An additional amount (0.33 g) of crude **22** was isolated from the alcohol filtrates.

Ethyl *N*-[7-(2-Benzylidene-1-methylhydrazino)-3*H*-imidazo[4,5-*b*]pyridin-5-yl]carbamate (23). A solution of **29** (1.18 g, 3.30 mmol) in a 10:3 mixture of EtOH and DMAC (65 mL) containing Raney nickel (3 g, weighed wet, washed with H_2O and EtOH) was hydrogenated at room temperature and atmospheric pressure for 75 min. The catalyst was removed by filtration (Celite) and washed with DMAC (5 mL). The combined filtrate and wash was evaporated to dryness in vacuo and crude **30**, a colored oil (1.57 g), was dissolved in $(EtO)_3CH$ (10 mL). This solution was treated with concentrated HCl (0.3 mL) and the resulting mixture was stirred at room temperature for 16 h. The mixture was diluted with Et_2O, and the solid was collected by filtration and recrystallized from 2-propanol (900 mL): yield, 535 mg.

Ethyl *N*-[1,4-Diamino-2-(benzylthio)imidazo[4,5-*c*]pyridin-6-yl]carbamate (24). A solution of **15** (1.00 g, ~3.23 mmol)[12] in H_2O (50 mL) and 1 N NaOH (3.25 mL) containing excess benzyl chloride (0.415 mL) was stirred at room temperature for 1.5 h. The precipitate was collected by filtration and washed with H_2O (50 mL) and Et_2O (40 mL): yield, 1.05 g.

Ethyl *N*-[1,4-Diamino-2-[(4-methoxybenzyl)thio]imidazo[4,5-*c*]pyridin-6-yl]carbamate (25). A solution of **15** (400 mg, ~1.49 mmol)[12] in H_2O (25 mL) and 1 N NaOH (1.5 mL) containing excess 4-methoxybenzyl chloride (0.22 mL) was stirred at room temperature for 1.5 h to deposit a mixture of **15** and **25**. The precipitate (426 mg) was retreated as described above and the product was washed with 0.02 N NaOH (15 mL) and recrystallized from propanol: yield, 174 mg.

Ethyl *N*-[1,4-Diamino-2-[[3,4-(methylenedioxy)benzyl]thio]imidazo[4,5-*c*]pyridin-6-yl]carbamate (26). To a solution of **15** (388 mg, ~1.45 mmol)[12] in H_2O (25 mL) and 1 N NaOH (1.5 mL) was added portionwise with stirring a solution of 3,4-(methylenedioxy)benzyl bromide (500 mg, 2.33 mmol)[13] in dioxane (5 mL). After 1.5 h, the acidic mixture was treated with 1 N NaOH (1.5 mL), and the product was collected by filtration and recrystallized from propanol: yield, 289 mg.

Ethyl *N*-[6-Amino-4-(1-methylhydrazino)-5-nitropyridin-2-yl]carbamate (28). Methylhydrazine (7.80 g, 170 mmol) was added with stirring to a hot suspension of **27** (4.90 g, 18.8 mmol) in EtOH (150 mL). The clear solution was cooled and the precipitate was collected by filtration: yield, 4.54 g.

Ethyl *N*-[6-Amino-4-(2-benzylidene-1-methylhydrazino)-5-nitropyridin-2-yl]carbamate (29). A solution of **28** (939 mg, 3.48 mmol) in DMAC (10 mL) containing benzaldehyde (397 mg, 3.75 mmol) was stirred at room temperature for 16 h. After dilution with H_2O (40 mL), the product was collected by filtration, and washed with H_2O: yield, 1.18 g.

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Registry No. **4**, 109182-40-3; **5**, 123753-53-7; **6**, 123753-54-8; **7**, 123753-55-9; **8**, 123753-56-0; **9**, 123753-57-1; **10**, 123753-58-2; **10**·XHCl, 123753-74-2; **11**, 123753-59-3; **12**, 123753-60-6; **12**·DMAC, 123775-15-5; **13**, 37660-66-5; **14**, 123753-61-7; **15**, 123753-62-8; **16**, 123753-63-9; **17**, 123753-64-0; **19**, 37436-94-5; **20**, 123753-65-1; **21**, 123775-16-6; **22**, 123753-66-2; **23**, 123753-67-3; **24**, 123753-68-4; **25**, 123753-69-5; **26**, 123753-70-8; **27**, 6506-86-1; **28**, 123753-71-9; **29**, 123753-72-0; **30**, 123753-73-1; PhCHO, 100-52-7; *o*-$MeOC_6H_4CHO$, 135-02-4; *p*-$MeOC_6H_4CH_2Cl$, 824-94-2; 3,4-$(OCH_2O)C_6H_3CH_2Br$, 2606-51-1; $MeNHNH_2$, 60-34-4; ethyl *N*-(4,5,6-triaminopyridin-2-yl)carbamate, 123753-52-6; piperonal, 120-57-0.

(12) This sample of **12** was homogeneous by TLC, but the degree of solvation was unknown.

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Synthesis and Biological Activity of Atrial Natriuretic Factor Analogues: Effect of Modifications to the Disulfide Bridge

John DiMaio,*,† Jorge Jaramillo, Dominik Wernic, Louis Grenier, Ewald Welchner, and Julian Adams

Bio Mega Laboratories, 2100 Cunard, Laval, Quebec, Canada H7S 2G5. *Received January 13, 1989*

A series of atrial natriuretic factor (ANF) analogues with modifications to the disulfide bridge and lacking the exocyclic N-terminal sequence was synthesized. The native cystine residue was substituted by isofunctional deamino carba, β,β-dimethyl carba and dehydro dicarba spanners that bridge residues 106 and 120. The compounds were prepared by segment condensation coupling using the base-labile (9-fluorenylmethyl)carboxyl protecting group. Biological evaluation revealed that the exocyclic N-terminal segment of ANF is not necessary for expression of high biological activity. The compounds retained high affinity for ANF receptors in bovine adrenal zona glomerulosa cells and were found to be potent antihypertensive and diuretic agents, indicating that the native disulfide bridge can be mimicked by isosteric spanning residues. It was noted that the reported analogues, unlike the endogenous hormone, show marked reduced inhibitory activity on PGE_1-stimulated aldosterone secretion from adrenal zona glomerulosa cells. This lack of inhibition may be a contributing element to the low saluresis in spite of the high level of diuresis observed with some analogues.

