(MEM) containing 10% bovine calf serum at 37 °C for 24 h in a 97% humidified atmosphere of 5.5% CO_2. The medium was then replaced with fresh medium containing taxol or its derivatives in concentrations ranging from 5×10^{-9} M to 75×10^{-9} M. The final concentration of DMSO in the cell medium was 0.5% or less. This amount of DMSO did not have any effect on cell proliferation as determined from control experiments. After 40 h, the cells were released by trypsinization and counted in a Coulter counter. To study cell recovery after an acute treatment of the drug, cells were incubated with 10^{-5} and 10^{-6} M taxol or its analogue for 8 h. The medium was removed, and the cells were rinsed three times with fresh medium and allowed to grow in medium free of drug for 48 h.

Chromatin Fluorescence Staining. Following drug treatment and recovery, the medium was removed, and the cells were fixed with 4% formaldehyde in 0.05 M phosphate buffer, pH 7.3, and permeabilized with cold 95% ethanol for 30 min at 0 °C. Between each step the cells were washed once with PBSA (phosphate-buffered saline, without Ca^{2+} and Mg^{2+}) and twice with PBS (phosphate-buffered saline, with Ca^{2+} and Mg^{2+}). Chromosome staining was done using 1 mL of Hoechst 33342 DNA stain (0.2 μg/mL in PBS) per coverslip for 1 h in the dark at room temperature. The coverslips were mounted on glass slides and viewed under a Leitz fluorescence microscope.

Stability of Drugs during Microtubule Assembly and Cell Growth Studies. The stability of the taxol derivatives was examined under the same conditions used for assembly (PEM buffer, pH 6.9, 30 °C) and for cell growth (MEM with 10% serum, 37 °C) by chromatography on a C-8 Synchropak reversed-phase HPLC column. The compounds were eluted with 0.02 M ammonium acetate, pH 7-acetonitrile (50:50) and detected by their absorbance at 254 nm.

Electron Microscopy. For negative staining, aliquots of the assembly mixture diluted 15-fold in PEM buffer containing 0.25% glutaraldehyde were placed on Formvar- and carbon-coated grids, washed three times with H_2O, and stained with 2% uranyl acetate. The grids were viewed on a Phillips 300 transmission electron microscope.

In Vivo MX-1 Mammary Screen. A study of a limited number of derivatives in the subrenal capsule human mammary carcinoma (MX-1) xenograft screen in mice was performed by the National Cancer Institute. The study was performed according to procedures described in NIH Publication No. 84-2635 (February 1984). All the derivatives were administered ip as aqueous solutions except for taxol.

Acknowledgment. This work was supported by National Cancer Institute contracts N01-CM-67912 and N01-CM-97576 and in part by NIH grant GM 36953. M.R.M. is a Wesley Foundation (Wichita, KS) Postdoctoral Scholar. We thank Sandi Thompson and Jeanne Ellermeier for their excellent technical help. The assistance of Dr. Plowman and her staff at NCI in performing the MX-1 assays is greatly appreciated.

Registry No. **2**, 137234-34-5; **3**, 131966-80-8; **4**, 117527-60-3; **4a**, 132073-89-3; **5**, 132073-90-6; **5a**, 131966-75-1; **5b**, 132073-91-7; **6**, 131966-78-4; **6a**, 132073-93-9; **7**, 100431-55-8; **8**, 131966-76-2; **9**, 131966-77-3; **9a**, 132073-92-8; **10**, 131966-83-1; **11**, 137234-35-6; **12**, 131966-79-5; **12a**, 132073-94-0; L-$HO_2CCH(CH_3)NHCOOC(CH_3)_3$, 15761-38-3; $HO_2CCH_2N(CH_3)_2$, 1118-68-9; $HO_2C(CH_2)_2N(C_2H_5)_2$·HCl, 15674-67-6; $HO_2C(CH_2)_2N(C_2H_5)_2$·CH_3SO_3H, 131919-23-8; $ClCOOCH_2CCl_3$, 17341-93-4.

Preparation and Biological Activities of Potential Vasopressin Photoaffinity Labels

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Received March 13, 1991

Several potential photoaffinity analogues of the peptide hormone vasopressin (VP) were prepared by classical solid-phase peptide synthesis using two different pathways. Peptide sequences were built by introduction of (a) N^{ar}-protected aminophenylalanine or (b) nitrophenylalanine in the photolabeling position. Conversion to the azido peptide was completed in pathway a after cleavage and before purification and in pathway b from small quantities of purified nitrophenylalanine-containing precursor peptides. V_1 receptor binding properties were measured using membranes prepared from rat liver cells. The binding potential of agonistic VP structures was abolished by the introduction of an azido or a nitro group into the aromatic side chain at position 3. Cyclo desamino-β,β-dialkyl-Cys[1]-type VP antagonist structures were prepared with the photoactivable moiety in position 2 and an iodination residue in position 9. One particular compound, [Dmpa[1],Phe(N_3)[2],Val[4],Lys[8],D-Tyr[9]]VP (**8**), containing β,β-dimethyl-β-mercaptopropionic acid in position 1, had excellent binding properties, both in the radioiodinated ($K_d = 4.8 \pm 1.9 \times 10^{-10}$ M) and noniodinated form ($K_d = 6.4 \pm 0.98 \times 10^{-10}$ M). The analogues with long-chain β-alkylation (diethyl and pentamethylene) and the linear antagonist photolabel showed significantly less affinity. Optimal binding properties were obtained within a very narrow range of hydrophobicity; greater or lesser hydrophobicity was correlated to less potent binding. The precursor analogues, containing nitrophenylalanine, displayed a structure–activity relationship similar to that of the azido peptides. The most potent analogues will be used for receptor labeling studies. A linear antagonist structure having a photosensitive group in position 1 has also been prepared, but this compound displayed much less affinity than the cyclic antagonists. The most potent compounds were also highly selective for the V_1 receptor and did not recognize the V_2 receptor from other preparations.

Introduction

Peptide hormone receptors have been a favored research target, both in the past and today. Many efforts have been made to prepare highly specific ligands for labeling and, eventually, for biochemical isolation of their respective receptors. In particular, the receptors for the peptide hormone vasopressin (VP) have been investigated with selective ligands, but both the vascular receptor V_1 and the renal receptor V_2 have so far eluded isolation. In the past, several studies have been carried out with photosensitive analogues of VP and the other neurohypophyseal hormones, but all studies with photoaffinity analogues of VP produced unsatisfactory results because the molecular

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0022-2623/92/1835-0151$03.00/0

weights found for the presumed VP receptors were too small to be those of a typical G-protein-mediated receptor of the β-adrenergic receptor super family.

