Inhibitors of Tripeptidyl Peptidase II. 2. Generation of the First Novel Lead Inhibitor of Cholecystokinin-8-Inactivating Peptidase: A Strategy for the Design of Peptidase Inhibitors‡

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The cholecystokinin-8 (CCK-8)-inactivating peptidase is a serine peptidase which has been shown to be a membrane-bound isoform of tripeptidyl peptidase II (EC 3.4.14.10). It cleaves the neurotransmitter CCK-8 sulfate at the Met−Gly bond to give Asp-Tyr(SO_3H)-Met-OH + Gly-Trp-Met-Asp-Phe-NH_2. In seeking a reversible inhibitor of this peptidase, the enzymatic binding subsites were characterized using a fluorimetric assay based on the hydrolysis of the artificial substrate Ala-Ala-Phe-amidomethylcoumarin. A series of di- and tripeptides having various alkyl or aryl side chains was studied to determine the accessible volume for binding and to probe the potential for hydrophobic interactions. From this initial study the tripeptides Ile-Pro-Ile-OH (K_i = 1 μM) and Ala-Pro-Ala-OH (K_i = 3 μM) and dipeptide amide Val-Nvl-NHBu (K_i = 3 μM) emerged as leads. Comparison of these structures led to the synthesis of Val-Pro-NHBu (K_i = 0.57 μM) which served for later optimization in the design of butabindide, a potent reversible competitive and selective inhibitor of the CCK-8-inactivating peptidase. The strategy for this work is explicitly described since it illustrates a possible general approach for peptidase inhibitor design.

Introduction

Cholecystokinins (CCKs) comprise a family of hormonal and neuronal peptides that act on the gastrointestinal tract and central nervous system.[1] Multiple biologically active forms are known (e.g. CCK-58, CCK-39, CCK-33, CCK-8, and CCK-4), and the octapeptide CCK-8 in its sulfated form functions as a neurotransmitter.[2]

CCK is released from intestinal endocrine cells in response to nutrient ingestion and participates in the control of food digestion processes through regulation of gallbladder contraction,[3] pancreatic secretion,[3] and contraction of the pyloric sphincter to delay gastric emptying.[4] CCK also functions as a satiety signal acting through CCK-A receptors[5] that stimulate vagal afferents which signal feeding centers in the brain.[6]

The sulfated C-terminal octapeptide (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-PheNH_2), CCK-8, appears to be the minimum sequence required for bioactivity[1] and is reportedly some 100 times more potent than the nonsulfated form in the rat.[7] Studies have demonstrated that exogenous CCK-8 can shorten meal duration and reduce meal size in several species[8,9] including lean[10] and obese[11] humans. The metabolic instability and poor bioavailability (lack of oral activity), however, limits the potential of sulfated CCK-8 as a satiety agent for assisting the treatment of obesity.

Attempts to devise CCK-8 analogues as agonists for use as satiety agents have also been limited by lack of oral bioavailability.[12−15] These compounds have been peptides or peptoids, e.g. an octapeptide[9] U-67827E, heptapeptides,[13c] a hexapeptide[12] AR-R 15849, the tetrapeptide[13,14] A-71623 and analogues, or a peptoid with blocking groups having a total of five amidic NH groups.[15] Very recently, however, 1,5-benzodiazepines have been described[16] (e.g. GW 5823) as being orally active satiety agents (in the rat) acting as CCK-A receptor agonists. It remains to be seen whether these will yield clinically useful drugs.

Another approach to this problem would be to design selective peptidase inhibitors to protect the endogenous neuropeptide (CCK-8) against inactivation and thereby amplify the biological responses it triggers. A precedent, demonstrating the successful potentiation of an endogenous neuropeptide for drug design, is provided by the enkephalins. The inactivating ectopeptidase 'enkephalinase' (neprilysin, EC 3.4.24.11) was identified,[17] and inhibitors were designed[18,19] to protect the enkephalins[20] which subsequently yielded drugs for use in gastroenterology.[21]

For CCK, however, the problem has been to identify the true physiological peptidase(s) from a variety of candidates shown to cleave CCK-8 in vitro.[22−25] As a result of studying the inactivation of endogenous CCK-8 released from depolarized brain slices, it was proposed that one or more serine peptidases, which cleave the two peptide bonds where the carboxyl group is donated by a methionine residue (Figure 1), are the physiologically relevant peptidases.[26,27] An artificial substrate

‡ For part 1, see ref 28.

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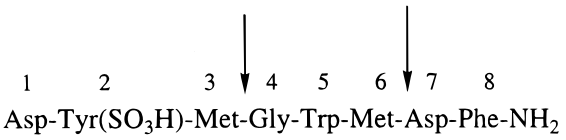


Figure 1. Major cleavage bonds for proteolysis of sulfated CCK-8.

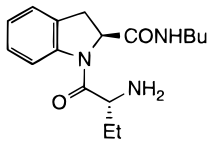


Figure 2. Butabindide.

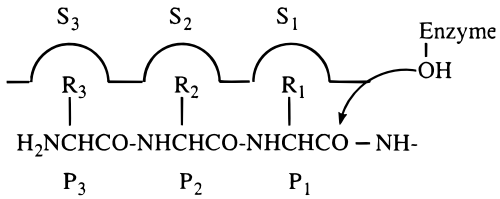


Figure 3. Designation of peptide sequence (P_1, P_2, P_3, ...) and enzyme subsites (S_1, S_2, S_3, ...) according to ref 32.

(described below) was used with a series of dipeptides to generate a lead. The search for, and subsequent exploitation of, potential hydrophobic bonding led to the design of butabindide (Figure 2) which was shown to be a selective reversible competitive inhibitor[28] (K_i = 7 nM) of the enzyme. Since this approach may have a more general application to the design of peptidase inhibitors, the strategy is explicitly identified below and exemplified with the early structure−activity studies and results.

A single enzyme was shown to be responsible for both cleavages of CCK-8, which at the time was thought most likely to be an *endo*peptidase. The enzyme corresponded to a serine peptidase of relative molecular mass 135 000 and was subsequently identified as having a high degree of homology with human tripeptidyl peptidase II (TPP II) (EC 3.4.14.10).[28] This is a subtilisin-like *exo*peptidase that was initially purified from cytosolic extracts of rat liver and human erythrocytes and is found in a variety of tissues, but its physiological role was essentially unknown.[29,30]

Strategy for the Design of Inhibitors

Serine proteases have similar catalytic centers, but they possess different substrate specificities based upon the noncovalent interactions of substrates with the molecular architecture surrounding this center[31] (the so-called binding subsites). Clearly, an inhibitor has to be recognized by the active site and bind to it very strongly so as to prevent access of the enzyme to CCK.

Most serine protease inhibitors synthesized so far comprise: (1) a variable peptide sequence (P_1, P_2, P_3 ...) designed to interact noncovalently with the particular subsites (designated S_1, S_2, S_3 ... according to ref 32) (Figure 3) and which endows them with specificity and (2) a serine-reactive group (for example, chloromethyl ketone or boronate), which endows them with potency (affinity), usually by covalent interaction with the catalytic center or formation of a transition-state analogue. In our approach to obtaining a *reversible* inhibitor of the CCK-inactivating peptidase with therapeutic potential, we deliberately avoided the use of serine-reactive groups, despite the advantage it provides for conferring affinity, to achieve the necessary selectivity and lower toxicity.

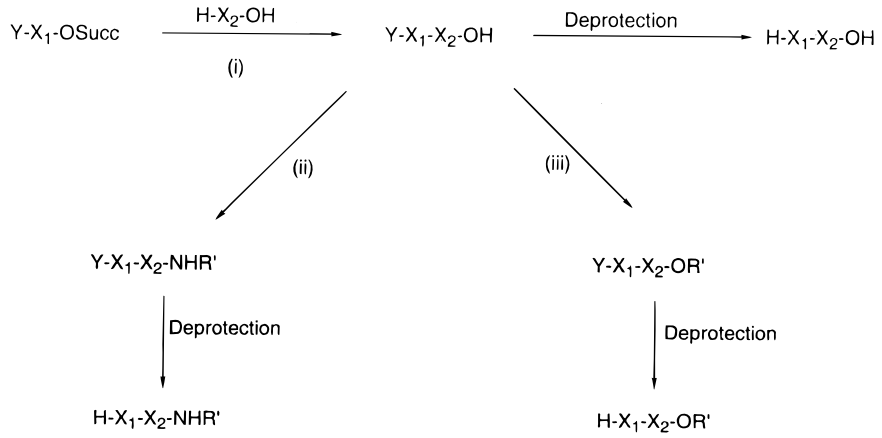
The enzyme recognizes CCK-8 sulfate, but this only binds briefly ($K_i = 2\ \mu$M, this work), otherwise it would block the enzyme. Hence CCK-8 is not a good lead structure for an antagonist, and in any case a much smaller molecule is required as a potential drug. Alternatively one may use molecular probes to seek noncovalent interactions surrounding the enzyme active site; the aim is to achieve closely matched stereospecific interactions between the enzyme and the putative inhibitor. The strategy is similar to that employed in the design of the neprilysin (enkephalinase) inhibitor thiorphan,[18,19] that is to first characterize the binding opportunities of the enzyme subsites using a series of systematically varied dipeptides. These are not necessarily the same amino acids that make up CCK-8. In the absence of an accessible peptide library[33] our approach was to screen some commercially available compounds and some which we had synthesized. After the initial screen the amino acids for the peptides were selected from those with alkyl or aryl side chains in order to determine the accessible volume for binding and to probe the potential for hydrophobic interaction. Amino acids with polar side-chain functionality were of less interest since these would have a reduced ability to penetrate membranes and also would complicate the synthesis of derivatives (by requiring use of additional protecting groups); furthermore, they would not bind hydrophobically. The compounds were studied in vitro for their ability to inhibit the CCK-inactivating peptidase activity determined fluorimetrically from the hydrolysis of the artificial substrate Ala-Ala-Phe-amidomethylcoumarin[34] (AAF-Amc; Amc = 4-methylcoumarin-7-ylamide).

