

clopropyl CH), 1.32 (dt, $J = 2.7$ and 13.4 Hz, 1 H, C-8 H), 1.4-1.5 (m, 1 H, C-15 H), 1.61 (dt, $J = 3.3$ and 13.6 Hz, 1 H, C-8 H), 1.7-1.8 (m, 1 H, C-7 H), 1.92 (dq, $J = 2.3$ and 12.9 Hz, 1 H, C-17 H), 2.11 (dq, $J = 3.2$ and 11.7 Hz, 1 H, C-16 H), 2.21 (dt, $J = 4.4$ and 12.2 Hz, 1 H, C-15 H), 2.35 (d, $J = 6.5$ Hz, 2 H, NCH₂-cyclopropyl), 2.5-2.7 (m, 2 H, C-10 α H and C-16 H), 3.00 (d, $J = 18.2$ Hz, 1 H, C-10 β H), 3.06 (d, $J = 5.5$ Hz, 1 H, C-9 H), 3.15-3.25 (m, 1 H, C-6 H), 3.83 (s, 3 H, CO₂CH₃), 4.14 (d, $J = 13.6$ Hz, 1 H, allylic CH), 4.45 (d, $J = 6.6$ Hz, 1 H, C-5 H), 4.48 (d, $J = 13.7$ Hz, 1 H, allylic CH), 5.7 (br s, movable, 1 H, OH), 5.80 (s, 1 H, *E*-vinylic CH), 6.26 (s, 1 H, *Z*-vinylic CH), 6.53 (d, $J = 8.2$ Hz, 1 H, C-1 H), 6.70 (d, $J = 8.2$ Hz, 1 H, C-2 H); ¹³C NMR (CDCl₃) δ 3.79, 3.90, 9.44, 22.57, 23.63, 29.74, 30.35, 43.77, 47.66, 52.15, 59.07, 62.16, 68.09, 70.09, 79.04, 94.48, 117.07, 118.60, 123.82, 127.24, 131.51, 137.69, 139.84, 141.99, 167.63; FTIR (KBr) 3700-3100, 2948, 1718, 1636, 1452, 1321, 1097 cm⁻¹. FABMS calcd for [M + H]⁺ C₂₅H₃₂NO₆ 442.2226, obsd 442.2244. Anal. Calcd for C₂₅H₃₁NO₆: C, H, N.

Opioid Receptor Binding. The binding assay was carried out essentially as described by Lin and Simon.¹⁵ Crude membranes were prepared from bovine striatum and stored at -70 °C until needed. The labeled ligands used were [³H]bremazocine (18.5 Ci/mmol) for total opioid receptors and [³H]DAGO (33.8 Ci/mmol) for μ -receptors. The concentrations of labeled ligand were 0.5 nM for [³H]bremazocine and 1 nM for [³H]DAGO. Five concentrations of each drug to be tested were used for competition against labeled ligands. Nonspecific binding was measured in the

presence of 10 μ M naloxone. The samples were incubated in 50 mM Tris-HCl or potassium phosphate buffer, pH 7.4, containing 1 mM EDTA for 45 min at 25 °C. Samples were rapidly filtered through Whatman GF/B filters, rinsed twice with 4 mL of cold buffer, dried, and counted in a toluene-based scintillation cocktail in a scintillation counter.

Values reported are averages of duplicate determinations (± 10 -15%) or means of triplicates with standard deviations of 15% or less.

Irreversibility and Protection Studies. Membrane preparations were incubated with drug to be tested for 45 min at 25 °C. For protection studies naloxone was added at a concentration of 1 μ M (recovery was checked with naloxone alone). After incubation, the samples were diluted 4-fold with buffer and centrifuged for 15 min at 20000g. The supernatant was removed and the pellet was resuspended in 3 times the original volume of buffer and incubated at 37 °C for 15 min, centrifuged again, and resuspended in the original volume of buffer. A binding assay using [³H]bremazocine (0.5 nM) was carried out as described above.

Acknowledgment. We acknowledge the support of this work by the National Institute on Drug Abuse through research Grants DA-3933 to W. L. Nelson and DA-0017 to E. J. Simon.

Registry No. 9, 124154-46-7; 10, 117332-64-6; 11, 124154-47-8; 12, 124154-48-9; 13, 124154-49-0; 13 ketone, 96453-52-0; 14, 49625-89-0; 15, 124154-50-3; 16, 124154-51-4; 17, 124154-52-5; 18, 124266-28-0; methacryloyl chloride, 920-46-7; methyl α -(bromo-methyl)acrylate, 4224-69-5.

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Functionalized Congener Approach for the Design of Novel Muscarinic Agents. Synthesis and Pharmacological Evaluation of *N*-Methyl-*N*-[4-(1-pyrrolidinyl)-2-butynyl] Amides

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A functionalized congener approach was used to design ligands for muscarinic cholinergic receptors (mAChRs). A series of ω -functionalized alkyl amides of *N*-methyl-4-(1-pyrrolidinyl)-2-butyamine (**22**) were prepared as functionalized analogues of UH 5 [*N*-methyl-*N*-[4-(1-pyrrolidinyl)-2-butynyl]acetamide], a muscarinic agonist related to oxotremorine. Intermediate **22** was coupled to a series of Boc-protected ω -amino acids, and the resulting amides were deprotected and acylated. Intermediate **22** was also acylated with succinic anhydride and derivatized. The synthetic intermediates and final compounds were evaluated *in vitro* for their effects on the turnover of phosphatidylinositides in SK-N-SH human neuroblastoma cells that express m₃AChRs, and on the production of cyclic AMP in NG108-15 neuroblastoma x glioma cells that express only m₄AChRs. The displacement of [³H]-*N*-methylscopolamine was also measured in membrane preparations from each of these cell lines. Conjugates of glycine and β -alanine were agonists at m₄AChRs, having little or no activity at m₃AChRs. The potency in displacement of [³H]-*N*-methylscopolamine from both m₃- and m₄AChRs generally increased with increasing chain lengths of the ω -aminoalkyl congeners. The amides of 7-aminoheptanoic acid and 8-aminooctanoic acid, and their Boc-protected derivatives, had comparable affinities to UH 5 ($K_i = 5.0$ and 4.5 μ M at m₃AChRs and at m₄AChRs, respectively) at both receptors but lacked any agonist effects.

The potential therapeutic benefit of central muscarinic cholinergic agonists in the treatment of Alzheimer's disease (AD) has recently received considerable attention.^{1,2} However, the currently available therapeutic cholinergic agents suffer from serious side effects, toxicity, and narrow therapeutic windows.^{1,2} Recent molecular biological studies of muscarinic cholinergic receptors^{3,4} (mAChRs) have now raised the possibility of designing subtype-specific agonists that, by virtue of their improved selectivity, should be devoid of many of these side effects. Although several

selective muscarinic antagonists have been defined pharmacologically, relatively few selective agonists are known. In the current work we describe a new series of muscarinic agents and their pharmacological properties.