Atrial natriuretic factor (ANF) is an important regulatory hormone secreted by atrial myocytes[1] whose role as an endocrine, renal, and hemodynamic modulator renders it therapeutically attractive.[2,3] The pharmacological activity of ANF in normal and pathologic states has been the subject of considerable investigation. Its peripheral effects oppose the renin–angiotensin–aldosterone system[4] and indicate a primary role in the homeostatic regulation of extracellular fluid volume and electrolyte excretion. Ac-

† Present address: Biotechnology Research Institute of Montreal, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2.

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Chart I. Primary Amino Acid Sequence of Rat (a) and Human (b) Circulating Atrial Natriuretic Factor

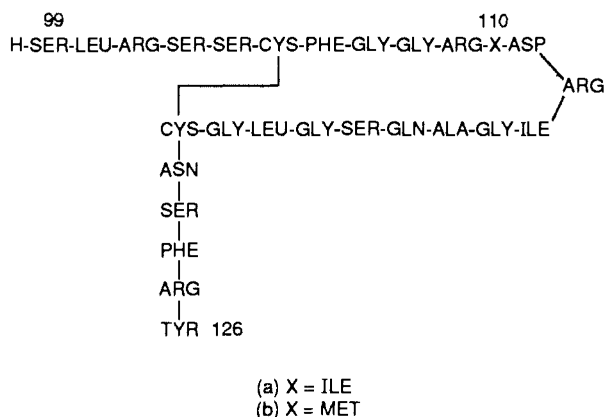
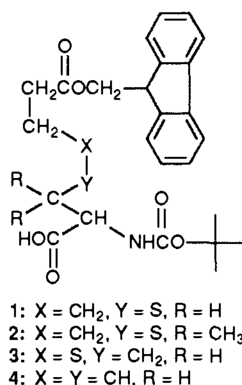


Chart II. Orthogonally Protected Spanning Residues Used to Bridge Positions 106 and 121 in the Reported ANF Analogues



cordingly, it has been shown that ANF is a powerful diuretic and natriuretic agent[5] that inhibits renin secretion[6] and angiotensin II (AII) stimulated aldosterone release in vitro.[7] In addition, the hormone is a potent vasodilator and relaxes precontracted vascular tissue by an endothelium-independent mechanism.

The primary structure of ANF is highly conserved among various species. Circulating human ANF (h-ANF) comprises the amino acid sequence 99–126 of the prohormone and differs from the rat sequence by a single residue in position 110 (Chart I). Early investigations indicated that an intact disulfide bridge is essential for expression of biological activity.[8] On the other hand, the native disulfide may be a potential source of lability contributing to the rapid clearance of the native hormone through disulfide interchange reactions with serum proteins. In addition, previous studies have shown that the disulfide bridge of other cyclic peptide hormones such as vasopressin, oxytocin, or somatostatin is not required for expression of high biological activity and that isofunctional replacements are tolerated.[9,10]

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Scheme I

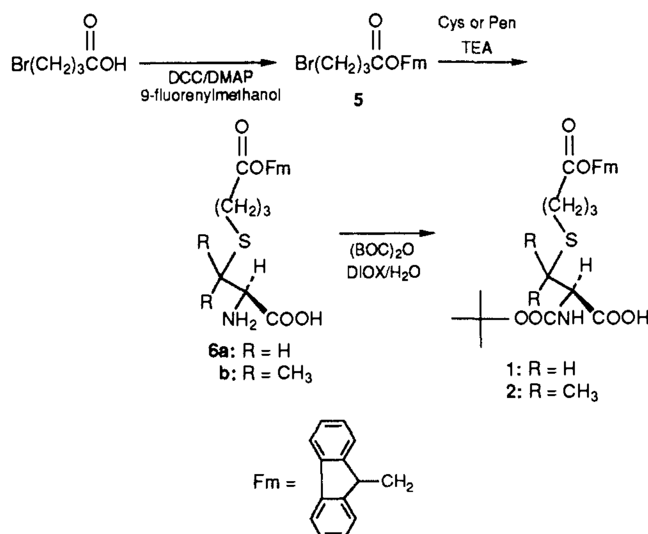
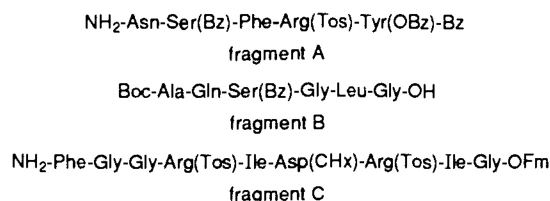


Chart III. Side Chain Protected Fragments Synthesized for Segment Condensation Coupling



Given these considerations, we investigated the functional role of the disulfide bridge of rat ANF (r-ANF) and report the synthesis and biological activity of a series of analogues with modified bridges and lacking the N-terminal exocyclic sequence.

Chemistry

Synthesis of Spanners. The variable spanners were secured by synthesizing the orthogonally protected carba (1–3) and dehydro dicarba (4) spanner analogues (Chart II) of the native cystine residue. The synthesis of the bridging residues **1** and **2** is shown in Scheme I. Reaction of 4-bromobutyric acid with 9-fluorenylmethanol in the presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) afforded 9-fluorenylmethyl ester **5**. The bromo ester was reacted with either L-cysteine or L-penicillamine in aqueous ethanol providing the thioether adducts **6a** and **6b**, respectively. Reaction with di-*tert*-butyl dicarbonate afforded the respective N^{α}-BOC protected spanners **2** and **4**.

For carba residue **3** the required 3-bromopropionic 9-fluorenylmethyl ester was obtained by acid-catalyzed esterification in benzene at reflux. Subsequently L-homocysteine was first reduced with Na in liquid NH_3 and then reacted with the 3-bromo ester as described above.

The synthesis of N^{α}-BOC L-γ,δ-dehydro-α-aminosuberic acid ω-9-fluorenylmethyl ester (spanner **4**) and its dehydro congener are reported elsewhere.[11]

Synthesis of Fragments. The necessary fragments required for segment condensation synthesis of the reported analogues are shown in Chart III. Fragment A served as starting material for N-terminal extention in

