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# Synthesis and Bioactivity Studies of 1-Adamantanamine Derivatives of Peptides

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Abstract: Small enkephalin-related peptides containing a 1-adamantanamine moiety coupled through an amide linkage at the C-terminus were synthesized. Several of the compounds showed high $\mu$ opioid activity and $\mu$ receptor selectivity. The new adamantanamine derivatives were also examined for antiviral activity against HIV-1 in a cell culture system. Some of them inhibited syncytia formation even when the antigen assay gave evidence for viral replication.

Keywords: 1-Adamantanamine; peptide synthesis; opioid compounds; antiviral agents; HIV-1

A low molecular weight subfraction derived from human leukocyte dialysates has been shown to enhance cell-mediated immunity (now under clinical evaluation as an immunosupportive agent in AIDS patients) and two peptides (H-Tyr-Gly-OH, H-Tyr-Gly-Gly-OH) were found to be the main components responsible for the immunological activity [1]. These peptides have not previously been demonstrated to affect the immune system, and it is of interest to note that they are identical to the amino terminal ends of endogenous opioid peptides, such as the enkephalins (H-Tyr-Gly-Gly-Phe-Leu/Met-OH).

Studies on the effect of the introduction of a bulky hydrophobic amide moiety at the C-terminal carboxyl group of some biologically active peptides have shown that derivatization with 1-adamantanamine produced potent analogues with peripheral and central opioid activities comparable to or higher than those of the parent peptides [2, 3] or stimulated host immune response [4].

1-Adamantanamine (amantadine, Symmetrel®) has been reported to possess a wide range of biological activities and it is used or considered for treatment of viral diseases [5–7], Parkinson's disease [8], depression [9] and heredo-degenerative diseases [10].

These findings prompted us to synthesize 1-adamantanamine derivatives of peptides corresponding to N-terminal segments of enkephalins and their D-Ala$^2$-analogues, in order to combine the opioid activity and cell-mediated immunity enhancement of these peptide sequences with already reported beneficial effects of 1-adamantanamine presence in host immune response.

## Peptide Synthesis

The peptide amides were prepared by classical solution-phase methods of peptide synthesis with 1-adamantanamine as the starting material (Scheme 1). Despite the continuous improvement in peptide synthesis methodology, because of differences in reactivity of peptide precursors, no general strategy was available for the synthesis of 1-adamantanamine

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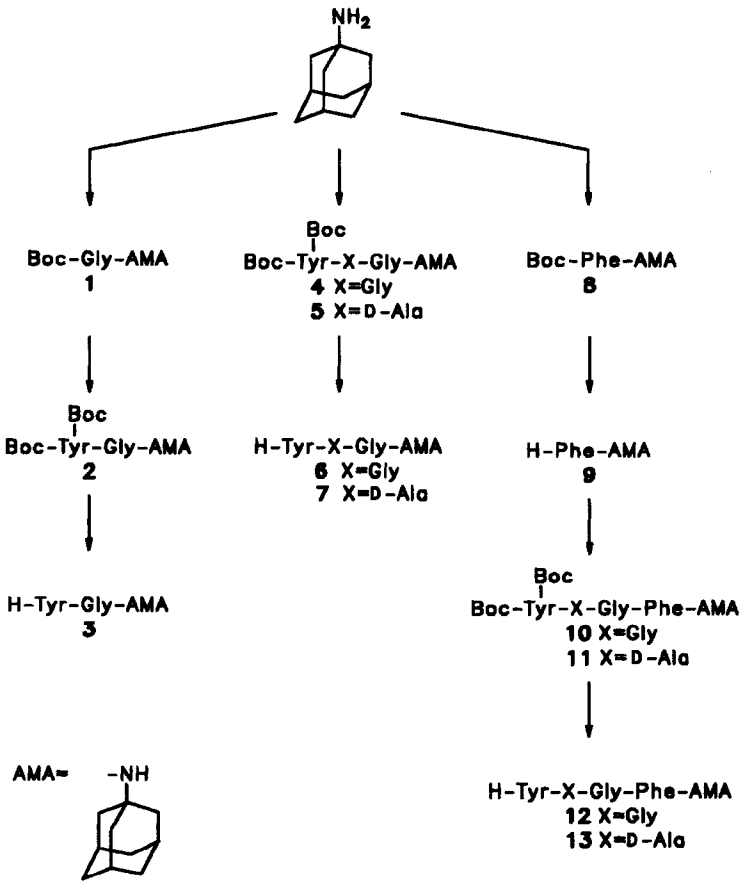
derivatives. Thus, the coupling of the peptide segments was achieved by using either different active esters of the N-terminally protected compounds or the mixed anhydride method with isobutyl chloroformate. In order to complete the peptide chain, the Boc-protecting group was cleaved by trifluoroacetic acid and the obtained compounds were condensed with the corresponding amino acid or peptide derivative. Finally, deprotection of the N-terminal amino acid residue and subsequent purification by gel chromatography gave the desired 1-adamantanamine peptide derivatives. The purity and identity of the synthetic peptides were assessed by RP-HPLC, TLC, NMR and elemental analysis.

