Preparation of Constrained Unnatural Aromatic Amino Acids *via* Unsaturated Diketopiperazine Intermediate

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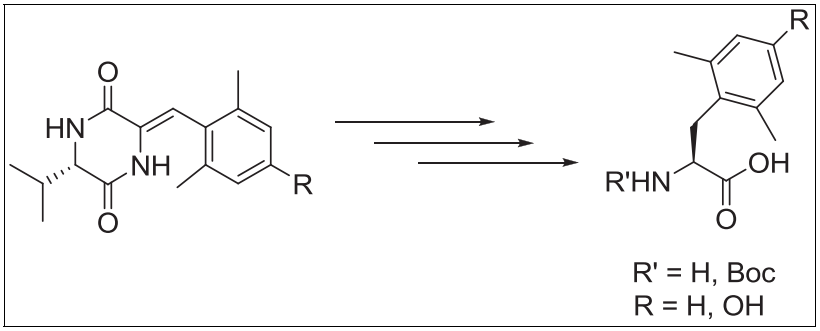

Unnatural aromatic amino acids are useful tools in drug discovery, since their insertion in bioactive peptide sequences can change the side chains spatial orientation, the backbone conformation and above all, their bioactivity. In this communication, we propose a straightforward method to synthesize 2′,6′-dimethyl-tyrosine and 2′,6′-dimehylphenyl-alanine derivatives as handling building blocks for peptide synthesis *via* unsaturated diketopiperazine (DKP) intermediate.

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INTRODUCTION

It is well-known that a small change of the sequence of a native peptide ligand, such as the incorporation of unnatural amino acids, may contribute markedly to the biological profile of the peptide. As an example, it has been demonstrated that the introduction of 2′,6′-dimethyl-tyrosine (DMT) in the place of the native Tyrosine[1] and 2′,6′-dimethyl-phenylalanine (DMP) in place of Phenylalanine[3] in the neuropeptide endomorphin-2 yielded very potent analogs, and in the latter case to an enhancement of the binding affinity and selectivity to μ opioid receptor (Table 1) [1,2]. This change in the biological profile may be explained with the concept of χ space [3–5]. Side chains of natural amino acids, usually possess a certain degree of rotational freedom around χ dihedral angles (Fig. 1), giving to the native peptide ligands the possibility to adapt their shape to the binding pocket of a wide range of receptor subtypes, as in the case of enkephalins and endomorphins, which have flexible structures capable to bind to both μ and δ receptors with the selectivity ranging from none to good [6–8]. In nature, selectivity and duration of action of native peptides are usually controlled by a site-directed biosynthesis and *in situ* degradation. In peptidomimetics design, to achieve a high potency and selectivity, one approach could be the reduction of native residues conformational freedom, in order to obtain the most favorable topology for the binding to one preferred receptor subtype [9]. This approach can be virtually applied to all bioactive peptidomimetics, but the extremely high cost of such unnatural amino acids and the complexity of the previously reported preparations can easily discourage the researchers to the use of these tools in their design. In this paper, we report the development of a simple, general method to obtain unnatural constrained aromatic amino acids as building blocks suitable for peptide synthesis as partially reported by us in the patent titled "Sintesi enantioselettiva di amminoacidi aromatici non-naturali", Application Number: RM2015A000091, date: 27/02/2015.

A crucial step of the synthesis is the control of the stereochemistry of the final product which is generally achieved by preparative HPLC (in case of intermediate diastereoisomers) [10] or by using stereoselective hydrogenation with [Rh(1,5-COD)-(*R,R*-DIPAMP)]BF_4 as catalyst [11,12]. The latter yielded the unnatural amino acid 2′,6′-dimethyl-*L*-tyrosine in high purity on the kilogram scale, but it required high catalyst concentrations and prolonged reaction times [13] with respect to our proposed method.

Table 1

Biological effect of the insertion of DMT and DMP in the endomorphin-2 sequence [1,2].