Chemistry

Formulas, melting points, and crystallization solvents are given in the structure−activity Tables 1−5. The synthetic routes to the compounds are given in Schemes 1−8. The majority of the compounds were prepared according to Scheme 1 (Table 6). In this method, an *N*-protected amino acid succinimide ester (Y-X_1-OSucc) is condensed with a second amino acid in polar basic medium to give the protected dipeptide acid Y-X_1-X_2-OH. When a dipeptide acid was the desired product, this intermediate was deprotected immediately. Dipeptide esters were prepared from Y-X_1-X_2-OH by esterification followed by deprotection by hydrogenolysis (Scheme 1). Dipeptide amides were prepared from Y-X_1-X_2-OH by converting the acid to a mixed anhydride, then in situ reaction with amine RNH_2 followed by deprotection (Scheme 1).

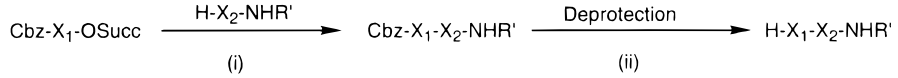
The benzyloxycarbonyl (Cbz) and *tert*-butoxycarbonyl (*t*-Boc) protecting groups were used almost exclusively. When side-chain functionality at X_2 would be sensitive to hydrogenolysis, *t*-Boc protection was used. When protected side-chain functionality was required to be unmasked (**6**), the *N*-protecting group was chosen such that the deprotection of both functions could be carried out simultaneously. In the case of **9** however, the

Scheme 1[a]



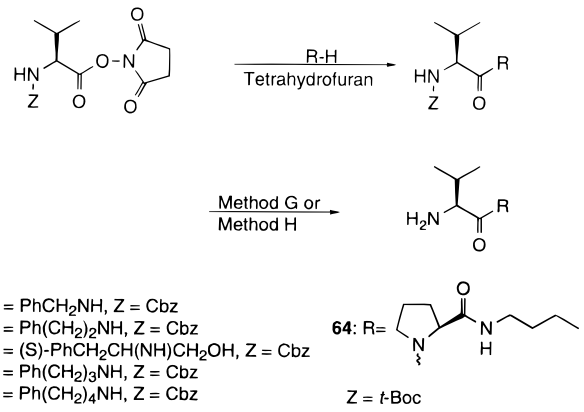
[a] OSucc, succinimide ester; R′, various; X_1, X_2, amino acid residues; Y, *N*-protecting group; (i) see Table 6; (ii) *i*-BuOCOCl/*N*-ethylmorpholine/tetrahydrofuran/R′NH_2 (method J); (iii) see Table 6.

Scheme 2[a]

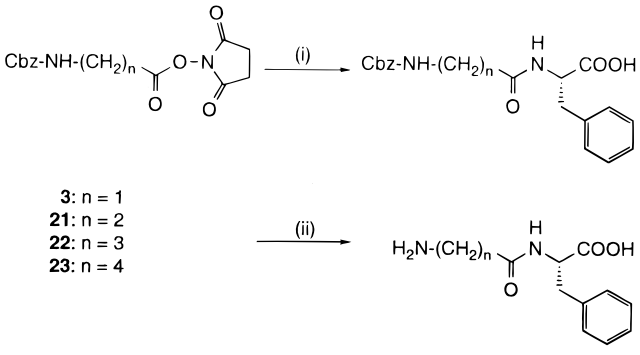


[a] OSucc, succinimide ester; R′, various; X_1, X_2, amino acid residues; (i) DME/H_2O/TEA or THF/H_2O/TEA; (ii) H_2/Pd/C/MeOH/acetic acid (optional).

Scheme 3



Scheme 4[a]

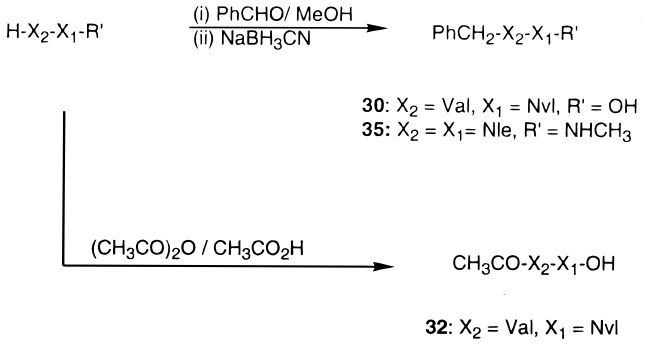


[a] (i) L-Phenylalanine/tetrahydrofuran/water/triethylamine (method B); (ii) H_2/Pd/C/methanol (method F).

O-benzyl group at X_2 was to be retained in the final product so 9-fluorenylmethoxycarbonyl (Fmoc) was used as the protecting group.

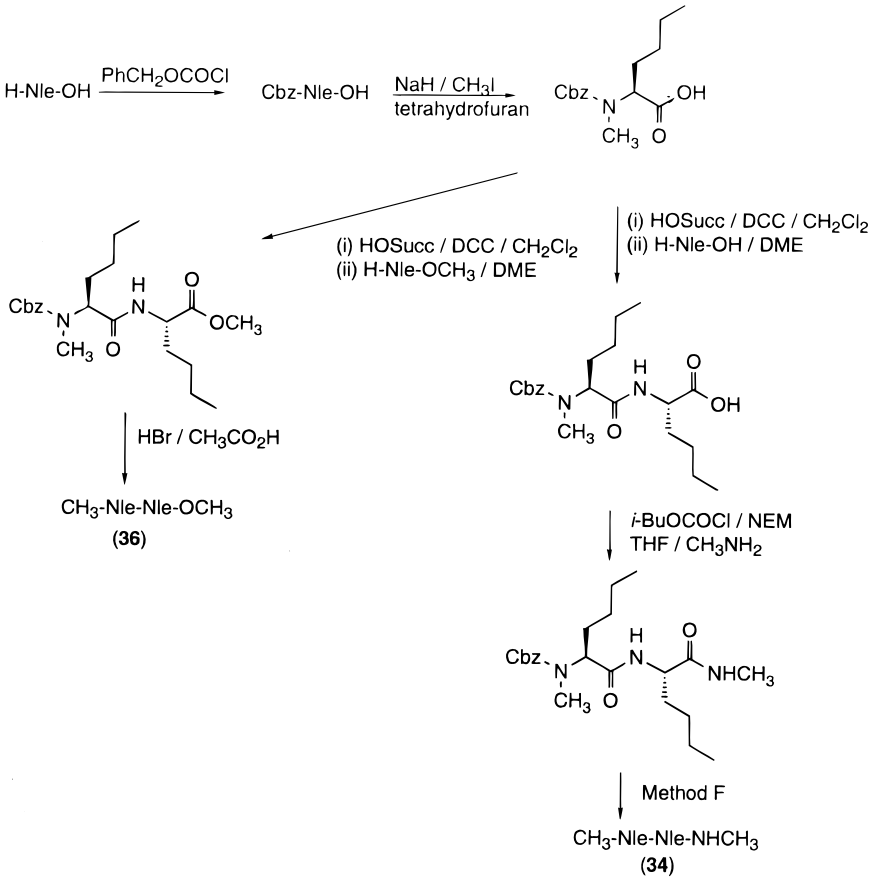
An alternative method of preparing dipeptide amides was also used (Scheme 2) in which an *N*-protected amino acid succinimide ester was condensed with an amino acid amide in polar medium to give an intermediate protected dipeptide amide which was then deprotected. The series of valylamides and the series of *N*-aminoacylphenylalanines were prepared as given respectively in Scheme 3 (**24**−**29**) and Scheme 4 (**21**−**23**). *N*-Substituted dipeptides **30**−**40** were synthesized according to Scheme 1, 2, 5, or 6 as identified in Table 6. Val-Phe-OCH_3 (**48**) was prepared by esterification of the parent dipeptide **1** (Scheme 7). The tripeptide acids **57** and **58** were synthesized (Scheme 8) analogously to the depeptide acids in Scheme 1.

Scheme 5

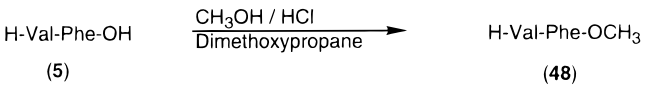


Purity of the compounds for testing was mainly assessed by NMR and HPLC. It was subseqently found, however, that the HPLC column in general use, Lichrosorb rp select B, was not able to separate the diastereomers. Later work demonstrated that a Kromasil column was able to effect separation. Several of the compounds were therefore reexamined to confirm the level of stereoisomeric purity. Examples from Schemes 1, 2, 5, and 6 were analyzed (**6**, **17**, **32**, **34**, **36**, **46**); none of these compounds showed any evidence for the presence of a second epimer. In the case of **64** and **65**, however, a small amount of a second epimer was detected.

