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NOVEL *IN VITRO* AND *IN VIVO* INHIBITORS OF PROLYL ENDOPEPTIDASE

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Abstract. Inhibition of prolyl endopeptidase by Z-cyclohexyl prolinal and Z-indolinyl prolinal occurs with slow, tight binding inhibition and K_i values of 2 - 3 nM. *In vivo* enzyme inhibition is also observed with a half time for recovery of enzyme activity of 3 - 4 h.

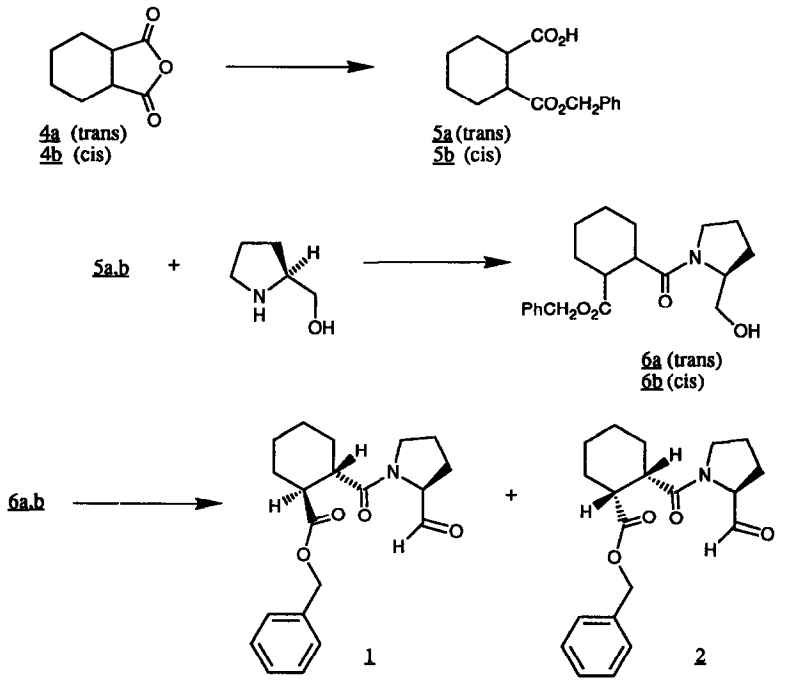
Prolyl endopeptidase (PEP) is a serine protease that specifically cleaves peptidyl proline bonds[1]. The enzyme hydrolyzes many biologically active peptides, including thyrotropin releasing hormone (TRH), substance P, vasopressin, angiotensin II, oxytocin and bradykinin[2, 3]. Vasopressin may facilitate learning and memory[4, 5]; since prolyl endopeptidase cleaves vasopressin, it is possible that inhibition of this enzyme could lead to a beneficial increase in CNS vasopressin levels. Yoshimoto *et al.*[6] report that the PEP inhibitor N-benzyloxycarbonyl prolyl-prolinal (Z-pro-prolinal), reverses scopolamine-induced amnesia in the passive avoidance learning test in rats. The anti-amnesic effect of a series of compounds correlated with their *in vitro* enzyme inhibition potency. Thus, compounds which inhibit prolyl endopeptidase may be useful as palliative agents in dementia. Recently, we have shown that Z-pro-prolinal is a slow, tight binding inhibitor of prolyl endopeptidase[7]. Here we report on the *in vitro* and *in vivo* inhibition of prolyl endopeptidase by two novel Z-pro-prolinal derivatives, 2-[(2-formyl-1-pyrrolidinyl) carbonyl]-cyclohexanecarboxylic acid, phenylmethyl ester (Z-cyclohexyl prolinal; 1 and 2) and 2-[(2-formyl-1-pyrrolidinyl) carbonyl]-2,3-dihydro-1H-indole-1-carboxylic acid, phenylmethyl ester (Z-indolinyl prolinal; 3).

Compounds 1 - 3 were selected as targets in an effort to explore whether or not the Z-prolyl moiety of the known inhibitor Z-pro-prolinal could be mimicked by alternative functional groups. Previously, this proline moiety has been replaced with various amino acids which generally resulted in significant loss of enzyme inhibition activity[8], or with L-thioproline, which appears to afford equivalent activity[9]. Replacement of L-proline with D-proline (to give Z-D-prolyl-L-prolinal) leads to a complete loss in enzyme inhibition activity[10]. It is not clear from these observations what are the optimum size and configuration of this amino acid. We therefore prepared compounds 1 - 3 with a proline replacement, in order to maximize the chance of finding superior *in vitro* and *in vivo* activi-

ty.