The relative hydrophobicity of peptide amides **3**, **6**, **7**, **12** and **13** was estimated by HPLC. In a $C_{18}$ bonded reverse-phase column, retention times of all peptides conjugated to 1-aminoadamantane moiety were higher than the retention of [Leu$^5$]enkephalin (Table I). The effect was especially pronounced in tetrapeptides **12** and **13**, leading to a considerably stronger retention on reversed-phase column. Interestingly, the replacement of Gly$^2$ with D-Ala residue has rather little influence on retention behaviour.

The physical properties of the synthesized peptides are listed in Table 1.

### Determination of Opioid Activities *In Vitro*

In Table 2 the opioid activities of the di- (**3**), tri- (**6, 7**) and tetrapeptide (**12, 13**) amides in the guinea pig ileum (GPI) assay ($\mu$-receptor representative) and the mouse vas deferens (MVD) assay ($\delta$-receptor representative) are presented. Compound **3** behaved as a partial agonist in the GPI assay, since no more than 40% inhibition of the electrically evoked contractions could be achieved. The effect of the latter compound on the GPI was not naloxone-reversible, indicating that it was not due to interaction with opioid receptors. The low $K_e$-values observed with com-




Scheme 1

Table 1. Physical Properties and Elemental Analyses of Peptide Derivatives **1–13**