Scheme 6



Scheme 7



Biological Assay

Rat cerebral membranes were prepared from cerebral cortex which was homogenized (Polytron) in 10 vol of 50 mM K_2/K phosphate buffer (pH 7.4) containing 10% glycerol. After centrifugation (500000*g*), the pellet was washed three times in the same buffer and finally recovered in 50 mM K_2/K phosphate buffer (pH 7.4) containing 10% glycerol, 0.1% Brij 35, and 1 mM dithiothreitol, at a protein concentration of 25 μg/μL. TPPII activity was evaluated in a final volume of 100 μL of K_2/K phosphate buffer containing 10^{-4} M bestatin, 10^{-7} M thiorphan, and 0.1% Brij 35, using 50 μM of the artificial fluorescent substrate AAF-Amc[34] and 25 μg of membrane proteins. Incubations were performed at 37 °C during 30 min, and the release of Amc was measured using a microfluor reader (Dynatech). When increasing concentrations of inhibitors were added, decreasing amounts of Amc were released from which the IC_{50} was determined. Concentration−inhibition curves were established with 2−3 replicates. K_i values were derived from the IC_{50} using the Cheng−Prussoff[42] equation (K_m AAF-Amc = 25 μM); SEMs were generally around 10% of the mean.

Results and Discussion

Some dipeptide partial structures of CCK-8 were screened of which only Gly-Trp-OH (**2**) had a submillimolar K_i. Other dipeptides were tested and several acted as inhibitors (K_i < 1 mM), but none stood out sufficiently well to provide a clear lead (a selection is given in Table 1 and Figure 4a). However, since the substrate CCK-8 has Tyr at P_2 and Val-Phe-OH (**5**) was one of the best of the dipeptides as an inhibitor (K_i = 350 ± 36 μM), it was taken as an initial lead along with Val-Nvl-OH (**18**) (K_i = 433 ± 40 μM). During this early exploratory phase of the study we had assumed that the

Scheme 8

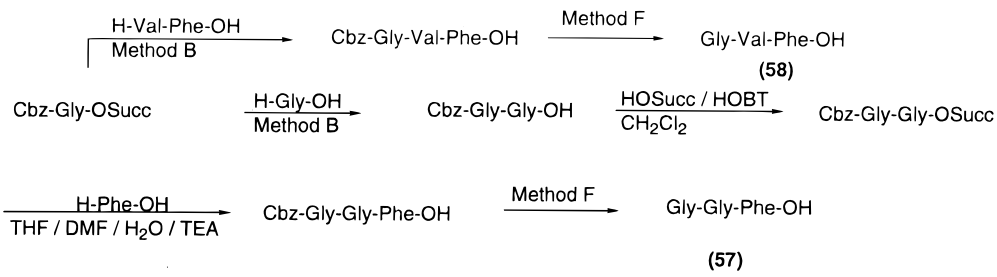
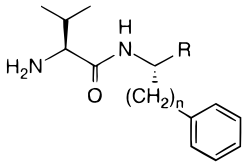


Table 1. Inhibition Potencies of a Selection of Dipeptide Acids Having an Unsubstituted Amino Terminus*

compd	structure	K_i (μM)	molecular formula	mp (°C)	crystallization solvents	scheme no.
1	Asp-Tyr-OH	>5000				
2	Gly-Trp-OH	650				
3	Gly-Phe-OH	470	$C_{11}H_{14}N_2O_3 \cdot 0.25H_2O$	265−267[a]	EtOAc/petrol	4
4	Ala-Phe-OH	1140				
5	Val-Phe-OH	350				
6	Val-Tyr-OH	857	$C_{14}H_{20}N_2O_4 \cdot 0.75CF_3COOH$	152−153	*b*	1
7	Val-Phg-OH[c]	600	$C_{13}H_{18}N_2O_3 \cdot 0.75H_2O$	209−210	MeOH/EtOAc	1
8	Met-Tyr-OH	2000				
9	Val-(4-PhCH$_2$O)Phe-OH[d]	210	$C_{21}H_{26}N_2O_4 \cdot 1.75H_2O$	135−136	*e*	1
10	Val-(β-naphthyl)Ala-OH[d]	60	$C_{18}H_{22}N_2O_3 \cdot 0.75H_2O$	244	MeOH	1
11	Ala-Ala-OH	5000				
12	Abu-Abu-OH[e]	1670				
13	Nle-Nle-OH	1430	$C_{12}H_{24}N_2O_3$	265−268	EtOH	1
14	Met-Met-OH	>5000				
15	Val-Val-OH	1670	$C_{10}H_{20}N_2O_3 \cdot 0.25H_2O$	>300	EtOH/H$_2$O	1
16	Val-Met-OH	2000				
17	Val-Nle-OH	860	$C_{11}H_{22}N_2O_3$	242−245	MeOH	1
18	Val-Nvl-OH	433	$C_{10}H_{20}N_2O_3 \cdot 0.25C_2H_5OH$	267−268	EtOH/H$_2$O	1
19	Val-Ala-OH	9000				
20	Abu-Nvl-OH[f]	1000				

*Compounds synthesized are indicated by molecular formula, melting point, solvent, and schemes. Other compounds were obtained commercially. [a] Lit.[38] mp 266−268 °C. [b] Purified by chromatography. [c] Phg = phenylglycine. [d] **9** and **10** were not part of the initial screening. [e] Purified by trituration with petrol. [f] Abu = 2*S*-aminobutyric acid.

Table 2. Effect on Inhibition Potency of Removal of the Terminal Carboxylic Acid Group: Variation of R and *n*



compd	R	*n*	K_i (μM)	molecular formula	mp (°C)	crystallization solvents	scheme no.
7	COOH	0	600				
24	H	0	80	$C_{12}H_{18}N_2O \cdot (COOH)_2$	185−186	EtOH/Et$_2$O	3
5	COOH	1	350				
25	H	1	114	$C_{13}H_{20}N_2O \cdot (COOH)_2$	137.5	EtOH/Et$_2$O	3
26	CH$_2$OH	1	2100	$C_{14}H_{22}N_2O_2 \cdot (COOH)_2$	95−96	EtOH/Et$_2$O	3
27	COOH	2	570	$C_{15}H_{22}N_2O_3 \cdot CF_3COOH$	110−111	*a*	1
28	H	2	86	$C_{14}H_{22}N_2O \cdot (COOH)_2 \cdot 0.5H_2O$	154−156	EtOH/Et$_2$O	3
29	H	3	33	$C_{15}H_{24}N_2O \cdot (COOH)_2$	120−122	EtOH/Et$_2$O	3

[a] Purified by preparative HPLC.

Table 3. Effect on Inhibition Potency of Substitution at the Nitrogen Terminus of Dipeptides

compd	structure	K_i (μM)	molecular formula	mp (°C)	crystallization solvents	scheme no.
18	Val-Nvl-OH	433				
30	PhCH$_2$-Val-Nvl-OH	>5000	$C_{17}H_{26}N_2O_3 \cdot (COOH)_2 \cdot 0.5H_2O$	214−216	Et$_2$O/EtOAc	5
31	PhCH$_2$OCO-Val-Nvl-OH	>5000	$C_{18}H_{26}N_2O_5$	181−182	*i*-PrOH/EtOAc	1
32	CH$_3$CO-Val-Nvl-OH	>500	$C_{12}H_{22}N_2O_4$	224−225	*a*	5
13	Nle-Nle-OH	1430				
33	Nle-Nle-NHCH$_3$	9	$C_{13}H_{27}N_3O_2 \cdot 0.5H_2O$	153	MeOH/EtOAc	2
34	CH$_3$-Nle-Nle-NHCH$_3$	>3000	$C_{14}H_{29}N_3O_2$	132−133	EtOAc/Et$_2$O	6
35	PhCH$_2$-Nle-Nle-NHCH$_3$	>3000	$C_{20}H_{33}N_3O_2 \cdot 0.25H_2O$	109−110	EtOAc/petrol	5
36	CH$_3$-Nle-Nle-OCH$_3$	>5000	$C_{14}H_{28}N_2O_3 \cdot HBr$	124−126	*b*	6
15	Val-Val-OH	1666				
37	PhCH$_2$OCO-Val-Val-OH	>5000	$C_{18}H_{26}N_2O_5 \cdot 0.25H_2O$	139−141[c]	EtOAc/petrol	1
17	Val-Nle-OH	860				
38	BuOCO-Val-Nle-OH	>5000	$C_{16}H_{30}N_2O_5$	151−153	EtOAc	1
39	Val-Nle-O(CH$_2$)$_3$CH$_3$	290	$C_{15}H_{30}N_2O_3 \cdot (COOH)_2$	161−163	CHCl$_3$/MeOH	1
40	PhCH$_2$OCO-Val-Nle-O(CH$_2$)$_3$CH$_3$	>3000	$C_{23}H_{36}N_2O_5$	104−106	Et$_2$O	1

[a] Triturated with EtOAc. [b] Triturated with CH$_2$Cl$_2$/petrol. [c] Lit.[39] mp 139.5−140 °C.

