Modification of the Enkephalin "Message" with an Artificial Polycationic C-Terminus†,‖

Alan R. Jacobson,‡ S. William Tam,§ and Lawrence M. Sayre*,‡

Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106-7078, and CNS Diseases Research, The Du Pont Merck Pharmaceutical Company, Wilmington, Delaware 19880-0400. *Received May 13, 1991*

The C-terminal "address" sequences of prodynorphin-derived opioid peptides contain an unusually high proportion of basic residues, which are known to be crucial for conferring high activity and selectivity for κ-opioid receptors. In an effort to investigate the possibility that the polycationic "tails" may be involved in a coulombic interaction with a complementary polyanionic receptor domain, we attached a series of achiral peptide-like cationic fragments to the C-terminus of the opioid peptide "message", Tyr-Gly-Gly-Phe. Binding of the various compounds to opioid receptor types in guinea pig brain membranes was weak, and the pharmacologic activities in the guinea pig ileum were marginal. These results indicate either that the chosen ligand design does not satisfy the structural requirements of the hypothesized coulombic interaction or that the latter is a minor criterion governing receptor recognition.

Introduction

Opioid peptides derived from the proenkephalin B (prodynorphin) gene product[1] include (Figure 1) dynorphin A,[2,3] α- and β-neoendorphin,[4] and both leumorphin[5] and its N-terminal tridecapeptide rimorphin (dynorphin B). Although they all contain the δ-preferring [Leu^5]enkephalin sequence at their N-termini, these peptides were found to exhibit unusually high potency as well as produced selectivity for κ-opioid receptors.[4-8] This finding was initially quite surprising, since all previously studied natural or synthetic opioid peptides had displayed preference for either μ- or δ-receptors, and high κ-activity had been observed only for alkaloid- and piperidine-based opiates. Thus, several studies have been concerned with the nature of the post-enkephalin sequences responsible for conferring κ-selectivity.

An obvious initial focus was the unusual wealth of basic amino acids, including in every case an Arg^6-Arg/Lys^7 pair immediately following the invariant Tyr-Gly-Gly-Phe-Leu "message" (Figure 1). The importance of basic residues in conferring κ-activity to Dyn A_{1-17} was first established by Chavkin and Goldstein, who showed that sequential removal of C-terminal residues resulted in a gradual loss of κ-selectivity/activity, and that activity drops were more pronounced upon the removal of Lys or Arg residues.[9] Subsequent studies by others expanded on these initial findings.[10-16]

A comparison of the post-enkephalin sequences among the various proenkephalin B peptides shows that neither the choice between Arg and Lys nor the exact placement of the basic residue in the amino acid sequence are conserved (Figure 1). This appears inconsistent with a sequential matching of amino acid side-chains with complementary receptor subsites, assuming these peptides interact with a common receptor (competitive binding has been demonstrated). A possible importance of sequence position was implied by the observations of a 10-fold potency decrease associated with two "register-shift" dynorphin analogues (a Gly^6 insert or a Arg^6 deletion),[9] but this could merely reflect the importance of the Arg^6-Arg^7 fragment.

We considered the possible involvement of a non-sequence-specific coulombic interaction between the polycationic peptide tail and a complementary polyanionic locus on the κ-receptor, one possibility for the latter being the phospholipid surface of a proteolipid receptor–membrane complex.[17,18] An electrostatic binding of the Dyn A_{1-13} "address" to liposomes,[19] and the role of the positive charges in governing the interaction of Dyn A_{1-13} with anionic (acidic) lipids[20,21] has been documented. Certainly

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‡ Case Western Reserve University.

§ Du Pont Merck Pharmaceuticals.

‖ Abbreviations: Standard three letter abbreviations were used for the amino acids. DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; HOBt, 1-hydroxybenzotriazole; CBZ, benzyloxycarbonyl; BOC, *tert*-butoxycarbonyl; EDA, ethylenediamine; Ac, acetyl; OpNP, *p*-nitrophenol; THF, tetrahydrofuran; GPI, guinea pig ileum; EKC, ethylketocyclazocine; DADLE, [D-Ala^2,D-Leu^5]enkephalin.

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Table I. Biological Activity of Hybrid Enkephalin Analogues

compound[a]	binding; K_i,[b] nM			GPI; % inhibn[c]	
	κ	μ	δ	+ bestatin	+ cocktail[d]
Tyr-Gly-Gly-Phe-$NHCH_2CH_2NHR$ (1a)	36700 ± 3240	16500 ± 740	10100 ± 4630	29.5	28.5
$-NHCH_2CH_2N(R)CH_2CH_2NHR$ (2a)	8750 ± 488	14400	22700	13.5	17.5
$-NHCH_2CH_2N(R)CH_2CH_2N(R)CH_2CH_2NHR$ (3a)	3730	6760	>10000	11.0	9.0
$-NHCH_2CH_2CH_2N(R)CH_2CH_2CH_2NHR$ (4a)	5510	7300	8430	13.0	12.5
$RNHCH_2CH_2N(R)CH_2CH_2NHR$ (5) $R = -C(O)CH_2CH_2CH_2NH_2$	ND[e]	ND	ND	0	0
dynorphin A(1-13)amide	3.6	50	16	ND	ND