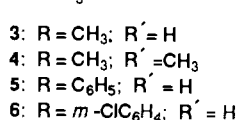
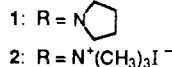
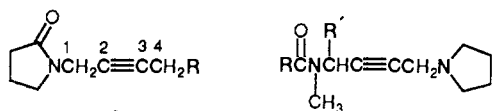
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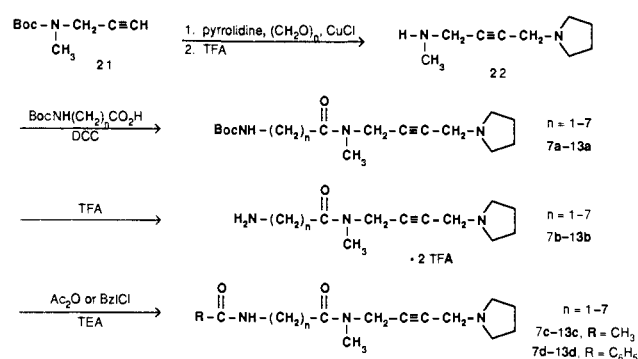
Recent studies on the molecular biology, regional distribution, affinity states, and second messenger coupling of mAChRs has complicated the classification of mAChR subtypes. This has further complicated the definition of mAChR selectivity and the methodologies used to determine the subtype specificity of muscarinic agents. The earliest definition of mAChR subtypes was defined *pharmacologically* on the basis of their affinities for the nonclassical antagonist pirenzepine.⁵ The M₁AChRs have a high affinity for pirenzepine, whereas M₂AChRs have a low affinity.⁶ Recent studies have demonstrated the existence of five distinct genes for mAChRs, each coding for a separate protein, which define five new subtypes, m₁AChR through m₅AChR.^{3,7-9} Pharmacologically, m₁- and m₄AChRs demonstrate a high affinity for pirenzepine, m₃- and m₅AChRs have intermediate affinity, and the m₂AChRs have low affinity.^{3,7} These subtypes have been correlated to two of the several intracellular biochemical processes which are activated by mAChRs. The m₁-, m₃-, and m₅AChRs couple preferentially to phosphatidylinositol (PI) turnover, whereas m₂- and m₄AChRs couple preferentially to adenylate cyclase inhibition.^{7,10} The lack of well-defined agonists or antagonists for these mAChR subtypes has prompted us to characterize the selectivity of potential muscarinic agents by determining the binding affinity and activation of second messengers in systems containing exclusively one mAChR. We have used two cultured cell lines that each express a specific mAChR subtype: SK-N-SH human neuroblastoma cells, which express only m₃AChRs coupled to PI turnover,^{11,12} and NG108-15 neuroblastoma x glioma hybrid cells, which express only m₄AChRs coupled to the inhibition of adenylate cyclase.^{8a,12} The purpose of this study was to design selective muscarinic agonists as pharmacological tools for the study of mAChRs, and as potential therapeutic agents for the treatment of the cognitive impairments associated with AD.

An *N*-(4-amino-2-butynyl) amide substructure is found in certain muscarinic agonists, most notably the potent and moderately selective (M₂ > M₁) central muscarinic agonist oxotremorine (1).¹³ Several reviews on the SAR of mus-



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Scheme I

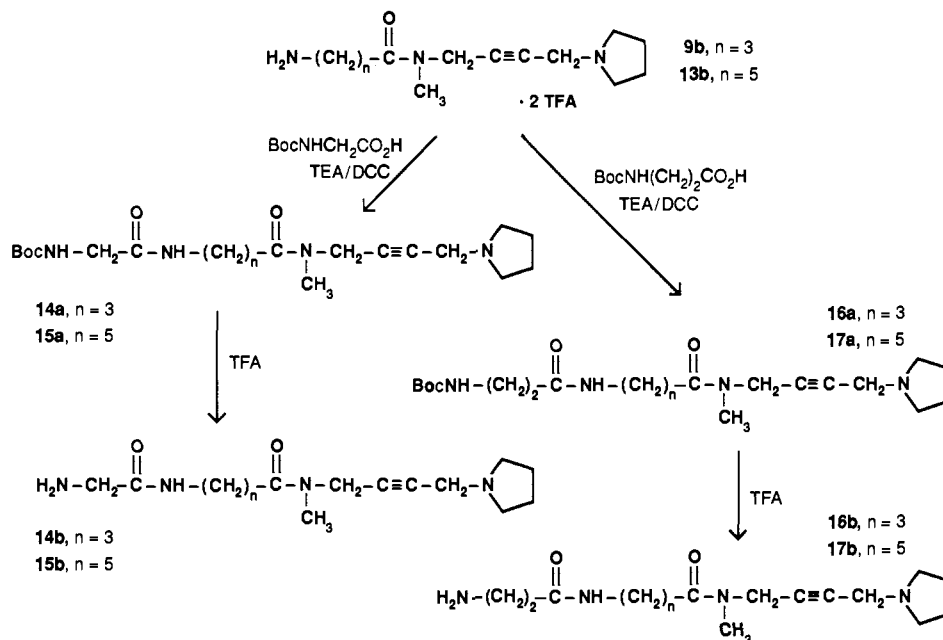


carinic agents similar in structure to 1 can be found.¹⁴⁻¹⁶ While modification at either end of the linear central chain often has profound effects on biological activity, an open-ring analogue of 1, UH 5 (3), maintains agonist activity and is nearly equipotent to 1.^{14a,17} The α -methyl analogue of UH 5, BM 5 (4), is also a potent muscarinic agent which has been described as a partial agonist at postsynaptic mAChRs and an antagonist at presynaptic mAChRs.¹⁸ This profile of muscarinic activity may be useful for the treatment of AD.^{18,19}

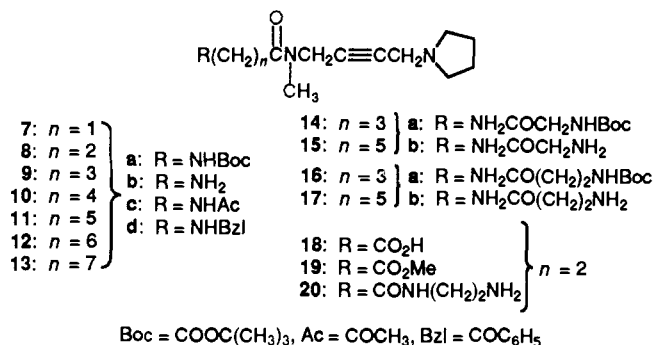
We chose to synthesize a series of UH 5 homologues using the "functionalized congener" approach²⁰ to drug design. This approach has been successfully established in the field of adenosine receptor ligands²¹ and catecholamines.²² By this approach, a chain terminating in a functional group (e.g., an amine or carboxylic acid) is appended to a known receptor ligand at a site that allows modification with retention of biological activity. The chain-extended functional group may enhance receptor affinity and selectivity and serves as a site for further functionalization to develop irreversible inhibitors, prodrugs, or labeled receptor probes.²³ This study will explore the feasibility of this drug design approach through modifications of a CNS-active muscarinic pharmacophore. We initially focused on a series of *N*-methyl-*N*-[4-(1-pyrrolidinyl)-2-butynyl] amides as homologues of 3. An alkyl spacer group separating the functional group from the central pharmacophore was chosen in favor of a bulkier

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Scheme II



aryl group spacer on the basis of our initial binding studies of compounds 3–6. (See Pharmacology below.) Aryl amides 5 and 6 had significantly lower affinities than the alkyl amides 3 and 4, suggesting an intolerance for bulky groups near the amide carbonyl. Thus, a homologous set of alkyl amide linked functionalized congeners (7–13) were syn-

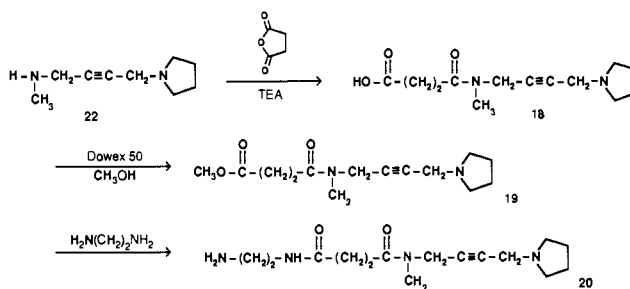


thesized with an amine or modified amine variably spaced from the parent pharmacophore for SAR analyses. Several bis(amino acid) congeners (14–17) and a series of succinamide congeners (18–20) were also prepared. These compounds were tested in competitive binding assays and for the activation of second messengers in cell lines expressing unique mAChR subtypes as described above.

Results and Discussion

Chemistry. A Mannich-type condensation²⁴ of pyrrolidine, paraformaldehyde, and Boc-*N*-methylpropargylamine (21), followed by deprotection of the resultant product, gave the pivotal intermediate *N*-methyl-4-(1-pyrrolidinyl)-2-butyamine (22) in 57% overall yield. (See Scheme I.) Coupling of 22 to the appropriate Boc-protected *n*-alkylamino acid using DCC gave compounds 7a–13a, which served as both synthetic intermediates and potential muscarinic agents. Deprotection with trifluoroacetic acid (TFA) gave the primary amines 7b–13b, which were isolated and tested as the bis(TFA) salts.