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Table I. Binding of r-ANF Analogues to Zona Glomerulosa Cells B Receptor and Mouse Fibroblast Cells C Receptor Using [^{125}I]r-ANF(99–126)

compound	IC_{50},[a] nM B receptor	IC_{50},[a] nM C receptor
8, r-ANF(99–126)	0.043 ± 0.004	0.13 ± 0.03
9, r-ANF(103–126)	1.37 ± 0.06	0.35 ± 0.14
10, r-ANF(105–126)	3.60 ± 0.35	0.50 ± 0.04
11, deamino[Mpr^{105},Cys^{121}][b]r-ANF(105–126)	1.03 ± 0.25	0.22 ± 0.03
12, deamino[carba^{105},Pen^{121}]r-ANF(105–126)	7.80 ± 0.92	0.35 ± 0.14
13, deamino[carba^{105},Cys^{121}]r-ANF(105–126) methyl ester	8.67 ± 0.58	0.40 ± 0.19
14, deamino[carba^{105},Cys^{121}]r-ANF(105–126)	6.17 ± 1.90	0.56 ± 0.16
15, deamino[carba^{121}]r-ANF(105–126)	7.80 ± 2.08	0.21 ± 0.07
16, deamino[dicarba^{105,121}][c]r-ANF(105–126)	28.77 ± 2.30	0.78 ± 0.09
17, deamino[γ,δ-dehydrodicarba^{105,121}]r-ANF(105–126)	51.00 ± 16	0.63 ± 0.20

[a] Mean of three determinations ± SEM [b] Reference 20. [c] Reference 21.

order to incorporate spanning residues (1–4). The preparation of the fragments followed standard solution methods using in most cases either diphenyl phosphorazidate (DPPA) or dicyclohexylcarbodiimide/hydroxybenzotriazole (DCC/HOBT) mediated coupling of appropriately side-chain protected L-N^{α}-BOC amino acids. Side chain functional groups were protected as follows: Arg, tosyl; Asp, cyclohexyl; Ser, benzyl; Tyr, benzyl. The fragments were characterized (see the Experimental Section) by TLC, HPLC, amino acid analysis, and FAB mass spectrometry.

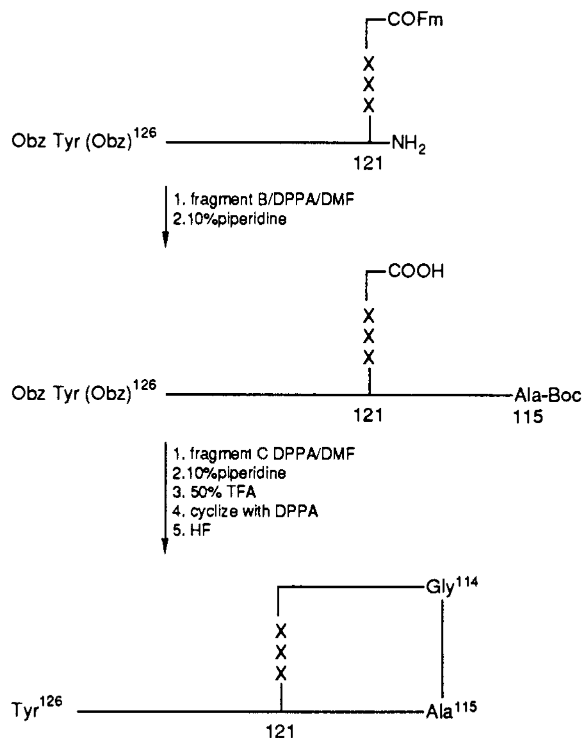
In all cases fragment condensations were mediated by DPPA in dimethyl formamide (DMF) at 4 °C. Segment couplings were monitored by HPLC and were usually complete after 15 h. Scheme II shows the sequence of segment condensations. The base-labile 9-fluorenylmethyl group[12,13] served as an excellent carboxyl protecting group which could be effectively removed in the presence of other ester-protecting functions with 10% piperidine in DMF at 0 °C. The N^{α}-BOC group was removed with 50% trifluoroacetic acid (TFA) in CH_2Cl_2. Intramolecular cyclization was affected between Gly^{114} and Ala^{115} with DPPA and was found to be spontaneous and quantitative in all cases.

Side chain protecting groups were removed by standard methods using hydrogen fluoride and the crude products were obtained as lyophilized powders. The products were purified to homogeneity by a two-step procedure using medium-pressure reverse-phase chromatography on Vydac 30 Å or Vydac 10-15 Å C_{18} silica. Product purity (minimum 95%) was assessed by analytical HPLC at 215 nm and TLC. Final compounds were also subjected to amino acid analysis and FAB mass spectrometry for characterization.

Biology

The reported analogues were evaluated for their ability to bind to ANF receptors in bovine adrenal zona glomerulosa cells[14] and mouse fibroblast cells.[15] The former has been shown to have predominantly B receptors, which are coupled to particulate guanylate cyclase.[16] Cultured mouse fibroblast cells (NIH 3T3) have been reported to contain exclusively clearance or C receptors.[17]

Scheme II



In vitro vasorelaxation was assessed by the capacity of the reported analogues to antagonize the phenylephrine-induced contractions of superfused rabbit thoracic aortic rings. Antihypertensive activity was evaluated in deoxycorticosterone (DOCA)–saline hypertensive rats[18] and 5/6 nephrectomized rats.[19] Diuretic and saluretic activity was measured in conscious normotensive rats. Aldosterone inhibition was determined by the method of DeLean et al.[14] in cultured bovine adrenal zona glomerulosa cells.

The biological activities of the bridge-modified analogues were compared to the circulating hormone r-ANF(99–126) and N-terminal truncated fragments r-ANF(103–126) [APIII] and r-ANF(105–126). In addition, we also evaluated two recently reported analogues as controls: deamino[Mpr^{105},Cys^{121}]r-ANF(105–126)[20] and deamino-[Asu^{105,121}]r-ANF(105–126).[21] Both analogues lack the

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Table II. Potencies of ANF Analogues in the Rabbit Aorta Assay and Antihypertensive Activity in DOCA and 5/6 Nephrectomized Rats

	rabbit aorta:[a] EC_{50}, mol/50 μL	antihypertensive activity[b] DOCA ED_{50}	DOCA corr coeff[c]	5/6 ED_{50}	5/6 corr coeff
8	3.8 ± 0.30 × 10^{-11}	0.10	0.994	0.14	0.988
9	8.9 ± 0.05 × 10^{-11}	0.16	0.999	0.13	0.966
10	3.1 ± 0.10 × 10^{-10}	0.10	0.968		
11	1.1 ± 0.20 × 10^{-10}	1.10	0.974	1.50	0.988
12	6.8 ± 0.70 × 10^{-10}	0.14	0.967	0.11	0.966
13	1.4 ± 0.10 × 10^{-10}	>4		>2	
14	2.6 ± 0.50 × 10^{-10}	0.72	0.984	1.10	0.999
15	3.5 ± 0.30 × 10^{-10}	0.83	0.977	1.20	0.973
16	4.8 ± 0.80 × 10^{-10}			5.70	0.993
17	5.5 ± 0.70 × 10^{-10}			1.20	0.997

[a] Superfused aortic rings precontracted with phenylephrine; doses are based on peptide content; mean of three determinations ± SEM. [b] ED_{50} values are expressed as nmol/kg per min; average of six experimental animals. [c] Correlation coefficient.

