Partially Modified Retro-Inverso Pseudopeptides as Non-natural Ligands for the Human Class I Histocompatibility Molecule HLA-A2

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Received December 27, 1995[⊗]

Syntheses of a series of partially modified retro-inverso analogues of the antigenic peptide M58-66 derived from the influenza virus matrix protein are reported. The retro-inverso modification Ψ(NH−CO) was obtained by replacement of two successive amino acid residues with a 2-substituted malonate derivative and *gem*-diaminoalkyl residue. The resulting compounds **1**−**8** were tested for their binding to the human histocompatibility class I molecule HLA-A2 in an assembly assay using lysates of peptide transporter-deficient cells T2. Specific peptide-dependent HLA-A2 assembly was revealed by an enzyme-linked immunosorbent assay. Significant HLA-A2 assembly was detected in the presence of analogues [gGly^{58}-(*S*)mLeu^{59}]-M58-66 **(1a)**, [gGly^{61}-(*R*,*S*)mPhe^{62}]M58-66 **(4)**, [gVal^{63}-(*R*,*S*)mPhe^{64}]M58-66 **(6)**, and [gPhe^{64}-(*R*,*S*)mAla^{65}]M58-66 **(7)**. The introduction of the retro-inverso modification between P2−P3, P3−P4, P5−P6, and P8−P9 (compounds **2**, **3**, **5**, and **8**, respectively) however led to a dramatic reduction in peptide binding to HLA-A2. Interestingly, compound **1a** which contains modification between P1−P2 was found to be the most potent analogue, being able to retain the original HLA-A2 binding profile of the parent peptide M58-66. Taken together, these results and recent binding data obtained in the context of murine MHC class I molecule H-2K^{d} suggest that the incorporation of peptide bond surrogates in MHC class I-restricted epitopes is a useful approach to design molecules having both increased stability and high MHC-binding capacity. Depending on their agonist or antagonist effects at the T-cell receptor, such non-natural MHC ligands are likely to find many applications in the development of peptide-based vaccines or as potential therapeutic agents in the treatment of allergies and autoimmune diseases.

Introduction

CD8+ cytotoxic T cells (CTL) play a key role in the immune response against intracellular pathogens—such as viruses—and tumors. T-cell activation and subsequent lysis of target cells require recognition by the T-cell receptors (TCR) of complexes formed between class I major histocompatibility complex (MHC) molecules and antigenic peptides derived, in most cases, from intracellular processing of endogeneous proteins.[1]

MHC class I molecules are cell surface glycoproteins that consist of a highly polymorphic heavy chain associated noncovalently with the invariant β2-microglobulin. Only a few different class I MHC proteins—no more than six—are expressed in the tissue of any given individual. As a consequence each single allele has to bind a sufficiently broad array of peptides to allow the immune system to both recognize foreign antigen and remain tolerant to self-derived peptides. Indeed, a single MHC class I allele can bind over 10 000 different peptides. However, MHC class I molecules are highly polymorphic, and the particular set of bound peptides is specific for each allele.[2] The 3D structure determination of several human and murine class I molecules crystallized and complexed either with a mixture of endogeneous ligands or with single peptides has been performed. It offers some insight into the molecular mechanisms for specific peptide/MHC molecule interaction and antigen presentation.[3−11] The MHC peptide binding site is a groove formed by two α-helices lying across an eight-stranded β-pleated sheet which accommodates peptides of 8−10 residues long in an extended conformation. The amino and carboxyl termini of the peptide are positioned in two pockets at each end of the cleft through extensive hydrogen bonding to highly conserved MHC residues. The allelic specificity of the binding is introduced by additional pockets containing MHC polymorphic residues which accommodate a few peptide anchor side chains. For example, HLA-A2 which is one of the most frequent class I alleles—expressed by 46% of the Caucasian population—preferentially binds nonapeptides with leucine, methionine, or isoleucine at position P2 and valine, leucine, or isoleucine at position P9.[2,12,13] As suggested by the 3D structure of class I MHC complexes, the central part of the peptide arches away from the floor of the binding site, and it is assumed that it is particularly exposed for T-cell recognition.[7−9]

The use of peptides corresponding to MHC class I-restricted epitopes to induce a protective CTL response against tumors or viruses is of particular interest in the development of peptide-based vaccines.[14,15] MHC-binding peptides have also been shown to be attractive as potential therapeutic agents in the treatment of allergies and autoimmune diseases. It is assumed that the presentation and the recognition of self-derived peptides by T cells are involved in the pathogenesis of many autoimmune diseases. In a first approach, peptides

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1996.

S0022-2623(95)00951-4 CCC: $12.00

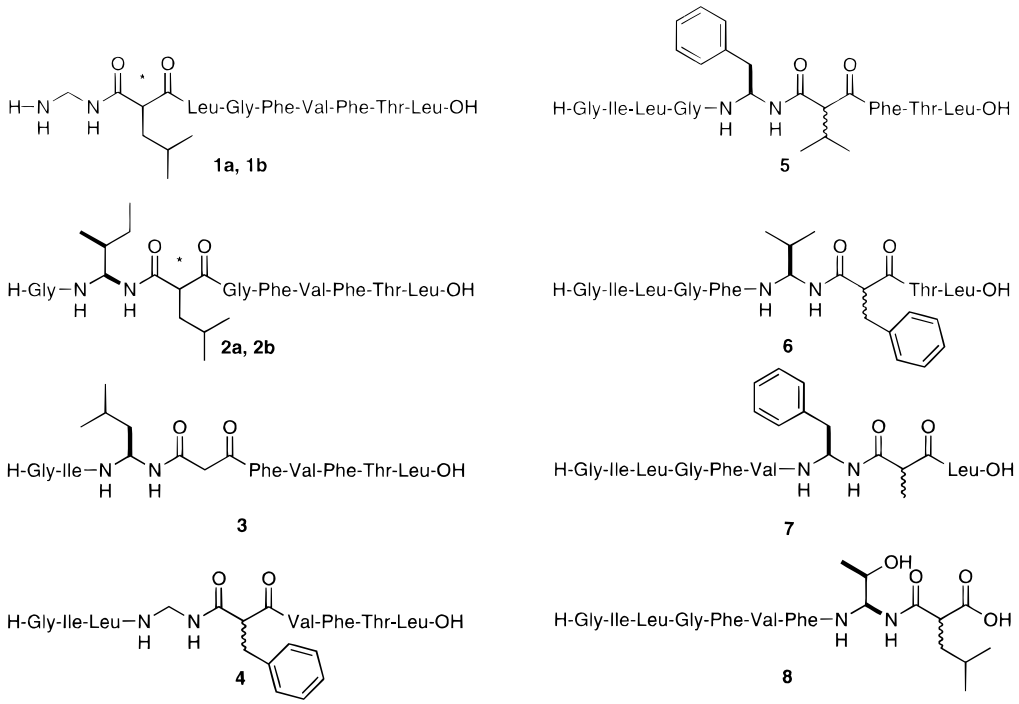

Figure 1. Partially modified retro-inverso analogues of influenza virus matrix protein-derived peptide M58-66.

with high MHC-binding capacity were designed and used to block the peptide binding site and hence inhibit antigen presentation.[16−18] More recently, antigen analogues containing subtle changes in peptide sequence were found to act as TCR antagonists or partial agonists.[19−22] This phenomenon, because of its high efficiency and specificity, represents a promising alternative for antigen specific immunomodulation.[22−26]

The potential applications of peptides as immunogens or immunomodulators, however, are strongly impaired by their high susceptibility toward enzymatic degradation and their rapid clearance from circulation.[27,28] Pseudopeptides have been widely used in pharmacology to convert biologically active peptides into more stable molecules which could retain—or even enhance—the original biological activity.[29−32] Moreover, isosteric replacement of the amide bond also led in some cases to the discovery of potent antagonists of the parent peptide.[33−38] Recent investigations have also described the potential interest of pseudopeptides in immunology. We have thus reported the use of retro-inverso peptidomimetics and reduced peptide bond pseudopeptides for mimicking antigenic sites and as potential synthetic vaccines,[39−43] and only a very few recent studies have investigated the immunological impact of peptide backbone modifications in the context of peptide−class I or II MHC molecule interaction.[44−48]

In a rational attempt to design TCR agonists or antagonists of increased stability using pseudopeptides, we evaluated, in a preliminary study, the ability of various peptide analogues to bind MHC class I molecules. This approach should allow us to first examine the contribution of each peptide amide bond in the binding of peptide to MHC class I molecule before testing the influence of these changes at the level of the TCR. In this study, we synthesized a series of partially modified retro-inverso analogues of the influenza matrix peptide 58-66 (M58-66) of sequence H-Gly^{58}-Ile-Leu Gly-Phe-Val -Phe-Thr-Leu^{66}-OH known to be a HLA-A2-restricted epitope.[49] The resulting analogues were tested in vitro for their ability to associate with HLA-A2 heavy chains and form stable peptide/HLA-A2 complexes.