Compounds	K_i^{μ}	K_i^{δ}	K_i^{κ}	δ/μ ratio
EM-2	1.33 ± 0.15	6085 ± 1215	>4000	>3000
[DMT][1]-EM2	0.26 ± 0.91	99.2 ± 7.9	489 ± 135	1880
[DMP][3]-EM2	0.044 ± 0.003	1440 ± 94	>4000	>91 000

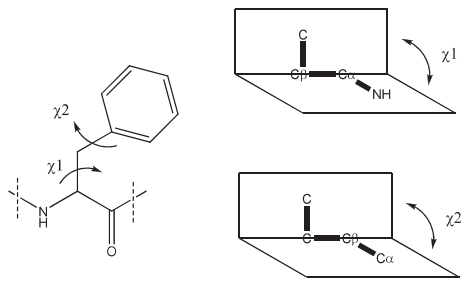
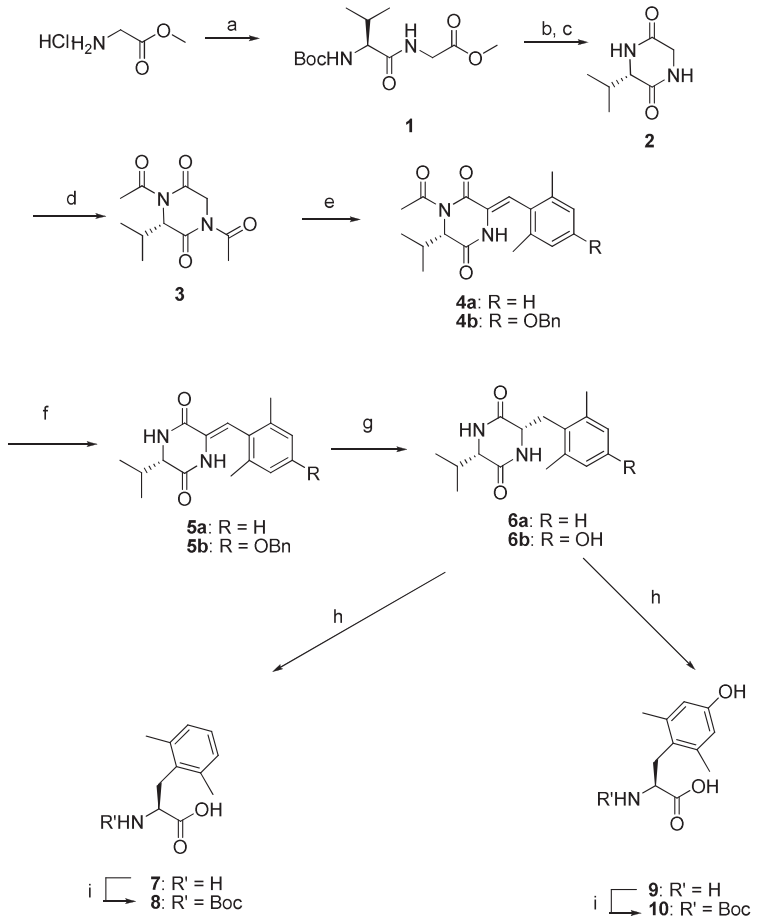


Figure 1. Definition of χ1 and χ2 dihedral angles.

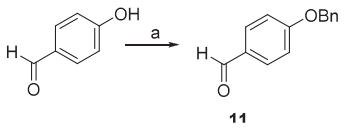
DISCUSSION

Asymmetric synthesis of sterically constrained amino acid is characterized by a high cost and the use of expensive Rh catalysts and Ni(II)-complexes of chiral Schiff base of glycine or alanine. The here reported synthetic strategy for compounds **7–10** is characterized by α,β unsaturated 2,5-diketopiperazines (**5a,b**) as the key intermediate products (Scheme 1). Reagents and solvents were conveniently purchased from the common commercial sources and the experimental procedures are straightforward and with high chemical yields. The procedure starts with the coupling between Boc-Valine (**1**) to HCl·Glycine methyl ester and the resulting dipeptide was easily converted to the DPK (**2**) in high overall yield. The DKP (**2**) was further diacetylated by treatment with acetic anhydride [14]. Reaction of the *N′*,*N*-diacetyl derivative (**3**) with potassium *tert*-butoxide in DMF in the presence of an appropriate commercial 2,6-dimethylbenzaldehyde (in the case of DMP) or newly synthesized (**11**) aldehyde (in the case of DMT, as outlined in Scheme 2) yielded the corresponding

