Synthesis of the 60 Amino Acid Homeo Domain and Smaller Fragments of the *Drosophila* Gene Regulatory Protein *Antennapedia* by a Segment Synthesis–Condensation Approach

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The synthesis of the 60-amino acid homeo domain of the *Antennapedia* gene regulatory protein is described starting with 10 highly purified, fully side-chain-protected peptide fragments of 4–10 amino acid residues. These fragments were condensed on a *p*-nitrobenzophenone oxime resin to give three medium-sized segments, 17–20 amino acids in length, which were further coupled in solution to give the full length homeo domain protein 1. This strategy was also applied to two smaller fragments of the homeo domain containing 29 and 40 residues, which encompass the helix-turn-helix, DNA-binding portion of the protein. Circular dichroism data for the three *Antennapedia* peptides is described.

The chemical synthesis of biologically active proteins in high purity is still a major objective of protein chemistry. The advent of Merrifield's solid-phase technique accompanied by the improvement of high-performance liquid chromatography (HPLC) makes possible the sequential synthesis of peptides containing 30–40 amino acid residues in high purity.[2,3] The preparation of larger peptides and proteins becomes problematic, although syntheses of proteins with up to 150 residues have been reported.[4] A major problem with the sequential approach is difficulty in separating the microheterogeneous products produced in long protein syntheses (i.e., single amino acid deletions), along with difficulty in purity assessment. In addition, making any change in the protein sequence would require an entirely new synthesis if the sequential approach is used.

A desirable approach toward the synthesis of proteins larger than 40–50 amino acid residues would be convergent, whereby smaller peptide fragments could be purified to homogeneity before coupling to form the larger protein. In this paper we describe the preparation of the 60 amino acid homeo domain 1 from the *Drosophila* gene regulatory protein *Antennapedia* (*Antp*) by a segment synthesis–condensation approach.[5] The *Antp* homeo domain sequence is highly conserved in many proteins involved in differential gene expression during early *Drosophila* development,[6,7] and its structure contains a helix-turn-helix motif as determined in solution by nuclear magnetic resonance.[8] We also describe the synthesis of two smaller peptides corresponding to residues 21–60 (**2**) and 27–55 (**3**) of the *Antp* homeo domain which contain the helix-turn-helix region by the same approach and compare their circular dichroism (CD) spectra to the intact homeo domain 1.

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                10        20        30        40        50        60
(1) RKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEN
(2)                 HFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEN
(3)                       TRRRRIEIAHALCLTERQIKIWFQNRRMK
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Two factors which are inherent to the success of the segment synthesis-condensation strategy are the ability to prepare fully side-chain-protected peptide fragments and the methods by which these fragments may be coupled. The synthesis of fully side-chain-protected peptide fragments has previously been achieved using solid phase methodology on a copolymer of *p*-nitrobenzophenone oxime bound to polystyrene-1% divinylbenzene (oxime resin).[9–12] In this paper we report the coupling of side-chain-protected fragments in the synthesis of 60-, 40-, and 29-amino acid residue peptides of the *Antp* homeo domain. In this way protein 1 was constructed in a completely convergent manner from 10 peptide fragments, varying from 4 to 10 amino acids in length.

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(2) All amino acids except glycine are of L-configuration. Standard abbreviations for amino acids, peptides, and protecting groups follow the recommendations of the IUPAC-IUB Commission on biochemical nomenclature. Other abbreviations used in the test are the following: A, alanine; C, cysteine; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; Q, glutamine; R, arginine; T, threonine; W, tryptophan; Y, tyrosine; AcOH, acetic acid; *Antp*, *Antennapedia*; Boc, *tert*-butyloxycarbonyl; Bom, benzyloxymethyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; Bzl, benzyl; Cl-Z, 2-chlorobenzyloxycarbonyl; Cl_2Bzl, 2,6-dichlorobenzyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIEA, *N*,*N*′-diisopropylethylamine; Ecnox, ethyl 2-hydroxyimino-2-cyanoacetate; EDC, 1-ethyl-3-[(dimethylamino)propyl]carbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, *N*,*N*′-dimethylformamide; HOPip, 1-hydroxypiperidine; MeBzl, methylbenzyl; NMM, *N*-methylmorpholine; Pam, phenylacetamidomethyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Tos, tosyl; Z, benzyloxycarbonyl; CD, circular dichroism; HPLC, high-performance liquid chromatography.

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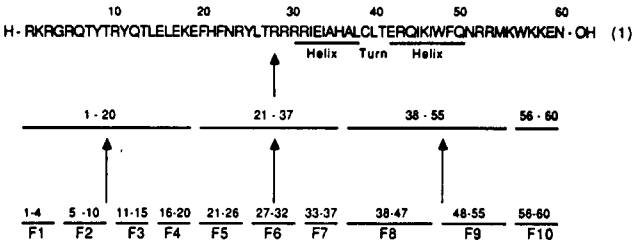

Figure 1. Strategy for the synthesis of the 60-amino acid *Antp* homeo domain 1. The putative helix-turn-helix portion is indicated.

Results

General Strategy. The *Antp* homeo domain 1 was divided into 10 small segments for segment synthesis–condensation on the oxime resin (Figure 1). The retrosynthetic strategy is as follows: Protein 1 was divided into three large segments and one small segment. The latter small segment was designed as a spacer on the resin for the final medium-sized segment condensations on the resin. The three large segments were further divided into two to four small protected segments which would be synthesized in a stepwise manner on the oxime resin.

When dividing the 60-amino acid protein into 10 segments, the following points were taken into consideration: (1) peptides were selected with no more than 10 amino acids to insure easy purification by reverse-phase HPLC and which could be coupled to each other on the resin in high yield; (2) Boc-Asn and Boc-Gln were generally not used in the third position of the peptide fragments because of difficulties with diketopiperazine formation which resulted in cleavage of the first two amino acids from the resin, although by using (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) as the activating agent these amino acids have been used as the third amino acid;[13] (3) the first amino acid of the peptide fragment should be soluble in CH_2Cl_2 to obtain reasonable substitution levels on the resin.[14] Peptides 2 and 3, which correspond to residues 21–60 and 27–55 of the *Antp* homeo domain, respectively, and contain the putative helix-turn-helix DNA binding region, were also obtained using this synthetic strategy.

