Synthesis and biological evaluation of two chemically modified peptide epitopes for the class I MHC protein HLA-B*2705

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The T-cell receptor of a $CD8^+$ T-cell recognises peptide epitopes bound by class I major histocompatibility complex (MHC) glycoproteins presented in a groove on their upper surface. Within the groove of the MHC molecule are 6 pockets, two of which mostly display a high degree of specificity for binding amino acids capable of making conserved and energetically favourable contacts with the MHC. One type of MHC molecule, HLA-B*2705, preferentially binds peptides containing an arginine at position 2. In an effort to increase the affinity of peptides for HLA-B*2705, potentially leading to better immune responses to such a peptide, we synthesised two modified epitopes where the amino acid at position 2 involved in anchoring the peptide to the class I molecule was replaced with the α-methylated β,γ-unsaturated arginine analogue 2-(*S*)-amino-5-guanidino-2-methyl-pent-3-enoic acid. The latter was prepared *via* a multi-step synthetic sequence, starting from α-methyl serine, and incorporated into dipeptides which were fragment-coupled to resin-bound heptameric peptides yielding the target nonameric sequences. Biological characterisation indicated that the modified peptides were poorer than the native peptides at stabilising empty class I MHC complexes, and cells sensitised with these peptides were not recognised as well by cognate $CD8^+$ T-cells, where available, compared to those sensitised with the native peptide. We suggest that the modifications made to the peptide have decreased its ability to bind to the peptide binding groove of HLA-B*2705 molecules which may explain the decrease in recognition by cytotoxic T-cells when compared to the native peptide.

Introduction

Our immune system protects us against infectious agents and malignancies through the recognition of specific antigens derived from pathogens. Specific cell-mediated immune responses involve T-lymphocytes that respond to peptide epitopes, typically of between 8 and 20 amino acids in length, derived from these agents.[1] The peptides are recognised by $CD8^+$ T-cells only when bound to cell-surface protein receptors known as major histocompatibility complex (MHC) molecules. Our particular interest is in class I MHC molecules, which are on the surface of all nucleated cells and present peptides of 8–10 amino acids in length, derived mostly from intracellular proteins. Class I MHC molecules show a strong preference for peptides with particular residues—termed anchor residues—in certain positions along the chain. X-Ray crystallographic evidence has shown that the peptides bind in a long groove in the protein, with the anchor residues fitting into specific pockets in the binding groove.[2]

Recent advances in our understanding of the cellular immune response at the molecular level have led to a new strategy for vaccine development. The strategy involves the chemical modification of viral epitopes to make them more immunogenic, a process termed epitope enhancement.[3] Epitope enhancement can act by increasing the affinity of the epitope for an MHC molecule, by increasing the affinity of the peptide–MHC complex for the T-cell receptor, or by stimulating a broader T-cell response against the pathogen. The approach which has been most widely adopted, and which has been shown to produce significantly improved vaccines, is to increase the affinity of the epitope for the MHC molecule.[4] To increase the peptide affinity for MHC molecules, one can take advantage of known sequence motifs for peptide binding[5] and modify the anchor residues that provide much of the specificity of binding to the MHC molecule.

The human allele HLA-B*2705 is one of the most extensively studied class I MHC proteins, with a very clear motif for binding peptides. Its association with a variety of autoimmune diseases meant that HLA-B*2705–peptide complexes were amongst the first to be crystallised. HLA-B*2705 has a high specificity for peptides of 9 residues in length with an arginine at position 2, and analysis of the X-ray structure has shown that the side chain of this arginine (Arg 412 in crystallographic numbering) is bound in a polar pocket in the protein formed by His 9, Thr 24, Glu 45 and Cys 67 (Fig. 1).[2]

To increase the affinity of epitopes for HLA-B*2705, we designed an arginine isostere containing a double bond to restrict the side-chain flexibility.[7,8] We envisaged that by introducing a conformational restriction within the P2 anchor residue we could reduce the entropic cost of binding a peptide to HLA-B*2705, thereby enhancing the peptide–MHC affinity and possibly, in a vaccination setting, leading to a stronger immune response.[9] We chose two peptide epitopes on which to test this hypothesis:

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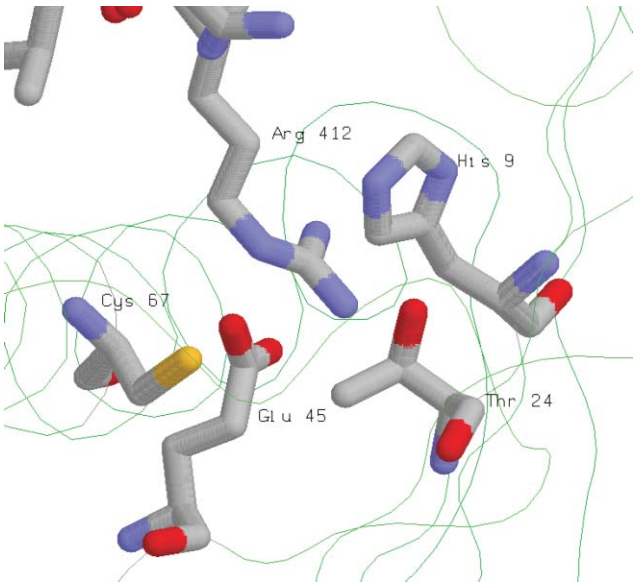


Fig. 1 Interactions of Arg 412 with residues in the binding pocket. Figure prepared using Rasmol[6] with data from Madden *et al.*[2]

GRAFVTIGK, residues 314–322 of the HIV envelope protein gp120;[10] and RRIYDLIEL, residues 258–266 of the Epstein-Barr virus protein EBNA3C.[11]

Design

Inspection of the published crystal structures of HLA-B*2705 containing bound peptides[2,12] showed that the P2 arginine was bound in an extended conformation, making the analogue **1** containing a double bond essentially isosteric (Fig. 1 and 2). Although there are a number of reported syntheses of amino acids containing β,γ-unsaturation,[13] they are prone to isomerisation of the double bond into conjugation with the carbonyl group, making their incorporation into peptides very challenging.[14] To circumvent this problem we decided to introduce an α-methyl substituent,[14] making our target the amino acid **2**. As our starting point for the synthesis we chose the known orthogonally protected amino acid (*S*)-*N*(Boc)-α-methylserine methyl ester **3**, which we reasoned could be elaborated towards the target β,γ-unsaturated isostere by oxidation to the aldehyde and subsequent Wittig chemistry.

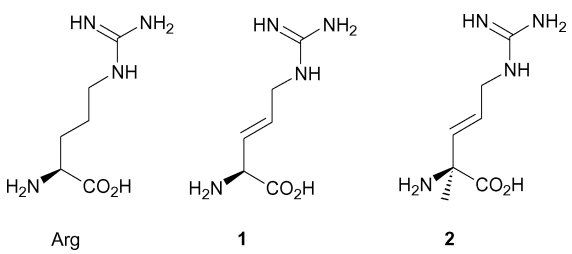
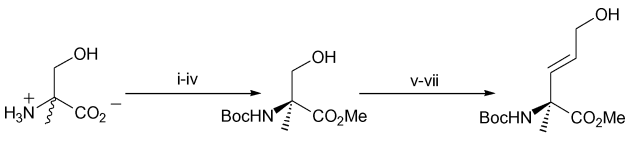


Fig. 2 P2 arginine, analogue **1** and target amino acid **2**.

Results and discussion

Our synthetic route required a significant quantity of (*S*)-*N*(Boc)-α-methylserine methyl ester **3**. Although several syntheses of this compound have been reported,[15] none appeared to be amenable to scale-up, and so we decided to explore a route involving resolution of the commercially available (±)-α-methylserine **4**. The racemic amino acid is inexpensive, and we believed that resolution by diastereomeric salt formation with camphorsulfonic acid should be possible.

Esterification of (±)-α-methyl-serine **4** was achieved using methanol and thionyl chloride[16] (Scheme 1), and the resulting hydrochloride salt was exchanged for the camphorsulfonic acid salt by heating with (+)-camphorsulfonic acid in CH_2Cl_2 and acetone. Crystallisation overnight from the same solvent mixture gave an excellent recovery of the α-methylserine methyl ester–(+)-camphorsulfonic acid salt, enriched in the (*S*)-enantiomer of the amino ester. The crystals were redissolved in CH_2Cl_2 and acetone and the fractional crystallisation procedure was repeated. The enantiomerically enriched amino ester was isolated by carbamate protection with $(Boc)_2O$, affording **3** in 34% overall yield from α-methylserine methyl ester, *i.e.* 68% based on recovery of a single enantiomer. The resolution was routinely performed on a 20 g scale.