Scheme 1. Reactions and conditions: a) Boc-Val-OH, EDC·HCl, HOBt, NMM, DMF, r.t., overnight; b) TFA/CH_2Cl_2 1:1, r.t., 1 h, under N_2; c) AcOH/2-butanol, NMM, 120 °C, 3 h; d) Ac_2O, reflux, overnight; e) **11** or 2,6-dimethylbenzaldehyde, *t*BuOK, DMF, r.t., 4 h; f) NH_2–NH_2·H_2O, MeOH, 2 h; g) H_2, Pd/C 10% 6 atm; h) 6N HCl, reflux, overnight; i) Boc_2O, $NaHCO_3$, dioxane/H_2O.



Scheme 2. Reactions and conditions: a) BnBr, DMF, Na_2CO_3, 12 h, r.t.



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mono-acetylated unsaturated DKP with *Z*-configuration (**4a** and **4b**). *E*-isomer is not easily accessible in this condition, due to its thermodynamic instability [15]. The residual acetyl group was removed in mild condition by methanolic solution of hydrazine monohydrate providing the formation of the corresponding deacetylated unsaturated DKPs **5a** and **5b**. These intermediates were reduced under 6–8 atm of H_2 over Pd-C at 60 °C using a Parr 4843 apparatus.

As regards the reaction mechanism, the steric course of the synthesis has been justified by assuming that the DKP ring was nearly planar and that the side chain of the valine moiety protruded from the molecular plane, forcing the DKP to be preferentially adsorbed from the less bulky side (*Re* face) of the molecule. Thus the catalytic hydrogenation resulted in the formation of (*S*)-amino acid (Fig. 2). The catalytic hydrogenation of **5a** and **5b** gave **6a** and **6b** with very high chiral induction as reported for other optically active DKPs demonstrating a very effective asymmetric hydrogenation and the pivotal role played by the rigid skeleton of DKP ring. As reported by Morrison [16], there could be some effects that operate prior to the stereoselective adsorption (*Re* or *Si* face) of the substrate on the catalyst that make the bulkiness of the side chain (out of the plane) of the amino acid important for high chiral induction. For example, a C=O (or NH) group of the DKP may first be adsorbed preferentially on the catalyst surface vertically from the less bulky face.

The asymmetric center of the valine residue is held close to the catalytic surface and can exert an important influence in determining which face of the unsaturated moiety will be preferentially adsorbed. In addition, starting from (*R*)-valine, the proposed procedure should also afford the corresponding (*R*)-DMP and DMT.

The resulting 2,5-diketopiperazines *c*[Val-DMP] (**6a**) and *c*[Val-DMT] (**6b**) were hydrolyzed in refluxing 6N HCl overnight, leading to a homogeneous mixture of **7** or **9** and valine. At this point, the amino acids were *N*-Boc protected by reaction with Boc_2O in Schotten–Baumann condition and then separated as Boc derivatives **8** and **10** in RP-HPLC. The enatiomeric excess was calculated by the optical rotatory power and resulted to be 51% (76:24, L:D) for Boc-DMP-OH and 55% (77:23, L:D) for Boc-DMT-OH. The partial loss of enatiomeric purity could be attributed to the harsh acidic conditions required for the DKP hydrolysis.

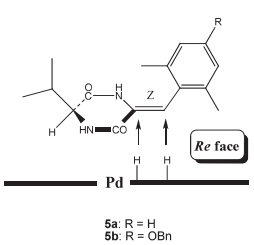

Figure 2. Proposed mechanism for the catalytic hydrogenation of unsaturated DKPs.