Synthesis of the Small Fragments

For the synthesis of the small fragments F1–F10, *tert*-butyloxycarbonyl (Boc) was used for semipermanent α-amine protection, and benzyl-based protecting groups were used for the side-chain functionality due to their stability to repeated trifluoroacetic acid (TFA) exposure and the conditions used to cleave the fragments from the oxime resin. The protecting groups used for side-chain functionality were as follows: amine, 2-chlorobenzyloxycarbonyl (ClZ); carboxyl and hydroxyl, benzyl (Bzl); guanidine, tosyl (Tos); imidazole, benzyloxymethyl (Bom); indole, formyl; sulfhydryl, methylbenzyl (MeBzl); phenol, 2,6-dichlorobenzyl (Cl_2Bzl). The 10 small segments were synthesized on the oxime resin in a stepwise manner by the symmetrical anhydride method[15] for most of the Boc-amino acid derivatives, except for Boc-Asn, -Gln, and -His(Bom) which used the HOBt method,[16] followed by treatment with Zn/AcOH or cleaved from the resin with an amino acid benzyl ester. No significant racemization accompanied these procedures.[11] These side-chain-protected small segments were purified to homogeneity by preparative C_4 reverse-phase HPLC and characterized by amino acid analysis, ^{252}Cf fission-fragment ionization mass spectroscopy,[17] and ^1H-NMR.

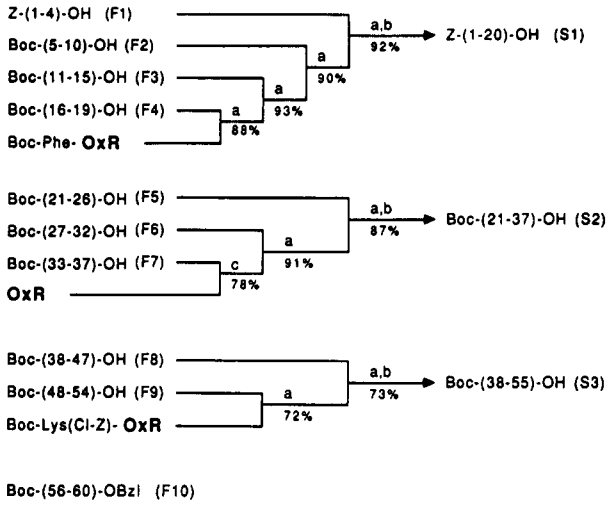

Figure 2. Fragment condensation on the oxime resin to synthesize the medium-sized segments: (a) (1) 25% TFA, (2) DCC–HOBt, (3) acetic anhydride; (b) (1) HOPip, (2) Zn/5% TFA–DMF, (3) Sephadex LH-60 (DMF); (c) (1) DCC–Ecnox, (2) acetic anhydride; OxR, the oxime resin. Yields represent those for the coupling procedure.

We have also examined the utility of BOP for the stepwise synthesis of protected segments on the oxime resin.[18,19] The small segments were synthesized using the BOP reagent (3 equiv) and Boc-amino acid derivatives (3 equiv), and the purity and yields of the peptides obtained were the same as those synthesized by the symmetrical anhydride method. This reagent was also used for the coupling of Boc-Asn and -Gln to avoid side-chain dehydration to the nitrile.[13]

Synthesis of the Medium-Sized Segments

The small fragments were reattached to an amino acid oxime resin with DCC–HOBt in DMF–CH_2Cl_2 (F4 and F9) or directly reattached to the oxime resin with DCC–ethyl 2-hydroximino-2-cyanoacetate (Ecnox)[12,20] in CH_2Cl_2. (Direct attachment of the protected peptide in the presence of DMF gave low yields and racemization.[11]) The other small fragments were reacted with these oxime resins in a stepwise fashion with DCC–HOBt to give the medium-sized segments (17–20 amino acids in length) as shown in Figure 2.

High coupling yields (between 72–93%) were obtained in the fragment condensations as determined at each step

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by amino acid analysis. The medium-sized segments were removed from the resin with HOPip, and the hydroxypiperidyl peptide esters were purified by size-exclusion chromatography on Sephadex LH-60 with DMF as the solvent. Reduction of the peptide esters with Zn in 5% TFA/DMF (v/v) provided the desired medium-sized segments as determined by amino acid analysis and fission-fragment mass spectroscopy (Table I). No significant racemization was detected during the coupling and deprotection procedures.[11]

Synthesis of the *Antp* Homeo Domain Peptides 1 and 2

At first we had intended to assemble the medium-sized segments on a polymer to avoid the general solubility problems associated with large side-chain-protected peptides. The C-terminal pentapeptide of the *Antp* homeo domain, which we had designed to use as a spacer on the Pam resin,[21] was synthesized by coupling the tetrapeptide Boc-(56–59)-OH to the Asn-Pam resin (97% yield). Boc-(38–55)-OH was coupled to the H-(56–60)-Pam resin to give the Boc-(38–60)-Pam resin (70% yield after the first coupling, 90% after the second coupling), and further coupling of Boc-(21–37)-OH to the Boc-(38–60)-Pam resin gave the Boc-(21–60)-Pam resin (17% yield after the first coupling, 28% after the second). The low yields obtained in the second medium-sized segment couplings indicated that the polystyrene resin was not suitable for segment condensations of large peptides. Therefore, we adopted a solution-phase method for coupling our medium-sized segments.

The medium-sized segments were successfully assembled starting from the C-terminal pentapeptide benzyl ester (F10) in solution using the EDC–HOBt method to give the fully side-chain-protected peptides 1 and 2 (Figure 3).[22,23]

After each segment condensation, the protected peptides were purified by size exclusion chromatography on Sephadex LH-60 (DMF) as shown in Figure 4. Coupling yields ranged from 50 to 83% after purification, and each protected peptide was characterized by amino acid analysis and fission-fragment mass spectroscopy (Table II).