Results and Discussion

Chemistry. Partially modified retro-inverso analogues **1**−**8** (Figure 1) of the influenza virus matrix protein-derived peptide M58-66 were synthesized by conventional methods in solution. The retro-inverso modification Ψ(NH−CO) was obtained by replacement of two sequential amino acids with a (*R*,*S*)-2-substituted malonate derivative and a *gem*-diaminoalkyl residue. Because the replacements of Ile by Leu at position 59 and Thr by Ala at position 65 were shown not to affect the HLA-A2 binding,[50] we decided for easier syntheses to incorporate a (*R*,*S*)-2-isobutylmalonic acid in compound **1** and a (*R*,*S*)-2-methylmalonic acid in compound **7**.

The synthesis of compound **1** was carried out as follows. The dipeptide carboxamide EtO-(*R*,*S*)mLeu-Gly-NH_2 **(13)**, which was obtained from (*R*,*S*)-2-isobutylmalonic acid monoethyl ester and HCl·H-Gly-NH_2, was treated with [bis(trifluoroacetoxy)iodo]benzene and converted through a Hofmann rearrangement to the trifluoroacetate salt of the corresponding *gem*-diaminoalkyl derivative EtO-(*R*,*S*)mLeu-gGly-H·TFA **(14)**. Benzyloxy carbonylation of **14** using Z-OSu and subsequent saponification led to the partially modified retro-inverso dipeptide Z-Gly-(*R*,*S*)mLeu-OH **(16)** which was further condensed with TFA·Leu-Gly-OMe to give the protected tetrapeptide Z-Gly-(*R*,*S*)mLeu-Leu-Gly-OMe **(18)**. Saponification of **18** and reaction with TFA·H-Phe-Val-Phe-Thr(Bzl)-Leu-OBzl using BOP as a coupling agent led to the fully protected nonapeptide **20**. Catalytic hydrogenation using Pd/C afforded **1** with a diastereoisomeric mixture of 46/54. The shorter retained isomer **1a** could be separated by HPLC; however, we were unable to isolate the longer retained isomer in

Table 1. Analytical Data of M58-66 Analogues **1**−**8**

no.	compound	mp (°C)	HPLC t_R (min)[a]	enantiomeric ratio[b]	FAB-MS[d]
1a	[gGly^{58}-(*S*)mLeu^{59}]M58-66	155 dec	11.92	100:0	966.5
1b	[gGly^{58}-(*R,S*)mLeu^{59}]M58-66	165 dec	11.96, 12.59	19:81	966.5
2a	[gIle^{59}-(*S*)mLeu^{60}]M58-66	220−230	12.00	100:0	966.6
2b	[gIle^{59}-(*R*)mLeu^{60}]M58-66	202 dec	12.96	0:100	966.6
3	[gLeu^{60}-mGly^{61}]M58-66	190 dec	11.63		966.4
4	[gGly^{61}-(*R,S*)mPhe^{62}]M58-66	207 dec	11.79, 12.16	29:71	966.5
5	[gPhe^{62}-(*R,S*)mVal^{63}]M58-66	177 dec	11.46, 11.97	42:58	966.5
6	[gVal^{63}-(*R,S*)mPhe^{64}]M58-66	215−225	11.87, 11.92	*c*	966.5
7	[gPhe^{64}-(*R,S*)mAla^{65}]M58-66	165 dec	11.93, 12.30	36:64	936.5
8	[gThr^{65}-(*R,S*)mLeu^{66}]M58-66	162 dec	11.77, 11.90	47:53	966.5

[a] Linear gradient of A:0.1% TFA and B:acetonitrile containing 0.08% TFA, 20−80% B, 20 min. In the case of diasteroisomeric mixture, the t_R values of both the shorter and the longer retained isomer are reported. [b] Shorter retained isomer:longer retained isomer ratio as determined by HPLC. [c] The poor separation of the two diastereoisomers did not allow the diastereoisomeric ratio to be determined. [d] Fast atom bombardment mass spectroscopy.

pure isomeric form, and compound **1b** was shown to be contaminated by 19% of compound **1a**.

The absolute configuration of mLeu residue in each isomer was deduced from biological and chromatographic data. Chromatographic studies have shown that peptides containing L- and D-residues are more hydrophobic than peptides composed of a single chirality.[32,51] In addition, the correct stereochemistry at the main anchor side chain was found to be critical for binding in the context of a MHC class II molecule.[45] We thus assume that the shorter retained isomer **1a** contains the (*S*)mLeu derivative. Monoacyl *gem*-diaminoalkyls are susceptible to hydrolysis,[52] and thus we investigated the stability of **1a** at 37 °C at pH = 6.0 and 8.0. No significant hydrolysis was observed after 48 h. In addition, **1a** was found to be configurationally stable, as no racemization occurred at the malonyl residue after 48 h (data not shown).

Compounds **2**−**7** were obtained similarly. The *gem*-diaminoalkyl residues were generated by conversion of dipeptide or tripeptide amides using the [bis(trifluoroacetoxy)iodo]benzene and further coupled to 2-substituted malonic acid monoethyl esters to give the corresponding protected pseudopeptide intermediate. Fragment condensation and stepwise elongation of the peptide chains using BOP led to the fully protected nonapeptides, which after HF cleavage or hydrogenolysis over Pd/C yielded compounds **2**−**7**. Because the 2-substituted malonate derivative was incorporated into the peptide chain as a racemate, compounds **2**, **4**−**7** were obtained as diastereoisomeric mixtures. Attempts to separate the diasteoisomers by reverse phase HPLC failed for compounds **4**−**7** probably because of the chiral instability of the malonate. In the case of compound **2**, however, the two diastereoisomers, namely, compounds **2a**,**b**, were isolated in pure isomeric form.

Boc-Phe-gThr(Bzl)-(*R,S*)mLeu-OBzl **(67)** was the key intermediate in the synthesis of compound **8**. Reductive alkylation of Meldrum's acid (2,2-dimethyl-1,3-dioxane-4,6-dione) with isobutyraldehyde and borane·dimethylamine[53] gave the corresponding 2,2-dimethyl-5-isobutyl-1,3-dioxane-4,6-dione **(65)**. Ring opening of the Meldrum derivative **65** in the presence of an excess of benzyl alcohol, as described by Chorev and co-workers,[54] followed by column chromatography afforded pure (*R,S*)-2-benzylmalonic acid monobenzyl ester **(66)**. Coupling of the 2-substituted malonate derivative **66** to Boc-Phe-gThr(Bzl)-H·TFA **(63)**, obtained from the corresponding dipeptide amide **64**, led to Boc-Phe-gThr(Bzl)-(*R,S*)-mLeu-OBzl **(67)**. Stepwise elongation of the peptide chain or fragment condensation led to the fully protected nonapeptide **69**. HF cleavage and HPLC purification on a C18 column afforded **8** as a pure diastereoisomeric mixture. As in the case of compounds **4**−**7**, attempt to purify the two diastereoisomers was unsuccesful.

All final products **1**−**8** were identified by 500 MHz ^{1}H-NMR spectroscopy and fast atom bombardment spectrometry, and their homogeneity was assessed by analytical reverse phase HPLC. Physical and analytical data are given in Table 1. As an example, experimental ^{1}H-NMR data of compound **1a** in DMSO-d_6 are given in Table 2.

HLA-A2 Binding. Partially modified retro-inverso peptides **1**−**8** (Figure 1) were tested for their ability to promote the assembly of HLA-A2 molecules in T2 cell lysates. T2 is a mutant of cell line deficient in peptide transporters, and it constitutes a source of empty HLA heavy chains. Stable peptide/heavy chain/β2m complexes can be formed by adding synthetic peptides to the cell lysates. Specific assembly of HLA-A2 was then detected by an enzyme-linked immunosorbent assay as previously described by Connan et al.[55] The key reagent is a monoclonal antibody (mAb) that only recognizes HLA structures similar to those existing at the cell surface, i.e., containing peptide. The results are listed in Figure 2. In the same experiment pseudopeptide analogues were compared with the original HLA-A2-restricted antigenic peptide H-Gly-Ile-Leu-Gly-Phe-Val-Phe-Thr-Leu-OH (M58-66) derived from influenza virus matrix protein. The ability of M58-66-derived peptides [Leu^{59}]M58-66 and [Ala^{65}]M58-66 to promote HLA-A2 assembly was also included to allow a direct comparison with the corresponding analogues **1a**,**b** and **7** (Figure 2). As expected,[50] no significant difference was found between M58-66 and the two controls [Leu^{59}]M58-66 and [Ala^{65}]M58-66 in their capacity to form stable complex with HLA-A2. Broad differences were observed in the ability of the different partially modified retro-inverso analogues to promote HLA-A2 assembly: Analogues **1a**,**b**, **4**, **6**, and **7** were efficient, whereas analogues **2a**,**b**, **3**, **5**, and **8** were not (Figure 2).

