Synthetic Bovine Prothrombin Precursor 13-29 for Studies of Vitamin K Dependent Carboxylase

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The synthesis of the amino acid sequence found in bovine prothrombin precursor 13-29 (PTP 13-29) has been achieved by solid-phase synthesis of the bis(acetamidomethyl)-protected linear peptide followed by cyclization to the monomeric disulfide. Synthesis of the disulfide bond was achieved by deprotection with mercuric acetate in acetic acid followed by oxidation with potassium ferricyanide. Experimental conditions for closure of the disulfide bond were identified by obtaining the circular dichroism spectra of the linear precursor in a variety of solvent systems. Cyclization in organic solvent systems was not successful but led to the formation of insoluble polymers. Synthetic PTP 13-29 was tested as a substrate for the vitamin K dependent carboxylase. Neither the linear nor cyclic synthetic 17 amino acid peptides were carboxylated as well as the standard, Boc-Glu-Glu-Leu-OMe, at mM concentrations. The estimated K_m of synthetic PTP 13-29 is greater than 1 mM. Thus, bovine prothrombin precursor 13-29 is not an unusually effective substrate for the carboxylase as reported by Soute et al.

A key step in the intracellular processing of the vitamin K dependent blood coagulation factors is the coversion of several specific glutamate (Glu)[1] residues into γ-carboxyglutamate (Gla) residues by a specific carboxylation reaction. The liver microsomal enzyme catalyzing this reaction, the vitamin K dependent carboxylase, requires oxygen, reduced vitamin K, and carbon dioxide.[2]

The synthetic peptide substrate H-Phe-Leu-Glu-Glu-Val-OH[3] (corresponding to bovine prothrombin precursor 5-9; PTP 5-9) and a large number of other small peptides have been synthesized[4] and utilized to study many aspects of the carboxylation reaction. These substrates, similar substrates synthesized by others[4a,b], and even somewhat larger synthetic fragments (PTP 18-23)[5] of the prothrombin precursor are not carboxylated as efficiently ($K_m \approx 3$-8 mM) as the natural substrate ($K_m \approx \mu$M). However, Soute et al.[6] have reported that a peptide called Fragment Su, which was obtained by partial enzymatic digestion of bovine plasma descarboxyprothrombin and identified (amino acid analysis and partial sequencing) as the segment 13-29 of the prothrombin precursor (PTP 13-29; Figure 1), is a remarkably efficient substrate for vitamin K dependent carboxylase ($K_m \approx 1$ μM).

The low-K_m value of Fragment Su is remarkable when compared with the K_m of the shorter synthetic peptide PTP 18-23 ($K_m = 3$ mM), and these differences suggested that some peptide structures contained within PTP segments 13-17 and 24-29 greatly enhanced the binding of this peptide to the carboxylase. We, therefore, decided to synthesize the peptide sequence 13-29 found in bovine prothrombin precursor (1) to see if the report of Soute et al. was correct and to determine which specific peptide segments in 1 enhance binding. We report here that synthetic 1 and its linear precursor, 2 (Figure 1), are poor substrates for the carboxylase. In addition, synthetic closure of the cyclic disulfide ring in 1 is not achieved under standard cyclization conditions, most likely because of the conformation of the linear precursor in the reaction media. Circular dichroism spectroscopy was used to identify a solvent system in which cyclization to the cyclic monomer 1 was possible.

Chemistry. The synthesis of 1 was carried out by conventional solid-phase peptide synthetic procedures.[7] A typical synthetic cycle for this peptide is shown in Table I. The *S,S'*-bis(acetamidomethyl) (Acm) protected peptide 2 was purified after anhydrous HF treatment by gel filtration and reverse-phase HPLC and obtained in 24% yield.