Table III. Diuretic and Saluretic Activity of ANF Analogues in Conscious Normotensive Rats

	ratio of urine volume:[a] treated/control	electrolyte excretion: treated − control, μmol/sample Na^+	K^+	Cl^-
8	3.1	57.4	16.2	72.4
9	2.7	79.6	23.0	98.6
10	2.2	15.9	12.9	28.5
11	1.7	30.4	22.6	52.9
12	3.0	17.9	24.8	39.6
13	3.4	38.1	23.5	52.1
14	2.8	34.0	10.2	41.1
15	1.7	7.2	3.5	15.9
16	2.1	4.8	0	10.0
17	2.5	0.4	3.2	7.3

[a] Ratio of volumes for treated over control animals; volume in a 10-min sample and average of three consecutive samples in six experimental animals; all compounds were infused at a dose of 0.5 μg/kg per min.

N-terminal exocyclic segment present in r-ANF(99–126) and, in the latter, the disulfide bridge is replaced by an ethylene unit.

Results and Discussion

Table I shows the results of the receptor binding based on the displacement of [^{125}I]r-ANF(99–126) from binding sites on bovine adrenal zona glomerulosa cell membranes and mouse fibroblast (NIH 3T3) cells. The data demonstrates that systematic deletion of the exocyclic N-terminal residues causes up to 100-fold reduction in affinity to receptor sites on adrenal zona glomerulosa cells in agreement with the results of Thibault et al.[22] Removal of the N-terminal amino group from r-ANF(105–126) affords deamino[Mpr^{105},Cys^{121}]r-ANF(105–126) (**11**) reported by Schiller et al.,[20] which, in our hands, exhibits receptor affinity comparable to that of ANF(103–126) or APIII. Modification of the disulfide bridge as exemplified by the deamino carba analogues **12–15** causes a further uniform drop in affinity on the B receptor binding assay. Replacement of the cystine residue in r-ANF(105–126) by L-α-aminosuberic acid[21] or dehydro-L-α-aminosuberic acid (**16** and **17**, respectively) affords analogues with even greater reduced receptor affinity. It has been reported that ANF binds to a heterogeneous population of receptors.[23] Bovine adrenal zona glomerulosa cells have predominantly B receptors (guanylate cyclase coupled) while receptor sites on mouse fibroblast cells are predominantly of the C type (clearance receptors). Indeed the data in Table I demonstrates that while modifications to the disulfide bridge causes up to a 1000-fold reduction in affinity to B receptors, binding to C receptors is only moderately affected and supports the hypothesis that B and C receptors have different structural requirements.[23] Clearly the requirements of the C receptor are independent of the disulfide moiety and bridging residue.

In the bioassay based on the inhibition of the stimulated contractions of the superfused rabbit thoracic aortic rings, all the reported compounds exhibited uniform reduced potency compared to that of the circulating hormone r-ANF(99–126) (Table II). They were found to be equipotent to the dicarba analogue [Asu^{105,121}]r-ANF(105–126) (**16**) reported by Chino et al.[21] and to deamino-[Mpr^{105},Cys^{121}]r-ANF(105–126)[20] (**11**).

Intravenous infusion of the modified-bridge analogues lowered blood pressure in a dose-dependent manner in both DOCA and 5/6 nephrectomized rats (Table II). The reduced vasorelaxant activity in the in vitro bioassay is in agreement with the potency decrease in the in vivo antihypertensive assay. This effect can be attributed directly to modifications to the disulfide bridge since N-terminal deletions alone [r-ANF(105–126)] do not affect antihypertensive activity. The bridge-modified analogue deamino[carba^{105},Pen^{121}]r-ANF(105–126) (**12**) represents an exception, being equipotent to the endogenous hormone r-ANF(99–126) (**8**) while exhibiting decreased potency on the in vitro bioassay and also lowered receptor affinity.

It is difficult to explain the observed discrepancies between the reduced receptor binding (which varies between 1 and 3 orders of magnitude for the synthetic analogues) on one hand and the uniform moderate drop in vasorelaxant activity in vitro and antihypertensive activity in vivo. The observation that N-terminal deleted analogues r-ANF(103–126) (**9**) and r-ANF(105–126) (**10**) are equipotent antihypertensive agents compared to r-ANF(99–126) (Table III) could indicate that the exocyclic N-terminal sequence contributes more to accessory binding than to receptor activation. However, since the closely related disulfide [Mpr^{105},Cys^{121}]r-ANF(105–126) exhibits a moderate 20-fold reduction in receptor binding, it follows that modifications to the disulfide bridge are detrimental to receptor binding but maintain efficacy, an effect previously observed in other hormonal systems.[28] The analogue deamino[carba^{105},Pen^{121}]r-ANF(105–126) (**12**) exemplifies this property.

All of the modified-bridge analogues including the previously reported compounds **11** and **16** were found to be potent diuretics (Table II), increasing urine volume

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excretion from 2-fold to approximately 4-fold over control values. Interestingly electrolyte excretion was substantially reduced compared to the native hormone r-ANF **8** or its N-terminal deleted analogue **9** [APIII]. Most notable is the deamino[dehydro-Asu[105,121]]r-ANF(105–126) analogue **17**, which was devoid of saluretic activity despite the high level of diuresis at a typical dose of 0.5 μg/kg per min. The data also reveals that the carba[121] analogue is also weakly saluretic compared to its carba[105] counterpart albeit with concomitant lowered diuretic activity. The renal mechanisms of ANF-induced natriuresis are poorly understood. It has been postulated that natriuresis could be due to increased glomerular filtration rate (GFR) or inhibition of sodium reabsorption in the distal tubule.[29] Our results on the bioassay based on the inhibition of PGE_1-stimulated secretion of aldosterone in cultured zona glomerulosa cells some of the modified-bridge analogues were inactive up to a concentration of 10^{-7} M whereas r-ANF(103–126) has an $IC_{50} = 1.0 \times 10^{-9}$ M.[17] This observation suggests that a mechanism based on suppression of aldosterone secretion may contribute to the saluretic properties of ANF and that an intact disulfide bridge is required for maximal effect.

It has been reported that left atrial distention causes a decrease in plasma arginine vasopressin (AVP) concentration.[30] Since the present observations do not rule out a mechanism involving inhibition of AVP secretion we are conducting experiments to measure immunoreactive AVP during the infusion periods.

