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Studies on the Solid-Phase Synthesis of Bovine Pancreatic Trypsin Inhibitor (Kunitz) and the Characterization of the Synthetic Material

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A resin-bound protected linear polypeptide of 58 residues with the sequence of the bovine pancreatic trypsin inhibitor (Kunitz) was synthesized by the solid-phase method. The polypeptide was removed from the solid support by cleavage with HF and purified, and the three disulfide bonds were formed by air oxidation of the reduced form. The synthetic inhibitor was purified by gel filtration, trypsin–Sepharose affinity chromatography, and finally by ion-exchange chromatography. A highly purified inhibitor which inhibited trypsin stoichiometrically was isolated. The synthetic trypsin inhibitor was indistinguishable from natural trypsin inhibitor by chromatography on CM-Sephadex, polyacrylamide gel electrophoresis, amino acid analysis, peptide maps of tryptic digests, and circular dichroism spectra. The dissociation constant for the trypsin–synthetic trypsin inhibitor complex also agreed well with that for the trypsin–native trypsin inhibitor complex.

The methodology of Merrifield solid-phase synthesis[1-3] has gained considerable acceptance, particularly in the preparation of peptides of moderate molecular weight. This has encouraged a number of investigators to attempt the synthesis of large biologically active polypeptides by the solid-phase approach. However, the difficulties associated with a stepwise solid-phase strategy are expected to increase in magnitude with increases in the length of the polypeptide chain.

We have undertaken studies on solid-phase peptide synthesis with a view toward eventually using this method in the design of polypeptide model enzymatic catalysts. In this paper, we present the results of our solid-phase synthesis of a polypeptide with the amino acid sequence of bovine pancreatic trypsin inhibitor (Kunitz) and of our characterization of the synthetic species. We selected this inhibitor (BPTI) for synthesis because its amino acid chain length (58) is in the same range as the model enzymes we plan to prepare and because it is a very stable, well-characterized protein which has been studied in great detail in many laboratories.[4-6] Both its amino acid sequence[7-10] and crystallographic structure[11-13] have been determined. Also, it has been established with the native inhibitor that the denatured, reduced peptide chain can be reoxidized and refolded into a structure possessing full inhibitory activity.[14-16] Furthermore, the extraordinarily low dissociation constant (6×10^{-14} M t pH 8, 25 °C) which has been measured for the trypsin inhibitor–trypsin complex[17] provides a stringent criterion by which the purity of synthetic material can be assessed. Finally, if success in the solid-phase synthesis of a peptide possessing the amino acid sequence of native BPTI and meeting high standards of purity can be achieved, this could open the way to the preparation of analogues with variations in the amino acid composition in the vicinity of the "reactive site" which would be useful in mechanistic studies of the inhibition process.

Results

Solid-Phase Synthesis. The yield of crude uncleaved peptide we obtained (77%, Table I) suggests that the average yields of the individual steps in our synthesis were very high and that little peptide must have been lost from the resin during the course of synthesis. The conventional protecting groups for the hydroxyl function of tyrosine (Bzl) and for the ϵ-amino function of lysine (Z) are not very satisfactory for the stepwise synthesis of long peptide chains and their loss during synthesis can easily lead to extensive chain branching.[18-20] To reduce this problem the Bzl (2,6-Cl_2) and ϵ-Z (2-Cl) protecting groups were used for tyrosine and lysine, respectively.

In our synthetic work the individual coupling steps and deprotecting steps were monitored by the ninhydrin test.[21] Repeated coupling was used to ensure complete reaction when the ninhydrin test was positive after the initial coupling had occurred. This was found to be a necessary procedure only in the cases of three residues. Thus, it appears that the long, growing peptide chain was able to react rapidly and in high yield with added Boc-amino acids.

Purification of the Synthetic Peptide. The most powerful purification step employed in this synthesis is that involving trypsin–Sepharose affinity chromatography. Gel filtration on Sephadex G-50 of an acetic acid extract of the resin–peptide mixture obtained after the HF cleavage step gave a solution containing a polypeptide of approximately the correct molecular weight. After treatment with β-mercaptoethanol and passage again through a Sephadex G-50 column, the solution was diluted to a concentration of 0.01 mg of the polypeptide per ml. The latter solution was air oxidized for

Table I. Summary of Yields and Inhibitory Activities Obtained for Synthetic Material [a,b]

Stage of synthesis or purification	Yield, %	Inhibitory activity against trypsin, %
1. Protected BPTI-resin	77	
2. Crude, cleaved peptide	75	
3. BPTI monomer isolated by gel filtration through Sephadex G-50	58	10
4. BPTI isolated by affinity chromatography	9.5	80–90
5. BPTI isolated after ion-exchange chromatography	90	100

[a] The overall yield at the end of the fifth stage was 2.9% of material which was 100% active as a β-trypsin inhibitor. [b] When HF treated sulfonated native BPTI was purified by the procedure outlined in the table a final yield of 50% of material which was 100% active as a β-trypsin inhibitor was obtained.

Table II. Amino Acid Composition of Purified Synthetic Trypsin Inhibitor

Amino acid	Number of residues found: Expected[4]	Native inhibitor	Synthetic inhibitor
Arg	6	5.89	5.97
Lys	4	4.12	4.25
Asx	5	5.10	4.98
Thr	3	3.11	2.95
Ser	1	0.97	0.89
Glx	3	3.11	2.95
Pro	4	4.15	3.96
Gly	6	6.11	6.25
Ala	6	6.21	6.30
Val	1	0.75	0.79
Met	1	0.78	0.85
Ile	2	0.98	0.98
Leu	2	2.00	2.00
Tyr	4	3.69	3.57
Phe	4	4.15	4.05
Cys	6	Not determined	Not determined

4 days and applied to a trypsin–Sepharose column, yielding an inhibitor after lyophilization which was 80–90% active. The inactive fractions obtained from the affinity column presumably consisted of peptides with failure sequences and of peptides with the correct amino acid sequence but incorrectly paired disulfide bonds. No attempt was made to recycle the inactive material.

The overall yield of active inhibitor in our synthesis (see Table I) might have been higher if the proper pairing of the sulfhydryl groups (Cys 5–55, 14–38, 30–51) could have been ensured. We tried to avoid intermolecular side reactions by using an anaerobic system during the handling of the synthetic inhibitor in the reduced form and by employing high dilution during the air oxidation. However, even though our observation that the synthetic inhibitor was 100% active and exhibited essentially the correct dissociation constant in its complex formation with β-trypsin provides extremely strong support for the tenet that the tertiary structure of a protein is determined by its primary sequence,[22] in the absence of an efficient disulfide reshuffling process the incorrect pairing of disulfide bonds must have certainly played a significant role in lowering the final yield. To assist the disulfide reshuffling process we tried to use the oxidized glutathione-reduced glutathione system,[23] but the yield was even lower here. Application of the disulfide reshuffling enzyme[24] would probably be very helpful.