Table I. Steps for Solid-Phase Synthesis of Peptides[a]

reagents	no. of cycles, time
(1) CH_2Cl_2	3 × 2 min
(2) 25% TFA, 7.5% thioanisole/CH_2Cl_2	1 × 5 min, 1 × 30 min
(3) CH_2Cl_2	2 × 5 min
(4) 5% DIEA/CH_2Cl_2	1 × 1 min, 1 × 5 min
(5) CH_2Cl_2	2 × 2 min
(6) 50% DMF/CH_2Cl_2	3 × 2 min
(7) Boc-amino acid + HOBt (2.5 equiv each)	1 × 2 min
(8) DCC (2.5 equiv)	1 × 120 min
(9) 50% DMF/CH_2Cl_2	3 × 2 min
(10) CH_2Cl_2	3 × 2 min
(11) 50% *i*-PrOH/CH_2Cl_2	1 × 2 min
(12) *i*-PrOH	3 × 2 min
(13) CH_2Cl_2	3 × 2 min

[a] Recoupling conditions utilized steps 3-13, except that 1 equiv of Boc-amino acid, HOBt, and DCC were used instead of 2.5 equiv of these reagents.

Attempts to simultaneously remove the Acm protecting group and oxidize the thiols by reaction with iodine in aqueous methanol[5,9] failed to give the anticipated cyclic disulfide. Instead, a highly insoluble product that is probably a high molecular weight polymer of 1 was obtained. Successful oxidation to the monomeric cyclic disulfide 1 was achieved by treating the free thiol peptide

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(1) Abbreviations used follow IUPAC-IUB nomenclature. Additional abbreviations used are: Gla, γ-carboxyglutamic acid; Acm, acetamidomethyl; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; HPLC, high-pressure liquid chromatography; CD, circular dichroism; DIEA, diisopropylethylamine; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; PTP, prothrombin precursor.

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15 20 25
H-Asn-Leu-Glu-Arg-Glu-Cys-Leu-Glu-Glu-Pro-Cys-Ser-Arg-Glu-Glu-Ala-Phe-OH
R R

R = S-S 1

S-Acm 2

Figure 1. Sequence of bovine prothrombin precursor 13–29, 1, and the principle precursor, *S,S'*-bis(acetamidomethyl) derivative 2.

Table II. Amino Acid Analysis of Synthetic Prothrombin Precursors (PTP)

	bis-Acm-PT 18–29	bis-Acm-PT 13–29	Pt 13–29
Asp		0.89 (1)	0.91 (1)
Ser	0.91 (1)	0.98 (1)	0.98 (1)
Glu	3.97 (4)	5.96 (6)	6.26 (6)
Pro	1.07 (1)	1.06 (1)	1.06 (1)
$(Cys)_2$			0.58 (1)
Ala	1.00 (1)	1.00 (1)	1.03 (1)
Leu	0.92 (1)	1.86 (2)	2.00 (2)
Phe	1.05 (1)	0.95 (1)	0.97 (1)
Arg	0.88 (1)	1.94 (2)	1.84 (2)

with potassium ferricyanide in ammonium acetate at pH 8.5. Circular dichroism (CD) spectra of the protected peptide 2 in different solvents were used to identify an appropriate solvent in which the reduced peptide could be cyclized cleanly to 1 without extensive dimerization. In 2,2,2-trifluoroethanol (TFE) the conformation of peptide 2 is highly ordered as indicated by the strong negative ellipticities at 208 and 222 nm and the appearance of a positive ellipticity below 200 nm,[8] suggestive of α-helix conformers. In contrast, the CD spectra of peptide 2 taken in two other solvent systems, aqueous methanol and ammonium acetate, lack the strong negative ellipticity at 222 nm characteristic of an α-helix. The CD spectra (218 nm, $[\theta] = -2800$ and $[\theta] = -1000$ deg cm^2 dmol^{-1}, respectively) are similar to those of essentially random coil peptides with the least ordered structure present in the ammonium acetate buffer.

Gel filtration of the product through a Sephadex G-25 column established the presence of two major components. The first component, present in a small amount, had an estimated molecular weight of 4500; therefore, it is most likely a dimer of 1. The major component was shown to have an estimated molecular weight of 2000. Furthermore, amino acid analysis (Table II), HPLC (Figure 2), and thin-layer chromatography (TLC) indicated that the peptide was nearly homogeneous and has the correct composition of amino acids.