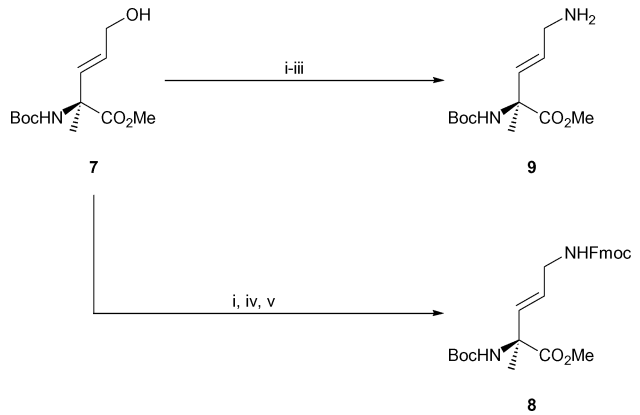


Scheme 1 Reagents and conditions: i. $SOCl_2$, MeOH, Δ, 16 h, 95%; ii. (+)-CSA, Δ, CH_2Cl_2, acetone; iii. fractional crystallisation; iv. $(Boc)_2O$, Et_3N, $CHCl_3$, Δ, 16 h, 34% over 3 steps, 68% based on recovery of single enantiomer; v. PCC, CH_2Cl_2, 16 h, 54%; vi. $Ph_3PCHCHO$, C_6H_6, 4 days, 70%; vii. $NaBH_4$, 0.4 M $CeCl_3$, MeOH, 50%.

The enantiopurity of the (*S*)-*N*(Boc)-α-methylserine methyl ester **3** was determined by formation of the Mosher's ester.[17] Examination of the ^1H NMR revealed a dr of >70 : 1, and the absolute stereochemistry of the product was confirmed as *S* by comparison of the specific optical rotation with literature values.[18] As the specific rotation of **3** at 589 nm was so small, we also measured it at a range of wavelengths to provide additional reference data (see Experimental section).

With multigram quantities of the enantiomerically enriched precursor **3** available, we set out to install the unsaturated side-chain using Wittig chemistry (Scheme 1). PCC oxidation of alcohol **3** under standard conditions proceeded smoothly to afford aldehyde **5** in a 54% yield, and this was followed by a Wittig reaction with the commercially available (formylmethylene)triphenyl phosphorane, giving the alkene **6** in 70% yield, exclusively as the *E*-stereoisomer. Diisobutylaluminium hydride reduction of **6** proceeded smoothly on a small scale, but the yields were not reproducible on a larger scale. Switching to the Luche reduction furnished the required allylic alcohol **7** in 58% yield, and this procedure could be reliably scaled to several grams.[19]

The next step was to introduce the guanidino function required for the arginine isostere. Since guanidinylation of an amine is one of the most straightforward methods for the introduction of the guanidino group,[20] we set about the synthesis of the triprotected ornithine analogue **8** (Scheme 2). Mesylation of alcohol **7**, followed by treatment with sodium azide gave the corresponding azide in

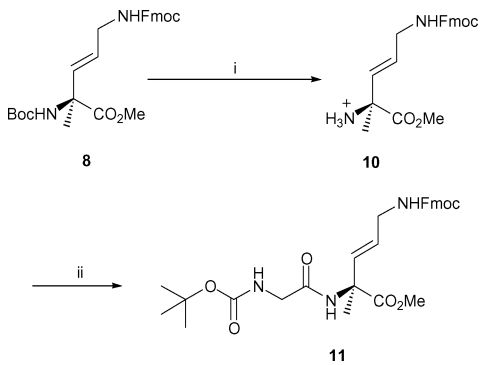


Scheme 2 Reagents and conditions: i. MsCl, Et_3N, CH_2Cl_2, 16 h; ii. NaN_3, CH_3CN, Δ, 16 h, 75% over 2 steps; iii. Ph_3P, THF–H_2O, Δ, 2 h, 20%; iv. 28% aq. NH_3, CH_2Cl_2, 4 h; v. FmocOSu, $NaHCO_3$, acetone–H_2O, 16 h, 25% for 3 steps.

75% yield over two steps, but the Staudinger reduction to give amine **9** was low-yielding (20%), and separation of the amine from residual triphenylphosphine oxide was difficult, prompting us to explore an alternative approach. Instead, treatment of the mesylate with aqueous ammonia gave the desired amine **9**, which was Fmoc-protected to afford **8** in 25% yield over 3 steps.

