Synthesis of a Nonadecapeptide Corresponding to Residues 37–55 of Ovine Prolactin.[1] Detection and Isolation of the Sulfonium Form of Methionine-Containing Peptides

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Abstract: The syntheses of two methionine-containing peptides, prolactin-(37–55) and Leu-Gly-Arg-Leu-Gly-Met-Phe, have been accomplished by the solid-phase method. The preformed symmetrical anhydride and active ester coupling methods were used exclusively. Both syntheses resulted in a side product which underwent facile conversion in the solid state to the respective desired peptide. The heptapeptide side product was isolated in high yield and shown to be the *S-tert*-butylsulfonium form of the heptapeptide. The nonadecapeptide was found to exhibit immunoreactivity in complement fixation experiments.

Ovine prolactin is a protein consisting of 198 amino acids in a single polypeptide chain with three disulfide bridges, and its complete amino acid sequence has been elucidated.[2] Immunochemical studies have shown that the hormone is a potent antigen and highly species specific.[3,4] During the course of investigating the immunoreactivity of various peptide fragments obtained from cyanogen bromide cleavage of ovine prolactin, it was found that a fragment with sequence corresponding to amino acid residues 37–53 possessed significant immunological activity in radioimmunoassay and complement-fixation experiments using antisera to ovine prolactin.[5] In order to substantiate this observation, synthesis of the fragment was undertaken using the solid-phase method.[6] This communication reports the synthesis and complement-fixation activity of a nonadecapeptide with a sequence identical with residues 37–55 of the ovine prolactin molecule (Figure 1). In the course of synthesis, it was observed that the methionine residue was modified. Details of this observation will also be reported herein.

The incorporation of methionine in solid-phase peptide synthesis has generally been without protection of the thioether group. It has been assumed that subsequent side reactions could occur resulting in the alkylation of the sulfur and formation of the sulfonium ion. One form of protection that has been reported is the use[7] of methionine sulfoxide which cannot undergo formation of the sulfonium ion and yet can be reduced to methionine during the isolation and purification of the peptide. Where methionine has not been protected, scavengers for carbonium ions, such as anisole, have been employed to prevent this type of side reaction. In this study, the thioether group of methionine was not protected and anisole was used as scavenger during the HF cleavage of completed peptide from resin in the syntheses of the nonadecapeptide and a methionine-containing model peptide. Isolation of the highly purified sulfonium form of the model peptide in high yield showed that alkylation of methionine occurred even in the presence of anisole.

For synthesis of prolactin-(37–55), the starting Boc-Leu polymer was prepared by the Loffet method[8] with modifications previously reported.[9] With the exception of the asparagine residue, all other residues were coupled by use of symmetrical anhydrides[10] preformed with DCC.[11-13] The finished protected peptide resin was treated in 50% TFA–CH_2Cl_2 to remove the Boc group. Removal of the solid support as well as the side-chain protecting groups was accomplished in HF in the presence of anisole.[14] Gel filtration on Sephadex G-10, in which only one peak was detected, was followed by chromatography on CM-cellulose[15] (Figure 2, A). Further purification of fraction A by partition chromatography[16] on Sephadex G-50 (Figure 3, A) gave prolactin-(37–55) in highly purified form with 47% overall yield based on the starting Boc-Leu resin. Partition chromatography of this material on Sephadex G-50 gave a single symmetrical peak with R_f 0.48 (Figure 3, B). The product was homogeneous on paper electrophoresis in three buffer systems and on TLC in two solvent systems. Amino acid analyses[17] of acid and enzyme hydrolysates were in agreement with expected values (Table I).

In the synthesis described above, the Boc group was removed from the protected peptide resin with TFA before proceeding to the HF step. When the Boc group was not removed in this manner but was removed along with all the other protecting groups with HF, the crude peptide material from gel filtration gave a pattern on CM-cellulose chromatography shown in Figure 2, B. Prolactin-(37–55) (fraction A) was obtained in diminished yield. The major side product (fraction B) had an amino acid composition identical with prolactin-(37–55). Two spots were observed when this material was examined on paper electrophoresis in 5% aqueous acetic acid: one corresponding to prolactin-(37–55) and the other traveling like a more basic substance. This suggested that the side product was unstable and could be converted to prolactin-(37–55) under suitable conditions. To test this, the crude peptide material (after gel filtration on Sephadex G-10) was heated in the dry state. The resulting chromatographic pattern on CM-cellulose was similar to that of Figure 2, A. A greatly diminished amount of fraction B and a corresponding increase in fraction A were observed. It appeared that the side product (fraction B) could be a sulfonium form of prolactin-(37–55) resulting from alkylation of methionine. The possibility of partial reversal in solution is known,[18] but the reversal observed here appears to occur in the solid state.