Biological Assays. Both peptides 1 and 2 were evaluated as substrates for the rat liver microsomal vitamin K dependent carboxylase by using the previously described methods[3] (Table III). Surprisingly, the peptides were found to be poor substrates compared to the standard substrate. When assayed at different concentrations, peptide 2 exhibited 15–20% of the activity of the standard substrate Boc-Glu-Glu-Leu-OMe and peptide 1 had only 7–8% activity. Because of this poor activity K_m values were not determined for the peptides. However, on the basis of our experience with other peptides, these peptides probably have K_m values in the mM range instead of the μM range that was reported by Soute et al.[6] for an isolated peptide assigned this sequence.

Discussion

The solid-phase synthesis of the S-protected linear 17-peptide 1 was achieved without difficulty. However, initial

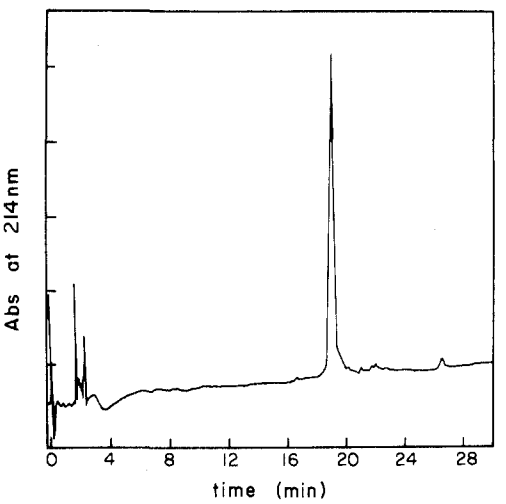

Figure 2. Analytical HPLC of 1 on a 0.39 × 30 cm μBondapak C_{18} column. Elution conditions were a 20–40% CH_3CN linear gradient for 30 min with 0.1% TFA added, 1.6 mL/min, detection at 214 nm.

Table III. Substrate Activity of Bovine Prothrombin Precursor Fragments[a]

substrate	dpm incorporated 0.25 mM	0.125 mM	0.0625 mM
Boc-Glu-Glu-Leu-OMe	11430	5770	3021
2	2380	920	480
1	810	480	240

[a] Data are an average of duplicate 60-min, 17 °C incubations and a no vitamin K blank incubation value has been subtracted.

attempts to form the disulfide bond between residues Cys-18 and Cys-23 in several solvent systems were unsuccessful and only insoluble, polymeric materials were obtained. It is probable that the difficulty at this cyclization step is due to the conformation of the reduced linear peptide PTP 13–29 in the solvent systems used to effect cyclization. This difficulty in forming the disulfide ring is not a consequence of the amino acid sequence 18–23 within the ring system because in the synthesis of the shorter peptide, PTP 18–23,[5] the disulfide formed without difficulty by treating the fully protected peptide with iodine[9] in methanol. However, when 2 was treated under similar reaction conditions, a monomeric cyclic disulfide was not obtained.

The dithiol-containing hexapeptide segment 18–23 in 2 does not cyclize in aqueous methanol because the peptide is largely in a highly ordered structure, possibly containing α-helical segments, in which the two thiols are held apart. Such an ordered conformation is predicted for reduced 1 by using Chou–Fasman rules.[10] Cys-18 may be part of an α-helix ending at Pro-22, and Cys-23 may be part of a β-turn or part of another α-helix. In either calculated

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conformation, the thiol groups would be unable to approach each other as is necessary for forming the intramolecular disulfide bond. We determined by CD that the conformation of peptide **2** is highly ordered in TFE, a known helix-promoting solvent.

CD spectroscopy of the linear precursor **2** in several solvent systems was used to screen for a solvent system suitable for oxidizing the peptide to the monomeric cyclic disulfide. The least ordered structure for **2** was found in aqueous ammonium acetate so this solvent system was selected for additional cyclization studies. The Acm groups were removed from **2** by using a two-step procedure.[9] First, the Acm protecting group was removed by reaction with $Hg(OAc)_2$ followed by cleavage of the mercuriomercaptide bond[11] to give cleanly the reduced form of **1**. Oxidation of the thiols was accomplished by treating the crude peptide with ferricyanide at high dilution in ammonium acetate solution.[12] The best yield of disulfide product obtained was only 14%. The use of other oxidizing conditions (data not shown) did not improve the yield. Nevertheless, sufficient purified peptide **2** was obtained to test the activity reported for this sequence.