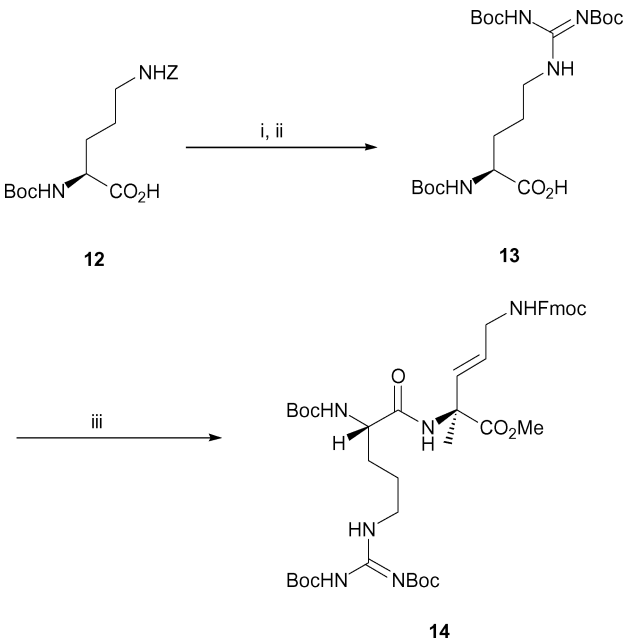
We were concerned that the incorporation of a sterically congested α-methylated amino acid into a peptide could be low-yielding, and that this steric crowding could also affect subsequent peptide coupling steps. Since the quaternary stereogenic centre is not susceptible to racemisation, we elected to employ a fragment coupling, first coupling our isostere at the N-terminus to form a dipeptide, and then attaching this to a heptameric sequence on resin to form the target nonameric peptide. To minimise protecting group manipulations, we decided to couple the protected ornithine analogue **8** to give a dipeptide, and then guanidinylate the δ-amino group to form the arginine isostere.

Two dipeptides were required to make our target nonapeptides, one incorporating glycine and the other incorporating arginine. Synthesis of the first dipeptide was straightforward. Boc group removal from **8** with TFA, giving **10**, was followed by reaction with the acid fluoride of *N*-Boc-glycine[21] to give the coupled product **11** in 66% yield over two steps (Scheme 3). A range of amino acid fluorides have been reported, and they have found application as highly activated coupling partners in peptide bond formation.[22]



Scheme 3 Reagents and conditions: i. TFA, CH_2Cl_2, 16 h; ii. Et_3N, CH_2Cl_2, BocGlyF, 16 h, 66% over 2 steps.

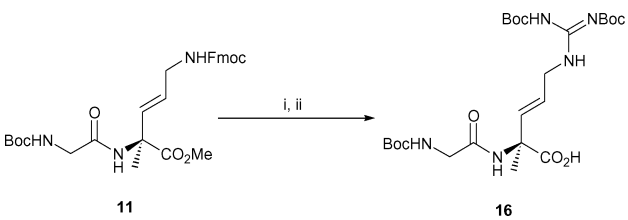
There are no reported examples of arginine acid fluorides, and so we attempted to prepare the acid fluoride of tri-Boc-protected arginine. Our synthesis started with the commercially available (*S*)-α-*N*(Boc)-δ-*N*(Cbz)-ornithine **12**. Hydrogenolysis to remove the Cbz group was followed by guanidinylation using bis(*tert*-butoxycarbonyl)triflylguanidine[23] to form tri-Boc-protected arginine **13** in 76% yield after chromatography (Scheme 4). Unfortunately, treatment with cyanuric fluoride under the reported conditions[21] did not yield any of the desired acid fluoride, with only unreacted starting material recovered.



Scheme 4 Reagents and conditions: i. H_2, Pd–C, MeOH, 16 h, 99%; ii. Bis(*tert*-butoxycarbonyl)triflylguanidine, MeOH, 3 days, 76%; iii. **10**, iPr_2EtN, HATU, HOAt, 16 h, 13%.

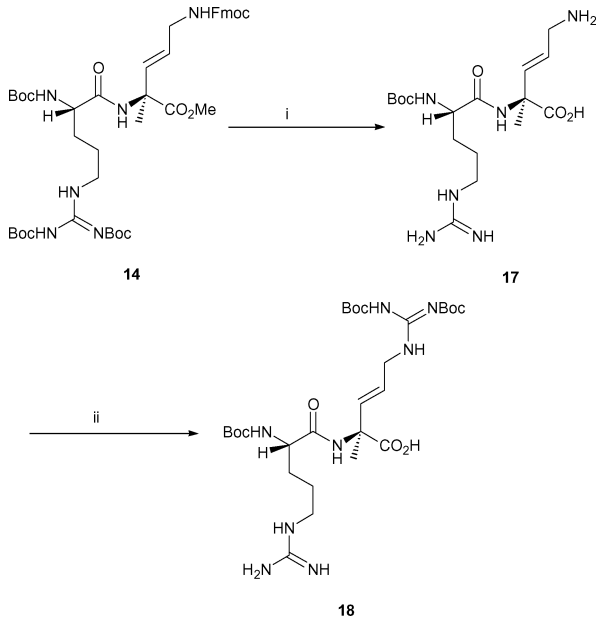
Our failure to prepare the protected arginine fluoride led us to turn to the coupling of **10** and **13** using HATU and HOAt, which did produce the desired dipeptide **14**, but in a disappointing 13% yield.