Scheme III



The *N*-acetyl derivatives, 7c–13c, were synthesized from 7b–13b, respectively, to determine if the primary amines could be acylated without significant loss of biological activity. Benzoyl derivatives 9d and 11d were made and tested to determine the tolerance of bulk at the distal end of the functionalized chain.

Compounds 9b and 11b were coupled to a second urethane-protected ω -amino acid and deprotected as above to give congener series 14a–17a and 14b–17b. (See Scheme II.) The succinoyl amide zwitterion 18 was prepared from 22 and succinic anhydride (Scheme III) to provide an alternate functional group (CO₂H) that is readily derivatized. This intermediate was esterified to give 19, which was then heated with ethylenediamine to yield congener 20.

The ¹H NMR of each *N*-methyl-*N*-[4-(1-pyrrolidinyl)-2-butyryl] amide revealed, in most cases, the presence of *Z* and *E* amide conformers in a ratio of approximately 2/1 (undetermined assignment of isomers) by the appearance of two singlets each for the methyl group and for the α -methylenes of the amide nitrogen. The methylene α to the amide carbonyl also gave two distinct signals. The existence of two conformers separated by a relatively high-energy rotational barrier complicates the correlation of functional group separation distances, based on a hypothetical active conformation, with biological activity. However, previous studies of a series of structurally related compounds^{25,26} suggested that the *Z* con-

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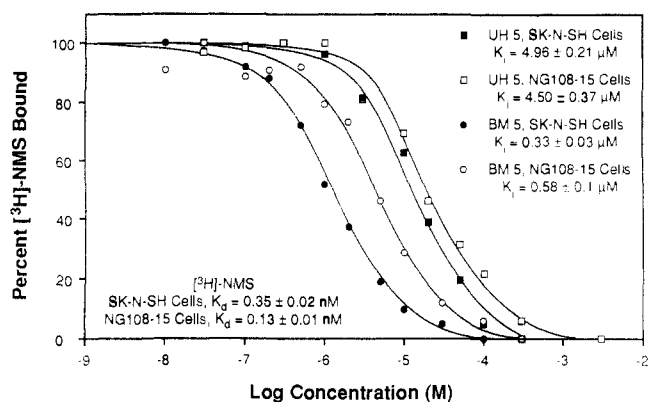


Figure 1. Affinity of UH 5 and BM 5 for SK-N-SH and NG108-15 cells.

formation predominates and is primarily associated with their muscarinic activity.

Interestingly, a unique conformation for compounds **12c** and **13c** in chloroform is suggested by ^1H NMR. The signals for the hydrogens on the carbons α to the pyrrolidine nitrogen typically appear as singlets in the range of 3.32–3.38 and 2.52–2.59 ppm. For **12c** and **13c**, these signals are each split into two separate singlets with a small (0.05–0.10 ppm) downfield shift. The ratio (2/1) of these signals suggests that one of the tertiary amide conformers is capable of folding back to allow hydrogen bonding between the acetamide hydrogen and the pyrrolidine nitrogen. A similar phenomenon was observed for the amino acid congener **18**.

Pharmacology. The *N*-methyl-*N*-[4-(1-pyrrolidinyl)-2-butynyl] amides were tested for their effects on second messengers and for their inhibition of [^3H]-*N*-methylscopolamine ([^3H]NMS) binding in two separate cultured cell lines. The cells employed were SK-N-SH human neuroblastoma cells, which express only m_3 AChRs coupled to PI turnover, and NG108-15 neuroblastoma x glioma hybrid cells, which express only m_4 AChRs coupled to adenylate cyclase inhibition. The results of these assays are shown in Table I.

The displacement curves of [^3H]NMS binding to membranes from SK-N-SH cells and NG108-15 cells for the comparative standards UH 5 (**3**) and BM 5 (**4**) are shown in Figure 1. These data were analyzed by using the LIGAND computerized characterization approach²⁷ and were found to fit a one-site binding model. The K_i values were calculated by using the Cheng–Prusoff equation²⁸ from the dissociation constants for [^3H]NMS binding in each system. These K_i values demonstrate that the affinities of either **3** or **4** for receptors from SK-N-SH cells are not significantly different than those for receptors from NG108-15 cells. Thus, neither of these compounds is selective for the displacement of [^3H]NMS from these receptors. Furthermore, the affinity of **4** in each system is approximately 1 order of magnitude greater than that of **3**.

For purposes of screening, the inhibition of [^3H]NMS binding to membranes from each cell line was compared by using a single concentration (100 μM) of each compound and are reported in Table I as the percent displacement of [^3H]NMS. The degree of displacement of [^3H]NMS

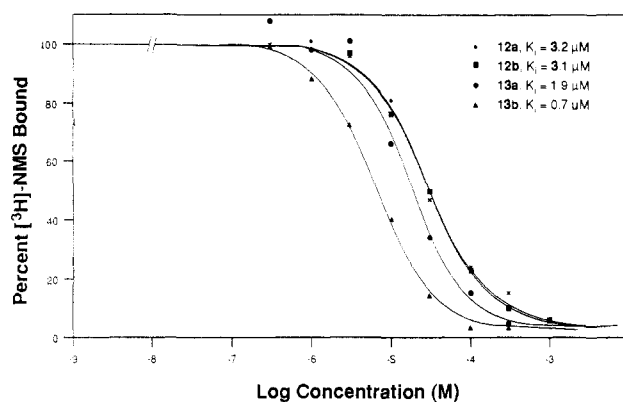


Figure 2. Affinity of **12a**, **12b**, **13a**, and **13b** for NG108-15 cells.

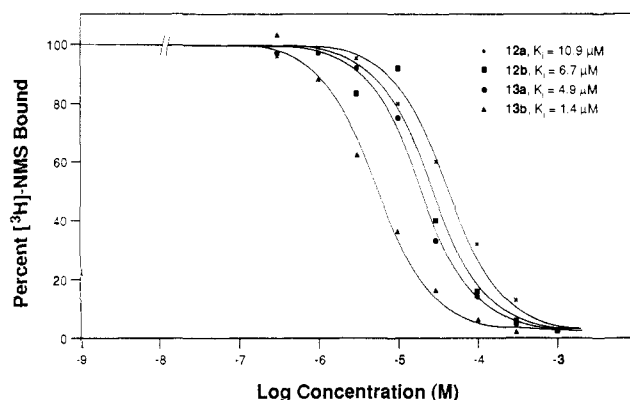


Figure 3. Affinity of **12a**, **12b**, **13a**, and **13b** for SK-N-SH cells.

reflects the relative affinity of the compounds for the specific mAChR subtype present if one assumes parallel binding curves. To determine if these curves were indeed parallel, full binding curves were determined for compounds **12a**, **12b**, **13a**, and **13b** and are shown in Figures 2 and 3. These displacement curves all had Hill coefficients of close to 1, indicative of a single binding site. The IC_{50} values ranged from 5.1 μM for **13b** to 41 μM for **12a** with a 2–4-fold selectivity in the SK-N-SH cell line membranes. While the curves for these four compounds are parallel (curves were fitted as 3 and 4), it should be noted that the comparisons of affinity stated below are based on a single concentration (100 μM).

