Articles

Nonapeptide Analogues Containing (*R*)-3-Hydroxybutanoate and *β*-Homoalanine Oligomers: Synthesis and Binding Affinity to a Class I Major Histocompatibility Complex Protein

Sorana Poenaru,[‡,§] José R. Lamas,[†] Gerd Folkers,[||] José A. López de Castro,[†] Dieter Seebach,*[,‡] and Didier Rognan*[,||]

Laboratory for Organic Chemistry, Swiss Federal Institute of Technology, Universitätstrasse 16, CH-8092 Zürich, Switzerland, Centro de Biologia Molecular "Severo Ochoa", Facultad de Ciencias, Universidad Autonoma de Madrid, E-28049 Madrid, Spain, and Department of Pharmacy, Swiss Federal Institute of Technology, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

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Crystal structures of antigenic peptides bound to class I MHC proteins suggest that chemical modifications of the central part of the bound peptide should not alter binding affinity to the MHC restriction protein but could perturb the T-cell response to the parent epitope. In our effort in designing nonpeptidic high-affinity ligands for class I MHC proteins, oligomers of (*R*)-3-hydroxybutanoate and(or) *β*-homoalanine have been substituted for the central part of a HLA-B27-restricted T-cell epitope of viral origin. The affinity of six modified peptides to the B*2705 allele was determined by an in vitro stabilization assay. Four out of the six designed analogues presented an affinity similar to that of the parent peptide. Two compounds, sharing the same stereochemistry (*R*,*R*,*S*,*S*) at the four stereogenic centers of the nonpeptidic spacer, bound to B*2705 with a 5−6-fold decreased affinity. Although the chiral spacers do not strongly interact with the protein active site, there are configurations which are not accepted by the MHC binding groove, probably because of improper orientation of some lateral substituents in the bound state and different conformational behavior in the free state. However we demonstrate that *β*-amino acids can be incorporated in the sequence of viral T-cell epitopes without impairing MHC binding. The presented structure−activity relationships open the door to the rational design of peptide-based vaccines and of nonnatural T-cell receptor antagonists aimed at blocking peptide-specific T-cell responses in MHC-associated autoimmune diseases.

Introduction

Class I major histocompatibility complex (MHC)-encoded proteins play a key role in the intracellular immune surveillance by selectively binding to intracellular peptide antigens and presenting them at the cell surface to T-cell receptors (TCRs) of cytotoxic T-lymphocytes (CTL).[1] Due to the genetically encoded discrepancy between the limited number of class I alleles (about six) expressed by each individual and the infinite number of potential antigenic peptides (usually nonamers), class I MHC molecules must bind diverse sets of foreign peptides with a broad specificity but a high affinity. Numerous structural data on class I MHC−peptide complexes are nowadays available at the three-dimensional level[2] and provide an explanation for that paradigm. The 27 reported X-ray structures (for nine different class I MHC molecules) illustrate a peptide-independent recognition in which both terminal ends of the peptide backbone are tightly bonded to conserved residues of the MHC binding groove. Allele specificity is ensured by the interaction of anchoring side chains,[3,4] usually at positions P2, P3 (P*n* standing for position *n*), and the C-terminus with polymorphic pockets[5] of the host MHC protein. The central part of the bound peptides (from positions 4 to 8) generally zigzags[6] or bulges[7] out of the binding groove and thus allows variation in the length of the bound peptides (from 8 to 11 amino acids). Systematic peptide mutation[8] and X-ray structure of MHC−peptide−TCR ternary complexes[9−12] show that this central part whose conformation is not complementary to that of the MHC protein is the major contact area for *αβ* TCRs that trigger the T-cell response to the foreign peptide.

The tight association observed between MHC expression and susceptibility or resistance to autoimmune disorders led us to consider class I MHC proteins as particularly interesting targets for the selective immunotherapy of autoimmune diseases. At least two ways of shunting the T-cell response to autoantigens using small-molecular-weight molecules have been proposed. The first one involving MHC blockade by a high-affinity

* To whom correspondence should be addressed. D. Seebach: fax, +41.1.632 11 44; e-mail, seebach@org.chem.ethz.ch. D. Rognan: fax, +41.1.635 68 84; e-mail, didier@pharma.ethz.ch.
‡ Laboratory for Organic Chemistry, SFIT.
† Universidad Autonoma de Madrid.
|| Department of Pharmacy, SFIT.
§ Present address: Department of Chemistry, University of California, Berkeley, CA 94720-1460.

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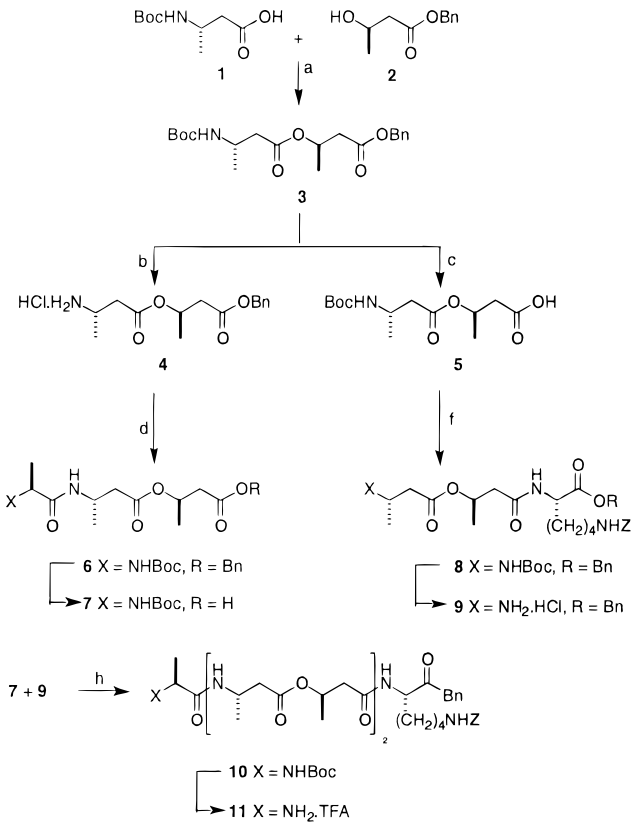
competitor[13] is unlikely as MHC-bound peptides at the cell surface are almost impossible to displace.[14] The only way to overcome this drawback would be to supply the peptide competitor in liposomes[15] or as lipopeptides[16] into the endoplasmic reticulum where assembly of the class I MHC−peptide complexes takes place. The second inhibiting pathway relying on TCR antagonism[17] suggests that the presentation of the epitope to autoreactive T-cells would be antagonized by a modified peptide analogue. This approach is much more promising since only a few TCRs at the surface of CTLs need to be targeted,[18] whereas MHC blockade requires saturation of all MHC binding sites at the cell surface.[19] Two prerequisites are however necessary for designing TCR antagonists: (i) a good affinity to the MHC−restriction protein, (ii) a fast dissociation of the corresponding MHC−ligand complex to the TCR.[20,21] The few TCR antagonists known to date are all peptide analogues for which one TCR-anchoring amino acid has been mutated.[22] Unfortunately, the poor stability and pharmacokinetic properties inherent to their peptidic nature preclude their general use as immunosuppressors. Thus, there is a need for designing high-affinity nonpeptide ligands for class I MHC proteins. Rather few variations around the canonical nonapeptide structure have been described up to date.[23] Peptides bearing unnatural L- or D-α-amino acids at MHC-anchoring positions,[24−29] reduced peptide bond pseudopeptides,[30] retroinverso analogues,[31] poly-N-acylated amines,[32] or incorporation of a β-homoglycine residue at the peptide N-terminus[33] have been reported. An alternative strategy we initiated 3 years ago is to replace the central TCR-binding amino acids by various nonpeptidic spacers: oligomers of aminoalkanoates,[24,34] phenanthridine derivatives,[35] or poly(ethylene glycol) loops.[36] All these chemical modifications led to ligands that could associate with class I MHC proteins but always with a slight decrease in binding affinity when compared to that of the parent peptides.

We recently described the replacement of a natural pentameric peptide sequence (from positions P4 to P8) by (*R*)-3-hydroxybutanoate (*R*-HB) oligomers in HLA-B27-binding nonapeptides[37] while enhancing 5-fold the binding affinity for the MHC restriction protein.[34] However, the partial hydrolysis of oligo-HB ester bonds, observed during the synthesis, suggests that these analogues should have very poor in vivo pharmacokinetic properties because of their high sensitivity to esterases and peptidases. Recent reports on the remarkable enzymatic stability of β-peptides[38,39] led us to consider oligomers of β-amino acids as potential surrogates for the TCR-binding residues of class I MHC-binding peptides. Since β-homoalanine (β-HAla) is an isostere of HB, binding to HLA-B27 should thus be retained in light of our previous results on poly(ester peptides) (PEPs).[34] However, the low solubility of protected β-HAla oligomers in any solvent[40] could be a drawback to the synthesis and biological evaluation of these compounds. To increase the solubility in water of compounds containing four β-HAla units,[41] a positively charged peptide epitope from the HIV-1 gp120 protein (G^{314}RAFVTIGK^{322}, one-letter amino acid code), known to bind well to HLA-B*2705,[34] was chosen as template for the reported chemical modifications (Table 1). Furthermore, combining β-HAla and HB oligomers should enhance the solubility of the resulting spacers in chlorinated solvents[42] and thus facilitate their synthetic access. Most of the designed peptide analogues bind indeed with a high affinity to a class I MHC protein (HLA-B*2705 allele), whose expression is associated with susceptibility to severe autoimmune diseases.[43]

Scheme 1[a]



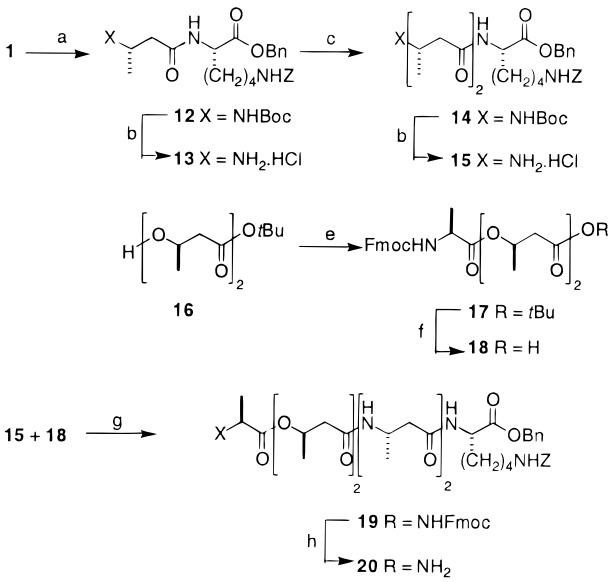
[a] (a) DCC, DMAP, CH_2Cl_2; (b) HCl/ether (satd); (c) H_2, Pd/C, MeOH; (d) Boc-Ala-OH, HOBt, EDC, Et_3N, CH_2Cl_2; (e) H_2, Pd/C, MeOH; (f) HCl•H-Lys(Z)-OBn, HOBt, EDC, Et_3N, CH_2Cl_2; (g) HCl/dioxane (satd); (h) HOBt, EDC, DIEA, CH_2Cl_2; (i) TFA/CH_2Cl_2, 1:1.