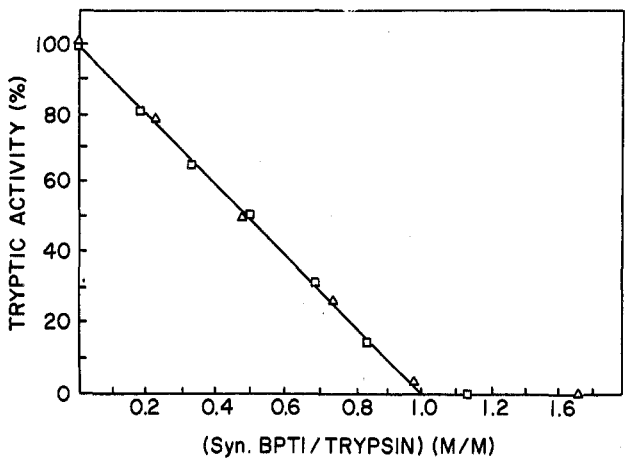

Figure 1. Assay of highly purified synthetic BPTI. The titration of synthetic BPTI was carried out employing increasing inhibitor to β-trypsin radios. The titrations were monitored by assaying the remaining β-trypsin activity. BPTI was incubated with β-trypsin for 3 min, followed by addition of the appropriate substrate. The remaining trypsin activity was measured by (a) rate assay (△); (b) NPGB active site titration (□).

Characterization of the Synthetic Peptide. Amino Acid Analysis of the Synthetic Inhibitor. The highly purified synthetic BPTI had the overall amino acid composition (Table II) expected of BPTI except for Ile, which gave only 1 (theory 2) molar equiv after 24 h acid hydrolysis, as already discussed by Dlouha et al.[25]

Inhibitory Activity of Synthetic BPTI. The trypsin concentration employed was 4.00×10^{-7} M when *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) was used as the substrate and 5.33×10^{-6} M when *p*-nitrophenyl *p'*-guanidinobenzoate (NPGB) was used as an active site titrant. The stoichiometry of inhibition was followed by adding increasing quantities of synthetic BPTI and by measuring the residual activity after 3 min of incubation (Figure 1 and Table III). For our highly purified synthetic BPTI, both methods gave excellent 1:1 stoichiometry with pure β-trypsin. Thus, the highly purified synthetic BPTI is 100% active.

Circular Dichroism Spectra of the Natural and the Synthetic Inhibitor. The CD spectra of synthetic and natural BPTI are shown in Figure 2. The solvent was a pH 5.0 acetate buffer (0.01 M sodium acetate, 0.14 M NaCl). The molecular ellipticity is given by eq 1 where M is the gram-molecular weight of the sample, c is the concentration of the peptide in g cm^{-3}, and l is the pathlength of sample solution in cm.[26] The sample solution contained ca. 0.2 mg/ml.

$$[\theta] = \frac{\theta}{10}\frac{M}{lc} \text{ (deg cm}^2\text{/dmol)} \quad (1)$$

The fact that the CD spectra of synthetic and native BPTI are virtually identical indicates that they must have very similar, if not the same, conformation and that a substantial degree of racemization of the various amino acids present in the synthetic peptide had not occurred.

Polyacrylamide Gel Electrophoresis. The synthetic BPTI was indistinguishable from native BPTI on disk polyacrylamide gel electrophoresis (7.5% gel, stacked at pH 4.0, run at pH 2.3). When a mixture of the synthetic and native inhibitors was developed on a disk gel the combined protein moved as a single band and behaved identically with pure native BPTI. This indicated a high degree of homogeneity for the highly purified synthetic inhibitor.

Table III. Stoichiometric Titration of Trypsin by Highly Purified Synthetic Inhibitor

A. Using BAPNA Rate Assay to Monitor Residual Tryptic Activity

Trypsin concn 4.00×10^{-7} M

Inhibitor concn $\times 10^6$, M	Inhibitor to trypsin ratio	Residual activity, %
0.000	0.00	100
0.099	0.24	77
0.199	0.50	51
0.248	0.74	26
0.397	0.98	3
0.595	1.47	0

B. Using Active Site Titrant NPGB to Monitor Residual Tryptic Activity

Trypsin concn 5.33×10^{-6} M

Inhibitor concn $\times 10^6$, M	Inhibitor to trypsin ratio	Residual activity, %
0.00	0.00	100
0.88	0.17	78
1.76	0.34	65
2.65	0.51	52
3.53	0.67	33
4.45	0.85	15
5.95	1.13	0

Peptide Maps of Native and Synthetic BPTI. Samples of native and synthetic BPTI were oxidized with performic acid.[27] The oxidized derivatives were then subjected to trypsin digestion, and the peptide maps obtained for natural and synthetic material were identical.[28]

Determination of the Dissociation Constant of the Trypsin–Synthetic Inhibitor Complex.[29] One of the most notable features of the trypsin–BPTI complex is the unusually tight binding of the two proteins to each other. The dissociation constant is 6.0×10^{-14} M at pH 8.0, 25 °C.[6,17] The magnitude of the dissociation constant (K_I) is very sensitive to alterations in the structure of the inhibitor. It was found that selective reduction of the Cys 14–38 bridge in the inhibitor raised the value of K_I to 1.8×10^{-9} M,[17] 3×10^4 times higher than the dissociation constant of the trypsin–native inhibitor complex. Modification of the selectively reduced inhibitor by iodoacetamide lowered the K_I value to 1.7×10^{-10} M. Thus, measurement of the K_I value for the complex of synthetic BPTI with trypsin should provide strong evidence with regard to the identity of the structure of the synthetic inhibitor with that of the native inhibitor, at least in the region of the reactive site.