Incorporation of the retro-inverso modification between Ile^{59} and Leu^{60} (P2−P3), Leu^{60} and Gly^{61} (P3−P4), Phe^{62} and Val^{63} (P5−P6), and Thr^{65} and Leu^{66} (P8−P9) was thus deleterious for binding. The reversal of the direction of the amide bond modifies the relative position of the main chain oxygen and nitrogen atoms available for hydrogen bonding and hence may alter the intermolecular hydrogen-bonding pattern of the peptide bound to the HLA molecule. In the case of compounds

Table 2. Experimental ^{1}H-NMR Data for Compound **1a** in DMSO-d_6 Solution

	NH		Hα	Hβ		others
	δ (ppm)	^3J (Hz)	δ (ppm)	δ (ppm)	^3J (Hz)	δ (ppm)
gGly	8.67	6.0/6.0	4.17			
			4.10			
(*S*)mLeu			3.31	1.59		γCH, 1.46
				1.46		$\delta\delta'$CH$_3$, 0.84/0.82
Leu	8.08	6.9	4.27	1.56		γCH, 1.44
				1.44		$\delta\delta'$CH$_3$, 0.85/0.80
Gly	8.12	5.8	3.69			
		6.0	3.58			
Phe	7.99	8.3	4.56	2.92	4.1	
				2.71	9.7	
					($^2J_{\beta\beta'} = 14.0$)	
Val	7.92	8.9	4.13	1.91		γCH$_3$, 0.77 ($^3J = 6.8$ Hz)
						γ'CH$_3$, 0.76 ($^3J = 6.8$ Hz)
Phe	8.07	7.8	4.68	3.05	4.1	
				2.82	9.6	
					($^2J_{\beta\beta'} = 14.0$)	
Thr	7.93	8.6	4.23	3.95		γCH$_3$, 1.04 ($^3J = 6.3$ Hz)
Leu	7.84	8.0	4.25	1.66		γCH, 1.53
				1.53		$\delta\delta'$CH$_3$, 0.89/0.96

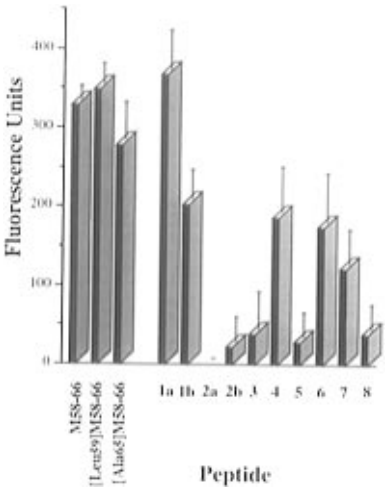

Figure 2. Induction of HLA-A2 assembly in the presence of 10^{-6} M peptide M58-66 from influenza matrix, [Leu^{59}]M58-66, [Ala^{65}]M58-66, and analogues **1**−**8**. Results are expressed as fluorescence arbitrary units measured at 340/460 nm.

2a,**b** and **8**, this observation is consistent with recent crystallographic studies of different human class I molecules complexed with either endogeneous mixtures or single peptides.[4−7,9] The structures of five different peptides—including M58-66—bound to HLA-A2 reveal a major contribution for polar peptide main chain atoms in the binding interaction.[9] A similar mode of peptide binding was also found for HLA-B27 complexed with a mixture of endogeneous ligands[4,7] and HLA-Aw68 complexed with a peptide derived from the nucleoprotein of influenza virus.[6] Conserved side chains of the HLA molecule were shown to be hydrogen bonded to main chain polar atoms at both ends of the peptide, therefore tethering the peptide in the cleft in an extended conformation. Additional hydrogen bonds between the peptide backbone and polymorphic MHC residues were also visualized in these complexes. In the structure of HLA-A2 complexed with M58-66 peptide, polymorphic MHC residues Lys^{66} and Tyr^{99} were found to be hydrogen bonded to the carbonyl oxygen of Ile^{59} at P2 and to the amide nitrogen of Leu^{60} at P3, respectively.[9] This hydrogen bond pattern is likely to be altered in analogues **2a**,**b**, and accordingly they were unable to bind to and stabilize HLA-A2. Similarly, the side chain of the polymorphic residue Asp^{77} was shown to hydrogen bond directly to the amide nitrogen of Leu^{66} at P9. In addition, the nitrogen atom of the indole ring in the highly conserved Trp^{147} forms a hydrogen bond with the main chain carbonyl oxygen between P8−P9. The replacement in compound **8** of the Thr^{65}−Leu^{66} amide bond by a Ψ(NH−CO) bond abolished peptide−HLA-A2 binding. Similarly, a major drop in binding affinity to the mouse H2-K^d molecule was observed with a PbCs peptide analogue containing a Ψ(CH$_2$−NH) amide bond surrogate at P8−P9.[48] Interestingly, the results obtained in these two studies may indicate a critical role for the two hydrogen bonds in the stabilization of MHC class I molecule−peptide complexes. Conversely, the crystal structure of the HLA-A2 molecule complexed with M58-66 reveals that the central peptide residues P4−P7 do not make direct hydrogen bonds to the HLA molecule. The detection of HLA-A2 assembly in the presence of analogues **4**, **6**, and **7** tends to support this observation. By contrast, the lack of detection of HLA-A2 assembly in the presence of analogues with retro-inverso modifications at P3−P4 and P5−P6 (compounds **3** and **5**, respectively) was surprising. This suggests that some retro-inverso modifications might alter the overall structure of the analogues which are no longer able to mimic the extended bioactive conformation of M58-66.

Most interestingly, compound **1a** was found to be the most potent analogue in the HLA-A2 assembly assay. It showed an efficiency for HLA-A2 stabilization similar to that of the parent peptide and the corresponding control [Leu^{59}]M58-66. Figure 3 shows the effect of peptide concentration on HLA assembly: Analogue **1a** was found to retain the full original binding profile of [Leu^{59}]M58-66, being able to stabilize HLA-A2 molecule at a concentration of 10 nM or less. The high efficiency for HLA-A2 assembly shown by peptide **1a** was relatively unexpected. Indeed, the crystal structure of HLA-A2 complexed to M58-66 reveals that the carbonyl oxygen and the amide nitrogen at the P1−P2 peptide bond are respectively hydrogen bonded to the hydroxyl group of the highly conserved Tyr^{159} and the side chain of the more polymorphic Glu^{63}. In the same manner, the incorporation of a reduced peptide bond at P1−P2 position in the PbCs peptide was not detrimental for

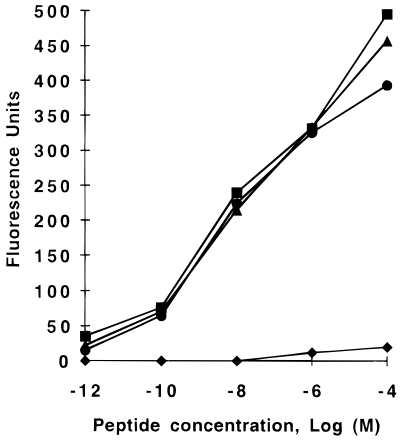

Figure 3. Induction of HLA-A2 assembly in the presence of different concentrations (10^{-4}–10^{-12} M) of peptide: (●) [Leu[59]]M58-66, (▲) M58-66, (■) analogue **1a**, and (◆) analogue **2b**.

the association to H2-K^d molecule, although a 5-fold decrease in binding affinity was observed.[48] This indicates either that the two hydrogen bonds may not be as critical as suggested by structural studies or that, in the case of peptide **1a**, the reversal of the amide bond at P1–P2 creates another hydrogen-bonding pattern susceptible to participate in the stabilization of the complex. For HLA-A2 binding, peptide side chains at P2 (Leu or Ile) and P9 (Val, Leu, or Ile) are the dominant anchors that define allele specific binding motif. If the stereochemical requirements at the main anchor positions have not been defined for HLA molecules, it has been shown, in the context of human MHC class II molecule DRB1*0101 and DRB1*0401, that the introduction of a D-Phe instead of the L-Phe resulted in a decrease of the binding affinity to the MHC molecule of 2 orders of magnitude.[45] Compound **1a** which contains the (*S*)mLeu[59] was the most efficient isomer, and despite the fact that compound **1b** contains 19% of **1a**, the inversion of configuration of mLeu[59] residue at P2 decreased its reactivity. Similarly, the apparent decrease in potency found for compounds **4**, **6**, and **7** (Figure 2) in their ability to promote HLA-A2 assembly as compared to the parent peptide might be related to the fact that these analogues are diastereoisomeric mixtures. However, in the absence of data demonstrating the importance of each chiral center in MHC class I ligands, one can only speculate about the possible difference in biological activity of the diastereoisomers.