[a] All compounds were tested as the HCl salts. [b] The numbers represent either single experiments in duplicate or means ± SEM of three experiments in duplicate. See Experimental Section for other details. [c] Compounds were tested at 10^{-6} M final concentration in the GPI. Values represent the average of two or three experiments in tissues in which morphine gave an average of 50% inhibition of electrically evoked contractions at 10^{-7} M. [d] Comprised of 10 μM bestatin, 10 μM captopril, 0.3 μM thiorphan, and 10 mM L-leucylleucine. See ref 40 for details. [e] ND = not determined.

dynorphin A(1–13):
Tyr-Gly-Gly-Phe-Leu-Arg$^+$-Arg$^+$-Ile-Arg$^+$-Pro-Lys$^+$-Leu-Lys$^+$

α-neoendorphin: Tyr-Gly-Gly-Phe-Leu-Arg$^+$-Lys$^+$-Pro-Lys$^+$

dynorphin B: Tyr-Gly-Gly-Phe-Leu-Arg$^+$-Arg$^+$-Gln-Phe-Lys$^+$-Val-Val-Thr

Figure 1. Amino acid sequences of some prodynorphin-derived opioid peptides.

any such coulombic interaction cannot be an absolute criterion governing opioid receptor type preference, as there exist many non-peptide ligands for the κ-receptor (e.g., ethylketocyclazocine, tifluodom, and the U-50, 488 benzeneacetamide series of analogues) which lack a polycationic fragment. However, the relationship between peptidergic and nonpeptidergic ligands for opioid receptor types is not always straightforward, one possibility being that such ligands interact with different receptor subpopulations (as many as four subtypes of κ-receptors have been implicated[22]) or with different domains of the same recognition site.

If a coulombic interaction involving the "address" segments of prodynorphin-based peptides does play a role in receptor recognition, it should be possible to reproduce the required ligand–receptor interaction with an artificial peptide-like polycationic tail. In an effort to test this hypothesis we synthesized the series of hybrid compounds listed in Table I involving the appendage of an achiral *N*-(4-aminobutyryl)-functionalized polyethylenimine chain to the N-terminal enkephalin-recognition tetrapeptide. This design was chosen to maintain peptide-like physicochemical properties and the proper three-atom spacing of side chains. Although attachment of the polycationic tail directly to Tyr-Gly-Gly-Phe deleted the Leu[5] residue, the spacing of the first cationic side chain is within one atom of where it should be (see Figure 2). In one case (**4a**), the di*propylene*triamine backbone was used to increase the spacing between cationic side chains in order to better approximate the lower "charge density" situation when Lys

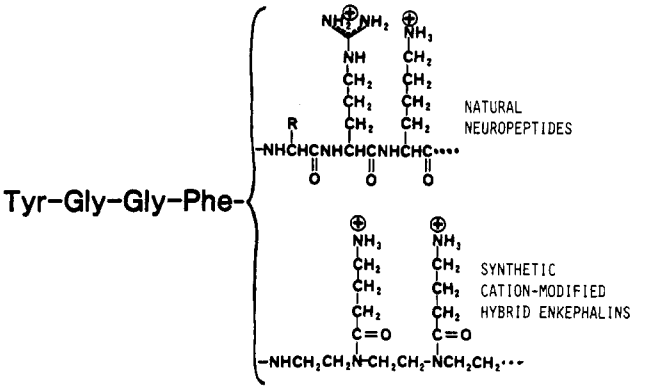
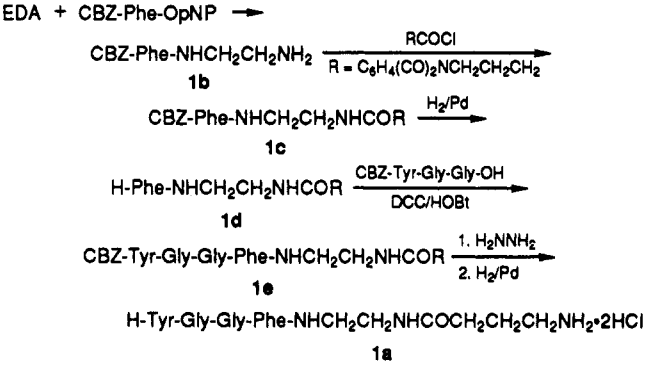


Figure 2. Comparison between natural peptides and cation-modified enkephalins.

Scheme I



and/or Arg are separated by a neutral amino acid. If a non-sequence-specific recognition were involved, we expected that activity would not be too sensitive to the exact spacing. The despeptide analogue **5** was synthesized as a "control" to check for possible nonopioid effects of the polycationic hybrid peptides. The biological activity was evaluated on the basis of binding studies (guinea pig brain) and pharmacologic evaluation in the guinea pig ileum (GPI), which responds well to typical κ (as well as μ) agonists.

Chemistry

Our initial approach to the synthesis of the desired analogues was to develop a strategy for the selective monoacylation of the appropriate symmetric polyamine. In the case of ethylenediamine, we were able to take advantage of the amine volatility and a statistical rate advantage when using a large excess of the diamine (e.g., as the solvent), to achieve a high yield (>80%) of monoacylation.[23] In this way, the synthesis of the first member

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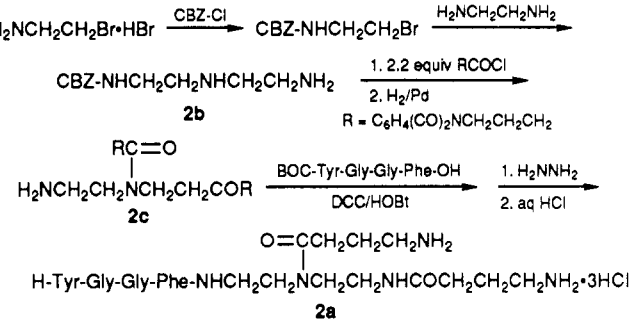
Scheme II



of our target series **1a** is summarized in Scheme I.

