Titanium(III) as a Selective Reducing Agent for Nitroarginyl Peptides: Synthesis of Arginine Vasotocin

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Nitroarginyl peptides are selectively reduced by titanium(III) to the corresponding arginyl peptides. Thus, the nitro protecting group, which is labile toward nucleophilic reagents, may be retained through part of a synthesis, but may be removed selectively prior to treatment with nucleophiles such as hydrazine and ammonia. The selectivity of titanium(III) for removal of the nitro functionality increases the flexibility of this protecting group in the synthesis of arginyl peptides. A synthesis of the molluscan cardiac stimulant H-Phe-Met-Arg-PheNH_2, which demonstrates these features, is described. A synthesis of arginine vasotocin using a combination of solid phase and solution techniques which incorporate this method is also reported.

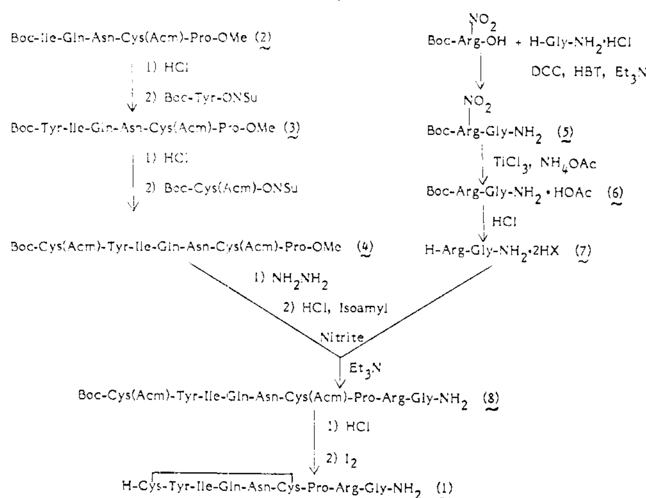
Arginine vasotocin (1), a nonapeptide structurally related to both oxytocin and vasopressin, has been identified within the pineal gland of several mammalian species[1] and has been shown to have potent effects on the secretion of several pituitary hormones.[2] No radioimmunoassay has been described for this natural product. We have undertaken the synthesis of arginine vasotocin of high purity in a collaborative effort to permit the development and utilization of a radioimmunoassay by Dr. Richard Wurtman, Dr. John Fernstrom, and their associates.[3] This led to the generation of antibodies which are highly selective for the subject compound vis a vis oxytocin and vasopressin.[4]

Syntheses of arginine vasotocin (AVT) have been reported which utilize classical solution methods or solid phase techniques.[5] We report herein a synthesis of AVT by a fragment condensation approach in which one of the fragments is prepared by a combination of solid phase and solution chemistry. The synthesis was designed to minimize side chain protection and allow purification of intermediates at convenient stages. In the course of this work, the potential of titanium trichloride in peptide synthesis was explored for the first time, and it was found to be a useful reagent for removing the nitro protecting group from nitroarginyl peptides. We have explored the scope of this method in peptide synthesis.

Results and Discussion

The route of synthesis of AVT is outlined in Scheme I. Protected pentapeptide 2 was prepared by the solid phase method using base-catalyzed transesterification for removal from the solid support. The acetamidomethyl (Acm) group[6] was employed to protect the sulfhydryl of cysteine; Boc-asparagine and Boc-glutamine were introduced as their *p*-nitrophenyl esters. Subsequently, coupling reactions for the introduction of Boc-tyrosine and N^{α}-Boc-*S*-Acm-cysteine were carried out in solution using the *N*-hydroxysuccinimide esters[7] to give the hexapeptide 3 and the heptapeptide 4, respectively. The latter compound was purified by silica gel chromatography. Protection of the phenolic hydroxyl group of tyrosine was unnecessary in this approach, thereby avoiding the troublesome rearrangement of *O*-benzyltyrosine under acidic cleavage conditions.[8] The protected dipeptide 5 was obtained from the dicyclohexylcarbodiimide (DCC) mediated coupling of N^{α}-Boc-N^{G}-nitroarginine and glycinamide hydrochloride in the presence of 1-hydroxybenzotriazole (HBT). We found that the addition of HBT reduced formation of byproducts which, in this case, had limited initial yields to 30%.[9] Treatment of 5 with titanium trichloride removed the nitro group to provide the partially protected dipeptide 6. This method of deprotecting nitroarginyl peptides is novel, and its utility is discussed below. Removal of the Boc group from compound 6 with trifluoroacetic acid gave dipeptide 7. Alternatively, treatment of 5 with anhydrous hydrogen fluoride furnished a sparingly soluble salt which was immediately converted via the free amine to the trifluoroacetate salt of 7 which could be dissolved in DMF for the subsequent coupling reaction. Ornithine formation was not detected in this case, although it is a frequently observed side reaction during treatment of nitroarginyl peptides in liquid HF.[10]