enzyme was an endopeptidase, and by comparison with the substrate (CCK-8 sulfate) we attributed Val-Phe-OH to represent P_3P_2 (binding to subsites S_3S_2) and Val-Nvl-OH to represent P_2P_1 (binding to S_2S_1, since Nvl simulates Met). Hence these were treated as two separate leads which were followed up in parallel. The effect on potency of modifying the phenyl group was then investigated (Figure 4b). The molecule was probed for potential electronic effects on binding by introducing electron-withdrawing (Cl, NO_2) or electron-releasing (NH_2, OH, OBu, OCH_2Ph) substituents in the aromatic ring, but this had little effect on activity (K_i = 140−860 μM) suggesting little electronic or hydrogen-bonding contribution to affinity. There appeared to be a substantial volume available in the region of the 4-position of the ring since a 4-benzyloxy group (**9**, K_i = 210 μM)

Table 4. Effect on Inhibition Potency of Changes in Terminal Carboxylic Acid Functionality of Dipeptides

compd	formula	K_i (μM)	molecular formula	mp (°C)	crystallization solvents	scheme no.
17	Val-Nle-OH	860				
41	Val-Nle-$NHCH_3$	66	$C_{12}H_{25}N_3O_2 \cdot 0.25H_2O$	178−180	$CHCl_3$	2
42	Val-Nle-$NH(CH_2)_3CH_3$	14	$C_{15}H_{31}N_3O_2 \cdot 2.25H_2O$	213-215	EtOAc/MeOH	1
43	Val-Nle-$NH(CH_2)_3Ph$	29	$C_{20}H_{33}N_3O_2 \cdot 0.25H_2O$	96−98	CH_2Cl_2/petrol	2
39	Val-Nle-$O(CH_2)_3CH_3$	290				
18	Val-Nvl-OH	433				
44	Val-Nvl-$NHCH_3$	28	$C_{11}H_{23}N_3O_2 \cdot CF_3COOH$	219−220	*a*	1
45	Val-Nvl-$NH(CH_2)_3CH_3$	3	$C_{14}H_{29}N_3O_2$	151−153	EtOAc/petrol	1
13	Nle-Nle-OH	1430				
33	Nle-Nle-$NHCH_3$	9				
5	Val-Phe-OH	350				
46	Val-Phe-$NHCH_3$	22	$C_{15}N_{23}N_3O_2 \cdot 1.25CF_3COOH$	177	*a*	2
47	Val-Phe-$NH(CH_2)_3CH_3$	20	$C_{18}H_{29}N_3O_2 \cdot CH_3COOH$	198−199	$EtOH/Et_2O$	1
48	Val-Phe-OCH_3	260	$C_{15}H_{22}N_2O_3 \cdot HCl \cdot 0.25H_2O$	201−202	$MeOH/Et_2O$/petrol	7
49	Val-Phe-$O(CH_2)_3CH_3$	230	$C_{18}H_{28}N_2O_4 \cdot 1.25CF_3COOH \cdot 0.5H_2O$	150.5−151.5	*a*	1
7	Val-Phg-OH	600				
50	Val-Phg-$NHCH_3$	29	$C_{14}H_{21}N_3O_2 \cdot 0.5H_2O$	177−180	EtOAc	1
6	Val-Tyr-OH	857				
51	Val-Tyr-$NHCH_3$	11	$C_{15}H_{23}N_3O_3 \cdot 0.25H_2O$	214−216	EtOAc/MeOH	1
15	Val-Val-OH	1666				
52	Val-Val-$NHCH_3$	46	$C_{11}H_{23}N_3O_2$	194−196	*b*	2
53	Val-Cha-OH[c]	3000	$C_{14}H_{26}N_2O_3 \cdot CF_3COOH$	128−130	$MeOH/Et_2O$	1
54	Val-Cha-$NH(CH_2)_3CH_3$[c]	57	$C_{18}H_{35}N_3O_2 \cdot (COOH)_2 \cdot 0.5H_2O$	140−142	$EtOH/Et_2O$	1

[a] Purified by preparative HPLC. [b] Purified by trituration with ethyl acetate. [c] Cha: (*S*)-cyclohexylalanyl.

Table 5. Inhibition Potency of Some Tripeptide and Related Homologous Acids

compd[a]	structure	K_i (μM)	molecular formula	mp (°C)	crystallization solvents	scheme no.
55	Asp-Tyr-Met-OH	50				
56	Gly-Trp-Met-OH	20				
57	Gly-Gly-Phe-OH	110	$C_{13}H_{17}N_3O_4$	228−229[b]	$MeOH/H_2O$	8
58	Gly-Val-Phe-OH	29	$C_{16}H_{23}N_3O_4 \cdot 0.4H_2O$	275−276	$EtOH/H_2O$	8
59	Ala-Ala-Ala-OH	14				
60	Ala-Pro-Ala-OH	3.0				
61	Ile-Pro-Ile-OH	1.0				
11	Ala-Ala-OH	5000				
62	Ala-Pro-OH	1400				
63	Ala-Ala-Pro-Ala-OH	114				

[a] Compounds **55**, **56**, and **59**−**63** were obtained commercially from Sigma or Bachem. [b] Lit.[42] mp 228−230 °C.

Table 6. Experimental Data for Compounds Made by Scheme 1

no.	X	*a*	*b*	*c*	notes	no.	X	*a*	*b*	*c*	notes
6	Cbz	B		F	*d*	**40**	Cbz	A	X		
7	Cbz	B		F		**42**	Cbz	A		E	
9	Fmoc	*e*		*f*		**44**	Cbz	A		E	
10	Cbz	B		F		**45**	Cbz	A		F	
13	Cbz	A		E		**47**	Cbz	A		E	
15	Cbz	A		E		**49**	Cbz	A	Y	E	
17	Cbz	A		E		**50**	Cbz	A		F	
18	Cbz	A		E		**51**	Cbz	B		F	*d*
27	Cbz	B		F		**53**	Cbz	B		F	
31	Cbz	A				**54**	Cbz	B		F	
37	Cbz	A				**65**	*t*-Boc	B		G	
39	Cbz	A	X	*g*							

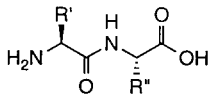
[a] Reaction medium for step (i) of Scheme 1: A, DME/H_2O/TEA; B, THF/H_2O/TEA; C, DMF/DME/TEA; D, H_2O/TEA. [b] Esterification conditions for step (iii) of Scheme 1: X, 4-toluenesulfonic acid/benzene/*n*-butanol; Y, *n*-butanol/$SOCl_2$. [c] Deprotection method: E, H_2/Pd/C/MeOH/acetic acid; F, H_2/Pd/C/MeOH; G, TFA. [d] Tyrosine incorporated as its *O*-benzyl ether. [e] See text. [f] Piperidine. [g] HBr/CH_3COOH.

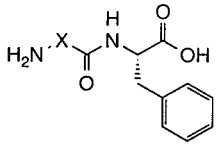
was easily accommodated. Replacing Phe by another aromatic α-amino acid resulted in an increase in activity when the aromatic system was extended to naphthyl, as in valyl-β-naphthylalanine (**10**, $K_i = 60$ μM).

Another series of compounds was made (H_2N-$(CH_2)_n$-CO-Phe-OH) (Figure 4c) in which the chain between the phenyl ring and the NH_2 was lengthened ($n = 1-4$) in order to probe whether there might be a better location for a hydrophobic interaction with the enzyme than in Phe. The optimum was indeed Gly-Phe-OH (**3**) ($n = 1$, $K_i = 340$ μM) (cf. $n = 2-4$, $K_i = 2300-3000$ μM). Another approach to probing for hydrophobic interaction was to alter the chain length linking the phenyl group to the amino acid in the dipeptide Val-Phe-OH (Table 2). The optimum was Val-Phe-OH (**5**), but it was of great interest to find that removal of the carboxylic acid group *increased* affinity (**24**, **25**, **28**) in every case; i.e., the substituted valylamides were more potent than the corresponding dipeptides, the best being the amide Val-$NH(CH_2)_4Ph$ (**29**) which had a $K_i = 33$ μM. In contrast, reduction of the acid function to give CH_2OH (**26**) dramatically lowered activity ($K_i = 2100$ μM).

The assumption is made that the compounds are acting on the same subsites of the enzyme. It may be that the acids are acting at a different subsite. However, taking the simplest interpretation (i.e. applying Occam's razor in the first instance) we have used these results together with the subsequent findings (below) as an empirical set from which to construct the best lead.

Some derivatives of the dipeptides were synthesized and tested, and very important information was derived from this initial phase of the active site characterization. As indicated by the dipeptides listed in Table 3, acyl derivatives [e.g. Cbz (**31**, **37**, **40**), Ac (**32**), *n*-Boc (**38**)] were not active and neither were compounds having an alkyl substituent [e.g. $PhCH_2$ (**30**, **35**), CH_3 (**34**, **36**)] on the NH_2 terminus.





3, 21-23

(a) R' = H, alkyl, aryl, CH_2COOH, CH_2OH, CH_2CONH_2, CH(Me)OH, CH_2CH_2SMe, $(CH_2)_3NHC(=NH)NH_2$, $(CH_2)_4NH_2$
R" = H, CH_2CONH_2, CH_2OH, CH(Me)OH, $(CH_2)_3NHC(=NH)NH_2$, $(CH_2)_4NH_2$, CH_2CH_2SMe, alkyl, CH_2-aryl, CH_2-imidazolyl

(b) R' = $CH(Me)_2$
R" = Ph, CH_2-Ph, CH_2-aryl, CH_2-imidazolyl, CH_2-cyclohexyl

(c) X = $-(CH_2)_n-$
n = 1 - 4

Figure 4. Initial series of compounds tested showing (a and b) the variation in side-chain groups R′ and R″ and (c) the increase in separation of the N-terminal amino group.