For the preparation of the higher members of the polyethyleneimine analogue series (**2a** and **3a**), the direct monoacylation strategy was problematic, owing to the nonvolatility of the requisite polyamines. Obtaining the desired monoacyl products by relying on a chromatographic separation was found to be tedious, and this approach was abandoned. In addition, attempts to build up the chain via a terminal *N,N*-dibenzylpolyamine or via hydride ($LiAlH_4$ or B_2H_6) reduction of oligoglycine units gave undesirably low yields. Instead, strategies were developed (Schemes II and III) which involved the stepwise introduction of ethylenediamine units to extend the backbone, wherein high yields of monoacylation or monoalkylation were ensured by using this volatile diamine in large excess.[23]

The synthesis of the dipropylenetriamine analogue **4a** was accomplished through a similar strategy (Scheme IV), involving a direct monoalkylation of 1,3-diaminopropane in high yield through use of this volatile amine in excess.

Results

The data in Table I shows that binding of the synthetic compounds to opioid receptor types in guinea pig brain membranes is very weak compared to dynorphin A(1–13)amide. Binding appears to improve somewhat with increasing length of polycationic tail (from monocation **1a** to dications **2a** and **4a** to trication **3a**), but the weak affinity mediates against placing too much significance in this observation, and the selectivity displayed for κ-receptors was marginal. In the last two columns of Table I are given data on the ability of the compounds to inhibit electrically stimulated contractions in the longitudinal muscle of the GPI in the presence of either 10 μM of the aminopeptidase inhibitor bestatin or a "cocktail" of peptidase inhibitors commonly employed in enkephalin bioassays. The C-terminal monocation **1a** displayed <10% of the inhibitory activity of morphine, and increasing the length of the polyethyleneimine backbone resulted in a steady decrease rather than increase in activity. Due to the weak potencies of these compounds, we did not attempt to obtain concentration–response curves nor did we investigate the possible existence of opioid receptor selectivity. In fact, our inability to demonstrate good naloxone sensitivity of the GPI activities, coupled with the binding data, suggests that the observed agonist activity in GPI may be at least partially nonopioid in nature. Compounds **1a–4a** were also screened in the GPI for possible antagonist activity against the standard κ-agonist ethylketocyclazocine (EKC). At 1 μM, none of the compounds caused a significant diminution of the 50–60% inhibition of contractions produced by 1 nM EKC. Finally, compound **5**, the despeptide triamine analogue, was devoid of any pharmacologic activity in the GPI.

Discussion

Since its initial formulation in 1977 by Schwyzer,[24] the "message/address" concept has been useful in analyzing opioid receptor type preferences. According to this hypothesis, the N-terminal tetrapeptide Tyr-Gly-Gly-Phe provides the principal recognition locus that determines opioid activity in general (the "message"), whereas the various C-terminal extensions ("addresses") determine receptor type preference.

The basic side-chain residues found in the post-enkephalin sequences of the prodynorphin-derived neuropeptides are thought to be crucial for κ-selectivity, but the structural basis of recognition is unclear. Work by Schiller[25,26] and others has focused on the question of conformation, the idea being to correlate type selectivity with preferred extended or various folded forms of opioid peptides. Covalent cyclization of peptides offers an indirect method for probing the preferred secondary structure of ligands recognized at receptors. Hruby and coworkers recently prepared dynorphin A analogues with cyclization in the "address" sequence in order to investigate the possibility of a crucial reverse turn at residues 8 or 10.[27] Cyclization of Dyn A_{1-13} in the "message" sequence appears to result in high μ-activity.[26]

Schwyzer has advanced an alternative analysis which focuses on the nature of peptide secondary structure induced upon interaction with lipid bilayer membrane interfaces.[28,29] He proposes that the various opioid receptor types are associated with distinct receptor–membrane organizations which are complementary to specific combinations of peptide charge and amphiphilic potential. In particular, the "address" sequence of Dyn A is proposed to conform to an amphiphilic α-helix, the membrane interaction of which is thought to result in a perpendicular projection of the enkephalin "message" to the appropriate depth of residence of the κ-recognition locus in the membrane.[28] Substantial physicochemical data on dynorphin peptides[21,30] and the activity of minimally homologous secondary structure mimics[31] support the notion of a membrane-assisted opioid receptor selection.[29]

The "address" sequence may also govern other issues, such as the stability toward peptidases and how this feature is tied into the question of whether the opioid peptide serves a neurohormonal or a neurotransmitter function. For prodynorphin-derived peptides, it has been proposed that fragments of 10 amino acids or greater are more κ-like, are relatively resistant to peptidases, and act hormonally (viz., at a distance from the site of release); whereas the shorter fragments are less κ-selective, are more readily cleaved by peptidases, and serve a transmitter role in vivo.[11] Dynorphin itself exhibits only weak analgesic ac-