Owing to the unusually strong binding of BPTI to trypsin, the dissociation constant cannot be evaluated by conventional methods.[5] In analogy to the work of Vincent and Lazdunski, competition for trypsin between the synthetic BPTI and reduced, ^{14}C-carboxyamidomethylated BPTI (abbreviated RCAM*BPTI) was used to obtain the ratio $K_I/K_{I'}$, where K_I represents the dissociation constant of the trypsin–synthetic inhibitor and $K_{I'}$, the dissociation constant for the trypsin–RCAM*BPTI complex.[6,17] Equations 2–4 below define K_I, $K_{I'}$, and their ratio. From this ratio and the known value of $K_{I'}$, it is possible to calculate K_I for the synthetic inhibitor.

$$\text{trypsin} + \text{syn-BPTI} \underset{K_I}{\rightleftharpoons} \text{trypsin-syn-BPTI} \quad (2)$$

$$\text{trypsin} + \text{RCAM*BPTI} \underset{K_{I'}}{\rightleftharpoons} \text{trypsin–RCAM*BPTI} \quad (3)$$

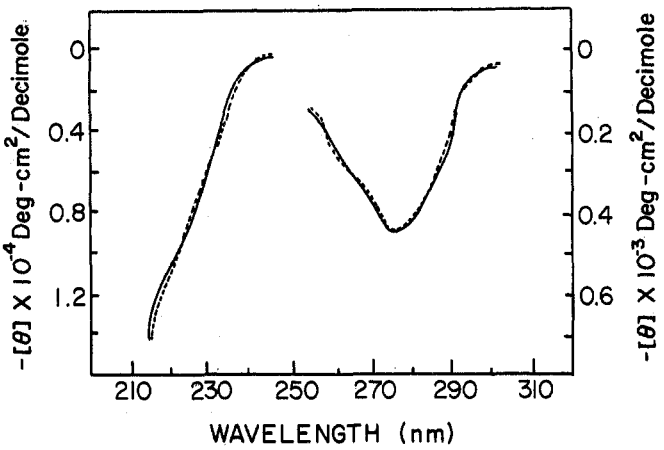

Figure 2. Circular dichroism spectra of natural (solid line) and synthetic (broken line) BPTI. The solvent was a pH 5 buffer containing 0.01 M sodium acetate and 0.14 M NaCl. The left-hand scale is for the far uv region, and the right-hand scale is for the near-uv region.

$$\frac{K_I}{K_{I'}} = \frac{(\text{syn-BPTI})(\text{trypsin-RCAM*BPTI})}{(\text{RCAM*BPTI})(\text{trypsin-syn-BPTI})} \quad (4)$$

Since we could not be sure at the outset whether our synthetic material would bind to trypsin as well as native BPTI does, to avoid wasting ^{14}C-RCAM*BPTI we carried out the following preliminary experiment first. At 25 °C in 0.05 M Tris (pH 8.0, 0.1 M NaCl, 0.05 M $CaCl_2$), 0.15 μmol of β-trypsin was incubated with 0.15 μmol of labeled RCAM*BPTI (0.98 mg). After an incubation period of 15 min, 0.15 μmol of synthetic BPTI was added. The total volume was 15 ml with a 10 μM concentration of each of the partners in complex formation. At times 0, 10 days, and 17 days, aliquots of 4 ml each were passed through a Sephadex G-75 column (2.5 × 72 cm). Since almost all of the labeled RCAM*BPTI was displaced from its complex with β-trypsin, this indicated that the synthetic inhibitor formed a very strong complex indeed with the enzyme.

To determine the dissociation constant for the β-trypsin–synthetic BPTI complex accurately, 0.15 μmol of purified β-trypsin (3.6 mg) was incubated with 15 μmol of ^{14}C-RCAM*BPTI (97.5 mg). After 15 min, 0.15 μmol (0.98 mg) of synthetic BPTI was added. An aliquot of 4 ml of the mixture (containing 0.04 μmol each of β-trypsin and synthetic BPTI and 4.0 μmol of ^{14}C-RCAM*BPTI) was taken and passed through a Sephadex G-75 column, as mentioned above. From the elution profile of Figure 3 it was possible to determine the concentration of trypsin–synthetic BPTI and trypsin–RCAM*BPTI after 10 and 17 days. Equilibrium was attained after 10 days. The concentrations of RCAM*BPTI and of synthetic BPTI were also easily determined.

Unfortunately, in the radioactivity elution pattern shown in Figure 3 the peaks of the complex and the free inhibitor were not completely separated. The total counts of radioactivity in the complex were estimated by assuming a Gaussian distribution for both peaks. The value thus obtained for zero time was checked by applying 0.04 μmol of trypsin–RCAM*BPTI complex to the same column and by determining that the measurement made in the latter experiment was within 5% of that in the former.

From Figure 3 we calculate that $K_I/K_{I'}$ is 4.9×10^{-4}. Since $K_{I'} = 1.7 \times 10^{-10}$ M,[17] we find that $K_I = 8.4 \times 10^{-14}$ M for the complex of β-trypsin with the synthetic inhibitor, a value in very good correspondence with the value of 6×10^{-14} M found in the case of the native inhibitor.[17]

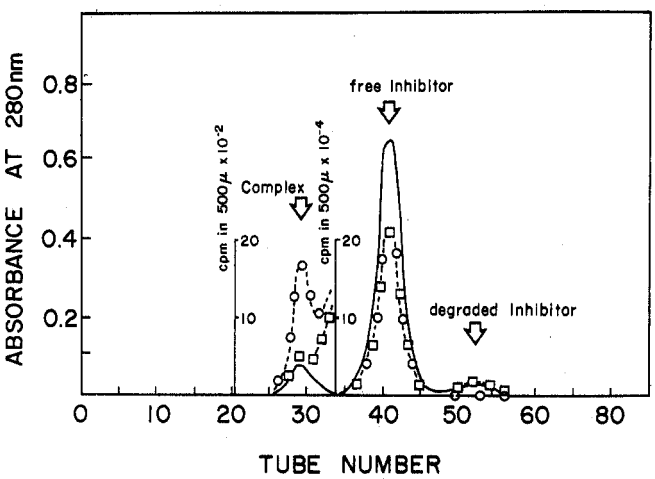

Figure 3. A competition experiment for the evaluation of the dissociation constant of the trypsin–synthetic BPTI complex. First, β-trypsin (0.15 μmol) was incubated with a 100-fold molar excess of ^{14}C-RCAM*BPTI at 25 °C. The association process was completed after 15 min had elapsed.[6,17] At that time, the dissociation of ^{14}C-RCAM*BPTI from the trypsin–^{14}C-RCAM*BPTI complex was initiated by adding synthetic BPTI to give a final concentration of 10.0 μM. Aliquots were taken at different times and gel filtration was performed on a 2.5 × 72 cm Sephadex G-75 column. Solid line: optical density profile at 280 nm. Broken line: radioactivity elution profile patterns at different times in the competition reaction; -O-O-O- time zero; □-□-□-10 days and 17 days. As shown in this figure, equilibrium was attained after 10 days.

