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Structural Factors Affecting the Kinetics of O,N-Acyl Transfer in Potential O-Peptide Prodrugs

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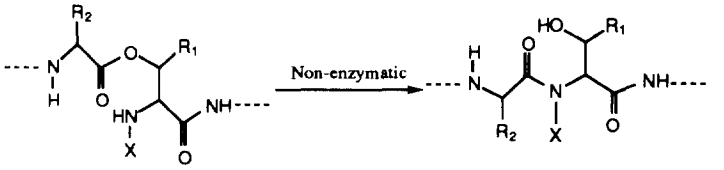
Abstract: In the present study, the conversion kinetics of a number of O-peptide prodrugs have been investigated to elucidate the importance of side chain bulkiness, backbone cyclization and the reactive amine moiety. The significant difference in the chemical reactivities of iso[Val-MeLeu (3-OH)-Abu] and iso[Val-Leu (3-OH)-Abu], indicated that the nature of amine moiety is important to the kinetics of O,N-acyl migration. The importance of side chain bulkiness and backbone cyclization were found to be less significant.

Introduction: The poor solubility of a drug molecule in an aqueous environment is often an undesirable characteristic which may severely limit the therapeutic potential of the drug entity. Many cyclic peptides and by design some of the newer experimental HIV protease and renin inhibitors possess high lipophilicity and therefore compromised aqueous solubility.[1-4] These compounds often display erratic oral bioavailability in animal models and are difficult to formulate as aqueous parenteral dosage forms. These difficulties have occasionally limited their usefulness as therapeutic agents.

The problems associated with a poor solubility profile may be improved by introduction of an ionizable moiety linked to derivatizable functionalities of the polypeptide.[5,6] We have studied factors affecting N,O- and O,N-acyl transfer reactions in β-hydroxy containing peptides[7-9] while other investigators's[10,11] have suggested the use of N,O-acyl reactions as a mean of introducing an ionizable moiety into β-hydroxyl containing polypeptides [Figure 1]. β-hydroxy containing

peptides can rearrange to their corresponding O-peptides under acidic conditions producing a protonated amine group that can impart more desirable solubility properties to the molecule.



O-peptide (Prodrug) N-peptide (Drug)

Figure 1

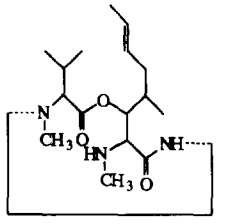
Under physiological conditions, the N,O-acyl prodrug (O-peptide) undergoes non-enzymatic conversion to form the parent polypeptide. The rate and the extent of N-peptide formation are the principle determinants to the applicability of N,O-acyl prodrugs.[5,6] The N,O-Acyl prodrug of cyclosporin A (CsA), isocyclosporin A (isoCsA), was shown[7,10] to quantitatively convert to the parent polypeptide (CsA) under simulated physiological conditions. However, the kinetics of conversion was too slow for the isoCsA to be considered as a prodrug of CsA.

Understanding the relative importance of primary and secondary structure on the rate and the extent of O,N-acyl migration will allow for better design and development of prodrugs that could utilize this chemistry. In the present study, the chemical reactivities of cyclic and linear analogs of isoCsA [Figure 2] were examined to establish the importance of a) side chain bulkiness, b) cyclic versus linear nature of the amide backbone, and c) the nature of amine moiety (primary versus secondary).

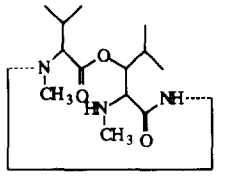
Experimental: Methanol and acetonitrile were both HPLC grade. The water was de ionized and glass distilled (Mega-pure system model mp-1, corning). All other chemicals were of reagent grade and used without further purification.

The synthesis of the parent N-peptides have been previously described.[9] The O-peptides isoCsA, iso[MeLeu (3-OH)][1]-CsA and iso[Val-MeLeu (3-OH)-Abu] were all generated from the parent N-peptide in aqueous acidic condition (pH 1.5, 50°C). The O-peptide iso[Val-Leu (3-OH)-Abu] was generated in Methanol containing 30 mg/ml methanesulfonic acid to minimize the extent of alternative hydrolytic pathways. The products were chromatographically isolated and shown to convert back to the corresponding N-peptides under neutral to basic conditions.