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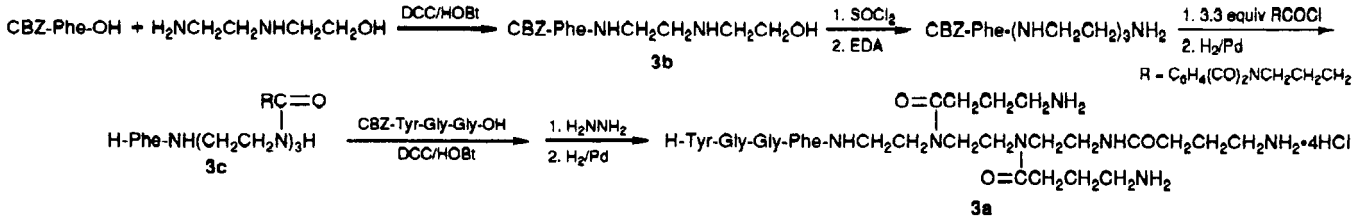
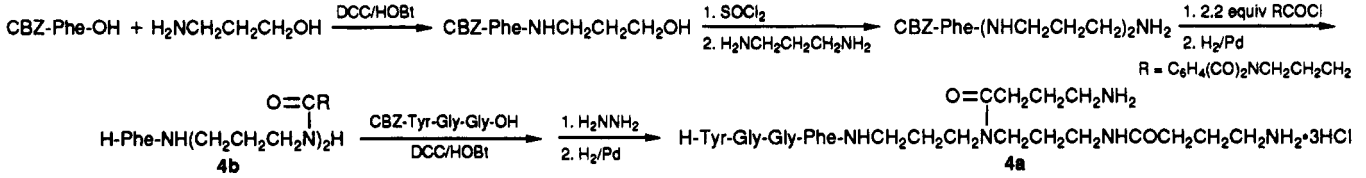
Scheme III



Scheme IV



tivity, and its physiological function is thought to be mainly one of modulating the response of the μ-opioid receptor.[32-34] The unusually persistent binding observed with Dyn A and Dyn A_{1-13},[35] perhaps "intentionally programmed" by the "address" sequence, may be a requirement of such neuromodulatory activity.

On the basis of all these studies, it seems clear that the way in which sequence specificity and secondary structure of opioid peptides govern *agonist activity at opioid receptor types* is complex and intertwined. There have been some successful attempts to direct receptor type selectivity of "hybrid" analogues by attaching receptor type-selective "address" sequences to generic "message" molecules;[36,37] however, other work has suggested an important role of the "message" in modulating receptor type preference.[38,39] Thus, additional structure–activity studies will be needed to clarify the overall requirements for receptor type selection.

The aim of our study was to investigate the possibility that the basic side-chain groups of prodynorphin-derived peptides might be involved in a non-sequence-specific polyvalent coulombic interaction with the κ-receptor. Although our synthetic compounds were not devoid of activity, the weak binding to opioid receptors in guinea pig brain membranes and the marginal agonist activity in the GPI (and complete lack of κ-antagonist activity) indicate a general lack of affinity for opioid receptors. Thus, it seems clear that if a general coulombic interaction is crucial for observing preferential κ-binding, our ligand design does not meet the necessary structural requirements. One explanation might be that a specific secondary structure of the dynorphin "address" sequence is crucial; although the *N*-(4-aminobutyryl)polyamine-based polycationic tail in our analogues is peptide-like in certain respects, the presence of tertiary rather than secondary amide units would prevent the adoption of a normal peptide-like secondary structure. Another possibility is that the stereochemistry of side-chain projections within the natural "address" sequences is important. A final possibility is that the coulombic role of the cationic side chains is a minor factor superimposed on major issues of conformation and complementarity. Despite the failure of the present effort to shed light on the role of the dynorphin "address" in conferring high activity and κ-selectivity, the overall concept of using peptide-like molecular fragments to construct hybrid analogues of biologically active peptides is a sensible approach, and the design chosen here may prove useful in other applications.

Experimental Section

General Procedures. Details of the general synthetic (e.g., DCC-mediated peptide bond formation) and analytical procedures have been previously described[40] and are not reproduced here. No attempts were made to maximize yields. In the case of HPLC (Waters Associates μ-BONDAPAK C-18, 3.9 mm × 150 mm column), the analytical conditions for which relative retention time (k' values) are reported were as follows: flow rate = 0.1 mL/min; 0.01% aqueous CF_3COOH with a linear gradient (0–40% v/v) of CH_3CN over 40 min; λ = 210 and 254 nm. All organic fine chemicals were from Aldrich Chemical Co., and amines were stored at least 24 h over NaOH pellets before use. Amino acid derivatives were purchased from Sigma Chemical Co. and/or prepared by standard procedures. Combustion analyses were determined by Galbraith Labs, Inc., Knoxville, TN.

1-(Tyr-Gly-Gly-Phe)-4-(4-aminobutyryl)-1,4-diazabutane Dihydrochloride (1a). EDA (25 mL, used as solvent) was cooled in an ice bath and 2.5 g (6.0 mmol) of CBZ-Phe-OpNP was added as a solid in small portions over a 5-min period. The mixture was allowed to warm to room temperature, and excess EDA was removed by repeated concentrations in vacuo using $PhCH_3$ as an azeotropic "chase". The gummy residue was diluted with 0.6 N HCl (50 mL) and extracted twice with $CHCl_3$. The aqueous layer was evaporated to dryness and the HCl salt was crystallized from CH_3CN/MeOH (10:1) yielding 1.9 g (85%) of CBZ-Phe-$NHCH_2CH_2NH_2$·HCl (**1b**) (TLC, MeOH/EtOAc/NH_4OH 1:1:0.01, R_f = 0.37, ninhydrin positive).