| Compound | Mp (°C) | $[\alpha]_D$ (c 1) | | Elemental analysis (upper, calc; lower, found) | | | RP-HPLC retention time (min)[a] |
|---|---|---|---|---|---|---|---|
| | | | | C | H | N | |
| Boc-Gly-AMA (**1**) | 121–123 | – | $C_{17}H_{28}N_2O_3$ | 66.20 | 9.15 | 9.08 | |
| | | | | 66.33 | 8.92 | 9.04 | |
| Boc-Tyr(Boc)-Gly-AMA (**2**) | 106–110 | – 5.3° ($CHCl_3$) | $C_{31}H_{45}N_3O_7$ | 65.13 | 7.93 | 7.35 | |
| | | | | 65.35 | 8.20 | 7.08 | |
| TFA × H-Tyr-Gly-AMA (**3**) | 135–145 | +37.0° (MeOH) | $C_{23}H_{30}F_3N_3O_5$ | 56.90 | 6.23 | 8.65 | 7.28 |
| | | | | 56.72 | 6.32 | 8.59 | |
| Boc-Tyr(Boc)-Gly-Gly-AMA (**4**) | 120–122 | + 1.5° ($CHCl_3$) | $C_{33}H_{48}N_4O_8$ | 63.04 | 7.69 | 8.91 | |
| | | | | 63.28 | 7.84 | 8.71 | |
| Boc-Tyr(Boc)-D-Ala-Gly-AMA (**5**) | 133–135 | – 29.0° (DMF) | $C_{34}H_{50}N_4O_8$ | 63.53 | 7.56 | 8.72 | |
| | | | | 63.72 | 7.56 | 8.50 | |
| TFA × H-Tyr-Gly-Gly-AMA (**6**) | 109–112 | +31.5° (MeOH) | $C_{25}H_{33}F_3N_4O_6$ | 55.34 | 6.13 | 10.33 | 6.80 |
| | | | | 55.28 | 6.32 | 10.54 | |
| TFA × H-Tyr-D-Ala-Gly-AMA (**7**) | 158–173 | +45.5° (MeOH) | $C_{26}H_{35}F_3N_4O_6$ | 56.11 | 6.34 | 10.06 | 6.89 |
| | | | | 55.90 | 6.14 | 10.19 | |
| Boc-Phe-AMA (**8**) | 118 | +4.8° (MeOH) | $C_{24}H_{34}N_2O_3$ | 72.33 | 8.59 | 7.02 | |
| | | | | 72.30 | 8.39 | 7.20 | |
| H-Phe-AMA (**9**) | 126–128 | +2.4° (MeOH) | $C_{19}H_{26}N_2O$ | 76.47 | 8.78 | 9.38 | |
| | | | | 76.26 | 8.89 | 9.48 | |
| Boc-Tyr(Boc)-Gly-Gly-Phe-AMA (**10**) | 131–141 | – 4.4° (MeOH) | $C_{42}H_{57}N_5O_9$ | 65.01 | 7.40 | 9.02 | |
| | | | | 64.83 | 7.48 | 9.22 | |
| Boc-Tyr(Boc)-D-Ala-Gly-Phe-AMA (**11**) | 150–151 | – 23.1° (DMF) | $C_{43}H_{59}N_5O_9$ | 65.38 | 7.53 | 8.86 | |
| | | | | 65.13 | 7.31 | 8.76 | |
| TFA × H-Tyr-Gly-Gly-Phe-AMA (**12**) | 119 (dec.) | +23.8° (MeOH) | $C_{34}H_{42}F_3N_5O_7$ | 59.21 | 6.14 | 10.15 | 18.68 |
| | | | | 59.15 | 6.33 | 10.30 | |
| TFA × H-Tyr-D-Ala-Gly-Phe-AMA (**13**) | 160 (dec.) | +26.4° (MeOH) | $C_{35}H_{44}F_3N_5O_7$ | 59.73 | 6.30 | 9.95 | 19.00 |
| | | | | 59.94 | 6.29 | 10.09 | |

[a]For RP-HPLC conditions see Experimental. Retention time of [Leu[5]]enkephalin: 5.25 min.

Table 2 Guinea Pig Ileum (GPI) and Mouse vas deferens (MVD) Assay of 1-Adamantanamine Peptide Derivatives

| Compound | GPI | | MVD | | MVD/GPI $IC_{50}$-ratio |
|---|---|---|---|---|---|
| | $IC_{50}$ (nM)[a] | Relative potency | $IC_{50}$ (nM)[a] | Relative potency | |
| H-Tyr-Gly-AMA (**3**) | partial agonist[b] | | 2280 ± 270 | 0.005 | – |
| H-Tyr-Gly-Gly-AMA (**6**) | 13 300 ± 290 | 0.02 | > 10 000 | < 0.001 | > 0.75 |
| H-Tyr-D-Ala-Gly-AMA (**7**) | 331 ± 14 | 0.74 | > 10 000 | < 0.001 | > 30.2 |
| H-Tyr-Gly-Gly-Phe-AMA (**12**) | 413 ± 10 | 0.59 | 1510 ± 340 | 0.007 | 3.65 |
| H-Tyr-D-Ala-Gly-Phe-AMA (**13**) | 4 ± 0.3 | 61.5 | 42 ± 3 | 0.271 | 0.5 |
| [Leu[5]]enkephalin | 246 ± 39 | 1 | 1.4 ± 1 | 1 | 0.05 |

[a]Mean of three determinations ± SEM: [b]Less than 50% inhibition of the electrically evoked contractions at $5 \times 10^{-5}$M.

pounds **6**, **7**, **12** and **13** in the GPI assay for naloxone as antagonist indicate that these peptide amides act on $\mu$-receptors in this assay and not on $\kappa$-receptors [11].