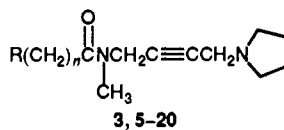
The aryl amides **5**²⁹ and **6** had significantly lower affinities than alkyl amide **3**, suggesting the intolerance of bulky substituents near the amide carbonyl. All of the functionalized alkyl amide congeners showed some inhibition of binding in each system. Moderate selectivity for the SK-N-SH cell membrane receptors was seen for several compounds. The inhibitory activities of the compounds in groups 7–11 (one to five methylene spacers) were significantly lower than that of the parent compound **3**. A trend of increasing affinity was observed as the chain length was lengthened from compounds **10** through **13** (four to seven methylene spacers), with compounds **13a** and **13b** having a greater inhibitory activity at 100 μM than that of **3** in both cell preparations. This increase may suggest a distal antagonist binding region on these receptors at which an extended functionalized chain interacts favorably. Acetylation of **11b**, **12b**, and **13b** significantly decreased the displacement of [^3H]NMS at both m_3 - and m_4 AChRs. Acetylation of the primary amine congeners

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Table I. Characterization and Biological Activity at m_3 and m_4 Receptors of *N*-Methyl-*N*-[4-(1-pyrrolidinyl)-2-butynyl] Amide Derivatives

no.	n	R	formula ^a	% yield	NG108-15 cells		SK-N-SH cells	
					activity, % ^b	affinity, % ^c	activity, % ^d	affinity, % ^e
2					64.1 ± 1.9	96.2 ± 2.1	100	52.1 ± 1.2
3	0	CH ₃	C ₁₁ H ₁₈ N ₂ O·0.25 oxalate ^e	81	63.2 ± 6.0	79.6 ± 2.1	48 ± 3	88.8 ± 3.2
4					36 ± 1.5	97.0 ± 2.0	i	99.0 ± 2.0
5	0	C ₆ H ₅	C ₁₆ H ₂₀ N ₂ O·0.5 oxalate·0.33H ₂ O	99	i	25	8 ± 5	40.0 ± 2.0
6	0	<i>m</i> -ClC ₆ H ₄	C ₁₆ H ₁₉ N ₂ OCl·0.5 oxalate·0.25H ₂ O	100	i	34	4 ± 3	55.0 ± 3.0
7a	1	NHBoc	C ₁₆ H ₂₇ N ₃ O ₃ ·0.5 oxalate	70	7.0 ± 2.9	32.6 ± 1.5	i	40.0 ± 2.1
7b	1	NH ₂	C ₁₁ H ₁₉ N ₃ O·2TFA	96	26.1 ± 1.6	23.5 ± 4.0	i	17.3 ± 3.3
7c	1	NHAc	C ₁₃ H ₂₁ N ₃ O ₂ ·0.5 oxalate·0.33H ₂ O	93	14.1 ± 2.3	24.0 ± 5.4	8 ± 3	12.5 ± 2.8
8a	2	NHBoc	C ₁₇ H ₂₉ N ₃ O ₃ ·0.25 oxalate	97	12.5 ± 2.7	36.3 ± 3.1	i	45.1 ± 2.5
8b	2	NH ₂	C ₁₂ H ₂₁ N ₃ O·2TFA	80	20.8 ± 1.7	15.1 ± 3.5	i	18.1 ± 2.8
8c	2	NHAc	C ₁₄ H ₂₃ N ₃ O ₂ ·oxalate·0.5H ₂ O	79	10.2 ± 4.8	67.7 ± 3.6	i	67.4 ± 3.3
9a	3	NHBoc	C ₁₈ H ₃₁ N ₃ O ₃ ·0.25 oxalate	98	3.5 ± 1.7	24.4 ± 2.5	i	32.1 ± 0.7
9b	3	NH ₂	C ₁₃ H ₂₃ N ₃ O·2TFA	72	3.7 ± 2.0	12.1 ± 2.2	i	20.0 ± 1.5
9c	3	NHAc	C ₁₅ H ₂₅ N ₃ O ₂ ·0.25 oxalate ^f	91	8.0 ± 3.7	19.1 ± 1.8	14 ± 5	15.6 ± 1.6
9d	3	NHBzl	C ₂₀ H ₂₇ N ₃ O ₂ ·0.5 oxalate	95	i	27.7 ± 4.2	i	30.3 ± 2.3
10a	4	NHBoc	C ₁₉ H ₃₃ N ₃ O ₃ ·0.25 oxalate	84	0.5 ± 0.3	26.7 ± 1.6	1 ± 1	32.1 ± 2.1
10b	4	NH ₂	C ₁₄ H ₂₅ N ₃ O·2TFA	94	15.8 ± 2.7	22.4 ± 3.2	i	34.4 ± 0.3
10c	4	NHAc	C ₁₆ H ₂₇ N ₃ O ₂ ·0.5 oxalate	89	5.2 ± 2.2	17.9 ± 7.9	i	29.4 ± 1.0
11a	5	NHBoc	C ₂₀ H ₃₅ N ₃ O ₃ ·0.25 oxalate	95	i	31.9 ± 0.9	i	39.1 ± 2.4
11b	5	NH ₂	C ₁₅ H ₂₇ N ₃ O·2TFA	94	14.3 ± 1.0	37.9 ± 3.2	i	55.6 ± 1.9
11c	5	NHAc	C ₁₇ H ₂₉ N ₃ O ₂ ·0.5 oxalate	96	3.0 ± 1.7	22.7 ± 2.8	1 ± 1	17.4 ± 3.6
11d	5	NHBzl	C ₂₂ H ₃₁ N ₃ O ₂ ·0.5 oxalate	89	i	52.8 ± 8.3	i	51.7 ± 2.4
12a	6	NHBoc	C ₂₁ H ₃₇ N ₃ O ₃ ·0.5 oxalate	84	i	77.9 ± 2.4	i	81.4 ± 2.4
12b	6	NH ₂	C ₁₆ H ₂₉ N ₃ O·2TFA	87	3.0 ± 1.2	72.1 ± 2.7	i	81.9 ± 1.0
12c	6	NHAc	C ₁₈ H ₃₁ N ₃ O ₂ ·0.5 oxalate·H ₂ O ^g	99	6.9 ± 2.9	20.4 ± 5.5	1 ± 2	24.6 ± 3.1
13a	7	NHBoc	C ₂₂ H ₃₉ N ₃ O ₃ ·0.25 oxalate	89	i	89.0 ± 2.5	i	92.1 ± 1.0
13b	7	NH ₂	C ₁₇ H ₃₁ N ₃ O·2TFA	80	i	92.3 ± 3.2	i	93.7 ± 1.8
13c	7	NHAc	C ₁₉ H ₃₃ N ₃ O ₂ ·0.5 oxalate·0.75H ₂ O ^h	93	i	50.4 ± 4.9	i	59.1 ± 0.8
14a	3	NHCOCH ₂ NHBoc	C ₂₀ H ₃₄ N ₄ O ₄ ·0.5 oxalate	91	i	24.4 ± 19.4	i	37.5 ± 2.5
14b	3	NHCOCH ₂ NH ₂	C ₁₅ H ₂₆ N ₄ O ₂ ·2TFA	100	i	31	7 ± 4	47.0 ± 2.0
15a	5	NHCOCH ₂ NHBoc	C ₂₂ H ₃₈ N ₄ O ₄ ·0.5 oxalate	87	i	52.3 ± 1.3	3 ± 1	52.6 ± 2.6
15b	5	NHCOCH ₂ NH ₂	C ₁₇ H ₃₀ N ₄ O ₂ ·2TFA	99	i	57	i	65.0 ± 1.0
16a	3	NHCO(CH ₂) ₂ NHBoc	C ₂₁ H ₃₆ N ₄ O ₄ ·0.5 oxalate	92	i	38.8 ± 13.8	i	39.2 ± 0.8
16b	3	NHCO(CH ₂) ₂ NH ₂	C ₁₆ H ₂₈ N ₄ O ₂ ·2TFA	100	i	29	7 ± 5	40.5 ± 5.5
17a	5	NHCO(CH ₂) ₂ NHBoc	C ₂₃ H ₄₀ N ₄ O ₄ ·0.5 oxalate	95	i	57.3 ± 6.3	i	59.1 ± 0.9
17b	5	NHCO(CH ₂) ₂ NH ₂	C ₁₈ H ₃₂ N ₄ O ₂ ·2TFA ⁱ	98	i	56.8 ± 3.2	7 ± 3	70.5 ± 4.5
18	2	CO ₂ H	C ₁₃ H ₂₀ N ₂ O ₃ ·0.33H ₂ O ^j	65	i	0	i	6
19	2	CO ₂ CH ₃	C ₁₄ H ₂₂ N ₂ O ₃ ·0.5 oxalate·0.67H ₂ O	66	i	7	i	28
20	2	CONH(CH ₂) ₂ NH ₂	C ₁₅ H ₂₆ N ₄ O ₂ ·0.5 oxalate·H ₂ O ^k	71	i	14 ± 2	i	25 ± 5