Scheme I



Intermediate 4 was converted to its hydrazide and coupled with 7 via the azide method to furnish the partially protected nonapeptide 8. After selective removal of the *tert*-butyloxycarbonyl (Boc) group from this compound, removal of the remaining protecting groups and cyclization were accomplished in a single step by the action of iodine in methanol.[11] The resultant crude AVT was purified by silica gel chromatography followed by preparative free flow electrophoresis.[12] The AVT (1) so obtained had a purity of greater than 98% as estimated by thin-layer chromatography.[13] The structure was confirmed by amino acid analysis, gel filtration, and comparison of the NMR spectrum at 300 MHz with a published 220 MHz spectrum.[14]

As mentioned above, a novel approach to the removal of the nitro protecting group from nitroarginine-containing peptides was developed using the one-electron reducing agent titanium trichloride,[15] a reagent which has not previously found application in peptide synthesis. This method was investigated because titanium(III) has greater potential for selectivity than some of the reagents currently in use for removal of the nitro group.[10] Several simple nitroarginine-containing compounds were reduced under a variety of conditions to establish the effects of solvent, pH, and substitution at carboxyl and/or amino termini on product yield and byproduct formation.

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Table I. Reduction of Nitroarginine Derivatives with $TiCl_3$

compd	registry no.	conditions[a]	arginine-derived products, %[b] Arg	Orn	unk.	% yield	registry no.
Boc-Arg(NO_2)-Gly-NH_2	67844-86-4	MeOH, H_2O, buffer[c]	97.6	2.4		83.6	67844-89-7
Boc-Arg(NO_2)-Gly-NH_2		MeOH, H_2O, 36 equiv of Et_3N	71.4	28.6[f]		62.4	
Boc-Arg(NO_2)-Gly-NH_2		DMF, H_2O, buffer[c]	78.0	21.2	0.8	73.4	
CF_3CO_2H·H-Arg(NO_2)-Gly-NH_2	67844-87-5	MeOH, H_2O, buffer[c]	91.5	8.5[g]		83.7	34367-76-5
CF_3CO_2H·H-Arg(NO_2)-Gly-NH_2		2 N CF_3CO_2H	90.8	6.6	2.6	77.5	
CF_3CO_2H·H-Arg(NO_2)-Gly-NH_2		2 N HCl	90.9[d]	3.7	4.1	74.5	
CF_3CO_2H·H-Arg(NO_2)-Gly-NH_2		H_2O, buffer[c]	80.7	17.6	1.9	71.8	
Boc-Arg(NO_2)-OH	2188-18-3	MeOH, H_2O, buffer[c]	91.2	8.8[h]		92.6	13726-76-6
H-Arg(NO_2)-OH	17035-90-4	2 N HCl	91.5	4.4	4.1	72.0	74-79-3
H-Arg(NO_2)-OH		85% HOAc	3.0	89.7[i]	7.3	69.1	
H-Arg(NO_2)-OH		H_2O, 2.5 equiv of HOAc	27.4	64.1	8.5	74.9	
Boc-Phe-Met-Arg(NO_2)-Phe-OCH_3	67844-88-6	MeOH, H_2O, buffer[c]	99.0	1.0		77.0[e]	67845-00-5

[a] Reactions were run for 45–60 min at ambient temperature under nitrogen atmosphere. Excess reagent was oxidized with air or Me_2SO. [b] Determined by amino acid analysis of total reaction products. Less than quantitative recoveries were attributed to absorption losses on insoluble Ti(IV) species formed in the reactions. [c] 4 M aqueous ammonium acetate; pH of reactions was 4–6. [d] 1.3% nitroarginine remained. [e] Yield determined by isolation. [f] Registry no., 67844-90-0. [g] Registry no., 67844-94-4. [h] Registry no., 21887-64-9. [i] Registry no., 70-26-8.