Val-Nvl-NHBu (**45**)
Ki = 3μM

Ile-Pro-Ile-OH (**61**)
Ki = 1μM

Val-Pro-NHBu (**64**)
Ki = 0.57μM

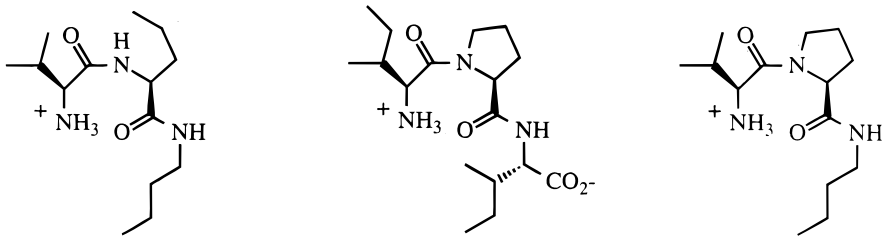


Figure 5. Comparison of the structural formulas of the dipeptide amide inhibitor UCL 1154 (**45**, Val-Nvl-NHBu) with the tripeptide acid inhibitor (**61**, Ile-Pro-Ile-OH) leading to the combined structure UCL 1324 (**64**, Val-Pro-NHBu). The structure of **60** (Ala-Pro-Ala-OH) is subsumed into that of **61**.

On the other hand, as demonstrated by the dipeptide derivatives in Table 4, in which free acids are compared with their corresponding terminal esters or amides, the esters (**39**, **48**, **49**) were slightly more active than the acids but methylamides (**33**, **41**, **44**, **46**, **50**−**52**) were consistently much more potent and, furthermore, butylamides (**42**, **45**) were more potent than methylamides suggesting a possible hydrophobic component to the binding affinity. Thus binding affinity increased in the order OH < OBu < NHMe < NHBu, and the most active Val-Nvl-NHBu (**45**) had $K_i = 3$ μM which represented over 100-fold potency increase relative to the free acid **18**. This therefore constituted an important lead which also emphasized the findings in Table 2 where it is clear that the presence of CO_2H is actually deleterious to binding.

The tripeptide hydrolysis products from CCK-8, Asp-Tyr-Met (**55**), and Gly-Trp-Met-OH (**56**) had appreciable affinities (K_i = 50 and 20 μM, respectively, Table 5). Other tripeptide acids were tested (Table 5), and some of these showed an increase in potency of 1−2 orders of magnitude (K_i = 1−29 μM) in comparison with any of the dipeptide acids examined (K_i > 350 μM). For example, compare Ala-Ala-Ala-OH (**59**, K_i = 14 μM) (presumed to be $P_3P_2P_1$) with Ala-Ala-OH (**11**, K_i = 5000 μM). Two tripeptides, **60** (Ala-Pro-Ala-OH) and **61** (Ile-Pro-Ile-OH), which contain Pro at the putative P_2 position, stood out as being particularly potent (K_i = 1−3 μM). In contrast, the dipeptide acid Ala-Pro-OH (**62**, K_i = 1,400 μM) and the tetrapeptide acid Ala-Ala-Pro-Ala-OH (**63**, K_i = 114 μM) had much lower affinities.

A comparison of the most potent tripeptide acids (Ile-Pro-Ile-OH, **61**, $K_i = 1.0 \pm 0.4$ μM; Ala-Pro-Ala-OH, **60**, $K_i = 3.0 \pm 0.3$ μM) with the most potent dipeptide amide (Val-Nvl-NHBu, **45**, $K_i = 3 \pm 0.2$ μM) revealed a striking similarity (Figure 5). It showed that the terminal carboxylic acid group of **60** and **61** could be omitted without loss of affinity, whence **45** could represent P_3P_2 in contrast to our initial assumption that it was P_2P_1. Thus several features of the peptide sequence were identified as being responsible for higher binding: (i) the absolute requirement for a nonsubstituted ammonium group, as shown by the dramatic loss of potency when alkylated or acylated and (ii) the major contribution of the amide function to affinity provided by P_1, so that a tripeptide $P_3P_2P_1$ could be simplified into a dipeptide amide P_3P_2. These results are consistent with TPPII which is an exopeptidase recognizing a primary amino group and two peptide bonds and cleaving the third peptide bond (Figure 3). In addition they showed the favorable influence of a Pro residue in P_2. This is a very important finding for at least four reasons: (1) the pyrrolidine ring in Pro provides protection against enzymatic proteolysis and is a classic way of enhancing stability of peptides in vivo, (2) the removal of an NH will assist penetration through membranes and also removes a site of potential *N*-glucuronidation so assisting oral absorption, (3) the removal of an NH

shows that at P_2 it is not critical to an intermolecular hydrogen-bonding interaction with the enzyme for providing inhibitor affinity, and (4) the rigidity of the pyrrolidine ring in Pro reduces conformational flexibility of the inhibitor and simplifies the analysis for determining the likely active three-dimensional structure.

From these observations, and taking into account the activity of compounds **45**, **60,** and **61**, a composite compound Val-Pro-NHBu (UCL 1324, **64**) (Figure 5) was identified for synthesis (Scheme 3). The K_i was found to be 0.57 ± 0.09 μM. In these compounds the natural amino acid chirality (*S*) has been maintained. As a check to determine whether the compounds were acting stereospecifically, **65**, the *S,R* epimer of **64,** was synthesized and tested; although it was shown by HPLC to contain ca. 1.9% of the active *S,S* diastereomer it was much less active (K_i = 190 μM), demonstrating a eudismic ratio of at least 300. A significant proportion of this activity is likely to be due to the active contaminant **64**. It is interesting to note that in the initial screening of dipeptides, Met-Pro-OH was tested and found to be only weakly active (K_i = 1000 μM); this emphasizes the importance of the amide group. Hence this approach has generated an inhibitor lacking any obvious serine-reactive group yet having a sub-micromolar K_i, which was to serve as an important lead for structure optimization, to yield the more potent nanomolar reversible competitive and selective inhibitor, butabindide.[28] The structural optimization studies will be reported subsequently.

Experimental Section

Melting points were determined using an Electrothermal melting point apparatus and are uncorrected. ^{1}H NMR were recorded on a JEOL PMX60SI, Varian XL-200, or Varian VXR-400 NMR spectrometer. HPLC (analytical and preparative) was carried out on a Gilson binary gradient system using a UV detector set to 215 nm unless otherwise stated. Mass spectra, EI or FAB, were recorded on a VG 7070H double focusing mass spectrometer with Finnigan Incos data system. Solvents requiring drying were purified by literature procedures.[36] Amino acids, *N*-protected amino acids, and their succinimide esters were obtained commercially (from Aldrich, Sigma, or Bachem) or prepared by literature proceedures.[37]

Method A. *N*-Protected amino acid succinimide ester (40 mmol) in dimethoxyethane (14 mL) was treated with amino acid or amide (40 mmol) in water (70 mL) containing triethylamine (60 mmol). The mixture was stirred at room temperature overnight. For neutral products, the reaction mixture was reduced to low volume and extracted with ethyl acetate (100 mL). The extract was dried (Na_2SO_4) and the solvent removed to give a residue which was purified. For acid products, the mixture was made basic (Na_2CO_3) and extracted with ethyl acetate (200 mL). The organic portion was discarded and the aqueous phase then acidified to pH 2 and extracted with ethyl acetate (2×200 mL); these extracts were combined and dried (Na_2SO_4) and the solvent was removed to give a residue which was purified.

Method B. *N*-Protected amino acid succinimide ester (0.02 mole) was dissolved in tetrahydrofuran (50 mL). Amino acid or amine (0.02 mole) in water (25 mL) and triethylamine (1 mL) were added and the mixture stirred at room temperature overnight. The mixture was made alkaline with 4 M NaOH and extracted with ether (20 mL). The aqueous phase was made acid (HCl) to pH 2; occasionally, the product precipitated out and was then collected, washed with water and dried. Otherwise, the acidic solution was extracted with ethyl acetate (3×50 mL). The combined ethyl acetate portions were dried ($MgSO_4$), the solvent was removed and the residue was purified.

Method C. *N*-Protected amino acid succinimide ester (6.7 mmol) was dissolved in a mixture of dimethoxyethane (10 mL) and dimethyl formamide (4 mL) and added to a solution of amino acid or amide (6.7 mmol) in water (5 mL) and triethylamine (0.94 mL, 6.7 mmol). The mixture was stirred at room temperature overnight, concentrated, acidified with dilute HCl to pH 2 (acid products only) and extracted with ethyl acetate (2×100 mL). The combined extracts were dried ($MgSO_4$) and the solvent was removed. The residue was then purified.

Method D. L-Proline (3.4 g, 29.9 mmol) was dissolved in water (100 mL) and triethylamine (4.5 g, 44.8 mmol) added. *N*-Protected amino acid succinimide ester (29.9 mmol) was dissolved in tetrahydrofuran (100 mL) and chilled in ice. The proline solution was added over 40 min and the mixture stirred at 0 °C for 1 h then overnight at room temperature. The volume of solvent was reduced by approximately one-half, made acidic (pH 2, HCl), and extracted with ethyl acetate (3×200 mL). The combined organic layers were dried (Na_2SO_4) and the solvent was removed to give an oil.

