Conformational and spacial preferences for substrates of PepT1

Patrick D. Bailey,*[a] C. A. Richard Boyd,[b] Ian D. Collier,†[c] John. G. George,[c] George L. Kellett,[d] David Meredith,[b] Keith M. Morgan,[c] Rachel Pettecrew,[a] Richard A. Price[a] and Robin G. Pritchard[a]

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The conformation at the first residue of dipeptide substrates for the peptide transporter PepT1 has been probed using constrained peptide analogues, and the active conformation has been identified.

PepT1 is an essential eukaryotic membrane protein that actively transports small peptides.[1–3] It is found in the brush borders of the small intestinal epithelium in mammals, and provides the main pathway for the absorption of dietary nitrogen.[4,5]

Although a number of preferred features had been identified for binding and transport by PepT1 (Fig. 1),[6] no model had been proposed that predicted whether substrates would be accepted by PepT1, until our template model published in 2000.[7] This provides a semi-quantitative method of predicting the binding to PepT1, by aligning potential substrates against a template structure, and assessing the correlation of key binding features. One feature of this model is the requirement of the *E*-stereochemistry for the peptide bond between residues 1 and 2, and we have recently confirmed this stereochemical requirement.

In this paper we address two key questions:

(a) What is the conformational preference of the α-centre of residue 1 (*i.e.* ψ_1 torsional angle) relative to the peptide bond?

(b) Is the lower binding of D-residues at position 1 due to conformational factors in the dipeptide, or spacial limitations in the protein?

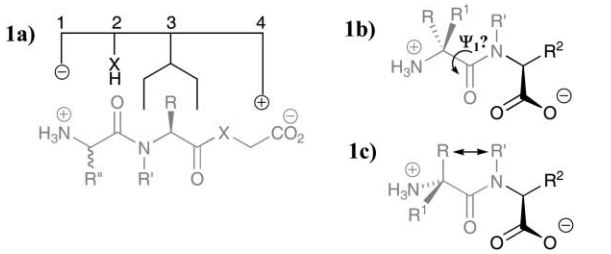
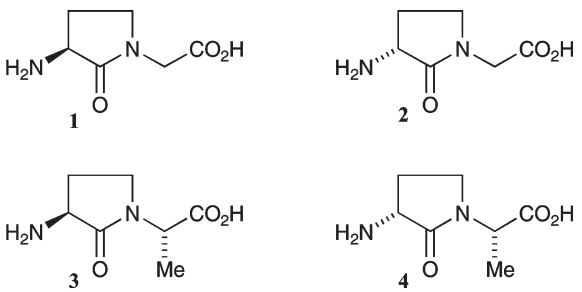


Fig. 1 (a) General features for binding/transport of di-/tri-peptides; (b/c) possible dipeptide conformation (b), or an alternative conformation (c) in which D-residue at position 1 would incur unfavourable interactions with N–R′.

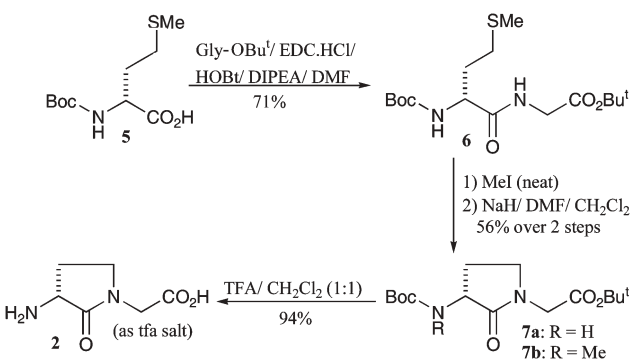
[a]*School of Chemistry, Faraday Building, The University of Manchester, Sackville Street, Manchester, UK M60 1QD. E-mail: pat.bailey@manchester.ac.uk; Fax: (0161) 306 4541; Tel: (0161) 306 4448*
[b]*Dept. of Human Anatomy and Genetics, University of Oxford, South Parks Road, Oxford, UK OX1 3QX*
[c]*Department of Chemistry, Heriot-Watt University, Edinburgh, UK EH14 4AS*
[d]*Department of Biology, University of York (Area 3), Heslington, York, UK YO1 5DD*
† In memory of Ian Collier (1960–2002).