Results and Discussion

Chemistry. Synthesis of the derivative **11** was achieved using a fragment-type coupling strategy (Scheme 1). Boc-protected β-homoalanine[38,44] and benzyl 3-hydroxybutanoate (**2**)[45] were coupled using DCC and DMAP as activating reagents[46] to give **3**. Deprotection of the amino group under acidic conditions gave the amino ester **4**, whereas hydrogenolysis of the benzyl ester gave acid **5**. Coupling of **4** with the commercially available Boc-protected alanine under traditional HOBt/EDC peptide conditions[47−49] gave the fully protected derivative **6** whose benzyl ester group was cleaved by H_2 (Pd/C) to yield acid **7**. ^{1}H NMR measurements led to assignment of all signals to the corresponding protons of the α-amino acid, of the HB unit, and of the β-HAla moiety.

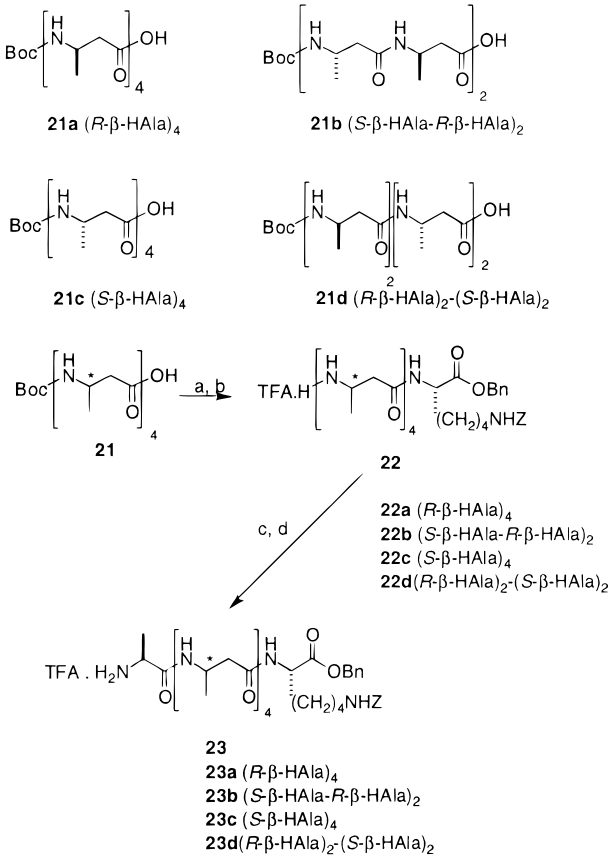
To obtain the second fragment **9** with free amino and protected carboxy group, the acid **5** was coupled with H-Lys(Z)-OBn, using the HOBt/EDC strategy to give **8** (80%), treatment of which with a saturated HCl/dioxane solution yielded the corresponding HCl salt **9**. Compound **10**, consisting of six building blocks, was then

Scheme 2[a]



[a] (a) HCl•H-Lys(Z)-OBn, HOBt, EDC, Et_3N; (b) HCl/dioxane (satd); (c) **1**, HOBt, EDC, DIEA, DMF; (d) HCl/dioxane (satd); (e) Fmoc-Ala-OH, DCC, DMAP, CH_2Cl_2; (f) TFA/CH_2Cl_2, 1:1; (g) HOBt, EDC, DIEA, CH_2Cl_2/DMF, 1:1; (h) Et_2NH/DMF, 1:4.

Scheme 3[a]

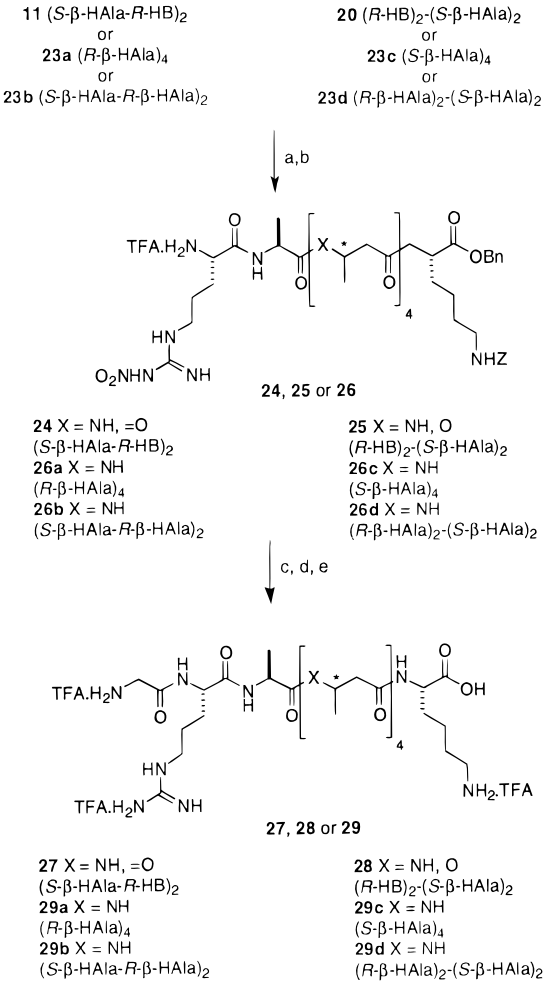


[a] (a) HCl•H-Lys(Z)-OBn, HOBt, EDC, DIEA, DMF; (b) TFA, 10 min; (c) Boc-Ala-OH, HOBt, EDC, DIEA, DMF; (d) TFA, 10 min.

obtained in 90% yield by coupling of **7** with **9**.[50] Subsequent cleavage of the Boc protecting group led to the amino ester **11** which was used for the next coupling with arginine, without further purification (Scheme 4).

The derivative **20**, with HB and β-HAla incorporated in different sequence (Scheme 2), was synthesized by

Scheme 4[a]



[a] (a) Boc-Arg(NO_2)-OH, HOBt, EDC, DIEA, DMF; (b) TFA, 10 min; (c) Boc-Gly-OH, HOBt, EDC, DIEA, DMF; (d) H_2, Pd/$BaSO_4$, TFE/AcOH, 4:1; (e) TFA, 10 min.

application of the same fragment coupling strategy. The fragment **15** was synthesized in a linear fashion, coupling first Boc-β-HAla-OH (**1**) with H-Lys(Z)-OBn to give dipeptide **12** (85%) which was deprotected to give the HCl salt **13** (HCl in dioxane). The amino functionality of **13**, set free in situ by the base present in the reaction mixture, was coupled with **1** to give the fully protected compound **14** (74% from **12**). The Boc group of **14** was cleaved to yield **15**. The fragment **17** was obtained by ester formation between the hydroxy dimer **16**[37,51] and Fmoc-protected alanine (using DCC, DMAP), and cleavage of the *tert*-butyl ester group (50% TFA) gave the desired compound **18**. It should be noted that by using a small amount of DMAP (0.05 equiv), no racemization was observed upon coupling.[37] The amino functionality, generated by in situ deprotonation of the HCl salt **15**, was coupled with the acid group of **18** (HOBt/EDC) to give the fully protected compound **19** in 92% yield. The Fmoc protecting group was then cleaved (20% diethylamine in DMF[52]) to give the amino ester **20**. It is noteworthy that the backbone of compound **19** varies from that of **10** only by the respective positions of HB and β-HAla in the sequence. However their respective solubilities in organic solvents are very different. Compound **10** is highly soluble in chlorinated

Table 1. Binding and Analytical Properties of Ligands **27**, **28**, and **29a−d**

compd	sequence	C_{50}[a] (μmol)	HPLC (t_R, min)[b]	MS[c] $(M+1)^+$
HIV gp120[d]	Gly-Arg-Ala-**[Phe-Val-Thr-Ile-Gly]**-Lys	2.8		
A68P1[e]	Glu-Val-Ala-Pro-Pro-Glu-Tyr-His-Arg	nb[f]		
27	Gly-Arg-Ala-**[(*S*-β-HAla-*R*-HB)$_2$]**-Lys[g]	2.8	18.7	773.3
28	Gly-Arg-Ala-**[(*R*-HB)$_2$-(*S*-β-HAla)$_2$]**-Lys	17.0	19.5	774.1
29a	Gly-Arg-Ala-**[(*R*-β-HAla)$_4$]**-Lys	2.8	16.4	771.9
29b	Gly-Arg-Ala-**[(*S*-β-HAla-*R*-β-HAla)$_2$]**-Lys	4.8	15.4	772.6
29c	Gly-Arg-Ala-**[(*S*-β-HAla)$_4$]**-Lys	6.0	15.3	771.9
29d	Gly-Arg-Ala-**[(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$]**-Lys	30.0	15.4	772.0

[a] Concentration of ligand at which HLA-B*2705 fluorescence (measured by FMC analysis with an anti-B27 monoclonal antibody) on RMA-S cells was half the maximum obtained with that compound (see Experimental Section). [b] HPLC purification using a gradient of A (0.1% trifluoroacetic acid in water) and B (acetonitrile): 0−100% B, 60 min. [c] MALDI-TOF spectra, recorded on a Bruker Biflex instrument (Bruker-Franzen Analytik, Bremen, Germany) in linear mode. [d] HIV-1 glycoprotein 120 (314−322). [e] Self-peptide eluted from the HLA-A68 allotype.[8] [f] No detectable binding at 10^{-4} M. [g] β-HAla, β-homoalanine; HB, 3-hydroxybutanoate.

solvents such as CH_2Cl_2, whereas **19** is poorly soluble even in DMF.

The configurational isomers (diastereoisomers) **21a−d** containing four β-HAla units (Scheme 3) have been previously prepared, starting from (*S*)- and (*R*)-Boc-Ala-OH and using a fragment coupling strategy.[40] These oligomers are difficult to handle because of their low solubility in any solvent tested so far. For example, the ^{1}H NMR spectra of protected β-HAla tetramers can only be measured using dimethyl-d_6 sulfoxide as solvent and show rather broad signals. During the synthesis, larger amounts of DMF were necessary to dissolve the β-HAla oligomers (c = 0.05 M), as compared to those required in α-peptide synthesis, and reaction times were consequently longer. Therefore, the yields of the reactions are not specified, since no purification is possible before the last deprotection step, due to the poor solubility of this class of compounds.

The acids **21** were coupled with H-Lys(Z)-OBn using HOBt/EDC, to provide the fully protected derivatives, the Boc protecting groups of which were cleaved (concentrated TFA) to give the TFA salts **22** which were precipitated with ether and dried in high vacuum before the next reaction step: deprotonation and reaction with Boc-Ala-OH to yield, after another deprotection step, the corresponding TFA salts **23**.