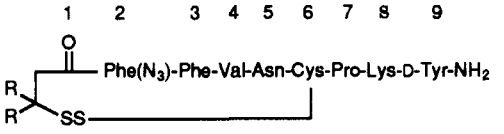
Fahrenholz and co-workers prepared VP photoaffinity labels with the photosensitive moiety at position 3, 4, or 8 of VP and with ^3H as the tracer isotope.[1-3] More recently, the same researchers prepared a radioiodinated analogue containing the photolabel at position 9.[4] In all photolabeling studies so far, VP receptor proteins with molecular weights in the range of 30 kDa have been reported. On the other hand, radiation inactivation studies by the same group indicate a molecular weight of around 76 kDa,[5] more than double the size found with photoaffinity labeling. Moreover, the yield of the covalently bound receptor–ligand complex thusly obtained was always less than 1% of the initial, noncovalent receptor–ligand complex. Several studies have shown that labeling yields of 40% and greater[6] are possible when appropriate labeling peptides were used. The apparent molecular weights of all similar, G-protein-mediated peptide receptors were consistently above 40 kDa, including angiotensin II,[7] ACTH,[8] αMSH,[9] LHRH,[10] substance P,[11] and somatostatin.[12] The recent sequencing of all three tachykinin receptors by Nakasnishi[13] has shown that the protein molecular weight is about 43 kDa, when carbohydrate residues are not included. Receptor glycosylation, however, is frequently responsible for a major increase in apparent molecular weight, e.g. a doubled molecular weight has been reported for the D_2 receptor.[14]

For the above reasons, our objective was to design alternative VP receptor labels in order (a) to increase the labeling yield, (b) to search for the higher molecular weight forms of the putative VP receptors which might have escaped detection so far, and (d) to investigate the interaction of solubilized, nondenatured VP receptors with G proteins. We therefore explored the possibility of simultaneous introduction of a photolabeling moiety and an iodination site in several groups of VP analogues, both agonistic and antagonistic structures, without seriously reducing the affinity of the analogue for the V_1 receptor.

Design and Syntheses

Fahrenholz and co-workers reported[1-3] the incorporation of photolabile groups at several positions within the VP molecule; positions 3, 4, and 8 in particular have been exploited and recently also position 9.[4] Their results were still not optimal, which motivated us to investigate positions 1–3. Position 1, a half-cystine, has not been considered at all until now, but the availability of V_1-specific, linear VP antagonists[15] has made this a reasonable modification, because of the relatively simple, hydrophobic non amino acid residues at position 1. Position 3 has been utilized in the past, but the absence of an iodination site in these agonistic molecules has made their utility problematic because of the low specific activity obtained with tritium labeling.[1] Therefore, the introduction of D-Tyr in position 2 of [Phe(N_3)^3]VP has been attempted, a modification which has led in the past to molecules[16] which permit iodination without loss of receptor recognition: H-Cys-D,L-Tyr-Phe(N_3)-Gln-Asn-Cys-Pro-Arg-Gly-NH_2.

Potent and selective antagonists lacking the N-terminal amino function have been described that were β,β-alkylated at the position 1 residue and contained methyl or ethyl ethers on Tyr.[17,18] In those antagonists, position 9 shows a particular tolerance to modification.[19] The photolabeling moiety, an aryl azido group, has pseudohalogen character and is almost isosteric to the aromatic methoxy group; we therefore attempted to introduce the photolabeling moiety in position 2 as Phe(N_3) instead of the well-accepted Tyr(OMe). As a potential iodination site, D-Tyr was introduced in position 9. The D isomer of Tyr was chosen for the C-terminal position in order to increase the resistance of the resulting peptide to enzymatic degradation. Because both modifications increase the hydrophobicity of the antagonist molecule (the N_3-residue is more hydrophobic[20] than both OMe and OEt), the optimal alkylation of position 1 had to be found by the synthesis of a series of analogues with increasing alkyl content of the β-position in the mercaptopropionyl residue at position 1: β-mercaptopropionic acid (MPa), β,β-dimethyl-β-mercaptopropionic acid (Dmpa), β,β-diethyl-β-mercaptopropionic acid (Depa), and β,β-pentamethylene-β-mercaptopropionic acid (Ppa).



Peptides were synthesized by two strategies using conventional solid-phase synthesis and the BOC–TFA–HF scheme.[21] The disulfide rings of the cyclic peptides were

(1) Fahrenholz, F.; Crause, P. *Biochem. Biophys. Res. Commun.* **1984**, *122*, 974.
(2) Boer, R.; Fahrenholz, F. *J. Biol. Chem.* **1985**, *260*, 15051.
(3) Fahrenholz, F.; Boer, R.; Crause, P.; Toth, M. V. *Eur. J. Biochem.* **1985**, *152*, 589.
(4) Fahrenholz, F.; Korjo, E.; Muller, M.; Boer, R.; Lohr, R.; Grzonka, Z. *Eur. J. Biochem.* **1986**, *161*, 321.
(5) Crause, P.; Boer, R.; Fahrenholz, F. *FEBS Lett.* **1984**, *175*, 383.
(6) Eberle, A. N.; De Graan, P. N. E. *Methods Enzymol.* **1985**, *109*, 121.
(7) Carson, M. C.; Leach-Harper, C. M.; Baukal, A. J.; Aquilera, G.; Cah, K. J. *Mol. Endocrinol.* **1986**, *1*, 147. Guillemette, G.; Escher, E. *Biochemistry* **1983**, *22*, 5591.
(8) Ramachandran, J.; Muramoto, K.; Kenez-Keri, M.; Keri, G.; Buckley, D. I. *Proc. Natl. Acad. Sci., U.S.A.* **1983**, *77*, 3967. Escher, E.; Mackiewicz, Z.; Sircar, S.; Bourque, D.; Bouthillier, F.; Lehoux, J.-G. In Peptides 1986; Theodoropoulos, D., Ed.; Water de Gruyter & Co., Berlin, 1987; p 473.
(9) Solca, F.; Siegrist, W.; Drodz, R.; Girard, J.; Eberle, A. N. *J. Biol. Chem.* **1989**, *264*, 14277.
(10) Hazum, E.; Keinan, D. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 116. Escher, E.; Mackiewicz, Z.; Lehoux, J.-G.; Gallo-Payet, N.; Bellabarba, D.; Bélisle, S. *J. Recept. Res.* **1988**, *8*, 391.
(11) Dam, T. V.; Escher, E.; Quirion, R. *Biochem. Biophys. Res. Commun.* **1987**, *149*, 297.
(12) Murthy, K. K.; Srikant, C. B.; Escher, E.; Patel, Y. C. *J. Clin. Invest.* Submitted.
(13) Shigemoto, R.; Yokota, Y.; Tsuchida, K.; Nakanishi, S. *J. Biol. Chem.* **1990**, *265*, 623.
(14) Grigoriads, D. E.; Niznik, H. B.; Jarvie, K. R.; Seeman, P. *FEBS. Lett.* **1988**, *227*, 220.
(15) Manning, M.; Przybylski, J.-P.; Olma, A.; Klis, W. A.; Kruszynski, M.; Wo, N. C.; Pelton, G. H.; Sawyer, W. H. *Nature* **1987**, *329*, 839.
(16) Jard, S.; Lombard, C.; Seyer, R.; Aumelas, A.; Manning, M.; Sawyer, W. H. *Mol. Pharmacol.* **1987**, *32*, 369.
(17) Kruszynski, M.; Lammek, B.; Manning, M.; Seto, J.; Haldar, J.; Sawyer, W. H. *J. Med. Chem.* **1980**, *23*, 364.
(18) Elands, J.; Barberis, C.; Jard, S.; Lammek, B.; Manning, M.; Sawyer, W. H.; de Kloet, R. *FEBS Lett.* **1988**, *229*, 251.
(19) Manning, M.; Olma, A.; Klis, W.; Kolodziejczyk, A.; Nawrocka, E.; Misicka, A.; Seto, J. *Nature* **1984**, *308*, 652.
(20) Hansch, C.; Leo, A.; Unger, S. H.; Kim, K. H.; Nikaitani, D.; Lien, E. J. *J. Med. Chem.* **1973**, *16*, 1207.
(21) Mackiewicz, Z.; Bélisle, S.; Bellarbarba, D.; Gallo-Payet, N.; Lehoux, J.-G.; Escher, E. *Helv. Chim. Acta* **1987**, *70*, 423.