The biological data obtained for fragment 13–29 (**1**) indicated that this synthetic peptide does not have the biological activity reported by Soute et al.[6] for Fragment Su isolated from an enzymic digest. Their isolated peptide was identified by amino acid sequence comparisons, and the purity of the isolated material was not established. It is possible that Fragment Su is contaminated with an unidentified fragment of prothrombin precursor that is an exceptional substrate or that additional, uncharacterized modifications are present on the isolated peptides. In either event, the well-characterized synthetic prothrombin precursor 13–29 reported here is not an efficient substrate for the vitamin K dependent carboxylase. It will be necessary to repeat the isolation of Fragment Su in order to compare its structure with **1** and to resolve the apparent discrepancy.

The biological activities of **1** and **2** are consistent with the previously reported biological activities of related analogues of fragment 18–23[13] in which the linear prothrombin segment is more active as a substrate than the cyclic hexapeptide 18–23. These results, together with those reported here, suggest that the cyclic disulfide 18–23 of prothrombin precursor is not an exceptional recognition site for the carboxylase. Additional binding interactions between vitamin K dependent carboxylase and endogenous substrate or its propeptide precursor appear needed for maximal substrate reactivity.

Experimental Section

General Procedures. All amino acids were coupled as their N^{α}-Boc derivatives, which were purchased from Bachem, Inc. or Vega Biochemicals. The side-chain functionalities were protected as follows: γ-benzyl ester for glutamic acid, *O*-benzyl ether for serine, N^{G}-*p*-toluenesulfonyl for arginine, and *S*-acetamidomethyl (Acm) for cysteine. Trifluoroacetic acid (TFA), and diisopropylethylamine (DIEA) were distilled prior to use. Dicyclohexylcarbodiimide (DCC) was vacuum distilled, dichloromethane (CH_2Cl_2) was distilled from P_2O_5, and dimethylforamide (DMF) was purchased as glass distilled (Burdick and Jackson) and stored over 4A molecular sieves. All other reagents and solvents were of reagent or HPLC grade.

The synthesis of the peptides was performed on a Beckman Model 990 automatic peptide synthesizer using solid-phase peptide synthesis procedures.[7] Coupling of the Boc-amino acids was performed via 2.5 equiv of DCC/HOBt[14] after deprotection and neutralization of the peptide-resin as detailed in Table I. All couplings were monitored by the Kaiser test,[15] and when necessary recouplings were carried out with 1 equiv of the Boc-amino acid, DCC, and HOBt.

Analytical and semipreparative HPLC was performed on a Waters HPLC system consisting of a Model 680 automatic gradient controller, two Model 510 pumps with extended flow pump heads, a Model U6K injector, and a Model 441Z UV detector operating at 214 nm. A 3.9 mm × 30 cm μBondapak C_{18} column was used for analytical runs. For purification, a 7.8 mm × 30 cm semipreparative C_{18} column was used for approximately 40-mg loadings at flow rates of 3 mL/min.

Thin-layer chromatography was performed on 0.25-mm silica gel 60. TLC plates with the following solvent systems: (I) 1-butanol, acetic acid, water ethyl acetate (1:1:1:1), (II) 1-butanol, formic acid, water (4:2:1). Spots were visualized by UV, ninhydrin, and the chlorine-peptide spray.[16]

Amino acid analysis was performed on a Beckman 119CL amino acid analyzer after hydrolysis of the peptides in 6 N HCl at 110 °C for 22 h. Ratios are uncorrected for decomposition.

Circular dichroism spectra were recorded on a JASCO J-40A automatic recording spectropolarimeter. Solution concentrations ranged from 0.05 to 0.1 mM, and the cell path length was 0.50 or 0.10 cm. CD data are reported as mean residue weight molar ellipticities (θ_{MRW}).

Boc-Phe-*O*-resin. A 2.002-g (1.40 mmol) sample of hydroxymethyl copoly(styrene-1% divinylbenzene), purchased from Peninsula Laboratories as 0.70 mequiv/g, was acylated by a modification of the procedure by Wang[17] with 0.797 g (3.00 mmol) of Boc-Phe-OH, 0.620 g (3.00 mmol) of DCC, and 0.185 g (1.51 mmol) of 4-(N,N-dimethylamino)pyridine in 50% DMF/CH_2Dl_2 for 2 h. After washing and drying of the resin, 2.346 g was obtained, which corresponds to an incorporation of 0.60 mequiv/g. A 1.210-g (0.726 mmol) sample of this resin was acetylated with 9.5 μL (0.100 mmol) of acetic anhydride and 17 μL (0.100 mmol) DIEA in 50% DMF/CH_2Cl_2 for 2 h.

