Epimerization of peptide nucleic acids analogs during solid-phase synthesis: optimization of the coupling conditions for increasing the optical purity

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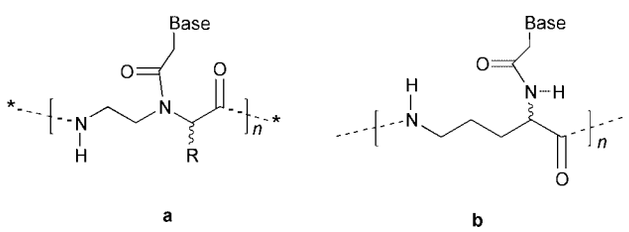
Peptide nucleic acid (PNA) analogs based on N^{α}-(thymin-1-ylacetyl)ornithine were previously shown to form triplexes with complementary RNA. In order to obtain optically pure compounds for hybridization experiments, chiral monomers based on D- or L-ornithine, N^{δ}-Fmoc-N^{α}-(thymin-1-ylacetyl)ornithine **2** and N^{δ}-Fmoc-N^{α}-(uracil-1-ylacetyl)ornithine **3** were synthesized either by a one-step or by a simple three-step procedure starting from N^{δ}-protected ornithine; the latter procedure led to enantiomerically pure products. Oligomerization of **2** and **3** was carried out either in solution, or by solid-phase peptide synthesis (SPPS) on an MBHA-Rink amide resin. The oligomers turned out to contain large amounts of epimerization products, especially those obtained by SPPS. Therefore, we examined carefully the parameters which may be involved in epimerization: the nature of the coupling reagent, of the base, and the addition mode. Coupling of the monomer **L-3** was performed under various conditions. Lower racemization was found to occur when using (7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as coupling agent and 2,4,6-trimethylpyridine (*sym*-collidine, TMP) as base, without preactivation, leading to a residual 4% of the D-enantiomer. By applying a procedure based on the stepwise addition of the base the D-enantiomer content was reduced to less than 1%. Using this procedure, a decamer of **L-3** was synthesized, which was shown to contain less than 2% of the D-ornithine derivative.

Introduction

Optical purity and methods for preventing epimerization are essential features for peptide synthesis, since the presence of diastereomeric impurities can considerably lower the yield and purity of products, and since biological activity is strictly related to stereochemistry.[1] The problem is particularly important in peptide synthesis carried out in solution, and could be a serious problem in the case of coupling with *N*-methyl amino acids,[2] in segment coupling,[3] in convergent solid-phase peptide synthesis (CSPPS),[4] or in the synthesis of conformationally constrained cyclic peptides.[5] By carefully choosing the coupling reagents, the base, and the reaction conditions, epimerization can be reduced to very small levels, as shown in several studies by Carpino and co-workers.[6]

Optical purity is a crucial point also for oligonucleotide analogs, since stereochemically impure products may contain many diastereoisomers, each interacting with complementary DNA or RNA with different affinity. For example, methylphosphonate or phosphorothioate oligonucleotides, which have been used as antisense drugs, are normally synthesized as mixtures of 2^n diastereoisomers (n = number of monomeric units), and it was shown that their binding properties strongly depend upon the absolute configuration of the phosphorus stereogenic center.[7]

Peptide nucleic acids are DNA mimics, first introduced by Nielsen, Buchardt, Berg and Egholm,[8] in which the sugar-phosphate backbone was replaced by a polyamide chain composed of aminoethylglycine covalently linked to DNA bases



a **b**

Chart 1

(Chart 1**a**, R = H). They were shown to form hybrids with complementary DNA or RNA strands of remarkable affinity and selectivity,[9] and are currently used as very powerful tools in molecular biology for the detection of defined DNA sequences or mutations,[10] and for the inhibition[11] and regulation of gene expression.[12] PNAs are also promising as antisense drugs.[13,14]

PNA oligomers can be easily obtained on a milligram-to-gram scale from monomeric units by solid-phase peptide synthesis (SPPS),[15] using standard procedures on automatic instruments. Since their discovery in 1991, many studies have been published describing modified PNA, with the aim of characterizing the best structural features for DNA complexation.[16,17] Among these, particular attention has been given to chiral PNA analogs,[18] since chirality could, in principle, induce some degree of pre-organization (helicity)[19,20] and thus favor the interaction with helically structured DNA. Furthermore, chirality has been used as a tool to increase sequence selectivity.[21] Preferential complexation of DNA by PNA derived

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from D- or L-amino acids could also shed some light on the origin of homochirality in nature,[22] since PNAs can act as templates for RNA synthesis.[23]

Chiral PNAs have been synthesized by replacement of the aminoethylglycine unit with L- or D-aminoethyl amino acids (Chart 1**a**)[24,25] or by more drastic changes in the original design.[26–29] However, we have recently demonstrated that chiral PNA monomers can undergo epimerization during SPPS.[30]

Some of the proposed chiral modifications have also shown DNA-complexation properties comparable with, or in some cases better than, those of achiral PNAs.[31] A substantial modification of PNA was introduced by Nielsen and collaborators,[32] and by others,[33] who used thymine-containing homochiral oligomers derived from L-ornithine (Chart 1, type **b**). They showed no affinity for DNA, while forming triplexes with RNA. Recently it was demonstrated that these products, when synthesized by common SPPS procedures exhibit a large amount of epimerization,[34] and a synthesis of optically pure PNA oligomers of this type has been described, based on a modified procedure using submonomer synthesis with ornithine protected with orthogonal groups (Boc for N^{α} and Fmoc for N^{δ}), although the number of coupling steps was doubled.[35] The optically pure oligomers showed increased affinity for complementary DNA and RNA. Therefore, the synthesis of these ornithine PNAs can be regarded as one of the most challenging models for the study of epimerization as a function of the coupling conditions.

In this paper we report the synthesis of thymine and uracil PNA oligomers based on D- and L-ornithine both in solution and in the solid phase, with an extensive study of the enantiomeric composition of the products as a function of the coupling conditions: the nature of the coupling reagent, of the base, and the coupling protocol were investigated. The enantiomeric analysis was based on the previous experience developed by some of us in the field of chiral recognition, in particular of amino acids,[36–38] and of PNAs[30] by chromatographic methods. The aim is to develop a synthetic method for extensively reducing the epimerization in the coupling of ornithine PNA monomers.