Table I. Structure and Physicochemical Properties of VP Analogues

no.	structure[a]	formula	FAB-MS (M + 1)	TLC R_f A	TLC R_f B
VP	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (1 2 3 4 5 6 7 8 9)	$C_{46}H_{65}O_{12}N_{15}S_2$	1084	0.42	0.58
1	[Phe(NO_2)^3]VP	$C_{46}H_{64}O_{14}N_{16}S_2$	1127	0.43	0.59
2	[Phe(N_3)^3]VP	$C_{46}H_{64}O_{12}N_{18}S_2$	1125	0.45	0.59
3	[D-Tyr^2,Phe(NO_2)^3]VP	$C_{46}H_{64}O_{14}N_{16}S_2$	1129	0.43	0.58
4	[D-Tyr^2,Phe(N_3)^3]VP	$C_{46}H_{64}O_{12}N_{18}S_2$	1125	0.45	0.58
5	[Mpa^1,Phe(NO_2)^2,Val^4,Lys^8,D-Tyr^9]VP	$C_{53}H_{70}O_{13}N_{12}S_2$	1147	0.54	0.76
6	[Mpa^1,Phe(N_3)^2,Val^4,Lys^8,D-Tyr^9]VP	$C_{53}H_{70}O_{11}N_{14}S_2$	1143	0.58	0.76
7	[Dmpa^1,Phe(NO_2)^2,Val^4,Lys^8,D-Tyr^9]VP	$C_{55}H_{74}O_{13}N_{12}S_2$	1175	0.54	0.76
8	[Dmpa^1,Phe(N_3)^2,Val^4,Lys^8,D-Tyr^9]VP	$C_{55}H_{74}O_{11}N_{14}S_2$	1171	0.60	0.76
9	[Depa^1,Phe(NO_2)^2,Val^4,Lys^8,D-Tyr^9]VP	$C_{57}H_{78}O_{13}N_{12}S_2$	1203	0.57	0.76
10	[Depa^1,Phe(N_3)^2,Val^4,Lys^8,D-Tyr^9]VP	$C_{57}H_{78}O_{11}N_{14}S_2$	1199	0.62	0.76
11	[Ppa^1,Phe(NO_2)^2,Val^4,Lys^8,D-Tyr^9]VP	$C_{58}H_{78}O_{13}N_{12}S_2$	1215	0.60	0.76
12	[Ppa^1,Phe(N_3)^2,Val^4,Lys^8,D-Tyr^9]VP	$C_{58}H_{78}O_{11}N_{14}S_2$	1211	0.60	0.76
13	[Npac^1,D-Tyr(OEt)^2,Val^4,Abu^6,Arg^{8,9}]VP	$C_{58}H_{83}O_{13}N_{17}$	1226	0.48	ND
14	[Apac^1,D-Tyr(OEt)^2,Val^4,Abu^6,Arg^{8,9}]VP	$C_{58}H_{83}O_{11}N_{19}$	1222	0.51	ND
15	[Ppa^1,Tyr(OMe)^2,Val^4,Lys^8,D-Tyr^9]VP	$C_{59}H_{81}O_{12}N_{11}S_2$	1200	0.55	0.76

[a] Mpa = β-mercaptopropionic acid. Dmp: β,β-dimethyl-β-mercaptopropionic acid. Depa: β,β-diethyl-β-mercaptopropionic acid. Ppa: β,β-pentamethylene-β-mercaptopropionic acid. Npac: 4′-nitrophenylacetic acid. Apac: 4′-azidophenylacetic acid. Abu: aminobutyric acid.

closed after HF cleavage by ferricyanide oxidation[22] in the first pathway. The photolabile group was introduced by synthesis of a precursor peptide containing nitrophenylalanine and its conversion, after cleavage, cyclization, and purification, into the photolabeling peptide containing azidophenylalanine.[23] In a second pathway, Z-protected aminophenylalanine was introduced instead of nitrophenylalanine, and after completion of the synthesis the crude peptide was converted into the azido peptide.[24] In this second scheme, the protecting group of the aromatic amino function was removed during HF cleavage, together with the other side-chain-protecting groups and the peptide–resin ester link. Immediately afterward, the azido group was introduced by diazotation of the aromatic amine followed by addition of sodium azide to the whole of the peptide. In the case of the linear antagonist **14**, the photolabeling function was introduced similarly to the first pathway during synthesis by N-terminal acylation with nitrophenylacetic acid (Npac) and subsequent conversion to azidophenylacetyl (Apac) and then purification of the azido peptide.

1 2 3 4 5 6 7 8 9

Acyl-D-Tyr(OEt)-Phe-Val-Asn-Abu-Pro-Arg-Arg-NH_2

The completed peptides were assessed for purity by HPLC and TLC and for structural identity by FAB-MS. Biological screening was carried out by determining their binding potencies on rat liver membranes, a preparation displaying the V_1 receptor.[16] The most potent analogues were also investigated for their biological activities by binding to the V_2 receptor[25] and by activation of the adenylate cyclase of V_2-expressing LLCPK cells,[26] while their antagonistic nature was assessed from V_1-stimulated phospholipase C activity.[27]

Peptide iodination procedures were carried out in diluted AcOH, which effectively suppresses the formation of diiodinated derivatives. Radioiodinated peptides were purified twice by HPLC from reversed-phase material using a gradient elution under conditions assuring base-line separation for iodinated and noniodinated VP's.[28] The observed specific radioactivity was not changed following a further HPLC elution; the radioligands were therefore the monoiodinated peptides, free from noniodinated and diiodinated forms.