The peptides were synthesized according to the procedures described in Table I. Recouplings were required for the addition of Glu-21, Leu-19, Glu-15, and Leu-14. Asn-13 was coupled and recoupled with 2.5 and 1 equiv, respectively, of the *p*-nitrophenyl ester with an equimolar amount of HOBt present. At residue 18, the peptide-resin was divided into two portions. One was used for the 18–29 fragment and the other for the synthesis of the 13–29 fragment.

After removal of the N-terminal Boc group, each peptide was cleaved from the resin and the protecting groups removed by treatment with 15 mL of anhydrous HF containing 10% anisole for 45 min at 0 °C.[18] The deprotected peptides were extracted from the resin with 50% aqueous acetic acid and lyophilized.

The *S,S'*-bis-Acm fragment 18–29 was obtained in a yield of 0.288 g (50%) of the crude peptide. Desalting of this peptide by gel filtration over a Sephadex G-25 column (2.3 × 60 cm) with 30% acetic acid gave 0.263 g of crude peptide. This was further purified by semipreparative HPLC using a gradient of 10–30% CH_3CN, 0.1% TFA/H_2O developed over a 60-min time period. The recovery of the pure peptide was 70% to give an overall yield of 33%. TLC: R_f I, 0.32.

The *S,S'*-bis-Acm fragment 13–29 was obtained in a yield of 0.448 g (55%) of the crude peptide. This peptide was desalted as described for the 18–29 fragment to give 0.348 g of peptide. This was further purified by semipreparative HPLC using a gradient of 15–40% CH_3CN, 0.1% TFA/H_2O developed over a 60-min time period. The recovery of pure peptide was approx-

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imately 50% for all runs to give an overall yield of 24%. TLC: R_f I, 0.15; II, 0.24.

Prothrombin Precursor 13–29 Cyclic Disulfide. A 13.1-mg (5.96 μmol) sample of the *S,S'*-bis-Acm fragment 13–29 was dissolved in 3 mL of 50% aqueous acetic acid. A 48-mg (150 μmol) sample of $Hg(OAc)_2$ was added to this solution and stirred for 4 h. The reaction mixture was then placed under an atmosphere of N_2, and 2 mL of mercaptoethanol was added to the reaction via syringe. After the mixture was stirred for 20 h, the mercuriomercaptide and excess mercaptoethanol were removed by gel filtration on a 1.7 × 30 cm Sephadex G-15 column equilibrated with 0.1 N acetic acid.[11] Fractions containing the peptide were lyophilized to give 7.1 mg (58%) of the deprotected peptide, which was immediately suspended in 160 mL of 0.1 N HOAc. The peptide completedly dissolved when the pH was adjusted to pH 8.5 with 3 N NH_4OH to give a peptide concentration of 0.02 mM. The solution was titrated with 3 mL of 0.010 N $K_3Fe(CN)_6$[12] and stirred for 1 h at room temperature. To remove the ferrocyanide, the pH was adjusted to 5.0 with 50% aqueous acetic acid and the solution stirred with 2 g of Amberlite IR-45 (Cl^- form) for 20 min. After filtration of the resin, the peptide was isolated by lyophilization. The peptide was desalted by gel filtration over a 2.6 × 65 cm Sephadex G-15 column equilibratated with 20% aqueous acetic acid. A 3.4-mg sample of product was obtained for an overall yield of 7%. TLC: R_f II, 0.31.

Molecular Weight Determination. The apparent molecular weight was estimated by gel permeation chromatography in 1.0 N acetic acid with a Sephadex G-25 column (1.1 × 45 cm). Molecular weight standards were ribonuclease A (13700), insulin (5500), oxidized insulin B chain (3496), bovine prothrombin precursor fragment -9–9[19] (2074), encephalomyocarditis virus protein fragment 1501–1515[20] (1765), and bovine prothrombin precursor fragment 1–9[18] (1006). All eluants were monitored at 254 nm. The molecular weight determination was carried out as described by Andrews.[21]

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Book Reviews

Molecular Connectivity in Structure–Activity Studies. By Lemont B. Kier and Lowell H. Hall. Research Studies Press, Lechtworth, Hertfordshire, England. 1986. xvii + 262 pp. 15 × 24 cm. ISBN 0471 909831. $59.95.