^a All compounds were analyzed for C, H, and N. The analytical results for those elements were within ±0.4% of the theoretical value except for those formula marked with a footnote. ^b Each compound was tested at 100 μM. Data are reported as the percent inhibition (±standard error) of cyclic AMP relative to a control with no compound added and are the average of three trials, each run in triplicate. i = inactive (<1%). ^c Each compound was tested at 100 μM. Data are reported as the percent displacement (±standard error) of bound [³H]NMS and are the average of two or three trials, each run in triplicate. Data without standard errors were determined on one trial run in triplicate. ^d Each compound was tested at 100 μM. Data are reported as the percent increase in [³H]-*m*yo-inositol (±standard error) relative to 2 (100%) and are the average of two or three trials, each run in triplicate. Data without standard errors were determined on one trial run in triplicate. i = inactive (<1%). ^e Anal. Calcd for C₁₁H₁₈N₂O₂·0.25 oxalate: C, 63.72. Found: C, 63.02. ^f Anal. Calcd for C₁₅H₂₅N₃O₂·0.5 oxalate: C, 59.24. Found: C, 58.62. ^g Anal. Calcd for C₁₈H₃₁N₃O₂·0.5 oxalate·H₂O: H, 8.91. Found: H, 8.46. ^h Anal. Calcd for C₁₉H₃₃N₃O₂·0.5 oxalate·0.75H₂O: H, 9.01. Found: H, 8.64. ⁱ Anal. Calcd for C₁₈H₃₂N₃O₂·2TFA: C, 46.81. Found: C, 47.52. ^j Anal. Calcd for C₁₃H₂₀N₂O₃·0.33H₂O: H, 8.07. Found: H, 7.59. ^k Anal. Calcd for C₁₅H₂₆N₄O₂·0.5 oxalate·H₂O: N, 15.68. Found: C, 13.53.

of 7-10 did not notably decrease binding, and for 8b, acetylation increased binding to greater than 65% at 100 μM. Benzoylation of 9b or 11b had little effect on binding in each preparation.

Di(amino acid) congeners 14 and 16 can be considered analogous to compounds 12 and 13, respectively, with an amide linkage replacing two of the methylene spacers. These compounds, however, had a much lower inhibitory activity than their corresponding analogues at 100 μM. Some selectivity for the SK-N-SH cells was also observed for these di(amino acid) congeners. Similarly compounds 15 and 17 can be considered to be analogous to congeners with eight and nine methylene spacers, respectively. These compounds had lower affinities than the seven-methylene

congeners 13, again showing some selectivity for the SK-N-SH cells. Furthermore, these congeners had higher affinities than the acylated derivatives of their synthetic precursor 11b. Compound 20 can be considered analogous to 12b with a reversed amide linkage replacing two methylene spacers. This compound also had a much lower affinity than the corresponding methylene analogue. The succinic acid congener 18 and its methyl ester 19 bound weakly to mAChRs in both cell lines at 100 μM.

The novel functionalized congeners were screened for biological activity by measuring their effects on the formation of coupled second messengers in the two cultured cell preparations (vide supra) and were compared to the muscarinic agents 2-4. All compounds were tested at a

single screening concentration (100 μ M) and are reported in Table I as percent activities relative to a control. Comparison of activities assumes parallel concentration-effect curves.

Several of the compounds were weak inhibitors of cyclic AMP production in NG108-15 cells (control = no compound), indicating that these compounds are m_4 AChR agonists. Of the compounds tested, compounds **7b** and **8b** were the most active ($26.1 \pm 1.6\%$ and $20.8 \pm 1.7\%$ inhibition relative to a zero control, respectively, at 100 μ M). The inhibition of cyclic AMP accumulation by **7b** at 100 μ M was completely blocked by 1 μ M atropine. The full agonist oxotremorine M (**2**) inhibited cyclic AMP formation by $65 \pm 3\%$ at an equivalent dose in this assay. Comparison of the ratios of affinity to activity suggest that **7b** and **8b** have a greater intrinsic efficacy than **4**. Several other compounds demonstrated some activity ($>10\%$ inhibition of cyclic AMP accumulation at 100 μ M) at m_4 AChRs, including compounds **7c**, **8a**, **8c**, **10b**, and **11b**. Within each family of functionalized congener having the same number of methylenes, the free amine compound generally had greater activity at m_4 AChRs than the Boc, acetyl, or benzoyl derivative. This trend was not attributable to the difference between the salt forms used for the primary amine congeners (TFA) and their functionalized derivatives (oxalates). The TFA salt of **7c** and the 0.5-oxalate salt of **8b** were prepared, and no significant change in activity was measured.

In contrast to the activity at m_4 AChRs, all of the compounds tested were either inactive or only slightly active (5%–15% relative to **2**) in stimulating PI turnover in SK-N-SH cells as m_3 AChRs agonists. Thus, compounds **7b**, **8b**, **8c**, **10b**, and **11b** are weakly selective agonists of m_4 AChRs versus m_3 AChRs, in that they are active at m_4 AChRs ($>10\%$ inhibition of cyclic AMP production) but not at m_3 AChRs. Compounds **12a** and **12b** (six methylenes) and **13a** and **13b** (seven methylene) lacked substantial agonist activity but had relatively high affinities ($>75\%$ displacement at 100 μ M) in both cell lines, indicating that these compounds are nonselective muscarinic antagonists. These compounds inhibited the stimulation of PI turnover of **2** (100 μ M) at 100 μ M by 20%, 43%, 44%, and 47%, respectively.

Conclusion

Several of the compounds made for this study are weak muscarinic agonists at m_4 AChRs with no agonist activity at m_3 AChRs. The parent ligand of the functionalized congeners, UH 5 (**3**), however, is active at both receptors. The pharmacological data show that BM 5 (**4**) is an agonist at m_4 AChRs and has no activity at m_3 AChRs. Nordström and co-workers¹⁸ have shown that **4** is a partial agonist at postsynaptic mAChRs and an antagonist at presynaptic mAChRs. Thus, our data may suggest that m_4 AChRs are postsynaptic and m_3 AChRs are presynaptic; however, further studies are necessary to fully characterize **4** at m_1 AChRs and m_2 AChRs. Comparison of the ratios of affinity (K_i) to activity (EC_{50}) suggest that **3** has a greater intrinsic efficacy than **4**. Although compounds **7b** and **8b** are relatively weak agonists, they have maintained a relation of affinity to activity at m_4 AChRs similar to that of **3**. In addition, these compounds have gained a selectivity of action for m_4 AChRs over m_3 AChRs similar to that of compound **4**. This profile may be advantageous in vivo by enhancing cholinergic transmission by (1) postsynaptic muscarinic agonist effects and (2) presynaptic muscarinic antagonism which can stimulate the release of acetylcholine. The potential therapeutic benefits of these compounds (e.g., the treatment of AD) can only be determined

through detailed in vivo pharmacological studies.

Several of the UH 5 congeners were antagonists at both m_3 - and m_4 AChRs, showing some selectivity for the m_3 AChR subtype. Generally, agonist activity at m_4 AChRs decreased and affinity at both receptors increased as the spacer chain was lengthened within each homologous series (R group remains the same) of functionalized ω -aminoalkyl amides (7–13). This trend of increasing affinity suggests a distal antagonist binding region for m_4 AChRs and m_3 AChRs at which the functionalized chain can interact. The high affinities of compounds **12a**, **12b**, **13a**, and **13b** are essentially equivalent to that of compound **3**, suggesting that the 1-position amide of **3** is a site for general derivatization by the "functionalized congener" approach. Although agonist activity is lost, these compounds may be candidates for functionalization with prosthetic groups to develop receptor probes or irreversible inhibitors.