Method E. *N*-Benzyloxycarbonyl-protected product was dissolved in methanol (100 mL) containing glacial acetic acid (0.4 mL) and 10% palladium on charcoal (25 wt % of substrate). The mixture was hydrogenated at 30 psi and 25 °C for 3 h. The catalyst was filtered off and the solvent removed. The residue was then purified.

Method F. *N*-Benzyloxycarbonyl-protected product (3 g) was dissolved in methanol (100 mL) and 10% palladium on charcoal (1.2 g, 50% wet) added. The mixture was hydrogenated at 30 psi and 25 °C for 3 h. The catalyst was removed and the solvent evaporated. The residue was purified.

Method G. *N-tert*-Butoxycarbonyl-protected product was dissolved in trifluoroacetic acid (5−10 mL) at 0 °C. The mixture was stirred for 2 h and the solvent acid removed in vacuum. The residue was then purified.

Method H. *N*-Benzyloxycarbonyl-protected product (208 mmol) was dissolved in methanol (100 mL) and palladium on charcoal (300 mg) was added. The mixture was hydrogenated at 30 psi for 3 h then the catalyst was filtered off and the solvent removed to give a pale oil. Oxalic acid (182 mg, 202 mmol) in ethanol (5 mL) was added and the oil dissolved. Ether (50 mL) was added to give a turbid solution which was refrigerated. Filtration yielded the desired product.

Method J. *N*-Protected dipeptide (2.9 mmol) was dissolved in tetrahydrofuran (30 mL), cooled to −10 °C and treated with isobutyl chloroformate (430 mg, 3.14 mmol) and *N*-ethylmorpholine (360 mg, 3.14 mmol). After 30 min, amine (3.14 mmol) was added and the mixture stirred at room temperature overnight. The mixture was diluted with ethyl acetate (200 mL), washed successively with dilute HCl (50 mL), dilute $NaHCO_3$ (50 mL), and water, and dried (Na_2SO_4) and the solvent removed. The product was then purified.

***N*-9-Fluorenylmethyloxycarbonyl-L-valyl-*O*-benzyl-L-tyrosine.** *O*-Benzyl-L-tyrosine (0.504 g, 1.86 mmol) was dissolved in water (20 mL) containing tetrahydrofuran (20 mL) and treated with triethylamine (0.91 mL, 6.5 mmol) and a solution of *N*-fluorenylmethyleneoxycarbonyl-L-valine pentafluorophenyl ester (0.94 g, 1.86 mmol) in tetrahydrofuran (15 mL). The solution was stirred at room temperature for 18 h. The solvent was removed and the residue dissolved in dilute HCl and extracted with ethyl acetate. The organic portions were dried ($MgSO_4$) and the solvent was removed. Crystallization from ethyl acetate/petroleum gave 0.402 g (37%) of the product: mp 186−188 °C; MS *m/z* 593 $[M]^+$.

L-Valyl-*O*-benzyl-L-tyrosine (9). *N*-9-Fluorenylmethyloxycarbonyl-L-valyl-*O*-benzyl-L-tyrosine (0.218 g, 0.34 mmol) was treated with piperidine at room temperature over 50 min. Water (50 mL) was added and the precipitate collected. The filtrate was concentrated to dryness, dissolved in methanol, filtered and concentrated again. Trituration with petroleum gave the product: yield 16.6%; mp 135−136 °C; ^{1}H NMR (200 MHz DMSO-d_6) δ 8.04 (1H, bs, NH), 7.25−7.45 (5H, m, Ar CH), 7.08 (2H, d, Ar CH), 6.83 (2H, d, Ar CH), 5.0 (2H, s, $PhCH_2O$), 4.25 (1H, bs, NCHCO), 3.65 (1H, d, NCHCO), 2.95 (2H, m, β-CH2 of tyrosine), 1.91 (1H, m, $(CH_3)_2C\mathbf{H}$), 0.81 (3H,

d, CH_3), 0.68 (3H, d, CH_3); MS *m/z* 371 $[M]^+$; HPLC purity (Lichrosorb rp select B; water + 0.1% TFA/methanol + 0.1% TFA 50:50) 98.4%. Anal. ($C_{21}H_{26}N_2O_4 \cdot 1.75H_2O$) C, H, N.

***N*-Benzyl-L-valyl-L-norvaline Oxalate (30).** L-Valyl-L-norvaline (0.65 g, 3 mmol) and benzaldehyde (0.38 g, 3.6 mmol) were dissolved in methanol (6 mL) and stirred for 2 h at room temperature. Sodium cyanoborohydride (0.23 g, 3.6 mmol) was added and the mixture stirred for 24 h. The mixture was diluted with water (10 mL) and extracted with chloroform (3 × 30 mL) and the aqueous layer evaporated to dryness. The residue was dissolved in hot ethanol and treated with oxalic acid (0.3 g, 3 mmol) and the solvent removed. This residue was crystallized from ethyl acetate/ether 1:1 to give 0.33 g (28%) of the desired product: mp 214−216 °C; ^{1}H NMR (200 MHz DMSO-d_6) δ 8.1−8.7 (4H, m, NH, NH_2, COOH), 7.32 (5H, m, Ph), 4.16 (1H, m, C**H**COOH), 3.76 & 3.94 (2H, q, $PhCH_2$), 3.24 (1H, m, NHC**H**CO of valine), 1.98 (1H, m, C**H**$(CH_3)_2$), 1.58 (2H, m, $CH_3CH_2C\mathbf{H_2}$), 1.26 (2H, m, $CH_3C\mathbf{H_2}$), 0.85 (9H, m, 3 × CH_3); MS *m/z* 307 $[M + H]^+$; HPLC purity (Lichrosorb rp select B; water + 0.1% TFA + 0.01% TEA/methanol + 0.1% TFA + 0.01% TEA 1:1) 99.06%. Anal. ($C_{17}H_{26}N_2O_3 \cdot (COOH)_2 \cdot 0.5H_2O$) C, H, N.

***N*-Acetyl-L-valyl-L-norvaline (32).** L-Valyl-L-norvaline (1 g, 4.4 mmol) in acetic acid (15 mL) was treated with acetic anhydride (6 mL) and stirred at 10 °C for 1 h. The mixture was taken to dryness, dissolved in 10% aqueous Na_2CO_3 solution (30 mL) and washed with chloroform (50 mL). The aqueous solution was acidified and extracted with ethyl acetate (100 mL). The organic extract was washed with water (2 × 100 mL), dried (Na_2SO_4), and concentrated whereupon the product crystallized out. It was collected and dried. The aqueous washings were concentrated and further product crystallized. It was collected, washed with water and ethyl acetate and dried: total yield 1.034 g (86%); mp 224−225 °C; ^{1}H NMR (DMSO-d_6) δ 8.14 (1H, d, NH), 7.86 (1H, d, NH), 4.20 (1H, dd, NCHCO), 4.14 (1H, m, NCHCO), 1.92 (1H, m, C**H**$(CH_3)_2$), 1.84 (3H, s, CH_3CO), 1.60 (2H, m, $CH_3CH_2C\mathbf{H_2}$), 1.32 (H, m, $CH_3C\mathbf{H_2}$), 0.82−0.92 (9H, m, 3 × CH_3); MS *m/z* 259 $[M + H]^+$; HPLC purity (Lichrosorb rp select B; water/acetonitrile 80:20 to 50:50 over 25 min) 98.8%; (Kromasil C18; water + 0.1% TFA/methanol + 0.1% TFA 60:40) 99.8%. Anal. ($C_{12}H_{22}N_2O_4$) C, H, N.

***N*-Benzyl-L-norleucyl-L-norleucine Methylamide (35).** Prepared in like fashion to **30** from l-norleucyl-L-norleucine methylamide and benzaldehyde. Purified by column chromatography over silica gel using ethyl acetate/petrol 5:2 as eluent then preparative HPLC (Lichrosorb B; water + 0.01%TEA/methanol + 0.01% TEA 30:70) then crystallization from ethyl acetate/petrol: yield 27%; mp 109−110 °C; ^{1}H NMR (200 MHz $CDCl_3$) δ 7.7 (1H, d, NH), 7.28 (5H, s, Ph), 6.20 (1H, bs, NH), 4.30 (1H, q, NCHCO), 3.65 (2H, dd, $PhCH_2$), 3.1 (1H, m, NCHCO), 2.7 (3H, m, NCH_3), 1.9−1.4 (4H, m, 2 × $C_3H_7C\mathbf{H_2}$), 1.1−1.3 (8H, m, 2 × $CH_3C\mathbf{H_2}C\mathbf{H_2}$), 0.7−0.9 (2 × $C\mathbf{H_3}CH_2$); MS *m/z* 348 $[M + H]^+$; HPLC purity (Lichrosorb rp select B; water + 0.01% TEA/methanol + 0.01% TEA 3:7) 98.45%. Anal. ($C_{20}H_{33}N_3O \cdot 0.25H_2O$) C, H, N.

