A Phosphinic Acid Dipeptide Analogue to Stabilize Peptide Drugs During Their Intranasal Absorption

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A major challenge in intranasal delivery of peptides is to overcome the enzymatic barrier that limits their absorption. Aminopeptidase inhibitors may be useful for improving systemic delivery of peptide drugs administered nasally. A phosphinic acid dipeptide analogue, a transition-state analogue aminopeptidase inhibitor in which the phosphinate moiety exists in a tetrahedral state mimicking peptides during their enzymatic hydrolysis, was synthesized and tested nasally *in situ* in rats. This inhibitor was found to inhibit greatly the degradation of the model peptide leucine-enkaphalin in the nasal perfusate at ≤2 μM concentrations. The nasal peptidase hydrolytic activity was reversible after exposure to the inhibitor. This inhibitor has the advantage of efficacy at very low concentrations and reversibility of effects.

KEY WORDS: phosphinic acid dipeptide analogue; nasal peptide delivery; transition-state analogue.

INTRODUCTION

Biologically active peptides are typically inactive when administered orally because of extensive metabolism in the gastrointestinal tract and poor membrane permeability. Other transmucosal routes of administration such as nasal, rectal, vaginal, and buccal have been examined as alternatives to dosing orally or parenterally. Among these routes, the nasal route has been the route most investigated for peptide delivery. Nasal bioavailability of peptides is generally much higher than oral bioavailability because the proteolytic digestive enzymes of the gut are circumvented; however, it is usually <100% and, in many cases, is very low because of the metabolism by the nasal mucosal peptidases, poor membrane penetration, or both. Peptidase inhibitors might be useful for inhibiting the presystemic metabolism of nasally administered peptide drugs.

One attractive class of inhibitors is the transition-state analogues because of their expected potency and reversibility. A transition-state analogue design is based on the hypothesis that molecules which resemble a substrate in its transition-state geometry have a much higher affinity for the active site of an enzyme than the substrate itself. We have recently investigated a class of transition-state analogue aminopeptidase inhibitors (aminoboronic acid derivatives) (1). These inhibitors were found to inhibit, at very low concentrations, the degradation of leucine-enkephalin (leu-enk) (1) and thymopentin (2) by the rat nasal mucosal peptidases. The rat nasal mucosa was found to recover from the effects of these inhibitors (3).

Phosphinic acid dipeptide analogues are another class of transition-state analogue inhibitors in which the phosphinate moiety exists in a tetrahedral state resembling peptides during their enzymatic hydrolysis (Scheme I). Phosphinic and phosphinic acid analogues of amino acids and peptides have previously been shown to be inhibitors of aminopeptidases (4). However, phosphinate dipeptide isosteres where $-C(O)NH-$ is replaced by $-P(O)(OH)CH_2-$ have not been described for this purpose. In this report, the synthesis and potential use of a phosphinic acid dipeptide analogue to stabilize the model peptide, leucine-enkephalin, in the rat nasal purfusate model are described.

EXPERIMENTAL

Materials

Leucine enkephalin (Tyr-Gly-Gly-Phe-Leu), destyrosyl-leucine enkephalin (Gly-Gly-Phe-Leu), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were obtained from Sigma Chemical Company.

Sodium pentobarbital injection (Nembutal) was purchased from Abbott Laboratories. All other reagents were obtained from Aldrich Chemical Company.

Chemistry

The enzyme inhibitor VI, H-Phe-Ψ[P(O)(OH)CH_2]-Phe-OCH_3, was synthesized according to Scheme II. Compound I was synthesized as described by Baylis *et al.* (5). The racemate was resolved by recrystallization of the (+)-α-methylbenzylamine salt of the *N*-benzyloxycarbonyl derivative to constant optical rotation ($[\alpha]^{24}$, $-59.8 \pm 0.8°$) for the levorotatory salt. The phosphinic acid analogue of (L)-phenylalanine, II, was isolated from the salt by acidification of its aqueous solution followed by extraction into ethylacetate. The free phosphinic acid was protected by forming the methyl ester, III, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in anhydrous methanol.