A solution of the above amine salt **1b** (2.8 g, 7.4 mmol) in 10 mL of THF and 1 mL of DMF was added dropwise to a cold solution of 4-phthalimidobutyryl chloride[41] (2.25 g, 8.9 mmol) in 30 mL of anhydrous THF containing Et_3N (2.5 mL, 17.8 mmol) under a N_2 atmosphere. After warming to room temperature, 10 mL of saturated aqueous $NaHCO_3$ was added to quench the reaction. The THF was removed in vacuo, and the reaction was diluted with 75 mL of $CHCl_3$. The organic layer was extracted successively with two 50-mL portions each of 0.6 N HCl, half-

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saturated aqueous Na_2CO_3, and distilled H_2O, then dried ($MgSO_4$), and evaporated to dryness. Trituration with Et_2O gave 2.5 g (62%) of the white CBZ-protected intermediate **1c**, which ran as a single spot on TLC ($CHCl_3$/10% MeOH, R_f = 0.66). Removal of the CBZ group (10% Pd on carbon, 760 mm of H_2, 25 °C, EtOH containing a trace amount of HOAc) afforded 1-(phenylalanyl)-4-(4-phthalimidobutyryl)-1,4-diazabutane (**1d**), isolated as the HCl salt. TLC (MeOH/EtOAc/NH_4OH 1:1:0.01, R_f = 0.35) showed a single ninhydrin-positive spot: ^1H NMR (CD_3OD) δ 1.70 (p, 2 H, $CH_2CH_2CH_2$, J = 7 Hz), 1.93 (t, 2 H, $NCH_2CH_2CH_2C{=}O$, J = 7 Hz), 2.93 (m, 8 H), 6.96 (m, 5 H, Phe Ar), 7.24 (m, 4 H, phthalyl ArH).

Compound **1d** was coupled directly to CBZ-Tyr-Gly-Gly-OH, prepared as reported,[42] by the following standard procedure. To 30 mL of DMF at 0–5 °C was added 1.9 g (4.2 mmol) of **1d**·HCl, 1.8 g (4.2 mmol) of the CBZ tripeptide, 0.58 mL (4.2 mmol) of Et_3N, 567 mg (4.2 mmol) of HOBt, and, after 5 min, 952 mg (4.6 mmol) of DCC. The solution was allowed to warm to room temperature with mixing overnight and was then filtered. The mother liquor was concentrated in vacuo, diluted with 75 mL $CHCl_3$, and extracted successively with two 50-mL portions each of 0.6 N HCl, half-saturated aqueous Na_2CO_3, and distilled H_2O. The organic layer was dried ($MgSO_4$) and concentrated, and the residue was purified by silica gel flash chromatography (EtOAc/10% MeOH) yielding 1.9 g (56%) of 1-(CBZ-Tyr-Gly-Gly-Phe)-4-(4-phthalimidobutyryl)-1,4-diazabutane (**1e**); R_f = 0.55, EtOAc/10% MeOH.

Final deprotections were accomplished by using known procedures. Compound **1e** (1.8 g, 2.16 mmol) was refluxed in EtOH containing 0.34 mL (10.8 mmol) of N_2H_4 under N_2. The reaction mixture was cooled to room temperature and acidified to pH ~ 4 with glacial HOAc, and the precipitated phthalhydrazide was filtered. The filtrate was concentrated in vacuo, resuspended in EtOH containing 1% (by weight) of 10% Pd on activated carbon, and placed under a gentle stream of H_2 (760 mm) overnight at room temperature. After filtration of catalyst and solvent evaporation, the product was converted to the 2HCl salt and precipitated from solution by the addition of Et_2O, giving 1.1 g (89%) of crude **1a**. The hygroscopic material was further purified by recrystallization from MeOH/CH_3CN (1:10), yielding 720 mg (52%) of a white solid: mp 212–215 °C dec; $[\alpha]_D^{25}$ = 22.4° (c = 1.5, MeOH); ^1H NMR (D_2O, CH_2Cl_2 int std) δ 1.79 (p, 2 H, J = 7.7 Hz, $CH_2CH_2CH_2$), 2.17 (t, 2 H, J = 7.7 Hz, $NCH_2CH_2CH_2C{=}O$), 2.95 (br m, 10 H), 3.77 (m, 4 H, gly CH_2), 4.13 (t, 1 H, J = 6.9 Hz, Tyr α-CH), 4.38 (t, 1 H, J = 6.7 Hz, Phe α-CH), 6.75 and 7.05 (2 d, 2 H each, J = 8.3 Hz, Tyr Ar H), 7.20 (m, 5 H, Phe Ar H). An analytical sample was prepared by semipreparative reverse-phase HPLC (k' = 3.01). Anal. ($C_{28}H_{39}N_7O_8$·2HCl·0.5H_2O) C, H, N.