After purification, the protected peptides corresponding to 1 and 2 were deprotected using a low and high HF procedure.[24] The crude deprotected materials were purified by gel-filtration chromatography and reverse-phase HPLC. The purities were determined by analytical HPLC (Figure 5) and SDS-polyacrylamide gel electrophoresis and characterized by amino acid analysis using acid and enzymatic hydrolyses, peptide sequencing by automated Edman degradation (1–30 residues) for peptide 1, and fission-fragment mass spectroscopy. Deprotection and purification yields for peptides 1 and 2 were 22 and 29%, respectively.

Synthesis of *Antp* Homeo Domain Peptide 3

Peptide 3 was prepared by reattaching fragment F9 to a Boc-Lys(ClZ) oxime resin, followed by coupling fragments F8–F6 (Figure 6). Coupling yields ranged from 68–93%. The terminal *t*-Boc α-amine protecting group was removed with TFA, followed by full deprotection by the low and high HF procedure. The crude peptide was purified to homogeneity on HPLC to give the desired 3 as determined by amino acid analysis and fission-fragment

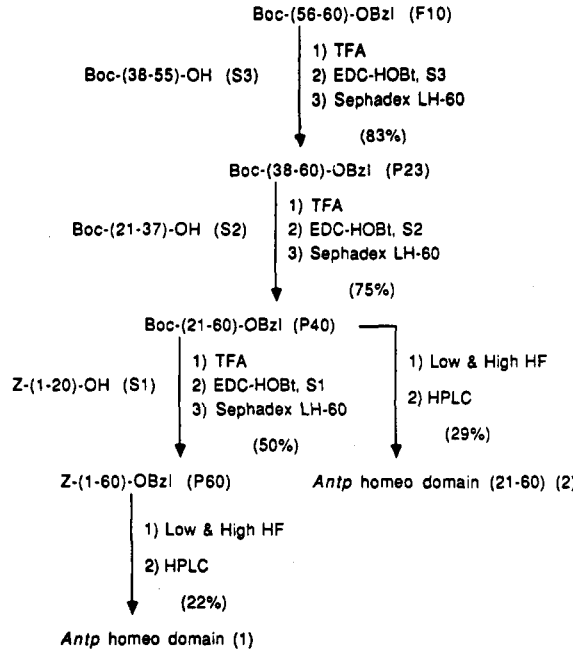

Figure 3. Condensation of the medium-sized segments in solution, followed by HF treatment. Yields represented are for both the coupling and purification.

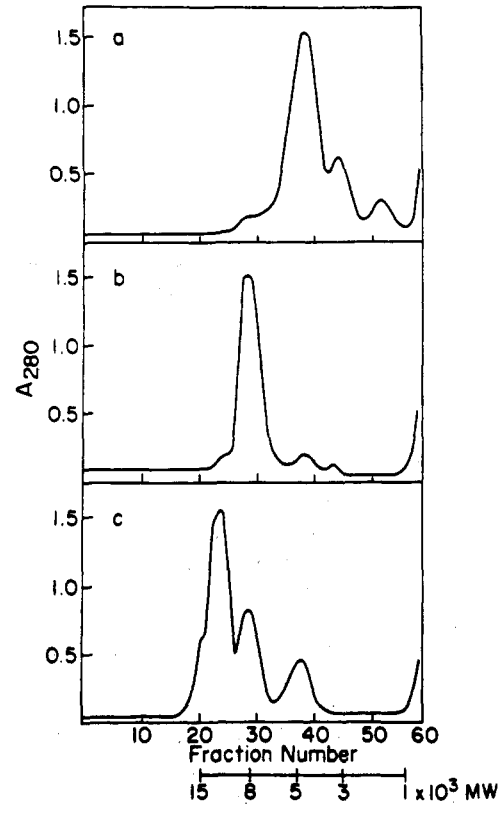

Figure 4. Gel filtration of the crude segments with a Sephadex LH-60 column (DMF): (a) Boc-(38–60)-OBzl (**P23**); (b) Boc-(21–60)-OBzl (**P40**); (c) Z-(1–60)-OBzl (**P60**). Molecular weight marker is shown at the bottom.

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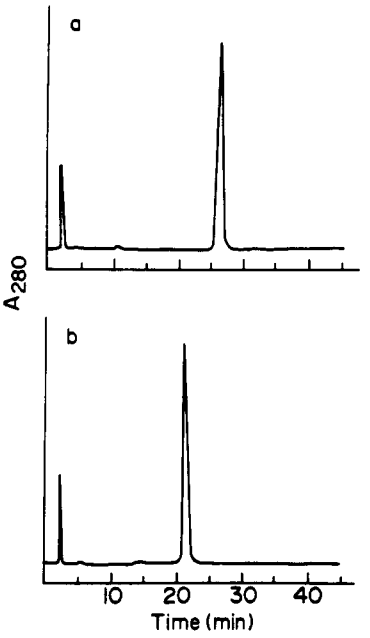

Figure 5. HPLC of the purified peptides 1 and 2. Column, TSK Phenyl-5PW RP (0.46 × 7.5 cm); solvent, 19–46% CH_3CN in 0.05% TFA (45 min); flow rate, 1 mL/min; (a) peptide 1, (b) peptide 2.

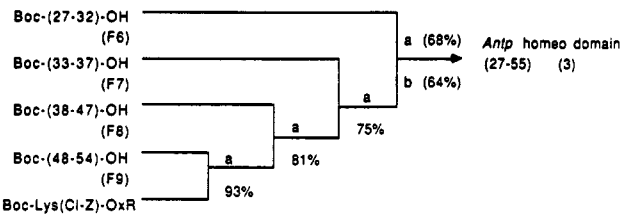

Figure 6. The fragment condensation synthesis of peptide 3. (a) (1) 25% TFA, (2) DCC–HOBt, (3) acetic anhydride; (b) (1) 25% TFA, (2) low–high HF treatment, (3) reverse-phase HPLC.

mass spectroscopy. Deprotection and purification yield for peptide 3 was 31%.