The D-Ala$^2$-substituted peptide amides **7** and **13** showed higher potency in both assay systems and increased $\mu$-receptor selectivity in comparison with the Gly$^2$-analogues **6** and **12**. In fact, compound **13** ranks among the most potent enkephalin-derived $\mu$ agonists reported to date.

The obtained data indicate that amidation of the C-terminal carboxyl group with 1-adamantanamine moiety produced a decrease in activity at the $\delta$ receptor. These results are in agreement with the general observation that enkephalin amides are more $\mu$-selective than corresponding enkephalins carrying a free C-terminal carboxyl group (for reviews, see [12] and [13]). Assuming that enzymatic degradation can be excluded as a factor affecting the potency relationships of compounds **12** and **13** in assay systems used [14], hydrophobic and steric factors [15] must play an important role in the interaction of these tetrapeptides with opioid receptors and cellular membranes. According to a new principle in peptide–receptor recognition mechanisms [16], the target cell membrane exerts translational, conformational and orientational constraints on regulatory peptides and enables the peptide to find its receptor and fit the binding site with greater ease. Thus, we suggest that hydrophobic peptide analogues **12** and **13** containing aminoadamantane moiety which is reported to penetrate the lipid bilayers [17], undergo spontaneous association with the membrane. When bound, D-Ala$^2$-peptide **13** most probably adopts the conformation characterized by a type II'$\beta$-turn around Gly$^3$-Phe$^4$ and a $\gamma$-turn around D-Ala$^2$ resulting in $\mu$-selective topography [16].

Interestingly, the tripeptide amide **7** was found to retain quite high $\mu$ agonist potency and to be considerably more $\mu$-selective than tetrapeptide **13**.

### Antiviral activity against HIV-1

The adamantanamine derivatives were evaluated for their antiviral activity using previously reported protocols [18]. The antiviral activities of compounds **3**, **6**, **7**, **12** and **13** against HIV-1, listed in Table 3, were determined in a cell culture system using peripheral blood lymphocytes. The anti-HIV activity of the compounds was determined on day 4 after virus infection by screening for syncytia formation (cytophatic effect, CPE) and quantitatively by measuring reverse transcriptase activity in the culture supernatant. None of the new adamantanamine derivatives was capable of providing full protection to the cells against HIV infection. In addition, tri- and tetrapeptide amides **6** and **12** proved to be toxic to the host cells at concentrations of 100 $\mu$g/ml. Although less syncytia formation was seen with compound **3** at 100 $\mu$g/ml and compounds **12** and **13** at 50 $\mu$g/ml, the antigen assay revealed that virus particles nonetheless had been synthesized. However, most interestingly, the D-Ala$^2$-tetrapeptide derivative **13** was able to inhibit syncytia formation even when the antigen assay gave evidence of viral replication. Whether the observed effect is a consequence of modified lymphocyte functions after interaction with opioid receptors present on the surface of this cells [19], or with some other molecules on the infected lymphocyte cell membrane, remains to be elucidated, however. In conclusion, these results show that opioid peptide analogues should be investigated further as an interesting class of biomolecules for the development of anti-HIV drugs.