It has been previously demonstrated that the disulfide bridge in h-ANF is essential for expression of full biological activity since reduced and carboxylated derivatives were inactive.[8] We have found that modifications to the native disulfide bridge exemplified by deamino carba or dehydro dicarba analogues confers quantitative potency shifts in receptor binding, in vitro vasorelaxation, and antihypertensive activity. In addition, qualitative changes in the biological profile are also observed; notably the saluretic activity is markedly reduced without affecting the level of diuresis. This property is also reflected by a decreased capacity to inhibit stimulated aldosterone secretion. In vivo the analogue deamino[carba[105],Pen[121]]r-ANF(105–126) exhibited antihypertensive activity comparable to that of the native hormone r-ANF(99–126) while receptor binding and vasorelaxant activity were decreased. Studies are currently ongoing in our laboratories to further characterize the pharmacological mechanisms of vasorelaxation, diuresis, and saluresis with some of the analogue reported.

Experimental Section

Melting points were determined on a Büchi 510 melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Brucker AC 200 MHZ spectrophotometer; chemical shifts are reported in ppm (δ) downfield from Me_4Si. Analytical HPLC experiments were performed on a Waters LC instrument equipped with a variable-wavelength detector and μ-Bondapak C_{18} column (4.5 mm × 30 cm). For amino acid analysis (AAA), peptides were hydrolyzed in 6 N HCl at 110 °C for 20 h. The hydrolyzates were analyzed on a Waters LC apparatus equipped with an ion-exchange column, postcolumn derivatizer using *O*-phthaldehyde, and a Model 4200AC fluorescence detector.

Elemental analyses were performed by the analytical department of Bio Mega. For TLC, precoated plates 5 × 10 cm (Kieselgel 60 250 μm Merk Darmstadt) were used in the following solvent systems (v/v): *n*-BuOH/AcOH/H_2O (BAW, 4:1:5, organic phase) and *n*-BuOH/pyridine/AcOH/H_2O (BPAW, 15:10:3:12). FAB mass spectra were measured at low resolution on a KRATOS MS-50TCT mass spectrometer provided by the Centre Regionale de Spectrometrie de Masse of the University of Montreal.

The analytical data for the side chain protected fragments A–C are as follows: HPLC conditions (A/B 0.05% TFA/CH_3CN) for the fragments were either (I) 50–90% B over 20 min or (II) 40–90% B over 20 min. Fragment A: TLC R_f (BAW) 0.70; AAA Asn 1.11, Ser 0.96, Phe 1.07, Arg 0.93, Tyr 0.92; FABMS *m/e* 1111 $(M + H)^+$; HPLC t_R = 10.9 min (I). Fragment B: TLC R_f (BAW) 0.45; AAA Ala 1.00, Gln 1.03, Ser 0.89, Gly 2.21, Leu 1.03; FABMS *m/e* 723 $(M + H)^+$; HPLC t_R = 10.2 min (II). Fragment C: TLC R_f (BAW) 0.65; AAA Asp 1.01, Gly 3.16, Ile 1.90, Arg 1.83, Phe 0.98; FABMS *m/e* 1756 $[M + 2H]^+$; HPLC t_R = 11.5 min (I). Deamino[Mpr[105],Cys[121]]r-ANF(105–126) was synthesized as described[20] except that the thiols were protected with an acetamidomethyl group and oxidative cyclization was performed with I_2 in AcOH.[27]

All final compounds were at least 95% pure based on UV absorption at 215 μm by analytical HPLC.

3-Bromopropionic Acid 9-Fluorenylmethyl Ester. 9-Fluorenylmethanol (1.77 g, 9 mmol) and 1.53 g (10 mmol) of 3-bromopropionic acid were dissolved in 50 mL of benzene. The solution was heated under reflux in the presence of a trace of *p*-toluenesulfonic acid while removing water with a Dean–Stark trap. After 2 h the solution was cooled to room temperature and then extracted with 3 × 20 mL of 1 M $NaHCO_3$ and 3 × 20 mL of H_2O. The solution was dried over $MgSO_4$ and filtered. The filtrate was evaporated to dryness in vacuo. The residue was recrystallized from ethanol, filtered, and dried over P_2O_5: yield 2.35 g (79%); mp 86–87 °C; NMR ($CDCl_3$) δ 3.0 (t, 2 H, CH_2C), 3.6 (t, 2 H, CH_2Br), 4.3 (t, 1 H, CH), 4.5 (d, 2 H, CH_2O), 7.2–7.8 (m, 8 H, aromatic). Anal. ($C_{17}H_{15}BrO_2$) found 61.30% C, 4.49% H.

4-Bromobutyric Acid 9-Fluorenylmethyl Ester. To a solution of 4-bromobutyric acid (5.0 g, 29.9 mmol), 4-dimethylaminopyridine (353 mg, 2.9 mmol), and 9-fluorenylmethanol (5.7 g, 25 mmol) in 150 mL of CH_2Cl_2 at 0 °C was added DCC (6 g, 29 mmol). The mixture was stirred at room temperature for 2 h, after which the precipitated urea was filtered over Celite. The filtrate was extracted with 50 mL of 1 N HCl, 2 × 50 mL of 1 M $NaHCO_3$, and H_2O. The organic phase was dried over $MgSO_4$ and filtered. The filtrate was evaporated to dryness in vacuum and the residue was recrystallized from ethanol. The solid was filtered and dried over P_2O_5: yield 7.1 g (73%); mp 47–49 °C; NMR ($CDCl_3$) δ 2.2 (m, 2 H, $-CH_2-$), 2.6 (t, 2 H, $CH_2C{=}O$), 3.4 (t, 2 H, CH_2Br), 4.2 (t, 1 H, CH aromatic), 4.4 (d, 2 H, CH_2O), 7.3–7.8 (m, 8 H, aromatic). Anal. ($C_{18}H_{17}O_2Br$) found 62.32% C, 4.89% H.

***S*-[4-(9-Fluorenylmethoxy)-4-oxobutyl]-L-cysteine (6a).** 4-bromobutanoic acid 9-fluorenylmethyl ester (3 g, 8.7 mmol) was added to a solution of L-cysteine (1 g, 8.3 mmol) and DIEA (4.4 mL, 24.7 mmol) in 100 mL of 1:1 EtOH/H_2O. The mixture was stirred vigorously for 18 h at room temperature and neutralized with 1 N HCl (pH 6). The precipitate was filtered and washed extensively with water and EtOH. The solid was recrystallized from aqueous EtOH, affording 1.2 g of a solid (32%): mp 164–167 °C dec; NMR (DMSO) δ 1.6–1.8 (m, 2 H, $-CH_2-$), 2.3–2.5 (m, 4 H, CH_2SCH_2), 2.6–3 (m, 2 H, $CH_2C{=}O$), 3.4 (m, 1 H, CH), 4.3 (t, 1 H, CH aromatic), 4.4 (d, 2 H, CH_2O), 7.2–8.0 (m, 8 H, aromatic); MS *m/e* 408 M^+ + Na. Anal. ($C_{21}H_{23}NO_4S$) found 62.53% C, 6.01% H, 3.43% N.