Circular Dichroism Measurements

CD spectra of peptides 1–3 were measured in aqueous buffer and in a mixture of 1:1 trifluoroethanol (TFE)/buffer (v/v) (Figure 7). All spectra showed typical α-helical CD patterns in both aqueous buffer and 50% TFE, which indicates that these peptides have a significant α-helical structure. The α-helicity estimated from CD data is as follows: peptide 1, 22% in buffer and 45% in 1:1 TFE/buffer, corresponding to 13 and 27 amino acid residues of the 60-amino acid protein which exist in a helical conformation; peptide 2, 24% in buffer and 68% in 1:1 TFE/buffer, corresponding to 10 and 27 amino acid residues of the 40-amino acid peptide which exist in a helical conformation; and peptide 3, 40% in buffer and 91% in 1:1 TFE/buffer, corresponding to 12 and 26 amino acid residues of the 29-amino acid peptide which exist in a helical conformation.[25]

Discussion

The present work has demonstrated that the small protein (60 amino acids) corresponding to the homeo domain of the homeotic protein *Antp* is readily accessible by our segment synthesis–condensation method using the oxime resin. Our previous study had showed that a 44-amino acid Apolipoprotein A-I peptide model constructed with Ala, Glu, Leu, Lys, and Pro could be successfully synthesized by segment condensation of a 21-amino acid peptide and a 22-amino acid peptide on the oxime resin.[12] The present study has confirmed that the method can be applied to the preparation of large peptides and small proteins with native sequences which would be difficult to obtain by stepwise solid-phase synthesis procedure.

The purpose of developing the segment synthesis–condensation procedure on the oxime resin was to avoid the microheterogeneity (one-amino acid deletions) of large peptides synthesized by the stepwise solid-phase method. The small protected peptides (in this case containing 4–10 amino acids) were readily prepared in a stepwise fashion on the oxime resin. The protected segments were detached from the resin with either HOPip or an amino acid ester, and the crude segments were purified by HPLC to avoid contamination of the peptides by single amino acid deletions. The purified small segments were reattached to the resin by methods which suppress racemization and coupled to each other on the oxime resin.[11] The medium-sized segments were detached from the resin and purified by size-exclusion chromatography on LH-60 Sephadex. Purification on LH-60 Sephadex with DMF as a solvent proved to be a very efficient way to separate the medium-sized, fully protected peptides which are only sparingly soluble in the normal solvents used for reverse-phase HPLC. In this way peptides differing in size by one or more segments were easily removed from the desired large segments by this method. Coupling yields for assembling these segments were high (>72%). In the case of peptide 3, in which four peptide fragments were condensed, the coupling efficiency decreased as the chain length on the resin grew probably due to steric hinderance in the coupling reactions as the peptides became longer. The medium-sized protected peptides (17–20 amino acids in length) were used further as segments to synthesize the larger peptides.

Preliminary experiments in which we attempted to condense medium-sized peptides on a polymer support were not successful. Similar results were also obtained during the coupling of other large fragments on a polymer support in our group.[13] It is possible that this problem could have been overcome by using a resin with a larger pore size[26] or a polyacrylamide polymer,[27] but we chose to assemble the medium-sized protected peptides in solution to give the desired peptides in relatively high yield. The peptides obtained from medium-sized segment couplings (23, 40, and 60 amino acids in length) were purified by Sephadex LH-60 chromatography, which in our experience is sufficient for purifying protected peptides with 60–70 amino acids (MW = 13000–15000) as shown in Figure 4.

The CD spectra of 1, 2, and 3 suggest that all three peptides contain a significant amount of α-helical character. It should be noted that the number of residues involved in the α-helical portion of all three peptides remains constant. This fact suggests that these peptides, each differing in length, contain the same helical segments

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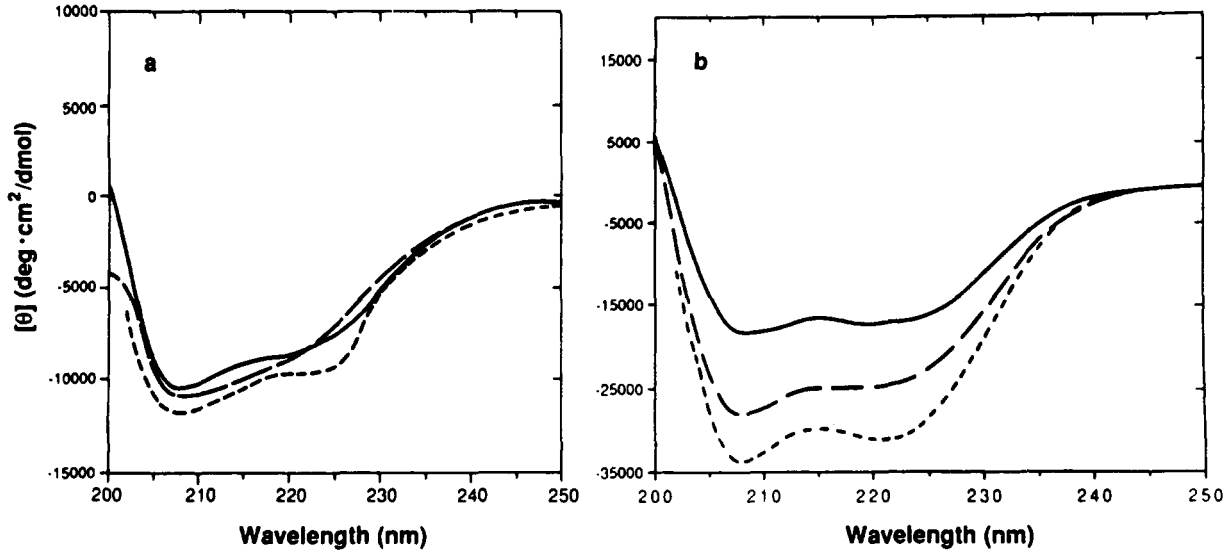