Table 3 Effect of 1-Adamantanamine Peptide Derivatives on HIV-1 Infectivity of T-lymphocytes[a]

| Compound | Cytophatic effect[b] | | | |
|---|---|---|---|---|
| | 10 $\mu$g/ml[c] | 25 $\mu$g/ml[c] | 50 $\mu$g/ml[c] | 100 $\mu$g/ml[c] |
| H-Tyr-Gly-AMA (**3**) | + + + | + + + | + + | + |
| H-Tyr-Gly-Gly-AMA (**6**) | + + + | + + | + + | toxic |
| H-Tyr-D-Ala-Gly-AMA (**7**) | + + + | + + | + + | + + |
| H-Tyr-Gly-Gly-Phe-AMA (**12**) | + + + | + + | (+) | toxic |
| H-Tyr-D-Ala-Gly-Phe-AMA (**13**) | + + + | + + + | + | − |
| Controls: positive + + + negative − | | | | |

[a]See [18] for details.
[b]Scoring of syncytia formation was based on graded level of no apparent syncytia ( − ) to > 30 syncytia (+ + +) in a single microscopic field.
[c]Concentration of the peptide derivative.

## EXPERIMENTAL PART

Melting points were determined in capillaries and are uncorrected. Optical rotations were measured using an Optical Activity LTD automatic AA-10 Polarimeter. Column chromatography was performed on Silica Gel (Merck, 0.040–0.063 mm) and TLC on Silica Gel 60 with detection with ninhydrin, the chlorine–iodine reagent, or charring with $H_2SO_4$. The solvents used were: (A) benzene-EtOAc (proportions are given in the text); (B) EtOAc-$H_2O$-HOAc (70:2:2); (C) benzene-EtOAc-HOAc (50:5:5); (D) EtOAc. Homogeneity and retention of the final compounds were determined on a Varian 9010 liquid chromatography utilizing an analytical RP $C_{18}$ column (Serva, Octadecyl Si 100, 4 mm × 25 cm) under isocratic conditions using 57.5% MeOH in 0.1% TFA. The flow rate was 0.8 ml/min; UV detection at 280 nm. The structures of the compounds were confirmed by microanalysis (C, H, N) and by NMR spectroscopy using a Varian Gemini 300 spectrometer operating at 75.5 MHz ($^{13}C$) and 300.1 MHz ($^{1}H$).

### H-Tyr-Gly-AMA (3)

1-Adamantanamine hydrochloride (Aldrich, USA) (1406 mg, 7.5 mmol) dissolved in $CH_2Cl_2$ (100 ml) was neutralized with NMM (0.82 ml, 7.5 mmol) and allowed to react with Boc-Gly-OPCP (3054 mg, 7.5 mmol). The reaction mixture was stirred overnight at room temperature, the solvent was evaporated and the crude product was purified by flash chromatography with solvent system (A) (4:1). The oily product was precipitated from hexane with petroleum ether to give pure Boc-Gly-AMA (**1**) (1350 mg, 58%). Compound **1** (1233 mg, 4 mmol) was treated with TFA/$H_2O$ (9:1) (10 ml) in the presence of anisole (0.2 ml) for 15 min at room temperature. The solvent was removed *in vacuo* and the residue was triturated with benzene and dried. The resulting white solid TFA × H-Gly-AMA (1290 mg, 100%) was taken up in $CH_2Cl_2$ (40 ml), neutralized with NMM (0.44 ml, 4 mmol), and then Boc-Tyr(Boc)-OH (1524 mg, 4 mmol), DCC (824 mg, 4 mmol) and HOBt (540 mg, 4 mmol) were added to the solution. After stirring overnight, the precipitated DCU was filtered off and the filtrate was washed with water, 10% citric acid, aq. $NaHCO_3$ and water, and was dried ($Na_2SO_4$). The solvent was evaporated and the residue was purified by flash chromatography with eluent (A), (2:1) and (1:2), respectively, yielding pure Boc-Tyr(Boc)-Gly-AMA (**2**) (1143 mg, 50%). Compound **2** (518 mg, 0.9 mmol) was treated with TFA/$H_2O$) (9:1) (10 ml) in the presence of anisole (0.2 ml) for 30 min at room temperature. After evaporation of the solvent, the residue was triturated several times with $Et_2O$ to give TFA × H-Tyr-Gly-AMA (431 mg, 98%). The TFA salt (100 mg, 0.2 mmol) was dissolved in a small amount of $H_2O$, desalted on a short (5 × 1 cm) Dowex 1 × 2200 (Ac) column and purified by gel filtration on a column (75 × 1.2 cm) of Sephadex G-15 with 1% aq. HOAc as solvent. Fractions containing pure product were lyophilized, yielding 41 mg of **3** (98% purity by RP HPLC). $^{13}C$-NMR ($D_2O$): $\delta$ 29.9 (AMA $\gamma$-CH), 36.5 (AMA $\delta$-$CH_2$), 37.1 (Tyr $\beta$-$CH_2$), 41.5 (AMA $\beta$-$CH_2$), 43.8 (Gly $CH_2$), 53.2 (AMA $\alpha$-C), 55.4 (Tyr $\alpha$-CH), 116.6 (Tyr $\varepsilon$-CH), 126.5 (Tyr $\delta$-CH), 131.6 (Tyr $\gamma$-C), 159.9 (Tyr-$\zeta$-C), 169.7, 170.9 (Tyr, Gly CO).