Conclusion

This work presents the syntheses of partially modified retro-inverso analogues of the peptide M58-66 derived from the influenza virus matrix protein (compounds **1**–**8**) and evaluates their ability to associate with the human leucocyte antigen HLA-A2. Broad differences were found in the capacity of the various analogues to bind to HLA-A2. While significant HLA-A2 assembly was observed in the presence of analogues modified at the P1–P2, P4–P5, P6–P7, and P7–P8 amide bond, the incorporation of the retro-inverso modification between P2–P3, P3–P4, P5–P6, and P8–P9 was found to be detrimental for binding to HLA-A2. These results confirm the major contribution in HLA-A2 binding of polar peptide main chain atoms especially at residues P2, P3, P8, and P9, as revealed by the crystal structures of HLA-A2 molecule complexed to M58-66.[9] Surprisingly, the reversal of the direction of the amide bond between P1–P2 gave the most potent analogue, **1a**, that, like the parent peptide, was able to stabilize HLA-A2 at a concentration of 10 nM. The results shown here, and in a previous work in the context of murine MHC class I molecule,[48] represent a preliminary step toward the design of peptide analogues with improved stability acting as TCR agonists or antagonists. With this aim, the four non-natural ligands for HLA-A2 found to be effective in this study will presently be tested for their ability to be recognized by HLA-A2-restricted cytotoxic T lymphocytes.

Experimental Section

Chemistry. Melting points were determined on a Büchi 530 apparatus (Flawil, Switzerland) in open capillary tubes. Optical rotations were measured at 25 °C on a Perkin Elmer 241 polarimeter (Saint Quentin Yvelines, France). Microanalyses were performed by the Service de Microanalyses, Institut de Chimie (Strasbourg, France). Ascending thin layer chromatographies (TLC) were performed on precoated silica gel 60 F_{254} (Merck, Darmstadt, Germany) with the following solvent systems (v/v): A, ethyl acetate/hexane, 1:2; B, ethyl acetate/hexane, 2:1; C, ethyl acetate; D, chloroform/methanol, 20:2; E, chloroform/methanol, 20:4; F, chloroform/methanol/acetic acid, 80:10:5; G, chloroform/methanol/acetic acid, 120:10:5. Peptide derivatives were visualized by UV light (254 nm), ninhydrin, or 5% sulfuric acid/methanol. Column chromatographic separations were performed with silica gel 60, 230–400 mesh ASTM (Merck, Darmstadt, Germany). Analytical HPLC were run on a Beckman instrument (Gagny, France) with a Nucleosil C18 column (5 μm, 3.9 × 150 mm) using a linear gradient of A:0.1% TFA and B:acetonitrile containing 0.08% TFA, at a flow rate of 1.2 mL/min with UV detection at 214 nm. Preparative reverse phase HPLC were performed using a Perkin-Elmer apparatus on an Aquapore ODS 20 μm column (100 × 10) by elution with a linear gradient of A:aqueous 0.06% TFA and B:80% acetonitrile/20% A, at a flow rate of 6 mL/min with UV detection at 220 nm.

NMR spectra of final compounds were recorded on a ARX 500 Bruker spectrometer (Bruker Spectrospin, Wissembourg, France) at 500 MHz under temperature control at 300 K. This machine is equipped with a Z gradient accessory, a 5 mm inverse ^{1}H probe, and a Z gradient coil. Both ROESY and COSY were acquired in States-TPPI mode increasing the sensitivity of these 2D experiments. The ROESY was recorded with 2.5 s interpulse delay and a 300 ms spin–lock at a power of 2.5 kHz,[56] whereas the COSY GRASP was recorded with the same 2.5 s relaxation delay and a 10:20 gradient sequence.[57] Both were recorded with 1K acquisition size and 512 increments and processed with a 2K × 2K zero filling prior transformation.

Mass spectra were obtained in FAB positive mode on a VG analytical ZAB-2SE double-focusing instrument and recorded on a VG 11-250 data system (VG Analytical, Manchester, U.K.) at the Service de Spectrométrie de Masse, Institut de Chimie (Strasbourg). The matrix was *m*-nitrobenzyl alcohol (*m*-NBA) or thioglycerol containing 1% TFA.

Amino acids and derivatives were from Neosystem (Strasbourg, France). All reagents and solvents were of analytical grade.

Boc-Thr(Bzl)-Leu-OBzl (9). To a solution of Boc-Thr(Bzl)-OH (4.63 g, 15 mmol) and BOP (6.63 g, 15 mmol) in DMF (30 mL) were sucessively added HCl·H-Leu-OBzl (6.49 g, 16,5 mmol) and DIEA (6.54 mL, 37.5 mmol). After 3 h of stirring at room temperature, the reaction product precipitated as a white solid upon addition of a saturated sodium hydrogen carbonate solution. It was filtered, washed with saturated sodium hydrogen carbonate solution, water, 1 M potassium hydrogen sulfate, and water, and finally dried *in vacuo* over phosphorus pentoxide: yield 7.32 g (95%); R_f (C) 0.89; mp 79–80 °C; $[\alpha]^{25}_D$ −6.99° (*c* 1.13, DMF). Anal. ($C_{29}H_{40}N_2O_6$) C,H,N.

Boc-Phe-Thr(Bzl)-Leu-OBzl (10). Compound **9** (7.17 g, 14 mmol) was treated with TFA for 30 min at room temperature. TFA was removed *in vacuo*, and the residue was triturated with ether−hexane (1:3, v/v), filtered, and dried *in vacuo* over KOH pellets to give TFA·H-Phe-Thr(Bzl)-Leu-OBzl: yield 7.0 g (95%). This compound was condensed with Boc-Phe-OH as described in the synthesis of **9**; 84% yield; R_f (C) 0.86; R_f (G) 0.87; mp 157−159 °C; $[\alpha]^{25}_D$ −2.29° (*c* 1.09, DMF). Anal. ($C_{38}H_{49}N_3O_7$) C,H,N.

Boc-Val-Phe-Thr(Bzl)-Leu-OBzl (11): prepared from Boc-Val-OH and TFA·Phe-Thr(Bzl)-Leu-OBzl (obtained from **10**) as described in the synthesis of **9**; 77% yield; R_f (C) 0.88; R_f (G) 0.84; mp 153−156 °C; $[\alpha]^{25}_D$ −2.49° (*c* 1.05, DMF). Anal. ($C_{43}H_{58}N_4O_8$) C,H,N.

Boc-Phe-Val-Phe-Thr(Bzl)-Leu-OBzl (12): prepared from Boc-Phe-OH and TFA·Val-Phe-Thr(Bzl)-Leu-OBzl (obtained from **11**) as described in the synthesis of **9**; 97% yield; R_f (G) 0.81; mp 189−191 °C; $[\alpha]^{25}_D$ −1.62 (*c* 1.05, DMF). Anal. ($C_{52}H_{67}N_5O_9$) C,H,N.

EtO-(*R,S*)mLeu-Gly-NH$_2$ (13). To a solution of 2(*R,S*)-isobutylmalonic acid ethyl ester (3.76 g, 20 mmol) and BOP (8.84 g, 20 mmol) in DMF (30 mL) were successively added HCl·H-Gly-NH$_2$ (2.45 g, 22 mmol) and DIEA (6.54 mL, 37.5 mmol). After 3 h of stirring at room temperature, a saturated hydrogen carbonate solution (100 mL) was added, and the resulting mixture was extracted with ethyl acetate (3 × 80 mL). The organic phases were combined, washed with saturated hydrogen carbonate solution, brine, 1 M potassium hydrogen sulfate, and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by column chromatography using $CHCl_3$−MeOH (20:4, v/v) as eluting solvent to give a residue which was cystallized from EtOH: yield 2.93 g (60%); R_f (C) 0.43; R_f (G) 0.54; mp 88−91 °C. Anal. ($C_{11}H_{20}N_2O_4$) C,H,N.