***N*-(*tert*-Butyloxycarbonyl)-*S*-[4-(9-fluorenylmethoxy)-4-oxobutyl]-L-cysteine (1).** Di-*tert*-butyl dicarbonate (2.2 g, 10 mmol) was added to a solution of **6a** (1.9 g, 4.9 mmol) in 100 mL of 50% aqueous dioxane containing DIEA (2.6 mL). The mixture was stirred at room temperature for 5 h and then diluted with H_2O (150 mL) and extracted three times with 50-mL portions of ether. The aqueous phase was made acidic with solid citric acid and extracted with EtOAc. The combined organic extracts were washed with 1 N HCl and H_2O, dried over $MgSO_4$, and evaporated under vacuum, affording 1.6 g of an oil (66%), which was used without further purification: NMR ($CDCl_3$) δ 1.4 (s, 9 H, $(CH_3)_3CO$), 1.8–2.2 (m, 2 H, $-CH_2-$), 2.5–2.8 (m, 6 H, CH_2SCH_2, $CH_2C{=}O$), 4.2 (t, 1 H, CH), 4.4 (b, 3 H, CH_2O, CH aromatic), 5.2 (b, 1 H, NH), 7.2–7.8 (m, 8 H, aromatic); MS *m/e* 486 $(M + H)^+$.

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N-(*tert*-Butyloxycarbonyl)-S-[3-(9-fluorenylmethoxy)-3-oxopropyl]-L-homocysteine (3). L-Homocystine (1 g, 3.7 mmol) was dissolved in 100 mL of liquid ammonia. The solution was treated with sodium chips until a blue color persisted for 30 s. The ammonia was evaporated and the solid residue was dried under high vacuum over P_2O_5. The solid mass was redissolved in 50 mL of degassed H_2O and the pH of the solution was adjusted to 3 with 1 N HCl followed by DIEA addition to pH 9. 3-Bromopropionic acid 9-fluorenylmethyl ester (2.6 g, 8 mmol) in 50 mL of dioxane was added and the resulting solution was stirred at room temperature for 3 h. The solution was made acidic with HCl, concentrated to a volume of 50 mL, and filtered over Celite. The filtrate was diluted with H_2O and extracted with ether. The aqueous phase was adjusted to pH 6 with DIEA and the solution stored at 4 °C. The resulting precipitate was collected by filtration and washed with 2-propanol. The solid was recrystallized from aqueous EtOH, affording 670 mg of a white solid that was dried over P_2O_5 (25%): mp 117–120 °C dec; NMR (DMSO d_6) δ 1.8–2.1 (m, 2 H, $-CH_2-$), 2.5–2.7 (m, 6 H, CH_2SCH_2, $CH_2C{=}O$), 3.3 (t, 1 H, CH), 4.3 (t, 1 H, CH aromatic), 4.4 (d, 2 H, CH_2O) 7.2–7.8 (m, 8 H, aromatic); MS m/e 386 $(M + H)^+$. Anal. ($C_{21}H_{23}NO_4S$) found 60.50% C, 5.94% H, 3.43% N.

The above compound (710 mg, 1.8 mmol) was suspended in 40 mL of 50% aqueous dioxane in an ice bath and treated with 0.9 mL of DIEA and di-*tert*-butyl dicarbonate (1.2 g, 5.5 mmol). The solution was stirred at 4 °C overnight and concentrated to a volume of 20 mL under high vacuum. The concentrate was diluted with H_2O and extracted with ether. The aqueous phase was acidified with solid citric acid and extracted with EtOAc. The combined organic extracts were dried over Na_2SO_4 and evaporated in vacuo, affording 435 mg (50%) of an oil sufficiently pure for subsequent reaction: NMR ($CDCl_3$) δ 1.5 (s, 9 H, $(CH_3)_3$), 1.9 (m, 2 H, $-CH_2-$), 2.5 (m, 4 H, CH_2SCH_2), 2.9 (m, 2 H, $CH_2C{=}O$), 4.3 (t, 1 H, CH), 4.4 (d, 2 H, CH_2O), 4.5 (b, 1 H, CH aromatic), 5.4 (d, 1 H, NH), 7.2–7.7 (m, 8 H, aromatic).

N-(*tert*-Butyloxycarbonyl)-S-[4-(9-fluorenylmethoxy)-4-oxobutyl]-L-penicillamine (2). L-Penicillamine (1.2 g, 8.3 mmol) and 4-bromobutanoic acid 9-fluorenylmethyl ester (3 g, 8.7 mmol) were reacted as described for **6a**. A solid corresponding to **6b** (1.2 g) was obtained after isolation and recrystallization from aqueous ethanol (36%): mp 151–153 °C; NMR (DMSO-d_6) δ 1.2 (s, 3 H, CH_3), 1.5 (s, 3 H, CH_3), 1.7 (m, 2 H, $-CH_2-$) 2.5 (m, 4 H, CH_2S, $CH_2C{=}O$), 4.2 (t, 1 H, CH), 4.3 (t, 1 H, CH aromatic), 4.5 (d, 2 H, CH_2O), 7.2–7.9 (m, 8 H, aromatic); MS m/e 414 $(M + H)^+$. Anal. ($C_{23}H_{27}NO_4S$) found 62.54% C, 6.62% H, 3.38% N.

Reaction of the above product with di-*tert*-butyl dicarbonate as described for **3** afforded 1 g (77%) of an oil corresponding to **2** which resisted crystallization and was used directly: NMR ($CDCl_3$) δ 1.2–1.5 (m, 15 H, $(CH_3)_3$, $(CH_3)_2$), 1.8 (m, 2 H, $-CH_2-$), 2.5 (m, 2 H, CH_2S), 2.8 (m, 2 H, CH_2O), 4.3 (b, 1 H, CH), 4.4 (b, 3 H, CH_2O, CH aromatic), 5.4 (b, 1 H, NH), 7.2–7.8 (m, 8 H, aromatic).