### H-Tyr-Gly-Gly-AMA (6)

To a chilled solution (− 15°C) of Boc-Tyr(Boc)-Gly-Gly-OH (2477 mg, 5 mmol) in $CH_2Cl_2$ (50 ml), NMM (0.56 ml, 5 mmol) and isobutyl chloroformate (0.68 ml, 5 mmol) were added. The resulting mixture was stirred for 2 min at the same temperature and a precooled solution of 1-adamantanamine hydrochloride (937 mg, 5 mmol) in $CH_2Cl_2$ (50 ml) containing NMM (0.56 ml, 5 mmol) was then added. The reaction mixture was stirred for 30 min at − 15°C and for 2 h at room temperature, and was then washed with water and dried ($Na_2SO_4$). The solvent was evaporated *in vacuo* and the residue was purified by silica gel flash chromatography with eluent (B). Trituration with diisopropyl ether gave homogeneous Boc-Tyr(Boc)-Gly-Gly-AMA (**4**) (1728 mg, 55%). To compound **4** (100 mg, 0.16 mmol) dissolved in $CH_2Cl_2$ (5 ml), TFA (1.5 ml) and anisole (0.2 ml) were added, and the solution was stirred at room temperature for 30 min. Addition of dry $Et_2O$ at 0°C and subsequent centrifugation gave TFA × H-Tyr-Gly-Gly-AMA (81 mg, 100%). Desalting and gel filtration, performed in the same way as described for **3**, afforded pure **6** (67 mg, 98%) (99% purity by RP-HPLC). $^{13}C$-NMR ($CD_3OD$): $\delta$ 29.2 (AMA $\gamma$-CH), 36.5 (AMA $\delta$-$CH_2$), 36.7 (Tyr $\beta$-$CH_2$), 41.5 (AMA $\beta$-$CH_2$), 43.2, 43.8 (Gly, Gly $CH_2$), 53.2 (AMA $\alpha$-C), 55.3 (Tyr $\alpha$-CH), 116.7 (Tyr $\varepsilon$-CH), 126.2 (Tyr $\delta$-CH), 131.6 (Tyr $\gamma$-C), 156.1 (Tyr $\zeta$-C), 170.0, 170.6, 171.7 (Tyr,Gly,Gly CO).