EtO-(*R,S*)mLeu-gGly-H·TFA (14). Compound **13** (2.61 g, 10.6 mmol) was suspended in a mixture of CH_3CN and water (1:1, 50 mL), and [bis(trifluoroacetoxy)iodo]benzene (4.79 g, 11.13 mmol) was added under nitrogen. After 4 h of stirring at room temperature, the solvents were removed *in vacuo* and compound **14** was recovered as a sticky solid which was used in the next step without further purification: yield 3.76 g (95%); R_f (F) 0.30.

EtO-(*R,S*)mLeu-gGly-Z (15). To a solution of compound **14** (3.76 g, 10.07 mmol) in $CHCl_3$ (25 mL) were successively added (benzyloxycarbonyl)-*N*-hydroxysuccinimide (2.64 g, 10.65 mmol) and DIEA (2.04 mL, 11.77 mmol). After 5 h of stirring at room temperature, the solvent was removed *in vacuo*, the residue was dissolved in ethyl acetate (80 mL), and a 1 M potassium hydrogen sulfate solution was added under stirring. The organic layer was collected, washed with saturated hydrogen carbonate solution, brine, 1 M potassium hydrogen sulfate, and brine, dried over $MgSO_4$, and concentrated *in vacuo*. Recrystallization from EtOH afforded **15** as white crystals: yield 2.45 g (70%); R_f (A) 0.27; R_f (G) 0.78; mp 89−100 °C. Anal. ($C_{18}H_{26}N_2O_5$) C,H,N.

HO-(*R,S*)mLeu-gGly-Z (16). To a cold 0 °C solution of compound **15** (1.75 g, 5 mmol) in EtOH (20 mL) was added 1 N aqueous NaOH (7 mmol). After 2 h of stirring, the solvent was removed, and a 1 M potassium hydrogen sulfate solution (100 mL) was added under stirring. The white precipitate which formed was filtered, washed with water, and dried *in vacuo* over phosphorus pentoxide: yield 1.07 g (66%); R_f (G) 0.75; mp 136−137 °C. Anal. ($C_{16}H_{22}N_2O_5$) C,H,N.

Boc-Leu-Gly-OMe (17): prepared from HCl·H-Gly-OMe and Boc-Leu-OH·H_2O as described for compound **9**; 85% yield; R_f (G) 0.75; mp 114−119 °C; $[\alpha]^{25}_D$ −14.95 (*c* 1.07, DMF). Anal. ($C_{14}H_{26}N_2O_5$) C,H,N.

Z-gGly-(*R,S*)mLeu-Leu-Gly-OMe (18): prepared from **16** and TFA·H-Leu-Gly-OMe (obtained from **17**) as described in the synthesis of **9** except that the reaction mixture was stirred for 12 h; 94% yield; R_f (C) 0.74; R_f (G) 0.81; mp 147−150 °C; $[\alpha]^{25}_D$ −14.6° (*c* 1.55, DMF). Anal. ($C_{25}H_{38}N_4O_7$) C,H,N.

Z-gGly-(*R,S*)mLeu-Leu-Gly-OH (19): prepared by saponification of compound **18** in MeOH following the procedure described for **16**; 92% yield; R_f (G) 0.70; mp 152−156 °C; $[\alpha]^{25}_D$ −13.5° (*c* 1.12, DMF). Anal. ($C_{24}H_{36}N_4O_7$) C,H,N.

Z-gGly-(*R,S*)mLeu-Leu-Gly-Phe-Val-Phe-Thr(Bzl)-Leu-OBzl (20): prepared from **19** and TFA·H-Phe-Val-Phe-Thr-(Bzl)-Leu-OBzl (obtained from **12**) as described in the synthesis of **9** except that the reaction mixture was stirred for 12 h: 88% yield. As a result of its high insolubility, **20** was subjected to final hydrogenolysis without further purification: R_f (G) 0.86.

H-gGly-(*R,S*)mLeu-Leu-Gly-Phe-Val-Phe-Thr-Leu-OH (1). Compound **20** (400 mg, 0.31 mmol) was dissolved in a mixture of DMF/AcOH/H_2O (6:1:1, 40 mL) and hydrogenated for 6 h in the presence of 10% Pd/C as a catalyst at room temperature and atmospheric pressure. The catalyst was removed by filtration and the filtrate concentrated *in vacuo* to leave a residue which precipitated upon addition of ether. It was collected, washed with ether, dried *in vacuo* over KOH pellets, and lyophilized: yield 200 mg (63%) with a diastereoisomeric mixture of 46/54. The shorter retained isomer **1a** was separated by HPLC (linear gradient, 30−70% B, 40 min) and lyophilized. However, we were unable to isolate the longer retained isomer in pure isomeric form, and compound **1b** was shown to be contaminated by 19% of compound **1a**. Analytical data are reported in Table 1.

Z-Gly-Ile-NH$_2$ (21): prepared from HCl·H-Ile-NH_2 and Z-Gly-OH as described for compound **9**; 79% yield; R_f (G) 0.58; mp 160−162 °C; $[\alpha]^{25}_D$ +7.5° (*c* 1.38, DMF). Anal. ($C_{16}H_{23}N_3O_4$·$0.5H_2O$) C,H,N.

Z-Gly-gIle-H·TFA (22): prepared from compound **21** as described for compound **14** to give a sticky solid which was used in the next step without further purification; yield 98%; R_f (G) 0.35.

Z-Gly-gIle-(*R,S*)mLeu-OEt (23): prepared from compound **22** and 2(*R,S*)-isobutylmalonic acid ethyl ester as described for compound **9**; 73% yield; R_f (C) 0.76; R_f (G) 0.73; HPLC t_R 12.0/12.6 min (linear gradient, 20−80% B, 20 min); mp 115−120 °C; $[\alpha]^{25}_D$ −11.69° (*c* 1.30, DMF). Anal. ($C_{24}H_{37}N_3O_6$) C,H,N.

Z-Gly-gIle-(*R,S*)mLeu-OH (24): prepared by saponification of compound **23** following the procedure described for **16**; 95% yield; R_f (G) 0.62; mp 118−140 °C; $[\alpha]^{25}_D$ −11.82° (*c* 1.1, DMF). Anal. ($C_{22}H_{33}N_3O_6$·$1.5H_2O$) C,H,N.

Boc-Gly-Phe-Val-Phe-Thr(Bzl)-Leu-OBzl (25): prepared from Boc-Gly-OH and TFA·Phe-Val-Phe-Thr(Bzl)-Leu-OBzl as described in the synthesis of **9**; 95% yield; R_f (C) 0.86; R_f (G) 0.75; mp 215−218 °C. Anal. ($C_{54}H_{70}N_6O_{10}$) C,H,N.

Z-Gly-gIle-(*R,S*)mLeu-Gly-Phe-Val-Phe-Thr(Bzl)-Leu-OBzl (26): prepared from **24** and TFA·Gly-Phe-Val-Phe-Thr-(Bzl)-Leu-OBzl (obtained from **25**) as described in the synthesis of **9**; 92% yield. As a result of its high insolubility, **26** was subjected to final hydrogenolysis without further purification: R_f (G) 0.78.

H-Gly-gIle-(*R,S*)mLeu-Gly-Phe-Val-Phe-Thr-Leu-OH (2). Compound **26** (400 mg, 0.32 mmol) was hydrogenated following the procedure described for **1** to give the title compound with a diastereoisomeric mixture of 29/71: yield 165 mg (51%). The two diastereoisomers (**2a**,**b**) were separated by HPLC (linear gradient, 30−70% B, 40 min) and lyophilized. Analytical data are reported in Table 1.

Boc-Ile-Leu-NH$_2$ (27): prepared from HCl·H-Ile-NH_2 and Boc-Ile-OH·$0.5H_2O$ as described for compound **9**; 89% yield; R_f (C) 0.69; R_f (F) 0.83; mp 199−200 °C; $[\alpha]^{25}_D$ −35.74° (*c* 1.15, DMF). Anal. ($C_{17}H_{33}N_3O_4$) C,H,N.

Boc-Ile-gLeu-H·TFA (28). Compound **27** (2.61 g, 10.6 mmol) was suspended in a mixture of CH_3CN and water (2:1, 50 mL), and [bis(trifluoroacetoxy)iodo]benzene (4.79 g, 11.13 mmol) was added under nitrogen. After 4 h of stirring at room temperature, the solvent were removed *in vacuo* and compound **28** precipitated upon addition of ether−hexane (1:1, v/v). It was filtered, washed with ether and dried *in vacuo* over KOH pellets: yield 3.76 g (95%); R_f (C) 0.42; R_f (G) 0.5; mp 120−123 °C; $[\alpha]^{25}_D$ −8.36° (*c* 1.1, DMF). Anal. ($C_{16}H_{33}N_3O_3$·$1.3CF_3COOH$) C,H,N.