These results are summarized in Table I. Yields of arginyl peptides were best and byproduct formation least when the nitroargininyl compounds were treated for 1 h in aqueous methanol containing ammonium acetate buffer (pH 4–6) with a slight excess of 20% aqueous titanium trichloride solution at 24 °C.[16] More acidic or basic conditions or other solvent combinations gave lower yields and more byproducts. Compounds in which the nitroarginine residue has neither a free carboxyl nor a free amino terminus tended to react more cleanly. As with other methods for removal of the nitro group, formation of ornithine was the dominant side reaction and could not be entirely eliminated under any of the conditions tested.

The stability of other protecting groups and sensitive functionalities to these reaction conditions was briefly surveyed. In the cases studied, loss of the Boc group was not observed. The isonicotinyloxycarbonyl (*i*-Noc) group is a useful new functionality for protection of the ϵ-amino group of lysine.[17] It may be removed by catalytic hydrogenation, electrolysis, or the two-electron reducing agent zinc. Since these procedures are commonly applied to the removal of the nitro group from nitroarginyl peptides, it was hoped that the *i*-Noc group would be stable to the one-electron reducing agent titanium(III), thus permitting selective removal of the nitro group from nitroarginine in the presence of *i*-Noc-lysine. Indeed, N^{α}-Boc-N^{ϵ}-*i*-Noc-lysine showed no detectable reaction, according to TLC, after exposure to titanium trichloride for the usual period (1 h), and only trace amounts of reaction were observed after 68 h. Similarly, treatment of tryptophan with titanium(III) produced traces of new products after 3 h, but none were detectable using the usual reaction conditions. Reduction of sulfoxides to sulfides with titanium trichloride has been reported,[18] and using the conditions for nitro group removal, methionine sulfoxide was converted to methionine, although at a much slower rate than the nitro group cleavage. Methionine sulfone was inert to the reaction conditions.

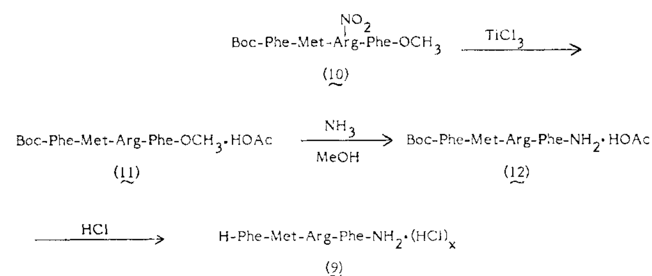
These results demonstrate the selectivity of titanium(III) for removing the nitro group from nitroarginine derivatives. We have not found another functionality commonly employed in peptide synthesis which is significantly affected by the reagent. In contrast, the usual conditions for removing the nitro group, including catalytic hydrogenation, anhydrous HF, zinc in acid, and electrolysis,[10] result in loss of one or more additional useful protecting groups. Titanium(III) may be most valuable when the fragment condensation strategy is employed. Selective removal would be advantageous when nucleophilic reagents such as hydrazine or ammonia, which react with the nitroguanidine, are likely to be used. Such an application is illustrated by our synthesis of the molluscan cardiac stimulant **9**[19] (Scheme II), in which a key step involves removal of the nitro protection from arginine in tetrapeptide ester **10** with titanium(III) prior to ammonolysis. Treatment of compound **10** with ammonia would be expected to result in substantial, if not complete, conversion of nitroarginine to ornithine. In a similar manner, fully protected nitroarginyl peptide fragments prepared using the solid phase could be selectively reduced to partially protected arginyl peptides with titanium(III) followed by conversion to the acyl hydrazides for subsequent coupling via the azide method.

The titanium(III) method for removing the nitro group from nitroarginyl peptides increases the utility and flexibility of this protecting group and permits useful strategies in fragment condensation syntheses which were not previously possible. Application of the nitro group for arginine in combination with other protecting groups such as Acm (cysteine) and *i*-Noc (lysine), which are also removable by chemically selective methods,[20] will make available formerly inaccessible partially protected peptides for biological evaluation. Titanium(III) will be a useful reagent whenever chemically selective removal of the nitro protecting group from nitroarginyl peptides is required.