One of the most abstract aspects of chemistry, especially organic chemistry as it relates to the design and study of biologically significant molecules, is the representation of structures in a manner relevant to the problem of biological activity. The objective of structure–activity studies in the general sense, and quantitative structure–activity relationships (QSAR) specifically, is to develop predictive constructs that can be used to design more effective compounds. This requires that structures of interest be reduced in some way to a numerical scale. The extension and use of graph theory and connectivity by Kier and Hall is a contemporary attempt at this very significant problem. These workers have published a number of papers and a book on this topic over the past decade and their work has been accepted by some and criticized by others. This book contains a review of the more significant papers, mostly by the authors, which have been published on the topic along with extensions of the use of connectivity in the expression of chemical bonding and structure.

The book begins with a preface by Professor Kier and a forward by Michael Tute of Pfizer Central Research. This is followed by nine chapters and appropriate indexes. Chapter 1 contains, to this reviewers knowledge, the most extensive discussion and development of the connectivity concept as proposed by Professors Kier and Hall. It begins with the idea of connection tables and is developed through Randic's use of connectivity as an index of branching in hydrocarbons. The idea of valence connectivity is then developed and this leads to the discussion in Chapter 2 of the correlation of the indexes with various physicochemical properties. Chapter 3 deals with topological information in the connectivity indexes and the use of specific connectivity indexes to rank compounds in terms of conformation, density, and flexibility is developed. Chapter 4 contains a discussion of electronic properties and connectivity. Here the Kier/Hall electronegativity scale is developed. The remainder of the book deals with connectivity and drug design. Chapter 5 is a very readable chapter dealing with statistical considerations. This is followed by strategies for the use of connectivity (Chapter 6), applications (Chapter 7), published studies (Chapter 8), and future directions (Chapter 9). This book represents considerable work on the part of the authors.

Since the book was written mainly for those involved in QSAR, comments based on more detailed considerations of the work are now presented. There is a phenomenological basis for QSAR studies and this is true of connectivity as developed by Professors Kier and Hall. There is certainly no theoretical basis for any of the material presented in this work. Most of the arguments presented for its validity are intuitive and in some cases border on conjecture and are rather shallow. For example, a flexibility index for alkanes is developed on pages 58 and 59 and then it is stated as not being useful for butane, 2-methylbutane, and pentane since the algorithm used to calculate the index has a minimum. On page 60 a table of values of the flexibility indexes is given for several alkanes. This table is footnoted with emperical rules for treating the index for several compounds to which the algorithm for various reasons cannot be applied in a straightforward manner.

Another difficult to understand discussion appears on pages 79–84. This deals with the use of connectivity to develop the Kier/Hall electronegativity scale. On page 82 a term E_{XY} is introduced, but not defined, as eq 10.

$$E^{\pi}_{xy} = .6\,E^{\sigma}_{x} + .6\,(E^{\sigma}_{y} - E^{\sigma}_{x}) \qquad (10)$$

This, of course, reduces to

$$E^{\pi}_{xy} = .6\,E^{\sigma}_{y}$$

which has not been defined. The identity in eq 10 is then substituted into a function that is used to interpret substituent effects in terms of connectivity. To this reviewers knowledge none of this material has been published and there are no journal references to work by Kier and Hall in the discussion that appears in the above referenced section.

It is accepted by everyone doing research in QSAR that this is a multivariable problem. So why do a large number of workers, including the authors of this book, still rely almost entirely on linear regression methods as the technique for model development? There are very appropriate uses of this technique in QSAR work but Chapter 5, "Statistical Considerations", while rather readable, has some very significant shortcomings and contains some very poor advice about the utility of multiple regression. There is a general rule of thumb that for such studies the ratio of independent variables to cases (compounds), *initially*, should be in the range of 1/5 or 1/4. This minimizes the possibility of