It should be noted that the L-stereochemistry had always been observed to be preferred at residue 1, with D-residues being generally accommodated with about a three-fold increase in K_i, but if the second residue is also *N*-alkylated, K_i increases by about 30-fold. We inferred that a conformation close to that depicted in Fig. 1(c) would account for these observations, with the low affinity of D *N*-alkyl dipeptides being due to the unfavourable interaction shown.[5]

We have now directly addressed the conformational question, by preparing the constrained analogues **1–4** in which the α-side-chain is linked to the amide nitrogen *via* a five-membered ring.



Although we explored a couple of other approaches,[8] we found that a modification of Freidinger's method[9] was most effective, as summarized in Scheme 1. For example, starting from D-methionine, and using *tert*-butyl type protection, we prepared **2**. Formation of the sulfonium salt, followed by base-induced cyclization, gave the protected constrained dipeptide **7a**, although considerable optimization was necessary to minimize the formation of *N*-methylated by-product **7b**. Deprotection with TFA gave the desired analogue *R*-**2**. A similar approach starting from L-methionine gave the enantiomer *S*-**1**.



Scheme 1

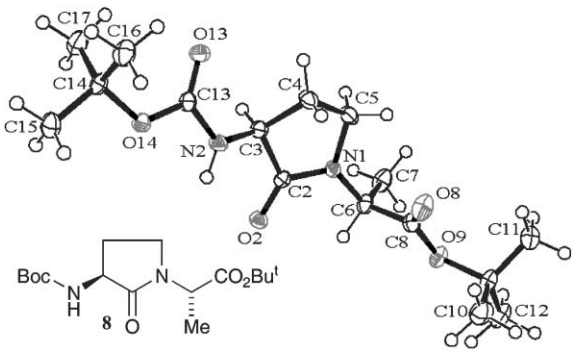


Fig. 2 X-Ray crystal structure of compound **8**.[10]

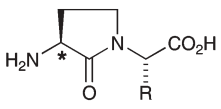
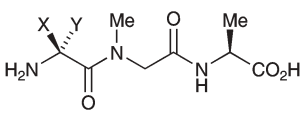
We also prepared the alanine analogues *S*-**3** and *R*-**4**, to reinforce our conformational deductions, and also allowing us to confirm that the synthetic method was stereochemically robust. Moreover, the alanyl analogues would be almost certain to bind in exactly the same way as natural substrates, because L-stereochemistry at position 2 is an essential feature for high affinity (<2 mM) substrates. The synthetic approach described above (Scheme 1, replacing Gly by Ala in step 1) was used, and the structure of protected *S*-**3** was confirmed by X-ray diffraction[10] (Fig. 2).

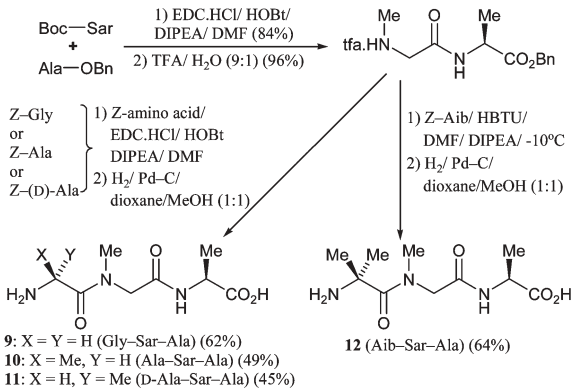
Table 1 summarizes the binding data for the four substrates. For both pairs of analogues, the D-isomer binds more tightly than the L-isomer by a factor of about three, closely matching the data for non-constrained D- and L-dipeptides, *but with opposite stereochemical preference*. This provides excellent evidence for the best substrates adopting a conformation similar to that shown in Fig. 1, as originally proposed by us.

Finally, we tried to probe the reason for the lower affinity of (non-constrained) D-residues at position 1. Our proposal had been that there was a binding site for the ammonium group (*N*-terminus), and that the L-isomer was able to adopt the required conformation in the free (and bound) dipeptide, whereas the D-isomer required eclipsing of the NH/side-chain substituents, and was therefore of higher energy; the substantially lower binding of *N*-alkyl dipeptides with a D-residue at position 1 was consistent with this. We therefore prepared the four tripeptides shown in Table 1, following the sequence shown in Scheme 2.