CONCLUSION

Aromatic constrained unnatural residues have been wisely and widely used in the field of opioid peptides. As discussed above, those building blocks are important tools to explore the topographical preferences of bioactive peptides. The only limits to the systematic exploration of the topographical requirements upon other bioactive peptides are their excessive cost or synthesis that require expensive chiral catalysts [11,17]. Our methodology, can be used to prepare a wide range of this type of residues, using a variety of commercial or synthetic aldehydes as described in the text, the synthesis provided DMP and DMT and their protected derivatives. Potentially any N or C terminal DMP and DMT derivatives are readily accessible from products **7** and **9**. Last, in this report, Boc protection has been preferred due to its broad application on both solid and solution peptide synthesis.

EXPERIMENTAL

General. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. The identity of the compounds was confirmed by ^{1}H NMR spectra recorded on a 300-MHz Varian Inova spectrometer (Varian Inc., Palo Alto, CA, USA). Chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me_4Si). The mass spectrometry system used consisted of an LCQ (Thermo Finnigan) ion trap mass spectrometer (San Jose, CA) equipped with an electrospray ionization (ESI) source. The capillary temperature was set at 300°C, and the spray voltage at 4.25 kV. The fluid was nebulized using nitrogen (N_2) as both the sheath gas and the auxiliary gas. Optical rotatory power were recorded on a Perkin-Elmer 241 Polarimeter. Homogeneity of the intermediates was confirmed by TLC on silica gel Merck 60 F254 (Merck, Germany). Chromatographic purifications were performed by high purity grade Merck 60 70–230 mesh silica gel column. Analytical thin-layer chromatography was carried out on Sigma-Aldrich® silica gel on TLA aluminum foils with fluorescent indicator 254 nm. Visualization was carried out under ultra-violet

irradiation (254 nm). Temperatures are reported in °C. All chemicals used were of the highest purity commercially available. 2,6-Dimethylbenzaldehyde and 2,6-dimethyl-4-hydroxybenzaldehyde were purchased from Fluorochem; all other reagents and solvents were purchased from Sigma-Aldrich (Italy) and VWR (Italy). Purification of Boc-protected final compounds was performed by RP-HPLC using a Waters XBridgeTM Prep BEH130 C18, 5.0 µm, 250 mm × 10 mm column at a flow rate of 5 mL/min on a Waters Binary pump 1525, using H_2O 0.1% TFA and acetonitrile 0.1% TFA as eluent.

CHEMISTRY

Boc-Val-Gly-OMe (1). To an ice-cooled mixture containing Boc-Val-OH (1.1 eq.) in DMF, EDC·HCl (1.1 eq.), HOBt (1.1 eq.), NMM (2.2 eq.) and HCl·H-Gly-OMe (1.0 eq.) were added. The reaction mixture was allowed to warm at r.t., stirred overnight and evaporated under reduced pressure. The residue was then dissolved in EtOAc and washed with three portions of 5% citric acid, saturated solution of $NaHCO_3$ and brine. The organic phases were collected and dried on Na_2SO_4, and the solvent evaporated under reduced pressure to give the desired product in quantitative yield.

^{1}H NMR ($CDCl_3$) δ: 6.65 (1H, t, Gly NH), 5.12 (1H, d, NHBoc), 4.32–4.25 (1H, m, Val αCH), 4.08–3.95 (2H, m, Gly CH_2), 3.75 (3H, s, OMe), 2.19 (1H, m, Val βCH), 1.35 (9H, s, Boc), 0.92–1.03 (6H, d, Val γCH_3). ^{13}C NMR ($CDCl_3$) δ 18.0, 19.2, 28.2, 30.8, 41.0, 52.3, 59.7, 80.0, 155.7, 170.1, 171.9. MS calcd.: 288.17 found: 288.40