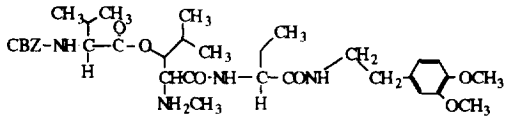
The pH of aqueous buffer solutions were adjusted at the experimental temperatures by measuring the pH of the solution using a Corning pH meter which was standardized at the experimental temperature with NBS buffer solutions. The ionic strength of the solutions were adjusted to 0.15 M with KCl.



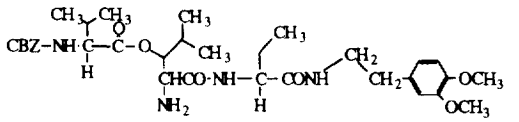
Isocyclosporin A



Iso[MeLeu (3-OH)]^{1}-CsA



Iso[Val-MeLeu (3-OH)-Abu]



Iso[Val-Leu (3-OH)-Abu]

Figure 2

An aliquot (10-30 µl) of the stock solution of the O-peptide was added to 4 ml of 0.04 M phosphate buffer (pH 6.0-7.4). The pH of the solution was measured at the start and the end of each kinetic run and the reported pH values correspond to the pH value upon addition of the O-peptide. At appropriate time intervals, samples were withdrawn and analyzed by HPLC. In the case where the analysis time exceeded the conversion kinetics, samples were withdrawn and quenched using 0.5 M acetic acid. Pseudo-first order rate constants for the apparent O,N-acyl transfer reaction were obtained by following the disappearance of the O-peptides for at least 3 half-lives. The appearance of the N-peptide was also followed to assess the quantitative nature of the reaction.

HPLC was performed using a system consisting of a Shimadzu SPD-6A variable wavelength detector operating at 278 nm; Shimadzu LC-6A pumps; a Shimadzu SIL-6B auto injector; and a Shimadzu CR601 integrator for peak processing. The HPLC studies were conducted using a reverse-phase analytical column C8 (15 cm x 3.9 mm) with mean particle diameter of 5 µm. For linear peptides, all the analyses were performed under an isocratic condition at ambient temperature. Flow rate was set at 1.0 ml/min. The mobile phase contained 50 parts

aqueous, 50 parts acetonitrile, 1 mM TBA and 1 mM TFA. Retention volumes for [Val-MeLeu (3-OH)-Abu], iso[Val-MeLeu (3-OH)-Abu], [Val-MeLeu-Abu], [Val-Leu (3-OH)-Abu] and iso[Val-Leu (3-OH)-Abu] were 6.2, 5.2, 5.5, 4.2 ml, respectively. For the cyclic peptide, all the analyses were performed under isocratic conditions at 70° C to minimize the peak broadening associated with cyclosporine analysis.[12] Flow rate was set at 1.5 ml/min. The mobile phase contained 26 parts 10 mM phosphate buffer (pH 4.6), 47 parts acetonitrile, and 27 parts methanol. Retention volumes MeLeu (3-OH)]^1-CsA and iso[MeLeu (3-OH)]^1-CsA were 5.3 and 6.8 ml respectively.

Conversion kinetics of potential O-peptide prodrugs: Figure 3 represents a partial pH-rate profile for the isomerization of iso[MeLeu (3-OH)]^1-CsA, iso[Val-MeLeu (3-OH)-Abu], iso[Val-Leu (3-OH)-Abu], and isoCsA at 37° C, where k_{obs} is the apparent first-order rate constant. The isomerization kinetics of iso[MeLeu (3-OH)]^1-CsA are nearly identical to that of isoCsA, indicating that the replacement of amino acid MeBmt with a less bulky N-methyl-β-hydroxyleucine does not significantly influence the rate of O,N-acyl migration.

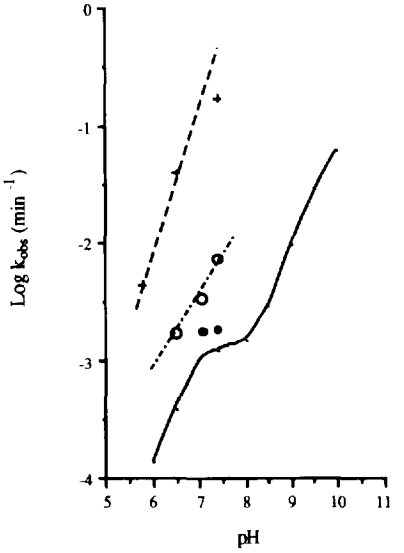


Figure 3. Partial pH-rate profile for the isomerization of isoCsA (solid line), iso[MeLeu (3-OH)]1-CsA (□), iso[Val-MeLeu (3-OH)-Abu] (o), and iso[Val-MeLeu (3-OH)-Abu] (+) at 37 °C.