During the cleavage of the Boc group, the Z protecting group of the lysine side chain was partially cleaved, giving rise to a byproduct (≤5%) which has been detected by mass spectrometry. Such debenzylations have been previously reported by Merrifield et al.[53] However, considering the much harsher conditions used by the Merrifield group, the observed loss of the Z group, in our case, was surprising. Due to solubility problems, it was impossible to purify the intermediates at this stage. Thus, we have carried the byproduct all along the following synthetic steps, with the consequence that some additional impurities were formed. After the last deprotection step, preparative HPLC purification still gave the desired pure compounds (**27**−**29**). The amino functionalities of the derivatives **11**, **20**, and **23a−d** (Scheme 4) were allowed to react with Boc-Arg(NO_2)-OH,[54] using the same coupling procedure as for the other coupling steps. Again, the Boc groups of the fully protected derivatives were cleaved with TFA to yield compounds **24**, **25**, and **26a−d** which, in turn, were coupled with Boc-Gly-OH to give the fully protected nonapeptides analogues. The NO_2 and Z protecting groups, as well as the benzyl ester group, were then simultaneously removed by hydrogenation, using Pd/$BaSO_4$ as catalyst.[56] Subsequent treatment with TFA led to cleavage of the Boc groups to give, after HPLC purification, the desired nonapeptide analogues **27**, **28**, and **29a−d** which were used for binding assays.

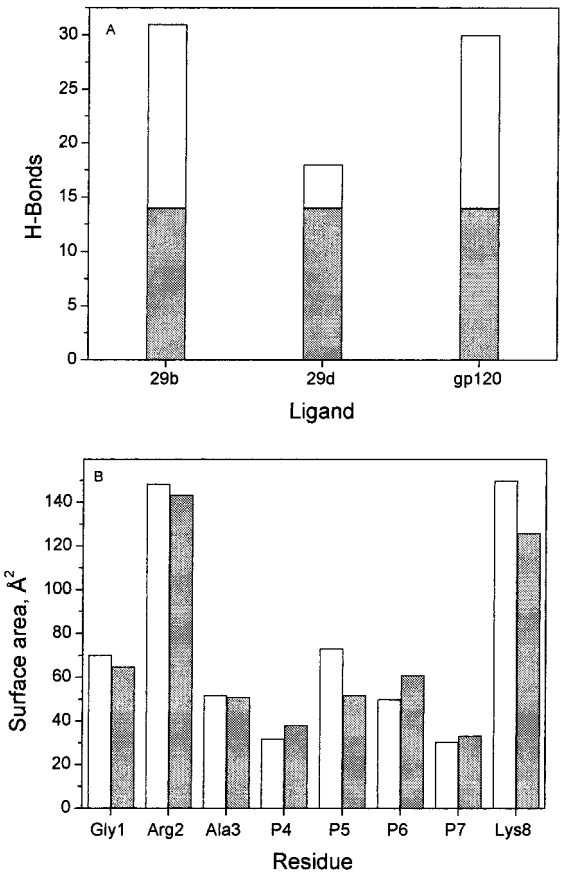
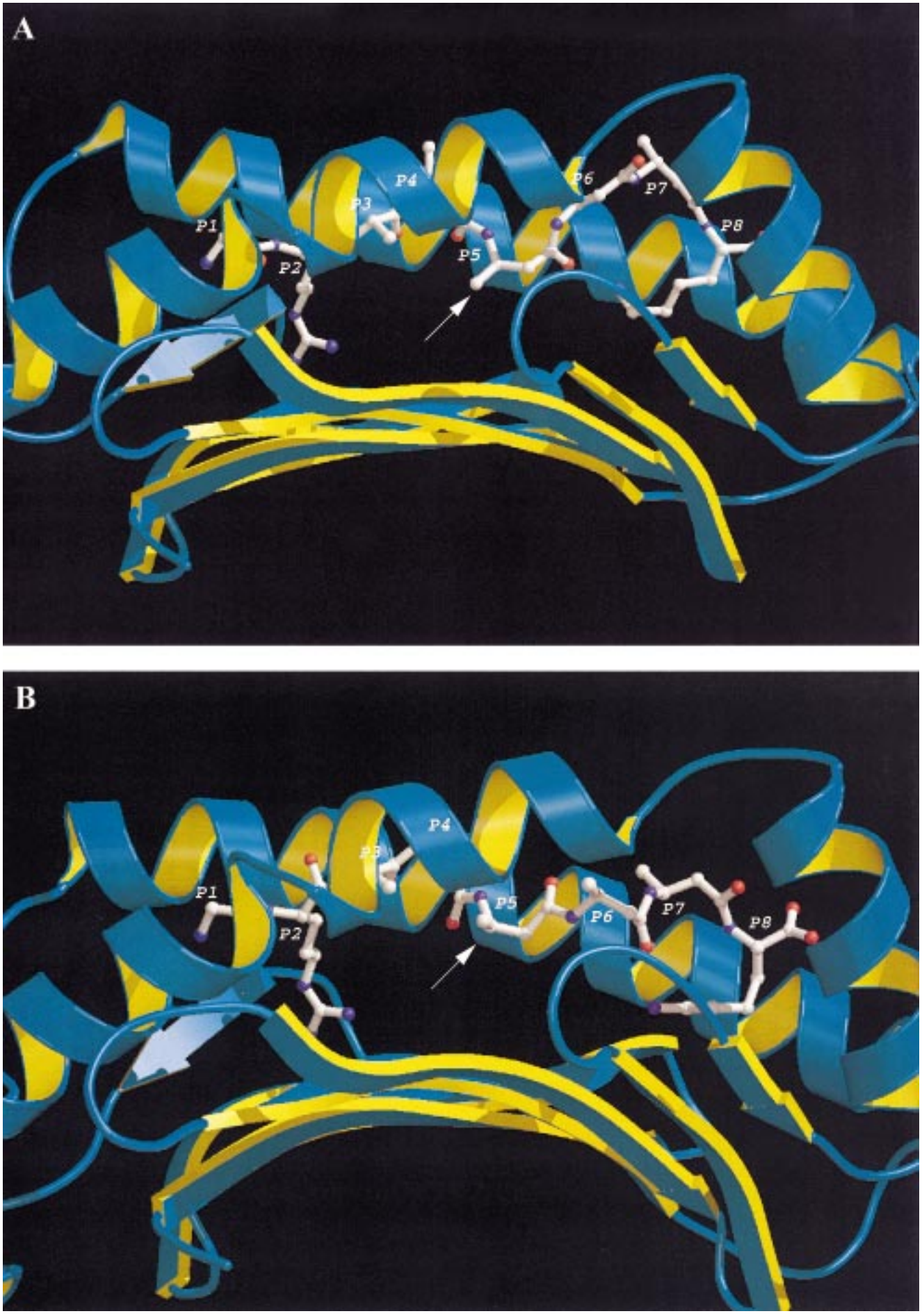


Figure 1. Dynamic properties of complexes of B*2705 with two modified peptides (**29b, 29d**) and the reference HIV-1 gp120 (314−322) peptide. (A) Intermolecular hydrogen-bond frequencies, recorded for the whole 500-ps trajectory of HLA-B27−ligand complexes over 1000 conformations. Frequencies between 25% and 50% and higher than 50% are displayed as white and gray columns, respectively. (B) Buried surface areas of HLA-B27-bound ligands **29b** (white columns) and **29d** (gray columns) calculated on energy-minimized time-averaged conformations. Surface areas were calculated using the MS program[74] with a 1.4-Å radius probe.

Binding Affinity to HLA-B*2705. The binding affinities of the modified peptides clearly show that the chirality of the spacer is important for recognition of the B*2705 protein. Compounds in which the chiral building block linked with the C-terminus (PC) has (*R*)-configuration (**27**, **29a−b**) were all potent ligands with


A
P1
P2
P3
P4
P5
P6
P7
P8
B
P1
P2
P3
P4
P5
P6
P7
P8

binding affinities similar to that of the parent HIV-1 gp120 peptide (Table 1). This observation is in agreement with our previous report on PEPs for which a penultimate (*R*)-HB unit was proposed to interact with the MHC binding groove.[34] Analogues bearing a moiety of (*S*)-configuration next to PC were less active, especially compounds **28** and **29d** sharing the same sequence of (*R*,*R*,*S*,*S*)-configuration of the spacing oligomers (Table 1). However, an (*S*)-chiral spacer attached to PC does not necessarily prevent binding (see compound **29c**, Table 1). Furthermore, it seems that certain configurations of the four spacing monomers are detrimental to binding. Thus, the two weakest binders (**28**, **29d**) share the same (*R*,*R*,*S*,*S*)-configuration at positions 4 to 7. Replacing ester by amide groups in the spacer (*cf.* **27** with **29b** and **28** with **29d**) did not affect binding for both high-affinity and low-affinity ligands. This result is in agreement with the available X-ray structure[56] of a B*2705−nonapeptide complex, showing weak contributions of peptide bonds, located between the P4 and P8 positions, to the binding of a nonapeptide to HLA-B27.

Apart from binding potencies, it should be noticed that HB-containing compounds **27** and **28** are probably still sensitive to esterases, although stability studies on these compounds have not been performed yet. We also expect that the replacement of HB oligomers by β-amino acids in analogues **29a**−**d** enhances the resistance of the modified peptides to enzymatic degradation.

Molecular Modeling of B*2705−Ligand Complexes. To find a rational explanation for the weak binding of compounds with (*R*,*R*,*S*,*S*)-configuration of the chiral spacer, a 500-ps molecular dynamics (MD) study of the binary complexes between B*2705 and three ligands was undertaken. Compound **29b** was chosen as representative of the high-affinity peptides, whereas **29d** was selected as representative of weak binding ligands. The parent HIV peptide was selected as reference. The trajectory of the three solvated complexes was stable after 350 ps, with rms deviations of the protein backbone from the starting X-ray coordinates of ca. 1.5 Å (data not shown). We previously used atomic fluctuations of the bound ligands, as a criterion, for discriminating high-affinity from low-affinity peptides.[24,33,57] In the present case, they were very similar for ligands **29b** and **29d**. Thus, subtle differences must cause the very different binding affinities of the two modified peptides. In fact, recording the frequency of the MHC−ligand hydrogen bonds allows to distinguish the two modified peptides. High-affinity ligands (HIV gp120, **29b)** present many more hydrogen bonds to the HLA-B27 binding groove than the weak binding compound **29d** (Figure 1A). Strong H-bonds with a frequency higher than 50% were remarkably identical in both cases, but medium H-bonds (with frequencies between 25% and 50%) are significantly in favor of **29b**. A very similar pattern has already been observed for a set of four natural peptides binding to two closely related HLA-B27 alleles.[33] The major differences between the two nonnatural complexes could be correlated with the H-bond-donating strength of the N-terminus, well-known to significantly contribute to the binding free energy of nonapeptides to class I MHC proteins.[58] The buried surface areas of each monomer of the protein-bound ligand were also very similar with the exception of two residues P5, and PC (Figure 1B). P5 corresponds to the second unit of the spacer (*R*-βHAla in both cases). Depending on its environment in the sequence of the modified peptide, it is more or less deeply buried in the HLA-B27 binding groove. With compound **29b**, the P5 position is significantly deeper inside the groove than with compound **29d** (compare Figure 2A,B). However, this feature is unlikely to induce nearly a 10-fold difference in the binding affinity of the corresponding ligands. The C-terminal amino acid (Lys) also shows a better fit in the case of the high-affinity ligand (Figure 1B). As the C-terminal residue is an important anchor to B*2705,[56] this structural difference should also contribute to the improved binding of **29b** versus **29d**.