Figure 7. CD spectra of peptides 1 (—), 2 (– – –), and 3 (- - -) in (a) 25 mM Tris·HCl (pH 7.5)/0.16 M KCl/1 mM DTT and in (b) TFE/buffer (1:1, v/v), [θ] denotes the mean residue molar ellipticity.

in each of the solvents used. The addition of TFE increased the helical content of these peptides as compared to buffer alone as follows: peptide 1, 23%, peptide 2, 44%, and peptide 3, 45%. Peptides 2 and 3 both had larger increases in helical content upon addition of TFE as compared to peptide 1 which may be due to the more flexible nature of the smaller peptide chains in aqueous solution. The maximum helical content in peptide 1 by CD is also lower than would be predicted from the solution NMR structure (45% by CD, 57% by NMR), which may be due to differences in the concentration (and hence aggregation) of peptide used in these experiments (25 and 50 μM for CD, 4 and 11 mM for NMR).[8]

Scaling up the synthesis of small proteins is also feasible with our procedure. The oxime resin itself is easily prepared from polystyrene-1% divinylbenzene copolymer and has been regenerated after peptide cleavage, and the protected small fragments are rapidly synthesized on the oxime resin. Half of the Boc-amino acid derivatives has been saved by using the BOP reagent for sequential coupling, as compared to the usual symmetrical anhydride method.

In conclusion, the synthesis of fragments of the *Antp* homeo domain by a segment synthesis–condensation approach has proven successful. This method has great potential for the synthesis of protein sequences which may be difficult to obtain in other ways. The synthesis of mutant proteins or proteins containing unnatural amino acids and nonpeptidic components can be accomplished easily with these methods by replacement of segments in a cassette mode. As methods improve for coupling longer protein sequences by either chemical or enzymatic means, the utility of the segment synthesis–condensation approach for the preparation of large proteins will be realized.

Experimental Section

Equipment, Materials, and Method. Amino acid analyses were performed with a Dionex D-300 amino acid analyzer. Side-chain-protected peptides and resin-bound peptides were hydrolyzed with a propionic acid/concentrated HCl (1:1, v/v) mixture in evacuated and sealed tubes at 130 °C for 2–3 h. Deprotected peptides were hydrolyzed with 6 M HCl at 110 °C for 24 h. For enzymatic hydrolysis, peptide (20 μg) was incubated with trypsin (2 μg) and leucine aminopeptidase (porcine kidney microsomes, 10 μg) in the presence of $CaCl_2$ (1 μg) in 0.2 M triethylamine–acetate buffer (pH 8.3, 100 μL) for 37 °C for 5 h. CD spectra were measured with an Aviv (Philadelphia) modification of a Cary Model 60 ds spectropolarimeter. ^{252}Cf fission-fragment mass spectra were measured by Dr. B. Chait at the Rockefeller University Biotechnology Mass Spectrometric Research Resource. HPLC was performed with a TSK Phenyl-5PW RP column (0.46 × 7.5 cm) for analytical purposes and with a VYDAC C-18 column (1.0 × 25 cm) for preparative separations or a VYDAC C-4 column (2.2 × 25 cm) for purication of side-chain-protected small peptides. A Sephadex LH-60 column (2.5 × 80 cm) was used for the purification of the medium-sized protected peptides with DMF as a solvent. The UV absorbance at 280 nm was monitored to detect the peptides. Amino acid derivatives used were Boc-Asp(OBzl), Glu(OBzl), Arg(Tos), Cys(MeBzl), Lys(ClZ), Ser(Bzl), Thr(Bzl), Trp(HCO), and Tyr(Cl_2Bzl), which were purchased from Peninsula Laboratories and Boc-His(Bom) from Bachem Biosciences. Stepwise solid-phase peptide syntheses on the oxime resin were carried out with a Beckman Model 990 peptide synthesizer or manually. Peptide segment condensations on the resin were performed manually. The *p*-nitrobenzophenone oxime resin was prepared according to the previously reported procedure.[9] The substitution levels of amino acids on the resin was on an average of 0.55 mmol/g of resin.

Attachment of Boc-Amino Acids to the Oxime Resin (4). Boc-amino acid (1 mmol) and DCC (1 mmol) were added to the oxime resin (1 g) in CH_2Cl_2 (10 mL). The mixture was shaken at room temperature overnight and then worked up according to the previously described procedure[11] to give Boc-amino acid oxime resin ester 4. In the case of Boc-Arg(Tos), double coupling was used in the presence of HOBt, because substitution levels of Boc-amino acids with DMF/CH_2Cl_2 as a solvent were lower (less than 0.2 mmol/g of resin) than those reacted in CH_2Cl_2 (0.55 mmol/g resin). Substitution level of Boc-Arg(Tos) was 0.33 mmol/g of resin.[28]

Stepwise Peptide Synthesis on the Oxime Resin (5). Boc-amino acid oxime resin ester 4 was placed in a reaction vessel, and the synthesis was carried out on an automated Beckman synthesizer or manually as described previously.[11] The Boc-amino acid symmetrical anhydride (3 equiv) was used for all amino acids with the exception of Boc-Asn, -Gln, and -His(Bom) which used the Boc-amino acid HOBt active ester (4 equiv). When coupling with the BOP reagent, the Boc-amino acid (3 equiv) in DMF (5 mL/g of resin), and BOP (3 equiv) in DMF (5 mL/g of resin) were added directly to the resin, followed by DIEA (5

(28) This low substitution level may be due to the specific side reaction of Boc-Arg(Tos) oxime resin (lactam formation). Nishino, N.; Xu, M.; Mihara, H.; Fujimoto, T. *Tetrahedron Lett.* **1992**, *33*, 1479–1482.