***c*(Val-Gly) (2).** Boc-Val-Gly-OMe (**1**) was deprotected by a 50% mixture of TFA in CH_2Cl_2 at r.t. for 1 h. The intermediate TFA salt was used for subsequent reactions without further purification. TFA·dipeptide methyl ester (1 eq.) was dissolved in 0.1 M AcOH/2-butanol (1.5 eq.), and NMM (1 eq.) was added. The resulting weakly acidic solution was refluxed in an oil bath (120°C) overnight. The solvent was evaporated *in vacuo*, the crude was suspended in acetone and the product was collected by filtration, washed with small amounts of cold H_2O and recrystallized from a mixture of H_2O/2-butanol to afford the pure product in quantitative yield.

^{1}H NMR (DMSO-d_6) δ: 7.45 (1H, s, NH), 7.38 (1H, s, NH), 4.60–4.55 (1H, d, Val αCH), 4.36–4.22 (2H, dd, Gly CH_2), 2.05 (1H, m, Val βCH), 0.95–1.10 (6H, d, Val γCH_3). ^{13}C NMR (D_2O) 16.6, 18.8, 33.7, 44.6, 60.7, 169.7, 171.1. MS calcd.: 156.09 found: 156.3.

***N,N′*-diacetyl-c(Val-Gly) (3).** A mixture of DKP (**2**) in acetic anhydride was stirred under reflux for 12 h. The solvent was removed by azeotropic distillation with methanol and toluene under reduced pressure. The residue was crystallized from EtOAc/Et_2O to yield (**3**) as a brown oil in 80% yield.

^{1}H NMR ($CDCl_3$) δ: 5.07–5.14 (1H, d, Gly CH_2, J = 19 Hz), 5.01 (1H, d, Val αCH, J = 9.9 Hz), 4.06–4.12 (1H, d, Gly CH_2, J = 19 Hz), 2.60–2.57 (6H, s, 2 × N-Ac), 2.04 (1H, d, Val βCH, J = 9.9 Hz), 1.11–0.97 (6H, d, Val γCH_3, J = 6.3 Hz). MS calcd.: 240.11 found: 240.5.

***c*(Δ^z-DMP-*N*-acetyl-Val) (4a).** Compound (**3**) (1 eq.) was dissolved in DMF, then 2,6-dimethylbenzaldehyde (1.5 eq.) and *t*BuOK 0.5 M in *t*BuOH (1.5 eq.) were added dropwise at 0°C. The mixture was allowed to warm to r.t. and stirred for 7 h. Then the reaction was quenched with aqueous NH_4Cl solution and extracted three times with EtOAc (3 × 50 mL). The combined organic layers were dried on anhydrous Na_2SO_4, filtered and evaporated. The crude was purified by silica gel chromatography using CH_2Cl_2/EtOAc 95:5 as eluent to obtain the final product as a colorless oil in 40% yield.

^{1}H NMR ($CDCl_3$) δ: 7.23–7.09 (4H, m, Ar, CH=C), 6.67 (1H, s, NH), 4.98 (1H, d, Val αCH, **J = 6.3** Hz), 2.63 (3H, s, N-Ac), 2.20 (6H, s, Dmp CH_3), 2.08 (1H, m, Val βCH), 1.08–1.01 (6H, d, Val γCH_3, J = 6.9 Hz). MS calcd.: 314.16 found: 314.6.

***c*(Δ^z-DMP-Val) (5a).** Compound (**4a**) (1 eq.) was dissolved in MeOH and hydrazine monohydrate (2 eq.) was added. The product began to precipitate from the solution. After 2 h the solution was filtered, and the pure final product was obtained as a white powder in quantitative yield.