Synthesis of *cis*- and *trans*-Z-cyclohexyl-prolinal was accomplished as follows (Scheme 1): *trans*-1,2-cyclohexanedicarboxylic anhydride was refluxed in benzyl alcohol to afford, after standard work-up, a 33% yield of the mono-benzyl ester of *trans*-1,2-cyclohexanedicarboxylic acid. Similarly, the benzyl ester of *cis*-1,2-cyclohexanedicarboxylic acid was prepared in 68% yield. The respective acids were reacted with S-pyrrolidine-2-methanol in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, [1-hydroxybenzotriazole, CH_2Cl_2, room temp, 48 h] to afford the *cis*- (67% yield) and *trans*- (33% yield) S-pyrrolidine-2-methanol amides. Oxidation of the alcohols [DMSO, oxalyl chloride, TEA, CH_2Cl_2, -70 °C] led to the desired *trans*-Z-cyclohexyl prolinal [oil, 76% yield; ^{1}H-NMR (250 MHz,$CDCl_3$, δ) 1.00-2.00 (m, 12H), 2.14 (m, 1H), 2.65 (m, 1H), 2.82 (m, 1H), 3.30 - 3.8 (m, 2H), 4.16 - 4.30 (m, 1H), 5.08 (m, 2H), 7.30 (m, 5H), 9.16-9.38 (s, 1H); MS m/e 344.2 (M^+)] and *cis*-Z-cyclohexyl prolinal [oil, 50% yield; ^{1}H-NMR (250 MHz, $CDCl_3$, δ) 1.00-2.60 (m, 13H), 3.12 (m, 1H), 3.50 (m, 2H), 4.20 (m, 1H), 5.0 (m, 2H), 7.28 (m, 5H), 9.12-9.36 (s, 1H); MS m/e 344.1 (M^+)].

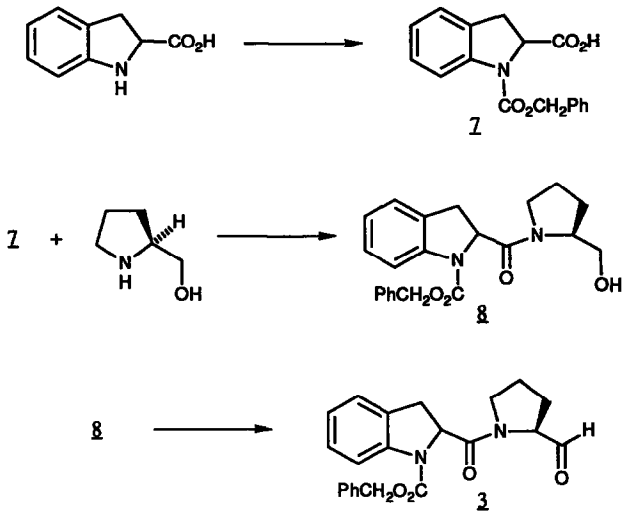
Scheme 1



Synthesis of Z-indolyl prolinal 3 (Scheme 2) was carried out by addition of indoline-2-car-

boxylic acid to a solution of carbobenzyloxychloride [H_2O, pH > 8] to afford N-carbobenzyloxyindoline-2-carboxylic acid (7) (51%). Subsequent reaction of this product with S-pyrrolidine-2-methanol [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 1-hydroxybenzotriazole, CH_2Cl_2, room temp, 16 h] gave the corresponding pyrrolidine amide in 49% yield. Oxidation of the pyrrolidine-2- alcohol [DMSO, oxalyl chloride, TEA, CH_2Cl_2, - 70 °C] yielded Z-indolinyl prolinal 3 as a white crystalline solid [^{1}H-NMR (250 MHz, $CDCl_3$, δ) 1.30-2.00 (m, 4H), 3.00 - 4.6 (m, 7H), 4.8 - 5.4 (m, 2H), 4.8 - 7.4 (m, 8H), 7.90 (d, 1H), 9.10 - 9.44 (m, 1H); MS m/e 378.1 (M^+); m.p.= 144°-146°C.

Scheme 2



In vitro inhibition of prolyl endopeptidase by Z-indolinyl prolinal, *trans* -Z-cyclohexyl prolinal and *cis* Z-cyclohexyl prolinal was studied under conditions described by Bakker *et al.*[7]. Slow, tight binding inhibition was observed with all three compounds. The kinetic parameters associated with inhibition are given in Table 1. Slow-binding inhibition[11] was apparent in the inhibition progress curves upon addition of 5 - 50 nM of each compound. Each progress curve was fit to the equation $y = Ae^{-kt} + B + Ct$ by non-linear least squares regression[12,13], in which the steady state (final) rate equals C, the initial rate is -Ak+C, and the observed rate constant is k. The kinetic mechanism of inhibition observed for all three compounds is identical to that observed in PEP inhibition by Z-pro-prolinal[7], and is consistent with simple one-step inhibition (Scheme 3).