However, the computed properties of the two analogues bound to their target protein can only explain a part of the experimentally determined difference of binding affinities. The modeling study presented here takes into account only enthalpic contributions to the binding of each ligand to HLA-B*2705. As desolvation energies and rotational/translational entropy losses upon binding (assuming a conserved binding mode of the two compounds) should be very similar due to the structural analogy of all modified peptides listed in Table 1, the 10-fold decreased binding of two analogues (**28**, **29d**), having the same configuration, may be due to different association rates and different conformational populations in the free state. This feature has already been experimentally described for two related PEPs,[34] for which the length of the polyester spacer varies. Hydrophobic β-peptides are known to have conformations strongly depending on the chirality of their residues and on the nature of their side chains.[38,59] The (*R*,*R*,*S*,*S*)-configuration of four chiral monomers in low-affinity ligands might lead to a conformational space arrangement in the free state that is different from that of high-affinity compounds (**27, 28, 29a**−**c**). The higher "strain energy" necessary to bring ligands **28** and **29d** from the free to the bound state may partially contribute to the weaker binding of these two analogues. Unfortunately, simulating the free ligands, although computationally easier, is very risky because they adopt no stable secondary structure, as concluded from their CD or NMR spectra.

Conclusion

Replacing the central amino acids of class I MHC-binding peptides by (*R*)-3-hydroxybutanoate and(or) β-homoalanine oligomers leads to still high-affinity ligands. Up to now, β-amino acids have hardly been used in medicinal chemistry. Some natural β-amino acids (taurine, β-aminobutyric acid, β-aminoisobutyric acid)

Figure 2. Three-dimensional structure of HLA-B*2705 in complex with **29b** (A) and **29d** (B). Peptide positions are labeled at the Cα atom from 1 (P1) to 8 (P8). The backbone trace of the MHC antigen-binding domain (α1, α2) of the B*2705 protein is represented as bands (α helices), arrows (β strands), and tubes (coil). Altered peptides are displayed by a ball-and-stick model. A white arrow indicates the position of the second β-HAla unit in both chiral spacers. The figure has been prepared using the MOLSCRIPT[75] and RASTER3D[76] programs.

have been reported as agonists of the inhibitory glycine receptor.[60] Substituted β-amino acids have also been described as fibrinogen receptor GIIb/IIIa antagonists,[61] β-lactamase inhibitors,[62] μ-opioid receptor agonists,[63] or enkephalin-degrading enzyme inhibitors.[64] Furthermore, various β-amino acids are found in natural antibiotics, fungicides, and antineoplastic compounds.[65] However, to the best of our knowledge, this is the very first report of biologically active molecules containing β-amino acid oligomers. The present study demonstrates that β-amino acids are valuable tools, indeed, for designing peptidomimetics of bioactive peptides. By contrast to most α-amino acid surrogates, the H-bonding properties of backbone atoms, the backbone direction, or the side chain directionality might be similar in natural and β-peptides, at the condition that the β-peptide can adopt the biologically active conformation of its natural α-peptide analogue. Thus, if all side chains are not mandatory for biological activity, β-amino acids and, more generally, β-peptides undoubtedly represent new promising tools in medicinal chemistry. In the special case of class I MHC ligands, one might imagine to use β-amino acids for replacing MHC anchors and(or) TCR contact residues. Such altered peptides may thus lead to peptide-based vaccines and TCR antagonists, which would be stable to all common peptidases tested so far, including pronase, 20S proteasome, and proteinase K.

Experimental Section

Abbreviations: (*R*)-β-homoalanine (*R*-β-HAla), (*S*)-β-homoalanine (*S*-β-HAla), dicyclohexylcarbodiimide (DCC), diisopropylethylamine (DIEA), 4-(dimethylamino)pyridine (DMAP), dimethylformamide (DMF), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), 1-hydroxy-1*H*-benzotriazole (HOBt), trifluoroacetic acid (TFA), trifluoroethanol (TFE), (*R*)-3-hydroxybutanoate (*R*-HB).

Chemistry. Dichloromethane (CH_2Cl_2) was dried over 4-Å molecular sieves. Solvents for chromatography and workup procedures were distilled from Sikkon (anhydrous $CaSO_4$, Fluka). Triethylamine (Et_3N) was distilled from CaH_2 and stored over KOH. Amino acid derivatives were purchased from Bachem or Senn. All other reagents were used as received from Fluka.

^1H (300-MHz) and ^{13}C (75-MHz) NMR spectra were recorded on a Varian Gemini 300 spectrometer and are reported in ppm on the δ scale from TMS. Coupling constants are reported in hertz (Hz). FAB-MS spectra were obtained with a Hitachi Perkin-Elmer RHU-6M using a 3-nitrobenzyl alcohol (3-NOBA) matrix. Elemental analyses were performed by the Microanalytical Laboratory of the Laboratorium für Organische Chemie, ETH-Zürich (only analyses above 0.4% were given).

Chromatography generally refers to flash silica gel 60 (Fluka 40−63 mm) and TLC (Merck Kieselgel 60 F_{254} plates), detection with UV and ninhydrin. HPLC analyses were carried out on a C18 analytical column on a Knauer HPLC system (pump type 64, EuroChrom 2000 integration package, degaser, UV detector (variable-wavelength monitor)) using a linear gradient of (A) 0.1% CF_3COOH in H_2O and (B) MeCN at a flow rate of 1 mL/min with UV detection at 220 nm. HPLC purification was carried out on a C8 preparative column on a Knauer HPLC system (pump type 64, programmer 50, UV detector (variable-wavelength monitor)) using a gradient of (A) 0.1% CF_3COOH in H_2O and (B) MeCN at a flow rate of 4 mL/min with UV detection at 214 nm. Retention times (t_R) are given in min.

General Procedure A: Amino Acid Coupling. The free amine or the appropriate salt (1 equiv) was dissolved in CH_2Cl_2 or 50% CH_2Cl_2/DMF (0.1 M) under argon and cooled to 0 °C. The reaction mixture was treated with a base (Et_3N or DIEA, 3 equiv). HOBt (1.25 equiv), the acid (1 equiv), and EDC (1.25 equiv) were then successively added. The reaction mixture was allowed to warm to room temperature and then stirred for 18 h. The mixture was diluted with CH_2Cl_2 and washed with 1 N HCl, saturated $NaHCO_3$ solution, and brine. The organic layer was dried over anhydrous $MgSO_4$, filtrated, and concentrated. The resulting residue was purified on silica gel to afford the pure product.

General Procedure B: Amino Acid Coupling. The free amine or the appropriate salt (1 equiv) was dissolved in DMF (0.15 M) under argon and cooled to 0 °C. The reaction mixture was treated with DIEA (3 equiv). HOBt (1.25 equiv), the acid (1 equiv), and EDC (1.25 equiv) were then successively added. The product was precipitated by the addition of a saturated $NaHCO_3$ solution. The precipitate was washed several times with saturated $NaHCO_3$, 1 M $KHSO_4$ solutions and H_2O and dried 24 h under high vacuum to give the crude product which was utilized in the next step without further purification.

General Procedure C: Boc Cleavage Using a HCl Solution. Under argon and at 0 °C, the Boc-protected compound was dissolved in a saturated HCl/(EtO_2 or dioxane) solution. The mixture was stirred for 15 min to 1 h and then evaporated. The resulting HCl salt was precipitated in ether, dried under high vacuum, and used for the next step without further purification.

General Procedure D: Boc Cleavage Using a TFA Solution. Under argon and at 0 °C, the Boc-protected compound was dissolved in a TFA/CH_2Cl_2 (50−100%) solution. The mixture was stirred for 10 min to 1 h and then evaporated. The resulting TFA salt was precipitated in Et_2O, dried under high vacuum, and used for the next step without further purification.

General Procedure E: Final Deprotection. The fully protected compound was dissolved in TFE/CH_3COOH (3/1), and a catalytic amount of 10% Pd/$BaSO_4$ was added. The apparatus was evacuated and flushed three times with H_2, and the mixture was stirred under an atmosphere of H_2 for ca. 15 h. The mixture was then filtrated though Celite, concentrated, and precipitated from Et_2O. The resulting yellow-white CH_3COOH salt was then treated with concentrated TFA. After 15 min, the crude product was precipitated from Et_2O and purified by HPLC.

Boc-*S*-β-HAla-*R*-HB-OBn (3). To a solution of the (*R*)-3-hydroxybutanoic benzyl ester (**2**)[45] (1.90 g, 9.8 mmol) in CH_2Cl_2 (30 mL) was added a solution of the acid **1**[38] (2.00 g, 9.8 mmol) in CH_2Cl_2 (30 mL) under argon, cooled to −5 °C. DCC (2.12 g, 10.3 mmol) and DMAP (0.09 g, 0.49 mmol) were added. The resulting mixture was allowed to warm to room temperature and then stirred for 24 h. The mixture was diluted with Et_2O and washed with 1 N HCl, saturated $NaHCO_3$ solution, and brine. The organic layer was dried over anhydrous $MgSO_4$, filtrated, and concentrated. The residue was purified on silica gel (20% Et_2O/pentane) and gave compound **3** (3.25 g, 88%) as a colorless oil. ^1H NMR (300 MHz, $CDCl_3$): δ 7.37−7.33 (m, 5H ar), 5.36−5.26 (m, 1H, CHO), 5.14 (AB, J = 12.1, 1H, OC*H*$_2$Ph), 5.09 (AB, J = 12.1, 1H, OC*H*$_2$Ph), 5.00−4.90 (m, 1H, NH), 4.10−3.96 (m, 1H, CHN), 2.66 (dd, ABX, J = 7.5, 15.6, 1H, C*H*$_2$CHO), 2.54 (dd, ABX, J = 5.3, 15.6, 1H, C*H*$_2$CHO), 2.43 (dd, ABX, J = 5.3, 14.9, 1H, C*H*$_2$CHN), 2.37 (dd, ABX, J = 5.9, 14.9, 1H, C*H*$_2$CHN), 1.43 (s, 9H, tBu), 1.29 (d, J = 6.2, 3H, Me of HB), 1.17 (d, J = 6.5, 3H, Me of β-HAla). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.00, 170.42, 155.36, 135.93, 128.84, 128.60, 67.58, 66.65, 43.60, 40.77, 28.46, 20.31, 19.96. FAB-MS: m/z 759 {26, $(2M + 1)^+$}, 308 {64, $(M + 1)^+$}, 280 (100).

HCl•H-*S*-β-HAla-*R*-HB-OBn (4). According to general procedure C, compound **3** (227 mg, 0.6 mmol) was treated with a saturated HCl/Et_2O solution (6 mL). The resulting HCl salt **4** was obtained as a white precipitate and used in the next coupling step without further purification.

Boc-*S*-β-HAla-*R*-HB-OH (5). The benzyl-protected compound **3** (900 mg, 2.9 mmol) was dissolved in MeOH (20 mL); catalytic amounts of 10% Pd/C (90 mg) and acetic acid (0.1

mL) were added. The apparatus was evacuated and flushed three times with H_2, and the mixture was stirred under an atmosphere of H_2 for ca. 8 h. Subsequent filtration though Celite and concentration under reduced pressure yielded the acid **5** (558 mg, 84%) as a colorless oil which was identified by NMR and used for the next coupling step without purification.