Results and discussion

Synthesis of chiral monomers and oligomers

Fmoc-protected T- (**2**) and U-containing (**3**) monomers were synthesized by treating N^{δ}-Fmoc-ornithine with 1-(carboxymethyl)thymine (CMT) or 1-(carboxymethyl)uracil (CMU)[39] *N*-hydroxysuccinimidyl (OSu) esters (Scheme 1, route a), without protection of the carboxy group. The overall yields obtained with this strategy were excellent (70–80% based on ornithine), although the optical purity was not completely preserved (ee = 92–98%). Therefore, we protected the carboxylic function of N^{δ}-Fmoc-ornithine as its methyl ester before treating it with CMT and CMU, using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethlyluronium tetrafluoroborate (TBTU) as coupling agent, and removed the methyl group by mild hydrolysis using barium hydroxide in THF–water in the last step (Scheme 1, route b). In this way optically pure monomers **2** and **3** were obtained (ee > 98%).

The oligomers synthesized in the present study (**7**–**10**) are reported in Chart 2.

The pentamers L- and D-(ThyOrn)$_5$GlyOEt **L-** and **D-7** (Chart 2) were synthesized in solution using 2-(benzotriazol-l-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)–Et_3N and precipitation after each step (see Experimental section). The two pentamers were obtained in satisfactory yields, but with a low ee (68%). Epimerization was measured either on a tetraamidic chiral stationary phase (Phe-3-O-TA) column prepared in our laboratories,[40,41] or on a commercial Chirasil-Val column.[42] However, attempts to prepare longer oligomers failed, due to the low solubility of the products and low coupling yields.

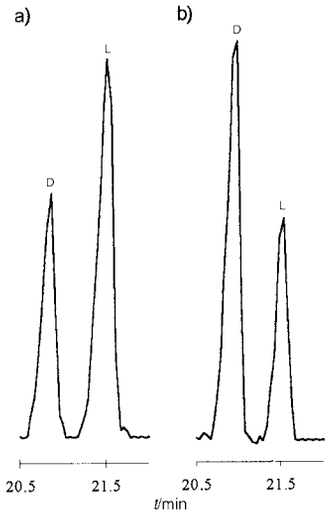
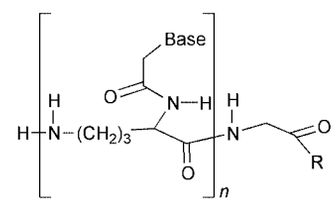


Fig. 1 Enantiomeric analysis by GC of the decamers (a): **L-9** and (b): **D-9** after hydrolysis with 6 M HCl and derivatization with HCl–propan-2-ol and trifluoroacetic anhydride. Chiral phase Phe-3-O-TA; temperature program: 120 °C (3 min), 120–160 °C (4 °C min^{-1}), 160 °C final isotherm; detector: FID.



L-, D-**7** : *n* = 5, Base = T, R = OEt
L-, D-**8** : *n* = 10, Base = T, R = NH_2
L-, D-**9** : *n* = 10, Base = U, R = NH_2
L-**10** : *n* = 2, Base = U, R = NH_2

Chart 2

The decamers D- and L-(ThyOrn)$_{10}$GlyNH$_2$ **8** and D- and L-(UrOrn)$_{10}$GlyNH$_2$ **9** were prepared by standard SPPS on an MBHA-Rink amide resin, using HBTU–diisopropylethylamine (DIEA) with 5 min preactivation (see Experimental section). This resulted in extensively epimerized products (ee = 10% for **L-8**, 6% for **D-8**, 34% for **L-9** and 28% for **D-9**). The gas chromatograms obtained for the decamers **L-9** and **D-9** are reported as an example in Fig. 1.

In order to establish which step of the peptide synthesis was responsible for the very high epimerization observed during the synthesis of the decamers, we analyzed the enantiomeric composition of ornithine after each step of the solid-phase synthesis of the dimer **L-10** under the same conditions used for the synthesis of decamers. The results showed that epimerization occurred in the coupling step of the first chiral monomer to the solid phase to an extent comparable to that observed for the decamers (ee = 20%), and was not substantially increased by either the capping or the deprotection steps. After the introduction of the second chiral monomer, the overall enantiomeric composition was almost the same. We therefore concluded that each step led to the same extent of epimerization during the coupling reaction. As a consequence, the synthesis of the decamers by standard SPPS should give rise to 1024 (= 2^{10}) diastereoisomers.

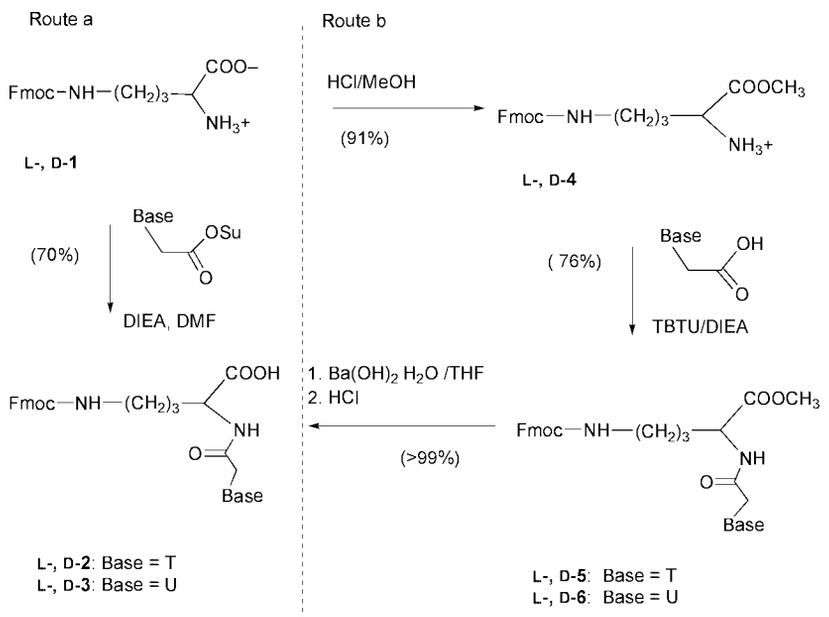
Effect of the coupling conditions on epimerization