Table I. Amino acid Analyses and Fission-Fragment Mass Spectra of the Small Segments

peptide	amino acid analysis[a]	$(M + Na)^+$ found	$(M + Na)^+$ calcd
F1	Gly(1) 1.0, Lys(1) 1.02, Arg(2) 2.14	1149.4	1149.2
F2	Thr(2) 1.60, Glu(1) 1.03, Tyr(1) 1.0, Arg(2) 1.98	1594.6	1594.65
F3	Thr(1) 0.87, Glu(2) 2.08, Leu(1) 1.0, Tyr(1) 1.09	1114.7	1115.1
F4	Glu(2) 2.20, Leu(1) 1.0, Lys(1) 1.06	989.4	989.5
F5	Asp(1) 0.93, Leu(1) 1.0, Tyr(1) 1.03, Phe(1) 0.97, His(1) 1.00, Arg(1) 0.95	1405.6	1405.9
F6	Thr(1) 0.90, Ile(1) 1.0, Arg(4) 3.92	1686.9	1686.9
F7	Glu(1) 1.04, Ala(2) 2.0, Ile(1) 1.01, His(1) 0.96	872.5	872.4
F8	Thr(1) 1.00, Glu(2) 2.25, Ile(2) 1.81, Leu(2) 2.23 Lys(1) 0.98, Arg(1) 1.0, (Cys(1))	1924.0	1923.6
F9	Asp(1) 1.06, Glu(1) 1.10, Met(1) 0.95, Phe(1) 1.0, Arg(2) 2.13 (Trp(1))	1496.6	1496.6
F10	Asp(1) 1.03, Glu(1) 1.10, Lys(2) 2.0, (Trp(1))	1372.1	1372.2

[a] Hydrolyzed with propionic acid/concentrated HCl (1:1, v/v).

Table II. Amino Acid Analyses and Fission-Fragment Mass Spectra of the Medium-Sized Segments

segment	amino acid analysis[a]	$(M + Na)^+$ found	$(M + Na)^+$ calcd
Z-(1–20)-OH (S1)	Thr(3) 2.64, Glu(5) 5.22, Gly(1) 1.0, Leu(2) 2.08, Tyr(2) 2.03, Phe(1) 0.99, Lys(2) 1.92, Arg(4) 3.93	4571.9	4573.0
Boc-(21–37)-OH (S2)	Asp(1) 1.00, Thr(1) 0.90, Glu(1) 1.10, Ala(2) 2.0, Ile(2) 1.92, Leu(1) 1.10, Tyr(1) 1.03, Phe(1) 0.99, His(2) 2.11, Arg(5) 5.23	3683.6	3683.2
Boc-(38–55)-OH (S3)	Asp(1) 1.08, Thr(1) 0.85, Glu(3) 3.00, Met(1) 1.10, Ile(2) 1.84, Leu(2) 1.85, Phe(1) 1.0, Lys(2) 2.19, Arg(3) 3.30	3599.3	3599.6

[a] Hydrolyzed with propionic acid/concentrated HCl.

equiv). The reaction was shaken for 2 h at rt. The resin was washed with DMF before and after coupling with the BOP reagent.

Removal of Protected Peptide Segments from the Oxime Resin (6) (a) with HOPip and Zn/AcOH. The protected peptide was cleaved from the oxime resin with HOPip (3 equiv) in CH_2Cl_2) (10 mL/g of resin) at rt for 20 h. The resin was filtered and washed with CH_2Cl_2, and the filtrate was concentrated in vacuo to give the peptide 1-piperidyl ester. The 1-piperidyl ester was treated with Zn (30 equiv) in 90% AcOH (10 mL/g of starting resin) for 30 min at rt. The zinc was filtered, washed with AcOH, and H_2O was added to the combined filtrates to precipitate the crude protected peptide. **(b) with Amino Acid Ester.** To a suspension of Boc-Trp(HOC)-Lys(ClZ)-Lys(ClZ)-Glu(OBzl)-oxime resin (1 equiv) in DMF were added HCl·H-Asn-OBzl (4 equiv), DIEA (4 equiv), and AcOH (4 equiv) in that order. The mixture was shaken at room temperature for 1 day, filtered, and concentrated to give crude Boc-Trp(HCO)-Lys(ClZ)-Lys(ClZ)-Glu(OBzl)-Asn-OBzl (F10).

The crude peptides were dissolved in DMF and purified by HPLC (VYDAC C4 preparative column (2.2 × 25 cm)) with an appropriate mixture of CH_3CN/0.1% AcOH or *i*PrOH–CH_3CN (2:1, v/v)/0.1% AcOH as a elution solvent. Each segment was characterized by amino acid analysis, ^{1}H-NMR, and ^{252}Cf fission-fragment ionization mass spectroscopy (Table I).

Attachment of the First Segments to the Oxime Resin (7). The first segment (1 equiv) was reattached in two different ways, i.e., (a) direct attachment to the oxime resin (1.5 equiv) with DCC (1 equiv) and Ecnox (2 equiv) in CH_2Cl_2 or (b) coupled to the amino acid oxime resin ester (2 equiv) with DCC (1 equiv) and HOBt (2 equiv) in 7:3 DMF/CH_2Cl_2 (10 mL/g of resin). The mixtures were shaken at 4 °C for 4 h and at room temperature for 20 h and then worked up as follows: (1) wash, DMF (3×); (2) wash, DMF/CH_2Cl_2 (1:1, v/v) (2×); (3) wash, CH_2Cl_2 (3×); (4) dry under vacuum; (5) check the substitution level by amino acid analysis (substitution levels were generally ca. 0.2 mmol/g of resin). Yields are shown in Figure 2.

