Application of N^{im}-2,6-Dimethoxybenzoyl Histidine in Solid-Phase Peptide Synthesis[‡]

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Fmoc-histidine derivatives protected with 2,6-dimethoxybenzoyl (2,6-Dmbz) units, either on the τ- (**1**) or on the π-position (**2**) of the imidazole residue, have been prepared and applied to the synthesis of the dipeptide Fmoc-His(2,6-Dmbz)-Val-OMe, with high coupling efficiencies (99 and 95%, respectively) and low racemization (0.3 and 0.1%, respectively), as ascertained by HPLC analysis on the fully protected dipeptides. In addition, the hexapeptide H-Ala-Ser-Val-His-Val-Phe-OH (**I**) has been synthesized on a solid support employing the novel building blocks **1** and **2** as well as commercially available Fmoc-His(τ-Trt)-OH (**3**). The crude deprotected products were analyzed by reverse-phase HPLC and mass spectrometry, which indicated that when the τ-protected derivatives **1** and **3** were employed the products were of comparable quality (93–94% major peak). The hexapeptide synthesized with the π-protected histidine **2** was contaminated with a product corresponding to 2,6-Dmbz-His-Val-Phe-OH (**12**), which was probably formed through the intramolecular acyl transfer of a 2,6-Dmbz unit from the π-nitrogen atom to the α-amino group of the N-terminal histidine unit upon removal of the Fmoc group. This side reaction was insignificant (less than 0.5%) when using the τ-protected derivative **1**.

Introduction

In recent years, sequence-recognizing oligonucleotide scaffolds tethered with imidazole-bearing structures capable of cleaving target RNA in a sequence-specific manner have been the subjects of considerable scientific attention.[1−3] Among the possible appendages through which imidazole functions can be incorporated in the oligonucleotide strands are histidine-containing peptides, such as Gly-Gly-His and His-Gly-His, which have been shown to possess phosphodiester cleaving ability.[4]

The preparation of these oligonucleotide−peptide conjugates (also called nucleopeptides) in a stepwise procedure requires a protection strategy where the protecting groups should be compatible with both oligonucleotide and peptide synthesis. The choice, however, of suitable protecting groups for the preparation of nucleopeptides is not straightforward. For example, the strongly acidic conditions that are commonly used in the final deprotection of peptide synthesis are prohibited since they would cause extensive depurination in oligonucleotides. Therefore, a suitable protecting group for the imidazole residue of histidine in nucleopeptides should not only withstand the conditions of oligonucleotide synthesis, but also be removed in the final stage of the synthesis under mild conditions and preferably with no additional steps.

Although a number of protecting groups for the imidazole function of histidine have been developed, only a few of them have found widespread application. Some of these protecting groups (mostly of the alkyl type)[5−7] are not compatible with the assembly of nucleopeptides since they are cleaved under quite harsh conditions,[8,9] for example with concentrated strong acids such as trifluoroacetic acid (TFA), trifluoromethanesulfonic acid, and liquid HF. Others, such as the urethane-[10,11] and sulfonyl-based groups,[12] normally block the τ nitrogen and, despite their electron-withdrawing character, may not provide sufficient suppression of racemization during the coupling step.[13,14] Furthermore, their histidine derivatives are, in general, not very stable during prolonged storage and they are very sensitive to nucleophiles, like the amino groups of amino acids[15] and other reagents used in standard peptide chemistry.[13]

In a previous paper[16] we explored the stability of various *N*-acylimidazoles to acidic and basic conditions that can be used for the removal of MMTr from hydroxyl functions and of 9-fluorenylmethyloxycarbonyl (Fmoc) from amino functions, respectively. The results of the above investigations indicated that the 2,6-dimethoxybenzoyl (2,6-Dmbz) group could be suitable for the synthesis of peptide−oligonucleotide conjugates.

[‡] Acyl Groups as Prospective Protection for Imidazole, II. Part I: Ref.[16]

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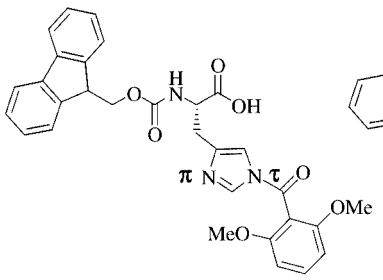
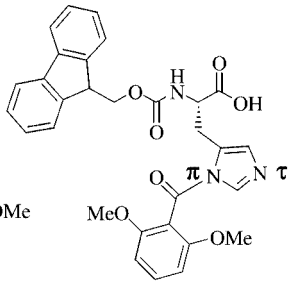
As a first step towards the preparation of nucleopeptides, the applicability of the 2,6-Dmbz group in the solid-phase synthesis of the peptide segment had to be carefully studied and evaluated. In this paper we report on the solid-phase peptide synthesis (SPPS) of a histidine-containing hexapeptide H-Ala-Ser-Val-His-Val-Phe-OH (**I**) using the 2,6-Dmbz group, together with the results of a thorough investigation of the side reactions that can occur during the various chemical steps.



Fmoc-His(τ-2,6-Dmbz)-OH (**1**)



Fmoc-His(π-2,6-Dmbz)-OH (**2**)

Results and Discussion

The first step towards evaluating the applicability of the 2,6-Dmbz group in the solid-phase synthesis of peptides, using Fmoc-chemistry, was to prepare the N^{im}-acyl-protected histidine derivatives **1** and **2**. The synthesis of the N^{τ}-acyl-protected histidine (**1**) was accomplished by regioselective acylation of Fmoc-His-OH with 2,6-dimethoxybenzoyl chloride, in presence of the proton scavenger *N*,*N*-diisopropylethylamine (DIEA) in DMF. This method was expected to provide the τ-blocked derivative as a major product.[9,17,18] Indeed, compound **1** was obtained in 82% yield, with a purity of 98%, contaminated with 1.4% of the π-acylated isomer, according to RP-HPLC analysis. The N^{π}-acyl-protected isomer (**2**) was obtained unexpectedly as a side product (13%) during the preparation of **1** through an alternative procedure, namely silylation with chlorotrimethylsilane in dichloromethane followed by acylation. The formation of compound **2** may be a consequence of the transient blockade of the τ-nitrogen atom by the silyl group. The isolated product **2** was 99% pure and contained 0.4% of the isomer **1**. Characterization of the compounds was performed by means of RP-HPLC, NMR spectroscopy, and mass spectrometric analysis.

Prior to application of derivatives **1** and **2** to SPPS, we studied the coupling reactions in solution of these compounds with H-Val-OMe under conditions identical to those of the automated solid-phase procedure. We were interested in determining not only the coupling efficiencies but also the extent of racemization that can occur upon activation, as well as the stability of the imidazole-protecting group during the condensation reaction. In the coupling tests, compounds **1**, **2**, and the commercially available Fmoc-His(τ-Trt)-OH (**3**) as a reference compound, were the carboxyl components. They were treated with the hydrochloride salt of H-Val-OMe as the amino component, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) and 1-hydroxybenzotriazole (HOBt) as the coupling reagents, DIEA as the base, in DMF as the solvent. Prior to coupling, the carboxyl component was preactivated in the absence of the amino component for 70 s. Aliquots of the coupling mixture were withdrawn at time intervals and subjected to HPLC analysis (Figure 1). Couplings of compounds **1**−**3** afforded the dipeptides Fmoc-His(τ-2,6-Dmbz)-Val-OMe (**4**), Fmoc-His(π-2,6-Dmbz)-Val-OMe (**5**), and Fmoc-His(τ-Trt)-Val-OMe (**6**), respectively.