To support the suggestion that fraction B (Figure 2, B) was the sulfonium form of the nonadecapeptide, a methionine-containing model peptide, Leu-Gly-Arg-Leu-Gly-Met-Phe (I), was synthesized with an attempt to isolate its sulfonium form (II). The placement of methionine near the carboxyl terminus afforded conditions for its repeated exposure to possible alkylation during removal of the Boc group in TFA. The arginine residue was chosen to impart sufficient polarity and charge to make purification convenient. Preliminary experiments gave variable proportions of two peptides depending on the manner of work-up; therefore, various routes were devised as shown in Figure 4.

For isolation of peptide I route 1 in Figure 4 was selected. The "normal lyophilization", synonymous with "lyophilization" in this paper, was performed in a vessel exposed to the ambient temperatures (ca. 25°). Final purification was effected by partition chromatography on Sephadex G-25, and

H - Phe - Asn - Glu - Phe - Asp - Lys - Arg - Tyr - Ala - Gln -

Gly - Lys - Gly - Phe - Ile - Thr - Met - Ala - Leu - OH

Figure 1. Amino acid sequence of prolactin-(37-55).

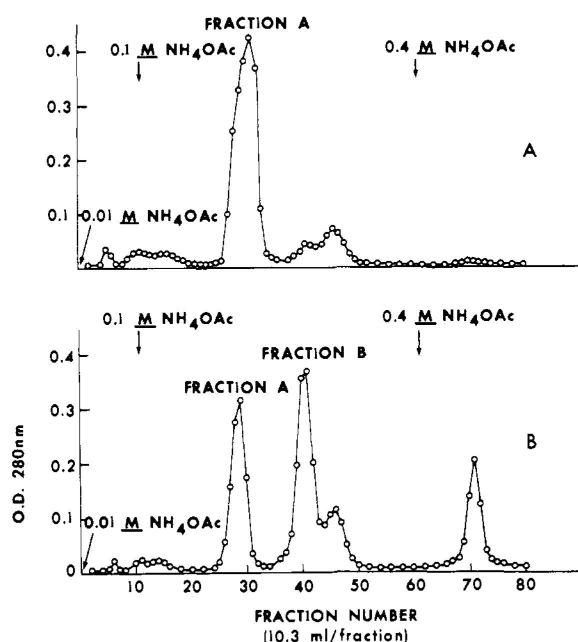

Figure 2. Carboxymethylcellulose chromatography of partially purified prolactin-(37-55) obtained by HF treatment of (A) TFA-deblocked peptide resin and (B) Boc-peptide resin.

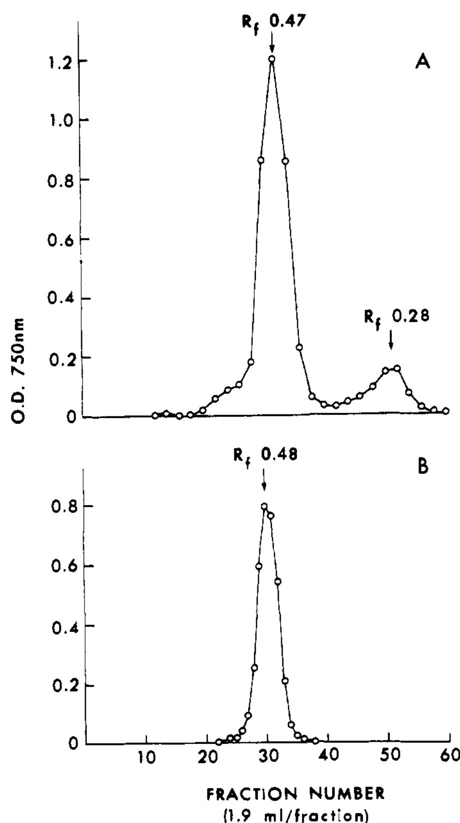

Figure 3. Partition chromatography of fraction A on Sephadex G-50: (A) 60 mg on a 1.89 × 35.4 cm column, yield (tubes 28-37), 50.5 mg; (B) 20.5 mg of highly purified prolactin-(37-55), yield (tubes 28-33), 19.0 mg.

isolation by lyophilization gave I in 68% overall yield based on starting Boc-Phe-resin.