Segment Condensation on the Oxime Resin (8). Protected peptide resin 7 was placed in a reaction vessel, and the synthesis was carried out manually according to the following protocol: (1) wash, CH_2Cl_2 (2×); (2) acetylate, Ac_2O (5 equiv) and NMM (5 equiv) in CH_2Cl_2 (1 × 60 min); (3) wash, CH_2Cl_2 (4×); (4) prewash, 25% TFA/CH_2Cl_2 (v/v) (1 × 1 min); (5) deprotect, 25% TFA/CH_2Cl_2 (1 × 30 min); (6) wash, CH_2Cl_2 (2×); (7) wash, *i*-PrOH (1×); (8) wash, CH_2Cl_2 (2×); (9) wash, *i*-PrOH (1×); (10 wash), CH_2Cl_2 (2×); (11) neutralize, 1.1% NMM/CH_2Cl_2 (3 × 1 min); (12) wash, CH_2Cl_2 (4×); (13) couple, protected peptide acid (1.2–1.5 equiv) and HOBt·H_2O (2.4–3.0 equiv) in DMF/CH_2Cl_2 (1:1, v/v), DCC (1.2–1.5 equiv) at 4 °C for 4 h and at room temperature, 20 h; (14) wash, DMF (3×); (15) wash, DMF/CH_2Cl_2 (1:1, v/v) (2×); (16) wash, CH_2Cl_2 (3×); (17) dry under vacuum; (18) check the coupling yield by amino acid analysis. The solvent volume for all wash steps was 15 mL/g of resin, and the time was 1 min. The yields of each segment condensation on the resin are shown in Figure 2.

Removal of Protected Medium-Sized Segments from the Oxime Resin and Subsequent Reduction with Zinc Dust (9). Protected peptide oxime resin 8 (1 equiv) was suspended in DMF (10 mL/g of resin), and the mixture was shaken with HOPip (3 equiv) at room temperature for 1 day. The resin was filtered and washed with DMF, DMF/CH_2Cl_2 (1:1, v/v), and CH_2Cl_2. The combined filtrate was evaporated under vacuum. The residual peptide 1-piperidyl ester was dissolved in DMF and applied to a column of Sephadex LH-60 with DMF. Fractions comprising the major peak were pooled and the solvent was evaporated under vacuum. The purified peptide 1-piperidyl ester (1 equiv) was dissolved in 5% TFA/DMF (v/v), and Zn dust (ca. 30 equiv) was added to the stirring solution. After stirring at room temperature for 1 h, Zn was removed by filtration and washed with DMF. The combined filtrate was evaporated under vacuum and the peptide acid was precipitated with H_2O. The results of amino acid analysis and mass spectroscopy are shown in Table II.

Segment Condensation in Solution. Boc-(38–60)-OBzl (P23). Boc-(56–60)-OBzl (F10) (60 mg, 0.044 mmol) was treated with TFA (1 mL) at 0 °C for 30 min. TFA was removed by a stream of nitrogen, and the residue was triturated with ether and dried under vacuum to give TFA·H-(56–60)-OBzl. The TFA salt was dissolved in DMF (1.5 mL), and neutralized to pH 8 with DIEA. Boc-(38–55)-OH (100 mg, 0.028 mmol), HOBt·H_2O (9 mg, 0.06 mmol), and EDC·HCl (11 mg, 0.06 mmol) were added to the solution at 0 °C. The mixture was stirred at 0 °C for 1 day, diluted with DMF, and applied to a column of Sephadex LH-60 (2.5 × 80 cm) with DMF (see Figure 4a). Fractions of the major peak corresponding to the molecular weight of Boc-(38–60)-OBzl were pooled, evaporated, and precipitated with H_2O: 111 mg (83%); amino acid analysis, Asp(2) 2.18, Thr(1) 0.82, Glu(4) 4.38, Met(1) 0.97, Ile(2) 1.91, Leu(2) 2.0, Phe(1) 1.10, Lys-(4) 4.18, Arg(3) 2.92, (Cys 1, Trp 2); mass spectrum $(M + Na)^+$ 4829.1 (calcd 4830.3), $(M + 2\ Na)^{2+}$ 2426.4 (calcd 2426.7).

Boc-(21–60)-OBzl (P40). Boc-(38–60)-OBzl (82 mg, 0.017 mmol) was treated with TFA (1 mL) at 0 °C for 1 h as described above. TFA·H-(38–60)-OBzl was dissolved in DMF (1 mL) and neutralized with DIEA. To this solution of Boc-(21–37)-OH (62

mg, 0.017 mmol) in DMF were added at 0 °C HOBt·H_2O (6 mg, 0.04 mmol) and EDC·HCl (8 mg, 0.04 mmol). The mixture was stirred at 0 °C for 2 days, diluted with DMF, and applied to a column of Sephadex LH-60 with DMF (Figure 4b). Fractions of the major peak corresponding to the molecular weight of Boc-(21–60)-OBzl were pooled, evaporated, and precipitated with H_2O: 106 mg (75%); amino acid analysis, Asp(3) 3.19, Thr(2) 1.85, Glu(5) 5.35, Ala(2) 2.0, Met(1) 0.99, Ile(4) 3.90, Leu(3) 3.11, Tyr(1) 0.98, Phe(2) 1.99, His(2) 1.90, Lys(4) 4.24, Arg(8) 8.24 (Cys 1, Trp 2); mass spectrum $(M + H)^+$ 8353.0 (calcd 8350.3), $(M + 2H)^{2+}$ 4176.9 (calcd 4175.7).

Z-(1–60)-OBzl (P60). Boc-(21–60)-OBzl (100 mg, 0.012 mmol) was treated with TFA (1 mL) at 0 °C for 1 h as described before. To the neutralized solution of H-(21–60)-OBzl in DMF (1 mL) were added at 0 °C Z-(1–20)-OH (55 mg, 0.012 mmol), HOBt·H_2O (3.7 mg, 0.024 mmol), and EDC·HCl (4.6 mg, 0.024 mmol). The mixture was stirred at 0 °C for 2 days, diluted with DMF, and applied to a column of Sephadex LH-60 with DMF (Figure 4c). Fractions corresponding to the molecular weight of Z-(1–60)-OBzl were pooled, evaporated, and precipitated with H_2O: 77 mg (50%); amino acid analysis, Asp(3) 3.30, Thr(5) 4.01, Glu(10) 9.95, Gly(1) 0.90, Ala(2) 2.0, Met(1) 0.91, Ile(4) 4.01, Leu(5) 5.02, Tyr(3) 3.03, Phe(3) 3.30, His(2) 2.16, Lys(6) 6.20, Arg(12) 12.17; mass spectrum, $(M + H)^+$ 12500–12800 (calcd 12781), $(M + 2H)^{2+}$ 6300–6400 (calcd 6392).