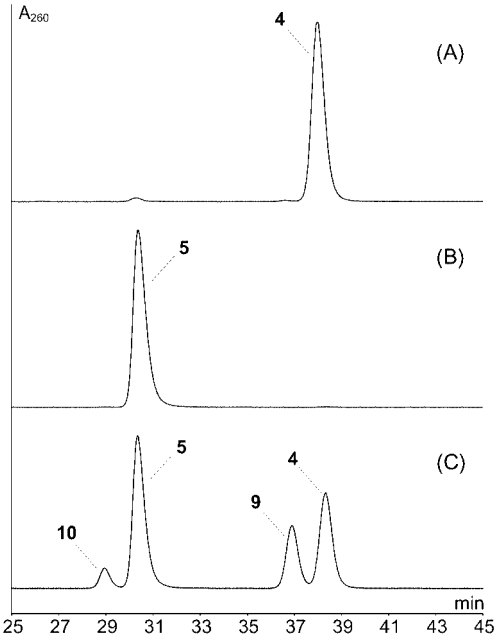


Figure 1. HPLC profiles of the coupling mixtures after a reaction time of 11 min. (A) Coupling of **1**; (B) coupling of **2**; (C) artificial mixture of LL-dipeptides (**4** and **5**) and DL-dipeptides (**9** and **10**). RP-HPLC analysis was carried out on a Thermo Hypersil C18 column with elution by a linear gradient (40−50%, 60 min) of 0.05% TFA in CH_3CN and 0.05% aq. TFA, flow rate 1.5 mL/min, temperature 50 °C, UV detection at 260 nm

The results of the condensation tests (Table 1) indicate a rapid conversion of the τ-protected isomer **1** into dipeptide **4**, while the π-protected isomer **2** appeared to be slightly less reactive in the formation of **5**, the starting materials being 99 and 94% consumed, respectively, after 11 min. The reaction of the reference trityl derivative **3** to give dipeptide **6** reached 97% completion after 11 min. These results can be explained by the potentially closer proximity of the N^{π}-acyl group in **2** to the α-carboxyl center, which may lead to some steric hindrance.

HPLC analysis of the crude reaction mixtures allowed the determination of the abundance of dipeptide diastereoisomers (Figure 1). Authentic samples of Fmoc-D-His(τ-Trt)-L-Val-OMe (**7**), Fmoc-D-His(τ-2,6-Dmbz)-L-Val-OMe (**9**), and Fmoc-D-His(π-2,6-Dmbz)-L-Val-OMe (**10**) were prepared and co-injected. The peak areas correspond-

Table 1. Synthesis of protected dipeptides Fmoc-His(*R*)-Val-OMe (**4**−**6**) by HBTU/HOBt-mediated coupling of the histidine derivatives **1**−**3**

Histidine protection (R)	Coupling yield [%][a]	DL-Dipeptide [%][b]
τ-2,6-Dmbz (**1**)	99 (94)	0.3
π-2,6-Dmbz (**2**)	94 (90)	< 0.1
τ-Trt (**3**)	97 (92)	0.2

[a] Coupling yields after 11 min of reaction were calculated as 100 × [% peak area dipeptide/(% peak area dipeptide + % peak area monomer)]; the numbers in parentheses are the yields of isolated product. [b] Values were determined by integration of the corresponding UV signals at 260 nm of the peaks of the crude products. At this wavelength, the Fmoc and benzoylimidazole (or the Trt) residues are the main contributors to the absorbance. Consequently, the differences in extinction coefficient between the histidine monomer and the parent LL- and DL-dipeptide can be considered negligible.

ing to the epimerized products accounted for 0.3, < 0.1, and 0.2% of the crude mixture for the couplings of **1**, **2**, and **3**, respectively (Table 1).

The next aspect we evaluated was the reactivity of the N^{im}-acyl moiety of the histidine monomers **1** and **2** towards the α-amino group of the amino component. Such reactivity would lead to intermolecular $N^{im} \rightarrow N^{\alpha}$ transacylation, which would result in irreversible chain termination occurring during peptide elongation. Fortunately, the product of this side reaction, 2,6-Dmbz-Val-OMe (**11**), was not observed in the coupling mixture, as was ascertained by co-injecting an authentic sample of the compound.

Stimulated by the results of the above investigations, we then used compounds **1** and **2** in the solid-phase synthesis of hexapeptide H-Ala-Ser-Val-His-Val-Phe-OH (**I**). Preparation of the peptide was accomplished on an automated synthesizer following Fmoc-chemistry procedures, starting from pre-loaded Wang resin and using HBTU/HOBt/DIEA in DMF/NMP as the coupling cocktail. At the end of the assembly, the peptide prepared with Fmoc-His(τ-Trt)-OH (**3**) was deblocked with a mixture of TFA/H_2O/TIS (95:2.5:2.5), whereas the peptides synthesized with the histidine derivatives **1** and **2** were cleaved from the support by acidolysis as above and then subjected to an ammonolytic treatment. Aqueous ammonia mixtures are normally used in oligonucleotide chemistry for deblocking from a solid support and side-chain deprotection, and it has been demonstrated that aq. ammonia treatments do not cause racemization of peptides during the preparation of oligonucleotide−peptide conjugates.[19] The HPLC profile of the crude product from **1** after acidolysis showed a major peak (93%) at 23.3 min, with a mass corresponding to the imidazole-protected hexapeptide, and no detectable traces of the fully deprotected hexapeptide. It is, thereby, demonstrated that the 2,6-Dmbz group remains intact upon exposure to the conditions of concentrated TFA that normally remove acid-labile side-chain protections. To ensure complete removal of the acyl group, two different ammonolytic treatments were tested: (a) 32% aq. ammonia/dioxane (1:1) and (b) 32% aq. ammonia/ethanol (3:1). Complete deprotection was observed in less than 6 and 2 h with (a) and (b), respectively. Characterization of the three hexapeptides obtained above was performed by means of HPLC (Figure 2) and mass spectrometry.

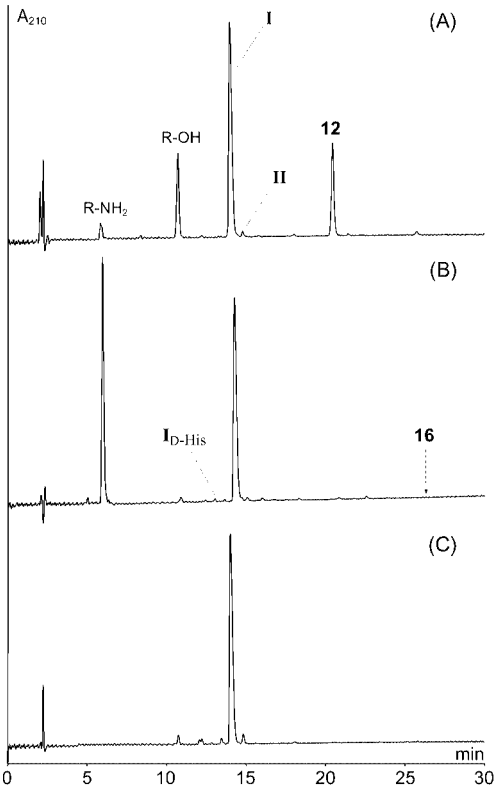
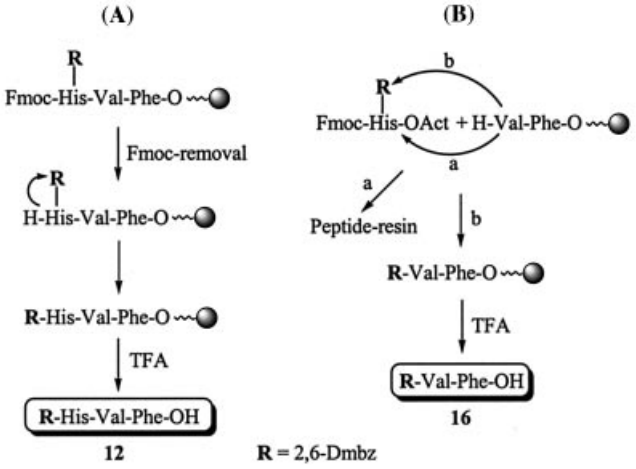


Figure 2. HPLC profiles of the crude hexapeptide H-Ala-Ser-Val-His-Val-Phe-OH (**I**) synthesized with **2** (A), with **1** (B), and with **3** (C). The peaks marked with R-NH_2 and R-OH correspond to the amide and the acid, respectively, of 2,6-Dmbz obtained as by-products after ammonolysis. $\mathbf{I}_{D\text{-His}}$ is H-Ala-Ser-Val-D-His-Val-Phe-OH, **II** is H-Ala-Ser-Val-Val-Phe-OH, and **12** is 2,6-Dmbz-His-Val-Phe-OH. The dashed arrow in panel B indicates the position of 2,6-Dmbz-Val-Phe-OH (**16**) if it had formed during the synthesis. RP-HPLC analysis was carried out on a Thermo Hypersil C18 column with elution by a linear gradient (10−50%, 40 min) of 0.1% TFA in CH_3CN and 0.1% aq. TFA, flow rate 1.0 mL/min, temperature 30 °C, UV detection at 210 nm