Experimental Section

General. All melting points were determined on a Thomas-Hoover Uni-Melt apparatus and are uncorrected. All ^1H NMR spectra were recorded by using a Varian XL-300 FT-NMR spectrometer, and all values are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Chemical ionization MS spectra using ionized NH_3 gas were recorded on a Finnigan 1015D mass spectrometer modified with EXTREL electronics. IR spectra were recorded on a Beckmann 4230 IR spectrophotometer. Thin-layer chromatography (TLC) analyses were carried out by using Analtech 250- μ m silica gel GF "Uniplates" or EM Kieselgel 60 F254, DC-Alufolien, 200- μ m plates and were visualized in an iodine chamber and/or with 1% ninhydrin in ethanol. Silica gel columns used MN-Kieselgel 60, 0.063–0.2-mm silica gel. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, GA. The term in vacuo refers to a water aspirator (15–30 mmHg) rotary evaporator. Percent yields are rounded to the nearest whole number. An asterisk (*) for the ^1H NMR data indicates the higher integration signal for the pair of signals which represent the same proton(s) of the amide tautomers.

N-Methyl-4-(1-pyrrolidinyl)-2-butynamine (22). Di-*tert*-butyl dicarbonate (27.0 mL, 117 mmol) was slowly added to a stirring solution of *N*-methylpropargylamine (10.0 mL, 118 mmol) in 50 mL of methanol at 25 $^\circ\text{C}$. The mixture was allowed to stir for 1 h or until complete by TLC analysis. All volatiles were removed in vacuo to yield 19.2 g (97%) of the crude Boc-*N*-methylpropargylamine (**21**) as a light yellow oil: MS (CI/ NH_3) m/e 186 (M - NH_3^+), 170 (MH $^+$), 86 (base); ^1H NMR (CDCl_3) δ 1.46 (s, 9H), 2.20 (s, 1H), 2.90 (s, 3H), 4.03 (br s, 2H). Following the procedure of Amstutz et al.²⁵ **21** (16.9 g, 100 mmol) was then dissolved in 200 mL of dioxane. To this solution was added pyrrolidine (10.4 mL, 125 mmol), paraformaldehyde (3.8 g, 125 mmol), and a catalytic amount of CuCl (400 mg, 4 mmol). The mixture was allowed to react for 4 h at 25 $^\circ\text{C}$, and then it was stirred at 50 $^\circ\text{C}$ for 1 h. The mixture was then cooled to 25 $^\circ\text{C}$, poured into a 6 M citric acid solution (100 mL), and washed with CH_2Cl_2 (3 \times 50 mL). The product was made basic (pH 10) with Na_2CO_3 , and the product was extracted into CH_2Cl_2 (3 \times 100 mL). All volatiles were removed in vacuo to give 23.0 g (91%) of crude Boc-*N*-methyl-4-(1-pyrrolidinyl)-2-butynamine as a yellow oil: MS (CI/ NH_3) m/e 253 (MH $^+$), 197, 153 (base); ^1H NMR (CDCl_3) δ 1.44 (s, 9H), 1.78 (br s, 4H), 2.58 (br s, 4H), 2.88 (s, 3H), 3.38 (s, 2H), 4.03 (br s, 2H). This crude oil was then cooled to 0 $^\circ\text{C}$, and excess trifluoroacetic acid (TFA, \approx 50 mL) was slowly added with stirring. The excess TFA was removed in vacuo, and 2 M Na_2CO_3 was added until basic (pH 10). The crude diamine was extracted into CH_2Cl_2 (3 \times 100 mL) and then vacuum distilled (0.1 mmHg) to give 9.0 g (66%, 57% from *N*-methylpropargylamine) of **22** as a clear, colorless oil: bp 50 $^\circ\text{C}$ (0.1 mmHg); MS (CI/ NH_3) m/e 153 (MH $^+$); ^1H NMR (CDCl_3) δ 1.63 (br s, 1H, exc) 1.79 (br s, 4H), 2.46 (s, 3H), 2.59 (br s, 4H), 3.40 (s, 4H); IR (neat) 3280, 2980, 2800, 1450, 1350, 1325, 1125 cm^{-1} .

General Coupling Procedure. To a solution of the appropriate Boc-protected amino acid in CH_3CN was added 1.1 equiv of dicyclohexylcarbodiimide (DCC). The mixture was stirred for 15 min at 25 $^\circ\text{C}$ before a solution of 0.75 equiv of **11** in CH_3CN

was slowly added. After 4 h, or until complete by TLC (more Boc-amino acid and DCC were added if necessary), the suspension was filtered. The filtrate was evaporated in vacuo and then redissolved in ethyl acetate (EtOAc) and washed with 2 M Na₂CO₃ (3 × equal volume). The product was then extracted into a 6 M citric acid solution and washed with CH₂Cl₂ (3 × equal volume). The acidic aqueous layer was made basic (pH 10) with solid Na₂CO₃ or 4 N NaOH, and the product was extracted into CH₂Cl₂. The organic phase was then dried over Na₂SO₄ and evaporated in vacuo to give the corresponding Boc-amino acid congener. All of the Boc-amino congeners were thick oils or amorphous solids, except **7a**, which was a crystalline solid (mp 66–68 °C).

N-Methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-2-(N-Boc-amino)acetamide (7a): mp 66–68 °C; TLC (CHCl₃/CH₃OH/NH₄OH, 40/10/1) *R_f* 0.67; ¹H NMR (CDCl₃) δ 1.39 (s, 9 H), 1.74 (br s, 4 H), 2.52 (br s, 4 H), 2.95 (s, 3 H), 3.32 (s, 2 H), 3.88* and 3.97 (s, 2 H), 3.94 and 4.20* (s, 2 H).

General Procedure for Preparation of Oxalate Salts. Oxalate salts were prepared by dissolving the compounds in CH₃OH, adding oxalic acid dihydrate as a 0.5 M solution in CH₃OH, and evaporating to dryness. Stock solutions (10 or 20 mM) of the 0.5-oxalate salts or 0.25-oxalate polymorphs in 50% ethanol were stored at –20 °C until used for pharmacological testing. All of the oxalate salts of the Boc-amino congeners were thick oils except the 0.5-oxalate of **7a**, which melted at 59–60 °C.

General Deblocking Procedure. Excess TFA was added slowly to the neat Boc-protected ω-aminoalkyl amide congener and stirred for 1 h or until complete by TLC. The excess TFA was removed under a stream of N₂ and dried for 24 h at 50 °C under high vacuum (0.1 mmHg) to yield the bis(TFA) salts, all of which were thick orange oils. For spectral analysis, an aliquot was neutralized with 2 M Na₂CO₃ solution, extracted into EtOAc (3 × equal volume), dried over Na₂CO₃, and evaporated in vacuo. Stock solutions (10 or 20 mM) of the bis(TFA) salts in 50% ethanol were stored at –20 °C until used for pharmacological testing.

N-Methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-2-aminoacetamide (7b): TLC (CHCl₃/CH₃OH/TEA, 15/10/1) *R_f* 0.53; MS (CI/NH₃) *m/e* 210 (MH⁺); ¹H NMR (CDCl₃) δ 1.73 (s, 2 H), 1.79 (br s, 4 H), 2.58 (br s, 4 H), 2.98* and 3.00 (s, 3 H), 3.38 (s, 2 H), 3.43* and 3.51 (s, 2 H), 3.98 and 4.26 (s, 2 H).