Results and Discussion

In this study, two pathways were utilized for the synthesis of photoaffinity labeling peptides: (a) introduction of nitrophenylalanine at the photolabeling position and conversion into the labeling peptide after synthesis and purification or (b) introduction of protected aminophenylalanine during synthesis and conversion to the azido peptide, prior to purification. For both pathways the syntheses were successful with comparable yields. A major disadvantage of the nitrophenylalanyl pathway a was use of the modification step from the purified nitro peptide into the azido peptide, especially the exposure of a disulfide cyclized peptide to catalytic hydrogenation followed by acidic diazotation, but we found no desulfurization nor permanent ring opening. (TLC coloration of concentrated samples was negative by the Ellman test; the compounds showed the authentic structure by FAB-MS, coeluted upon HPLC with those prepared by pathway b, and also had the same TLC R_f values.) On the other hand, our experience shows that azido peptides are less stable than nitrophenylalanyl peptides during prolonged storage. Therefore, transformation of only the required quantity has the advantage that fresh azido peptide can be regenerated from the stable precursor within a short time. Furthermore, the availability of an additional pure and characterized analogue of a given peptide can be interesting for SAR considerations,[29] which will be specifically addressed in this contribution. In the second pathway (b), when utilizing N^{ar}-protected Z-aminophenylalanine,[24] an

(22) Schiller, P. W.; Eggimann, B.; Di Maio, J.; Lemieux, C.; Nguyen, T. M. *Biochem. Biophys. Res. Commun.* **1981**, *101*, 337.
(23) Laczko, E.; Escher, E. *Helv. Chim. Acta* **1981**, *64*, 621.
(24) Fahrenholz, F.; Thierauch, K.-H. *Int. J. Pept. Protein Res.* **1980**, *15*, 323.
(25) Roy, C.; Ausiello, D. A. *J. Biol. Chem.* **1981**, *256*, 3415.
(26) Roy, C.; Hall, D.; Karish, M.; Ausiello, D. A. *J. Biol. Chem.* **1981**, *256*, 3423.
(27) Kirk, C. J.; Guillon, G.; Balestre, M. N.; Jard, S. *Biochem. J.* **1986**, *240*, 197.
(28) Moore, M. L.; Huffman, W. F.; Roberts, G. D.; Rottschaefer, S.; Sulat, L.; Stefankiewicz, J. S.; Stassen, F. *Biochem. Biophys. Res. Commun.* **1984**, *121*, 878.
(29) Escher, E.; Nguyen, T. M. D.; Robert, H.; St-Pierre, S. A.; Regoli, D. *J. Med. Chem.* **1978**, *21*, 860.

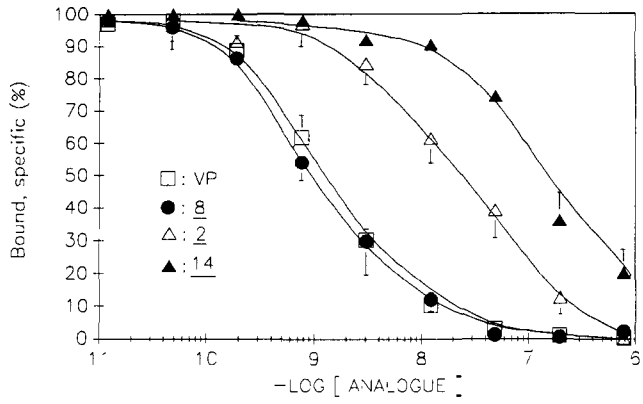


Figure 1. Representative dose–displacement curves of some VP analogues against ^{3}H-VP. Rat liver membranes (50 μg of protein) were incubated with increasing concentrations of unlabeled ligands (up to 10^{-6} M). Incubations were performed in the absence of UV irradiation at room temperature for 30 min in presence of 5×10^3 cpm of ^{3}H-VP; separation was achieved by filtration. Each point is the mean of at least three independent experiments carried out in duplicate. The ordinate in the percent of bound radioactive label compared to the total specific binding capacity.

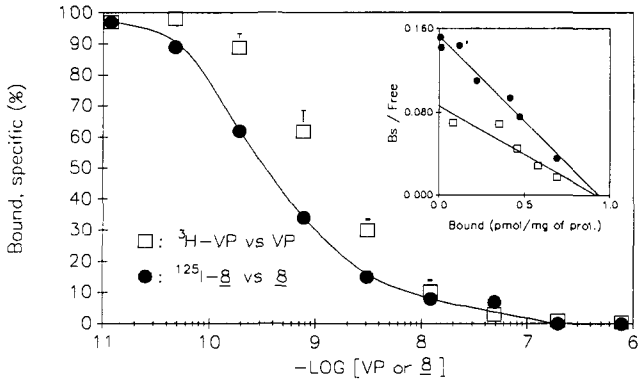


Figure 2. Displacement and Scatchard analysis of radioiodinated photolabel. Radioiodinated 8 (^{125}I-8) (442 Ci/mmol, $2,5 \times 10^4$ cpm/tube) was displaced in absence of UV radiation with increasing concentrations of unlabeled 8. Superposed is shown the displacement of ^{3}H-VP (68.5 Ci/mmol) by VP from Figure 1. Inset: Scatchard analysis of the displacements of ^{3}H-VP by VP and of ^{125}I-8 by 8 indicate the same binding capacity of the membranes K_d values, calculated with the respective specific radioactivities of the tracers, were determined at 0.48 nM for ^{125}I-8 and at 0.64 nM for ^{3}H-VP.

immediate transformation of the crude, cyclized peptide into the azido peptide is necessary because free aminophenylalanyl peptides are very sensitive to oxidation, as are all other primary aniline derivatives. Relatively mild oxidation is, however, applied for intramolecular disulfide bond formation during the cleavage step. In our view, the disadvantage of having to convert the whole batch of synthetic peptide immediately into the azidophenylalanyl peptide outweighs the drawbacks of the nitro–azido modification pathway because the finished peptide is less stable than the nitro precursor peptide.

Biological testing was carried out by the binding assay on rat liver membranes, which display the V_1-type of VP receptor. This assay was chosen because the antagonist structures (5–14, Table I) are generally better recognized by the V_1 receptor than by the renal-type receptor V_2.[15]

The results of the binding experiments, which are presented in Table II and Figures 1 and 2, have several surprising elements. The introduction of an azido group or a nitro group in the 4′-position of a phenylalanyl residue has been reasonably well accepted in other peptide hormone receptors, and in some cases even affinity increases were noted,[30] but the minor modification of Phe to Phe(N_3) or Phe(NO_2) in position 3 virtually abolished receptor recognition of analogue 1 and, to a lesser extent, azido peptide 2 (see Figure 1). This observation was confirmed in analogues 3 and 4 with D-Tyr in position 2 and modified Phe in position 3, which showed even less affinity for the V_1 receptor.

Table II. Binding Potencies of VP Analogues

compd[a]	K_d[b] (nM)	pK_d[b]	RA[c]	n
VP	0.64 ± 0.04	9.19	100	7
1	41.0 ± 2.7	7.39	1.6	3
2	16.4 ± 5.58	7.79	4	3
3	53.6 ± 16.4	7.27	1.2	3
4	33.3 ± 0.60	7.48	2	3
5	27.0 ± 4.6	7.57	2.3	3
6	5.0 ± 0.85	8.31	13	3
7	1.8 ± 0.6	8.74	36	3
8	0.60 ± 0.1	9.22	107	3
9	0.19 ± 0.014	9.72	340	6
10	2.55 ± 0.12	8.59	25	3
11	3.2 ± 0.7	8.49	20	3
12	35.5 ± 5.10	7.45	2	3
14	75.4 ± 15.0	7.12	0.8	3
15	0.18 ± 0.08	9.74	360	3
^{125}I-7	1.63 ± 0.5	8.78	39	3
^{125}I-8	0.48 ± 0.19	9.32	133	5
^{125}I-9	2.64 ± 0.23	8.58	24	3
^{125}I-15	0.234 ± 0.025	9.63	275	3
[Ppa^1,MeAla^7,Arg^8, Lys(N_3PhCNH)^9]VP	0.33 ± 0.16	9.48	194	ref 4
^{125}I-[Ppa^1,MeAla^7,Arg^8, Lys(N_3PhCNH)^9]VP	0.92 ± 0.20	9.04	70	ref 4

[a] Ppa: β,β-pentamethylene-β-mercaptopropionic acid. [b] K_d is the dissociation constant calculated from the binding experiments; pK_d is the negative log of K_d. [c] RA is the relative affinity in percent compared to VP = 100%. n is the number of experiments.