Additional tests were performed to examine the degree of racemization of the hexapeptide. Since **I** contains the amino acids serine and histidine, which are considered racemization-sensitive, three reference peptides where prepared to be used in establishing the degree of racemization of oligopeptide **I** that was prepared by using compounds **1**−**3**. These three peptides were as follows: a peptide containing D-Ser, a peptide with D-His ($\mathbf{I}_{D\text{-His}}$), and a peptide having both D-Ser and D-His units. We found that only $\mathbf{I}_{D\text{-His}}$ was present in a detectable amount in the crude hexapeptides. Next, peptide **I** was also assembled using Fmoc-His-OH in order to obtain a better insight into the type of side reactions that can occur with histidine bearing an unprotected imidazole residue. This experiment provided a mixture of the target peptide **I** (42%) and of the *des*-His sequence Ala-Ser-Val-Val-Phe **II** (54%). Side product **II** is indicative of an

inefficient coupling of histidine, which can be due either to solubility problems of the imidazole-unprotected histidine, or to the acylation of the imidazole residue by the activated histidine monomers that reduces the number of amino acid equivalents available for coupling, thus reducing the coupling yields. Surprisingly, the epimerized hexapeptide $\mathbf{I}_{D\text{-His}}$ was detected only in a relatively small amount (2.2%). These results were further confirmed after analyzing the pentapeptide H-Phe-Gly-Gly-His-Phe-OH, which was prepared with Fmoc-His-OH following Fmoc-chemistry protocols. The target pentapeptide (56%) and the *des*-His peptide Phe-Gly-Gly-Phe (41%) were the main products according to analysis by HPLC and mass spectrometry.

Moreover, considering the possibility that acylated histidines **1** and **2** could undergo intra- and intermolecular acyl-group transfer,[20−23] we also investigated these side reactions in the hexapeptide synthesis. These $N^{im} \rightarrow N^{\alpha}$ acyl-group transfers are most likely to occur after the removal of the Fmoc group. During the preparation of peptide **I**, intramolecular $N^{im} \rightarrow N^{\alpha}$ acyl-group transfer (Scheme 1, panel A) would produce 2,6-Dmbz-His-Val-Phe-OH (**12**). When hexapeptide **I** was assembled with derivative **2**, compound **12** made up to 25% of the crude product, according to integration of the HPLC peak at 210 nm, although this quantification does not take into account the difference in extinction coefficients between **I** and **12**. On the other hand, when compound **1** was used, we detected only traces of side product **12** (less than 0.5%) (see Figure 2 and Table 2). The difference reported above in the extent of intramolecular acylation obtained from the π- and the τ-blocked histidine-containing peptides presumably reflects the ease of formation of the two different reaction intermediates: a very-favored six-membered ring in the former case, a less-favored seven-membered ring in the latter case.



Scheme 1. Possible side products obtained by the intramolecular (A) and intermolecular (B) $N^{im} \rightarrow N^{\alpha}$ acyl-group transfer during SPPS of hexapeptide **I**. The OAct unit represents a generic activating species at the α-carboxy center

As it is depicted in panel B of Scheme 1, the intermolecular acyl-group transfer could take place between the incoming protected histidine unit and the resin-bound α-amino group. However, the product of this side reaction, 2,6-Dmbz-Val-Phe-OH (**16**), was not detected by RP-HPLC analysis in any of the crude reaction mixtures and, therefore, it is safe to assume that the activated α-carboxyl group reacts faster than the acylimidazole moiety, leading exclusively to the formation of the desired amide bond.

Table 2. Product distribution in the synthesis of the peptide H-Ala-Ser-Val-His-Val-Phe-OH (**I**) with the histidine derivatives **1**−**3**

Histidine protection (R)	Product distribution [%][a][b] **I**	$\mathbf{I}_{D\text{-His}}$	**II**	**12**	Others[c]
Unprotected	42	2.2	54	−	2.1
τ-2,6-Dmb (**1**)	94	< 0.5	1.3	< 0.5	3.9
π-2,6-Dmb (**2**)	72	n.d.[d]	1.1	25	2.1
τ-Trt (**3**)	93	0.1	2.5	−	4.1

[a] Values were determined from the integration of the peak areas at 210 nm of the corresponding UV signals; the areas of the peaks corresponding to the amide and the acid of 2,6-Dmbz were not included in the calculation. [b] Compound labeling is as follow: **I** = H-Ala-Ser-Val-His-Val-Phe-OH, $\mathbf{I}_{D\text{-His}}$ = H-Ala-Ser-Val-D-His-Val-Phe-OH, **II** = H-Ala-Ser-Val-Val-Phe-OH, **12** = 2,6-Dmbz-His-Val-Phe-OH. [c] Peaks originating from unidentified compounds. [d] n.d.: not detected.

Conclusion

The results presented in this paper show that the 2,6-dimethoxybenzoyl group located on the τ-nitrogen atom can be used successfully as protecting group for the imidazole function of histidine in the synthesis of peptides, both in solution and on a solid-support following the Fmoc-based strategy. The coupling efficiencies were high and the racemization levels were low and similar to those obtained from commercially available Fmoc-His(Trt)-OH. On the other hand, the π-blocked histidine derivative, although leading to a very low degree of racemization, suffered to a large extent from intramolecular N^{α}-acylation and is, consequently, not suitable for standard Fmoc-based chemistry. These findings provide the basis for further evaluation of the 2,6-dimethoxybenzoyl group for imidazole protection in the synthesis of oligonucleotide−peptide conjugates.

Experimental Section

General Remarks: Solvents were purchased from Merck Eurolab. DIEA (Fluka) and piperidine (Fluka) were refluxed over CaH_2, distilled at atmospheric pressure, and stored over CaH_2 pellets. DBU (Fluka) was distilled in vacuo. 2,6-Dimethoxybenzoyl chloride (Aldrich) was distilled under reduced pressure and stored at 5 °C in presence of a desiccant. HBTU, HOBt, and all amino acid derivatives, were purchased from Novabiochem, except for Fmoc-His-OH (Bachem). Amino acid derivatives were dried in vacuo in the presence of P_2O_5. The automated peptide synthesis was performed on an Applied Biosystems 433A synthesizer with custom-modified chemistry protocols. Solvents and ancillary reagents for SPPS were of peptide-synthesis grade (Applied Biosystems); DMF, NMP, and CH_2Cl_2 were stored under nitrogen over 4-Å molecular

sieves activated by heating over a free flame and cooled in vacuo. Concentration of solutions was generally carried out by evaporation under reduced pressure at a water-bath temperature not exceeding 40 °C. All glassware used during syntheses was dried in an oven at 120 °C prior to use. The glassware was assembled while hot and cooled under a flow of dry nitrogen. All moisture-sensitive reactions were carried out under dry nitrogen. NMR spectra were recorded on a Bruker Avance DRX 400 spectrometer at 400 and 100 MHz for ^{1}H and ^{13}C, respectively. ^{1}H and ^{13}C NMR spectroscopic chemical shifts are reported in ppm downfield from tetramethylsilane and were determined using residual non-deuterated solvent as an internal standard. High-resolution mass spectrometry (HRMS) was performed on a Micromass LCT (ES-TOF) mass spectrometer in positive-ion mode, using leucine enkephalin as an internal mass standard. Reverse-phase HPLC analysis was carried out on a Jasco HPLC system equipped with a Thermo Hypersil C18 analytical column (5 μm, 250 × 4.6 mm). Two different linear gradients of 0.1% aq. TFA (eluent A) and 0.1% TFA in CH_3CN (eluent B) were used with a flow rate of 1.0 mL/min at 30 °C. Gradient 1 (for the analysis of the dipeptides): 16% B for 2 min hold, then 16−80% B over 40 min. Gradient 2 (for the analysis of the hexapeptides): 10% B for 2 min hold, then 10−50% B over 40 min. To separate the DL- and LL-dipeptides, a linear gradient (gradient 3: 40% B for 2 min hold, then 40−50% B in 60 min) of 0.05% aq. TFA (eluent A) and 0.05% TFA in CH_3CN (eluent B) was applied with a flow rate of 1.5 mL/min at 50 °C. UV detection was carried out at 260 and 210 nm for the dipeptides and hexapeptides, respectively. Thin-layer chromatography (TLC) was performed on analytical Merck silica gel plates with F_{254} indicator. Two mobile phase systems were used: system A = $CHCl_3$/MeOH/HOAc, 90:8:2 (v/v/v) and system B = $CHCl_3$/MeOH, 9:1 (v/v). Visualization of the spots was accomplished by UV-shadowing (254 nm), by charring with methanolic 10% sulfuric acid in the case of trityl-containing compounds, by staining with a solution of 0.05% ninhydrin in 4% HOAc/*n*BuOH, or by the chlorine/*o*-tolidine test where appropriate.