1-(Tyr-Gly-Gly-Phe)-4,7-bis(4-aminobutyryl)-1,4,7-triazaheptane Trihydrochloride (2a). A solution of 2-bromoethylamine hydrobromide (10.0 g, 49 mmol) in 40 mL of 1 N aqueous NaOH/dioxane (3:1) was cooled to 0 °C, and CBZ-Cl (7.7 mL, 54 mmol) in 5 mL of dioxane was added dropwise under Schotten–Baumann conditions using 5 N NaOH to maintain the pH at 10–11. After warming to room temperature the intermediate CBZ-$NHCH_2CH_2Br$ was extracted into $CHCl_3$ and directly chromatographed ($CHCl_3$, R_f = 0.35). The yield of viscous oil was 11.7 g (93%). This bromide (5.0 g, 19.2 mmol) was diluted with 10 mL of $CHCl_3$ and added dropwise with vigorous stirring to 30 mL of EDA at 0 °C, and the resulting mixture was allowed to warm to room temperature overnight. The free base of the product CBZ-$(NHCH_2CH_2)_2NH_2$ (**2b**) was liberated by diluting the reaction with 20 mL of $CHCl_3$ and adding solid anhydrous K_2CO_3. After filtration, excess EDA was removed by repeated concentrations in vacuo, using a toluene azeotropic "chase". Purification of **2b** was by crystallization of the 2 HCl salt from anhydrous 2-propanol, yielding 3.52 g (59%).

Acylation of **2b** using 2.2 equiv of 4-phthalimidobutyryl chloride[41] was carried out as for **1b**, giving 1-(benzyloxycarbonyl)-4,7-bis-(4-phthalimidobutyryl)-1,4,7-triazaheptane: ^1H NMR ($CDCl_3$) δ 1.98 (br p, 4 H, $CH_2CH_2CH_2$, J = 5.4 Hz), 2.18 (app q, 2 H, $NCH_2CH_2CH_2(C{=}O)NH$, app J = 5.4 Hz), 2.36 (t, 2 H, $NCH_2CH_2CH_2(C{=}O)N$, J = 5.4 Hz), 3.43 (m, 8 H), 3.59 (m, 4 H), 5.03 (d, 2 H, CH_2 of CBZ group, J = 12.1 Hz), 7.29 (m, 5 H, ArH of CBZ group), 7.68 (m, 8 H, phthalyl ArH). Removal of CBZ was as reported for **1c**. Coupling of the deprotected amine (**2c**) to BOC-Tyr-Gly-Gly-Phe-OH, the latter prepared by coupling BOC-Tyr-OH to H-Gly-Phe-OCH_2Ph, followed by debenzylation of the ester using H_2 and Pd on activated carbon, gave 1-(BOC-Tyr-Gly-Gly-Phe)-4,7-bis-(4-phthalimidobutyryl)-1,4,7-triazaheptane (8.70 g, 38%): ^1H NMR ($CDCl_3$) δ 1.37 (s, 9 H), 1.96 (m, 4 H, $CH_2CH_2CH_2$), 2.20 (m, 4 H, $NCH_2CH_2CH_2C{=}O$), 3.69 (br s, 12 H), 4.19 and 4.70 (2 br s, 1 H each, α-CH), 6.74 and 6.96 (d, 2 H, J = 8.0 Hz, Tyr ArH), 7.67 (m, 5 H, Phe ArH), 7.76 (m, 8 H, phthalyl ArH).

Removal of the phthalyl groups was as for **1d**, and deprotection of the BOC group was accomplished with 4 N HCl/dioxane (1:4), as reported.[43] The final product was crystallized as the 3HCl salt from anhydrous i-PrOH, giving 310 mg (41%) of **2a** as a white solid: mp 220–225 °C dec; $[\alpha]_D^{25}$ = 25.4° (c = 2.3, MeOH); ^1H NMR (D_2O, CH_2Cl_2 int std) δ 1.79 (m, 4 H, $CH_2CH_2CH_2$), 2.19 (app q, 2 H, app J = 6.5 Hz, $NCH_2CH_2CH_2(C{=}O)NH$), 2.36 (br app s, 2 H, $NCH_2CH_2CH_2(C{=}O)N$), 2.88–3.20 (m, 16 H), 3.74 and 3.78 (2 s, 2 H each, CH_2 of Gly), 4.14 (t, 1 H, J = 6.8 Hz, Tyr α-CH), 4.40 (t, 1 H, J = 6.6 Hz, Phe α-CH), 6.70 and 7.04 (2 d, 2 H each, J = 6.8 Hz, Tyr ArH), 7.20 (m, 5 H, Phe ArH). An analytical sample of **2a** was prepared by reverse-phase HPLC (k' = 3.02). Anal. ($C_{34}H_{51}N_9O_7$·3HCl·H_2O) C, H, N.

1-(Tyr-Gly-Gly-Phe)-5,9-bis(4-aminobutyryl)-1,5,9-triazanonane Trihydrochloride (4a). To a solution of CBZ-Phe-OH (10.0 g, 34 mmol), 3-amino-1-propanol (2.5 mL, 34 mmol), and HOBt (4.6 g, 34 mmol) in 150 mL of CH_2Cl_2 was added DCC (7.7 g, 37 mmol) in 50 mL of CH_2Cl_2 at 5 °C, and the mixture was allowed to stir to room temperature overnight. The mixture was filtered to remove DCU and extracted successively with 0.6 N HCl, half-saturated aqueous Na_2CO_3, and distilled H_2O. Removal of the solvent in vacuo and recrystallization of the residue from 1-propanol/Et_2O afforded 7.8 g (65%) of CBZ-Phe-$NHCH_2CH_2CH_2OH$. The latter (3.0 g, 8.8 mmol) was converted to the alkyl chloride through reaction with $SOCl_2$ (0.8 mL, 10.6 mmol) in 30 mL of anhydrous THF containing 1.5 mL (10.6 mmol) of Et_3N. After stirring overnight under N_2 at room temperature, the mixture was concentrated in vacuo, diluted with $CHCl_3$ (30 mL), and extracted once with half-saturated aqueous Na_2CO_3. The organic layer was dried ($MgSO_4$) and concentrated in vacuo to dryness. Recrystallization from $CHCl_3$/petroleum ether yielded 1.7 g (54%) of CBZ-Phe-$NHCH_2CH_2CH_2Cl$.