***N*-Benzyloxycarbonyl-*N*-methyl-L-norleucine.** *N*-Benzyloxycarbonyl-L-norleucine (1.33 g, 5 mmol) and methyl iodide (5.7 g, 40 mmol) were dissolved in tetrahydrofuran and cooled to 0 °C. Sodium hydride (0.36 g, 15 mmol) was suspended in tetrahydrofuran (5 mL) and added to the first solution. The mixture was stirred for 2 days at room temperature. Ethyl acetate (25 mL) and water (10 mL) were added and the solvent was removed. The residue was dissolved in ether (15 mL) and extracted with water (50 mL) and aqueous $NaHCO_3$ solution (25 mL). The combined aqueous extracts were acidified to pH 2 and extracted with ethyl acetate (3 × 30 mL). The combined organic extracts were washed with water (25 mL), aqueous sodium thiosulfate (2 × 25 mL) and water (25 mL) and dried ($MgSO_4$) and the solvent was removed to give the product in 82% yield: ^{1}H NMR (200 MHz $CDCl_3$) δ 7.30−7.36 (5H, m, Ph), 5.16 (2H, m, $PhC\mathbf{H_2}$), 4.64−4.82 (1H, m, NCHCO), 2.86 (3H, s, NCH_3), 1.60−2.08 (2H, m, $C_3H_7C\mathbf{H_2}$), 1.3 (4H, m, $CH_3C\mathbf{H_2}C\mathbf{H_2}$), 0.88 (3H, m, $C\mathbf{H_3}CH_2$).

L-Norleucine Methyl Ester Hydrochloride. A mixture of L-norleucine (2.63 g, 20 mmol) and thionyl chloride (6 g, 50 mmol) was stirred in methanol (20 mL) at room temperature for 24 h. The solvent was removed and the residue suspended in ether. The product was filtered off: yield 97%; mp 55−57 °C; ^{1}H NMR (DMSO-d_6) δ 8.82 (3H, m, NH_3), 3.94 (1H, t, NCHCO), 3.72 (3H, s, OCH_3), 1.70−1.90 (2H, m, $C_3H_7C\mathbf{H_2}$), 1.12−1.48 (4H, m, $CH_3C\mathbf{H_2}C\mathbf{H_2}$), 0.80−0.93 (3H, m, $C\mathbf{H_3}CH_2$).

***N*-Benzyloxycarbonyl-*N*-methyl-L-norleucinyl-L-norleucine Methyl Ester.** *N*-Benzyloxycarbonyl-*N*-methyl-L-norleucine (1.14 g, 4.08 mmol) and *N*-hydroxysuccinimide (0.47 g, 4.08 mmol) were dissolved in methylene chloride (8 mL), cooled to 0 °C and treated with dicyclohexyl carbodiimide (0.84 g, 4.08 mmol). The reaction was stirred at room temperature for 2 h and filtered and the solvent removed. The residue was reacted with L-norleucine methyl ester hydrochloride according to method A. It was purified by column chromatography over silica gel using ether/petrol as eluant: yield 42%; ^{1}H NMR ($CDCl_3$) δ 7.36 (5H, s, Ph), 6.6 (1H, m), 5.18 (2H, s), 4.6 (2H, m) 3.74 (3H, s, OCH_3), 2.96 (3H, s, NCH_3), 1.56−2.06 (4H, m, 2 × $C_3H_7C\mathbf{H_2}$), 1.28 (8H, m, 2 × $CH_3C\mathbf{H_2}C\mathbf{H_2}$), 0.88 (6H, m, 2 × CH_3).

***N*-Methyl-L-norleucyl-L-norleucine Methyl Ester Hydrobromide (36).** *N*-Benzyloxycarbonyl-*N*-methyl-L-norleucyl-L-norleucine methyl ester was treated with HBr in glacial acetic acid for 1 h at room temperature. Ether was then added and the solvents were removed. The residue was suspended in CH_2Cl_2/ether 1:3 and the product isolated by filtration: yield 90%; mp 124−126 °C; ^{1}H NMR (200 MHz $CDCl_3$) δ 9.28 (1H, m, $CH_3N\mathbf{H}(H)^+$), 8.60 (1H, m, $CH_3NH(\mathbf{H})^+$), 8.18 (1H, d, CONH), 4.45 (1H, q, NCHCO), 4.30 (1H, m, NCHCO), 3.75 (3H, s, OCH_3), 2.76 (3H, m, NCH_3), 2.12 (2H, m, $C_3H_7C\mathbf{H_2}$), 1.88 (3H, m, $C_3H_7C\mathbf{H_2}$), 1.4 (8H, m, 2 × $CH_3C\mathbf{H_2}C\mathbf{H_2}$), 0.9 (6H, m, 2 × $C\mathbf{H_3}CH_2$); MS *m/z* 273 $[M + H]^+$; HPLC purity (Lichrosorb rp select B; water + 0.01% TEA/methanol + 0.01% TEA 1:4) 99.43%; (Kromasil C18; water + 0.1% TFA/methanol + 0.1% TFA 50:50) 96.8%. Anal. ($C_{14}H_{28}N_2O_3 \cdot HBr$) C, H, N.

L-Valyl-L-norleucine Butyl Ester Oxalate (39). To *N*-benzyloxycarbonyl-L-valyl-L-norleucine butyl ester (**40**; 1.26 g, 3 mmol) was added 4 M HBr in glacial acetic acid (5.4 mL) at room temperature. After 45 min, the reaction was neutralized with $NaHCO_3$ and extracted with ethyl acetate (3 × 30 mL). The extracts were dried ($MgSO_4$) and the solvent was removed. The residue was dissolved in hot ethanol and treated with oxalic acid (0.3 g, 3 mmol) and the solvent then removed. This residue was crystallized from chloroform/methanol 9:1 to give the product: yield 79%; mp 161−163 °C; ^{1}H NMR (DMSO-d_6) δ 8.72 (1H, d, NH), 7.15−7.8 (3H, m, NH_3^+), 4.25 (1H, m, NCHCO), 4.04 (2H, m, OCH_2), 3.63 (1H, m, NCHCO), 2.08 (1H, m, $(CH_3)_2C\mathbf{H}$), 1.6 (4H, m, 2 × CH_2), 1.29 (6H, m, 3 × CH_2), 0.88 (12H, m, 4 × CH_3): HPLC purity (Lichrosorb rp select B; methanol + 0.1% TFA/water + 0.1% TFA 70:30) 98.8%. Anal. ($C_{15}H_{30}N_2O_3 \cdot (COOH)_2$) C, H, N.

***N*-Benzyloxycarbonyl-L-valyl-L-norleucine Butyl Ester (40).** *N*-Benzyloxycarbonyl-L-valyl-L-norleucine (1.83 g, 5 mmol), *n*-butanol (0.56 g, 7.5 mmol), and *p*-toluenesulfonic acid (0.1 g, 0.5 mmol) in benzene (10 mL) were refluxed for 2 h 30 min. The mixture was cooled, washed with 10% K_2CO_3 solution (2 × 50 mL), and dried ($MgSO_4$) and the solvent removed. The residue was crystallized from diethyl ether to give 1.58 g (75%) of the product: mp 104−106 °C; ^{1}H NMR (200 MHz $CDCl_3$) δ 7.24 (5H, s, Ph CH), 6.6 (1H, m, NH), 5.5 (1H, d, NH), 5.1 (2H, s, $PhCH_2$), 4.58 (1H, m, NCHCO), 4.1 (3H, m, NCHCO, OCH_2), 2.1 (1H, m, $(CH_3)_2C\mathbf{H}$), 1.5−1.9 (4H, m, 2 × CH_2), 1.3 (6H, m, 3 × CH_2), 0.92 (12H, m, 4 × CH_3); MS *m/z* 420 $[M]^+$; HPLC purity (Lichrosorb rp select B; water + 0.1% TFA/methanol + 0.1% TFA 30:70) 99.52%. Anal. ($C_{23}H_{36}N_2O_5$) C, H, N.

L-Valyl-L-phenylalanine Methyl Ester (48). L-Valyl-L-phenylalanine (0.56 g, 2.12 mmol) was dissolved in dimethoxypropane (34 mL) containing methanol (9 mL) and concentrated HCl (2.1 mL). The mixture was stirred at room temperature overnight. The solvent was removed and the residue triturated with ether. The resulting solid was crystallized from methanol/

ether/petroleum: yield 0.19 g, 28.8%; mp 201−202 °C; ^{1}H NMR (DMSO-d_6) δ 9.00 (1H, d, NH), 8.18 (2H, bs, NH_2), 7.28 (5H, bs, Ar CH), 4.53 (1H, d, NCHCO), 3.65 (1H, d, NCHCO), 3.52 (3H, s, OCH_3), 3.04 (2H, d, CH_2), 2.13 (1H, m, $(CH_3)_2$C**H**), 0.93 (6H, d, (C**H$_3$**)$_2$CH); MS *m*/*z* 278 $[M]^+$; HPLC purity (Lichrosorb rp select B; water + 0.1% TFA/acetonitrile + 0.1% TFA 80:20) 97.7%. Anal. ($C_{15}H_{22}N_2O_3$·HCl·0.25H_2O) C, H, N.