General Procedure for N-Acetylation. The bis(trifluoroacetate) salt of the ω-aminoalkyl amide congener was dissolved in CH₃CN, and 1.1 equiv of triethylamine (TEA) and 1.1 equiv of Ac₂O were added. After 15 min, or until complete by TLC, the CH₃CN was removed under a stream of N₂, and the residue was dissolved in EtOAc and washed with 2 M Na₂CO₃ saturated with NaCl (3 × equal volume). The organic phase was dried over Na₂SO₄, and all volatiles were removed in vacuo. The crude product was chromatographed (CHCl₃/CH₃OH/NH₄OH, 40/10/1) to give the corresponding *N*-acetylamino congeners, all of which were viscous oils. Oxalate salts or polymorphs were prepared as described above, and all were thick oils or amorphous solids. Stock solutions (10 or 20 mM) in 50% ethanol were stored at –20 °C until used for pharmacological testing.

N-Methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-2-(N-acetyl-amino)acetamide (7c): TLC (CHCl₃/CH₃OH/NH₄OH, 10/10/1) *R_f* 0.60; MS (CI/NH₃) *m/e* 252 (MH⁺); ¹H NMR (CDCl₃) δ 1.80 (br s, 4 H), 2.04 (s, 3 H), 2.56 (br s, 4 H), 3.03 (s, 3 H), 3.38 (s, 2 H), 4.04* and 4.12 (d, *J* = 3.9 Hz, 2 H), 4.02 and 4.26* (s, 2 H), 6.56 (br s, 1 H).

N-Methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-8-(N-acetyl-amino)octanamide (13c): MS (CI/NH₃) *m/e* 336 (MH⁺), 283, 267, 197; ¹H NMR (CDCl₃) δ 1.33 (br s, 6 H), 1.49 (br m, 2 H), 1.63 (br m, 2 H), 1.83 (br s, 4 H), 1.97 (s, 3 H), 2.30* and 2.36 (t, *J* = 7.4 Hz, 2 H), 2.62 and 2.69* (br s, 4 H), 2.97 and 3.05* (s, 3 H), 3.22 (dt, *J* = 6.9, 6.0 Hz, 2 H), 3.42 and 3.46* (s, 2 H), 4.05 and 4.24* (s, 2 H), 5.52 (br s, 1 H).

General Procedure for N-Benzoylation. The bis(trifluoroacetate) amine congener was stirred in CH₃CN with 3.1 equiv of TEA and 1.1 equiv of benzoyl chloride. After 15 min, or until complete by TLC, the CH₃CN was removed under a stream of N₂, and the residue was dissolved in EtOAc and washed with 2 M Na₂CO₃ saturated with NaCl (3 × equal volume). The organic phase was dried over Na₂SO₄, all volatiles were removed in vacuo, and the crude product was chromatographed (CHCl₃/CH₃OH/

NH₄OH, 40/10/1) to give the corresponding *N*-benzoylamino congeners (oils). Half-oxalate salts were prepared as described above and were also thick oils. Stock solutions (10 or 20 mM) in 50% ethanol were stored at –20 °C until used for pharmacological testing.

N-Methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-4-(N-benzoylamino)butanamide (9d): TLC (CHCl₃/CH₃OH/NH₄OH, 40/10/1) *R_f* 0.53; MS (CI/NH₃) *m/e* 342 (MH⁺), 273, 121; ¹H NMR (CDCl₃) δ 1.74 (br s, 4 H), 1.97 (m, 2 H), 2.43* and 2.53 (overlapping triplet and broad singlet) (t, *J* = 6.3 Hz, 2 H), 2.53 (overlapping triplet and broad singlet) (br m, 4 H), 2.92 and 2.99* (s, 3 H), 3.30 (s, 2 H), 3.44 (dt, *J* = 5.1, 7.2 Hz, 2 H), 3.99 and 4.18* (s, 2 H), 7.20 (s, 1 H), 7.26 (br s, 1 H), 7.38 (m, 2 H), 7.76 (d, *J* = 8.1 Hz, 2 H).

N-Methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-4-[N-2-(N-Boc-amino)ethanoyl]amino]butanamide (14). Compound 14 was prepared by using the general procedure for DCC coupling, except 2 equiv of TEA were added prior to the addition of DCC and the amino acid. TLC (CHCl₃/CH₃OH/NH₄OH, 40/10/1) *R_f* 0.41; MS (CI/NH₃) *m/e* 395 (MH⁺), 321, 295 (base), 121; ¹H NMR (CDCl₃) δ 1.45 (s, 9 H), 1.82 (br s, 4 H), 1.89 (m, 2 H), 2.39* and 2.46 (t, *J* = 6.7 Hz, 2 H), 2.61 (br s, 4 H), 2.98 and 3.04* (s, 3 H), 3.32 (dt, *J* = 6.0, 6.3 Hz, 2 H), 3.40 (s, 2 H), 3.76 (d, *J* = 5.7 Hz, 2 H), 4.04 and 4.24* (s, 2 H), 5.23 and 5.46* (br s, 1 H), 6.71 (br s, 1 H).

N-Methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-3-carboxypropanamide (18). Intermediate **22** (557.0 mg, 3.67 mmol) was added to a solution of succinic anhydride (366.1 mg, 3.67 mmol) and triethylamine (0.51 mL, 3.67 mmol) in CH₃CN (50 mL) and stirred for 2 h, or until complete by TLC analysis. The CH₃CN was removed under a stream of N₂, and the crude product was chromatographed on a silica gel column (CHCl₃/CH₃OH/NH₄OH, 10/40/1) to give 540.4 mg of **18** (65%) as a light orange oil. TLC (CHCl₃/CH₃OH/NH₄OH, 10/40/1) *R_f* 0.67; MS (CI/NH₃) *m/e* 253 (MH⁺), 172, 153; ¹H NMR (CDCl₃) δ 1.82 (br s, 4 H), 2.53 (br m, 4 H), 2.73 and 2.80* (br s, 4 H), 2.91 and 3.02* (s, 3 H), 3.48 and 3.51* (s, 2 H), 4.06 and 4.17* (s, 2 H), 9.91 (br s, 1 H).

N-Methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-3-(methoxycarbonyl)propanamide (19). Dowex 50W cation-exchange resin (100 mg) which had been activated with HCl was added to a solution of **18** (63.9 mg, 0.25 mmol) in CH₃OH (3 mL), and the suspension was vigorously stirred overnight. The resin was filtered off and washed thoroughly with TFA. The combined CH₃OH and TFA washes were evaporated under a stream of N₂ and neutralized with 2 M Na₂CO₃ solution, and the product was extracted into EtOAc (3 × equal volume). The organic layer was dried over Na₂CO₃, evaporated in vacuo, and chromatographed (CHCl₃/CH₃OH/NH₄OH, 40/10/1) to give 44.4 mg of **19** (66%) as a light yellow oil: MS (CI/NH₃) *m/e* 267 (MH⁺), 186, 172; ¹H NMR (CDCl₃) δ 1.79 (br s, 4 H), 2.58 (br s, 4 H), 2.64 (m, 4 H), 2.98 and 3.07* (s, 3 H), 3.38 (s, 2 H), 3.68 (s, 3 H), 4.08 and 4.24* (s, 2 H).

N-Methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-2-[[N-(2-aminoethyl)amino]carbonyl]propanamide (20). Excess ethylenediamine (EDA) was added to **19** (23.3 mg, 0.087 mmol) and heated at 50 °C for 4 h. The excess EDA was removed under a stream of N₂, and the product was chromatographed (CHCl₃/CH₃OH/NH₄OH, 10/90/3) to give **19** (18.3 mg, 71% yield) as a thick yellow oil: MS (CI/NH₃) *m/e* 295 (MH⁺), 203 (base), 143; ¹H NMR (CDCl₃) δ 1.73 (br s, 4 H), 2.08 (br s, 2 H), 2.47 (t, *J* = 6.2 Hz, 2 H), 2.52 (br s, 4 H), 2.62* and 2.69 (t, *J* = 6.2 Hz, 2 H), 2.75 (t, *J* = 5.8 Hz, 2 H), 3.23 (dt, *J* = 5.8, 6.0 Hz, 2 H), 3.31 (s, 2 H), 2.91 and 3.00* (s, 3 H), 4.03 and 4.15* (s, 2 H), 6.45 (br s, 1 H).