Fmoc-His(τ-2,6-Dmbz)-OH (1): Fmoc-His-OH (1.89 g, 5.0 mmol) was dissolved in anhydrous DMF (10 mL) and DIEA (1.9 mL, 11 mmol). The mixture was cooled in an ice-bath and a solution of 2,6-dimethoxybenzoyl chloride (1.10 g, 5.5 mmol) in anhydrous DMF (2.5 mL) was added dropwise over a period of 30 min. The mixture was stirred for 4 h at room temperature. DMF was evaporated in vacuo and the resulting thick yellow oil was poured into a mixture of EtOAc (30 mL) and ice-chilled 10% citric acid (30 mL). The aqueous phase was separated and extracted with EtOAc (2 × 20 mL). The combined organic phases were washed with water (2 × 20 mL) and brine (20 mL), before being dried with Na_2SO_4, filtered, and concentrated under reduced pressure. The residual solution was slowly poured into *n*-hexane under vigorous stirring, which caused the precipitation of crude compound **1**. The residue was then collected by filtration and crystallized from EtOAc/*n*-hexane. White solid (2.22 g, 82%). RP-HPLC (gradient 1): t_R = 21.0 min. TLC (system A): R_f = 0.34. ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.85 (dd, J' = 14.9, J'' = 10.0 Hz, 1 H, C_β−H), 2.96 (dd, J' = 14.9, J'' = 4.2 Hz, 1 H, C_β−H), 3.71 (s, 6 H, OCH_3), 4.19−4.29 (m, 4 H, OCH_2CH + C_α−H), 6.82 (d, J = 8.5 Hz, 2 H, Ar−H), 7.29−7.34 (m, 3 H, Fmoc + im), 7.42 (t, J = 7.5 Hz, 2 H, Fmoc), 7.51 (t, J = 8.5 Hz, 1 H, Ar−H), 7.66−7.71 (m, 3 H, Fmoc + im), 7.87 (br., 1 H, NH), 7.90 (d, J = 7.5 Hz, 2 H, Fmoc) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 30.3, 47.4, 54.1, 56.9, 66.5, 105.4, 111.8, 114.4, 121.0, 126.1, 128.0, 128.5, 133.9, 138.3, 141.2, 141.5, 144.6, 156.8, 157.9, 163.5, 174.1 ppm. HRMS (ES-TOF): *m*/*z* calcd. for $C_{30}H_{28}N_3O_7$ [M + H]$^+$: 542.1927; found 542.1925.

Fmoc-His(π-2,6-Dmbz)-OH (2): Chlorotrimethylsilane (1.35 mL, 10.5 mmol) was slowly added to an ice-chilled solution of Fmoc-His-OH (1.89 g, 5.0 mmol) and DIEA (2.0 mL, 12 mmol) in anhydrous CH_2Cl_2 (5.0 mL) and the resulting clear solution was stirred at room temperature for 15 min. DIEA (1.0 mL, 6.0 mmol) was added and the mixture was again cooled with an ice-bath and a solution of 2,6-dimethoxybenzoyl chloride (1.10 g, 5.5 mmol) in dry CH_2Cl_2 (5.0 mL) was slowly added with stirring over a period of 30 min. Stirring was continued at room temperature for 6 h. CH_2Cl_2 (20 mL) was added and the reaction mixture was washed with ice-chilled 10% citric acid (40 mL). The resulting precipitate was filtered off and set aside. The organic phase was further washed with water (3 × 30 mL), dried with Na_2SO_4, filtered, and the solvents evaporated to dryness to yield a yellowish residue. Trituration with EtOAc/*n*-hexane afforded a white powder. NMR spectroscopic analysis of this residue showed it to be identical to the τ-blocked histidine derivative **1** (1.88 g). The precipitate from the acidic extraction (0.95 g) was suspended in boiling EtOAc and the slurry filtered while hot. The insoluble residue mostly contained Fmoc-His-OH. A white residue precipitated upon cooling of the warm filtrate, which was identified by NMR spectroscopy as the π-protected derivative **2**. White powder (0.35 g, 13%). RP-HPLC (gradient 1): t_R = 18.8 min. TLC (system A): R_f = 0.34. ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 3.15 (dd, J' = 15.7, J'' = 10.8 Hz, 1 H, C_β−H), 3.44 (dd, J' = 15.7, J'' = 3.8 Hz, 1 H, C_β−H), 3.71 (s, 6 H, OCH_3), 4.18−4.27 (m, 3 H, OCH_2CH), 4.33 (ddd, J' = 10.3, J'' = 8.6, J''' = 3.9 Hz, 1 H, C_α−H), 6.80 (d, J = 8.5 Hz, 2 H, Ar−H), 6.87 (s, 1 H, im), 7.27−7.33 (m, 2 H, Fmoc), 7.38−7.42 (m, 2 H, Fmoc), 7.50 (t, J = 8.5 Hz, 1 H, Ar−H), 7.54 (s, 1 H, im), 7.66 (t, J = 7.9 Hz, 2 H, Fmoc), 7.80 (d, J = 8.5 Hz, 1 H, NH), 7.88 (d, J = 7.5 Hz, 2 H, Fmoc) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 28.4, 47.4, 53.3, 56.9, 66.5, 105.4, 112.8, 121.0, 126.1, 128.0, 128.5, 129.4, 131.5, 133.8, 140.6, 141.5, 144.6, 156.8, 157.7, 165.4, 173.9 ppm. HRMS (ES-TOF): *m*/*z* calcd. for $C_{30}H_{28}N_3O_7$ [M + H]$^+$: 542.1927; found 542.1932.

Coupling Tests: The carboxyl component (0.5 mmol), HBTU (0.5 mmol), and HOBt (0.5 mmol) were weighed in a vial bearing a septum and then DMF (2.5 mL) was added. DIEA (1.5 mmol) was injected and the mixture was reacted for 70 s at room temperature. The solution was taken up with a syringe and transferred to a sealed vial containing the amino component as the hydrochloride salt (0.5 mmol). The coupling was carried out at room temperature for 2 h. Aliquots (5 μL) were withdrawn at time intervals, diluted in starting buffer (1.0 mL) and subjected to RP-HPLC analysis with gradient 1. Coupling yields at a given time were calculated as 100 × [% peak area dipeptide/(% peak area dipeptide + % peak area monomer)]. DMF was evaporated under reduced pressure and the thick oil obtained was dissolved in EtOAc (15 mL) and washed with 10% citric acid (15 mL), 5% $NaHCO_3$ (15 mL), H_2O (15 mL), and brine (15 mL), and then the solution was dried with Na_2SO_4, filtered, and the solvents evaporated to dryness. Trituration of the residue with EtOAc/*n*-hexane afforded the pure fully protected dipeptide as an amorphous white solid.