Discussion

Chemical and Physical Evidence for the Purity of the Synthetic Inhibitor and Its Similarity to the Native Inhibitor. The various data recorded and discussed here strongly support the view that our synthetic inhibitor is highly homogeneous and shows virtual identity with the native inhibitor with respect to all chemical and physical properties examined. The evidence for this identity can be summarized as follows. The purified synthetic inhibitor had the overall amino acid composition expected for BPTI. The circular dichroism spectra of the synthetic inhibitor and the native inhibitor were identical within experimental error, indicating that the conformation of the synthetic inhibitor was indistinguishable from that of the native inhibitor. Although, predictably, the synthetic trypsin inhibitor was very resistant to trypsin, it was readily digested after performic acid oxidation. Peptide maps of the digests showed, as expected, ten spots that corresponded very well with the positions of the peptides derived from the native inhibitor. However, the possibility exists that peptides arising from very small amounts of chains with different sequences would have gone undetected. The chromatographic behavior on CM-Sephadex of the highly purified synthetic inhibitor corresponded exactly with that of native BPTI. Furthermore, the synthetic inhibitor was indistinguishable from the native inhibitor on polyacrylamide gel electrophoresis, providing evidence for the similarity of the net charge, size, and shape of the two species. The most important criterion for the identity of the synthetic inhibitor with the native inhibitor, particularly in the region of the crucial reactive site, is the good agreement of the dissociation constant of the β-trypsin–synthetic inhibitor complex with that of the β-trypsin–native inhibitor complex. Our value (8×10^{-14} M) is practically the same as the literature [6,17] value (6×10^{-14}M). The small difference between the values is within the experimental error of our determination and is probably due to the incomplete separation of the peaks in the radioactivity elution pattern of the complex and free inhibitor species.

Conclusions

In 1971, Noda et al.[30] reported the synthesis using the Merrifield solid-phase method[1-3] of a polypeptide with 35–39% of the inhibitory activity of BPTI against trypsin, as determined by a rate assay method. Later, Yajima et al.[31,32] used fragment condensation on a solid support to synthesize a polypeptide which had 82% of the inhibitor activity of BPTI against trypsin, using the same rate assay technique. On this basis the latter workers argued that fragment condensation is a better procedure for the preparation of peptides than the simple stepwise solid-phase synthesis. However, a major problem with this argument is that the inhibitory activity of the synthetic inhibitor measured by a rate assay technique with a good substrate like α-*N*-tosyl-L-arginine methyl ester (see Noda et al.[30] and Yajima et al.[31,32]) provides very weak evidence indeed for the identity of the reactive site region of the synthetic and native inhibitor or for the homogeneity of the synthetic material. This statement is made because the binding of the native inhibitor to trypsin is very tight (dissociation constant = 6×10^{-14} M at pH 8, 25 °C). Since even the most sensitive rate assays for trypsin require trypsin concentrations of at least 10^{-9} M, this means that any synthetic trypsin inhibitor which has a dissociation constant appreciably below 10^{-10} M could appear to be 100% pure using an assay substrate like α-*N*-tosyl-L-arginine methyl ester. Hence, if the synthetic inhibitor were heterogeneous or had an incorrect sequence around the reactive site, as long as the average dissociation constant was significantly less than 10^{-10} M, the synthetic inhibitor would appear to be 100% active, using the rate asaay criterion. On the basis of the published information it is not possible to tell whether Noda et al.[30] obtained an inhibitor in their synthesis which was 35–39% pure BPTI or whether they synthesized another inhibitor with a much higher dissociation constant than BPTI. A similar question arises with regard to the work of Yajima et al.[31,32]

In our synthesis we have been able to determine the dissociation constant of the synthetic BPTI–trypsin complex which is 8×10^{-14} M under conditions where a value of 6×10^{-14} M has been reported in the literature[6,17] for the native inhibitor trypsin complex. This observation, in addition to the peptide mapping, polyacrylamide gel electrophoresis, circular dichroism, and amino acid analysis data, indicates that we do have a synthetic inhibitor which has properties identical with those of the native inhibitor within experimental error. In conclusion, we feel that the present status of work on the synthesis of BPTI does not provide support for the contention of Yajima et al.[31] that "fragment condensation in peptide synthesis is better than simple stepwise solid phase synthesis".

Experimental Section

Materials and Methods. Glass-distilled dichloromethane and chloroform from Burdick and Jackson Laboratories were distilled before use. Trifluoroacetic acid (Aldrich) was distilled and stored in amber bottles with polyethylene-lined screw caps. Analytical grade dimethylformamide from J. T. Baker Chemical Co. was purified and tested according to the procedure of Stewart and Young[3] and was stored under a nitrogen atmosphere at 4 °C in brown bottles over molecular sieves (Linde Type 4A). Triethylamine (Eastman Kodak) was distilled from calcium hydride. Dicyclohexylcarbodiimide from Aldrich Chemical Co. was distilled under reduced pressure.

Chloromethylated styrene–divinylbenzene (DVB) copolymer (1% cross-linked) was purchased from Schwarz/Mann. The *tert*-butyloxycarbonyl amino acid derivatives used were the following: Ala, Asp(β-benzyl), Asn-*p*-nitrophenyl ester, Glu(γ-benzyl), Gln-*p*-nitrophenyl ester, Gly, Lys(ε-2-chlorobenzyloxycarbonyl), Phe, Thr(benzyl), Ser(benzyl), Tyr(2,6-dichlorobenzyl), Cys(*p*-methoxybenzyl), Met, Val, Leu, Ile, and Pro. These were all purchased from Bachem Inc., Calif. The purity of each amino acid derivative was checked by thin layer chromatography on silica gel chromatogram sheets (Eastman) before use.[3]

Sodium acetate, sodium sulfite, sodium carbonate, sodium bicar-

bonate (Baker Analyzed grade), β-mercaptoethanol (Eastman Kodak), Tris (Tris base, Schwarz/Mann), *N*-benzoyl-DL-arginine *p*-nitroanilide (Sigma), *p*-nitrophenyl *p*-guanidinobenzoate HCl (Schwarz/Mann), calcium chloride (Baker), benzamidine HCl, cyanogen bromide, and ninhydrin (Aldrich) were used without further purification. Reagent grade urea (Fisher Scientific Co.) was recrystallized from 95% ethanol prior to use.[33] Sephadex G-50, CM Sephadex C-25, Sephadex SEC-50, and Sepharose 4B were obtained from Pharmacia Fine Chemicals.