Table I. Amino Acid Analyses of Synthetic Prolactin-(37-55)

Amino acid	Theoretical	Acid hydrolysate	Enzyme digest[a]
Lys	2	2.1	2.0
Arg	1	1.0	1.0
Asp	1	2.0	1.1
Asn	1		
Thr	1	1.0 (Asn + Thr + Gln)	3.1 (Asn + Thr + Gln)
Gln	1		
Glu	1	2.1	1.0
Gly	2	1.9	1.9
Ala	2	2.0	2.1
Met	1	1.0	1.0
Ile	1	0.9	0.9
Leu	1	1.0	1.0
Tyr	1	1.1	1.0
Phe	3	3.0	3.0

[a] Digestion with chymotrypsin for 24 h at 37°, followed by 24-h digestion with leucine aminopeptidase.

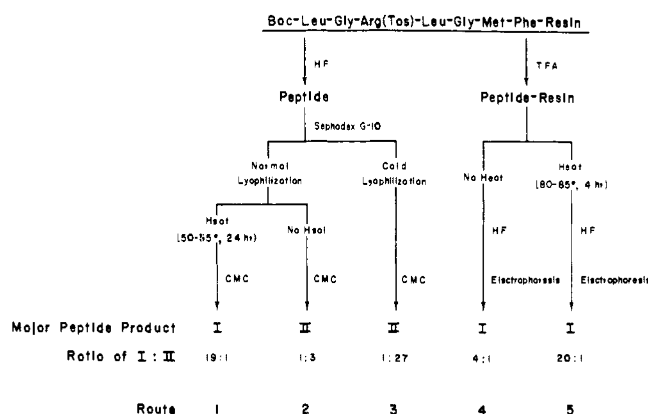

Figure 4. Outline of the various routes investigated (see text) which resulted in different proportions of the desired methionyl peptide I and a side-product peptide II.

The yield of I was drastically diminished (to 21%) when the heat treatment was omitted (route 2, Figure 4). In the chromatography on CM-cellulose, a major side product was detected which was eluted as a symmetrical peak much later than I, suggesting that it was a more positively charged substance. The amino acid composition of an acid hydrolysate was identical with I. Experiments indicated that the side product was relatively stable while in solution but was partially converted to I when the solvent in which it was dissolved was removed as during spotting for thin-layer chromatography or paper electrophoresis, and even during lyophilization or storage of the solid at room temperature. Thus, when the side product was isolated by normal lyophilization, stored for 3 days at room temperature, and rechromatographed on CM-cellulose, the pattern shown in Figure 5 was obtained. The first peak corresponds to the position of I and the second peak to the position of the side product. By paper electrophoresis, the peptide material in the first peak is identical with peptide I.

For isolation of an analytical sample of the side product, route 3 in Figure 4 was devised. The "cold lyophilization" was accomplished in a vessel immersed in an ice-water bath at all times. Chromatography on CM-cellulose gave a major peak corresponding to the previously observed position of the side product, and cold lyophilization gave peptide II in 66% overall yield based on starting Boc-Phe resin. The peptide showed homogeneity on thin-layer chromatography and paper electrophoresis only after careful spotting and immediate development. In order to confirm that II