Fmoc-His(τ-2,6-Dmbz)-Val-OMe (4): White solid (308 mg, 94%). RP-HPLC (gradient 1): t_R = 25.4 min. TLC (system A): R_f = 0.61. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.85 (d, J = 5.8 Hz, 6 H, C_γ^{Val}−H), 2.13−2.15 (m, 1 H, C_β^{Val}−H), 3.03 (dd, J' = 15.0, J'' = 6.1 Hz, 1 H, C_β^{His}−H), 3.18 (dd, J' = 15.0, J'' = 4.7 Hz, 1 H, C_β^{His}−H), 3.71 (s, 3 H, $COOCH_3$), 3.78 (s, 6 H, OCH_3), 4.27 (br. t, J = 7.2 Hz, 1 H, OCH_2CH), 4.39 (d, J = 7.3 Hz, 2 H, OCH_2CH),

4.50 (dd, $J' = 8.6$, $J'' = 4.9$ Hz, 1 H, C_α^{Val}−H), 4.58−4.63 (br. m, 1 H, C_α^{His}−H), 6.65 (d, $J = 8.5$ Hz, 2 H, Ar−H), 6.69 (d, $J = 7.2$ Hz, 1 H, NH), 7.27 (br. s, 1 H, im), 7.32 (br. t, $J = 7.4$ Hz, 2 H, Fmoc), 7.42 (br. t, $J = 7.4$ Hz, 2 H, Fmoc), 7.45 (t, $J = 8.5$ Hz, 1 H, Ar−H), 7.57−7.64 (br. m, 3 H, Fmoc + NH), 7.78 (d, $J = 7.4$ Hz, 2 H, Fmoc), 7.87 (br. s, 1 H, im) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 18.0, 19.2, 30.8, 31.4, 47.5, 52.5, 54.9, 56.4, 57.8, 67.7, 104.4, 111.7, 114.8, 120.4, 125.6, 127.5, 128.1, 133.4, 137.6, 140.1, 141.7, 144.2, 156.6, 158.1, 163.3, 171.4, 172.4 ppm. HRMS (ES-TOF): *m*/*z* calcd. for $C_{36}H_{39}N_4O_8$ $[M + H]^+$: 655.2768; found 655.2771.

Fmoc-His(π-2,6-Dmbz)-Val-OMe (5): White solid (295 mg, 90%). RP-HPLC (gradient 1): $t_R = 23.9$ min. TLC (system A): $R_f = 0.57$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.93−0.97 (m, 6 H, C_γ^{Val}−H), 2.15−2.25 (m, 1 H, C_β^{Val}−H), 3.23 (dd, $J' = 14.9$, $J'' = 9.6$ Hz, 1 H, C_β^{His}−H), 3.68 (dd, $J' = 15.1$, $J'' = 3.6$ Hz, 1 H, C_β^{His}−H), 3.74 (s, 3 H, $COOCH_3$), 3.78 (s, 6 H, OCH_3), 4.21 (t, $J = 7.3$ Hz, 1 H, OC*H*CH_2), 4.27−4.32 (m, 2 H, OCHC*H*$_2$), 4.54 (dd, $J' = 8.5$, $J'' = 5.0$ Hz, 1 H, C_α^{Val}−H), 4.65−4.70 (m, 1 H, C_α^{His}−H), 5.73 (d, $J = 8.2$ Hz, 1 H, NH), 6.63 (d, $J = 8.4$ Hz, 2 H, Ar−H), 6.80 (d, $J = 8.6$ Hz, 1 H, NH), 6.93 (s, 1 H, im), 7.26−7.31 (br. m, 2 H, Fmoc), 7.39 (t, $J = 7.5$ Hz, 2 H, Fmoc), 7.44 (t, $J = 8.5$ Hz, 1 H, Ar−H), 7.54−7.57 (br. m, 3 H, Fmoc + im), 7.76 (d, $J = 7.5$ Hz, 1 H, Fmoc) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 18.2, 19.4, 31.5, 32.0, 47.4, 52.6, 54.7, 56.5, 57.8, 67.6, 104.4, 112.8, 120.3, 125.6, 127.5, 128.1, 128.6, 131.9, 133.3, 141.0, 141.6, 144.2, 156.5, 158.0, 165.9, 171.6, 172.4 ppm. HRMS (ES-TOF): *m*/*z* calcd. for $C_{36}H_{39}N_4O_8$ $[M + H]^+$: 655.2768; found 655.2769.

Fmoc-His(τ-Trt)-Val-OMe (6): White solid (348 mg, 92%). RP-HPLC (gradient 1): $t_R = 29.5$ min. RP-HPLC (gradient 3): $t_R = 48.0$ min. TLC (system A): $R_f = 0.55$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.88 (d, $J = 6.6$ Hz, 6 H, C_γ^{Val}−H), 2.14−2.19 (m, 1 H, C_β^{Val}−H), 3.03 (dd, $J' = 15.0$, $J'' = 6.3$ Hz, 1 H, C_β^{His}−H), 3.13 (dd, $J' = 15.0$, $J'' = 4.9$ Hz, 1 H, C_β^{His}−H), 3.68 (s, 3 H, $COOCH_3$), 4.24−4.28 (br. m, 1 H, OCH_2CH), 4.33−4.41 (m, 2 H, OCH_2CH), 4.51 (dd, $J' = 8.3$, $J'' = 4.9$ Hz, 1 H, C_α^{Val}−H), 4.61 (dd, $J' = 11.9$, $J'' = 6.3$ Hz, 1 H, C_α^{His}−H), 6.72 (br. s, 1 H, im), 6.90 (br. s, $J = 6.6$ Hz, 1 H, NH), 7.11−7.13 (m, 6 H, Trt), 7.28−7.35 (m, 11 H, Trt + Fmoc), 7.39−7.43 (m, 3 H, Fmoc + im), 7.64 (br. d, $J = 7.0$ Hz, 2 H, Fmoc), 7.78 (d, $J = 7.5$ Hz, 2 H, Fmoc), 7.84 (br. d, $J = 8.0$ Hz, 1 H, NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 18.3, 19.5, 31.0, 31.5, 47.5, 52.4, 55.3, 57.9, 67.7, 75.8, 119.9, 120.3, 125.7, 127.5, 128.1, 128.5, 130.2, 137.4, 138.8, 141.6, 142.7, 144.3, 144.4, 156.7, 171.8, 172.5 ppm. HRMS (ES-TOF): *m*/*z* calcd. for $C_{46}H_{45}N_4O_5$ $[M + H]^+$: 733.3390; found 733.3391.

Fmoc-D-His(τ-Trt)-L-Val-OMe (7): Fmoc-D-His(τ-Trt)-OH (620 mg, 1.0 mmol) and HBTU (380 mg, 1.0 mmol) were weighed in a vial bearing a septum and then DMF (2.5 mL) was added. DIEA (190 μL, 1.1 mmol) was injected and the mixture was reacted for 1 min at room temperature. The solution was taken up with a syringe and transferred into a sealed vial containing HCl·H-L-Val-OMe (183 mg, 1.1 mmol) and DIEA (190 μL, 1.1 mmol) in DMF (1.0 mL). The coupling reaction was carried out at room temperature for 2 h. DMF was evaporated in vacuo and the oily residue was partitioned between EtOAc (12 mL) and 10% citric acid (10 mL). The organic phase was washed with H_2O (10 mL), brine (10 mL), 5% $NaHCO_3$ (15 mL), H_2O (15 mL), and brine (15 mL), dried with Na_2SO_4 and the solvents evaporated to dryness. The oily residue was chromatographed on a silica-gel column (75 × 30 mm) with a mobile phase of EtOAc/*n*-hexane, 1:1 (v/v). The fractions containing the product were concentrated to yield a white powder (509 mg, 70%). RP-HPLC (gradient 3): $t_R = 46.7$ min. TLC (system A): $R_f = 0.53$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.87 (d, $J = 6.9$ Hz, 3 H, C_γ^{Val}−H), 0.92 (d, $J = 7.0$ Hz, 3 H, C_γ^{Val}−H), 2.16−2.20 (m, 1 H, C_β^{Val}−H), 3.03 (dd, $J' = 15.0$, $J'' = 6.4$ Hz, 1 H, C_β^{His}−H), 3.11 (dd, $J' = 14.9$, $J'' = 5.1$ Hz, 1 H, C_β^{His}−H), 3.68 (s, 3 H, $COOCH_3$), 4.25−4.26 (br. m, 1 H, OC*H*$_2$CH), 4.32−4.36 (br. m, 2 H, OCH_2C*H*), 4.55 (dd, $J' = 8.6$, $J'' = 4.9$ Hz, 1 H, C_α^{Val}−H), 4.59−4.62 (m, 1 H, C_α^{His}−H), 6.71 (s, 1 H, im), 6.86 (br. d, $J = 5.0$ Hz, 1 H, NH), 7.11−7.14 (m, 6 H, Trt), 7.30−7.34 (m, 11 H, Trt + Fmoc), 7.40 (t, $J = 7.4$ Hz, 2 H, Fmoc), 7.45 (s, 1 H, im), 7.63 (br. d, $J = 7.1$ Hz, 2 H, Fmoc), 7.77 (d + br., $J = 7.5$ Hz, 3 H, Fmoc + NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 18.2, 19.4, 31.0, 31.7, 47.5, 52.5, 55.5, 57.7, 67.6, 75.8, 119.9, 120.4, 125.7, 127.5, 128.1, 128.5, 130.2, 137.1, 138.8, 141.7, 142.7, 144.3, 156.7, 171.7, 172.5 ppm. HRMS (ES-TOF): *m*/*z* calcd. for $C_{46}H_{45}N_4O_5$ $[M + H]^+$: 733.3390; found 733.3383.