Experimental Section

Boc-Ile-Gln-Asn-Cys(Acm)-Pro-OMe (2). Boc-prolyl resin (27.5 g, 43.75 mmol, of proline) was treated in a four cycle procedure according to the following scheme: (1) methylene chloride wash, 3 × 2 min; (2) 1:1 (v/v) trifluoroacetic acid–methylene chloride deprotection (ethanedithiol added as scavenger), 5 + 25 min; (3) methylene chloride wash, 3 × 2 min; (4) chloroform wash, 3 × 2 min; (5) 1:9 (v/v) triethylamine–chloroform neutralization, 10 min; (6) chloroform wash, 3 × 2 min; (7) methylene chloride wash, 3 × 2 min; (8) Boc-amino acid (2.5 molar excess) in a minimum volume of methylene chloride followed after 5 min by dicyclohexylcarbodiimide (2.5 molar excess) in

Scheme II



methylene chloride (1:1 v/v), 2 h; (9) alternating methanol and methylene chloride washes, 3 each × 2 min. For incorporation of asparagine and glutamine, a 4-fold excess of the Boc-protected *p*-nitrophenyl esters in a minimum volume of dimethylformamide (DMF) was reacted for 4 h. In these cases, the methylene chloride wash immediately preceding the coupling step was changed to DMF and DMF washes (3 × 2 min) were added after the coupling. All washes were 550 mL. Coupling efficiency was monitored with the Kaiser test,[21] and repeat couplings were run for asparagine and isoleucine. The completed Boc-pentapeptide was transesterified from the resin with triethylamine (300 mL) in methanol (1.1 L) to provide 19.8 g of crude product. This material from two identical runs was combined and recrystallized from methanol–ethyl acetate to yield 36.2 g (55%) of Boc-Ile-Gln-Asn-Cys(Acm)-Pro-OMe: mp 200–201 °C dec; $[\alpha]^{24}_{589}$ −93.7° (*c* 0.656, 50% methanol–acetic acid).

Anal. Calcd for $C_{32}H_{54}N_8O_{11}S \cdot H_2O$: C, 49.47; H, 7.27; N, 14.42; S, 4.13. Found: C, 49.42; H, 7.04; N, 14.31; S, 4.10.

Amino acid analysis showed Ile 0.97, Glu 0.98, Asp 1.06, Pro 1.08, Cys 1.04, and NH_3 2.90.

Boc-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-OMe (3). The protected pentapeptide 2 (34 g, 44.8 mmol) was slurried in ethyl acetate (1.2 L), and HCl gas was bubbled through the mixture with ice bath cooling for 40 min followed by nitrogen for 1.5 h. The insoluble solid was filtered, washed with ethyl acetate, and dried in vacuo to give 33.9 g of the terminal amino hydrochloride salt, one spot by TLC.

The entire quantity of hydrochloride salt and Boc-tyrosine hydroxysuccinimide ester (18.6 g, 49.3 mmol) were dissolved in degassed DMF (240 mL) under nitrogen, and triethylamine (12 mL) was added to attain an apparent "pH" 8 as measured by moistened narrow range pH paper. The "pH" was readjusted periodically with triethylamine (2.8 mL) until it remained constant. The reaction mixture was stirred overnight and filtered to remove $Et_3N \cdot HCl$. The filtrate was concentrated in vacuo, and the residue was crystallized from methanol to provide 37.0 g (90%) of Boc-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-OMe: mp 194–198 °C dec; $[\alpha]^{24}_{589}$ −76.5° (*c* 0.51, methanol).

Anal. Calcd for $C_{41}H_{63}N_9O_{13}S \cdot CH_3OH$: C, 52.87; H, 7.08; N, 13.21. Found: C, 52.69; H, 6.72; N, 13.14.

Amino acid analysis showed Tyr 0.97, Ile 1.00, Glu 1.03, Asp 1.01, and NH_3 3.07; Pro 1.00 and Cys 0.91 (separate determination after air oxidation of cysteine to cystine at pH 10.4).

Boc-Cys(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-OMe (4). The protected hexapeptide (42.8 g, 44.8 mmol) was deprotected with HCl in ethyl acetate as described for Boc-pentapeptide 2 to give 39.2 g of hexapeptide methyl ester hydrochloride.