The phosphinic acid dipeptide analogue, IV, was obtained by Michael addition of III to methyl benzacrylate, using the procedure described by Parsons *et al.* (6). The product was extracted into ethyl acetate from a neutral aqueous solution; on evaporation, a clear oil was obtained. ^{31}P-NMR (CD_3OD) δ 55 ppm (overlapping 2d); ^{1}H-NMR (CD_3OD) δ 1.95 (m, 1 H), 2.25 (m, 1 H), 2.65–3.25 (overlapping m, 4 H), 3.55–3.80 (overlapping m and s, 7 H), 4.22 (m, 1 H), 4.96 (m, 2 H), 7.10–7.48 (overlapping m, 15 H); MS (FAB), *m/e* 510.2 (M + H), 532.2 (M + Na).

The phosphinic acid moiety of IV was deprotected by treatment with bromotrimethylsilane in deuterochloroform (the reaction was monitored by ^{1}H-NMR); excess reagent was quenched by methanol, which also regenerated the free phosphinic acid. The product was precipitated from methanol/ether as a white powder. ^{31}P-NMR ($CDCl_3$) δ 42–45 ppm (overlapping, broad, 4m); ^{1}H-NMR ($CDCl_3$) δ 1.92 (m, 1 H),

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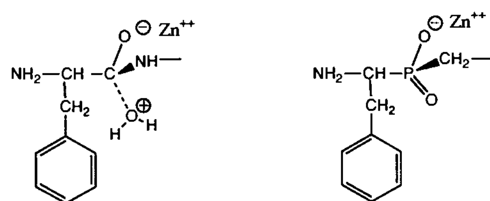
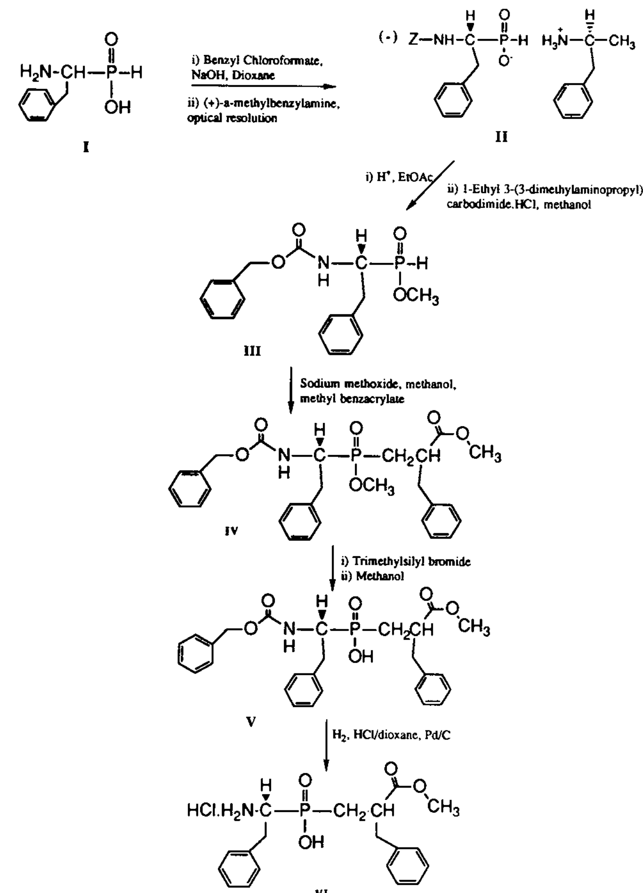


Scheme I. Transition state of peptide hydrolysis and proposed inhibition mechanism of aminopeptidase by a phosphinate inhibitor.

2.17 (m, 1 H), 2.72–2.93 (overlapping m, 2 H), 3.10–3.35 (overlapping m, 3 H), 3.56–3.71 (2 s, 3 H), 4.14 (m, 1 H), 4.95 (m, 2 H), 7.08–7.42 (overlapping m, 15 H), MS (FAB), *m/e* 496.2 (M + H), 518.2 (M + Na).