Trypsin was purchased from Sigma and purified by Sephadex SEC-50 ion exchange chromatography.[34] β-Trypsin was used throughout the whole experiment. A trypsin–Sepharose column was prepared according to Chauvet and Acher.[35] The native BPTI was a gift from Bayer Co., with part of it isolated from bovine lung.[4] The protein was pure[36] as judged by polyacrylamide gel electrophoresis and stoichiometry of the inhibition with trypsin. ^{14}C-Carboxamidomethylated BPTI selectively reduced at S–S bond 14–38 was a gift from Professor M. Lazdunski and Dr. J. P. Vincent. (Radioactivity was 4.4×10^6 dpm/μmol.)

The optical densities of column effluents were measured with a Gilford spectrophotometer. Circular dichroism spectra were measured on a Cary 60 spectropolarimeter. Trypsin assays and active site titration were carried out on a Cary 15 spectrophotometer. A Packard Tri-Carb liquid scintillation spectrometer was used for counting of radioactive samples. Amino acid analyses were performed using the 0.2 and 2.0 scales on a Beckman Spinco Model 121 automatic amino acid analyzer. The Beckman Model 990 peptide synthesizer was used for the synthetic work.

All pH measurements were carried out on a Beckman Research pH meter with combined glass–calomel electrodes (Thomas no. 4094 L60). The meter was standardized against Fisher Certified standard buffers prior to use. Deionized water was obtained by passing distilled water through a Continental demineralizer.

BPTI and trypsin concentrations were estimated on the basis of the absorbance of their solutions at 280 nm with a conversion factor to mg/ml of 1.206 and 0.67, respectively.[37]

Assay of Trypsin Inhibitor. The inhibitory activity of trypsin inhibitor was measured as a function of the inhibitor to proteinase ratio.[7] As described below, two methods were employed to monitor the titration of trypsin's active site with the inhibitor.

A. Rate Assay Employing *N*-α-Benzoyl-DL-arginine *p*-Nitroanilide (BAPNA)[4] to Measure the Remaining Tryptic Activity. To 3 ml of 0.1 M Tris buffer (pH 7.8, 0.02 M $CaCl_2$) 100 μl of β-trypsin solution (ca. 10^{-5} M) was added. An appropriate amount of inhibitor was added and the resultant solution was incubated for 3 min at 25 °C. Then 100 μl of BAPNA solution (10 mg/ml of DMF) was added. The ΔOD_{405} per min was measured with a Cary 15 spectrophotometer. The final concentrations of the enzyme and inhibitor were ca 10^{-7} M.

B. Active Site Titration to Measure the Remaining Tryptic Activity.[38,39] An aliquot of trypsin solution (in 10^{-3} M HCl, 0.02 M $CaCl_2$) was diluted with 0.1 M veronal buffer (pH 8.3, 0.02 M $CaCl_2$) to give 1.0 ml of 5×10^{-6} M trypsin solution. An appropriate amount of trypsin inhibitor was added. The resultant solution in a 1 ml capacity cuvette was placed in the sample cell position of the spectrophotometer and the instrument balanced against a reference cell containing the same buffer. Then a solution of 10 μl of 0.01 M NPGB (*p*-nitrophenyl *p'*-guanidinobenzoate) in DMF was added to the reference cuvette, the contents mixed, and the cell replaced in the instrument; the same procedure was followed with the sample cuvette and the instrument turned on. The "burst" was measured and the concentration of active trypsin calculated by assuming ϵ_{410} = 16 595.

Anaerobic Column Chromatography.[40] In order to minimize the undesirable intermolecular cross-linking of free thiol groups during the column chromatography of the concentrated reduced inhibitor, an anaerobic system was used. It consisted of a column and an elution buffer vessel, both modified to facilitate anaerobic assembly and operation. Oxygen-free nitrogen gas was used for flushing and bubbling throughout the experiment.

Synthesis of the Protected Linear 58 Amino Acid Residue Polypeptide Chain of Bovine Pancreatic Trypsin Inhibitor. Boc-Ala-resin ester was prepared essentially as described in the literature.[41] The hydrolysis of 20 mg of the sample with 6 M HCl in dioxane yielded 0.28 mmol of Ala per gram of resin.

Synthesis of the Protected Peptide. Boc-Ala-resin ester (1.2 mequiv, 4.28 g) was placed in the small vessel (5 g capacity) of the automated Beckman Model 990 peptide synthesizer. The instrument was programmed to perform the remainder of the synthesis automatically.

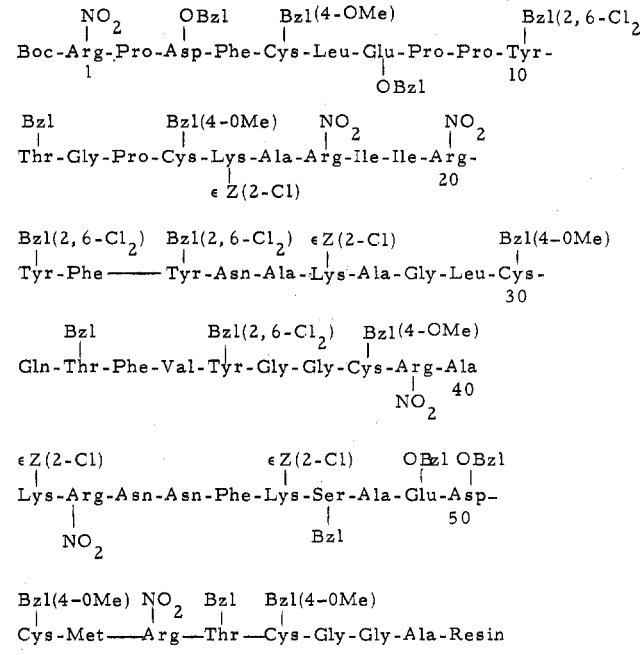

Figure 4. Structure of fully protected BPTI resin.

One cycle of the synthesis (DCC-mediated coupling) consisted of (1) CH_2Cl_2 wash (2 × 40 ml); (2) TFA–CH_2Cl_2 (1:1) prewash (20 ml); (3) TFA–CH_2Cl_2 (1:1, 40 ml) for 30 min; (4) CH_2Cl_2 wash (3 × 40 ml); (5) methanol wash (25 ml); (6) CH_2Cl_2 wash (3 × 40 ml); (7) 10% triethylamine in chloroform (40 ml); (8) CH_2Cl_2 wash (4 × 25 ml); (9) Boc-amino acid (2.5-fold excess in CH_2Cl_2, the Boc derivatives of ε-2-chloro-Z-lysine and nitroarginine were dissolved in a minimum amount of DMF before dilution to 10 ml with CH_2Cl_2); (10) dicyclohexylcarbodiimide (5 ml, threefold excess), 4–6 h; (11) CH_2Cl_2 wash (3 × 25 ml); (12) DMF wash (2 × 25 ml); (13) CH_2Cl_2 wash (3 × 25 ml). The time for all washing steps was set at 1 min.