Both monomers **2** and **3**, as well as other chiral PNAs, are *N*-alkanoyl amino acids and therefore, similarly to *N*-acetyl amino

Table 1 Effect of the reaction conditions on epimerization during the coupling of the monomer L-**3** to the MBHA-Rink amide resin

Coupling agent	Base	**Protocol**	Average yield [a] (%)	%D-Enantiomer (std. dev.) [b]
HBTU	DIEA	**a**	>99	48.5 (0.3)
HBTU	DIEA	**b**	95	22.3 (2.1)
HATU	DIEA	**a**	>99	44.6 (0.5)
HATU	DIEA	**b**	97	17.0 (2.7)
HATU	DIEA	**c**	99	6.2 (0.6)
HATU	TMP	**a**	98	8.7 (1.1)
HATU	TMP	**b**	98	3.9 (0.9)
HATU	TMP	**c**	92	1.0 (0.3)

[a] Measured by the absorption, at 301 nm, of the piperidine–dibenzofulvene adduct obtained after deprotection (referred to that obtained in the deprotection of the Fmoc-protected MBHA-Rink amide resin). [b] Measured by chiral analysis using GC-MS with Chirasil-Val column, single ion detection at 166 amu.



Scheme 1

acids or to dipeptides, are more liable to racemization than are the carbamate-protected amino acids commonly used in SPPS.[43] In a previous work on ornithine PNA, van Boom and co-workers utilized a model reaction in solution to test the effect of various coupling agents (HBTU, BOP, CF_3NO_2Py-BOP, DCC–HOBt) on the epimerization. The yields were found to be low (75–80%) and racemization rather high (10–25%).[35]

In preliminary experiments, the monomers D- and L-**3** were directly coupled to the MBHA-Rink amide resin using HBTU and DIEA, then cleaved and analyzed as reported above. In agreement with the literature when using dipeptides as synthons,[3] it was shown that racemization could be reduced by decreasing the amount of base, and by eliminating the preactivation step before coupling (results not shown). Attempts to decrease the equivalents of the base under the 1 : 1 ratio (based on monomer) did not give satisfactory results, as indicated by positive Kaiser tests. Mixed solvents, such as DMF–CH_2Cl_2 [3] could not be used in this case, due to the lack of solubility of the monomers **2** and **3** in CH_2Cl_2. The addition of 1-hydroxybenzotriazole (HOBt) was avoided, since it has been reported to increase epimerization.[3]

Using the same test reaction we then evaluated the effect of the coupling agent, of the base used for neutralization, and of the addition mode of the base on the epimerization of the monomer L-**3**. According to Carpino *et al.* the use of the coupling agent HATU[44,45] and of the base *sym*-collidine (2,4,6-trimethylpyridine, TMP)[46] is a convenient combination for obtaining high yields and for preventing epimerization. We therefore focused our attention on these conditions.

At least three tests were performed for each coupling condition, and yields were measured on the basis of the dibenzofulvene–piperidine adduct absorption (301 nm) after deprotection.[47] In all protocols, 4 equivalents of the monomer, of the coupling agent, and of the base (based on the resin active sites) were used. The results are reported in Table 1.

First it can be noted that for each protocol the use of HATU instead of HBTU led to a lower degree of epimerization, and even better results were obtained by substituting DIEA with collidine (TMP). The coupling protocol was found to be important also.

In **protocol a** the monomer L-**3**, the coupling agent, and the base in 1 : 1 : 1 proportions were mixed together and stored at rt for 5 min before being introduced into the reactor and stirred for 30 min. These conditions were chosen in order to reproduce those used in solid-phase synthesis; a large degree of racemization was observed, as a consequence of the action of the base on the monomer in its activated form. Accordingly, using HATU as coupling agent, the stronger base DIEA gave rise to larger quantites of the D-isomer (44.6%) than when using the weaker base TMP (8.7%).

In **protocol b** the monomer L-**3** and the coupling agent in a 1 : 1 ratio were mixed and stored at rt for 5 min. The mixture was then introduced into the reactor and the base was immediately added. The results obtained by this protocol improved, clearly showing that the preactivation step is critical and should be avoided, though in some cases this can lead to lower yields. When using this protocol, a competition occurs between acylation and racemization of the active ester, and,

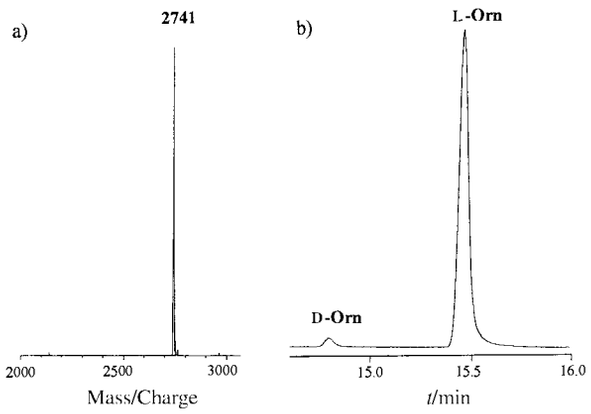


Fig. 2 a) MALDI-TOF and b) GC-MS enantiomeric analysis (column: Chirasil-Val; temperature program: 120 °C (1 min), 120–175 °C, 4 °C min^{-1}, 175 °C final isotherm) of the decamer **L-9** synthesized by **protocol c** in combination with HATU and TMP.

also in this case, the use of the weaker base TMP gave the best result (3.9% of D-enantiomer), with only a little loss of the coupling efficiency (98% as compared with 99% with DIEA). However, the degree of epimerization was still not optimal.

Protocol c was designed according to the following considerations: by comparing the results of **protocols a** and **b**, it was clear that the base should be added as late as possible, in order to prevent the formation of the active ester in the presence of an excess of base. Therefore, it is better to add the base in subsequent steps during the coupling reaction, since this provides only the quantity required for the neutralization of the HOBt once the coupling reaction has occurred. Accordingly, we devised a protocol based on the stepwise addition of the base: the monomer and the coupling agent were mixed in a 1 : 1 ratio and then introduced into the reactor; then 1/4 of the total amount of base (hence in a 1 : 1 ratio with the resin active sites) was immediately added, to initiate the reaction; the other three (1/4) portions of the base were added stepwise, every 15 min. The effect of this procedure is remarkable if one compares the results obtained using HATU and DIEA for **protocols b** and **c**: the D-enantiomer content was lowered from 17% to 6.2%, without loss of coupling efficiency. By using this protocol, in combination with the use of HATU and TMP, we were able to obtain coupling reactions with the formation of only 1% of the D-enantiomer (less than 1/40 of that obtained with previous procedures), within a reasonable reaction time (1 h). The yields obtained with this method, though lower than those obtained with **protocol b**, were still high (92%), thus indicating that the partial reaction of the coupling agents with the amino groups of the growing peptide to give the guanidinium derivatives is only a minor problem in this type of coupling.