Much better results were observed with the analogue series 5–12 with the Manning-type structural modifications. In earlier publications, analogues similar to peptide 15 with a highly alkylated position 1 residue displayed greater affinities than the nonalkylated or short-chain alkylations in the β-position of the mercaptopropionyl residue.[17] The two additional modifications in our analogue series, the azido or nitro modifications instead of the methoxy or ethoxy residue in the para position of the aromatic amino acid in position 2 and the introduction of a D-tyrosine in position 9, may explain the greater affinity of the position 1 dimethyl alkylated azido analogue 8 or the diethyl alkylated nitro analogue 9. Optimal receptor recognition and binding with most long-acting or high-affinity analogues is coupled to their increased hydrophobicity. Typical examples are the β-alkylated desamino antagonists of VP, such as peptide 15, with the pentamethylene substitution in the most potent analogues. This correlation with hydrophobicity must however have its limits, and this could explain why the corresponding diethyl and pentamethylene analogues of our azido photoaffinity labels are less effective ligands than the dimethyl analogue. The azido function at position 2 and the Gly to D-Tyr modification at position 9 both produced a more hydrophobic derivative and this might be held accountable for the observed effects. An additional argument for this view is the affinity of compound 9, the highest among the new compounds, and also that of peptide 11; both are more highly alkylated nitro analogues and are more than 10-fold more potent than their corresponding azido analogues 10 and 12. Iodination of 9 to ^{125}I-9 however reduces its af-

(30) Escher, E. *Pharmacol. Ther.* 1988, *37*, 37.

Table III. Partitioning Coefficients of Radioactive VP Analogues

peptide	log P	RA
^{3}H-VP	−1.62	100
^{125}I-7	+1.03	39
^{125}I-8	+1.40	133
^{125}I-9	+1.48	24
^{125}I-15	+1.41	275

finity 10-fold, to approximate that of compound **10**. It appears thus that a very fine balance of hydrophobic interaction governs affinity for the V_1 receptor, and optimal interactions are obtained with compounds **9**, **15**, and ^{125}I-**15** at a K_d of 0.2 nM. Iodination of the less hydrophobic analogues (**7** to ^{125}I-**7** and **8** to ^{125}I-**8**) tends to increase their affinity, although not significantly (see Figure 2 and Table II), while on the other hand iodination of more hydrophobic analogues (**9** to ^{125}I-**9**) tends to reduce their affinity. This assumption is well illustrated by analysis of the partitioning behavior of the radioactive compounds (see Table III).

The selectivity of the new compounds was also screened by their biological activity mediated by the V_1 receptor and the V_2 receptor (renal). The most interesting derivatives, the photolabel **8** and the nonphotosensitive but similar analogue **15**, were investigated as representative compounds. The inhibition of the VP stimulation of phospholipase C in cultured WRK cells was used as a V_1-mediated assay.[27] Compounds **8** and **15** were both pure antagonists, devoid of any agonistic properties up to concentration of 10^{-7} M, and both compounds had similar K_i's: 1.9 ± 0.6 (**8**) and 1.4 ± 0.6 nM (**15**), estimated from the inhibition of 3 nM VP stimulation of phospholipase C. The biological activity of the V_2 system was evaluated by the adenylate cyclase activity in LLCPK cells.[26] Neither agonistic (VP-like) nor antagonistic properties were evident for compound **8** or **15** at a concentration of 10^{-7} M. A very weak partial agonism (2.5%), compared to that of VP (100% intrinsic activity), and no significant antagonism against VP-stimulated cAMP accumulation was observed for both compounds in the range of 10^{-7} to 10^{-5} M. Binding of **8** and **15** was also tested using LLCPK cells;[25] both compounds displayed very weak affinities to this V_2-receptor preparation with K_d's $> 1 \times 10^{-7}$ M. In conclusion, it is therefore reasonable to claim that the described analogues are highly selective V_1-receptor ligands with virtually no recognition of the V_2 receptor.

Oxidative iodination of peptide **8** with ^{125}I and Iodogen produced an excellent V_1-labeling ligand in high yields which showed good binding parameters. In the absence of ultraviolet radiation, this analogue seems to be the best radioactive photolabel available for V_1-receptor studies (K_d of 4.84 ± 1.90 × 10^{-10} M). Reports on VP antagonists with D-Tyr in position 9 for radiolabeling which proposed very similar structures have appeared during this study.[16,18] Comparable compounds with the pentamethylene residue in position 1 (**15** and ^{125}I-**15**) have therefore been synthetized that have Tyr(OMe) in position 2 but are otherwise identical to our analogue series **5–12** included in this study. It is interesting to note that in a previous publication[4] another photolabeling peptide was reported, with comparable but somewhat lower binding affinities; the functional groups were in interchanged positions with the iodination site at position 2 and the photolabeling residue at position 9 (see Table II, bottom).

In conclusion, we have synthesized a novel and highly selective ligand with antagonistic properties toward the V_1 receptor, which will be of great interest for studies upon and isolation of the receptor. Photolabeling experiments with this goal in view are currently under investigation and will be published later.