^{1}H NMR (DMSO-d_6) δ: 9.38 (1H, s, NH), 8.52 (1H, s, NH), 7.07–7.01 (3H, m, Ar), 6.62 (1H, s, CH=C), 3.79 (1H, m, Val αCH), 2.10 (6H, s, DMP CH_3), 2.02 (1H, m, Val βCH), 0.94–0.84 (6H, d, Val γCH_3, J = 6.3 Hz). ^{13}C NMR (DMSO-d_6) δ: 17.8, 19.1, 20.3, 34.3, 61.4, 114.5, 128.6, 129.4, 130.0, 134.5, 161.5, 167.4. MS calcd.: 272.15 found: 272.3.

***c*(DMP-Val) (6a).** Compound (**5a**) was dissolved in MeOH and Pd/C (20%) was added. Then the mixture was hydrogenated for 24 h in a Parr 4843 apparatus at 6–8 bar of H_2 and 45°C. The mixture was filtered to remove the catalyst and the solvent was evaporated to give the reduced product as a white powder in quantitative yield.

^{1}H NMR (DMSO-d_6) δ: 8.28 (1H, d, NH, J = 3 Hz), 7.93 (1H, d, NH, J = 3.3 Hz), 6.99–6.98 (3H, m, Ar), 3.83 (1H, m, Val αCH), 3.51 (1H, m, DMP αCH), 3.22–2.88 (2H, m, DMP βCH_2, AB part of an ABX system J^{AB} = 14.1, J^{AX} = 6.3, J^{BX} = 8.7 Hz), 2.25 (6H, s, DMP CH_3), 2.03 (1H, m, Val βCH), 0.98–0.92 (6H, d, Val γCH_3, J = 6.3 Hz). ^{13}C NMR (DMSO-d_6) δ: 16.4, 18.1, 21.6, 31.5, 38.4, 56.0, 60.4, 127.1, 129.0, 131.4, 137.0, 166.2, 166.0. MS calcd.: 274.17 found: 274.5.

Boc-DMP-OH (8). The DKP was dissolved in 6N HCl (50 mL for 100 mg), and the solution was stirred at 100°C for 24 h. Then the solvent was evaporated under reduced

pressure, and the obtained powder was used for the next step without further purification. The mixture of HCl·H-DMP-OH and HCl·H-Val-OH was dissolved in dioxane/H_2O 1:1. Successively, $NaHCO_3$ (2 eq.) and Boc_2O (1.2 eq.) dissolved in dioxane were added at 0°C. The mixture was allowed to warm to r.t. and stirred for 18 h. The dioxane was evaporated, pH was adjusted to 12 by adding 1N NaOH and the aqueous solution was washed two times with Et_2O (2 × 50 mL). Then the aqueous phase was acidified to pH 2–3 by adding 2N HCl, and three extractions with EtOAc (3 × 50 mL) were performed. The combined organic layers were dried on anhydrous Na_2SO_4, filtered and evaporated *in vacuo*. The obtained crude was purified by semi-preparative HPLC (see General section) to give pure Boc-DMP-OH as a white powder. $[\alpha]_D^{20}$ −9.2° (c = 0.8 MeOH e.e. 51 %).

^{1}H NMR ($CDCl_3$) δ: 7.25–6.99 (3H, m, Ar), 5.01 (1H, d, BocNH), 4.53 (1H, m, αCH), 3.49–3.062 (2H, m, βCH_2, AB part of an ABX system $J^{AB} = 14.1$, $J^{AX} = 3.6$, $J^{BX} = 10.8$ Hz), 2.37 (6H, 2 × s, 2 × CH_3), 1.35 and 1.061 (9H, s, Boc).

***O*-Bn-2,6-dimethylbenzaldehyde (11).** 2,6-Dimethyl-4-hydroxybenzaldehyde (1 eq.) was dissolved in DMF and Na_2CO_3 (1.2 eq.) and benzyl bromide (1.2 eq.) were added. The mixture was stirred at 60°C, and the course of the reaction was monitored by TLC. When complete, the solvent was evaporated, and the obtained product (90% yield) was used for the next reaction without further purification.

^{1}H NMR ($CDCl_3$) δ: 10.48 (1H, s, CHO), 7.42–7.40 (7H, m, Ar), 5.10 (2H, s, CH_2—Ar), 2.60 (6H, s, 2 × CH_3).