By using these reaction conditions (HATU, TMP and **protocol c**), we synthesized the decamer L-$(UrOrn)_{10}GlyNH_2$ **L-9**, which was purified by HPLC and analyzed by MALDI-TOF (Fig. 2a). A small amount of the decamer, hydrolyzed and analyzed as reported above, showed the presence of 1.7% of D-ornithine (Fig. 2b). Unfortunately, it was not possible to compare the optical purity obtained by us with that previously obtained by van Boom and co-workers,[35] since the enantiomeric composition of their T_{10} oligomers was not reported. The slightly higher degree of epimerization, as compared with that observed during the monomer coupling with the same method, and the lower coupling yields (average 83%) could indicate that during the synthesis of homochiral oligomers coupling could be not equally easy for all monomers, leading to better competition by the epimerization reaction. However, by using HATU, TMP, and **protocol c**, it was possible to decrease the D-monomer content from 34% to 1.7%, providing a product of high optical purity.

Conclusions

In the present work we have demonstrated that ornithine-based chiral PNA analogs undergo extensive epimerization which is strongly dependent on the coupling conditions. This issue has not been addressed as much as for ordinary peptide synthesis, since the first PNAs described were achiral. It is therefore very important to carefully determine the enantiomeric purity of chiral PNAs before performing hybridization experiments with nucleic acids. By carefully examining the effect of several parameters on the epimerization rate of ornithine-based monomers we found that the stepwise addition of a weak base (TMP), together with the use of HATU, dramatically decreases epimerization during the coupling steps, by favoring the acyl nucleophilic substitution *versus* proton abstraction. The method reported herein is particularly suitable not only for chiral PNA analogs, but also for peptide couplings with amino acids or segments that present severe epimerization problems.

The protocol developed allowed us to synthesize decamers containing either D- or L-ornithine of high optical purity, which will give more clear-cut results about the effect of chirality on the stability and conformational properties of complexes formed by these molecules with complementary RNA or DNA. Preliminary hybridization results obtained with these enantiomerically pure compounds are very promising.

Experimental

Melting points: Electrothermal apparatus. ^1H and ^{13}C NMR spectra: Bruker AC 100 and AC 300; *J*-values are given in Hz. Mass spectra: Finnigan spectrometer Mat SSQ 710. Optical rotation: Autopol polarimeter, Rudolph Research. $[\alpha]_D$-Values are given in 10^{-1} deg cm^2 g^{-1}. UV measurements: UVIKON 941 double-beam spectrophotometer. CD spectra: JASCO J-715 spectropolarimeter. All the commercially available reagents were used without purification. DMF used in the synthesis and in the coupling rections was distilled under vacuum and stored on molecular sieves. 1-(Carboxymethyl)thymine (CMT) and 1-(carboxymethyl)uracil (CMU) were synthesized as reported in the literature.[37]

N^{δ}-Fmoc-ornithine 1

D- or L-Ornithine hydrochloride (0.909 g, 5.39 mmol) and copper(II) acetate (0.538 g, 2.69 mmol) were dissolved in 16 mL of 10% aq. sodium carbonate, and the solution was vigorously stirred for 45 minutes. To the stirred solution were added 180 mL of water and 200 mL of 1,4-dioxane, followed by slow addition of a solution of Fmoc-succinimidyl carbonate (2 g, 5.93 mmol) in 80 mL of 1,4-dioxane. After 45 minutes the reaction was acidified with 6 M HCl, then extracted twice with diethyl ether and twice with ethyl acetate. The aqueous solution was treated with H_2S (CAUTION!) for 15 minutes and stirred for about 1 hour. After filtration to remove CuS, the solution was concentrated under vacuum, and the pH was adjusted to 5.5 with 1 M NaOH. The solution was stored at 5 °C overnight, then the precipitate was filtered off, and dried under vacuum over P_2O_5 to yield 1.83 g of the title compound as a white solid.

L-1 (96%) mp 140 °C (decomp.) (from water); $[\alpha]_D^{25}$ +1.25 (*c* 1 in DMF). **D-1** (96%) mp 140 °C (decomp.) (from water); $[\alpha]_D^{25}$ −1.25 (*c* 1 in DMF) (Found: C, 64.20; H, 6.16; N, 7.45. Calc. for $C_{20}H_{22}N_2O_4 \cdot H_2O$: C, 64.50; H, 6.50; N, 7.52%); ν_{max} (KBr)/cm^{-1} 3332 (NH), 3500–2500br (NH_3^+), 2956 (CH), 1690 (C=O), 1596 (asymm. COO^-), 1538 (NH), 1410 (symm. COO^-), 1263 (C-O); δ_H [300 MHz; DMSO d_6 (2% TFA)] 1.40–1.60 (2H, m, CH_α-C*H*$_2$-CH_2-CH_2-NH), 1.70–1.90 (2H, m, CH_α-CH_2-C*H*$_2$-CH_2-NH), 3.00 (2H, q, *J* 5.6, CH_α-CH_2-CH_2-C*H*$_2$-NH), 3.90–4.00 (1H, m, C*H*$_\alpha$-CH_2-CH_2-CH_2-NH), 4.20–4.30 (1H, m, C*H*-CH_2 Fmoc), 4.30–4.40 (2H, m, CH-C*H*$_2$ Fmoc), 7.30 (2H, t, *J* 7.4, aromatic C-H Fmoc), 7.30–7.40 (1H, br m, Fmoc-N-H), 7.41 (2H, t, *J* 7.4 aromatic C-H Fmoc), 7.68 (2H, d, *J* 7.3,

aromatic C-H Fmoc), 7.88 (2H, d, *J* 7.4, aromatic C-H Fmoc), 8.25 (3H, br s, NH_3^+); δ_C [75 MHz; DMSO-d_6 (2% TFA), assignments based on DEPT] 25.2 (CH_2), 25.6 (CH_2), 39.8 (CH_2), 46.9 (CH), 51.9 (CH), 65.4 (CH_2), 120.2 (CH_{Arom}), 125.2 (CH_{Arom}), 127.2 (CH_{Arom}), 127.7 (CH_{Arom}), 140.9 ($C_{quat.\ arom.}$), 144.0 ($C_{quat.\ arom.}$), 156.3 (C=O), 171.1 (C=O); *m/z* (CI) 355 (MH^+, traces), 207 (20%), 179 (90), 178 (100), 115 (20), 81 (55), 79 (100), 63 (100), 62 (80).