Table 1

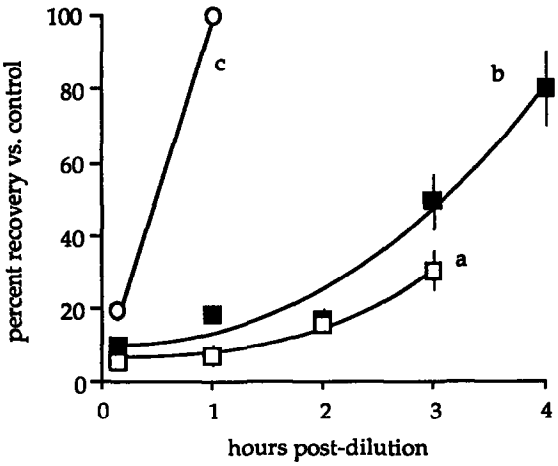
compound	K_i	k_{on} ($M^{-1}sec^{-1}$)	k_{off} (sec^{-1})
trans Z-cyclohexyl prolinal (1)	3 nM	2.3×10^5	0.7×10^{-3}
cis Z-cyclohexyl prolinal (2)	3 nM	7×10^5	2×10^{-3}
Z-indolinyl prolinal (3)	2.4 nM	1.4×10^5	0.3×10^{-3}

Scheme 3

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

Ex vivo enzyme inhibition studies were performed to assess the degree of CNS availability and PEP inhibition after peripheral compound administration. Male CD-1 mice (20-40 gm) received an i.p. injection of vehicle (5:5:90::DMSO: Emulphor™: saline) or compound (in vehicle). The mice received a single i.p. injection of Z-indolinyl prolinal, *cis* Z-cyclohexyl prolinal or *trans* Z-cyclohexyl prolinal at 32 mg/kg, and were decapitated at fixed time intervals thereafter (10-360 min). The brain was immediately removed, weighed, and homogenized with an equal volume of ice-cold buffer (0.1 M Tris·HCl, 0.5 mM EDTA, pH 8.3). A 1 µL aliquot of the homogenate was added to an assay cuvette containing 2.0 mL buffer (0.1 M Tris·HCl, 1 mM DTT; pH 7.4) and 1.25 µM substrate (Z-gly-pro-AMC). The return of activity was measured fluorometrically (380 nm excitation, 460 nm emission) at 25 °C. The initial enzyme rate from a test animal was used to express the results as percent of control. As shown in Figure 1, all compounds cross the blood/brain barrier and inhibit the enzyme at levels greater than 80% within 10 min. Enzyme inhibition by Z-indolinyl prolinal and *trans* Z-cyclohexyl prolinal returns with half times ($t_{1/2}$) of approximately 3 and 4 h, respectively, while $t_{1/2}$ for *cis* Z-cyclohexyl prolinal is < 1 hr. In contrast, recovery from Z-pro-prolinal inhibition in similar *ex vivo* experiments has $t_{1/2} >> 5$ h after a dose of 3.2 mg/kg. Although Z-pro-prolinal is 10-fold more potent *in vitro* than 1 or 3, the increase in $t_{1/2}$ is more than one would expect from enzyme inhibition kinetics alone. Thus, pharmacokinetic parameters not associated with enzyme inhibition are very important in determining drug efficacy, since compounds that show excellent *in vitro* potency may be poor inhibitors *in vivo*.

Figure 1



Return of enzyme activity after *in vivo* administration: Mice were treated as described in the text with (a) *trans* Z-cyclohexyl prolinal, (b) Z-indolinyl prolinal, or (c) *cis* Z-cyclohexyl prolinal. The x-axis shows the time between i.p. administration and rapid sacrifice/homogenization/enzyme assay.

Thus, we have shown that both Z-cyclohexyl prolinal and Z-indolyl prolinal are slow, tight binding inhibitors of prolyl endopeptidase which cross the blood brain barrier (in mice) to inhibit enzyme *in vivo*. Differences observed in the half time for return of *in vivo* enzyme activity between *trans* Z-cyclohexyl prolinal and *cis* Z-cyclohexyl prolinal appear to be due to pharmacokinetic properties, e.g., faster metabolic processing of the *cis* isomer via degradation or excretion. Although one can design novel, potent inhibitors which inhibit *in vitro* enzyme activity, designing compounds that cross the blood-brain barrier and are metabolically stable is still poorly understood. The resolution and testing of these compounds as potential anti-amnesic agents is presently in progress.

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