***N*-Benzyloxycarbonyl-L-valyl-L-phenylalanine *n*-Butyl Ester.** *N*-Benzyloxycarbonyl-L-valyl-L-phenylalanine (0.5 g, 1.25 mmol) was suspended in *n*-butanol (8 mL, dried, distilled) and cooled in ice. Thionyl chloride (2.03 g, 17 mmol) was added and the mixture allowed to warm to room temperature. It was then heated to 40 °C with stirring. The solvent was removed and the residue twice dissolved in ethanol which was removed in vacuum. The residue was then triturated with hexane and a solid isolated: yield 79.4%. It was purified by preparative HPLC (Lichrosorb rp select B; methanol + 0.1% TFA/water + 0.1% TFA 80:20): ^{1}H NMR (200 MHz $CDCl_3$) δ 7.30−7.44 (1H, d, NH), 7.36 (5H, s, Ar CH), 7.16 (5H, s, Ar CH), 5.00 (2H, s, $PhCH_2O$), 4.50−4.58 (1H, m, NCHCO), 3.82−3.98 (2H, m, $COOCH_2$), 2.90−3.00 (2H, m, PhC**H$_2$**CH), 1.82−1.92 (1H, m, $(CH_3)_2$C**H**), 1.36−1.48 (2H, m, $COOCH_2$C**H$_2$**), 1.08−1.27 (2H, m, CH_3C**H$_2$**), 0.74−0.88 (9H, m, 3 × CH_3); MS *m*/*z* 455 $[M + H]^+$. Anal. ($C_{26}H_{34}N_2O_5$·0.05CF_3COOH) C, H, N.

L-Valyl-L-phenylalanine *n*-Butyl Ester Trifluoroacetate (49). Prepared by method E from *N*-benzyloxycarbonyl-L-valyl-L-phenylalanine *n*-butyl ester. Crystallized from ether/hexane and purified by preparative HPLC (Lichrosorb rp select B; methanol + 0.1% TFA/water + 0.1% TFA 50:50): yield 77%; mp 150.5−151.5 °C; ^{1}H NMR (400 MHz DMSO-d_6) δ 8.81−8.83 (1H, d, NH), 7.21−7.31 (5H, m, Ph CH), 4.53−4.55 (1H, m, NCHCO) 3.97−4.0 (2H, m, $COOCH_2$), 3.56−3.58 (1H, m, NCHCO), 2.94−3.06 (2H, m, $PhCH_2$), 2.08−2.10 (1H, m, $(CH_3)_2$C**H**), 1.41−1.46 (2H, m, $COOCH_2$C**H$_2$**), 1.18−1.26 (2H, m, CH_3C**H$_2$**), 0.8−0.94 (9H, m, 3 × CH_3); MS *m*/*z* 321 $[M + H]^+$; HPLC purity (Lichrosorb rp select B; methanol + 0.1% TFA/water + 0.1% TFA 50:50) 97.3%. Anal. ($C_{18}H_{28}N_2O_4$·1.25CF_3COOH·0.5H_2O) C, H, N.

***N*-Benzyloxycarbonyl-L-glycyl-L-glycine.** Prepared by method B from *N*-benzyloxycarbonyl-L-glycine succinimide ester and glycine: yield 50%.

***N*-Benzyloxycarbonyl-L-glycyl-L-glycine Succinimide Ester.** Prepared by the method of Anderson.[37] Crystallized from ethanol: yield 100%; ^{1}H NMR (200 MHz $CDCl_3$) δ 8.5 (1H, bs, NH), 7.5 (1H, bs, NH), 7.3 (5H, m, Ar CH), 5.5 (1H, d, NH), 5.0 (2H, s, $PhCH_2$), 4.2 (2H, d, CH_2), 3.6 (2H, d, CH_2), 2.8 (4H, s, 2 × CH_2).

***N*-Benzyloxycarbonyl-L-glycyl-L-glycyl-L-phenylalanine.** *N*-Benzyloxycarbonyl-L-glycyl-L-glycine succinimide ester (3.0 g, 8.26 mmol) was dissolved in a mixture of tetrahydrofuran (30 mL) and dimethylformamide (20 mL). It was treated with phenylalanine (1.36 g, 8.2 mmol) in water (20 mL) containing triethylamine (1 mL). The mixture was stirred at room temperature for 16 h, made acid to pH 1 (concentrated HCl) and extracted with ethyl acetate (3 × 50 mL). The combined extracts were dried (Na_2SO_4) and concentrated to give an oil which was purified by chromatography over silica gel using ethyl acetate/methanol/acetic acid 8:2:0.5 as eluant: yield 2.0 g (58%); ^{1}H NMR (200 MHz DMSO-d_6) δ 8.1 (1H, d, NH), 8.0 (1H, d, NH), 7.4 (5H, m, Ar CH), 5.0 (2H, s, $PhCH_2$), 4.4 (1H, m, NCHCO), 3.7 (2H, d, CH_2), 3.6 (2H, d, CH_2), 2.95 (2H, d, CH_2).

L-Glycyl-L-glycyl-L-phenylalanine (57). Prepared by method F from *N*-benzyloxycarbonyl-L-glycyl-L-glycyl-L-phenylalanine. The residue was washed with hot methanol and then crystallized from methanol/water: yield 16%; mp 228−229 °C (lit.[42] mp 228−230 °C); ^{1}H NMR (200 MHz DMSO-d_6) δ 8.5 (1H, d, NH), 7.9 (1H, d, NH), 7.2 (5H, m, Ar CH), 4.2 (1H, dd, NCHCO), 3.6 (2H, dd, CH_2), 3.25 (2H, s, CH_2), 3.0 (1H, dd, CH(**H**)), 2.8 (1H, dd, C**H**(H)); MS *m*/*z* 280 $[M + H]^+$; HPLC purity (Lichrosorb rp select B; methanol + 0.1% TFA/water + 0.1% TFA 20:80) 99.6%. Anal. ($C_{13}H_{17}N_3O_4$) C, H, N.

***N*-*tert*-Butoxycarbonyl-L-valyl-L-proline.** Prepared by method D from *N*-*tert*-butoxycarbonyl-L-valine succinimide ester and L-proline. Purified by crystallization from ethyl acetate/petrol 1:4: yield 45%; ^{1}H NMR (200 MHz $CDCl_3$) δ 5.30 (1H, d, NH), 4.57 (1H, dd, NCHCO of val), 4.26 (1H, t, NCHCO of pro), 2.19 (1H, q, $(CH_3)_2$C**H**), 2.03 (4H, m, NCH_2C**H$_2$**C**H$_2$**), 1.39 (9H, s, *t*-butyl), 0.97 (3H, d, CH_3), 0.92 (3H, d, CH_3); MS *m*/*z* 314 $[M]^+$.

***N*-*tert*-Butoxycarbonyl-L-valyl-L-proline *n*-Butylamide.** Prepared by method B from *N*-*tert*-butoxycarbonyl-L-valine succinimide and L-proline butylamide. Purified by chromatography over silica gel using ethyl acetate/petrol 1:1 as eluant: yield 41%; ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.9 (1H, bs, NH), 5.2 (1H, d, NH), 4.5 (1H, dd, NCHCO), 4.25 (1H, dd, NCHCO), 3.6 (2H, m, NCH_2), 3.2 (2H, q, NCH_2), 1.1 (5H, $(CH_3)_2$C**H**, 2 × CH_2), 1.4 (9H, 2, *t*-Bu), 1.3 (4H, m, 2 × CH_2), 0.9 (6H, dd, (C**H$_3$**)$_2$CH), 0.8 (3H, t, CH_3).

L-Valyl-L-proline *n*-Butylamide Oxalate (64). Prepared by method G from *N*-*tert*-butoxycarbonyl-L-valyl-L-proline *n*-butylamide. Purified by preparative HPLC and converted to its oxalate salt. Purified again by crystallization from methanol/ethyl acetate/ether 1:1:3: yield 42%; mp 165−166 °C; ^{1}H NMR (200 MHz DMSO-d_6) δ 7.9 (t, 1H, NH), 7.3 (2H, bs, NH_2), 4.25 (1H, t, NCHCO), 3.95 (1H, d, NCHCO), 3.45−3.65 (2H, m, NCH_2 of pro), 3.0 (2H, m, NCH_2 of butyl), 1.5−2.2 (5H, $(CH_3)_2$C**H**, 2 × CH_2), 1.3 (4H, m, 2 × CH_2), 0.9 (6H, dd, (C**H$_3$**)$_2$-CH), 0.8 (t, 3H, CH_3); MS *m*/*z* 269 $[M]^+$; HPLC purity (Lichrosorb rp select B; methanol/water/trifluoroacetic acid 30:70:0.1) 100%; (Kromasil C18; water + 0.1% TFA/methanol + 0.1% TFA 60:40) 95.9%. Anal. ($C_{14}H_{27}N_3O_2$·1.25$(COOH)_2$) C, H, N.

L-Valyl-D-proline Butylamide Oxalate (65). Prepared from *N*-*tert*-butoxycarbonyl-L-valine succinimide ester and D-proline by method B then method J then method G. The product was isolated as the oxalate salt and crystallized from ethanol/diethyl ether: mp 104 °C; ^{1}H NMR (200 MHz DMSO-d_6) δ 8.0 (1H, t, NH), 4.3 (1H, d, CH), 3.9 (1H, d, CH), 3.75 (1H, m, CH(**H**)), 3.5 (1H, m, C(**H**)H), 3.0 (2H, m, CH_2), 2.0 (5H, m, 2 × CH_2, CH), 1.3 (4H, m, 2 × CH_2), 0.9 (6H, dd, 2 × CH_3), 0.85 (3H, t, CH_3); MS *m*/*z* 269 $[M]^+$; HPLC purity (Kromasil C18; water + 0.1% TFA/methanol + 0.1% TFA 60:40) 96%. Anal. ($C_{14}H_{27}N_3O_2$·1.25$(COOH)_2$) C, H, N.

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Supporting Information Available: Three further tables of biochemical results, one synthetic scheme, and preparative details of 47 additional compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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