Boc-Ile-gLeu-mGly-OEt (29): prepared from compound **28** and malonic acid ethyl ester as described for compound **9**; 79% yield; R_f (C) 0.81; R_f (G) 0.83; mp 105 °C dec; $[\alpha]^{25}_D$ +2.48° (*c* 1.13, DMF). Anal. ($C_{21}H_{39}N_3O_6$·H_2O) C,H,N.

Boc-Ile-gLeu-mGly-OH (30): prepared by saponification of compound **29** following the procedure described for **16**; 82%

yield; R_f (G) 0.6; mp 175−180 °C; $[\alpha]^{25}_D$ +1.11° (*c* 1.17, DMF). Anal. ($C_{19}H_{35}N_3O_6$) C,H,N.

Boc-Ile-gLeu-mGly-Phe-Val-Phe-Thr(Bzl)-Leu-OBzl (31): prepared from compound **30** and TFA·Phe-Val-Phe-Thr-(Bzl)-Leu-OBzl as described for compound **9**; 95% yield; R_f (G) 0.86; mp 227−230 °C dec; $[\alpha]^{25}_D$ +1.35° (*c* 0.96, DMF). Anal. ($C_{66}H_{92}N_8O_{12}\cdot 2H_2O$) C,H,N.

Boc-Gly-Ile-gLeu-mGly-Phe-Val-Phe-Thr(Bzl)-Leu-OBzl (32): prepared from Boc-Gly-OH and TFA·Ile-gLeu-mGly-Phe-Val-Phe-Thr(Bzl)-Leu-OBzl (obtained from **31**) as described for compound **9**; 90% yield. As a result of its high insolubility, **32** was subjected to HF cleavage without further purification: R_f (G) 0.83.

H-Gly-Ile-gLeu-mGly-Phe-Val-Phe-Thr-Leu-OH (3). Compound **32** (350 mg, 0.28 mmol) was treated with HF (10 mL) in the presence of anisole (1 mL) for 45 min at 0 °C. After removal of HF, the peptide was precipitated in ether, filtered, and dried *in vacuo*: yield 291 mg (95%). It was purified by HPLC (linear gradient, 30−100% B, 40 min) and lyophilized. Analytical data are reported in Table 1.

Boc-Leu-Gly-NH_2 (33): prepared from Boc-leu-OH·H_2O and HCl·H-Gly-NH_2 as described for compound **13** to give **33** as a sticky solid; 90% yield; R_f (C) 0.29; R_f (G) 0.45.

Boc-Ile-Leu-Gly-NH_2 (34): prepared from Boc-Ile-OH·0.5H_2O and TFA·H-Leu-Gly-NH_2 (obtained from **33**) as described for compound **13**; 50% yield; R_f (C) 0.49; R_f (F) 0.78; mp 118−122 °C; $[\alpha]^{25}_D$ −24.05° (*c* 1.85, DMF). Anal. ($C_{19}H_{36}N_4O_5\cdot 1.5H_2O$) C,H,N.

Boc-Ile-Leu-gGly-H·TFA (35): prepared from compound **34** following the procedure described for **28**; 72% yield; R_f (C) 0.18; R_f (F) 0.44; mp 118−120 °C; $[\alpha]^{25}_D$ −20.96° (*c* 1.04, DMF). Anal. ($C_{18}H_{36}N_4O_4\cdot 1.3CF_3COOH$) C,H,N.

Boc-Ile-Leu-gGly-(*R,S*)mPhe-OEt (36): prepared from compound **35** and 2(*R,S*)-benzylmalonic acid ethyl ester as described for compound **9**; 87% yield; R_f (C) 0.84; R_f (F) 0.82; mp 120−130 °C; $[\alpha]^{25}_D$ −24.48° (*c* 1.65, DMF). Anal. ($C_{30}H_{48}N_4O_7\cdot H_2O$) C,H,N.

Boc-Ile-Leu-gGly-(*R,S*)mPhe-OH (37): prepared by saponification of compound **36** following the procedure described for compound **16**; 88% yield; R_f (G) 0.78; mp 195−200 °C; $[\alpha]^{25}_D$ −14.95 (*c* 1.11, DMF). Anal. ($C_{28}H_{44}N_4O_7\cdot 0.25H_2O$) C,H,N.

Boc-Ile-Leu-gGly-(*R,S*)mPhe-Val-Phe-Thr(Bzl)-Leu-OBzl (38): prepared from compound **37** and TFA·H-Val-Phe-Thr(Bzl)-Leu-OBzl as described for compound **9**; 96% yield; R_f (F) 0.84; mp 242−246 °C. Anal. ($C_{66}H_{92}N_8O_{12}\cdot 0.5H_2O$) C,H,N.

Boc-Gly-Ile-Leu-gGly-(*R,S*)mPhe-Val-Phe-Thr(Bzl)-Leu-OBzl (39): prepared from Boc-Gly-OH and TFA·H-Ile-Leu-gGly-(*R,S*)mPhe-Val-Phe-Thr(Bzl)-Leu-OBzl (obtained from **38**) as described for compound **9**; 90% yield. As a result of its high insolubility, **39** was subjected to HF cleavage without further purification: R_f (G) 0.68.

H-Gly-Ile-Leu-gGly-(*R,S*)mPhe-Val-Phe-Thr-Leu-OH (4). Compound **39** (350 mg, 0.28 mmol) was treated with HF following the procedure described for **3**: yield 277 mg (92%). Compound **4** was purified by HPLC (linear gradient, 30−70% B, 40 min) and lyophilized. Analytical data are reported in Table 1.

Boc-Gly-Phe-NH_2 (40): prepared from Boc-Gly-OH and HCl·H-Phe-NH_2 as described for compound **13**; 70% yield; R_f (C) 0.47; R_f (G) 0.65; mp 169−171 °C; $[\alpha]^{25}_D$ −5.45° (*c* 0.88, DMF). Anal. ($C_{16}H_{23}N_3O_4$) C,H,N.

Boc-Gly-gPhe-H·TFA (41): prepared from compound **40** following the procedure described for **28**; 95% yield; R_f (C) 0.34; R_f (G) 0.36; HPLC t_R 6.1 min (linear gradient, 20−80% B, 20 min); mp 113−114 °C; $[\alpha]^{25}_D$ −14.18° (*c* 1.1, DMF). Anal. ($C_{15}H_{23}N_3O_3\cdot CF_3COOH$) C,H,N.

Boc-Gly-gPhe-(*R,S*)mVal-OEt (42): prepared from compound **41** and 2(*R,S*)-isopropylmalonic acid ethyl ester as described for **9**; 69% yield; R_f (C) 0.78; R_f (G) 0.79; mp 121−123 °C; $[\alpha]^{25}_D$ −5.37° (*c* 1.08, DMF). Anal. ($C_{23}H_{35}N_3O_6$) C,H,N.

Boc-Gly-gPhe-(*R,S*)mVal-OH (43): prepared by saponification of compound **42** following the procedure described for **16**; 67% yield; R_f (G) 0.58; mp 153−157 °C; $[\alpha]^{25}_D$ −10.18° (*c* 1.13, DMF). Anal. ($C_{21}H_{31}N_3O_6$) C,H,N.

Boc-Gly-gPhe-(*R,S*)mVal-Phe-Thr(Bzl)-Leu-OBzl (44): prepared from compound **43** and TFA·H-Phe-Thr(Bzl)-Leu-OBzl as described for **9**; 69% yield; R_f (C) 0.79; R_f (G) 0.70; mp 180 °C dec; $[\alpha]^{25}_D$ +2.19° (*c* 1.05, DMF). Anal. ($C_{54}H_{70}N_6O_{10}\cdot H_2O$) C,H,N.

Boc-Leu-Gly-gPhe-(*R,S*)mVal-Phe-Thr(Bzl)-Leu-OBzl (45): prepared from Boc-Leu-OH·H_2O and TFA·H-Gly-gPhe-(*R,S*)mVal-Phe-Thr(Bzl)-Leu-OBzl (obtained from **44**) as described for **9**; 90% yield; R_f (C) 0.71; R_f (G) 0.79; mp 178 °C dec; $[\alpha]^{25}_D$ −3.26° (*c* 1.29, DMF). Anal. ($C_{60}H_{81}N_7O_{11}\cdot 0.5H_2O$) C,H,N.