Boc-Ala-*S*-*β*-HAla-*R*-HB-OBn (6). According to general procedure A, to a solution in CH_2Cl_2 (26 mL) of HCl salt **4** (1 equiv, 2.61 mmol) was added Et_3N (1.45 mL, 10.4 mmol). HOBt (440 mg, 3.3 mmol), Boc-Ala-OH (4.94 mg, 2.61 mmol), and then EDC (623 mg, 3.3 mmol) were successively added to the reaction. The residue was purified by recrystallization (Et_2O/pentane, 2/5) to give compound **6** (994 mg, 85%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35−7.326 (m, 5H ar), 6.70 (br d, J = 6.8, 1H, NH), 5.33−5.27 (m, 1H, CHO), 5.12 9 (s, 2H, OCH_2Ph), 5.12−5.06 (m, 1H, NH), 4.36−4.22 (m, 1H, CHN), 4.18−4.06 (m, 1H, CHN), 2.74−2.66 (m, 1H, CH_2CHO), 2.58 (dd, ABX, J = 5.0, 15.57, 1H, CH_2CHO), 2.42 (d, J = 5.3, 2H, CH_2CHN), 1.44 (s, 9H, tBu), 1.34−1.29 (m, 6H, 2 Me), 1.17 (d, J = 6.8, 3H, Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.12, 170.87, 135.80, 128.86, 128.67, 128.62, 67.87, 66.78, 42.02, 40.69, 40.33, 28.38, 19.98, 19.66, 18.64. FAB-MS: *m*/*z* 901 {11, $(2M + 1)^+$}, 451 {100, $(M + 1)^{+9}$}, 351 (48).

Boc-Ala-*S*-*β*-HAla-*R*-HB-OH (7). The benzyl-protected compound **6** (675 mg, 1.5 mmol) was dissolved in MeOH (10 mL); catalytic amounts of 10% Pd/C (70 mg) and acetic acid (0.1 mL) were added. The apparatus was evacuated and flushed three times with H_2, and the mixture was stirred under an atmosphere of H_2 for ca. 8 h. Subsequent filtration though Celite and concentration under reduced pressure yielded the acid **7** in almost quantitative yield as a colorless oil which was used for the next coupling step without purification.

Boc-*S*-*β*-HAla-*R*-HB-Lys(Z)-OBn (8). According to general procedure A, to a solution in CH_2Cl_2 (15 mL) of HCl·H-Lys-(Z)-OBn (784 mg, 1.9 mmol) was added Et_3N (1.08 mL, 7.7 mmol). HOBt (326 mg, 2.4 mmol), compound **5** (558 mg, 1.9 mmol), and then EDC (460 mg, 2.4 mmol) were successively added to the reaction. The residue was purified on silica gel (50% Et_2O/pentane) to give compound **8** (964 mg, 78%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.38−7.29 (m, 10H ar), 6.96−6.90 (m, 1H, NH), 5.27−5.17 (m, 1H, CHO), 5.22−5.09 (m, 2H, OCH_2Ph), 5.08 (s, 2H, OCH_2Ph), 4.97−4.94 (m, 1H, NH), 4.90−4.86 (m, 1H, NH), 4.66−4.58 (m, 1H, CHN), 4.16−4.05 (m, 1H, CHN), 3.16−3.09 (m, 2H, CH_2NHZ), 2.54−2.27 (m, 4H, CH_2CHN, CH_2CHO), 1.90−1.60 (m, 4H, 2 CH_2), 1.50−1.28 (m, 2H, CH_2), 1.40 (s, 9H, tBu), 1.30 (d, J = 6.2, 3H, Me), 1.13 (d, J = 6.8, 3H, Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.57, 170.84, 169.74, 156.83,155.65, 136.87, 135.69, 128.86, 128.76, 128.71, 128.55, 111.19, 79.61, 68.19, 67.16, 66.74, 52.19, 43.84, 42.30, 41.96, 40.61, 31.71, 29.46, 28.43, 22.42, 20.90, 19.46. FAB-MS: *m*/*z* 642 {12, $(M + 1)^+$}, 542 (100).

HCl•H-*S*-*β*-HAla-*R*-HB-Lys(Z)-OBn (9). According to general procedure C, compound **8** (712 mg, 1.1 mmol) was treated with a saturated HCl/dioxane solution (10 mL). After 15 min, the reaction was completed and the solvent was evaporated. The resulting HCl salt **9** was obtained in almost quantitative yield as a white precipitate and used in the next coupling step without further purification.

Boc-Ala-(*S*-*β*-HAla-*R*-HB)$_2$-Lys(Z)-OBn (10). According to general procedure A, to a solution in CH_2Cl_2 (15 mL) of the HCl salt **9** (1 equiv, 1.5 mmol) was added DIEA (1.0 mL, 6.0 mmol). HOBt (253 mg, 1.8 mmol), compound **7** (1 equiv, 1.5 mmol), and then EDC (358 mg, 1.8 mmol) were successively added to the reaction. The residue was purified on silica gel (ethyl acetate/hexane, 9/1) to give compound **10** (884 mg, 90%) as a white solid foam. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36−7.30 (m, 10H ar), 7.10−7.00 (m, 3H, NH), 5.34 (d, J = 7.5, 1H, NH), 5.30−5.10 (m, 3H, CHO, NH), 5.21−5.09 (m, 2H, OCH_2-Ph), 5.07 (s, 2H, OCH_2Ph), 4.60−4.53 (m, 1H, CHN), 4.44−4.31 (m, 2H, CHN), 4.17−4.08 (m, 1H, CHN), 3.16−3.08 (m, 2H, CH_2NHZ), 2.55−2.29 (m, 8H, CH_2CHN, CH_2CHO), 1.88−1.60 (m, 2H, CH_2), 1.58−1.22 (m, 4H, CH_2), 1.42 (s, 9H, tBu), 1.31 (d, J = 6.8, 3H, Me), 1.29 (d, J = 6.2, 3H, Me), 1.25 (d, J = 6.2, 3H, Me), 1.17 (d, J = 6.8, 3H, Me), 1.15 (d, J = 6.5, 3H, Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.49, 170.79, 170.08, 169.58, 156.98, 136.84, 135.64, 128.86, 128.78, 128.73, 128.54, 128.37, 128.29, 68.61, 68.27, 67.19, 66.74, 52.39, 42.47, 42.21, 41.24, 40.40, 31.42, 29.40, 28.41, 22.36, 20.06, 19.85, 19.71, 18.81. FAB-MS: *m*/*z* 906 {6, $(M + Na)^+$}, 884 {32, $(M + 1)^+$}, 784 (100). Anal. ($C_{45}H_{65}N_5O_{13}$•H_2O) C, H, N.

TFA•H-Ala-(*S*-*β*-HAla-*R*-HB)$_2$-Lys(Z)-OBn (11). According to general procedure D, compound **10** (654 mg, 0.74 mmol) was treated with a CH_2Cl_2/TFA (1:1) solution (6 mL). After 30 min, the reaction was completed and the solvent was evaporated. The TFA resulting salt **11** was obtained in almost quantitative yield as a white precipitate (from Et_2O) and used in the next coupling step without further purification.

Boc-*S*-*β*-HAla-Lys(Z)-OBn (12). According to general procedure A, to a solution in DMF (50 mL) of the HCl salt of Lys-(Z)-OBn (2.00 g, 4.9 mmol) was added Et_3N (2.04 mL, 14.7 mmol). HOBt (0.83 g, 6.1 mmol), the acid Boc-*β*-HAla-OH (**1**) (1.00 g, 4.9 mmol), and then EDC (1.17 g, 6.1 mmol) were successively added to the reaction. The residue was purified by recrystallization (ethyl acetate/hexane, 20/1) to give compound **12** (2.30 g, 85%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.37−7.26 (m, 10H ar), 6.48−6.36 (m, 1H, NH), 5.22−5.10 (m, 3H, OCH_2Ph, NH), 5.09 (s, 2H, OCH_2Ph), 4.95−4.87 (m, 1H, NH), 4.62−4.56 (m, 1H, CHN), 4.00−3.90 (m, 1H, CHN), 3.17−3.10 (m, 2H, CH_2NHZ), 2.46−2.32 (m, 2H, CH_2-CHN), 1.90−1.60 (m, 2H, CH_2), 1.50−1.25 (m, 4H, CH_2), 1.42 (s, 9H, tBu), 1.16 (d, J = 6.8, 3H, Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.44, 156.90, 136.85, 135.56, 128.89, 128.78, 128.65, 128.37, 67.29, 66.74, 52.01, 44.18, 42.52, 40.41, 31.61, 29.30, 28.46, 22.15, 20.60. FAB-MS: *m*/*z* 556 {28, $(M + 1)^+$}, 456 (100).

HCl•H-*S*-*β*-HAla-Lys(Z)-OBn (13). According to general procedure C, compound **12** (1.00 g, 1.8 mmol) was treated with a saturated HCl/dioxane solution (20 mL). After 30 min, the reaction was completed and the solvent was evaporated. The resulting HCl salt **13** was obtained in almost quantitative yield as a white precipitate (from Et_2O) and used in the next coupling step without further purification.

Boc-(*S*-*β*-HAla)$_2$-Lys(Z)-OBn (14). According to general procedure A, to a solution in DMF (5 mL) of the HCl salt **13** (1 equiv, 1.8 mmol) was added DIEA (0.92 mL, 5.4 mmol). HOBt (304 mg, 2.2 mmol), the acid Boc-*β*-HAla-OH (**1**) (365 mg, 1.8 mmol), and then EDC (430 mg, 2.2 mmol) were successively added to the reaction. The residue was purified by recrystallization (CH_3Cl/hexane, 20/1) to give compound **14** (850 mg, 74%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.38−7.26 (m, 10H ar), 6.68−6.65 (m, 2H, NH), 5.23−5.09 (m, 4H, OCH_2Ph, NH), 5.09 (s, 2H, OCH_2Ph), 4.58−4.50 (m, 1H, CHN), 4.30−4.20 (m, 1H, CHN), 4.00−3.90 (m, 1H, CHN), 3.18−3.10 (m, 2H, CH_2NHZ), 2.42−2.22 (m, 4H, CH_2CHN), 1.90−1.62 (m, 2H, CH_2), 1.52−1.25 (m, 4H, CH_2), 1.42 (s, 9H, tBu), 1.19 (d, J = 6.5, 3H, Me), 1.15 (d, J = 6.8, 3H, Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.44, 155.78, 128.88, 128.78, 128.62, 128.37, 67.30, 66.74, 52.27, 43.10, 40.29, 31.29, 28.56, 28.48, 22.21, 20.24. FAB-MS: *m*/*z* 663 {22, $(M + Na)^+$}, 641 {38, $(M + 1)^+$}, 541 (100).

HCl•H-(*S*-*β*-HAla)$_2$-Lys(Z)-OBn (15). According to general procedure C, compound **15** (712 mg, 1.1 mmol) was treated with a saturated HCl/dioxane solution (10 mL). After 30 min, the reaction was completed and the solvent was evaporated. The HCl resulting salt **15** was obtained in almost quantitative yield as a white precipitate (from Et_2O) and used in the next coupling step without further purification.