The benzyloxycarbonyl group of V was removed by catalytic hydrogenation on a Parr apparatus using an initial pressure of 40 psi. The reaction was performed in methanol, in the presence of 10% Pd/C and 1 equiv of anhydrous hydrogen chloride. The catalyst was removed by filtration, and the filtrate was evaporated to obtain a solid. The product was dissolved in dimethyl sulfoxide, filtered, and reprecipitated by adding chloroform to yield the desired product VI as an amorphous light yellow solid. ^{31}P-NMR (DMSO-d_6) δ 40 ppm (broad, s); ^{1}H-NMR (DMSO-d_6) δ 1.32 (m, 1 H), 1.59 (m, 1 H), 2.67–3.13 (overlapping m, 6 H), 3.43 (s, 3H), 7.08–7.31 (overlapping m, 10 H); MS (FAB), *m/e* 367.3 (M-d_4 + ^{2}H).



Scheme II. Synthesis of the phosphinate inhibitor VI.

Perfusion Studies and Analysis

The method for perfusion of the rat nasal cavity was as described previously (1,2). Male rats (CD, Charles River) weighing approximately 300 g were used for all studies reported here.

Studies examining recovery of the rat nasal mucosa from the inhibitor effect were performed in two phases. In phase I, the rat nasal mucosa was perfused with leucine enkephalin either with or without the aminopeptidase inhibitor VI at a 1 μ*M* concentration. After phase I, the nasal cavity was perfused for 30 min with 45 ml of saline, which was not recirculated. In phase II, the perfusate contained only leucine enkephalin, without the aminopeptidase inhibitor. The samples were immediately diluted with 2 vol of 0.1 *M* citric acid (pH 2.3) to quench further metabolism.

Leucine enkephalin and des-tyrosyl-leucine enkephalin concentrations were then determined by HPLC. The mobile phase was 0.1 *M* monobasic sodium phosphate:phosphoric acid:acetonitrile (916:5:275) and the flow rate was 1.7 ml/min. An octylsilane column (DuPont) was used. Detection was by UV absorbance at 210 nm. Typical retention times for leucine enkephalin and destyrosyl-leucine enkephalin were 7.3 and 4.8 min, respectively.

RESULTS AND DISCUSSION

The mucosal membrane of the nasal cavity is known to have various types of peptidase and protease activities (7). Knowing the mechanism by which a peptide drug degrade will allow the design of an inhibitor to optimize its delivery across the absorption site. A useful inhibitor for a peptide intended for intranasal delivery should be potent and reversible. The phosphinate ester VI was tested for this use. Leu-enk (Ty-Gly-Gly-Phe-Leu), a pentapeptide previously reported to undergo extensive hydrolysis on the N-terminus in the nasal cavity (1), was used as a model peptide.

Figure 1 shows the degradation of leu-enk in the nasal perfusate of rats and the appearance of the metabolite, Des-Tyr-leu-enk (Gly-Gly-Phe-Leu), in the absence of inhibitor. Fifty percent of leu-enk was hydrolyzed in 60 min, with con-

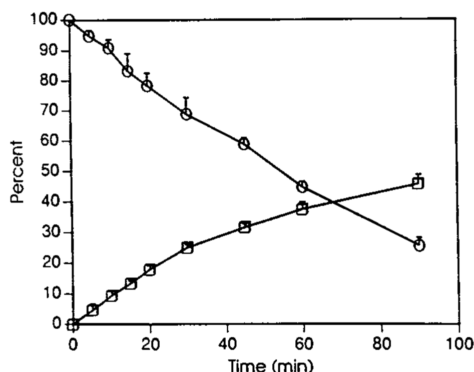


Fig. 1. *In situ* nasal disappearance of 0.1 m*M* leucine-enkephalin (○) and appearance of Des-Tyr-leucine-enkephalin (□) in the nasal perfusate (10 ml). Symbols represent the mean ± SD of three rats.

comitant appearance of its metabolite. However, in the presence of 2 μ*M* phosphinate inhibitor VI, 92% of leu-enk was still intact in the nasal perfusate after 90 min and the concentration of its metabolite was <10% even after 120 min (Fig. 2). Almost all the peptide was accounted for because the perfusion protocol delivers a large volume of solution to a proportionally small area of the membrane for a brief period of time permitting minimal absorption of the peptide. Figure 3 shows the inhibition of degradation of leu-enk in the nasal perfusate at different concentrations of VI compared to degradation in the absence of the inhibitor. In the presence of 0.5, 1, and 2 μ*M* concentrations of the inhibitor, the percentages of intact leu-enk in the nasal perfusate after 90 min were 75, 83, and 92%, respectively. However, in the absence of the inhibitor, only 30% was intact after 90 min.