N^{δ}-Fmoc-ornithine methyl ester hydrochloride 4

Into a suspension of N^{δ}-Fmoc-ornithine (1.5 g, 4.2 mmol) in 100 mL of methanol, cooled with an ice-bath, was bubbled gaseous HCl (**CAUTION!**) for 20 min. The mixture was then allowed to attain room temperature during 3 h, and the solvent was evaporated off. Methanol was added and evaporated thrice in order to eliminate excess of HCl. The crude product was dissolved in MeOH and recrystallized by addition of diethyl ether (1.48 g).

L-4 (91%) mp 124 °C (from Et_2O); $[\alpha]_D^{20}$ + 10.5 (*c* 1 in MeOH). **D-4** (91%) mp 120–122 °C (from Et_2O); $[\alpha]_D^{20}$ −10.6 (*c* 1 in MeOH) (Found: C, 60.00; H, 6.46; N, 6.69. Calc. for $C_{21}H_{24}N_2O_4 \cdot H_2O$: C, 59.64; H, 6.43; N, 6.62%); ν_{max} (KBr)/cm^{-1} 3339 (NH), 3200–2500br (NH_3^+), 2954 (CH), 1746 (C=O), 1693 (C=O), 1535 (NH), 1450 (CH), 1266 (C-O), 1149 (C-O); δ_H (400 MHz; DMSO-d_6) 1.40–1.60 (2H, m, CH_α-C*H*$_2$-CH_2-CH_2-NH), 1.70–1.90 (2H, m, CH_α-CH_2-C*H*$_2$-CH_2-NH), 3.00 (2H, q, *J* 6.4, CH_α-CH_2-CH_2-C*H*$_2$-NH), 3.74 (3H, s, OCH_3), 4.00–4.10 (1H, m, C*H*$_\alpha$-CH_2-CH_2-CH_2-NH), 4.21 (1H, t, *J* 6.8, C*H*-CH_2 Fmoc), 4.30 (2H, d, *J* 6.8, CH-C*H*$_2$ Fmoc), 7.34 (2H, t, *J* 7.4, aromatic CH Fmoc), 7.37 (1H, t, *J* 5.8, N*H*-Fmoc), 7.42 (2H, t, *J* 7.4, aromatic CH Fmoc) 7.69 (2H, d, *J* 7.4, aromatic CH Fmoc), 7.90 (2H, d, *J* 7.4, aromatic CH Fmoc), 8.53 (3H, br s, NH_3^+); δ_C (75 MHz; DMSO-d_6, assignments based on DEPT) 24.9 (CH_2), 27.4 (CH_2), 39.5 (CH_2), 46.7 (CH), 51.7 (CH), 52.7 (CH_3), 65.3 (CH_2), 120.1 ($CH_{Arom.}$), 125.1 ($CH_{Arom.}$), 127.0 ($CH_{Arom.}$), 127.6 ($CH_{Arom.}$), 140.7 ($C_{quat.\ arom}$), 143.9 ($C_{quat.\ arom}$), 156.1 (C=O), 169.8 (C=O); *m/z* (CI) 369 (MH^+, 15%), 207 (12), 179 (100), 165 (7), 113 (10), 79 (10).

N^{δ}-Fmoc-N^{α}-(thymin-1-ylacetyl)ornithine methyl ester 5 and N^{δ}-Fmoc-N^{α}-(uracil-1-ylacetyl)ornithine methyl ester 6

Fmoc-ornithine methyl ester hydrochloride (500 mg, 1.23 mmol) and CMT (0.251 g, 1.36 mmol) or CMU (0.255 g, 1.50 mmol) were dissolved in DMF (6 mL). TBTU (655 mg, 2.04 mmol) was added at room temperature, and DIEA was gradually added until basic pH (about 8) (total addition: 711 μL). After two hours under stirring at room temperature, the DMF was partly evaporated off and water was added to precipitate the product (**5**: 0.582 g, **6**: 0.503 g).

L-5 (87%) mp 164–166 °C (from water); $[\alpha]_D^{20}$ −5.8 (*c* 1 in DMF). **D-5** (87%) mp 163–165 °C (from water); $[\alpha]_D^{20}$ +5.0 (*c* 1 in DMF) (Found: C, 62.60; H, 5.67; N, 10.21. Calc for $C_{28}H_{30}N_4O_7$: C, 62.91; H, 5.66; N, 10.48%); ν_{max} (KBr)/cm^{-1} 3330 (NH), 3070 (CH), 2950–2920 (CH), 1680 (C=O), 1550 (δ NH), 1230 (C-O); δ_H (300 MHz; DMSO-d_6) 1.40–1.80 (4H, m, CH_α-C*H*$_2$-C*H*$_2$-CH_2-NH), 1.74 (3H, s, CH_3 thymine), 2.90–3.00 (m, 2 H, C*H*$_2$-NH), 3.63 (3H, s, CH_3O), 4.20–4.40 (4H, m, CH-CH_2 Fmoc + CH_α), 4.36 (2H, s, CH_2 acetyl linker), 7.25–7.35 (3H, m, aromatic CH Fmoc + NH-Fmoc), 7.35–7.45 (3H, m, CH thymine + aromatic CH Fmoc), 7.68 (2H, d, *J* 7.3, aromatic CH Fmoc), 7.88 (2H, d, *J* 7.4, aromatic CH Fmoc), 8.60 (1H, d, *J* 7.4, NH amide), 11.24 (1H, s, NH thymine); δ_C (75 MHz; DMSO-d_6, assignments based on DEPT) 11.9 (CH_3), 25.8 (CH_2), 28.4 (CH_2), 39.7 (CH_2), 46.8 (CH), 48.9 (CH_2), 51.78 (CH), 51.83 (CH_3), 65.3 (CH_2), 107.9 [$C_{Thy(5)}$], 120.1 ($CH_{Arom.\ Fmoc}$), 125.1 ($CH_{Arom.\ Fmoc}$), 127.1 ($CH_{Arom.\ Fmoc}$), 127.6 ($CH_{Arom.\ Fmoc}$), 140.7 ($C_{quat.\ arom}$), 142.4 [$C_{Thy(6)}$], 143.9 ($C_{quat.\ arom}$), 151.0 (C=O), 156.2 (C=), 164.5 (C=O), 167.2 (C=O), 172.2 (C=O); *m/z* (ESI) 535 (MH^+), 557 (MNa^+).