For an active ester coupling cycle (Asn or Gln), the Boc-amino acid *p*-nitrophenyl ester (ca. fourfold excess) was dissolved in DMF, and addition of the ester was preceded by washing twice with 25-ml portions of DMF. Of course, addition of DCC was omitted. The coupling time was usually 18 h.

The program was interrupted after steps 8 and 13, and a small sample of the resin (ca. 5–10 mg) was removed in order to carry out the ninhydrin color test.[21] When the ninhydrin test for step 13 was negative, the same cycle of reactions was repeated for the next amino acid residue. When a positive result was obtained, the resin was washed with absolute ethanol (2 × 40 ml) and the test repeated. If a positive result was still observed, the coupling reaction (steps 9–15) was repeated. Recoupling was found necessary for the following amino acid residues: Asn 44 (recoupled twice), Asn 43 (recoupled twice), and Asn 24. Following the second recoupling of Asn 43, the ninhydrin test was still slightly positive, and the peptide resin was then acetylated with a mixture of 2 ml of acetic anhydride (J. T. Baker Chemical Co.) and 1 ml of *N*-methylmorpholine (Aldrich) in 25 ml of DMF (30 min shaking), followed by through washing with DMF and CH_2Cl_2.

In addition to the small analytical samples a large sample (6.7 g) was removed after the peptide chain reached 42 residues for the synthesis of inhibitor analogues.

Upon completion of the synthesis, the resin was washed with ethanol (3 × 25 ml) and dried under vacuum. The final weight was 4.45 g. Taking into account the amounts removed in the synthesis the crude yield was 77%. The structure of the fully protected BPTI resin is illustrated in Figure 4.

HF Cleavage.[42] The apparatus was similar to that described by Sakakibara.[43] It consisted of three vessels (a reservoir, a reactor, and a trap) molded from Kel-F rods. All valves were made of Teflon. Liquid HF in stainless steel cylinders were obtained from Matheson Co. A typical HF cleavage procedure for a 1-g resin peptide sample is described below.

The reservoir was charged with 10 ml of HF and 50 mg each of tyrosine and tryptophan. The dried resin peptide sample was placed in the reactor, and 1 ml of anisole (Aldrich, 99%) and 200 mg of methionine were added. The reactor was kept at ice bath temperature (0–4 °C) and 5 ml of HF was distilled from the reservoir into the reactor over a period of a few minutes. The reaction mixture usually

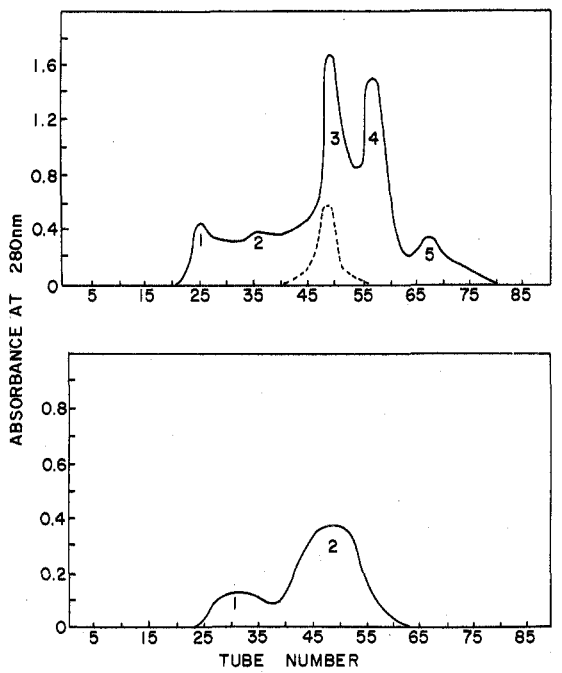

Figure 5. Gel filtration on Sephadex G-50 of the cleaved peptide. The column (4 × 50 cm) was eluted with 0.1 M acetic acid. Top—solid line: crude extract from 450 mg of resin peptide in ca. 25 ml of 10% acetic acid. Peaks 1 and 2, 51 mg; peak 3, 65 mg; peaks 4 and 5, 55 mg. Fraction size: 4.3 ml. Broken line: native BPTI applied to the same column. Bottom—the peptides present in the fractions under peaks 1 and 2 of the top chromatogram were reduced and then gel filtered on the same column. Peak 1, 16 mg; peak 2, 34 mg.

became orange-brown. After 40 min, HF was removed by distillation at room temperature over a period of about 15 min. The resin was broken up in ethyl acetate (250 ml) to wash out HF and anisole. The ethyl acetate filtrate was usually slightly hazy.

Purification of Synthetic BPTI. Sephadex G-50 Gel Filtration. Deaerated 10% HOAc (25 ml) was used to extract peptide from a 450-mg resin–peptide mixture. The solution was then fractionated on a Sephadex G-50 column (4 × 50 cm) by elution with deaerated 0.1 M acetic acid under anaerobic conditions. The synthetic product was separated into five peaks with the following yields: peak 1 and 2 combined, 51 mg; peak 3, 65 mg; peaks 4 and 5 combined, 55 mg.

Peak 3, emerging from the column between tube number 44 and 54, behaved similarly to native BPTI with respect to elution volume (Figure 5, top). Peaks 1 and 2 probably consisted of interchain disulfide aggregates of BPTI. Peaks 4 and 5 comprised a mixture of peptides with lower molecular weights. They presumably resulted from incomplete reaction during the synthesis. There was also some Met in peak 5.

The protein aggregates present under peaks 1 and 2 were lyophilized. They were reduced by dissolving in 10 ml of 1.4 M Tris buffer (pH 8.7, 8 M urea)[16] and incubated with about 1 ml of β-mercaptoethanol for 4 h under an atmosphere of nitrogen at room temperature. The solution was then applied to the 4 × 50 cm Sephadex G-50 column and was separated into two peaks (Figure 5, bottom). Peak 2 (34 mg) came out at the same elution volume as native BPTI. It was treated in the same way as peak 3 in the first fractionation.