***c*(*O*-Bn-Δ^z-DMT-*N*-acetyl-Val) (4b).** Compound (**4b**) was synthesized starting from *c*(Val-Gly) (**3**) and (**11**) following the same procedure of product (**4a**). After chromatographic purification the final product was obtained as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 7.45–7.33 (5H, m, OBn Ar), 7.12 (1H, s, CH=C), 7.05(1H, s, NH), 5.06 (2H, s, CH_2-Ar), 4.97 (1H, d, Val αCH, apparent J = 6.6 Hz), 2.61 (3H, s, N-Ac), 2.17 (6H, s, DMT CH_3), 2.07 (1H, m, Val βCH), 1.07–1.00 (6H, d, Val γCH_3). MS calcd.: 420.20 found: 420.6.

***c*(*O*-Bn-Δ^z-DMT-Val) (5b).** Compound (**4b**) was deacetylated following the same procedure of product (**4a**). The precipitated was obtained as a white powder in quantitative yield.

^{1}H NMR (DMSO-d_6) δ: 9.35 (1H, s, NH), 8.61 (1H, s, NH), 7.56–7.52 (5H, m, OBn Ar), 6.87 (2H, s, Ar), 6.67 (1H, s, CH=C), 5.26 (2H, s, CH_2—Ar), 3.76 (1H, m, Val αCH), 2.18 (6H, s, DMT CH_3), 2.05 (1H, m, Val βCH), 0.95–0.88 (6H, d, Val γCH_3). MS calcd.: 378.19 found: 378.7. ^{13}C NMR (DMSO-d_6) δ: 16.9, 18.2, 21.6, 33.3, 60.5, 114.0, 125.0, 125.7, 130.6, 158.9, 160.7, 166.3.

***c*(DMT-Val) (6b).** Hydrogenation of compound (**5b**) was performed in the same conditions of compound (**5a**), and the reduced product was obtained as a white powder in 92% yield.

^{1}H NMR (DMSO-d_6) δ: 9.01 (1H, s, DMT OH), 8.23 (1H, d, NH, J = 3.3 Hz), 7.80 (1H, d, NH, J = 3.6 Hz), 6.39 (2H, s, Ar), 3.76 (1H, m, Val αCH), 3.50 (1H, m, DMT αCH), 3.12–3.01 (2H, m, DMT βCH_2), 2.16 (6H, s, DMT CH_3), 2.04 (1H, m, Val βCH), 0.97–0.91 (6H, dd, Val γCH_3, J = 6.6 and 6.9 Hz). ^{13}C NMR (DMSO-d_6) δ: 16.5, 18.5, 23.4, 31.3, 37.4, 55.5, 59.4, 114.9, 126.5, 131.3, 156.3, 166.6, 166.9. MS calcd.: 290.16 found: 290.4.

Boc-DMT-OH (10). Hydrolysis and Boc protection of compound (**6b**) were performed following the same procedure of *c*(Val-DMP) (**6a**). After preparative HPLC purification Boc-DMT-OH was obtained as a white powder. $[\alpha]_D^{20}$ −6.5° (c = 0.8 MeOH; e.e. 55%).

^{1}H NMR ($CDCl_3$) δ: 6.51 (2H, s, Ar), 4.79 (1H, d, BocNH), 4.96–4.42 (1H, m, αCH), 3.15–2.96 (2H, m, βCH_2, AB part of an ABX system $J^{AB} = 14.4$, $J^{AX} = 5.7$, $J^{BX} = 9.3$ Hz), 2.31 (6H, s, 2 × CH_3), 1.45 and 1.37 (9H, s, Boc). ^{13}C NMR ($CDCl_3$) δ: 21.5, 25.9, 34.8, 55.3, 80.8, 126.4, 127.4, 133.3, 137.2, 155.3, 176.6. MS calcd.: 309.16 found: 309.5.

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