Experimental Section

Solvents were analytical grade redistilled before use. We utilized benzhydrylamine resin from IAF-Biochemicals (Montreal, Canada). Protected standard amino acids were purchased from IAF-Biochemicals (Montreal, Canada) or from Sigma. Other reagents, starting materials, and specific peptide reagents were from Aldrich and were of analytical grade. Photolabeling amino acid precursors and analogues of β-mercaptopropionic acid, *S*-(methylbenzyl)-β,β-dimethyl-β-mercaptopropionic acid, *S*-(methylbenzyl)-β,β-diethyl-β-mercaptopropionic acid, and *S*-(methylbenzyl)-β,β-pentamethylene-β-mercaptopropionic acid, were prepared in our laboratory following the procedures of the synthesis of the *S*-benzyl-protected analogues.[31-33] Peptide couplings were carried out by the symmetrical anhydride method for the following protected amino acids: Boc-Arg(Tos), Boc-Pro, and Boc-Gly; they were coupled at a 6-fold excess. The other amino acids and acylating agents were coupled by the DCC–HOBT method.[34] The syntheses were performed on an automatic peptide synthesizer Peptomat and every coupling was assessed for completion on a resin sample with ninhydrin.[35] If necessary, couplings were repeated and, if still unsatisfactory, aclyated with a 10-fold excess of acetic anhydride. Peptides were cleaved from the resin by liquid HF, in a Kel-F/Teflon instrument, together with the side-chain-protecting groups. Twenty milliliters of HF were distilled per 1 g of peptide resin; 1 mL of anisole and 150 μL of ethyl sulfide were added as scavengers. The cleavage reaction was carried out at 0 °C during 1 h, the liquid HF was then flushed out with a stream of dry N_2. The residue was dried in vacuo over solid KOH, for the first 15 min with a water pump and then for an additional 15 min with a mechanical pump in order to remove the last traces of HF and of volatile scavengers. The residue was washed twice with dry Et_2O and then twice with 20% aqueous acetic acid to extract the peptides. Peptides 1–11 (odd numbers) were cyclized by the addition of 400 mg of $K_3Fe(CN)_8$/mequiv of initially utilized resin. After 1 h of stirring at room temperature, the crude peptide extracts were lyophilized and subsequently subjected to gel filtration on Sephadex LH-20 (1.5 cm × 35 cm) and eluted with DMF. The peptide-containing fractions detected by UV absorption during the elution and also by TLC were combined, partially evaporated in vacuo, and lyophilized after dilution with water. The lyophilisate was dissolved in water and was then loaded onto a reversed-phase column [Michel-Miller columns, 1.5 cm × 30 cm (medium) or 0.8 × 20 cm (small)]; the stationary phase was 30 μm nucleosil-C18 (Macherey-Nagel, Darmstadt, Germany). Peptides were eluted with CH_3CN gradients in a buffer of 0.1% aqueous TFA. Peptide-containing fractions were detected by spotting the individual fractions on TLC and assessing their purity by analytical HPLC. Pure fractions were pooled, partially evaporated, diluted with water, and lyophilized twice. Peptide purity was assessed in TLC using the following media: A (butanol/AcOH/H_2O = 5:2:3) and B (1-butanol/AcOH/H_2O/pyridine = 30:6:20:12). Visualization was by UV quenching and a modified Reindel–Hoppe procedure.[36] The absence of free sulfhydryls was confirmed with Ellman's reagent.[37] Peptides were accepted as pure if homogeneity was achieved in TLC and the product peak in HPLC was equal to or greater than 98% of the total peaks. HPLC analyses were carried out on a Waters M-45 system, equipped with a UV monitor at 217 nm, and with a linear gradient of 0%–85% of CH_3CN in aqueous 0.05% TFA on Vydac 218TP-104 analytical reversed-

(31) Shulz, H.; du Vigneaud, V. *J. Med. Chem.* **1966**, *9*, 647.
(32) Vavrek, R. J.; Ferger, M. F.; Allen, G. A.; Rich, D. H.; Blomquist, A. T.; du Vigneaud, V. *J. Med. Chem.* **1972**, *15*, 123.
(33) Nestor, J. J.; Ferger, M. F.; du Vigneaud, V. *J. Med. Chem.* **1975**, *18*, 284.
(34) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788.
(35) Kaiser, J. E.; Colescott, R. L.; Bossinger, C. A.; Cook, P. I. Anal. Biochem. **1970**, *34*, 595.
(36) von Arx, E.; Faulpel, M.; Brugger, M. *J. Chromatogr.* **1976**, *120*, 224.
(37) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.

phase columns. FAB-MS analyses were carried out on a Hewlett-Packard 5988A quadruple mass spectrometer, equipped with a Xenon–FAB sample port, with peptide samples in a thioglycolate matrix. The reference samples of VP were of clinical grade (Pitressin, Parke-Davis), and ^{3}H-VP was obtained from Amersham-Searle with a specific radioactivity of 68.5 Ci/mmol. The partition coefficient of the radioactive peptides were determined by mixing with a magnetic stirrer 100 000 cpm of peptide in a 20-mL mixture of equal parts of H_2O and 1-octanol at room temperature for 30 min. After phase separation, 0.5–5-mL samples of each phase were withdrawn and analyzed for radioactivity. Partitioning behavior is expressed as the log of the octanol/water partitioning coefficient P.

Biological Activities. The binding potencies of all compounds were assessed by the binding assay on rat liver membranes.[16] Compounds **8** and **15** were assayed for V_2 specificity on the adenylate cyclase activity of LLCPK cells[26] and for the binding properties with the same cell type.[27] The two compounds **8** and **15** were also assayed for their potency for phospholipase C activation on the V_1 receptor expressing WRK cells.[25]

Cyclo-H-Cys-Tyr-Phe(NO_2)-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 ([Phe(NO_2)^3]VP, 1). The amino acids Boc-Gly, Boc-Arg(Tos), Boc-Pro, Boc-Cys(MeOBzl), Boc-Asn, Boc-Gln, and Boc-Phe(NO_2) were added sequentially to 1.5 g of BHA-resin. After the last coupling, the resin was divided and one part (smaller) was acylated with Boc-Tyr(Cl_2Z) and Boc-Cys(MeOBzl). After HF cleavage, cyclization, and purification, 32 mg of pure [Phe(NO_2)^{3}VP (**1**) was obtained.

Cyclo-H-Cys-Tyr-Phe(N_3)-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 ([Phe(N_3)]^{3}VP, 2). Ten milligrams of the above peptide **1** was hydrogenated in 1 mL of 2 N HOAc at ca. 8 atm over Pd/C (0.8 mg) for 1 h.[20] The catalyst was filtered; the vial and the filter were rinsed with 1.5 mL of 1 N HOAc containing 0.1% β-mercaptoethanol. After lyophilization (twice, or until no more mercaptan odor was present), the product was dissolved in 1 mL of 0.1 N HCl at 0 °C. Twenty microliters of freshly prepared 1 N $NaNO_2$ was added, with continuous stirring, until a positive iodine–starch test resulted. Excess nitrite was destroyed by addition of 20 μL of 1 N sulfamic acid, and after 10 min, the azide was formed by addition of 25 μL of 1 N NaN_3. The reaction mixture was diluted with 5 mL of water, buffered with 2 N $AcONH_4$, and loaded onto a small reversed-phase column. After elution, fraction pooling, and lyophilization, 5.7 mg of pure **2** was obtained.

Cyclo-H-Cys-D-Tyr-Phe(NO_2)-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 ([D-Tyr^2,Phe(NO_2)^3]VP, 3). The balance of the above heptapeptide resin was terminated with Boc-D-Tyr(Cl_2Z) and Boc-Cys(MeOBzl). After HF cleavage, cyclization, and purification, 75 mg of pure **3** was obtained.

Cyclo-H-Cys-D-Tyr-Phe(N_3)-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 ([D-Tyr^2,Phe(N_3)^3]VP, 4). Peptide **3** (10 mg) was treated identically to peptide **1** (above). After purification and lyophilization, 2.9 mg of pure **4** was obtained.