Determination of *K_i* Values. Dissociation constants (*K_d*) were determined by incubating membranes with various concentrations of [³H]NMS in Dulbecco's modified Eagle medium–Hepes buffer (DMEM–Hepes) for 10 min at 37 °C, followed by rapid filtration over GF/B filters. Nonspecific binding was determined in the presence of atropine and was subtracted from all values. The *K_i* values were determined by computer analysis of displacement curves obtained by incubating membranes with various concentrations of the compounds tested and with 1 nM [³H]NMS in DMEM–Hepes for 60 min at 37 °C, followed by rapid filtration over GF/B filters. The data represent means ± SEM from three determinations each made in duplicate.

Inhibition of [³H]NMS Binding. A crude membrane fraction was obtained as follows. Confluent cultures were rinsed three times with phosphate-buffered saline and then lysed in a solution of 2 mM Tris-HCl (pH 7.1) and 1 mM EDTA for 30 min at 2 °C. The cells were harvested by scraping and homogenized on a Polytron (10 s, 75% mix). Nuclei were removed in a low-speed centrifugation (400g, 5 min), and a crude membrane preparation was obtained by centrifugation of the supernatant at 50000g for 20 min. The resulting pellet was resuspended in the lysis buffer and recentrifuged at 50000g for 20 min. Membranes were stored frozen at -70 °C until needed. An aliquot of the membrane fraction (150–300 µg of protein) was incubated for 90 min at 37 °C with 0.5 nM [³H]NMS and 100 µM of agonist in DMEM-Hepes. The total volume was 1 mL. The incubation was terminated by rapid filtration over GF/B filters using a Brandel cell harvester. The filters were washed three times with ice-cold 0.9% NaCl, equilibrated in scintillation counting fluid, and counted on a Beckmann LS 5801 liquid scintillation counter at 47% efficiency. Nonspecific binding was determined by coinubation with 1 µM atropine and amounted to less than 15% of total counts. It was routinely subtracted from the total counts.

Stimulation of PI Turnover in SK-N-SH Cells. SK-N-SH human neuroblastoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in DMEM supplemented with 10% newborn calf serum (Advanced Biotechnologies, Columbia, MD). Cells were plated into 24-well plates at 100 000 cells per well. After 24 h, they were labeled overnight with 2 µCi per well of [³H]-*myo*-inositol (American Radiolabeled Chemicals, Inc., St. Louis, MO; 15 Ci/mM; 2 µL of stock/mL of media). The cells were then rinsed twice with 10 mM LiCl in DMEM-Hepes, and the cells were incubated in this solution for 5 min at 37 °C. The indicated compound was then added at a final concentration of 100 µM, and the cells were incubated for another 30 min at 37 °C. The reaction was stopped by aspirating the solution and adding cold methanol to the cells. After transferring the cells to a glass tube and sonicating briefly, chloroform and water were added to make a two-phase system. The upper aqueous phase was applied to 0.6-mL anion-exchange columns (AG X8, Bio-Rad), the columns were washed, and total inositol phosphates were eluted with 1 M ammonium formate and 0.1 M formic acid.

Inhibition of PGE₁-Stimulated cAMP Levels in NG108-15 Cells. NG108-15 neuroblastoma x glioma hybrid cells were obtained from Dr. M. Nirenberg and were grown at 37 °C in DMEM supplemented with 10% newborn calf serum, 0.1 µM hypoxanthine, and 0.016 µM thymidine. Cells were plated in 24-well plates and were grown to 90% confluence. The growth medium

was then replaced with a solution of 1 mM IBMX in DMEM-Hepes (0.5 mL per well) and the cells were incubated for 10 min at 37 °C. Agonists were then added at a final concentration of 100 µM, along with PGE₁ (final concentration 5 µM), and the cells were incubated for a further 15 min at 37 °C. The reaction was terminated by aspirating the solution and adding 0.5 mL of 0.1 N HCl. In order to extract the cAMP from the cells, they were incubated in 0.1 N HCl for 30 min at room temperature. An aliquot (35–50 µL) was removed, lyophilized, and assayed for cAMP by radioimmunoassay.

Registry No. 2, 3854-04-4; 3, 3854-05-5; 3¹/₄oxalate, 124045-76-7; 4, 83481-69-0; 5, 72570-06-0; 5¹/₂oxalate, 124045-77-8; 6, 124045-36-9; 6¹/₂oxalate, 124045-78-9; 7a, 124045-37-0; 7a¹/₂oxalate, 124045-79-0; 7b, 124045-52-9; 7b-2TFA, 124069-84-7; 7c, 124045-53-0; 7c¹/₂oxalate, 124045-80-3; 7d, 124045-73-4; 8a, 124045-38-1; 8a¹/₄oxalate, 124045-81-4; 8b, 124045-54-1; 8b-2TFA, 124069-85-8; 8c, 124045-55-2; 8c-oxalate, 124045-82-5; 8d, 124045-74-5; 9a, 124045-39-2; 9a¹/₄oxalate, 124045-83-6; 9b, 124045-56-3; 9b-2TFA, 124069-86-9; 9c, 124045-57-4; 9c¹/₄oxalate, 124045-84-7; 9d, 124045-58-5; 9d¹/₂oxalate, 124045-85-8; 10a, 124045-40-5; 10a¹/₄oxalate, 124045-86-9; 10b, 124045-59-6; 10b-2TFA, 124069-87-0; 10c, 124045-60-9; 10c¹/₂oxalate, 124045-87-0; 10d, 124045-75-6; 11a, 124045-41-6; 11a¹/₄oxalate, 124045-88-1; 11b, 124045-61-0; 11b-2TFA, 124069-88-1; 11c, 124045-62-1; 11c¹/₂oxalate, 124045-89-2; 11d, 124045-63-2; 11d¹/₂oxalate, 124045-90-5; 12a, 124045-42-7; 12a¹/₂oxalate, 124045-91-6; 12b, 124045-64-3; 12b-2TFA, 124069-89-2; 12c, 124045-65-4; 12c¹/₂oxalate, 124045-92-7; 12d, 124046-01-1; 13a, 124045-43-8; 13a¹/₄oxalate, 124045-93-8; 13b, 124045-66-5; 13b-2TFA, 124069-90-5; 13c, 124045-67-6; 13c¹/₂oxalate, 124045-94-9; 13d, 124046-02-2; 14a, 124045-44-9; 14a¹/₂oxalate, 124045-95-0; 14b, 124045-69-8; 14b-2TFA, 124069-91-6; 15a, 124045-45-0; 15a¹/₂oxalate, 124045-96-1; 15b, 124045-70-1; 15b-2TFA, 124069-92-7; 16a, 124045-46-1; 16a¹/₂oxalate, 124045-97-2; 16b, 124045-71-2; 16b-2TFA, 124069-93-8; 17a, 124045-47-2; 17a¹/₂oxalate, 124045-98-3; 17b, 124045-72-3; 17b-2TFA, 124069-94-9; 18, 124045-48-3; 19, 124045-49-4; 19¹/₂oxalate, 124045-99-4; 20, 124045-50-7; 20¹/₂oxalate, 124046-00-0; 21, 124045-51-8; 22, 75858-55-8; EDA, 107-15-3; HC≡CCH₂NHMe, 35161-71-8; BOCNHCH₂CO₂H, 4530-20-5; BOCNH(CH₂)₂CO₂H, 3303-84-2; BOCNH(CH₂)₃CO₂H, 57294-38-9; BOCNH(CH₂)₄CO₂H, 27219-07-4; BOCNH(CH₂)₅CO₂H, 6404-29-1; BOCNH(CH₂)₆CO₂H, 60142-89-4; BOCNH(CH₂)₇CO₂H, 30100-16-4; pyrrolidine, 123-75-1; (*tert*-butyloxycarbonyl)-*N*-methyl-4-(1-pyrrolidinyl)-2-butyamine, 124045-68-7; succinic anhydride, 108-30-5.