Boc-Ile-Leu-Gly-gPhe-(*R,S*)mVal-Phe-Thr(Bzl)-Leu-OBzl (46): prepared from Boc-Ile-OH·0.5H_2O and TFA·H-Leu-Gly-gPhe-(*R,S*)mVal-Phe-Thr(Bzl)-Leu-OBzl (obtained from **45**) as described for **9**; 88% yield; R_f (G) 0.74; mp 210 °C dec. Anal. ($C_{66}H_{92}N_8O_{12}\cdot H_2O$) C,H,N.

Boc-Gly-Ile-Leu-Gly-gPhe-(*R,S*)mVal-Phe-Thr(Bzl)-Leu-OBzl (47): prepared from Boc-Gly-OH and TFA·H-Ile-Leu-Gly-gPhe-(*R,S*)mVal-Phe-Thr(Bzl)-Leu-OBzl (obtained from **46**) as described for **9**; 94% yield. As a result of its high insolubility, **47** was subjected to HF cleavage without further purification: R_f (G) 0.79.

H-Gly-Ile-Leu-Gly-gPhe-(*R,S*)mVal-Phe-Thr-Leu-OH (5). Compound **47** (400 mg, 0.32 mmol) was treated with HF following the procedure described for **3**: yield 266 mg (88%). Compound **5** was purified by HPLC (linear gradient, 30−70% B, 40 min) and lyophilized. Analytical data are reported in Table 1.

Boc-Phe-Val-NH_2 (48): prepared from Boc-Phe-OH and HCl·H-Val-NH_2 as described for compound **9**: 92% yield; R_f (C) 0.73; R_f (F) 0.79; mp 194−195 °C; $[\alpha]^{25}_D$ −1.36° (*c* 1.1, DMF). Anal. ($C_{19}H_{29}N_3O_4$) C,H,N.

Boc-Phe-gVal-H·TFA (49): prepared from compound **48** following the procedure described for **28**; 88% yield; R_f (C) 0.45; R_f (F) 0.60; HPLC t_R 8.4 min (linear gradient, 20−80% B, 20 min); mp 144−145 °C; $[\alpha]^{25}_D$ −1.69 (*c* 1.3, DMF). Anal. ($C_{18}H_{29}N_3O_3\cdot 1.2CF_3COOH$) C,H,N.

Boc-Phe-gVal-(*R,S*)mPhe-OEt (50): prepared from compound **49** and 2(*R,S*)-benzylmalonic acid ethyl ester as described for **9**; 76% yield; R_f (C) 0.84; R_f (F) 0.89; mp 193−195 °C; $[\alpha]^{25}_D$ −6.69° (*c* 1.45, DMF). Anal. ($C_{30}H_{41}N_3O_6$) C,H,N.

Boc-Phe-gVal-(*R,S*)mPhe-OH (51): prepared by saponification of compound **50** in 1,4-dioxane following the procedure described for **16**; 94% yield; R_f (E) 0.61; R_f (F) 0.82; mp 205−207 °C; $[\alpha]^{25}_D$ +8.03° (*c* 1.57, DMF). Anal. ($C_{28}H_{37}N_3O_6\cdot 1.5H_2O$) C,H,N.

Boc-Phe-gVal-(*R,S*)mPhe-Thr(Bzl)-Leu-OBzl (52): prepared from compound **51** and TFA·H-Thr(Bzl)-Leu-OBzl as described for compound **9**; 81% yield; R_f (C) 0.87; R_f (F) 0.89; mp 174−176 °C; $[\alpha]^{25}_D$ −4.42° (*c* 1.13, DMF). Anal. ($C_{52}H_{67}N_5O_9\cdot 0.5H_2O$) C,H,N.

Boc-Ile-leu-Gly-OMe (53): prepared from Boc-Ile-OH·0.5H_2O and TFA·H-Leu-Gly-OMe as described for compound **13**; 72% yield; R_f (E) 0.80; R_f (F) 0.78; mp 135−145 °C; $[\alpha]^{25}_D$ −28.17 (*c* 1.15, DMF). Anal. ($C_{20}H_{37}N_3O_6\cdot H_2O$) C,H,N.

Boc-Gly-Ile-leu-Gly-OMe (54): prepared from Boc-Gly-OH and TFA·H-Ile-Leu-Gly-OMe (obtained from **53**) as described for compound **9**; 79% yield; R_f (C) 0.71; R_f (F) 0.83; mp 173−175 °C; $[\alpha]^{25}_D$ −20.21 (*c* 1.41, DMF). Anal. ($C_{22}H_{40}N_4O_7$) C,H,N.

Boc-Gly-Ile-leu-Gly-OH (55): prepared by saponification of compound **54** following the procedure described for compound **19**; 92% yield; R_f (F) 0.61; mp 214−215 °C; $[\alpha]^{25}_D$ −18.76 (*c* 1.13, DMF). Anal. ($C_{21}H_{38}N_4O_7$) C,H,N.

Boc-Gly-Ile-Leu-Gly-Phe-gVal-(*R,S*)mPhe-Thr(Bzl)-Leu-OBzl (56): prepared from compound **55** and TFA·H-Phe-gVal-(*R,S*)mPhe-Thr(Bzl)-Leu-OBzl (obtained from **52**) as described for compound **9**; 90% yield. As a result of its high insolubility, **56** was subjected to HF cleavage without further purification: R_f (G) 0.74.

H-Gly-Ile-Leu-Gly-Phe-gVal-(*R,S*)mPhe-Thr-Leu-OH (6). Compound **56** (400 mg, 0.32 mmol) was treated with HF following the procedure described for **3**: yield 260 mg (86%). Compound **6** was purified by HPLC (linear gradient, 30−70% B, 40 min) and lyophilized. Analytical data are reported in Table 1.

Boc-Val-Phe-NH_2 (57): prepared from Boc-Val-OH and HCl·H-Phe-NH_2 as described for compound **9**; 71% yield; R_f (C) 0.55; R_f (G) 0.84; mp 151−153 °C; $[\alpha]^{25}_D$ −33.8° (*c* 1.28, DMF). Anal. ($C_{19}H_{29}N_3O_4 \cdot 0.3H_2O$) C,H,N.

Boc-Val-gPhe-H·TFA (58): prepared from compound **57** following the procedure described for **28**; 83% yield; R_f (C) 0.51; R_f (F) 0.60; HPLC t_R 8.3 min (linear gradient, 20−80% B, 20 min); mp 137−139 °C; $[\alpha]^{25}_D$ −22.0° (*c* 1.14, DMF). Anal. ($C_{18}H_{29}N_3O_3 \cdot 1.2CF_3COOH$) C,H,N.

Boc-Val-gPhe-(*R,S*)mAla-OEt (59): prepared from compound **58** and 2(*R,S*)-methylmalonic acid ethyl ester as described for **9**; 95% yield; R_f (G) 0.86; HPLC t_R 8.3 min (linear gradient, 20−80% B, 20 min); mp 200−203 °C; $[\alpha]^{25}_D$ +6.4° (*c* 1.02, DMF). Anal. ($C_{24}H_{37}N_3O_6 \cdot H_2O$) C,H,N.

Boc-Val-gPhe-(*R,S*)mAla-OH (60): prepared by saponification of compound **59** in 1,4-dioxane following the procedure described for **16**; 78% yield; R_f (C) 0.32; R_f (G) 0.72; mp 181−183 °C; $[\alpha]^{25}_D$ +3.4° (*c* 1.06, DMF). Anal. ($C_{22}H_{33}N_3O_6 \cdot H_2O$) C,H,N.

Boc-Val-gPhe-(*R,S*)mAla-Leu-OBzl (61): prepared from compound **60** and TFA·H-Leu-OBzl as described for compound **9**; 79% yield; R_f (C) 0.71; R_f (F) 0.82; mp 185−190 °C; $[\alpha]^{25}_D$ −12.16° (*c* 1.11, DMF). Anal. ($C_{35}H_{50}N_4O_7 \cdot H_2O$) C,H,N.

Boc-Phe-Val-gPhe-(*R,S*)mAla-Leu-OBzl (62): prepared from Boc-Phe-OH and TFA·H-Val-gPhe-(*R,S*)mAla-Leu-OBzl (obtained from **61**) as described for compound **9**; 87% yield; R_f (B) 0.61; R_f (G) 0.79; mp 210−215 °C; $[\alpha]^{25}_D$ −12.66° (*c* 0.94, DMF). Anal. ($C_{44}H_{59}N_5O_8 \cdot 1.5H_2O$) C,H,N.