Reaction of the latter alkyl chloride with 1,3-diaminopropane, and the subsequent acylation with 2.2 equiv of 4-phthalimidobutyryl chloride[41] followed by removal of the CBZ group, were carried out according to the procedures used for **2b** and **1c**/**1d**, respectively, giving 1-(phenylalanyl)-5,9-bis(4-phthalimidobutyryl)-1,5,9-triazanonane (**4b**). Coupling of the latter to CBZ-Tyr-Gly-Gly and final deprotections were carried out as for **1e** and **1a**, respectively. The final product was obtained as the 3HCl salt by crystallization from anhydrous 2-propanol, giving 309 mg (48%) of **4a** as a white solid: mp 216–219°C dec; $[\alpha]_D^{25}$ = 25.3° (c = 2.2, MeOH); ^1H NMR (D_2O, CH_2Cl_2 int std) δ 1.43 and 1.56 (2 br p, 2 H each, J = 6.85 Hz, $NCH_2CH_2CH_2N$), 1.81 (br m, 4 H, $NCH_2CH_2CH_2C{=}O$), 2.23 (t, 2 H, J = 7.8 Hz, $NCH_2CH_2CH_2(C{=}O)NH$), 2.39 (dd, 2 H, J = 1.5 and 7.1 Hz, $NCH_2CH_2CH_2(C{=}O)N$), 2.8–3.2 (br m, 16 H), 3.76 and 3.79 (2 s, 2 H each, Gly CH_2), 4.14 (t, 1 H, J = 6.8 Hz, Tyr α-CH), 4.38 (t, 1 H, J = 6.4 Hz, Phe α-CH), 6.77 and 7.04 (2 d, 2 H each, J = 6.8 Hz, Tyr ArH), 7.20 (m, 5 H, Phe ArH). An analytical sample was prepared by reverse-phase HPLC (k' = 3.06). Anal. ($C_{36}H_{55}N_9O_7$·3HCl·H_2O) C, H, N.

1-(Tyr-Gly-Gly-Phe)-4,7,10-tris(4-aminobutyryl)-1,4,7,10-tetraazadecane Tetrahydrochloride (3a). The synthesis of this compound followed closely that of **4a**. Coupling of CBZ-Phe-OH with 2-[(2-aminoethyl)amino]ethanol using DCC/HOBt and crystallization of the HCl salt from absolute EtOH gave CBZ-Phe-$NHCH_2CH_2NHCH_2CH_2OH$ (**3b**) in 33% yield: ^1H NMR (free base, $CDCl_3$) δ 2.22 (br s, 2 H, NH and OH), 2.58 (app

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p, 4 H, app J = 4.1 Hz, CH_2NHCH_2), 3.08 (d, 2 H, J = 7.6 Hz, Phe CH_2), 3.28 (app q, 2 H, app J = 4.1 Hz, (C=O)-$NHCH_2CH_2NH$), 3.55 (t, 2 H, J = 5.4 Hz, $NHCH_2CH_2OH$), 4.42 (dd, 1 H, J = 7.6 and 17.2 Hz, α-CH), 5.01 (s, 2 H, CBZ CH_2), 5.70 (d, 1 H, J = 8.0 Hz, CBZ-NH), 6.54 (t, 1 H, J = 4.1 Hz, Phe-NH), 7.24 (m, 10 H, ArH).

Conversion of **3b** to the corresponding alkyl chloride with $SOCl_2$, reaction with EDA, acylation by 3.3 equiv of 4-phthalimidobutyryl chloride,[41] and removal of the CBZ group gave 1-(phenylalanyl)-4,7,10-tris(4-phthalamidobutyryl)-1,4,7,10-tetraazadecane (**3c**): ^{1}H NMR (D_2O/HOAc-d_4) δ 1.85 (m, 6 H, $CH_2CH_2CH_2$), 2.40 (m, 6 H, $NCH_2CH_2CH_2C$=O), 3.36 (m, 20 H), 4.25 (t, 1 H, J = 8.0 Hz, α-CH), 7.29 (m, 5 H, Phe ArH), 7.74 (m, 12 H, phthalyl ArH).

Coupling of **3c** to CBZ-Tyr-Gly-Gly-Gly followed by deprotection of phthalyl and CBZ groups, conversion to the HCl salt, and recrystallization from anhydrous 2-propanol/methanol, gave 1.0 g (6% overall yield) of **3a** as a white solid: mp 230-234°C dec; $[\alpha]_D^{25}$ = 20.7° (c = 3.3, MeOH); ^{1}H NMR (D_2O, CH_2Cl_2 int std) δ 1.79 (br m, 6 H, $CH_2CH_2CH_2$), 2.24 (app q, 2 H, J = 7.3 Hz, $NCH_2CH_2CH_2(C$=O)NH), 2.38 (br m, 4 H, $NCH_2CH_2CH_2(C$=O)N), 2.88-3.30 (br m, 22 H), 3.74 and 3.78 (2 s, 2 H each, Gly CH_2), 4.14 (t, 1 H, J = 6.8 Hz, Tyr α-CH), 4.40 (br t, 1 H, J = 6.5 Hz, Phe α-CH), 6.74 and 7.04 (2 d, 2 H each, J = 6.8 Hz, Tyr ArH), 7.20 (m, 5 H, Phe ArH). An analytical sample was prepared by reverse-phase HPLC (k' = 2.83). Anal. ($C_{40}H_{63}N_{11}O_8 \cdot 4HCl \cdot 5H_2O$) C, H, N.