Cyclo-(β-mercaptopropionyl)-Phe(NO_2)-Phe-Val-Asn-Cys-Pro-Lys-D-Tyr-NH_2 ([Mpa^1,Phe(NO_2)^2,Val^4,Lys^8,D-Tyr^9]VP, 5). To 2.6 g of BHA-resin (0.92 mequiv/g NH_2) were acylated the protected amino acids Boc-D-Tyr(Cl_2Z), N^α-Boc-N^ϵ-Cl_2Z-Lys, Boc-Pro, Boc-Cys(MeOBzl), Boc-Asn, Boc-Val, and Boc-Phe. Approximately 25% of the heptapeptide resin was removed and the rest was acylated with Boc-Phe(NO_2). This resin was split into four equal parts and one part was acylated with S-Boc-β-mercaptopropionic acid. After HF cleavage, cyclization, and LH-20 purification, 122 mg of crude peptide were obtained, producing after reversed-phase chromatography and lyophilization 31 mg of pure **5**.

Cyclo-(β-mercaptopropionyl)-Phe(N_3)-Phe-Val-Asn-Cys-Pro-Lys-D-Tyr-NH_2 ([Mpa^1,Phe(N_3)^2,Val^4,Lys^8,D-Tyr^9]VP, 6). A 9,2-mg sample of the above peptide **5** was hydrogenated, diazotized, and transformed to the azido peptide **6** according to the method for **2**. After final purification, 5.3 mg of pure **6** was obtained. In an alternative synthesis, the same peptide was prepared by coupling 8 mmol of N^α-Boc-Phe(4′-NHZ)[24] to 2.08 mequiv of the protected C-terminal heptapeptide H-Phe-Val-Asn-Cys-Pro-Lys-D-Tyr-BHA, with the resulting octapeptide being separated in six equal portions, one of which was acylated with S-(4-methylbenzyl)-β-mercaptopropionic acid. After cleavage, cyclization, gel filtration over LH-20, and lyophilization, the azido function was introduced into the crude peptide by diazotation in 10 mL of 0.1 N HCl at 0 °C with 350 μL of fresh 1 N $NaNO_2$, followed by 350 μL of 1 N sulfamic acid and finally 500 μL of 1 N NaN_3. The crude peptide was buffered with NH_4OAc and directly loaded on a reversed-phase column (medium size). After purification, 31.1 mg of pure **6** was obtained.

Cyclo-(β,β-dimethyl-β-mercaptopropionyl)-Phe(NO_2)-Phe-Val-Asn-Cys-Pro-Lys-D-Tyr-NH_2 ([Dmpa^1,Phe(NO_2)^2,Val^4,Lys^8,D-Tyr^9]VP, 7). A second portion of the octapeptide resin prepared in the method for **5** was acylated with β,β-dimethyl-S-(4-methylbenzyl)-β-mercaptopropionic acid. After cleavage, cyclization, and gel filtration, 101 mg of crude product was obtained, yielding after reversed-phase purification 25 mg of **7**.

Cyclo-(β,β-dimethyl-β-mercaptopropionyl)-Phe(N_3)-Phe-Val-Asn-Cys-Pro-Lys-D-Tyr-NH_2 ([Dmpa^1,Phe(N_3)^2,Val^4,Lys^8,D-Tyr^9]VP, 8). A portion of peptide **7** (6.2 mg) was transformed to azido analogue **8** according to the method for **2**, producing 3.5 mg of pure product. In the alternative synthesis, a portion of the peptide resin described in the procedure for **6** was acylated with the N-terminal acylation moiety of **7**. After cleavage, cyclization, azide transformation, and purification, 75 mg of pure **8** was obtained.

Cyclo-(β,β-diethyl-β-mercaptopropionyl)-Phe(NO_2)-Phe-Val-Asn-Cys-Pro-Lys-D-Tyr-NH_2 ([Depa^1,Phe(NO_2)^2,Val^4,Lys^8,D-Tyr^9]VP, 9). A portion of the octapeptide resin prepared during the synthesis of **5** was acylated with β,β-diethyl-S-(4-methylbenzyl)-β-mercaptopropionic acid. After cleavage, cyclization, and gel filtration, 50 mg of crude product was obtained and purified by preparative reversed-phase chromatography to yield 12.3 mg of pure **9**.

Cyclo-(β,β-diethyl-β-mercaptopropionyl)-Phe(N_3)-Phe-Val-Asn-Cys-Pro-Lys-D-Tyr-NH_2 ([Depa^1,Phe(N^3)^2,Val^4,Lys^8,D-Tyr^9]VP, 10). Peptide **9** (7.3 mg) was transformed to peptide **10** according to the method for **2**. After purification, 3.2 mg of **10** was obtained with the first synthesis variant. In the second variant, a portion of the octapeptide resin from the synthesis of **6** was acylated with the diethyl acylating moiety mentioned in the method for **9**. After cleavage, cyclization, azide formation, and purification, 37.8 mg of **10** identical to that synthesized with **9** was obtained.

Cyclo-(β,β-pentamethylene-β-mercaptopropionyl)-Phe(NO_2)-Phe-Val-Asn-Cys-Pro-Lys-D-Tyr-NH_2 ([Ppa^1,Phe(NO_2)^2,Val^4,Lys^8,D-Tyr^9]VP, 11). The last portion of the nitrophenylalanyl peptide resin prepared during the synthesis of **5** was acylated with 1-[(4-methylbenzyl)thio]-1-(carboxymethyl)-cyclohexane, cleaved, cyclized, and purified. Peptide **11** (28 mg) was recovered as pure product.

Cyclo-(β,β-pentamethylene-β-mercaptopropionyl)-Phe(N_3)-Phe-Val-Asn-Cys-Pro-Lys-D-Tyr-NH_2 ([Ppa^1,Phe(N_3)^2,Val^4,Lys^8,D-Tyr^9]VP, 12). Peptide **11** (6.3 mg) was transformed according to the procedure for peptide **2**, producing 2.9 mg of pure **12**. In the alternative synthesis, a portion of the resin described for peptide **6** was acylated with 1-[(4-methylbenzyl)thio]-1-(carboxymethyl)cyclohexane, producing after cleavage, cyclization, azide transformation, and purification 51.8 mg of pure **12** identical to that obtained with **11**.

(Nitrophenylacetyl)-D-Tyr(OEt)-Phe-Val-Asn-Abu-Pro-Arg-Arg-NH_2 ([Npac^1,D-Tyr(OEt)^2,Val^4,Abu^6,Arg^9]VP, 13). On 2.5 g of BHA-resin (0.43 mequiv/g) was built up the linear peptide sequence with the already mentioned protected amino acids Boc-Abu (α-aminobutric acid) and Boc-D-Tyr(OEt). Before coupling of phenylacetic acid, the N-terminal acylating moiety, the resin was separated into four equal portions and one was acylated. After cleavage, no cyclization was performed (linear peptide). After purification, 11.4 mg of the precursor peptide **13** was obtained.

(Azidophenylacetyl)-D-Tyr(OEt)-Phe-Val-Asn-Abu-Pro-Arg-NH_2 ([Apac^1,D-Tyr(OEt)^2,Val^4,Abu^6,Arg^9]VP, 14). Peptide **13** (5.3 mg) was transformed according to the method for peptide **2** into peptide **14**, producing 2.5 mg of pure peptide.