Deamino[carba^{105},Pen^{121}]r-ANF(105–126) (12). A solution of fragment A trifluoroacetate (1.5 g, 1.2 mmol) and compound **2** (770 mg, 1.5 mmol) in 50 mL of DMF was cooled to 0 °C in an ice bath and treated with diisopropylethylamine (0.64 mL, 3.6 mmol) and DPPA (687 mg, 2.5 mmol). After 15 h at 4 °C the solution was evaporated under vacuum and the residual oil was treated with ethyl acetate. The resulting solid was filtered, washed with cold ethanol, and dried under vacuum over P_2O_5 (1.3 g, 81% yield). A suspension of the protected peptide in 20 mL of CH_2Cl_2 was treated with 20 mL of TFA, and after it stirred at room temperature for 20 min, the solvent was evaporated under vacuum. The residue was treated with 50 mL of anhydrous ether whereupon the resulting solid was filtered and dried over P_2O_5. Fragment B (504 mg, 0.7 mmol) was dissolved in 50 mL of anhydrous DMF containing diisopropylethylamine (0.33 mL, 1.8 mmol) and the solution was cooled to 0 °C in an ice bath. The solution was treated with DPPA (825 mg, 3 mmol) and 1 g (0.61 mmol) of the above hexapeptide trifluoroacetate. The solution was allowed to stand at 4 °C for 18 h. After this time, HPLC in the gradient system I showed complete disappearance of the peak corresponding to the hexapeptide. The solution was concentrated to a viscous liquid and treated with ethyl acetate. The precipitate that formed was filtered, washed thoroughly with EtOAc, and dried under vacuum. The solid was suspended in 100 mL of ethanol, and the mixture was heated with vigorous stirring and cooled. The precipitated solid was collected by filtration and dried under vacuum over P_2O_5 (1.2 g, 90% yield): TLC R_f (BAW) 0.75; HPLC t_R = 16.4 min (I).

The above protected dodecapeptide (1.1 g) was redissolved in 50 mL of anhydrous DMF, cooled in an ice bath, and treated dropwise with 20 mL of 10% piperidine in DMF under nitrogen. After stirring for 20 min, the solution was treated with anhydrous ether, affording a precipitate. The precipitated solid was collected by filtration, washed thoroughly with ether, and resuspended in 100 mL of EtOH containing 1 mL of acetic acid. After stirring vigorously for 30 min, the resulting powder was filtered, washed with ethanol, and dried under vacuum over P_2O_5, affording 1 g of a white solid: TLC R_f (BAW) 0.70; HPLC t_R = 12.8 min (I).

The above acid (306 mg, 0.15 mmol) was dissolved in 50 mL of anhydrous DMF with vigorous stirring and the solution was cooled in an ice bath. To the resulting solution was added 0.11 mL (0.6 mmol) of DIEA and 550 mg (2 mmol) of DPPA followed by 327 mg (0.21 mmol) of fragment C. The reaction mixture was kept at 4 °C for 20 h, after which the reaction was judged to be complete on the basis of the disappearance of the peak corresponding to the dodecapeptide by HPLC. The solution was concentrated under high vacuum and treated with 100 mL of EtOAc, after which the precipitated solid was collected by filtration and dried. The solid was suspended in methanol (100 mL) with vigorous stirring, filtered, washed thoroughly with MeOH, and dried over P_2O_5, affording 350 mg of a white powder corresponding to the protected acyclic peptide; HPLC t_R = 22.5 min (I).

The above product was redissolved in 50 mL of anhydrous DMF with vigorous stirring and treated with 20 mL of 10% piperidine in DMF as described above for the removal of the fluorenylmethyl ester group. The isolated solid following work up was immediately treated with 20 mL of 50% trifluoroacetic acid in CH_2Cl_2 for 20 min at 0 °C and for 15 min at room temperature. The solvent was removed under vacuum and the product was isolated by addition of ethanol. The solid was filtered, washed thoroughly with ether and dried under high vacuum over P_2O_5 (300 mg).

For cyclization, the deprotected compound was dissolved in 50 mL of DMF at 0 °C and added via a syringe pump (1 mL/min) to a 500-mL solution of DMF containing 300 μL of DIEA and 600 mg of DPPA at 0 °C. The cyclization was followed by HPLC by monitoring the disappearance of the acyclic starting material (t_R = 10.8 min, ninhydrin positive) and the appearance of a ninhydrin-negative peak (t_R = 14.1 min). After 15 h, the solvent was removed under high vacuum and the product was isolated by addition of ether followed by filtration and thorough washing with ethanol.

The cyclized, protected peptide was treated with 10 mL of anhydrous hydrogen fluoride containing 1 mL of anisole for 30 min at −20 °C and for 30 min at 0 °C. Following evaporation of HF the residual gum was partitioned between 50 mL of 10% acetic acid and ether. The aqueous phase was extracted further with ether and lyophilized. The peptide (100 mg) was applied to an octadecyl silica glass column (2.5 × 40 cm) operating at a pressure of 40 psi and using a linear gradient of 0.05% TFA (A, 500 mL) and 70% MeOH (B, 1 L). The appropriate fractions corresponding to the principal peak were pooled and lyophilized, and the resulting powder was dried under high vacuum over P_2O_5: yield 20 mg; AAA Asp/Asn 1.91, Ser 1.91, Gln 1.02, Gly 5.16, Ala 0.98, Ile 1.76, Leu 1.10, Tyr 0.97, Phe 2.00, Arg 2.98; TLC R_f (BPAW) 0.65; FABMS for $C_{104}H_{160}N_{32}O_{30}S$, found m/e 2370 $(M + H)^+$.

Deamino[carba^{105},Cys^{121}]r-ANF(105–126) Methyl Ester (13). Compound **13** was obtained in the same way as described for **12** but substituting fragment A and incorporation of the methyl ester of tyrosine and also spanner 1: AAA Asp/Asn 2.01, Ser 1.72, Gln 0.99, Gly 5.38, Ala 1.04, Ile 1.77, Leu 1.06, Tyr 0.96, Phe 2.00, Arg 3.16; TLC R_f (BPAW) 0.70; FABMS for $C_{103}H_{158}N_{32}O_{30}S$, found m/e 2357 $(M + H)^+$.

Deamino[carba^{105},Cys^{121}]r-ANF(105–126) (14): AAA Asp/Asn 2.03, Ser 1.84, Gln 1.03, Gly 5.08, Ala 1.04, Ile 1.77, Leu 1.06, Tyr 0.96, Phe 2.00, Arg 3.16; TLC R_f (BPAW) 0.63; FABMS $C_{102}H_{156}N_{32}O_{30}S$, found m/e 2342 $(M + H)^+$.