1,4,7-Tris(4-aminobutyryl)-1,4,7-triazaheptane Trihydrochloride (5). To 1.5 g (6.0 mmol, 3.5 equiv) of 4-phthalimidobutyryl chloride[41] in 40 mL of anhydrous THF containing 2.4 mL (60.0 mmol) of Et_3N cooled to -78 °C was added dropwise over a 30 min period 0.18 mL (1.7 mmol) of diethylenetriamine in 5 mL of anhydrous THF under a N_2 atmosphere. After addition was complete the reaction was allowed to warm to room temperature, then filtered, and the filtrate was concentrated in vacuo. Recrystallization from $CHCl_3/Et_2O$ yielded 800 mg (63%) of 1,4,7-tris(4-phthalimidobutyryl)-1,4,7-triazaheptane: ^{1}H NMR ($CDCl_3$) δ 1.99 (br p, 6 H, J = 5.9 Hz, $CH_2CH_2CH_2$), 2.23 (dt, 2 H, J = 5.9 Hz, $NCH_2CH_2CH_2(C$=O)NH), 2.42 (t, 2 H, J = 5.9 Hz, $NCH_2CH_2CH_2(C$=O)N), 3.40 (br s, 8 H), 3.70 (m, 6 H, $NCH_2CH_2CH_2(C$=O)), 6.72 and 6.84 (2 br s, 1 H each, amide NH), 7.73 (m, 12 H, phthalyl ArH).

Removal of the phthalyl groups was as for **1d**. The final product was crystallized as the 3HCl salt from EtOH/i-PrOH, giving ~200 mg (25% overall yield) of **5** as a very hygroscopic material (no combustion analysis could be obtained): ^{1}H NMR (D_2O, $CHCl_3$ int std) δ 1.48 (p, 6 H, J = 7.6 Hz, $CH_2CH_2CH_2$), 1.90 (app q, 4 H, app J = 7.3 Hz, $CH_2CH_2CH_2(C$=O)NH), 2.12 (t, 2 H, J = 7.3 Hz, $CH_2CH_2CH_2(C$=O)N), 2.55 (app q, 6 H, J = 7.8 Hz, $CH_2NH_3^+$), 3.00 (m, 8 H).

Bioassays. The affinity of the synthetic compounds for opioid receptor types in brain membranes from male Hartley guinea pigs was assessed by standard radioligand competitive binding at 25 °C in the presence of 100 mM NaCl, as previously described.[44] The final concentrations of labeled ligands used were as follows: 0.5 nM [^{3}H]naloxone (μ-binding), 0.7 nM [^{3}H]DADLE in the presence of 4 nM sufentanil (δ-binding), and 1 nM (-)-[^{3}H]EKC in the presence of 500 nM DADLE and 20 nM sufentanil (κ-binding). The activity of the synthetic compounds was not changed upon addition of 50 μg/mL bacitracin (data not shown). The pharmacologic activity of the compounds was assessed with the electrically stimulated intact ileum from male albino Hartley guinea pigs at 37 °C as previously described.[40]

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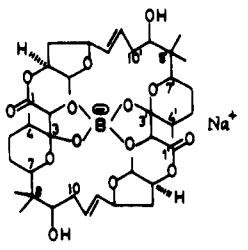
Monoterpenic Fragment Analogues of Aplasmomycin as Potential Antimalarials

N. Balu, Jacob V. Thomas, and Sujata V. Bhat*

Department of Chemistry, Indian Institute of Technology, Powai, Bombay 400 076, India. *Received October 23, 1990*

Seven analogues of monoterpenic fragment of aplasmomycin were synthesized as targeted antimalarial agents. The potency of the compound **6** was comparable with the sesquiterpene lactone artemisinin and the antibiotic aplasmomycin in vivo against *Plasmodium berghei yoelli*.

Aplasmomycin (**1**), a boron-containing ionophoric antibiotic isolated from marine *Streptomyces griseus*, was found to be active against *Plasmodium berghei* (NK 65) in vivo.[1] Several cyclic sesquiterpene peroxides[2,3] also exhibit antimalarial activity against a variety of parasite strains and the most notable are the endoperoxide sesquiterpene lactone artemisinin (**2**) and its derivatives.[4-7] The efficacy of these sesquiterpene derivatives was attributed in part on the observation that the parasitized red cells are selectively damaged by the oxidants, suggestive of an oxidative mechanism.[8,9] The presence of the terpenoidal moiety in **1** and **2** led us to the investigation of the structure-antimalarial activity relationship of monoterpenic fragment analogues of aplasmomycin.



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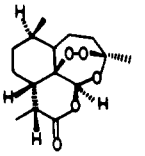

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In the present investigation seven analogues (**4-10**) of monoterpenic aplasmomycin fragment and a hemiterpenic ester (**3**) were synthesized and evaluated for antimalarial

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