Boc-Gly-Ile-Leu-Gly-Phe-Val-gPhe-(*R,S*)mAla-Leu-OBzl (63): prepared from compound **55** and TFA·H-Phe-Val-gPhe-(*R,S*)mAla-Leu-OBzl (obtained from **62**) as described for compound **9**; 90% yield. As a result of its high insolubility, **63** was subjected to HF cleavage without further purification: R_f (G) 0.66.

H-Gly-Ile-Leu-Gly-Phe-Val-gPhe-(*R,S*)mAla-Leu-OH (7). Compound **63** (337 mg, 0.30 mmol) was treated with HF following the procedure described for **3**: yield 270 mg (95%). Compound **7** was purified by HPLC (linear gradient, 30−70% B, 40 min) and lyophilized. Analytical data are reported in Table 1.

Boc-Phe-Thr(Bzl)-NH_2 (64): prepared from Boc-Phe-OH and HCl·H-Thr(Bzl)-NH_2 as described for compound **9**; 90% yield; R_f (C) 0.75; R_f (G) 0.77; mp 170−171 °C; $[\alpha]^{25}_D$ +10.84° (*c* 1.07, DMF). Anal. ($C_{25}H_{33}N_3O_5$) C,H,N.

Boc-Phe-gThr(Bzl)-H·TFA (65): prepared from compound **64** following the procedure described for **28**; 83% yield; R_f (C) 0.48; R_f (G) 0.56; HPLC t_R 12.6 min (linear gradient, 20−80% B, 20 min); mp 141−142 °C; $[\alpha]^{25}_D$ +15.6°(*c* 1.42, DMF). Anal. ($C_{24}H_{33}N_3O_4 \cdot 1.1CF_3COOH$) C,H,N.

2,2-Dimethyl-5-isobutyl-1,3-dioxane-4,6-dione (66). To a solution of borane·dimethylamine (2.36 g, 40 mmol) in methanol (50 mL) were sucessively dissolved under stirring 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid; 5.76 g, 40 mmol) and isobutyraldehyde (7.2 mL, 80 mmol).[53] After 10 min an additional portion of isobutyraldehyde (3.6 mL, 40 mmol) was added, and the reaction mixture was stirred for another 30 min; 100 mL of ice−water was then added, and the mixture was acidified with HCl. The precipitate which formed was filtered, washed with water, and dried *in vacuo* over phosphorus pentoxide: yield 4.64 g (58%); R_f (F) 0.89; mp 69−70 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.14 (d, 6H), 1.73 (s, 6H), 1.77 (m, 3H), 3.44 (t, 1H).

(*R,S*)-2-Isobutylmalonic Acid Monobenzyl Ester (67). Benzyl alcohol (5 mL) was added to a solution of compound **66** (4.64 g, 23 mmol) in toluene (10 mL), and the reaction mixture was heated at 70 °C overnight.[54] The solvent was removed under reduced pressure, and the residue was purified by column chromatography using $CHCl_3$−MeOH (20:1, v/v) as eluting solvent to give **67** as an oil: yield 4.6 g (80%); R_f (F) 0.76; ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.91 (d, 6H), 1.60 (m, 1H), 1.84 (t, 2H), 3.54 (t, 1H), 5.19 (s, 2H), 7.25−7.38 (m, 5H); FAB-MS *m/z* 251.1 ($M + H^+$).

Boc-Phe-gThr(Bzl)-(*R,S*)mLeu-OBzl (68): prepared from compound **65** and **67** as described for **9**; 96% yield; R_f (C) 0.90; mp 162−166 °C; $[\alpha]^{25}_D$ +1.16° (*c* 0.95, DMF). Anal. ($C_{38}H_{49}N_3O_7 \cdot 1.5H_2O$) C,H,N.

Boc-Val-Phe-gThr(Bzl)-(*R,S*)mLeu-OBzl (69): prepared from Boc-Val-OH and TFA·H-Phe-gThr(Bzl)-(*R,S*)mLeu-OBzl (obtained from **68**) as described for **9**. The residue was purified by silica gel column chromatography with chloroform−methanol−acetic acid (120:10:5, v/v/v) as eluant: 72% yield; R_f (C) 0.92; R_f (G) 0.74; mp 175−176 °C; $[\alpha]^{25}_D$ −3.97° (*c* 1.31, DMF). Anal. ($C_{43}H_{58}N_4O_8$) C,H,N.

Boc-Phe-Val-Phe-gThr(Bzl)-(*R,S*)mLeu-OBzl (70): prepared from Boc-Phe-OH and TFA·H-Val-Phe-gThr(Bzl)-(*R,S*)-mLeu-OBzl (obtained from **69**) as described for **9**; 85% yield; R_f (G) 0.80; mp 202 °C dec; $[\alpha]^{25}_D$ −1.25° (*c* 1.04, DMF). Anal. ($C_{52}H_{67}N_5O_9 \cdot 1.5H_2O$) C,H,N.

Boc-Gly-Ile-Leu-Gly-Phe-Val-Phe-gThr(Bzl)-(*R,S*)mLeu-OBzl (71): prepared from compound **55** and TFA·H-Phe-Val-Phe-gThr(Bzl)-mLeu-OBzl (obtained from **70**) as described for compound **9**; 90% yield. As a result of its high insolubility, **71** was subjected to HF cleavage without further purification: R_f (F) 0.69.

H-Gly-Ile-Leu-Gly-Phe-Val-Phe-gThr-(*R,S*)mLeu-OH (8). Compound **71** (350 mg, 0.28 mmol) was treated with HF following the procedure described for **3**: yield 277 mg (92%). Compound **8** was purified by HPLC (linear gradient, 30−70% B, 40 min) and lyophilized. Analytical data are reported in Table 1.

Detection of HLA-A2 Assembly. The capacity of peptides to associate with HLA-A2 heavy chains in the lysate of the mutant T2 cell line was tested as previously described.[55] T2 is a variant of the cell line T1 produced by fusion of the T lymphoma cell CEM and the B lymphoblastoid cell line 721.174.[58] It expresses reduced amount of HLA-A2 and no HLA-B or -C molecules at the cell surface because a lack of peptide transporters results in an accumulation of HLA heavy chains in the endoplasmic reticulum. Aliquots of 8×10^5 cells were lysed in 64 μL of 10 mM Tris-buffered saline, pH 7.5, containing 1% nonidet P40 in the presence of protease inhibitors (1 mM phenylmethanesulfonyl fluoride (PMSF), 100 μM iodoacetamide, 2 μg/mL aprotinin, 10 μM leupeptin, 10 μM pepstatin, and 10 μg/mL trypsin inhibitor from Sigma Chemical Co., St. Louis, MO). Cells were lysed in Eppendorf microfuge tubes with 1 μg/mL peptide (10^{-6} M) and incubated at 37 °C for 30 min. HLA-A2 peptide-dependent assembly was detected using the anti-HLA-A2 mAb BB7.2 (ATCC HB82, Rockville, MD). Wells of microtiter plates were first coated with 1 μg of mAb BB7.2 in 100 μL of phosphate-buffered saline (PBS) for 20 h at 4 °C and then saturated with 100 μL of PBS containing 10 mg/mL bovine serum albumin (BSA) for 1 h at 20−25 °C. Lysates of T2 cells were diluted with 136 μL of PBS containing 0.05% Tween 20, 3 mM sodium azide, 10 mg/mL BSA, and 1 mM PMSF; 100 μL of the mixture was placed in microtiter wells and incubated with mAb BB7.2 for 20 h at 4 °C. After washing, wells were incubated for 1 h at 37 °C with 2 mg/mL anti-β2-microglobulin antibodies M28[59] coupled to alkaline phosphatase. Alkaline phosphatase activity was detected using 4-methylumbelliferyl phosphate as substrate (M-883; Sigma), as described by Do Ngoc Lien et al.[60] The resulting fluorescence was measured at 340 nm excitation/460 nm emission (Cytofluor 2300, Millipore). Each peptide was tested in duplicate in at least three different experiments. Results are expressed as arbitrary fluorescence units obtained after 2 h of incubation with substrate. Background obtained with T2 lysates without exogenous peptide was substracted (mean value was 160 ± 35).

Acknowledgment. We are grateful to Dr. Marc H. V. Van Regenmortel for his constant support, Dr. Marc Rodriguez (Neosystem, Strasbourg, France) for advice and helpful discussion, and Dr. Michèle François (Institut de Chimie, Strasbourg, France) for microanalyses. This work was supported by Centre National de la Recherche Scientifique GCCB (Project 28D4).

Supporting Information Available: ^{1}H-NMR data of M58-66 analogues **2a** and **3**−**8** (7 pages). Ordering information is given on any current masthead page.

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JM9509511