Deamino[carba^{121}]r-ANF(105–126) (15): AAA Asp/Asn 2.00, Ser 1.78, Gln 1.01, Gly 5.18, Ala 1.00, Ile 1.80, Leu 1.02, Tyr 0.99,

Phe 2.00, Arg 3.45; TLC R_f (BPAW) 0.63; FABMS $C_{102}H_{156}N_{32}O_{30}S$, found m/e 2342 $(M + H)^+$.

Deamino[dicarba[105,121]]r-ANF(105–126) (16): AAA Asp/Asn 1.96, Ser 1.85, Gln 1.00, Gly 5.14, Ala 0.97, Ile 2.00, Leu 1.01, Tyr 0.94, Phe 1.98, Arg 3.14; TLC R_f (BPAW) 0.63; FABMS $C_{103}H_{158}N_{32}O_{30}$, found m/e 2325 $(M + H)^+$.

Deamino[γ,δ-dehydrodicarba[105,121]]r-ANF(105–126) (17): AAA Asp/Asn 1.99, Ser 1.81, Gln 0.98, Gly 5.18, Ala 1.00, Ile 1.90, Leu 0.98, Tyr 0.83, Phe 2.01, Arg 3.31; TLC R_f (BPAW) 0.63; FABMS $C_{103}H_{156}N_{32}O_{30}$, found m/e 2323 $(M + H)^+$.

Superfused Rabbit Aorta Rings. The descending thoracic aorta from New Zealand albino rabbits weighing approximately 1.5 kg was excised and placed in Krebs solution and bubbled with 5% CO_2 in O_2 to maintain the pH at 7.4. The aorta was cleaned of extraneous tissue and cut transversally into six 4 mm wide ring segments. Each segment was mounted on a force transducer (Grass Instruments, Model FT.O3) and connected to a polygraph for isometric recording according to the method described by Hooker et al.[24] The rings were placed under tension and readjusted until a stable 10-g resting tension was obtained. The tissues were superfused with Krebs solution at a rate of 15 mL/min with a multichannel perilstaltic pump (Piper, Model PL MT fitted with 3L-type pumpheads). After an equilibration period of 45 min, phenylephrine hydrochloride was added to the superfusate (1×10^{-7} M) causing a 40–60% increase in tension. This increase was maintained for the remainder of the experiment. A known concentration of the test compound (50 μL) was added to the superfusate 3–4 cm above the tissue. Once the tissue had recovered to the initial level of contractility a second dose of higher concentration was administered. A standard dose–response curve was constructed and the EC_{50} value was determined.

In Vivo Antihypertensive Studies. The antihypertensive effect of the ANF analogues was evaluated in DOCA–salt hypertensive rats[25] and in 5/6 nephrectomized rats.[26] Briefly, animals were anesthetized with halothane after infiltration through the skin with a 2% lidocaine solution, and the left femoral artery and vein were dissected and cannulated with PE50 and PE10 tubing, respectively. Both cannulas were exteriorized at a point near the tail and the skin wound was sutured. After a period of recovery, arterial blood pressure was measured with a Gould P50 transducer and a Gould Model 2800 S recorder. The test compounds were infused for 30-min intervals at increasing concentrations (0.1, 0.2, 0.5, and 1.0 μg/kg per min) at a rate of 70 μL/kg per min following a control period with saline. The animals were allowed to recover under saline infusion and a dose of hydralazine (100 μg/kg per min) was administered for standardization.

Diuresis and Natriuresis. Normotensive male Sprague–Dawley rats (300–325 g) were anesthetized with halothane. Following administration of a 2% lidocaine solution, the femoral artery was cannulated for measurement of blood pressure and the femoral vein was cannulated for administration of the test compound. The bladder was cannulated to measure urine flow. Following surgery the animals were allowed to recover for at least 1 h in plastic restraining cages, and administration of Ringer's solution 1.2 mL/h) was started. Urine samples were collected at 10-min intervals during a 30-min control period. The test compound was then infused at a rate of 0.5 μg/kg per min over 30 min, and urine samples were again collected at 10-min intervals. Ringer's solution was again infused during the next 30-min recovery period and urine samples were collected once again. Urine volume was determined and electrolyte concentrations were measured with a Nova Biomedical electrolyte analyzer. Systolic and diastolic blood pressures were recorded during the experiment to ascertain that an adequate perfusion pressure was maintained.

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Registry No. **1**, 123963-64-4; **2**, 123963-65-5; **3**, 123963-66-6; **3** (BOC-deblocked), 123963-72-4; **4**, 124095-60-9; **5**, 123963-67-7; **6a**, 123963-68-8; **6b**, 123963-73-5; **8**, 88898-17-3; **9**, 90817-13-3; **10**, 95079-20-2; **11**, 107865-22-5; **12**, 123992-46-1; **13**, 123992-47-2; **14**, 123963-69-9; **15**, 123963-70-2; **16**, 103340-30-3; **17**, 124093-79-4; fragment A, 122294-89-7; fragment B, 103339-80-6; fragment C, 122313-02-4; Fm-OH, 24324-17-2; $BrCH_2CH_2COO$-Fm, 123963-71-3; H-Cys-OH, 52-90-4; H-Hcy-OH, 6027-13-0; H-Pen-OH, 113-41-3.

Autoxidation of the Serotonergic Neurotoxin 5,7-Dihydroxytryptamine

Tahereh Tabatabaie, Monika Z. Wrona, and Glenn Dryhurst*

Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019. *Received April 20, 1989*

The indolic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) has been widely speculated to express its neurodegenerative effects as a result of intraneuronol autoxidation. Until recently, it was believed that autoxidation led to reactive electrophilic quinone imine species which alkylated neuronal membrane proteins and that byproducts of the autoxidation reaction were cytotoxic reduced-oxygen species. This study reveals that at physiological pH carbanions of 5,7-DHT act as the primary electron-donor species to yield C(4)- and C(6)-centered free radical superoxide complexes in a 1:2 ratio. The C(4)-centered complex reacts to yield, ultimately, 5-hydroxytryptamine-4,7-dione which has been shown to be a significantly more powerful neurotoxin than 5,7-DHT. The C(6)-centered radical superoxide complexes react to give 6,6′-bis(5-hydroxytryptamine-4,7-dione). It is likely that the latter reaction yields $O_2^{\bullet -}$ as a cytotoxic byproduct.

5,7-Dihydroxytryptamine (5,7-DHT) is used extensively for the selective chemical denervation of central serotonergic neurons.[1–8] The selectivity of 5,7-DHT probably results from its high affinity uptake by the membrane pump of serotonergic neurons. The toxicity of 5,7-DHT is widely believed to be related to its rapid oxidation by

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