Reduction and Reoxidation of the Synthetic Material.[16] The fractions under peak 3 obtained from the first Sephadex G-50 fractionation were combined. In the presence of 8 M urea, the pH of the solution was raised to 8.7 by adding Tris. β-Mercaptoethanol (5 ml) was then added and the solution incubated for 4 h at room temperature under a nitrogen atmosphere.

The protein was separated from the reagents with the 4 × 50 cm Sephadex G-50 column, using 0.1 M acetic acid as the eluent under anaerobic conditions. The solution containing the reduced peptide (100 mg) was then diluted to about 4 l., and the pH was raised to 4.5 by adding ammonium bicarbonate. The dilute peptide solution was then air oxidized for 4 days at room temperature.

Purification of the Synthetic Peptide by Affinity Chromatography. The solution containing the air-oxidized peptide (ca. 10% active as a trypsin inhibitor) was applied to the trypsin–Sepharose column. The column was then eluted with 0.1 M acetate buffer (pH 4.0, 0.3 M NaCl, 0.01 M $CaCl_2$) followed by 0.1 M HCl (pH 1.2, 0.5 M NaCl, 0.01 M $CaCl_2$). After lyophilization of the peptide fractions eluted with HCl, 9.5 mg of white powder which was about 80–90% active as a trypsin inhibitor was obtained.

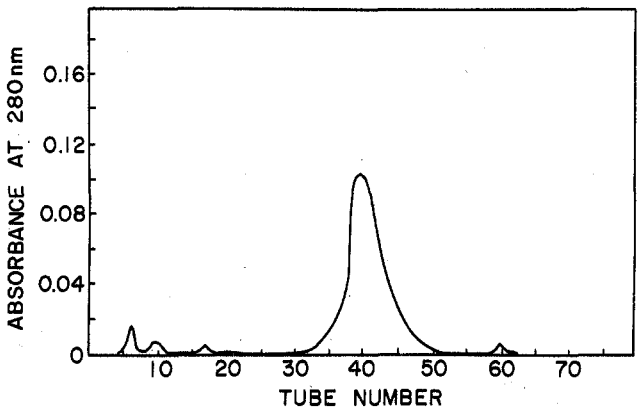

Figure 6. Chromatography of the synthetic BPTI on CM Sephadex C-25. Column, 1 × 15 cm; inhibitor load 4.3 mg; fractions, 4.3 ml each; flow rate about 15 ml/h. The mixing chamber consisted of a 500-ml flask containing 250 ml of 0.01 M phosphate buffer at pH 6.2. The influent to the mixing chamber was the same buffer solution which was also 1 M in NaCl. Yield: 3.9 mg of purified protein which inhibited trypsin stoichiometrically.

Further Purification of Synthetic Material by CM Sephadex C-25 Chromatography. CM Sephadex C-25 was equilibrated with 0.01 M phosphate buffer, pH 6.2. The synthetic inhibitor to be loaded on the column (4.3 mg) was dissolved in 1.5 ml of the same buffer. A 1 × 15 cm column was used. The protein was eluted with a NaCl linear gradient (0–0.25 M NaCl). The chromatogram obtained is shown in Figure 6. The only major peak constituted about 90% of the total protein, and its position agreed well with native BPTI with respect to elution volume. As described below, the highly purified synthetic BPTI showed 1:1 stoichiometry in its complex formation with trypsin.

Effect of HF treatment of Native BPTI. The native inhibitor (39.5 mg) was sulfonated,[44] subjected to HF treatment (in the presence of 2 mg of methionine, 0.4 ml of anisole, yield of recovered crude material 27.3 mg), and reduced.[44] The reduced native inhibitor was then air oxidized for 4 days (pH 4.5, concentration 0.01 mg/ml). After affinity chromatography and CM-Sephadex C-25 ion-exchange chromatography, fully active BPTI was obtained. A recovery of 50% (19.8 mg) of pure BPTI was achieved.

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Registry No.—Boc-Ala, 15761-38-3; Boc-Asp(β-benzyl), 7536-58-5; Boc-Asn-*p*-nitrophenyl ester, 4587-33-1; Boc-Glu(γ-benzyl), 13574-13-5; Boc-Gln-*p*-nitrophenyl ester, 15387-45-8; Boc-Gly, 4530-20-5; Boc-Lys(ε-2-chlorobenzyloxycarbonyl), 54613-99-9; Boc-Phe, 13734-34-4; Boc-Thr(benzyl), 54784-63-3; Boc-Ser(benzyl), 23680-31-1; Boc-Tyr(2,6-dichlorobenzyl), 40298-71-3; Boc-Cys(*p*-methoxybenzyl), 18942-46-6; Boc-Met, 2488-15-5; Boc-Val, 13734-41-3; Boc-Leu, 13139-15-6; Boc-Ile, 13139-16-7; Boc-Pro, 15761-39-4; BPTI, 12407-79-3.

References and Notes

(1) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
(2) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).
(3) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis", W. H. Freeman, San Francisco, Calif., 1969.
(4) B. Kassell, *Methods Enzymol.*, **19**, 844–852 (1970).
(5) M. Laskowski, Jr., and R. W. Sealock in "The Enzymes", Vol. III. P. D. Boyer,