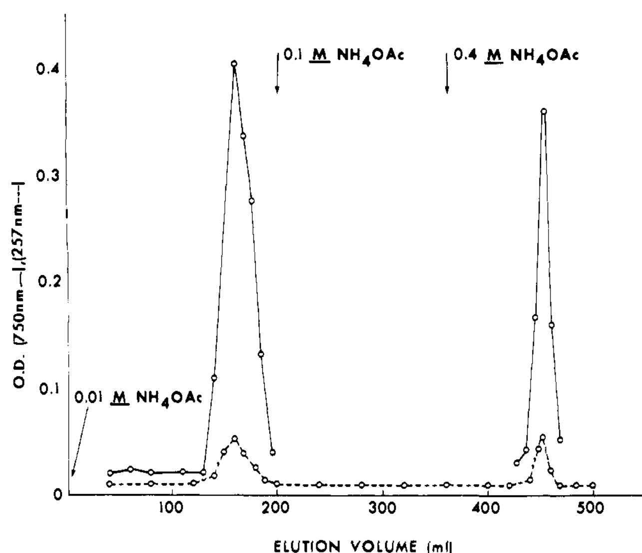

Figure 5. Rechromatography on carboxymethylcellulose of the side-product peptide II obtained by normal lyophilization (see text). Optical density at 750 nm obtained by Folin-Lowry analysis: O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265-275 (1951).

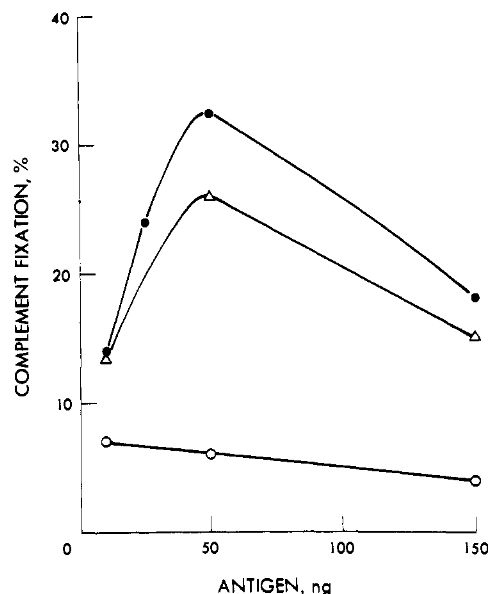

Figure 6. Microcomplement fixation curve obtained with natural prolactin-(37-53) (●—●), synthetic prolactin-(37-55) (△—△), and synthetic cyclic prolactin-(188-198) (○—○) using purified guinea pig antibodies to ovine prolactin.

was the *S-tert*-butylsulfonium form of peptide I,[19] proton NMR spectroscopy was carried out. The methyl peak of methionine located at δ 2.00 downfield from DSS for peptide I was shifted downfield to δ 2.75 for peptide II. Peptide II also gave a nine-proton *tert*-butyl peak at δ 1.52.

Alternatively, peptide II was synthesized by the reaction of peptide I with *tert*-butyl bromide in 50% TFA-CH_2Cl_2.

The amino acid composition of an acid hydrolysate of II was identical with peptide I. When I was first treated with performic acid and then acid hydrolyzed, all the methionine was converted to methionine sulfone. However, when peptide II was treated in like manner, the methioninesulfonium residue was almost completely destroyed to give a complex mixture of products. Thus, the *tert*-butylsulfonium form of methionine was affected by performic acid in a different manner than was methionine but was converted to methionine in acid hydrolysis.

Peptide II was converted to peptide I during storage of II in the solid form at room temperature, a fact in agreement with our preliminary observations on this conversion. Although the exact nature of this conversion needs further investigation, the results summarized in Figure 4 delineate routes for maximizing yields of either I or II. It is evident that the methionine residue in the crude peptide after HF treatment of the Boc-protected peptide resin is largely of the *tert*-butylsulfonium form. If the Boc group is first removed with TFA before the HF treatment, then the crude peptide consists largely of peptide I (routes 4 and 5 in Figure 4). This, in fact, was the route preferred for the synthesis and isolation of prolactin-(37-55).

The immunological activity of the synthetic prolactin-(37-55) was estimated by microcomplement fixation using purified guinea pig antibodies to ovine prolactin. As shown in Figure 6, the natural prolactin-(37-53) reached equivalence at 50 ng to fix 32% complement whereas the synthetic prolactin-(37-55) at the same equivalent dose fixed 26%. It may be noted that a synthetic cyclic prolactin-(188-198)[20] gave virtually no fixation of complement (Figure 6).