### H-Tyr-D-Ala-Gly-AMA (7)

To a cool (0°C) solution of Boc-Tyr(Boc)-D-Ala-Gly-OH (300 mg, 0.59 mmol) in $CH_2Cl_2$ (20 ml), *N*-hydroxy-5-norbornene-2,3-dicarboximide (106 mg, 0.59 mmol) and DCC (121 mg, 0.59 mmol) were added and the reaction mixture was stirred for 5 h at room temperature. The precipitated DCU was filtered off and the filtrate was added to a solution of 1-adamantanamine hydrochloride (111 mg, 0.59 mmol) in $CH_2Cl_2$ (10 ml) that had been neutralized with NMM (65 $\mu$l, 0.59 mmol). After stirring overnight, the solvent was evaporated *in vacuo* and the residue was purified by flash chromatography with eluent (C). After precipitation from $CHCl_3$/diisopropyl ether 248 mg (65%) of Boc-Tyr(Boc)-D-Ala-Gly-AMA (**5**) were obtained. Compound **5** (207 mg, 0.32 mmol) was deprotected with TFA as described in the preparation of **6**. The obtained TFA salt (112 mg, 63%) was desalted on a Dowex 1 × 2200 (Ac) column and purified by gel filtration on a column of Sephadex G-25 (fine) with 0.01 M aq. HOAc as solvent, yielding 64 mg (45%) of **7** (98% purity by RP-HPLC). $^{13}$C-NMR ($CD_3OD$): $\delta$ 17.5 (D-Ala $\beta$-$CH_3$), 30.8 (AMA $\gamma$-CH), 37.4 (AMA $\delta$-$CH_2$), 37.7 (Tyr $\beta$-$CH_2$), 42.2 (AMA $\beta$-$CH_2$), 43.8 (Gly $CH_2$), 50.6 (D-Ala $\alpha$-CH), 53.1 (AMA $\alpha$-C), 56.0 (Tyr $\alpha$-CH), 116.7 (Tyr $\varepsilon$-CH), 126.1 Tyr $\delta$-CH), 131.5 (Tyr $\gamma$-C), 158.2 (Tyr $\zeta$-C), 169.9, 170.0 (Tyr, Gly CO), 174.8 (D-Ala CO).

### H-Tyr-Gly-Gly-Phe-AMA (12)

Boc-Phe-AMA (**8**) was prepared from Boc-Phe-OH (1333 mg, 5 mmol) by using the method described for the synthesis of **2**. Chromatography on a silica gel column using eluent (A) (4:1) and subsequent precipitation from EtOH/$H_2O$ afforded pure **8** (1427 mg, 72%). **8** (453 mg, 1.13 mmol) was treated with 30% TFA in $CH_2Cl_2$ (5 ml) at 0°C for 30 min. The solvent was removed *in vacuo*, the residue was taken up in $CH_2Cl_2$ and was washed with aq. $Na_2CO_3$. After drying ($Na_2SO_4$), the solvent was evaporated and the product was precipitated from EtOH/$H_2O$, resulting in pure H-Phe-AMA (**9**) (260 mg, 76%). Boc-Tyr(Boc)-Gly-Gly-Phe-AMA (**10**) was prepared from **9** (480 mg, 1.6 mmol) and Boc-Tyr(Boc)-Gly-Gly-OH (796 mg, 1.6 mmol) in the same way as described for **4**. Chromatography on silica gel with eluent (D) and crystallization from diisopropyl ether-$Et_2O$ afforded homogeneous **10** (942 mg, 75%). Compound **10** (102 mg, 0.13 mmol) was treated with TFA following the procedure described for **9**. Evaporation of the solvent and crystallization from the solvent mixture EtOH-$Et_2O$-diisopropyl ether afforded the pure TFA salt of **12** (73 mg, 82%). Desalting and gel chromatography of the latter in the same manner as described for **7** gave pure compound **12** (50 mg, 67%) (96% purity by RP-HPLC). $^{13}$C-NMR ($CD_3OD$): $\delta$ 30.8 (AMA $\gamma$-CH), 37.4 (AMA $\delta$-$CH_2$), 39.4 (Tyr, Phe $\beta$-$CH_2$), 42.1 (AMA $\beta$-$CH_2$), 43.4, 43.9 (Gly, Gly $CH_2$), 53.0 (AMA $\alpha$-C), 56.5 (Tyr, Phe $\alpha$-CH), 56.5 (Tyr, Phe $\alpha$-CH), 116.6 (Tyr $\varepsilon$-CH), 127.7 (Tyr $\delta$-CH), 127.7 (Phe $\zeta$-CH), 129.4 (Phe $\varepsilon$-CH), 130.5 (Phe $\delta$-CH), 131.5 (Tyr $\gamma$-C), 138.3 (Phe $\gamma$-C), 157.8 (Tyr $\zeta$-C), 170.9, 171.8, 172.0, 174.2 (Tyr, Gly, Gly, Phe CO).