Fmoc-D-His-L-Val-OMe (8): Dipeptide **7** (220 mg, 0.3 mmol) was dissolved in TFA/H_2O (95:5, v/v; 2.0 mL) and stirred at room temperature for 1 h. The solvents were evaporated under a flow of nitrogen, and the residual oil was partitioned between EtOAc (10 mL) and 5% $NaHCO_3$ (15 mL), the aqueous layer was extracted with EtOAc (2 × 10 mL) and the organic layers were pooled and dried over Na_2SO_4. After filtration, the organic phase was concentrated in vacuo until pure compound **8** separated as a crystalline material, which was collected by filtration. White powder (134 mg, 91%). TLC (system A): $R_f = 0.19$. ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.79 (d, $J = 6.7$ Hz, 3 H, C_γ^{Val}−H), 0.80 (d, $J = 6.7$ Hz, 3 H, C_γ^{Val}−H), 1.96−2.04 (m, 1 H, C_β^{Val}−H), 2.81 (dd, $J' = 14.7$, $J'' = 8.9$ Hz, 1 H, C_β^{His}−H), 2.91 (dd, $J' = 14.7$, $J'' = 5.4$ Hz, 1 H, C_β^{His}−H), 3.63 (s, 3 H, $COOCH_3$), 4.17−4.27 (m, 4 H, OCH_2CH + C_α^{Val}−H), 4.39−4.42 (m, 1 H, C_α^{His}−H), 6.83 (s, 1 H, im), 7.32 (t, $J = 7.4$ Hz, 2 H, Fmoc), 7.42 (t, $J = 7.5$ Hz, 2 H, Fmoc), 7.57 (d, $J = 8.5$ Hz, 1 H, NH), 7.61 (s, 1 H, im), 7.67−7.70 (m, 2 H, Fmoc), 7.89 (d, $J = 7.5$ Hz, 2 H, Fmoc), 8.18 (d, $J = 8.5$ Hz, 2 H, NH) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 18.9, 19.7, 31.0, 47.4, 52.6, 55.3, 58.0, 66.6, 121.0, 126.1, 126.2, 128.0, 128.5, 135.5, 141.5, 144.6, 156.5, 172.5, 172.8 ppm. HRMS (ES-TOF): *m*/*z* calcd. for $C_{27}H_{31}N_4O_5$ $[M + H]^+$: 491.2294; found 491.2299.

Fmoc-D-His(τ-2,6-Dmbz)-L-Val-OMe (9) and Fmoc-D-His(π-2,6-Dmbz)-L-Val-OMe (10): Compound **8** (98 mg, 0.20 mmol) was suspended in dry CH_2Cl_2 (3 mL) in the presence of DIEA (38 μL, 0.22 mmol). 2,6-Dimethoxybenzoyl chloride (44 mg, 0.22 mmol) was added and the mixture was stirred at room temperature for 1 h, during which time the residual solid dissolved. The solution was diluted with CH_2Cl_2 (2 mL) and washed with 10% citric acid (5 mL), 5% $NaHCO_3$ (5 mL), and brine (5 mL). After standing over Na_2SO_4, the organic phase was filtered and the solvents were evaporated to dryness. The resulting white foam (119 mg) contained a mixture of **9** and **10** in approximately 7:2 molar ratio, according to RP-HPLC analysis. The residue was chromatographed on a silica-gel column (120 × 23 mm) with the mobile phase being EtOAc/*n*-hexane, 2:1 (v/v). The fractions containing **9** (46 mg) and the fractions containing a mixture of **9** and **10** (ca. 1:6 molar ratio; 44 mg) were collected and concentrated to yield white solid materials.

Fmoc-D-His(τ-2,6-Dmbz)-L-Val-OMe (9): RP-HPLC (gradient 3): $t_R = 36.9$ min. TLC (system A): $R_f = 0.61$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.85 (d, $J = 7.2$ Hz, 3 H, C_γ^{Val}−H), 0.89 (d, $J = 6.6$ Hz, 3 H, C_γ^{Val}−H), 2.12−2.16 (br. m, 1 H, C_β^{Val}−H), 3.01−3.14 (br. m, 2 H, C_β^{His}−H), 3.71 (s, 3 H, $COOCH_3$), 3.77 (s, 6 H, OCH_3), 4.25 (t, $J = 7.2$ Hz, 1 H, OCH_2C*H*), 4.38 (d, $J =$

7.2 Hz, 2 H, OCH_2CH), 4.52 (dd, J' = 8.6, J'' = 5.0 Hz, 1 H, C_α^{Val}−H), 4.59−4.64 (m, 1 H, C_α^{His}−H), 6.46 (br., 1 H, NH), 6.64 (d, J = 8.5 Hz, 2 H, Ar−H), 7.28−7.33 (m, 3 H, Fmoc + im), 7.39−7.47 (m, 4 H, Fmoc + Ar−H + NH), 7.62 (d, J = 7.4 Hz, 2 H, Fmoc), 7.77 (d, J = 7.5 Hz, 2 H, Fmoc), 7.84 (br. s, 1 H, im) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 18.1, 19.3, 31.2, 31.5, 47.5, 52.5, 55.0, 56.4, 57.7, 67.7, 104.4, 111.7, 114.6, 120.4, 125.6, 127.5, 128.1, 133.4, 137.8, 140.0, 141.7, 144.2, 156.6, 158.1, 163.3, 171.4, 172.4 ppm. HRMS (ES-TOF): *m/z* calcd. for $C_{36}H_{39}N_4O_8$ $[M + H]^+$: 655.2768; found 655.2762.

Fmoc-D-His(π-2,6-Dmbz)-L-Val-OMe (10): RP-HPLC (gradient 3): t_R = 28.9 min. TLC (system A): R_f = 0.57. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 0.83−0.86 (m, 6 H, C_γ^{Val}−H), 2.01−2.09 (m, 1 H, C_β^{Val}−H), 3.18 (dd, J' = 16.4, J'' = 9.7 Hz, 1 H, C_β^{His}−H), 3.31 (dd, J' = 16.4, J'' = 4.0 Hz, 1 H, C_β^{His}−H), 3.65 (s, 3 H, $COOCH_3$), 3.74 (s, 6 H, OCH_3), 4.23−4.33 (m, 4 H, $OCHCH_2$ + C_α^{Val}−H), 4.56−4.61 (m, 1 H, C_α^{His}−H), 6.82 (d, J = 8.5 Hz, 2 H, Ar−H), 6.88 (s, 1 H, im), 7.29−7.33 (m, 2 H, Fmoc), 7.39−7.44 (m, 2 H, Fmoc), 7.51 (t, J = 8.5 Hz, 1 H, Ar−H), 7.57 (s, 1 H, Ar−H), 7.68−7.72 (m, 2 H, Fmoc), 7.79 (d, J = 7.5 Hz, 1 H, NH), 7.89 (d, J = 7.5 Hz, 2 H, Fmoc), 8.17 (d, J = 8.4 Hz, 2 H, NH) ppm. ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 18.9, 19.8, 30.1, 31.0, 47.4, 52.6, 53.7, 56.9, 58.1, 66.7, 105.4, 112.8, 121.0, 126.2, 127.9, 128.5, 129.8, 130.4, 133.9, 140.4, 141.5, 144.6, 156.7, 157.7, 165.4, 172.2, 172.7 ppm. HRMS (ES-TOF): *m/z* calcd. for $C_{36}H_{39}N_4O_8$ $[M + H]^+$: 655.2768; found 655.2766.