A 37.4-g sample of this compound and N^{α}-Boc-*S*-Acm-cysteine (16.9 g, 43.4 mmol) were reacted in DMF (150 mL) as in the synthesis of Boc-hexapeptide to provide, after precipitation from methanol–ethyl acetate, 37.4 g of crude product. Chromatography on 3500 g of silica gel using the solvent system 20:20:7:5:3:3 $CHCl_3$–EtOAc–MeOH–pyridine–HOAc–H_2O and combining 80-mL fractions on the basis of TLC provided, after crystallization from methanol, 20.8 g (42%) of Boc-Cys(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-OMe: mp 216–218 °C dec; $[\alpha]^{24}_{589}$ −81.34° (*c* 0.92, 50% acetic acid).

Anal. Calcd for $C_{47}H_{73}N_{11}O_{15}S_2$: C, 51.49; H, 6.71; N, 14.05; S, 5.85. Found: C, 51.75; H, 6.62; N, 13.90; S, 5.90.

Amino acid analysis showed Cys 1.99, Tyr 1.00, Ile 0.94, Glu 1.04, Asp 1.02, and Pro 1.00; NH_3 3.97 (separate determination).

Boc-Arg(NO_2)-Gly-NH_2 (5).[22] N^{α}-Boc-N^{G}-nitroarginine (7.12 g, 22.3 mmol), glycinamide hydrochloride (2.46 g, 22.3 mmol), triethylamine (3.0 mL, 22.3 mmol), and hydroxybenzotriazole monohydrate (3.42 g, 22.3 mmol) were dissolved in a mixture of tetrahydrofuran (THF) (30 mL), acetonitrile (30 mL), and water (8 mL). Dicyclohexylcarbodiimide (4.66 g, 22.6 mmol) in 10 mL of 1:1 THF–acetonitrile was added dropwise during 10 min. The resultant mixture was stirred at room temperature for 17 h and filtered, and the solid was washed with 1:1 THF–acetonitrile. Most of the product was mixed with dicyclohexylurea in the filtered solids. Crystallization from MeOH–ether gave pure Boc-Arg(NO_2)-Gly-NH_2: 5.95 g (71% yield); mp 151–153 °C dec; $[\alpha]^{24}_{589}$ +0.21°, $[\alpha]^{24}_{463}$ +3.00° (*c* 1.4, MeOH).

Amino acid analysis showed Arg 0.99, Gly 1.01, and NH_3 1.36.[23]

Boc-Arg-Gly-NH_2 Acetate (6). Boc-Arg(NO_2)-Gly-NH_2 (5; 4.9 g, 13.1 mmol) in methanol (195 mL) was treated under nitrogen with a freshly prepared buffered solution of $TiCl_3$ made from 60 mL (78 mmol) of 20% aqueous $TiCl_3$ and 117 mL (468 mmol) of 4 M aqueous ammonium acetate. After 1 h, excess $TiCl_3$ was oxidized by bubbling through air until the purple color was dissipated (1 h). Titanium oxides were removed by centrifugation at 5000 rpm. The supernatant was decanted, and the solids were washed with methanol (2 × 100 mL). The combined supernatants were concentrated in vacuo. The residue was redissolved in water and reconcentrated four times to remove ammonium acetate. Most remaining salts were removed by precipitation from ethanol. Chromatography on 600 g of silica gel eluting with 10:5:1:3 ethyl acetate–pyridine–acetic acid–water and combination of 22-mL fractions on the basis of TLC gave 4.0 g (45%, corrected for peptide content) of Boc-Arg-Gly-NH_2 acetate, $[\alpha]^{24}_{589}$ −7.67° (*c* 2.92, 1 N acetic acid).

Amino acid analysis showed Arg 1.03, Gly 0.97, Orn 0.01, and NH_3 1.47.[23]

H-Arg-Gly-NH_2 Trifluoroacetate (7). Method A. Boc-Arg-Gly-NH_2 acetate (3.69 g, 9.45 mmol) was dissolved in 15 mL of ice-cold trifluoroacetic acid. After stirring at 15 °C for 15 min, the solution was concentrated in vacuo to give 4.28 g of the corresponding dipeptide.

Amino acid analysis showed Arg 0.92, Gly 1.04, Orn 0.04, and NH_3 1.33.[23]

Method B. Boc-Arg(NO_2)-Gly-NH_2 (4.99 g, 13.3 mmol) was dissolved in 50 mL of liquid HF in the presence of 5 mL of anisole at −78 °C. The temperature was raised to 0 °C for 50 min, after which the HF was removed using aspirator vacuum. The residue was dissolved in water and extracted with chloroform to remove insoluble material. Lyophilization of the aqueous phase gave H-Arg-Gly-$NH_2 \cdot 2HF$ (5.04 g). Conversion to the more soluble trifluoroacetate salt was accomplished by passing an aqueous solution of the product through 50 mL of IRA-400 anion exchange resin (OH^- form) and adding 1 mL of trifluoroacetic acid to the effluent. Lyophilization gave the amorphous salt (6.10 g) which contained less than 1% of impurities as estimated by TLC.[13]

Amino acid analysis of the HF salt showed Arg 1.00, Gly 1.00, and NH_3 1.06.