Fmoc-Ala-(*R*-HB)$_2$-OtBu (17). To a solution of the hydroxy derivative **16**[51] (1 equiv, 4.4 mmol) in CH_2Cl_2 (40 mL) was added Fmoc-Ala-OH (1.45 g, 4.4 mmol) under argon, and the mixture was cooled to − 5 °C. DCC (0.95 g, 4.62 mmol) and DMAP (0.04 g, 0.22 mmol) were added, and the resulting mixture was allow to warm to room temperature and then stirred for 24 h. The mixture was diluted with Et_2O and washed with 1 N HCl, saturated $NaHCO_3$ solution, and brine. The organic layer was dried over anhydrous $MgSO_4$, filtrated, and concentrated. The residue was purified on silica gel (Et_2O/

pentane, 2/3) to give compound **17** (700 mg, 30%) as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77−7.75 (m, 2H ar), 7.61−7.59 (m, 2H ar), 7.42−7.37 (m, 2H ar), 7.34−7.26 (m, 2H ar), 6.49 (d, J = 7.2, NH), 5.38−5.22 (m, 2H, CHO), 4.44−4.32 (m, 3H, CHN, CH_2 of Fmoc), 4.25−4.20 (m, 1H, CH of Fmoc), 2.69−2.37 (m, 4H, CH_2CHO), 1.43 (s, 9H, tBu), 1.30 (d, J = 6.2, 3H, Me), 1.29 (d, J = 6.2, 3H, Me), 1.26 (d, J = 6.5, 3H, Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.44, 169.69, 169.37, 155.91, 144.23, 144.12, 141.58, 127.94, 125.33, 120.20, 81.10, 68.68, 68.21, 67.09, 49.83, 47.27, 40.05, 40.88, 28.09, 19.80, 19.67, 18.67. FAB-MS: m/z 540 {6, $(M + 1)^+$}, 484 (100).

Fmoc-Ala-(*R*-HB)$_2$-OH (18). According to general procedure C, compound **17** (600 mg, 1.1 mmol) was treated with a TFA/CH_2Cl_2 (1:1) solution (6 mL). After 30 min, the reaction was completed and the solvent was evaporated. The acid **18** was obtained in almost quantitative yield as a yellow oil and used in the next coupling step without further purification.

Fmoc-Ala-(*R*-HB)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (19). According to general procedure A, to a solution in CH_2Cl_2/DMF (1:1, 12 mL) of the HCl salt **15** (1 equiv, 1.10 mmol) was added DIEA (0.75 mL, 4.40 mmol). HOBt (185 mg, 1.37 mmol), the acid **18** (1 equiv, 1.10 mmol), and then EDC (262 mg, 1.37 mmol) were successively added to the reaction. After 15 h reaction, some of the product precipitated in the reaction mixture. The CH_2Cl_2 was then evaporated, and the resulting oil was precipitated in a saturated $NaHCO_3$ solution. The precipitate was washed several times with saturated $NaHCO_3$, $KHSO_4$ (1 N) solutions and finally with H_2O and dried 15 h under high vacuum. The presence of compound **19** was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Compound **19** was used without further purification. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.28−8.22 (m, 1H, NH), 7.30−7.94 (m, 1H, NH), 7.80−7.74 (m, 1H, NH), 7.75−7.65 (m, 2H ar), 7.42−7.24 (m, 16H ar), 7.22−7.16 (m, 1H, NH), 5.23−5.02 (m, 3H, CHO, NH), 5.208 (s, 2H, OCH_2Ph), 5.96 (s, 2H, OCH_2Ph), 4.32−4.14 (m, 2H, CHN), 4.10−4.22 (m, 2H, CHN), 2.96−2.87 (m, 2H, CH_2-NHZ), 2.40−2.00 (m, 8H, CH_2CHN, CH_2CHO), 1.70−1.51 (m, 2H, $CH3_2$), 1.40−1.10 (m, 4H, 2 CH_2), 1.23 (d, J = 6.8, 3H, Me), 1.13 (d, J = 5.0, 3H, Me), 1.04 (d, J = 5.9, 3H, Me), 1.03−0.98 (m, 2 Me). ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.28, 170.52, 169.11, 168.09, 167.99, 156.26, 155.99, 144.02, 140.90, 137.43, 136.14, 128.55, 128.47, 128.15, 127.94, 127.84, 127.77, 127.21, 125.38, 120.25, 68.34, 67.77, 65.83, 65.64, 65.12, 63.44, 51.88, 49.42, 46.58, 44.25, 42.18, 42.05, 41.82, 41.64, 30.38, 28.86, 23.18, 22.55, 19.82, 19.75, 19.51, 19.38, 19.15. FAB-MS: m/z 1028 {23, $(M + Na)^+$}, 1006 {41, $(M + 1)^+$}, 809 (84), 713 (100).

H-Ala-(*R*-HB)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (20). The Fmoc-protected compound **19** (520 mg, 0.52 mmol) was dissolved in DMF/Et_2NH (9:1, 4 mL) under argon and cooled to 0 °C. The mixture was stirred for 1−2 h, and concentration under reduced pressure yielded the crude amine **20** which was identified by NMR and used without further purification.

TFA•H-(*R*-β-HAla)$_4$-Lys(Z)-OBn (22a). According to general procedure B, to a solution in DMF (11 mL) of HCl•H-Lys-(Z)-OBn (442 mg, 1.09 mmol) was added DIEA (0.56 mL, 3.27 mmol). HOBt (184 mg, 1.36 mmol), the acid Boc-(β-HAla)$_4$-OH (**21a**)[40] (1 equiv, 1.09 mmol), and then EDC (260 mg, 1.36 mmol) were successively added to the reaction. The residue was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-(*R*-β-HAla)$_4$-Lys(Z)-OBn (723 mg, 82% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (3.6 mL), according to the general procedure D, gave the TFA salt **22a**, which was used without further purification.

TFA•H-(*S*-β-HAla-*R*-β-HAla)$_2$-Lys(Z)-OBn (22b). According to general procedure B, to a solution in DMF (2 mL) of HCl•H-Lys(Z)-OBn (62 mg, 0.15 mmol) was added DIEA (0.08 mL, 0.45 mmol). HOBt (26 mg, 0.19 mmol), the acid Boc-(*S*-β-HAla-*R*-β-HAla)$_2$-OH (**21b**)[40] (1 equiv, 0.15 mmol), and then EDC (36 mg, 0.19 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-(*S*-β-HAla-*R*-β-HAla)$_2$-Lys(Z)-OBn (11 mg, 90% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (0.55 mL), according to the general procedure D, gave the TFA salt **22b**, which was used without further purification.

TFA•H-(*S*-β-HAla)$_4$-Lys(Z)-OBn (22c). According to general procedure B, to a solution in DMF (15 mL) of HCl•H-Lys-(Z)-OBn (515 mg, 1.27 mmol) was added DIEA (0.65 mL, 3.81 mmol). HOBt (214 mg, 1.59 mmol), the acid Boc-(*S*-β-HAla)$_4$-OH (**21c**)[40] (1 equiv, 1.27 mmol), and then EDC (304 mg, 1.59 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-(*S*-β-HAla)$_4$-Lys(Z)-OBn (819.1 mg, 79% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (4 mL), according to the general procedure D, gave the TFA salt **22c**, which was used without further purification.

TFA•H-(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (22d). According to general procedure B, to a solution in DMF (11 mL) of HCl•H-Lys(Z)-OBn (442 mg, 1.09 mmol) was added DIEA (0.56 mL, 3.27 mmol). HOBt (184 mg, 1.36 mmol), the acid Boc-(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OH (**21d**)[40] (1 equiv, 1.09 mmol), and then EDC (260 mg, 1.36 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$-Lys-(Z)-OBn (796 mg, 90% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (3.9 mL), according to the general procedure D, gave the TFA salt **22d**, which was used without further purification.

TFA•H-Ala-(*R*-β-HAla)$_4$-Lys(Z)-OBn (23a). According to general procedure B, to a solution in DMF (7 mL) of the TFA salt **22a** (1 equiv, 0.89 mmol) was added DIEA (0.61 mL, 3.56 mmol). HOBt (150 mg, 1.11 mmol), Boc-Ala-OH (202 mg, 1.06 mmol), and then EDC (212 mg, 1.1 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Ala-(*R*-β-HAla)$_4$-Lys(Z)-OBn (680 mg, 87% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (3.1 mL), according to the general procedure D, gave the TFA salt **23a**, which was used without further purification.

TFA•H-Ala-(*S*-β-HAla-*R*-β-HAla)$_2$-Lys(Z)-OBn (23b). According to general procedure B, to a solution in DMF (2 mL) of the TFA salt **22b** (1 equiv, 0.135 mmol) was added DIEA (0.092 mL, 0.540 mmol). HOBt (23 mg, 0.169 mmol), Boc-Ala-OH (31 mg, 0.162 mmol), and then EDC (32 mg, 0.169 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Ala-(*S*-β-HAla-*R*-β-HAla)$_2$-Lys(Z)-OBn (73 mg, 61% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (0.33 mL), according to the general procedure D, gave the TFA salt **23b**, which was used without further purification.

TFA•H-Ala-(*S*-β-HAla)$_4$-Lys(Z)-OBn (23c). According to general procedure B, to a solution in DMF (10 mL) of the TFA salt **22c** (1 equiv, 1.00 mmol) was added DIEA (0.68 mL, 4.00 mmol). HOBt (169 mg, 1.25 mmol), Boc-Ala-OH (227 mg, 1.20 mmol), and then EDC (239 mg, 1.25 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Ala-(*S*-β-HAla)$_4$-Lys(Z)-OBn (762 mg, 86% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (3.5 mL), according to the general procedure D, gave the TFA salt **23c**, which was used without further purification.

TFA•H-Ala-(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (23d).

According to general procedure B, to a solution in DMF (10 mL) of the TFA salt **22d** (770 mg, 0.93 mmol) was added DIEA (0.64 mL, 3.72 mmol). HOBt (157 mg, 1.16 mmol), Boc-Ala-OH (211 mg, 1.12 mmol), and then EDC (215 mg, 1.16 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Ala-(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (490 mg, 60% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (2.2 mL), according to the general procedure D, gave the TFA salt **23d**, which was used without further purification.