L-6 (76%) mp 159–161 °C (from water); $[\alpha]_D^{20}$ −5.9 (*c* 1 in DMF). **D-6** (76%) mp 155–157 °C (from water); $[\alpha]_D^{20}$ +5.6 (*c* 1 in DMF) (Found: C, 60.78; H, 5.76; N, 10.96. Calc. for $C_{27}H_{28}N_4O_7 \cdot \frac{1}{2}H_2O$: C, 61.24; H, 5.51; N, 10.58%); ν_{max} (KBr)/cm^{-1}3316 (NH), 3066 (CH), 2966 (CH), 1683 (C=O), 1550 (NH), 1266 (C-O); δ_H (300 MHz; DMSO-d_6) 1.30–1.80 (4 H, m, CH_α-C*H*$_2$-C*H*$_2$-CH_2-NH), 2.90–3.10 (2H, m, CH_α-CH_2-CH_2-C*H*$_2$-NH), 3.62 (3H, s, CH_3O), 4.10–4.30 (4 H, m, C*H*-C*H*$_2$ Fmoc + C*H*$_\alpha$), 4.39 (2H, s, CH_2-C=O), 5.55 (1 H, d, *J* 7.5, CH uracil), 7.27 (1H, s br, NH Fmoc), 7.32 (2H, t, *J* 7.3, aromatic CH Fmoc), 7.40 (2H, t, *J* 7.2, aromatic CH Fmoc), 7.51 (1H, d, *J* 7.5, CH uracil), 7.67 (2H, d, *J* 7.2, aromatic CH Fmoc), 7.86 (2H, d, *J* 7.3, aromatic CH Fmoc), 8.61 (1H, d, *J* 6.6, NH amide), 11.26 (1H, s, NH uracil); δ_C (75 MHz; DMSO-d_6, assignments based on DEPT) 25.7 (CH_2), 28.3 (CH_2), 39.6 (CH_2), 46.7 (CH), 49.1 (CH_2), 51.8 (CH), 51.9 (CH_3), 65.2 (CH_2), 100.4 [$CH_{Ur(5)}$] 120.0 ($CH_{Arom.}$), 125.1 ($CH_{Arom.}$), 127.0 ($CH_{Arom.}$), 127.6 ($CH_{Arom.}$), 140.7 ($C_{quat.\ arom}$), 143.8 ($C_{quat.\ arom}$), 146.6 [$CH_{Ur(6)}$)], 150.9 (C=O), 156.2 (C=O), 162.4 (C=O), 163.9 (C=O), 167.1 (C=O), 172.2 (C=O); *m/z* (ESI) 521 (MH^+, 100%).

N^{δ}-Fmoc-N^{α}-(thymin-1-ylacetyl)ornithine 2 and N^{δ}-Fmoc-N^{α}-(uracil-1-ylacetyl)ornithine 3

Route a. CMT (0.275 g, 1.5 mmol) or CMU (0.255 g, 1.5 mmol) and *N*-hydroxysuccinimide (0.170 g, 1.5 mmol) were dissolved in the minimum amount of DMF. The solution was cooled to 0 °C and DCC (0.310 g, 1.5 mmol) was added. After 15 minutes of stirring, the solution was allowed to warm at room temperature. After 5 hours the DCU was filtered off, washed with DMF, and N^{δ}-Fmoc-Orn **1** was added. The solution was stirred for 24 h, then was concentrated under vacuum. Water was added and the solution was stored at 5 °C. After several hours the precipitate was filtered off, washed with water, redissolved in DMF, and precipitated with diethyl ether to yield the pure product as a white solid (**2**: 0.550 g. **3**: 0.555 g. Average yield 70%).

Route b. To a suspension of 1.2 mmol of the methyl ester **5** (or **6**) in 39 mL of THF was added a solution of $Ba(OH)_2 \cdot 8H_2O$ (568 mg, 1.8 mmol) in water (39 mL). After 15 minutes the reaction was complete, as monitored by TLC (silica gel; eluent: methanol–dichloromethane 1 : 9). The pH was adjusted to 2 with HCl, the solvent was evaporated off, and the residue was dissolved in the minimum amount of DMF and precipitated with water. The solid was filtered off, dried under vacuum, redissolved in DMF (6 mL), filtered on a sintered glass funnel to eliminate insoluble residues, and dried under vacuum over P_2O_5. Yields: **2**: 0.813 g (99%). **3**: 0.752 g (99%). Gas chromatographic analysis (see below) showed the presence of 0.1–0.6% of the minor isomer.