Experimental Section

Thin-layer chromatography was run on silica gel in the following solvents: 1-butanol-acetic acid-water, 4:1:1; 1-butanol-pyridine-acetic acid-water, 15:10:3:12. Trifluoroacetic acid, *N,N'*-dicyclohexylcarbodiimide, and anisole were distilled before use. Dioxane was distilled from sodium and stored at 4°. Diisopropylethylamine was washed with water, dried over NaOH, and distilled. Dichloromethane was refluxed over P_2O_5 for 2 h and then distilled.

Paper electrophoresis on Whatman 3 MM paper was carried out in three buffer systems with ninhydrin for detection: (A) 5% aqueous acetic acid, 400 V, 2 h; (B) collidine acetate buffer, pH 6.7, 2 kV, 1 h; and (C) formic acid-acetic acid buffer, pH 2.1, 2 kV, 1 h. R_f values reported are with respect to lysine.

Carboxymethylcellulose chromatography[15] was performed in a 1.23 × 47 cm column with an initial buffer of 0.01 M NH_4OAc of pH 4.5. A gradient with respect to pH and salt concentration was effected by introducing the NH_4OAc buffers (as subsequently described) through a 500-ml mixing chamber containing the starting buffer.

Partition chromatography on Sephadex G-25 (1.89 × 23.3 cm) was performed by procedures previously described[16] using the solvent system A: 1-butanol-0.5 M acetic acid (1:1). Partition chromatography on Sephadex G-50 (1.89 × 34.7 cm) was performed by procedures previously described[9] using the solvent system B: 1-butanol-pyridine-0.1% aqueous acetic acid (5:3:11).

Protected Peptide Resin of Prolactin-(37-55). Boc-Leu resin, prepared from chloromethyl resin[21] by procedures previously described,[9] gave a substitution of 0.385 mmol/g as estimated by the method of Gisin.[22] The Boc-Leu resin (1.1 g, 0.426 mmol) was placed in a Beckman Model 990 peptide synthesizer and carried through the previously reported schedules[23] for incorporating the remaining 18 amino acid residues, with the following exceptions: 25% rather than 50% dioxane-CH_2Cl_2 in step 4; 33% rather than 50% EtOH-CH_2Cl_2 in step 17; and the "pre-mix" reaction mixture[11] containing the required symmetrical anhydrides was stirred for 30 min in step 14. N^{α}-Boc protection was used throughout with the following side-chain protecting groups: Lys, Z(*o*-Br);[9] Tyr, Z(*o*-Br);[24] Arg, tosyl;[25] Asp, OBzl; Thr, Bzl; and Glu, OBzl. Boc-Asn was coupled as the *p*-nitrophenyl ester[26] (10 equiv, 4.26 mmol for 10 h). The "pre-mix" reaction mixture used in all other couplings was prepared as previously described[23] with 6.5 equiv (2.77 mmol) of *tert*-butyloxycarbonylamino acid and 3.0 equiv (1.28 mmol) of DCC. Boc-Arg(Tos)-OH was dissolved in 1.2 ml of DMF and diluted with 3.6 ml of CH_2Cl_2 before the reaction with DCC. For the reaction of Boc-Gln-OH with DCC,[27] Boc-Gln-OH was dissolved in 2.4 ml of DMF and diluted with 2.4 ml of CH_2Cl_2. The finished peptide resin was dried under reduced pressure over P_2O_5 for 3 h to yield 2.41 g, 96% of theoretical weight gain, and stored at −20°.