TFA•H-Arg(NO$_2$)-Ala-(*S*-β-HAla-*R*-HB)$_2$-Lys(Z)-OBn (24). According to general procedure A, to a solution in CH_2Cl_2/DMF (4:3, 8 mL) of the TFA salt **11** (1 equiv, 0.74 mmol) was added DIEA (0.38 mL, 2.22 mmol). HOBt (125 mg, 0.92 mmol), Boc-Arg(NO$_2$)-OH (259 mg, 0.81 mmol), and then EDC (176 mg, 0.92 mmol) were successively added to the reaction. The resulting residue was purified on silica gel (CH_2Cl_2/MeOH, 9/1) to give compound Boc-Arg(NO$_2$)-Ala-(*S*-β-HAla-*R*-HB)$_2$-Lys(Z)-OBn (650 mg, 82%) as a fine white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.45−8.30 (m, 1H, NH), 7.80−7.60 (m, 3H, NH), 7.8−7.28 (m, 10H ar), 7.20−7.12 (m, 3H, NH), 5.70−5.60 (m, 1H, NH), 5.34−5.26 (m, 1H, NH), 5.26−5.06 (m, 4H, CHO, OCH_2Ph), 5.07 (s, 2H, OCH_2Ph), 4.60−4.50 (m, 1H, CHN), 4.46−4.2 (m, 4H, CHN), 3.36−3.24 (m, 2H, CH_2NHC), 3.16−3.07 (m, 2H, CH_2NHZ), 2.53−2.33 (m, 8H, CH_2CHN, CH_2-CHO), 1.90−1.60 (m, 6H, CH_2), 1.54−1.10 (m, 4H, CH_2) 1.41 (s, 9H, tBu), 1.34 (d, J = 6.8, 3H, Me), 1.28 (d, J = 6.2, 3H, Me), 1.24 (d, J = 6.5, 3H, Me), 1.20−1.16 (m, 6H, Me). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.61, 170.35, 170.27, 169.77, 157.09, 136.82, 135.61, 128.87, 128.80, 128.54, 128.37, 128.23, 80.46, 68.68, 68.40, 67.22, 65.96, 52.48, 49.54, 42.48, 42.16, 40.46, 31.36, 29.41, 28.39, 24.85, 22.41, 20.02, 19.88, 19.74, 19.66, 18.19. FAB-MS: m/z 1107 {60, $(M + Na)^+$}, 1085 {100, $(M + 1)^+$}, 985 (22), 713 (7). Further treatment with TFA (5 mL), according to the general procedure D, gave the TFA salt **24**, which was used without further purification.

TFA•H-Arg(NO$_2$)-Ala-(*R*-HB)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (25). According to general procedure B, to a solution in DMF (6 mL) of the TFA salt **20** (1 equiv, 0.52 mmol) was added DIEA (0.26 mL, 1.55 mmol). HOBt (87 mg, 0.65 mmol), Boc-Arg(NO$_2$)-OH (198 mg, 0.62 mmol), and then EDC (123 mg, 0.65 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Arg(NO$_2$)-Ala-(*R*-HB)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (409 mg, 77% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (1.4 mL), according to the general procedure D, gave the TFA salt **25**, which was used without further purification.

TFA•H-Arg(NO$_2$)-Ala-(*R*-β-HAla)$_4$-Lys(Z)-OBn (26a). According to general procedure B, to a solution in DMF (9 mL) of the TFA salt **23a** (1 equiv, 0.77 mmol) was added DIEA (0.53 mL, 3.08 mmol). HOBt (130 mg, 0.96 mmol), Boc-Arg(NO$_2$)-OH (295 mg, 0.92 mmol) and then EDC (183 mg, 0.96 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any solvent to be purified. The presence of the desired Boc-Arg(NO$_2$)-Ala-(*R*-β-HAla)$_4$-Lys(Z)-OBn (757 mg, 81% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (2.7 mL), according to the general procedure D, gave the TFA salt **26a**, which was used without further purification.

TFA•H-Arg(NO$_2$)-(*S*-β-HAla-*R*-β-HAla)$_2$-Lys(Z)-OBn (26b). According to general procedure B, to a solution in DMF (9 mL) of the TFA salt **23b** (1 equiv, 0.83 mmol) was added DIEA (0.057 mL, 0.33 mmol). HOBt (14 mg, 0.104 mmol), Boc-Arg(NO$_2$)-OH (32 mg, 0.099 mmol), and then EDC (20 mg, 0.104 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Arg(NO$_2$)-(*S*-β-HAla-*R*-β-HAla)$_2$-Lys(Z)-OBn (74 mg) was confirmed by FAB-MS. Further treatment with TFA (0.5 mL), according to the general procedure D, gave the TFA salt **26b**, which was used without further purification.

TFA•H-Arg(NO$_2$)-Ala-(*S*-β-HAla)$_4$-Lys(Z)-OBn (26c). According to general procedure B, to a solution in DMF (9 mL) of the TFA salt **23c** (1 equiv, 0.86 mmol) was added DIEA (0.59 mL, 3.44 mmol). HOBt (145 mg, 1.07 mmol), Boc-Arg(NO$_2$)-OH (329 mg, 1.03 mmol), and then EDC (205 mg, 1.07 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Arg(NO$_2$)-Ala-(*S*-β-HAla)$_4$-Lys(Z)-OBn (860 mg, 82% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (3.1 mL), according to the general procedure D, gave the TFA salt **26c**, which was used without further purification.

TFA•H-Arg(NO$_2$)-Ala-(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (26d). According to general procedure B, to a solution in DMF (9 mL) of the TFA salt **23d** (1 equiv, 0.45 mmol) was added DIEA (0.31 mL, 1.81 mmol). HOBt (76 mg, 0.56 mmol), Boc-Arg(NO$_2$)-OH (172 mg, 0.54 mmol), and then EDC (107 mg, 0.56 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Arg(NO$_2$)-Ala-(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (460 mg, 84% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra. Further treatment with TFA (2.2 mL), according to the general procedure D, gave the TFA salt **26d**, which was used without further purification.

H-Gly-Arg-Ala-(*S*-β-HAla-*R*-HB)$_2$-Lys-OH (27). According to general procedure B, to a solution in DMF (6 mL) of the TFA salt **24** (1 equiv, 0.50 mmol) was added DIEA (0.26 mL, 1.51 mmol). HOBt (85 mg, 0.63 mmol), Boc-Gly-OH (97 mg, 0.55 mmol), and then EDC (124 mg, 0.63 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any solvent to be purified. The presence of the desired Boc-Gly-Arg(NO$_2$)-Ala-(*S*-β-HAla-*R*-HB)$_2$-Lys-(Z)-OBn (447 mg, 68% crude) as a fine yellow-white powder was confirmed by ^{1}H and ^{13}C NMR and MS spectra.

According to general procedure E, Boc-Gly-Arg(NO$_2$)-Ala-(*S*-β-HAla-*R*-HB)$_2$-Lys(Z)-OBn (300 mg, 0.26 mmol) was dissolved in TFE/CH_3COOH (3:1, 4 mL) and hydrogenated in the presence of Pd/$BaSO_4$ (10%, 60 mg). The resulting precipitate was purified by HPLC C8 (5−40% B, 30 min), t_R 12.5 min, to give after lyophilization the pure compound **27** in about 40% yield. ^{1}H NMR (300 MHz, D_2O): δ 5.24−5.10 (m, 2H, CHO), 4.34−4.28 (m, 2H, CHN), 4.22−4.15 (m, 3H, CHN), 3.84 (s, 2H, CH_2N), 3.19 (t, J = 6.8, 2H, CH_2NHC), 3.00−2.95 (m, 2H, CH_2NH$_2$), 2.58−2.42 (m, 8H, CH_2CHO, CH_2CHN), 1.92−1.58 (m, 8H, CH_2), 1.50−1.40 (m, 2H, CH_2), 1.32 (d, J = 7.5, 3H, Me), 1.26 (d, J = 6.2, 3H, Me), 1.25 (d, J = 6.2, 3H, Me), 1.18−1.13 (m, 6H, Me). FAB-MS: m/z 811 {10, $(M + K)^+$}, 795 {32, $(M + Na)^+$}, 773 {100, $(M + 1)^+$}. Purity by analytical HPLC (0−100% B, 60 min, t_R 18.7 min) >99%.

H-Gly-Arg-Ala-(*R*-HB)$_2$-(*S*-β-HAla)$_2$-Lys-OH (28). According to general procedure D, to a solution in DMF (5 mL) of the TFA salt **25** (1 equiv, 0.37 mmol) was added DIEA (0.19 mL, 1.11 mmol). HOBt (62 mg, 0.46 mmol), Boc-Gly-OH (77 mg, 0.44 mmol), and then EDC (88 mg, 0.46 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Gly-Arg-(NO$_2$)-Ala-(*R*-HB)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (252 mg, 60% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra.

According to general procedure E, Boc-Gly-Arg(NO$_2$)-Ala-(*R*-HB)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (200 mg, 0.17 mmol) was dissolved in TFE/CH_3COOH (3:1, 3.5 mL) and hydrogenated in the presence of Pd/$BaSO_4$ (10%, 40 mg). The resulting

precipitate was purified by HPLC (10−40% B, 30 min), t_R 6.2 min, to give after lyophilization the pure compound **28** in about 25% yield. ^{1}H NMR (300 MHz, D_2O): δ 5.22−5.06 (m, 2H, CHO), 4.28−4.20 (m, 3H, CHN), 4.16−4.04 (m, 2H, CHN), 3.76 (s, 2H, CH_2N), 3.16−3.10 (m, 2H, CH_2NHC), 2.93−2.86 (m, 2H, CH_2NH$_2$), 2.65−2.49 (m, 2H, CH_2CHO), 2.43−2.32 (m, 2H, CH_2CHO), 2.36 (d, J = 7.2, 2H, CH_2CHN), 2.26 (d, J = 7.2, 2H, CH_2CHN), 1.85−1.53 (m, 8H, CH_2), 1.41−1.28 (m, 2H, CH_2), 1.35 (d, J = 7.2, 3H, Me), 1.20−1.14 (m, 6H, Me), 1.08−1.03 (m, 6H, Me). FAB-MS: m/z 811 {12, $(M + K)^+$}, 795 {23, $(M + Na)^+$}, 773 {100, $(M + 1)^+$}. Purity by analytical HPLC (0−100% B, 60 min, t_R 19.5 min) >99%.

H-Gly-Arg-Ala-(*R*-β-HAla)$_4$-Lys-OH (29a). According to general procedure B, to a solution in DMF (7 mL) of the TFA salt **26a** (1 equiv, 0.68 mmol) was added DIEA (0.47 mL, 2.71 mmol). HOBt (115 mg, 0.85 mmol), Boc-Gly-OH (143 mg, 0.82 mmol), and then EDC (163 mg, 0.85 mmol) were successively added to the reaction. The precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Gly-Arg(NO_2)-Ala-(*R*-β-HAla)$_4$-Lys-(Z)-OBn (622 mg, 80% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra.

According to general procedure E, Boc-Gly-Arg(NO_2)-Ala-(*R*-β-HAla)$_4$-Lys(Z)-OBn (200 mg, 0.18 mmol) was dissolved in TFE/CH_3COOH (3:1, 3.5 mL) and hydrogenated in the presence of Pd/$BaSO_4$ (10%, 40 mg). The resulting precipitate was purified by HPLC (5−40% B, 30 min), t_R 17.20 min, to give after lyophilization the pure compound **29a** in about 25% yield. ^{1}H NMR (300 MHz, D_2O): δ 4.32−4.24 (m, 2H, CHN), 4.21−4.07 (m, 5H, CHN), 3.85−3.80 (m, 2H, CH_2N), 3.20−3.14 (m, 2H, CH_2NHC), 2.98−2.92 (m, 2H, CH_2NH$_2$), 2.50−2.25 (m, 8H, CH_2CHN), 1.90−1.58 (m, 8H, CH_2), 1.47−1.36 (m, 2H, CH_2), 1.32 (d, J = 7.2, 3H, Me), 1.14−1.09 (m, 12H, Me). ^{13}C NMR (75 MHz, D_2O): δ 178.57, 176.08, 175.29, 56.37, 55.39, 52.77, 46.22, 46.11, 45.20, 43.36, 43.15, 41.95, 32.66, 31.13, 28.98, 27.05, 24.86, 22.00, 19.43. FAB-MS: m/z 1542 {9, $(2M + 2)^+$}, 771 {100, $(M + 1)^+$}. Purity by analytical HPLC (0−100% B, 60 min, t_R 16.4 min) >99%.