L-2 mp 240 °C (decomp.), (from Et_2O); $[\alpha]_D^{20}$ −4.1 (*c* 1 in DMF). **D-2** mp 240 °C (decomp.), (from Et_2O); $[\alpha]_D^{20}$ +4.0 (*c* 1 in DMF) (Found: C, 59.10; H, 5.78; N, 10.39. Calc. for $C_{27}H_{28}N_4O_7 \cdot 1.5\ H_2O$: C, 59.23; H, 5.70; N, 10.23%); ν_{max} (KBr)/cm^{-1} 3600–2800br (OH), 3324 (NH), 3283 (NH), 3063 (CH), 2950 (CH), 1698 (C=O), 1674 (C=O), 1538 (NH), 1264 (C-O); δ_H (300 MHz; DMSO-d_6) 1.40–1.80 (4H, m, CH_α-C*H*$_2$-C*H*$_2$-CH_2-NH), 1.74 (3H, s, CH_3 thymine), 2.90–3.10 (2H, m, C*H*$_2$-NH), 4.10–4.30 (4H, m, CH-CH_2 Fmoc + CH_α), 4.34 (2H, s, CH_2 acetyl linker), 7.25–7.35 (1H, m, NH-Fmoc), 7.33 (2H, t, *J* 7.3, aromatic CH Fmoc), 7.41 (1H, s, CH thymine), 7.41 (2H, t, *J* 7.3, aromatic CH Fmoc), 7.68 (2H, d, *J* 7.3, aromatic CH Fmoc), 7.88 (2H, d, *J* 7.4, aromatic CH Fmoc), 8.46 (1H, d, *J* 7.7, NH amide), 11.2 (1H, s, NH thymine); δ_C (75 MHz; DMSO-d_6, assignments based on DEPT) 12.0 (CH_3), 26.0 (CH_2), 28.8 (CH_2), 40.0 (CH_2), 46.9 (CH), 49.1 (CH_2), 52.1 (CH), 65.4 (CH_2), 108.0 [$C_{quat\ Thy(5)}$], 120.2 ($CH_{Arom.\ Fmoc}$), 125.3 ($CH_{Arom.\ Fmoc}$), 127.2 ($CH_{Arom.\ Fmoc}$), 127.7 ($CH_{Arom.\ Fmoc}$), 140.9 ($C_{quat.\ arom}$), 142.5 [$CH_{Thy(6)}$], 144.1 ($C_{quat.\ arom}$), 151.1 (C=O),

156.3 (C=O), 164.6 (C=O), 167.1 (C=O), 173.4 (C=O); *m*/*z* (CI) 521 (MH^+, 1%), 281 (4), 207 (8), 179 (100), 165 (8), 115 (4).

L-3 mp 200 °C (from Et_2O); $[\alpha]_D^{20}$ −3.4 (*c* 1 in DMF). **D-3** mp 200 °C (from Et_2O); $[\alpha]_D^{20}$ +3.4 (*c* 1 in DMF) (Found: C, 61.37; H, 5.38; N, 11.05. Calc. for $C_{26}H_{26}N_4O_7$: C, 61.65; H, 5.17; N, 11.06%); ν_{max} (KBr)/cm^{-1} 3286 (NH), 3063 (CH), 2951 (CH), 1670 (C=O), 1538 (NH), 1256 (C-O); δ_H (300 MHz; DMSO-d_6) 1.40–1.80 (4H, m, CH_α-CH_2-CH_2-CH_2-NH), 2.98 (2H, q, *J* 5.7, CH_2-NH), 4.20–4.30 (4H, m, CH-CH_2 Fmoc + CH_α), 4.38 (2H, s, CH_2-C=O), 5.54 (1H, d, *J* 7.8, CH uracil), 7.30–7.40 (1H, m, NH-Fmoc), 7.33 (2H, t, *J* 6.7, aromatic CH Fmoc), 7.41 (2H, t, *J* 7.3, aromatic CH Fmoc), 7.53 (1H, d, *J* 7.8, CH uracil), 7.68 (2H, d, *J* 7.3, aromatic CH Fmoc), 7.88 (2H, d, *J* 7.4, aromatic CH Fmoc), 8.47 (1H, d, *J* 7.7, NH amide), 11.25 (1H, s, NH uracil); δ_C (75 MHz; DMSO-d_6, assignments based on DEPT) 25.9 (CH_2), 28.6 (CH_2), 39.8 (CH_2), 46.8 (CH), 49.1 (CH_2), 51.8 (CH), 65.2 (CH_2), 100.4 [$CH_{Uracil(5)}$], 120.1 ($CH_{Arom.\ Fmoc}$), 125.1 ($CH_{Arom.\ Fmoc}$), 127.0 ($CH_{Arom.\ Fmoc}$), 127.6 ($CH_{Arom.\ Fmoc}$), 140.7 ($C_{quat.\ arom}$), 143.9 ($CH_{quat\ arom}$), 146.6 [$C_{Uracil(6)}$], 150.9 (C=O), 156.1 (C=O), 163.8 (C=O), 166.8 (C=O), 173.1 (C=O); *m*/*z* (CI) 507 (MH^+, traces), 266 (10%), 207 (20), 196 (25), 179 (100).

H-(L-ThyOrn)$_5$GlyOEt 7

The pentamer was obtained by homogeneous peptide synthesis, through deprotection–coupling cycles. Typical procedures for each step will be reported.

First coupling with glycine ethyl ester. D- or **L-2** (0.120 g, 0.23 mmol) was dissolved in the minimum amount of DMF. The solution was cooled to 0 °C and GlyOEt·HCl (0.04 g, 0.28 mmol) and Et_3N (87 μL, 0.28 mmol) were added. After 15 minutes the solution was allowed to warm to room temperature and HBTU (0.11 g, 0.28 mmol) was added. After 2 hours, a few mL of water were added to quench the reaction and precipitate the product, which was then purified by preparative HPLC (C18 column, eluent MeOH–water 72 : 28) to yield 0.11 g of N^δ-Fmoc-N^α-(thymin-1-ylacetyl)ornithylglycine ethyl ester as a white solid (80%)

Deprotection. The compound obtained from the coupling reaction (for the first step, Fmoc-L-ThyOrn-GlyOEt) was dissolved in 15% piperidine–DMF solution, and stirred at rt for 10 minutes. The solution was evaporated to dryness. The residue was taken up in MeOH–Et_2O and isolated by centrifugation. Typical yield >99%.

Coupling. The compound obtained from the deprotection reaction was dissolved in the minimum amount of DMF. Fmoc-L-ThyOrn (1 eq.), HBTU (1 eq.) and Et_3N (1 eq.) were added. The solution was stirred at rt for 3 hours, then evaporated to dryness. The residue was taken up in MeOH–Et_2O and isolated by centrifugation. Yields for each coupling step were as follows: 1) 72%, 2) 83%, 3) 70%, 4) 76%.