2,6-Dmbz-Val-OMe (11): HCl·H-Val-OMe (84 mg, 0.5 mmol) was dissolved in a mixture of dry CH_2Cl_2 (0.25 mL) and DIEA (0.18 mL, 1.0 mmol). A solution of 2,6-dimethoxybenzoyl chloride (99 mg, 0.5 mmol) in dry CH_2Cl_2 (0.25 mL) was poured into the stirred solution. The mixture was reacted at room temperature for 2 h. The solvent was evaporated under reduced pressure and the residual oil partitioned between EtOAc (5 mL) and 1 M $KHSO_4$ (5 mL). The organic phase was washed with H_2O (3 × 10 mL), 5% $NaHCO_3$ (10 mL), H_2O (10 mL), and brine (10 mL). After being dried with Na_2SO_4, the organic phase was filtered and the solvent was evaporated in vacuo. Crude compound **11** was crystallized from CH_2Cl_2/*n*-hexane to give colorless needle-like crystals, which were collected by filtration (113 mg, 77%). RP-HPLC (gradient 1): t_R = 13.3 min. TLC (system B): R_f = 0.64. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.96 (d, J = 6.9 Hz, 3 H, C_γ−H), 1.06 (d, J = 6.9 Hz, 3 H, C_γ−H), 2.22−2.34 (m, 1 H, C_β−H), 3.77 (s, 3 H, $COOCH_3$), 3.82 (s, 6 H, OCH_3), 4.86 (dd, J' = 9.1, J'' = 4.7 Hz, 1 H, C_α−H), 6.31 (br. d, J = 8.8 Hz, 1 H, NH), 6.57 (d, J = 8.4 Hz, 2 H, Ar−H), 7.29 (t, J = 8.4 Hz, 1 H, Ar−H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 18.1, 19.2, 32.2, 52.5, 56.3, 57.4, 104.4, 115.9, 131.2, 157.9, 165.6, 172.8 ppm. HRMS (ES-TOF): *m/z* calcd. for $C_{15}H_{22}NO_5$ $[M + H]^+$: 296.1498; found 296.1498.

Automated Solid-Phase Synthesis of H-Ala-Ser-Val-His-Val-Phe-OH (I): SPPS was performed on a 0.01-mmol scale following an Fmoc-chemistry protocol. Fmoc-Phe-preloaded 4-(hydroxymethyl)phenoxy-functionalized resin (Wang resin) (Novabiochem, initial loading of 0.62 mmol/g) was used as a support. Cartridges loaded with the Fmoc-amino acids were left overnight under high vacuum in the presence of P_2O_5, and were sealed under a flow of dry nitrogen. The β-hydroxyl group of serine was protected as its *t*Bu ether. The coupling reaction was conducted for 9 min with 20-fold molar excess of monomer with respect to the peptide resin; the molar excesses of HBTU, HOBt, and DIEA were 20-, 20-, and 40-fold, respectively. Fmoc-group removal was performed with 2% DBU/2% piperidine in NMP.[24] After the synthesis had been completed, the peptide resin was dried in vacuo overnight and transferred into a sealed vial for side-chain deprotection of Ser(*t*Bu) and His(Trt) and cleavage from the solid support, which were carried out with TFA/H_2O/TIS (95:2.5:2.5, v/v/v; 0.5 mL) at room temperature for 4 h. The filtrate from the cleavage mixture was partially concentrated under a stream of dry nitrogen, and the peptide was precipitated by addition of anhydrous Et_2O, followed by centrifugation and decanting of the supernatants. The addition of Et_2O, centrifugation and decanting was repeated three times. To compare rates of cleavage, the peptides assembled with **1** and **2** were further subjected to a 20-h treatment at room temperature with either 32% aq. ammonia/dioxane (1:1, v/v) or 32% aq. ammonia/ethanol (3:1, v/v). The ammonolysis mixtures were diluted with water and freeze-dried. The residues were then dissolved in 0.1% TFA in CH_3CN/H_2O (1:9, v/v) and analyzed by RP-HPLC with gradient 2.

2,6-Dmbz-His-Val-Phe-OH (12): The tripeptide His-Val-Phe was assembled on a solid support following the protocol described above for the hexapeptide **I**. The peptide resin (0.01 mmol) was swelled in dry DMF (0.25 mL) for 15 min, after which time it was reacted with 2,6-dimethoxybenzoyl chloride (22 mg, 0.11 mmol) in the presence of TEA (0.03 mL, 0.21 mmol) at room temperature for 1 h with occasional swirling. The peptide resin was collected on a filter, thoroughly washed with DMF and CH_2Cl_2, and dried in vacuo overnight. The resin was treated with TFA/H_2O/TIS (95:2.5:2.5, v/v/v; 0.25 mL) at room temperature for 2 h with occasional swirling. The slurry was filtered and the resin was washed with TFA. The filtrate was concentrated by evaporation under a flow of nitrogen, the resulting residue was triturated with diethyl ether and kept at 5 °C for 2 h. The title compound **12**, obtained as a white solid, was separated by centrifugation, dissolved in water, and freeze-dried. RP-HPLC (gradient 2) t_R = 20.4 min. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 0.82 (d, J = 6.7 Hz, 3 H, C_γ^{Val}−H), 0.88 (d, J = 6.7 Hz, 3 H, C_γ^{Val}−H), 1.95−2.00 (m, 1 H, C_β^{Val}−H), 2.89−2.97 (m, 2 H, C_β^{Phe}−H), 3.06 (dd, J' = 14.1, J'' = 5.5 Hz, 1 H, C_β^{His}−H), 3.22 (br. d, J = 15.0 Hz, 1 H, C_β^{His}−H), 3.70 (s, 6 H, OCH_3), 4.35−4.39 (m, 1 H, C_α^{Val}−H), 4.41−4.47 (m, 1 H, C_α^{Phe}−H), 4.65−4.70 (m, 1 H, C_α^{His}−H), 6.67 (d, J = 8.4 Hz, 2 H, Ar−H), 7.20−7.33 (m, 8 H, NH + Ar−H + Ph + im), 8.47 (d, J = 7.6 Hz, 1 H, NH), 8.63 (d, J = 8.4 Hz, 1 H, NH), 8.76 (br. s, 1 H, im) ppm. ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 18.2, 19.8, 27.3, 32.4, 37.3, 52.7, 54.4, 56.6, 57.6, 104.9, 116.5, 117.8, 124.1, 127.3, 129.1, 129.9, 131.3, 134.6, 138.3, 157.4, 165.7, 170.3, 171.4, 173.6 ppm. HRMS (ES-TOF): *m/z* calcd. for $C_{29}H_{36}N_5O_7$ $[M + H]^+$: 566.2614; found 566.2618.