Prolactin-(37–55). Protected peptide resin (1.00 g) was treated with 50% TFA-CH_2Cl_2 (10 ml) for 15 min at 25° and filtered. The resin was washed with 25 ml each of CH_2Cl_2, 5% DIEA-CH_2Cl_2, CH_2Cl_2, and absolute ethanol. After drying for 80 min under reduced pressure over P_2O_5, the partially protected peptide resin (971 mg) was treated with HF (10 ml) in the presence of anisole (1.0 ml) for 30 min at −30 to −20° and for 30 min at 0°.[28] After removal of the HF at 0° by a stream of nitrogen for 10 min, the peptide–resin mixture was dried under reduced pressure over NaOH for 30 min and then stirred with TFA (20 ml) for 20 min and filtered. The filtrate was evaporated in vacuo to give an oil which was twice reevaporated from glacial acetic acid (5 ml). The oily residue was then dissolved in 10% aqueous acetic acid (50 ml) and washed with two 20-ml portions of ether. The aqueous solution was lyophilized and the resulting material (359 mg) was subjected to gel filtration on Sephadex G-10 (2.2 × 28.5 cm) in 0.5 M acetic acid. The peptide material (elution volume of peak, 45 ml) was isolated by lyophilization to give 305 mg, 77% overall yield based on starting Boc-Leu resin. An aliquot (50.0 mg) was submitted to chromatography on CM-cellulose, Figure 2, A, and essentially three peaks were detected. Isolation by lyophilization yielded fraction A, 36 mg, and fraction B, 2.95 mg. Another aliquot (180 mg) chromatographed on CM-cellulose yielded 130 mg corresponding to the material in fraction A. Upon partition chromatography on Sephadex G-50 (1.89 × 35.4 cm), Figure 3, A, a 60-mg aliquot of fraction A resulted in a major peak with R_f 0.47 and a minor peak with R_f 0.28. Isolation of material represented by the major peak (R_f 0.47) gave 50.5 mg (47% overall yield) of highly purified prolactin-(37–55): TLC (BPAW), R_f 0.48; TLC (BAW), R_f 0.12; $[\alpha]^{24}\text{D}$ −50.03° (*c* 0.985, 1.0 M acetic acid).

A sample (20.5 mg) of the R_f 0.47 material was again subjected to partition chromatography on Sephadex G-50 and a single symmetrical peak with R_f 0.48 was obtained as shown in Figure 3, B. Paper electrophoresis in buffer systems A, B, and C each gave a single spot with R_f values of 0.62, 0.31, and 0.26, respectively. Amino acid analyses of acid and enzyme hydrolysates are shown in Table I.

When Boc-protected peptide resin (494 mg) was not treated with TFA but taken directly to HF treatment and worked up as described above, 153 mg (78% overall yield based on starting Boc-Leu resin) of peptide was isolated after the Sephadex G-10 step. An aliquot (50 mg) subjected to chromatography on CM-cellulose gave the pattern shown in Figure 2, B; yields, fraction A, 18 mg (28% overall yield), and fraction B, 23 mg. On paper electrophoresis in buffer system A, fraction A gave R_f 0.61 and fraction B gave two spots with R_f 0.61 and 0.76. At this stage of purity the overall yield of prolactin-(37–55) was 28%. Another aliquot of crude peptide (50 mg) was heated at 50–55° for 20 h and then subjected to chromatography on CM-cellulose. A pattern similar to that shown in Figure 2, A, was obtained, and the material isolated from the major peak (29 mg) was further purified (20-mg sample) by partition chromatography on Sephadex G-50 to give 16 mg of highly purified prolactin-(37–55) (36% overall yield).

Leu-Gly-Arg-Leu-Gly-Met-Phe (I). Boc-Phe resin was prepared by the method used for the Boc-Leu resin as above. Boc-Phe resin (1.0 g, 0.483 mmol) was placed in a Beckman Model 990 peptide synthesizer and essentially carried through the same procedures employed for the nonadecapeptide. About 10% of the resin was removed for analytical purposes during the synthesis. The finished peptide resin was dried under reduced pressure over P_2O_5 for 3 h at 25° to yield 1.25 g, 97% of the theoretical weight gain, and stored at −20°.

Protected peptide resin (300 mg) was treated with 10 ml of liquid HF for 30 min at 0° in the presence of 0.30 ml of anisole. The cleaved material was worked up and subjected to gel filtration as described for the nonadecapeptide. Isolation by lyophilization gave 91.2 mg. This material was heated at 50–55° for 24 h and then submitted to chromatography on CM-cellulose. The column was eluted with 200 ml of the initial buffer. The desired product emerged between elution volumes of 130 and 200 ml, and isolation by lyophilization gave 66.5 mg, 80% overall yield based on starting Boc-Phe resin. The column gradient was first raised with 160 ml of 0.10 M NH_4OAc, pH 6.7, and then with 0.40 M NH_4OAc, pH 6.7. A peak emerged between 88 and 124 ml after the start of the 0.40 M buffer, and isolation by lyophilization gave 3.5 mg of a peptide side product. The desired product (64 mg) was further purified by partition chromatography on Sephadex G-25 in solvent system A (R_f 0.26) to give 55 mg of peptide I (68% overall yield): TLC (BPAW), R_f 0.68; $[\alpha]^{24}\text{D}$ −22.07° (*c* 1.22, 1.0 M acetic acid). Paper electrophoresis in buffer systems A and B each gave a single spot with R_f values of 0.58 and 0.45, respectively. Amino acid analysis of an acid hydrolysate gave $Arg_{1.0}$ $Gly_{1.8}$ $Met_{1.0}$ $Leu_{2.1}$ $Phe_{1.0}$.