Boc-Cys(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Arg-Gly-$NH_2 \cdot$HOAc (8). Boc-heptapetide hydrazide (9.1 g, 8.3 mmol), from exposure of ester 4 to hydrazine in methanol, was slurried in 210 mL of degassed DMF under nitrogen at −10 °C. A 4 N solution of HCl in THF (9.3 mL, 37 mmol) was added dropwise, and after several minutes most of the solid had dissolved. The solution was further cooled to −25 to −20 °C, and 1:19 isoamyl nitrite–DMF (64 mL) was added until a positive starch–iodide test was maintained and azide formation was judged complete by TLC. A sample of H-Arg-Gly-NH_2 ditrifluoroacetate (5.1 g, 11.1 mmol) in 25 mL of degassed DMF was added, and the "pH" was adjusted to approximately 8 with 1:1 Et_3N–DMF (12 mL). The reaction mixture was placed in a freezer at −25 °C for 62 h, and additional Et_3N–DMF (3 mL) was added periodically to maintain the "pH" at 8. The reaction mixture was warmed to room temperature, and DMF was removed in vacuo at a bath temperature of 30 °C. Addition of methanol to the residue precipitated a solid (11.5 g). Crystallization of this material by addition of *n*-butyl alcohol to a solution in 10:3:12 pyridine–acetic acid–water gave 8.32 g (76%) of Boc-Cys(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Arg-Gly-$NH_2 \cdot$HOAc: mp 199 °C dec; $[\alpha]^{24}_{589}$ −69.32° (*c* 0.88, 50% HOAc).

Anal. Calcd for $C_{54}H_{87}N_{17}O_{16}S_2 \cdot C_2H_4O_2$: C, 49.66; H, 6.77; N, 17.58; S, 4.73. Found: C, 49.70; H, 6.81; N, 17.69; S, 4.80.

Amino acid analysis showed Arg 0.98, Asp 1.03, Glu 0.99, Pro 1.10, Gly 1.08, Cys 1.98, Ile 0.99, Tyr 1.01, and NH_3 5.09 (separate determination; hydrazine was added to scavenge formaldehyde from hydrolysis of Acm).

Arginine Vasotocin (1). The Boc protecting group was removed from the protected nonapeptide acetate 8 (8.0 g, 6.0 mmol) in HCl–ethyl acetate according to the procedure described for Boc-pentapeptide 2 to provide 8.34 g (>100%) of di-Acm-nonapeptide.

A sample of this compound (3.9 g, 3 mmol) was dissolved in 3.9 L of 4:1 water–methanol under nitrogen. Iodine (2.30 g, 9 mmol) in 150 mL of methanol was added rapidly at room temperature, and the resultant red solution was stirred for 21.5 h. The solution was concentrated in vacuo (bath temperature 30 °C) to a small volume with residual iodine being removed in the process. Chromatography of the crude product on 950 g of silica gel eluting with 50:20:6:24 *n*-butyl alcohol–pyridine–acetic acid–water and combining 25-mL fractions based on TLC gave 1.57 g of material of at least 90% purity by TLC.[13] Preparative free flow electrophoresis of a 1.1-g portion in 33% acetic acid yielded 514 mg (22%) of arginine vasotocin diacetate tetrahydrate, $[\alpha]^{24}_{589}$ −5.45° (*c* 0.55, 1 N acetic acid) [lit.[3] $[\alpha]^{20}_{589}$ −5° (*c* 2, 1 N acetic acid)].

Anal. Calcd for $C_{43}H_{67}N_{15}O_{12}S_2 \cdot 2C_2H_4O_2 + 4H_2O$: C, 45.44; H, 6.73; N, 16.91; S, 5.16. Found: C, 45.71; H, 6.59; N, 17.07; S, 5.13.