Cyclization of the peptide backbone usually leads to conformationally restricted peptides that often adopt a single or limited number of preferred conformations.[13,14] The linear peptide iso[Val-MeLeu (3-OH)-Abu] and the corresponding cyclic O-peptide, iso[MeLeu (3-OH)]^1-CsA, have identical structural features near the reactive site but differ in their peptide backbone characteristics (cyclic versus linear). Linear iso[Val-MeLeu (3-OH)-Abu] undergoes isomerization

at a faster rate than the corresponding cyclic O-peptides [Figure 3]. This observation indicates that the amide backbone flexibility is an important parameter in controlling the kinetics of the O-peptide isomerization, although the magnitude of the rate acceleration is quite modest. Whether this is a general conclusion or one restricted to these compounds requires further testing.

The N-alkylation of amine moiety has been shown to alter its nucleophilicity toward acyl groups.[15,16] The role that the nature of amine moiety plays in the conversion kinetics of O-peptides may be assessed by comparing the relative reactivities of iso[Val-MeLeu (3-OH)-Abu] and iso[Val-Leu (3-OH)-Abu]. The chemical structure of these two O-peptides differ only with respect to the nature of the reactive amine moiety [Figure 2], where iso[Val-MeLeu (3-OH)-Abu] and iso[Val-Leu (3-OH)-Abu] contain secondary and primary amines, respectively. The chemical reactivity of iso[Val-Leu (3-OH)-Abu] is significantly greater than the corresponding N-methylated iso[Val-MeLeu (3-OH)-Abu]. The amine nucleophilicity is a function of its basicity, steric demands and solvation characteristics. Based on the current data, it is difficult to suggest an unambiguous mechanistic explanation for the differences in the chemical reactivities between these two O-peptides. Nevertheless, the observed rate constants clearly suggest that the nature of the amine unit (primary versus secondary) is important.

There are two primary factors that predispose N,O-acyl transfer products (O-peptides) as potential prodrugs of cyclic and linear peptides. These factors include the extent and the kinetics of bioconversion of O-peptides to the parent N-peptides. For all the analogs studied here, the back conversions were nearly quantitative (about 95 to 100%) under simulate physiological conditions. Similar quantitative bioconversion profile may not occur in vivo, where the non-productive hydrolyses could conceivably compete with the O,N-acyl transfer reaction and drastically limit the extent of bioconversion of these O-peptides. Such non-productive hydrolyses are usually enzymatically mediated and can severely limit the applicability of a prodrug concept.[5,6] Therefore, a complete characterization of O-peptide prodrugs must contain *in vivo* as well as *in vitro* evaluations.

Table 1 summarizes the apparent half-lives for the conversion of the O-peptides under identical condition (37°C, pH 7.4, 0.04 M phosphate, and $\mu = 0.15$). On the basis of the relative rates of bioconversion, iso[MeLeu (3-OH)]^1-CsA undergoes O,N-acyl transfer at an identical rate to that of isoCsA. However, both iso[Val-MeLeu (3-OH)-Abu] and iso[Val-Leu (3-OH)-Abu] show greater conversion rates. In terms of the applicability for O-peptides as prodrugs of N-peptides, the kinetics of conversion of iso[Val-MeLeu (3-OH)-Abu] is not sufficiently fast, but iso[Val-Leu (3-OH)-Abu] displays a more than adequate conversion rate. The half-life of 4 minutes for the conversion of iso[Val-Leu (3-OH)-Abu] to its N-peptides is certainly short enough to allow for prodrug utility.[5,6]

Table 1. Half-lives for the conversion of various O-peptides to the corresponding N-peptide under psuedo physiological conditions (0.04 M phosphate, pH 7.4 and 37° C)

	Half-lives (min)
isoCsA	350
iso[MeLeu (3-OH)]^1-CsA	370
iso[Val-MeLeu (3-OH)-Abu]	95
iso[Val-Leu (3-OH)-Abu]	4

These observations are in conformity with the observed half-life for the conversion of N,O-acyl transfer prodrug of renin inhibitors[11] which also contain a primary amine moiety (37 °C and pH 7.4, $t_{1/2}$ is about 1 min). The data provided in the present study are not sufficient to allow for the generalized conclusion on the effect of amine moiety on the conversion kinetics of O-peptides, but suggest that O-peptides derived from secondary amides may have a shorter conversion half-life than those derived from tertiary amides, thus making them potentially more useful as prodrugs.

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