H-Gly-Arg-Ala-(*S*-β-HAla-*R*-β-HAla)$_2$-Lys-OH (29b). According to general procedure B, to a solution in DMF (2 mL) of the TFA salt **26b** (1 equiv, 0.068 mmol) was added DIEA (0.046 mL, 0.28 mmol). HOBt (12 mg, 0.085 mmol), Boc-Gly-OH (14 mg, 0.082 mmol), and then EDC (16 mg, 0.085 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Gly-Arg(NO_2)-(*S*-β-HAla-*R*-β-HAla)$_2$-Lys(Z)-OBn (70 mg) was confirmed by FAB-MS.

According to general procedure E, Boc-Gly-Arg(NO_2)-(*S*-β-HAla-*R*-β-HAla)$_2$-Lys(Z)-OBn (70 mg, 0.06 mmol) was dissolved in TFE/CH_3COOH (3:1, 1 mL) and hydrogenated in the presence of Pd/$BaSO_4$ (10%, 10 mg). The resulting precipitate was purified by HPLC (5−40% *A*, 30 min), t_R 8.6 min, to give after lyophilization the pure compound **29b** in about 25% yield. ^{1}H NMR (300 MHz, D_2O): δ 4.26−4.18 (m, 2H, CHN), 4.18−4.04 (m, 5H, CHN), 3.76−3.73 (m, 2H, CH_2N), 3.12−3.07 (m, 2H, CH_2NHC), 2.91−2.85 (m, 2H, CH_2NH$_2$), 2.40−2.32 (m, 2H, CH_2CHN), 2.30−2.22 (m, 6H, CH_2CHN), 1.85−1.50 (m, 8H, CH_2), 1.40−1.30 (m, 2H, CH_2), 1.26−1.22 (m, 3H, Me), 1.09−1.02 (m, 12H, Me). FAB-MS: m/z 771 (86, $[M + 1]^+$). Purity by analytical HPLC (0−100% B, 60 min, t_R 15.4 min) >99%.

H-Gly-Arg-Ala-(*S*-β-HAla)$_4$-Lys-OH (29c). According to general procedure B, to a solution in DMF (8 mL) of the TFA salt **26c** (1 equiv, 0.77 mmol) was added DIEA (0.53 mL, 3.1 mmol). HOBt (131 mg, 0.97 mmol), Boc-Gly-OH (163 mg, 0.93 mmol), and then EDC (185 mg, 0.97 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Gly-Arg(NO_2)-Ala-(*S*-β-HAla)$_4$-Lys(Z)-OBn (719 mg, 82% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra.

According to general procedure E, Boc-Gly-Arg(NO_2)-Ala-(*S*-β-HAla)$_4$-Lys(Z)-OBn (340 mg, 0.30 mmol) was dissolved in TFE/CH_3COOH (3:1, 4 mL) and hydrogenated in the presence of Pd/$BaSO_4$ (10%, 60 mg). The resulting precipitate was purified by HPLC (10−40% B, 30 min), t_R 6.8 min, to give after lyophilization the pure compound **29c** in about 30% yield. ^{1}H NMR (300 MHz, D_2O): δ 4.34−4.27 (m, 2H, CHN), 4.24−4.09 (m, 5H, CHN), 3.84−3.81 (m, 2H, CH_2N), 3.21−3.15 (m, 2H, CH_2NHC), 2.99−2.93 (m, 2H, CH_2NH$_2$), 2.45−2.41 (m, 2H, CH_2C9HN), 2.40−2.26 (m, 6H, CH_2CHN), 1.92−1.57 (m, 8H, CH_2), 1.47−1.35 (m, 2H, CH_2), 1.31 (d, J = 7.2, 3H, Me), 1.15−1.09 (m, 12H, Me). ^{13}C NMR (75 MHz, D_2O): δ 178.59, 176.58, 176.50, 176.08, 175.30, 170.09, 159.82, 56.22, 55.30, 52.71, 46.25, 45.22, 44.78, 43.37, 43.13, 42.00, 32.78, 31.13, 28.99, 27.02, 24.85, 22.16, 21.97, 19.50. FAB-MS: m/z 1542 {17, $(2M + 2)^+$}, 771 {100, $(M + 1)^+$}. Purity by analytical HPLC (0−100% B, 60 min, t_R 15.3 min) >99%.

H-Gly-Arg-Ala-(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$-Lys-OH (29d). According to general procedure B, to a solution in DMF (5 mL) of the TFA salt **26d** (1 equiv, 0.42 mmol) was added DIEA (0.29 mL, 1.68 mmol. HOBt (71 mg, 0.52 mmol), Boc-Gly-OH (88 mg, 0.50 mmol), and then EDC (100 mg, 0.52 mmol) were successively added to the reaction. The resulting precipitate was dried under high vacuum and used without further purification as it is not soluble in any flash chromatography or HPLC solvent. The presence of the desired Boc-Gly-Arg-(NO_2)-Ala-(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (333 mg, 70% crude) was confirmed by ^{1}H and ^{13}C NMR and MS spectra.

According to general procedure E, Boc-Gly-Arg(NO_2)-Ala-(*R*-β-HAla)$_2$-(*S*-β-HAla)$_2$-Lys(Z)-OBn (150 mg, 0.13 mmol) was dissolved in TFE/CH_3COOH (3:1, 3 mL) and hydrogenated in the presence of Pd/$BaSO_4$ (10%, 25 mg). The resulting precipitate was purified by HPLC (2−40% B, 30 min), t_R 20.5 min, to give after lyophilization the pure compound **29d** in about 20% yield. ^{1}H NMR (300 MHz, D_2O): δ 4.36−4.28 (m, 2H, CHN), 4.27−4.12 (m, 5H, CHN), 3.87−3.83 (m, 2H, CH_2N), 3.23−3.18 (m, 2H, CH_2NHC), 3.01−2.96 (m, 2H, CH_2NH$_2$), 2.346−2.43 (m, 2H, CH_2CHN), 2.41−2.31 (m, 6H, CH_2CHN), 1.92−1.60 (m, 8H, CH_2), 1.50−1.40 (m, 2H, CH_2), 1.35 (d, J = 7.5, 3H, Me), 1.17−1.11 (m, 12H, Me). FAB-MS: m/z 793 {15, $(M + Na)^+$}, 771 {100, $(M + 1)^+$}. Purity by analytical HPLC (0−100% B, 60 min, t_R 15.4 min) >80%.

Molecular Dynamics Simulations. Molecular mechanics and dynamics calculations were realized using the AMBER 5.0 package[66] using the parm96 parameter set and an all-atom force-field representation.[67] Force-field parameters for the ester bonds were taken from the literature.[68] Atomic charges for the new monomers (*R*-HB, *S*-β-HAla, *R*-β-HAla) were calculated using the GAUSSIAN94 package[69] and the HF/6-31G* basis set, by fitting atom-centered charges to an ab initio electrostatic potential, using the RESP method.[70] Initial coordinates for the MHC−ligand complexes were obtained from the X-ray structure of HLA-B*2705[56] (Protein Data Bank code 1hsa) as previously described.[33,34] The spacers were substituted for the natural pentapeptide sequence using the SYBYL 6.3 modeling package (TRIPOS Assoc., Inc., St. Louis, MO). From a starting fully extended conformation, dihedral angles of the main chain between P3 and P9 were modified in order to reproduce a correct trans geometry for the newly introduced amide or ester bonds. The ligand was first relaxed by 1000 steps of conjugate gradient energy minimization while maintaining the protein fixed. It was then submitted to a 100-ps Simulated annealing (SA) protocol in order to sample the broadest possible conformational space. Starting with random velocities assigned at a temperature of 1000 K, the peptide was first coupled to a heat bath at 1000 K using a temperature coupling constant T_τ of 0.2 ps and then linearly cooled to 50 K for the next 50 ps while strengthening T_τ to a value of 0.05 ps. During these 100 ps, no protein atom was allowed to move. As the simulated annealing was performed in vacuo, a distance-dependent dielectric function ($\epsilon = 4r$) was used. A twin cutoff (10.0, 15.0 Å) was used to calculate nonbonded electrostatic interactions at every minimization step and every nonbonded pair list update (10 steps), respectively.

From the last SA conformer, 13 counterions (9 Na^+ and 4 Cl^- ions) were then placed at electrostatic minima to neutralize the protein, using the CION routine of AMBER.[66] It was then solvated in a 10-Å thick TIP3P water shell. After the solvent was minimized by 1000 steps of steepest descent, the solvent (water and counterions) was equilibrated by 25-ps MD at 300 K. The solvent was minimized again, and the fully solvated complex was finally relaxed by 1000 steps of steepest descent. The obtained coordinates were then used as a starting point for a 500-ps MD simulation at 300 K. To avoid large drifts from the protein crystal structure, a weak positional harmonic constraint of 0.05 kcal·mol^{-1}·Å^{-1} was applied to backbone atoms of B*2705. As the solvent was implicitly taken into account, a constant dielectric function ($\epsilon = 1$) was utilized. For the whole trajectory, the same twin cutoff (10−15 Å) was used for calculating nonbonded interactions, and the nonbonded pair list was updated every 10 steps. The SHAKE algorithm was used on hydrogens with a tolerance of 0.00025 Å, a time step of 2 fs, and Berendsen temperature coupling with separate coupling of solute and solvent atoms to the heat bath. Coordinates, velocities, and energies were saved every 0.5 ps. All computations were done using the parallel version of AMBER5.0 implemented on a CRAY J90 cluster and an INTEL paragon machine. The analyses of molecular dynamics trajectories were achieved using in-house routines and the CARNAL module of AMBER.[66]

Epitope Stabilization Assay. The quantitative assay used was previously described.[71] Briefly, RMA-S transfectants expressing B*2705 were used. These are murine cells with impaired TAP-mediated peptide transport and low surface expression of (empty) class I MHC molecules, which can be induced at 26 °C[72] and stabilized at the cell surface through binding of exogenously added ligands. These cells were incubated at 26 °C for 24 h. After this time they were incubated for 1 h at 26 °C with 10^{-4}−10^{-9} M peptides, transferred to 37 °C, and collected after 4 h for flow microcytometry (FMC) analysis with the ME1 mAb (IgG1, specific for HLA-B27, -B7, and -B22).[73] The determinant recognized by ME1 is not affected by bound peptides (data not shown). Binding of a given ligand was measured as its C_{50}. This is its molar concentration at 50% of the fluorescence obtained with that ligand at 10^{-4} M. Ligands with $C_{50} \leq 5\ \mu$M were considered to bind with high affinity, as these were the values obtained for most of the natural B27-bound peptides. C_{50} values between 5 and 50 μM were considered to reflect intermediate affinity. $C_{50} \geq 50\ \mu$M indicated low affinity.

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