Ed., Academic Press, New York, N.Y., 1971, pp 375–473.
(6) M. Lazdunski, J. P. Vincent, H. Schweitz, M. Peron-Renner, and J. Pudles in "Proteinase Inhibitors", H. Fritz, H. Tschesche, L. J. Greene, and E. Truscheit, Ed., Springer-Verlag New York, New York, N.Y., 1974, pp 420–431.
(7) B. Kassel and M. Laskowski, Sr., *Biochem. Biophys. Res. Commun.*, **17**, 792 (1964).
(8) B. Kassell, M. Radicevic, M. J. Ansfield, and M. Laskowski, Sr., *Biochem. Biophys. Res. Commun.*, **18**, 255 (1965).
(9) B. Kassell and M. Laskowski, Sr., *Biochem. Biophys. Res. Commun.*, **20**, 463 (1965).
(10) B. Kassell and M. Laskowski, Sr., *Acta Biochim. Pol.*, **13**, 287 (1966).
(11) R. Huber, D. Kukla, A. Rühlmann, O. Epp, and H. Formanek, *Naturwissenschaften*, **57**, 389 (1970).
(12) R. Huber, D. Kukla, A. Rühlmann, and W. Steigemann, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 141 (1971).
(13) J. Deisenhofer and W. Steigemann in ref 6, pp 484–496.
(14) F. A. Anderer and S. Hörnle, *J. Biol. Chem.*, **241**, 1568 (1966).
(15) J. Chauvet and R. Acher, *Bull. Soc. Chim. Biol.*, **48**, 1248 (1966).
(16) D. Pospisilova, B. Meloun, I. Fric, and F. Sorm, *Collect. Czech. Chem. Commun.*, **32**, 4108 (1967).
(17) J. P. Vincent and M. Lazdunski, *Biochemistry*, **11**, 2967 (1972).
(18) B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3750 (1973).
(19) B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3757 (1973).
(20) D. Yamashiro and C. H. Li, *J. Am. Chem. Soc.*, **95**, 1310 (1973).
(21) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
(22) C. B. Anfinsen, *Biochem. J.*, **128**, 737 (1972).
(23) R. R. Hantgan, G. G. Hammes, and H. A. Scheraga, *Biochemistry*, **13**, 3421 (1974).
(24) R. F. Steiner, F. D. Lorenzo, and C. B. Anfinsen, *J. Biol. Chem.*, **240**, 4648 (1965).
(25) V. Dlouha, D. Pospisilova, B. Meloun, and F. Sorm, *Collect. czech. Chem. Commun.*, **30**, 1311 (1965).
(26) B. Jirgensons, M. Kawabata, and S. Capetillo, *Makromol. Chem.*, **125**, 126 (1969).
(27) C. H. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).
(28) B. Meloun, I. Fric, and F. Sorm, *Eur. J. Biochem.*, **4**, 112 (1968).
(29) Professor M. Lazdunski and Dr. J. P. Vincent provided us with ^{14}C-RCAM*BPTI for this experiment.
(30) K. Noda, S. Terada, N. Mitsuyasu, M. Waki, T. Kato, and N. Izumiya, *Naturwissenschaften*, **58**, 147 (1971).
(31) H. Yajima, Y. Kiso, Y. Okada, and H. Watanabe, *J. Chem., Soc., Chem. Commun.*, 106 (1974).
(32) Y. Kiso and H. Yajima, *Chem. Pharm. Bull.*, **22**, 1087 (1974).
(33) G. R. Stark, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **235**, 3177 (1960).
(34) D. D. Schroeder and E. Shaw, *J. Biol. Chem.*, **243**, 2943 (1968).
(35) J. Chauvet and R. Acher, *FEBS Lett.*, **23**, 317 (1972).
(36) E. Sach, M. Thely, and J. Choay, *C. R. Acad. Sci.*, **260**, 3491 (1965).
(37) R. Avineri-Goldman, I. Snir, G. Blauer, and M. Rigbi, *Arch. Biochem. Biophys.*, **121**, 107 (1967).
(38) T. Chase and E. Shaw, *Biochem. Biophys. Res. Commun.*, **29**, 508 (1967).
(39) J. C. Zahnley and J. G. Davis, *Biochemistry*, **6**, 1428 (1970).
(40) R. Repaske, *Methods Enzymol.*, **22**, 322–325 (1971).
(41) A. Marglin, *Tetrahedron Lett.*, **33**, 3145 (1971).
(42) These cleavages were performed by Mr. Jim Geever of the Armour Pharmaceutical Co.
(43) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).
(44) W. C. Chan, *Biochemistry*, **7**, 4247 (1968).

Nucleosides. 100. General Synthesis of Pyrimidine C-5 Nucleosides Related to Pseudouridine. Synthesis of 5-(β-D-Ribofuranosyl)isocytosine (Pseudoisocytidine), 5-(β-D-Ribofuranosyl)-2-thiouracil (2-Thiopseudouridine) and 5-(β-D-Ribofuranosyl)uracil (Pseudouridine)[1]

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A general procedure for the synthesis of pyrimidine C-5 nucleosides related to pseudouridine was developed. 5-(β-D-Ribofuranosyl)isocytosine (**7**, pseudoisocytidine), the first chemotherapeutically active synthetic C nucleoside, was prepared from readily available ethyl 2-(2,3-*O*-isopropylidene-5-*O*-trityl-D-ribofuranosyl)acetate (**3**). Compound **3** was formylated with ethyl formate and sodium hydride to the corresponding formylacetate sodium enolate **4** and methylated with methyl iodide in DMF to give 3-methoxy-2-(2,3-*O*-isopropylidene-5-*O*-trityl-D-ribofuranosyl)acrylate (**5**). Cyclization of **5** with guanidine afforded the protected isocytosine C-5 nucleoside **6**. Treatment of **6** with methanolic hydrogen chloride gave the desired crystalline β nucleoside, pseudoisocytidine (**7**). From the mother liquor, the α isomer **8** was obtained. Compound **8** can be epimerized effectively to **7** in methanolic hydrogen chloride so that a very high yield of the desired isomer **7** from **6** is readily achieved. The general applicability of this method to the syntheses of other C nucleosides was demonstrated by the synthesis of 2-thiopseudouridine (**10**) and pseudouridine (**13**). Condensation of the acrylate derivative **5** with thiourea followed by deblocking of the product afforded **10**. When **5** was treated with urea, the protected pseudouridine derivatives (**11** and **12**) were obtained. After deprotection of **11**, pseudouridine (**13**) was obtained.

Pseudouridine, the first C nucleoside found in nature, has attracted the interest of organic chemists and biochemists since its discovery in 1957.[2] Recently, other C nucleosides have also been isolated as nucleoside antibiotics from the culture filtrates of various *Streptomycetes*.[3] The unique structural characteristic of C nucleosides which distinguishes them from the ordinary nucleosides is the presence of a carbon to carbon linkage instead of a carbon to nitrogen bond between the aglycon and sugar moieties. This structural feature renders traditional approaches[4] for nucleoside synthesis of limited value.

Although several reports have appeared on the synthesis of pseudouridine[5] and pseudocytidine,[6] these methods involve the condensation of a suitably protected sugar with a preformed pyrimidine-5-yllithium derivative. These procedures are difficult to perform and are not suitable for large-scale preparations. More importantly these methods are specific for each C nucleoside, i.e., for the synthesis of a modified base analogue preparation of a particular pyrimidine 5-lithio derivative is required individually.

As a part of our efforts to develop a *general* method for the synthesis of pseudouridine and analogues thereof we reported in a recent communication[7] a synthesis of 5-(β-D-ribofuranosyl)isocytosine (**7**, pseudoisocytidine) in four steps from 2,3-*O*-isopropylidene-5-*O*-trityl-D-ribofuranose (**1**) via intermediates **3** → **4** → **6** (see Chart I).

Owing to the potential clinical importance[8] of pseudoisocytidine as an antileukemic agent, we now report our synthetic