As expected, both the Gly and L-Ala analogues bound to PepT1 with similar affinities, whereas the D-Ala analogue had a K_i about 10-fold higher. If our proposition were correct, then the Aib

Table 1 Binding data on di-/tri-peptide substrates of PepT1

Structures				Binding K_i/mM (s.e.)
	*	**R**		
	L	H	**1**	2.25 (1.85)
	D	H	**2**	0.37 (0.10)
	L	Me	**3**	1.04 (0.33)
	D	Me	**4**	0.32 (0.02)
	**X**	**Y**		
	H	H	**9**	0.54 (0.07)
	Me	H	**10**	1.19 (0.30)
	H	Me	**11**	5.58 (1.49)
	Me	Me	**12**	4.73 (0.56)



Scheme 2 Synthesis of tripeptides **9**–**12**.

analogue **12** should have bound with a K_i similar to the Gly analogue **9**; in actual fact, the K_i was almost identical to that for the D-Ala analogue **11**. This indicates that the D-isomers at position 1 (in non-constrained substrates) bind less well to PepT1 primarily because of spacial limitations within the binding pocket, rather than due to conformational factors for the free dipeptide.

In summary, we have identified the required conformation at residue 1 for substrates to have high affinity to PepT1, and have prepared the first analogues for which the D-isomer has higher affinity than the L-isomer. In addition, we have shown that D-isomers at position 1 (in non-constrained analogues) bind less well due to steric factors in the binding site, rather than (as previously suggested) due to conformational preferences. These results, when taken with other conformational/QSAR studies, allow us to propose a semi-quantitative template binding model for substrates of PepT1.

Notes and references

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6 By analysis of data from refs. 1–3.
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8 Alternative methods explored were: (*a*) cyclization of a homoserine derivative *via* the bromide, based on work by: N. M. Howarth and L. P. G. Wakelin, *J. Org. Chem.*, 1997, **62**, 5441; (*b*) cyclization of 2,4-diaminobutyric acid, based on work byR. Pellegata, M. Pinza and G. Pifferi, *Synthesis*, 1978, 614; both approaches were successful, but the method described in this paper was found to give the most reliable yields.
9 R. M. Freidlinger, D. S. Perlow and D. F. Veber, *J. Org. Chem.*, 1982, **47**, 104.
10 *Crystal data* for **8**: 0.25 × 0.2 × 0.04 mm, orthorhombic, space group $P2_12_12_1$, a = 5.90450(10), b = 16.7946(4), c = 18.1015(6) Å, V = 1795.01(8) Å^3, Z = 4, D_c = 1.215 Mg m^{-3}, $2\theta_{max}$ = 52.62°, molybdenum radiation, λ = 0.71073 Å, κCCD ϕ and ω scans to full Ewald sphere, temperature = 150(2) K, number of measured/independent reflections 9889/3566 (R_{int} = 0.0318), number of reflections included in the refinement 3566 (3216: $I > 2\sigma(I)$), Lorentz and polarization corrections were performed, absorption correction semi-empirical from equivalents (μ = 0.090 mm^{-1}, max./min. transmission: 0.996/0.978), the structure was solved by direct methods using SHELXS-97 ((*a*) G. M. Sheldrick, *SHELXS97: Program for*

crystal structure solution, University of Göttingen, Germany, 1997), refinement method full-matrix least squares on F^2 using SHELXL-97; (*b*) G. M. Sheldrick, *SHELXL97: Program for crystal structure refinement*, University of Göttingen, Germany, 1997), no. of parameters = 320, H atoms were subjected to isotopic refinement, final residuals refined against $|F^2|$ were $wR2 = 0.0712$ (all data), $R_1 = 0.0307$ ($I > 2\sigma(I)$), max. and min. residual electron density 0.14 and −0.19 e Å^{-3}. CCDC 278679. See http://dx.doi.org/10.1039/b510697d for crystallographic data in CIF or other electronic format.