Cyclo-(β,β-pentamethylene-β-mercaptopropionyl)-Tyr(OMe)-Phe-Val-Asn-Cys-Pro-Lys-D-Tyr-NH_2 ([Ppa^1,Tyr(OMe)^2,Val^4,Lys^8,D-Tyr^9]VP, 15). The heptapeptide resin prepared for the synthesis of **5** was acylated with Boc-Tyr(OMe)

and finally with 1-[(4-methylbenzyl)thio]-1-(carboxymethyl)-cyclohexane, cleaved, cyclized, and purified. Peptide 15 (35 mg) was recovered as pure product after purification.

Radioiodination of Peptides. A 0.2 N AcOH solution (50 μL) containing 20 μg of 8 was placed in a microfuge vial (polyethylene, 250 μL) coated with 5 μg of Iodogen.[38] After the addition of ca. 1 mCi of $Na^{125}I$ (Amersham, not completely carrier free) in 10 μL of aqueous solution, the vial was centrifuged and left for 30 min. With a disposable syringe containing 200 μL of 1 N KI and Na_2SO_3 the vial content was aspirated and rinsed twice with distilled water. The syringe content was injected onto a C-1.8 Sep-pack cartridge (Waters), washed with 5 mL of water and eluted with a gradient of 20%-80% acetonitrile in aqueous 0.05% trifluoroacetic acid. The radioactive fraction containing ^{125}I-8 was collected, pooled, partially evaporated, diluted with water, and injected onto reversed-phase analytical HPLC column, eluted with the same gradient. In the selected main fraction, ca. 300 μCi of labeled 8 were collected. The specific radioactivity of ^{125}I-8 was determined by Scatchard analysis and total binding capacity,[39] generally in the range of 900-400 Ci/mmol and adjusted hereafter according to the decay of ^{125}I. The radiolabeling of the other compounds to ^{125}I-7, ^{125}I-9, and ^{125}I-15 was carried out in an identical manner.

Acknowledgment. This work was supported by generous grants from the Quebec Chapter of the Canadian Kidney Foundation. D.B. holds a combined studentship from FRSQ, Ciba-Geigy, and the Faculty of Medicine. This work contains parts of the M.Sc. theses of D.B. and S.G. We are greatly indebted to Mrs. M.-R. Lefebvre for skillful and dedicated assistance, to Mr. R. Laprise for operating the peptide synthesizer, to Mrs. S. Leblond for preparing this report, and to Dr. D. Shapcott for proofreading. We would like to express our gratitude especially to the following researchers who measured the biological activities of compounds 8 and 15: Dr. G. Guillon, CCIP Montpellier, France, for phospholipase C stimulation; Drs. P. Lin and P. Golzman, McGill University, Montreal, Canada, for the determination of adenylate cyclase activity; and Drs. S. Larivière and H. H. Zingg, McGill University, Montreal, Canada, for the binding study on V_2-receptor preparations.

(38) Escher, E. In *Synthesis and Applications of Isotopically Labeled Compounds*; Duncan, W. P., Susan, A. B., Eds.; Elsevier-North Holland, Amsterdam, 1983; p 231.
(39) Bürgisser, E. *J. Recept. Res.* **1984**, *4*, 357.

New Conformationally Restricted ^{99m}Tc N_2S_2 Complexes as Myocardial Perfusion Imaging Agents

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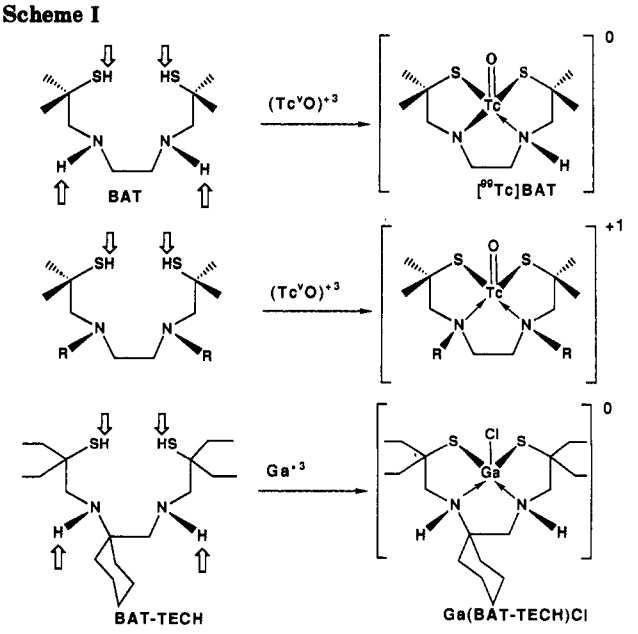
In developing ^{99m}Tc complexes as potential myocardial imaging agents, a new series of ligands based on a conformationally restricted N_2S_2 system were investigated. Using piperazine or homopiperazine as the starting material, two N_2S_2 ligands (**4a** and **4b**) with additional conformation restriction between the two nitrogen donor atoms were synthesized. The ^{99m}Tc complexes were prepared by a direct labeling method with tin(II) tartrate as the reducing agent for [^{99m}Tc]pertechnetate. The resulting ^{99m}Tc complexes were purified through a sulfonpropyl Sephadex column and further purified by HPLC with a reverse-phase column eluting with a solvent system of acetonitrile/buffer. Biodistribution studies in rats showed initial uptake in the heart (0.21%, 0.42% dose/order for [^{99m}Tc]**4a** and **4b** at 2 min postinjection). Carrier-added preparation of [^{99m}Tc]**4b** was successful. NMR, IR, UV, crystallographic, and elemental analysis of the [^{99}Tc]**4b** complex suggest that it contains a $Tc^{V}O^{3+}$ center core and is 1+ charged. The results suggest that this series of 1+ charged ^{99m}Tc complexes may have potential as myocardial imaging agents, and further study of the complexes is warranted.

Imaging of regional myocardial perfusion to separate ischemic from infarcted tissue is one of the most important diagnostic procedures for nuclear cardiology. The procedure is commonly performed with ^{201}Tl ($T_{1/2}$ = 73 h, 70-90 keV) in conjunction with a rest and stress test.[1,2] Based on the size/charge ratio, it is generally accepted that the thallium ion (Tl^{1+}) is a close analogue of potassium (K^+), and the ions are transported across the cell membrane by a Na^+/K^+-ATPase pump.[3] After iv injection, the agent displays a high initial first-pass extraction (>85%) into the myocardium, and distribution reflects regional myocardial blood flow. However, there are several disadvantages associated with ^{201}Tl. First of all, the γ-rays (70-90 keV, Hg X-rays) are too low for optimal γ-detection; a significant amount of the γ-rays is attenuated. Scattering also increases the noise level of the final images, both of which prevent quantitative interpretation of the images. Secondly, the physical half-life is too long; therefore, only a limited quantity (1-3 mCi) can be used for routine procedure. Finally, the isotope is relatively expensive. For these reasons, an agent labeled with ^{99m}Tc and possessing both a shorter half-life ($T_{1/2}$ = 6 h) and an optimal γ-ray energy (140 keV) would be preferable.

Scheme I



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0022-2623/92/1835-0157$03.00/0