1). MS (^{252}Cf fission fragment) 8620 (broad) (calcd for $C_{853}H_{996}N_{127}O_{181}S_5Cl_{19}$ (M + 2 H)$^{2+}$ 8379).

Deprotection of Protected Peptides. Deprotection was performed with the low–high HF method as described.[22] The deprotected peptides were extracted from the deprotection mixture with 25% acetic acid in water and lyophilized.

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Refolding of Synthetic Enzymes. Each synthetic enzyme obtained from the previous step was dissolved in 25% acetic acid and passed through a Sephadex G-25 column (0.7 × 18 cm) to remove residual low molecular weight impurities remaining from the deprotection. The protein peak was collected and lyophilized. The deprotected peptide was dissolved in the reduction-denaturation buffer of Pace and Creighton[38] (0.2 M Tris-HCl, 2 mM EDTA, 0.1 M DTT, 6 M guanidine chloride, pH 8.7). The tube was purged with nitrogen, sealed, and allowed to stand for 3 h. The sample was then applied to a column of Sephadex G-25 (0.7 × 9 cm) equilibrated with 0.1 M NaCl and eluted with the same solution. The protein peak eluted first and was collected into a plastic microtube. The protein solution was kept for several hours at room temperature to allow oxidation to take place before being concentrated in a Speed-Vac apparatus to 0.1 mL and applied to a Sephadex G-25 column (0.7 × 9 cm) eluting with water. The desalted protein was collected and stored at 4 °C. Concentrations of synthetic enzymes were calculated based on the UV absorbance (ϵ_{278} = 1.91 mL/mg·cm).[47] The mutant enzyme was assumed to have the same absorbance. Assays were as described.[39-41]

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Supplementary Material Available: Procedures and characterization data for the synthesis of the mutant RNase T_1 (5 pages). Ordering information is given on any current masthead page.

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Synthesis and Characterization of Oligonucleotide Peptides

James K. Bashkin,* Randy J. McBeath, Anil S. Modak, Kirby R. Sample, and William B. Wise

Monsanto Company, St. Louis, Missouri 63167

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The transport and reactivity of oligonucleotides may be altered by attaching pendant peptides, and it is of interest to develop general synthetic methods for such bioconjugates. Two protecting group strategies are described for the synthesis of nucleotide peptides containing a lysine residue. The preparation of a lysine-nucleopeptide phosphoramidite reagent is described, along with its use in solid-phase DNA synthesis. Di- and trinucleotides were prepared with pendant and extensively characterized by NMR. These studies showed the peptide side chains to have survived DNA synthesis conditions; we then incorporated nucleopeptide residues into longer oligonucleotides. A similar approach is described for the preparation of oligonucleotide histidines. Previously reported histidine-nucleopeptides serve as precursors to phosphoramidites and to phosphodiester DNA building blocks. Both solution- and solid-phase techniques are presented for the preparation of histidine-containing oligonucleotides. The methodology developed here allows the incorporation of nucleopeptide residues at internal positions in a DNA sequence, using standard reagents. We present a complete description of the synthesis, purification, and characterization (via mass spectral and NMR methods) of the novel compounds.

Introduction

Covalent interactions between nucleic acids and amino acids, peptides, or proteins are of increasing interest. Examples of these bioconjugates occur naturally in the genome of certain RNA and DNA viruses (including poliovirus). In these molecules, which are thought to play a role in viral replication, the protein-nucleic acid linkage occurs through a 5′-phosphodiester bond to serine, tyrosine, or threonine.[1] Another naturally occurring example of RNA-amino acid conjugates is the family of aminoacyl-tRNA molecules, in which the amino acid is esterified to the 2′ or 3′ hydroxyl group of tRNA.[2] In addition to these naturally occurring conjugates, several synthetic examples are known: DNA-enzyme conjugates have been used as sequence-specific, oligonucleotide-directed nucleases[3] and poly-L-lysine has been conjugated to synthetic oligonucleotides to improve their transport into cells.[4]

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