Boc-Val-Phe-OMe (13): Boc-Val-OH (216 mg, 1.0 mmol) and HCl·H-Phe-OMe (221 mg, 1.0 mmol) were dissolved in a mixture of THF/CH_3CN (5:2, v/v; 7 mL) and condensed with the aid of HBTU (379 mg, 1.0 mmol) and DIEA (0.52 mL, 3.0 mmol) over 3 h at room temperature. The solvent was evaporated and the residual oil was partitioned between EtOAc (20 mL) and ice-chilled 10% citric acid (10 mL). The organic phase was washed with H_2O (2 × 10 mL), brine (10 mL), 5% $NaHCO_3$ (10 mL), H_2O (10 mL), and brine (10 mL). After being dried with Na_2SO_4, the organic phase was filtered and concentrated under reduced pressure. Crystallization of the crude solid residue from EtOAc/*n*-hexane afforded pure dipeptide **13** as a white crystalline powder (335 mg, 89%). TLC (system B): R_f = 0.76. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.88 (br. d, J = 6.3 Hz, 3 H, Cγ^{Val}−H), 0.94 (d, J = 6.8 Hz, 3 H, Cγ^{Val}−H), 1.47 (s, 9 H, Boc *t*Bu), 2.07−2.13 (m, 1 H, C_β^{Val}−H), 3.09−3.19 (m, 2 H, C_β^{Phe}−H), 3.73 (s, 3 H, $COOCH_3$), 3.90−3.94 (br. m, 1 H, C_α^{Val}−H), 4.87−4.92 (m, 1 H, C_α^{Phe}−H), 5.02 (br. d, J = 8.1 Hz, 1 H, NH), 6.30 (br. d, J = 6.6 Hz, 1 H, NH), 7.12−7.14 (m, 2 H, Ph), 7.26−7.33 (m, 3 H, Ph) ppm. ^{13}C NMR (100 MHz,

$CDCl_3$): δ = 18.1, 19.6, 28.7, 31.3, 38.4, 52.7, 53.5, 60.3, 80.3, 127.6, 129.1, 129.6, 136.0, 156.1, 171.6, 172.1 ppm. HRMS (ES-TOF): *m/z* calcd. for $C_{20}H_{31}N_2O_5$ $[M + H]^+$: 379.2233; found 379.2232.

H-Val-Phe-OMe (14): Dipeptide **13** (335 mg, 0.89 mmol) was dissolved in CH_2Cl_2 (2.0 mL) and MeOH (0.2 mL). TFA (2.0 mL) was added and the mixture was stirred for 2 h at room temperature. The solvent was evaporated under a flow of nitrogen and the residual oil was triturated with *n*-hexane/Et_2O. Compound **14** was collected by filtration as a white solid (282 mg, 81%) and used without further purification. TLC (system A): R_f = 0.14. ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.91 (d, *J* = 6.9 Hz, 3 H, $C\gamma^{Val}$−H), 0.96 (d, *J* = 6.9 Hz, 3 H, $C\gamma^{Val}$−H), 2.08−2.16 (m, 1 H, C_β^{Val}−H), 2.98 (dd, *J′* = 14.0, *J″* = 8.6 Hz, 1 H, C_β^{Phe}−H), 3.08 (dd, *J′* = 14.0, *J″* = 5.7 Hz, 1 H, C_β^{Phe}−H), 3.60 (s, 3 H, $COOCH_3$), 3.63 (d, *J* = 5.1 Hz, 1 H, C_α^{Val}−H), 4.54−4.59 (m, 1 H, C_α^{Phe}−H), 7.23−7.33 (m, 5 H, Ph), 8.05 (br., 2 H, Val NH_2), 8.87 (d, *J* = 7.2 Hz, 1 H, Phe NH) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 17.9, 19.2, 30.7, 37.2, 52.8, 54.7, 57.9, 127.6, 129.3, 129.9, 137.6, 196.2, 172.2 ppm. HRMS (ES-TOF): *m/z* calcd. for $C_{15}H_{23}N_2O_3$ $[M + H]^+$: 279.1709; found 279.1709.

2,6-Dmbz-Val-Phe-OMe (15): Dipeptide **14** (196 mg, 0.50 mmol) was suspended in anhydrous CH_2Cl_2 (1.0 mL) and DIEA (0.2 mL, 1.10 mmol) and the mixture was cooled in an ice-bath. A solution of 2,6-dimethoxybenzoyl chloride (106 mg, 0.53 mmol) in anhydrous CH_2Cl_2 (1.0 mL) was added dropwise and the mixture was stirred for 4 h at room temperature. The mixture was partitioned between CH_2Cl_2 (10 mL) and 10% citric acid (10 mL). The organic phase was washed with 5% $NaHCO_3$ (10 mL), H_2O (2 × 10 mL), and brine (10 mL), dried with Na_2SO_4, and filtered. The solvent was evaporated under reduced pressure and the crude dipeptide **15** (227 mg) was crystallized from CH_2Cl_2/*n*-hexane to afford pure product **15** as a white solid, which was collected by filtration (214 mg, 97%). TLC (system B): R_f = 0.64. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.90 (d, *J* = 6.7 Hz, 3 H, $C\gamma^{Val}$−H), 1.03 (d, *J* = 6.8 Hz, 3 H, $C\gamma^{Val}$−H), 2.33−2.43 (m, 1 H, C_β^{Val}−H), 3.15 (dd, *J′* = 13.8, *J″* = 5.8 Hz, 2 H, C_β^{Phe}−H), 3.74 (s, 3 H, $COOCH_3$), 3.78 (s, 6 H, OCH_3), 4.59 (dd, *J′* = 9.1, *J″* = 5.5 Hz, 1 H, C_α^{Val}−H), 4.95 (td, *J′* = 7.9, *J″* = 5.9 Hz, 1 H, C_α^{Phe}−H), 6.05 (d, *J* = 9.1 Hz, 1 H, Val NH), 6.59 (d, *J* = 8.4 Hz, 2 H, Ar−H), 6.69 (d, *J* = 7.8 Hz, 1 H, Phe NH), 7.14−7.16 (m, 2 H, Ph), 7.24−7.28 (m, 3 H, Ph), 7.32 (t, *J* = 8.4 Hz, 1 H, Ar−H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.7, 19.5, 30.6, 38.5, 52.7, 53.6, 56.3, 58.6, 104.4, 115.8, 127.6, 129.0, 129.7, 131.4, 136.2, 157.8, 166.0, 171.0, 171.9 ppm. HRMS (ES-TOF): *m/z* calcd. for $C_{24}H_{31}N_2O_6$ $[M + H]^+$: 443.2182; found 443.2197.

2,6-Dmbz-Val-Phe-OH (16): Compound **15** (205 mg, 0.46 mmol) was suspended in 1 M aq. NaOH/dioxane (1:1, v/v; 10 mL). The mixture was stirred for 2 h at room temperature. The dioxane was evaporated, H_2O (5 mL) was added and the solution was acidified to pH 2 by addition of 10% HCl. A white precipitate was formed, which dissolved upon extraction with EtOAc (20 mL). The aqueous phase was separated and extracted with EtOAc (2 × 5 mL). The organic phases were combined and washed with H_2O (10 mL) and brine (10 mL), dried with Na_2SO_4, and filtered. The solution was concentrated under reduced pressure and the title compound **16** precipitated as a white powder, which was collected by filtration (186 mg, 94%). RP-HPLC (gradient 2) t_R = 26.8 min. TLC (system A): R_f = 0.33. ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.81 (d, *J* = 6.8 Hz, 3 H, $C\gamma^{Val}$−H), 0.83 (d, *J* = 6.8 Hz, 3 H, $C\gamma^{Val}$−H), 1.97−2.06 (m, 1 H, C_β^{Val}−H), 2.90 (dd, *J′* = 13.9, *J″* = 8.5 Hz, 1 H, C_β^{Phe}−H), 3.04 (dd, *J′* = 13.9, *J″* = 5.4 Hz, 1 H, C_β^{Phe}−H), 3.68 (s, 6 H, OCH_3), 4.32 (dd, *J′* = 9.3, *J″* = 6.4 Hz, 1 H, C_α^{Val}−H), 4.51−4.57 (td, *J′* = 8.0, *J″* = 5.4 Hz, 1 H, C_α^{Phe}−H), 6.67 (d, *J* = 8.4 Hz, 2 H, Ar−H), 7.20−7.27 (m, 5 H, Ph), 7.30 (t, *J* = 8.3 Hz, 1 H, Ar−H), 7.87 (d, *J* = 8.0 Hz, 1 H, Phe NH), 7.97 (d, *J* = 9.3 Hz, 1 H, Val NH) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 18.6, 20.0, 31.4, 38.0, 54.1, 56.6, 58.7, 105.1, 117.4, 127.3, 129.1, 130.0, 130.9, 138.0, 157.6, 165.2, 171.5, 173.5 ppm. HRMS (ES-TOF): *m/z* calcd. for $C_{23}H_{29}N_2O_6$ $[M + H]^+$: 429.2026; found 429.2030.

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