Another portion of the protected peptide-resin (323 mg) was treated with HF and worked up in the same manner. The material after gel filtration was not heated but taken directly to CM-cellulose chromatography. The yield of peptide I was 18.8 mg, 21% overall yield, while the yield of the slow-moving side product was 60 mg. The preparation of peptide I obtained in this run was identical with an authentic sample of peptide I by TLC, paper electrophoresis, and amino acid composition.

Another portion of the protected peptide resin (122 mg) was treated with 50% TFA-CH_2Cl_2 and neutralized as described above for the nonadecapeptide resin. One-half of the peptide resin was treated with HF and worked up as described above to give an oily residue. The other half of the peptide resin was heated at 80–85° for 4 h and processed as the first half to give an oily residue. On paper electrophoresis in system A, peptide I traveled with R_f 0.58 and the side product with R_f 0.81. By this criteria the material obtained from the unheated resin showed a peptide I to side product ratio of 4:1. The material from the heated resin showed a 20:1 ratio.

Peptide II, Sulfonium Salt of I. Protected peptide resin (102 mg) was treated with 5 ml of liquid HF for 30 min at 0° in the presence of 0.10 ml of anisole. The cleaved material was worked up and subjected to gel filtration as described for the nonadecapeptide. The crude peptide material was isolated by lyophilization in which the lyophilizing flask was immersed in an ice-water bath. Subjecting this material to chromatography on CM-cellulose gave a slow-moving major peak in the same position as the side product (see previous section). Isolation by lyophilization as described above yielded 47.4 mg of the sulfonium salt of I, 66% overall yield based on $\epsilon_{257\ nm}$ of Phe: TLC (BPAW), R_f 0.45, with a trace of I with R_f 0.68; paper electrophoresis in system A gave R_f 0.81, with a trace of I with R_f 0.58. Amino acid analysis of an acid hydrolysate gave $Arg_{1.0}$ $Gly_{1.9}$ $Met_{1.0}$ $Leu_{2.1}$ $Phe_{1.0}$. Amino acid analysis of a performic acid oxidized sample gave $Hsl_{0.1}$ $Arg_{0.9}$ $Met(O)_{0.1}$ $Met(O_2)_{0.2}$ $Hse_{0.1}$ $Gly_{1.8}$ $Met_{0.1}$ $Leu_{2.0}$, while I under the same conditions gave $Arg_{1.0}$ $Met(O_2)_{1.0}$ $Gly_{1.7}$ $Leu_{2.0}$. Under the conditions described here phenylalanine was altered by chlorination.[29]

NMR spectra were taken with a Varian T-60 with DSS as internal reference. The sulfonium salt II (47 mg) and the authentic peptide I (54 mg) were dissolved in D_2O, 0.25 and 0.35 ml, respectively.

The sample of peptide II in D_2O used in the NMR study was recovered by lyophilization at 0°. The solid (20 mg) was stored at room temperature for 3 months and then a sample (18.9 mg) was subjected to chromatography on CM-cellulose. Peptide I was recovered in good yield (17.1 mg) in highly purified form identical with an authentic sample of I by TLC (BAW and BPAW) and paper electrophoresis in system A.

Peptide I could be converted to the sulfonium salt by the following procedure. Peptide I (9.7 mg, 12 μmol) was dissolved in 0.1 ml of 50% TFA-CH_2Cl_2 and treated with *tert*-butyl bromide (125 μmol) for 60 min at room temperature. After evaporation, the product was subjected to CM-cellulose chromatography in the usual manner. Peaks corresponding to the positions of peptide I and the sulfonium salt II were detected and isolation by lyophilization gave 2.8 and 5.2 mg, respectively.