### H-Tyr-D-Ala-Gly-Phe-AMA (13)

Compound **11** was prepared by the mixed anhydride method from Boc-Tyr(Boc)-D-Ala-Gly-OH (443 mg, 0.87 mmol) and **9** (259 mg, 0.87 mmol) in the same way as described for **10**. Flash chromatography with solvent (D) afforded pure **11** (452 mg, 66%). H-Tyr-D-Ala-Gly-Phe-AMA (**13**) was prepared from **11** (110 mg, 0.14 mmol) following the same deprotection and purification procedure as described for **12**, yielding TFA × **13** (75 mg, 76%). Gel filtration of the crude product resulted in pure **13** (20 mg, 24%) (98% purity by RP-HPLC). $^{13}$C-NMR ($CD_3OD$): $\delta$ 16.8 (D-Ala $\beta$-$CH_3$), 30.8 (AMA $\gamma$-CH), 37.4 (AMA $\delta$-$CH_2$), 37.8, 39.5 (Tyr, Phe $\beta$-$CH_2$), 42.1 (AMA $\beta$-$CH_2$), 43.4 (Gly $CH_2$), 51.9 (D-Ala $\alpha$-CH), 53.2 (AMA $\alpha$-C), 56.1, 56.2 (Tyr,Phe $\alpha$-CH), 116.9 (Tyr $\varepsilon$-CH), 126.0 (Phe $\zeta$-CH), 127.7 (Tyr $\delta$-CH), 129.4 (Phe $\varepsilon$-CH), 130.6 (Phe $\delta$-CH), 131.6 (Tyr $\gamma$-C), 138.3 (Phe $\gamma$-C), 158.3 (Tyr $\zeta$-C), 170.92, 170.94, 172.3, 175.3 (Tyr, D-Ala, Gly, Phe, CO).

### Opioid Activity Assays

The opioid agonist activities of peptide amides were determined *in vitro* on electrically stimulated longitudinal muscle strip of guinea pig ileum (GPI) and mouse vas deferens (MVD) preparations carried out as reported previously [20]. The agonist activities of compounds were characterized by the 50% inhibitory concentration ($IC_{50}$) given uniformly in nM. A log-dose/response curve was determined with [Leu$^5$]enkephalin as standard for each preparation and $IC_{50}$ values of the peptide derivatives being tested were normalized as described in the literature [21]. In GPI preparation naloxone sensitivities of peptide amides were determined using procedure previously described [11].

### Antiviral Activity

The assay is described in detail in [18]. Briefly, peripheral blood lymphocytes from HIV-seronegative donors were stimulated for two days with phytohaemagglutinin and infected with HIV-1 for 1 h in the absence of the compounds. Unbound virus was removed by washing and the cells were then cultured in the presence or absence of the substances for four days. Compounds **3**, **6**, **7**, **12** and **13**, dissolved in cell culture medium, were tested in concentrations of 10, 25, 50 and 100 μg/ml. Cultures were screened for the formation of HIV-induced syncytia by light microscopy. For quantitative analysis, reverse transcriptase activity in the culture supernatant was determined as described in the literature [18]. Each treatment was carried out in duplicate and the results are averaged. Additionally, cultures were screened for drug toxicity.

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