The *Antp* Homeo Domain 1. Z-(1–60)-OBzl (30 mg, 0.0023 mmol) was treated with the low–high HF method.[24] The P60 fragment was premixed with Cys (10 mg), dimethyl sulfide (DMS) (3.25 mL), *p*-cresol (0.25 mL), and *p*-thiocresol (0.25 mL), and liquid HF (1.3 mL) was added at −70 °C. The mixture was stirred at 0 °C for 2 h. HF and DMS were removed under vacuum. HF (9.5 mL) was added to give a final ratio of HF/*p*-cresol/*p*-thiocresol (95:2.5:2.5, v/v). The mixture was stirred at 0 °C for 1 h, and HF was removed under vacuum. The residue was triturated with ethyl acetate, and the powder obtained was dissolved in 25% AcOH and lyophilized to give the crude homeo domain. The crude peptide was dissolved in 50 mM Tris·HCl pH 8.0/4 M Guanidine-HCl/0.1 M DTT and passed through a column of Sephadex G-25 (1.5 × 20 cm) with 10% AcOH. Fractions of the crude peptide were pooled and purified by HPLC (VYDAC C18, 1.0 × 2.5 cm) with a linear gradient of CH_3CN/0.05% TFA (10–40% of solvent B over 30 min followed by rechromatography with 20–40% of solvent B over 30 min. Solvent A contained 10% CH_3CN/0.05% TFA and solvent B was 100% CH_3CN/0.05% TFA). The major peak which eluted at 28% CH_3CN was collected and lyophilized to give 4.0 mg (22%). The purified peptide 1 was characterized by analytical HPLC (TSK phenyl-5PW RP, 0.46 × 7.5 cm) (Figure 5a), and 1 provided a single band corresponding to 8000 Da on SDS-PAGE and the correct amino acid sequence (residues 1–30) by automated Edman degradation; amino acid analysis, (acid hydrolysis) Asp(3) 2.97, Thr(5) 4.53, Glu(10) 9.50, Gly(1) 1.09, Ala(2) 2.0, Met(1) 0.95, Ile(4) 3.76, Leu(5) 5.08, Tyr(3) 2.60, Phe(3) 2.98, His(2) 2.08, Lys(6) 5.98, Arg(12) 12.30, (Cys 1, Trp 2); (enzymatic hydrolysis) Thr(5) 4.88, Gly(1) 1.07, Ala(2) 2.0, Met(1) 0.99, Ile(4) 3.90, Leu(5) 4.89, Tyr-(3) 2.90, Phe(3) 3.12, His(2) 1.91, Arg(12) 11.50; mass spectrum $(M + H)^+$ 7770–7960 (calcd 7826), $(M + 2H)^{2+}$ 3903–3981 (calcd 3914).

The *Antp* Homeo Domain 2 (21–60). The P40 fragment (23 mg, 0.0027 mmol) was treated with the low and high HF method as described above. The crude **2** was passed through a Sephadex G-25 column (10% AcOH) and purified by VYDAC C18 reverse-phase chromatography with the same gradient system of CH_3CN/0.05% TFA as described above. The major peak which eluted at 24% CH_3CN was collected and lyophilized to afford 4.1 mg (29%). The purified **2** was characterized by analytical HPLC (Figure 5b); amino acid analysis, (acid hydrolysis) Asp(3) 2.95, Thr(2) 2.15, Glu(5) 5.15, Ala(2) 2.0, Met(1) 1.09, Ile(4) 3.74, Leu-(3) 2.88, Tyr(1) 0.97, Phe(2) 2.09, His(2) 1.93, Lys(4) 4.05, Arg(8) 7.80, (Cys 1, Trp 2); (enzymatic hydrolysis) Thr(2) 1.99, Ala(2) 2.0, Met(1) 0.93, Ile(4) 3.94, Leu(3) 3.22, Tyr(1) 0.98, Phe(2) 2.05, His(2) 1.90, Arg(8) 8.57; mass spectrum $(M + H)^+$ 5192–5375 (calcd 5241), $(M + 2H)^{2+}$ 2601–2604 (calcd 2621).

The *Antp* Homeo Domain 3 (27–55). The small peptide fragments F9–F6 were sequentially coupled to Boc-Lys(ClZ) oxime resin as described above to yield the fully side-chain-protected form a peptide **3** (P29) (Figure 6). Peptide P29 (30 mg, 0.0052 mmol) was treated with the low and high HF method as described above. The crude **3** was passed through a Sephadex G-25 column (10% AcOH) and purified by VYDAC C18 reverse-phase chromatography with the same gradient system of CH_3CN/0.05% TFA as described above. The major peak which eluted at 20% CH_3CN was collected and lyophilized to afford 9.6 mg (64%). Amino acid analysis: (acid hydrolysis) Asp(1) 1.30, Thr-(2) 2.16, Glu(4) 4.56, Ala(2) 2.12, Met(1) 1.0, Ile(4) 3.54, Leu(2) 2.39, Phe(1) 1.20, His(1) 1.21, Lys(2) 2.13, Arg(7) 6.72, (Cys 1, Trp 1); mass spectrum $(M + H)^+$ 3724.4 (calcd 3724).

Circular Dichroism Studies. CD spectra of peptides **1–3** at the concentration of 5.0×10^{-5} M in 25 mM Tris·HCl (pH 7.5)/0.16 M KCl/1 mM DTT and 2.5×10^{-5} M in TFE/buffer (1:1, v/v), respectively, were measured from 260 to 195 at 25 °C (Figure 7).

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