Immunological Studies. Guinea pig antiserum to ovine prolactin was obtained as previously described for the porcine hormone.[4] Specific antibodies were prepared by affinity chromatography using a prolactin-Sepharose immunoadsorbent according to the procedure of Sairam et al.[30] The antibodies were eluted with 2 M sodium trichloroacetate. Microcomplement fixation was carried out as described by Wasserman and Levine.[31]

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(12) Abbreviations: TFA, trifluoroacetic acid; CM-cellulose, carboxymethylcellulose; TLC, thin-layer chromatography; BAW, 1-butanol-acetic acid-water, 4:1:1 (v/v); BPAW, 1-butanol-pyridine-acetic acid-water, 15:10:3:12 (v/v); DCC, *N,N'*-dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; Hsl, homoserine lactone; Hse, homoserine.
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Polycyclic K-Region Arene Oxides. Products and Kinetics of Solvolysis

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Abstract: K-Region oxides derived from four polycyclic aromatic hydrocarbons (phenanthrene 9,10-oxide (**1**), benz[*a*]anthracene 5,6-oxide (**2**), dibenz[*a,h*]anthracene 5,6-oxide (**3**), and 3-methylcholanthrene 11,12-oxide (**4**) solvolyze in 1:1 dioxane-water, 0.10 M in KCl (cf. ref 14 for oxide **1**). At pH's 3-6 the oxides **1-4** quantitatively produced 3:1 mixtures of K-region phenols and dihydrodiols, respectively. Oxide **4** gave the same ratio up to pH 10 and a 1:1 ratio at pH 12. Liquid chromatography of the dihydrodiols showed that the mole percent cis isomer varied with the oxide: **1**, 20% *cis-*; **2**, 31% *cis-*; **3**, 32% *cis-*; **4**, 75% *cis-*. Oxide **4** produced equal cis/trans dihydrodiol ratios at pH's 3, 4, and 5. Diazomethane methylation of the product phenols, followed by comparison of the NMR or the gas chromatogram of the mixtures with those of the authentic ethers, showed the relative amounts of the positional isomers to be: **2**, 60:40, 5:6; **3**, 85:15, 5.6; **4**, >98:2, 11:12. Uv spectral analysis of oxide solvolyses carried out in a pH stat at 36.8 °C indicated first-order disappearance of oxides **1-4** and simultaneous appearance of phenols and dihydrodiols at the same rate. The first-order rate constant for oxides **1-3** can be expressed as $k_{obsd} = k_H a_H$ and that of oxide **4** is $k_{obsd} = k_H a_H + k_0$. Values for k_H (M^{-1} sec^{-1}) were: **1**, 8.09; **2**, 14.8; **3**, 19.3; **4**, 990. The k_0 value for oxide **4** was 3.07×10^{-5} sec^{-1}. These results can be rationalized by a scheme which includes rate-limiting ring opening of the protonated or unprotonated oxide ring, followed by competitive water attack on or NIH shift of the intermediate benzylic carbocation. Rate-limiting formation of the 11*H*-11-hydroxy-3-methylcholanthren-12-yl cation may be accelerated by inductive stabilization of the delocalized incipient carbocation.

Introduction

Polycyclic arenes are an important class of carcinogenic compounds. The work of the Millers[1] and others has demonstrated that most chemical carcinogens, including polycyclic arenes, must be metabolically activated before they can be covalently bound to cellular macromolecules. Such binding is considered necessary for cell transformation.[2] DNA may be the target for binding, but this has not yet been proven. Evidence has accumulated that arene oxides are one type of reactive metabolite of polycyclic arenes.[3] The conversion of arenes to arene oxides is carried out by membrane-bound cytochrome P-450 monooxygenases.[4]

Several K-region[5] arene oxides, including phenanthrene 9,10-oxide[6] (**1**), benz[*a*]anthracene 5,6-oxide[6] (**2**), and dibenz[*a,h*]anthracene 5,6-oxide[7] (**3**), have been detected as metabolites when the parent arenes were added to fortified rat liver microsomes. The biological effects of these and other arene oxides have been studied in our laboratory. It was demonstrated that **3** and 3-methylcholanthrene 11,12-oxide (**4**) were more active than the parent arenes or other