7. MALDI-TOF: Calc. *M*, 1504.6. Found: MH^+, 1506.

Solid-phase synthesis of decamers H-(ThyOrn)$_{10}$-Gly-NH$_2$ 8 and H-(UrOrn)$_{10}$-Gly-NH$_2$ 9

The synthesis of **8** was carried out on a Shimadzu PSSM-8 peptide synthesizer and that of **9** manually in a glass reactor using the same Fmoc protocol. An MBHA-Rink amide resin (50 mg, 0.5 m eq. g^{-1}) was used with the following steps for each monomeric unit: 1) deprotection with 30% piperidine in DMF (500 μL; 8 min); 2) preactivation in the first step was obtained by adding to Fmoc-Gly (29.1 mg, 98 μmol) and TBTU (31.5 mg, 98 μmol) a 1 M solution of DIEA in DMF (147 μL, 147 μmol), a 0.5 M solution of HOBt in DMF (196 μL, 98 μmol) and mixing for 5 min at room temperature; in the following cycles the monomer **2** (51 mg, 98 μmol) or **3** (49.6 mg, 98 μmol) was used instead of Fmoc-Gly; 3) coupling for 30 min at rt; 4) capping with a solution of acetic anhydride (4.7%) and pyridine (4%) in DMF (1 min). All steps were repeated twice and the resin was washed five times with 500 μL of DMF after each step. Cleavage from the resin was achieved by treatment with a TFA–water 95 : 5 mixture for 4 h. The products were then precipitated with diethyl ether, centrifuged, and washed four times with diethyl ether [yield: **8** 63 mg (88%), **9** 63 mg (92%)]. The crude product was purified by HPLC using a reversed-phase RP-18 column Spherisorb 10 ODS (30 × 1 cm), by gradient elution (eluent A: water; eluent B: 0.05% TFA in acetonitrile, gradient from 90% A to 100% B).

8 MALDI-TOF: Calc.: *M*, 2876.9. Found: MH^+, 2875.

9 MALDI-TOF: Calc.: *M*, 2736.6. Found: MH^+, 2741.

Analysis of epimerization during solid-phase peptide synthesis

Analysis of steps. The steps reported above for the decamer synthesis were repeated on 50 mg of MBHA-Rink amide resin with Fmoc-Gly, and then twice with **L-3** to obtain the dimer **10**. After each step involving the chiral monomer, one-sixth of the resin was separated and treated with a TFA–water 95 : 5 mixture for 4 h. These solutions were dried under vacuum, and the residue was treated as reported in the following section for GC chiral analysis.

Monomer coupling. In a typical experiment 10 mg of the MBHA-Rink amide resin (corresponding to 5 μeq. of reactive sites) were used for the evaluation of racemization during monomer coupling. After treatment with 30% piperidine in DMF, the coupling reaction was carried out with the monomer **L-3** using the following protocols.

Protocol a. The monomer **L-3** (10 mg, 20 μmol) and the coupling agent (HBTU or HATU, 20 μmol) were dissolved in 100 μL of DMF; then 20 μL of a 1 M solution of the base (DIEA or TMP) in DMF were added. The mixture was stored at rt for 5 min before being introduced into the reactor, and was then stirred for 30 min.

Protocol b. The monomer **L-3** (10 mg, 20 μmol) and the coupling agent (HBTU or HATU, 20 μmol), were dissolved in 100 μL of DMF and stored at rt for 5 min; then it was introduced into the reactor. Immediately, 20 μL of a 1 M solution of the base (DIEA or TMP) were added, and the reaction mixture was stirred for 30 min.

Protocol c. The monomer **L-3** (10 mg, 20 μmol) and the coupling agent (HBTU or HATU, 20 μmol), were dissolved in 100 μL of DMF and stored at rt for 5 min; then it was introduced into the reactor. Immediately 5 μL of a 1 M solution of the base (DIEA or TMP) were added, to start the reaction, and the reaction mixture was stirred. Three other 5 μL portions of the 1 M solution of the base (DIEA or TMP) were added, one every 15 min. The overall reaction time was 1 h. After each reaction, the ornithine monomer was deprotected with 30% piperidine. The absorbance of the resulting solution was measured at 301 nm, and compared with that obtained by the deprotection of the resin, in order to calculate the yield. The monomer was then cleaved from the resin as reported above, and subjected to chiral analysis.

Synthesis of optically pure L-9. This was carried out by using standard coupling (HBTU, DIEA with 5 min preactivation) for the first glycine residue, and **protocol c** for all ornithine monomers. Yields for each step were: 1(gly): 95%; 2) 93%; 3): 82%; 4) >99%; 5) 63%; 6) 82%; 7) 78%; 8) >99%; 9) 71%; 10) 71%.

Enantiomeric analysis by GC. Hydrolysis of the monomers and of the products obtained by SPPS (typical sample: 2 mg) was performed in a 2 mL solution of 6 M HCl for 6 h at 100 °C. The solution was then dried under vacuum, and the residue was treated with 4 mL of 2 M HCl in propan-2-ol and heated in a screw-cap test-tube (90 °C for 1 h); after evaporation of the

mixture, the sample was treated with 0.3 mL of trifluoroacetic anhydride and 2 mL of CH_2Cl_2, and heated in a screw-cap test-tube (60 °C for 1 h); the solution was evaporated and the residue was redissolved in 0.5 mL of CH_2Cl_2.

Chiral GC analysis was performed on a Phe-3-O-TA column (20 m; ID 0.3 mm; film thickness 0.2 μm), on a DANI 3900 instrument (detector FID), with the following temperature program: 120 °C for 3 min, 120–160 °C (3 °C min^{-1}), 160 °C final isotherm; the retention times were t_D 20 min, and t_L 21 min. Identification of the peaks was performed by injection of standard samples of D- and L-ornithine derivatives, and confirmed by GC-MS. Standard deviation of D/(D + L) or L/(D + L)% for this chromatographic system was 1%.

Chiral GC-MS analyses were performed on a Hewlett-Packard HP 6890 instrument, equipped with a 5973 Mass Selective Detector (quadrupole analyzer), on a Chirasil-Val (25 m, ID 0.25 mm; film thickness 0.12 μm), in scan mode for the identification of peaks, and in SIM mode (*m/z* 166, 306, 355) for quantitation (in order to achieve best signal-to-noise ratio). The temperature program was the following: 120 °C for 1 min, 120–175 °C, (4 °C min^{-1}), 175 °C final isotherm. Retention times were t_D 14.8 min for D-ornithine and t_F 15.5 min for L-ornithine. Standard deviation of D/(D + L) or L/(D + L)% for this chromatographic system was 0.2%, with a detection limit of 0.1%.

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