In order to elicit a similar effect, higher concentrations of the known inhibitors, bestatin (an inhibitor of leucine aminopeptidase, aminopeptidase B, and aminopeptidase N) and puromycin (an inhibitor of aminopeptidase B and aminopeptidase N), would be needed. The concentrations of bestatin and puromycin needed to elicit a potency similar to that of VI were greater than 10× and 100×, respectively (1).

To test for reversibility of enzyme inhibition, perfusion studies were conducted in two phases. In phase I, the perfusion solution contained leu-enk along with the inhibitor. The control group of rats was perfused with leu-enk alone. At the end of phase I, a perfusion rinse with 45 ml of saline was carried out. In phase II, both groups of rats were perfused with buffer containing leu-enk as in phase I, but with no inhibitor.

Decay rate constants were calculated, for the control group with no inhibitor and for rats for which the perfusion solution also contained VI, as the slopes of individual concentrations versus time.

In the absence of the inhibitor (control rats) there was no difference in the rate of leu-enk disappearance between the two phases (0.55 ± 0.02 vs 0.51 ± 0.05 μg/ml/min). In the presence of VI (1 μ*M*) leu-enk metabolism was greatly inhibited in Phase I (0.16 ± 0.05 μg/ml/min.). After the nasal cavity was rinsed with saline, the leu-enk disappearance rate returned to levels similar to those of control rats (0.50 ± 0.03 μg/ml/min), indicating that the inhibition of aminopeptidases was reversible. Previous work with bestatin and puromycin showed that these two inhibitors were both much less potent in preventing leu-enk degradation and at higher concentrations caused an increase in the disappearance rate of leu-enk in Phase II, indicating a possible disruption of the nasal mucosa (3).

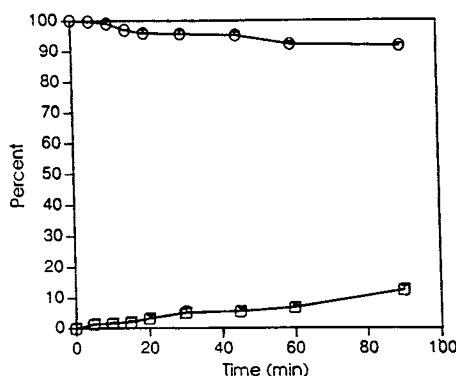


Fig. 2. *In situ* nasal disappearance of 0.1 m*M* leucine-enkephalin (○) and appearance of Des-Tyr-leucine-enkephalin (□) in the nasal perfusate in the presence of 2 μ*M* phosphinate inhibitor (VI). The symbols represent the mean ± SD of three rats.

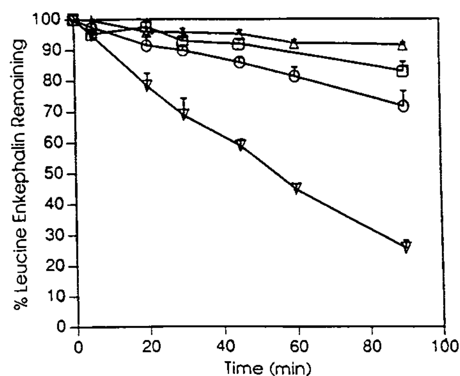


Fig. 3. *In situ* nasal disappearance of 0.1 m*M* leucine-enkephalin in the nasal perfusate (10 ml) in the absence of inhibitor (▽) and in the presence of 0.5 (○), 1.0 (□), and 2.0 (△) μ*M* phosphinate (VI). Symbols represent the means ± SD of three rats.

Although this inhibitor (*VI*) is more potent than bestatin and puromycin, it is less potent than boroleucine (1). The structural requirements for the substituents on the phosphinic acid that are critical for activity are being studied.

In conclusion, the phosphinate analogue VI is a potent inhibitor of the degradation of leu-enk in the nasal cavity by aminopeptidases. The inhibitor effect on the aminopeptidases activity is reversed when the inhibitor is removed.

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