Amino acid analysis showed Arg 1.05, Asp 1.07, Glu 1.05, Pro 0.99, Gly 1.03, Ile 0.97, Tyr 0.95, Lys 1.90, and NH_3 2.99.

Boc-Phe-Met-Arg-Phe-OCH_3 Acetate (11). A solid phase synthesis on the Beckman 990 synthesizer according to a procedure similar to that described for compound 2 followed by base-catalyzed

transesterification provided crude Boc-Phe-Met-Arg(NO_2)-Phe-OCH_3 (**10**) in quantitative yield.

A sample of compound **10** (1.52 g, 2 mmol) in 32 mL of methanol under nitrogen was treated with a freshly prepared buffered solution of $TiCl_3$ consisting of 8 mL (10.4 mmol) of 20% aqueous $TiCl_3$ and 18 mL (72 mmol) of 4 M aqueous ammonium acetate. After 45 min, 3.5 mL of dimethyl sulfoxide was added to oxidize excess $TiCl_3$. When the purple color was totally discharged (3.5 h), the mixture was centrifuged at 5000 rpm. The supernatant was decanted, and the solid titanium oxides were washed with methanol. The combined supernatants were concentrated in vacuo. Most remaining ammonium salts were precipitated by addition of isopropyl alcohol. Filtration and concentration in vacuo gave a crude product which was purified by preparative TLC on Quantum Q-1 silica gel plates (1000 μm) using 15:5:1:2 ethyl acetate–pyridine–acetic acid–water as developing solvent. Elution of the major band with 1:1 methanol–methylene chloride gave 1.2 g (77%) of Boc-Phe-Met-Arg-Phe-OCH_3 acetate: mp 210 °C dec; $[\alpha]^{24}{}_{589}$ −14.8° (*c* 0.40, methanol); IR (Nujol) 1740 cm^{-1} (ester carbonyl).

Amino acid analysis showed Phe 2.18, Met 0.86, Arg 0.96, and NH_3 0.19.

Boc-Phe-Met-Arg-Phe-NH_2 Acetate (12). A solution of tetrapeptide ester **11** (1.0 g, 1.3 mmol) in 50 mL of methanol in a pressure bottle was *saturated* at 0 °C *with ammonia. The bottle was sealed,* and the solution was stirred at room temperature for 24 h. Concentration of the solution in vacuo and precipitation of the residue from methanol–ether gave 710 mg (73%) of amorphous Boc-Phe-Met-Arg-Phe-NH_2 acetate: $[\alpha]^{24}{}_{589}$ −14.1° (*c* 1.0, methanol); IR (Nujol) 1645 cm^{-1} (no ester carbonyl).

Amino acid analysis showed Phe 2.06, Met 0.94, Arg 1.00, and NH_3 1.14.

H-Phe-Met-Arg-Phe-NH_2·$(HCl)_x$ (9). Protected tetrapeptide amide **12** was deprotected with HCl–ethyl acetate as described for pentapeptide **2**. The crude product (330 mg, 79%) was purified by preparative TLC on Quantum Q-1 silica gel plates using 10:5:1:3 ethyl acetate–pyridine–acetic acid–water as both developing and eluting solvents. The eluted solution was concentrated in vacuo to a film, triturated with ethyl acetate to remove pyridinium acetate, and freeze-dried from water to the hygroscopic solid H-Phe-Met-Arg-Phe-NH_2·$(HCl)_x$ (98 mg, 24%), $[\alpha]^{24}{}_{589}$ −4.4° (*c* 0.25, methanol).

Amino acid analysis showed Phe 2.00, Met 0.97, Arg 1.03, and NH_3 2.31.[23]

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Registry No.—1, 113-80-4; **2**, 67844-91-1; **3**, 67844-92-1; **4**, 67844-93-3; **6**, 67844-95-5; **7** $2CF_3CO_2H$, 67844-96-6; **7** 2HF, 67844-97-7; **8**, 67844-99-9; **9** 2HCl, 67872-50-8; **11**, 67845-01-6; **12**, 67845-03-8; Ile-Gln-Asn-Cys(Acm)-Pro-OMe, 67872-49-5; Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-OMe, 67845-04-9; Boc-Cys(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-NH-NH_2, 67845-05-0; Cys(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Arg-Gly-NH_2, 67845-06-1; Boc-tyrosine hydroxysuccinimide ester, 20866-56-2; N^{α}-Boc-*S*-Acm-cysteine, 19746-37-3.

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