With the protected dipeptides **11** and **14** in hand, we turned our attention to the guanidinylation. Treatment of **11** with lithium hydroxide in water–acetonitrile resulted in simultaneous ester hydrolysis and Fmoc cleavage to give dipeptide **15** in 94% yield (Scheme 5). Guanidinylation of **15** using bis(*tert*-butoxycarbonyl)triflylguanidine proceeded smoothly to afford the dipeptide **16** in 90% yield.



Scheme 5 Reagents and conditions: i. LiOH, MeCN–H_2O, 16 h, 94%; ii. Bis(*tert*-butoxycarbonyl)triflylguanidine, MeOH, 3 days, 90%.

Repeating the hydrolysis step on dipeptide **14** surprisingly led to removal of both Boc groups from the guanidino moiety, in addition to methyl ester hydrolysis and Fmoc cleavage, yielding the deprotected dipeptide **17** in 94% yield (Scheme 6). The use of arginine with an unprotected side-chain is not uncommon in peptide synthesis,[24] so, rather than attempt to re-protect the guanidino function, we simply went on to guanidinylate the amino group of our isostere, leading to **18** in 52% yield.



Scheme 6 Reagents and conditions: i. LiOH, MeCN–H_2O, 16 h, 94%; ii. Bis(*tert*-butoxycarbonyl)triflylguanidine, MeOH, 3 days, 52%.

Peptide synthesis

Fragment coupling of the dipeptide **16** to the resin-bound heptameric sequence AFVTIGK was attempted using HATU and HOAt, but under these conditions the only product identified following cleavage of the peptide from the support was the result of resin-capping by the coupling reagent.[25] Since epimerisation of the quaternary centres in **16** and **18** is not an issue, more vigorous coupling conditions may be used. In an attempt to increase the reactivity of the dipeptide fragments, we used DCC in association with the nucleophilic catalyst DMAP. The concentration was important, and it was found that a dipeptide concentration of 0.2 M in DMSO, with a 48 hour coupling time at 60 °C, was optimal. Under these conditions **16** coupled with resin-bound AFVTIGK and **18** coupled with resin-bound IYDLIEL to afford the desired nonameric peptides **19** and **20** in 42% and 47% yield, respectively, after resin cleavage and HPLC purification (Scheme 7). The two control sequences GRAFVTIGK **21** and RRIYDLIEL **22** were synthesised by standard Fmoc peptide synthesis protocols; the data for all four peptides are summarised in Table 1.

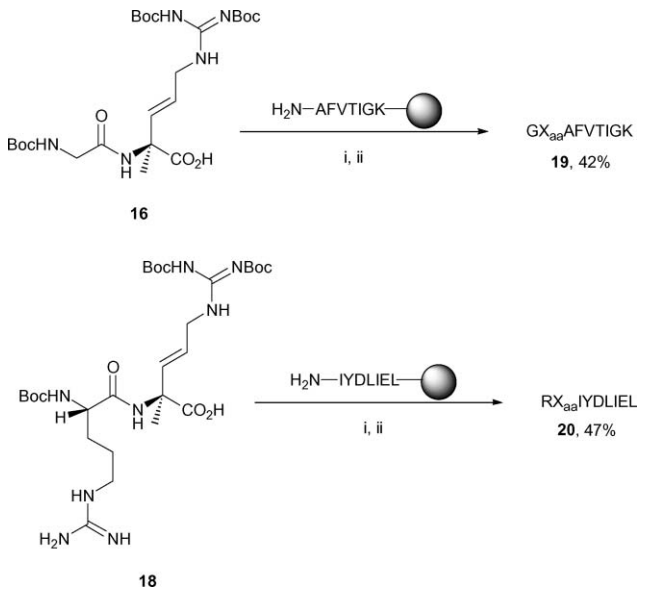
Immunological testing

The modified peptides **19** and **20** were tested alongside the native sequence peptides in two different immunological assays:

Table 1 Characterisation data for peptides

Peptide	t_r/min	m/z found[a]	m/z calc
GX_{aa}AFVTIGK **19**	25.83[d]	961.2	960.6
RX_{aa}IYDLIEL **20**	31.87[d]	1202.6	1202.7
GRAFVTIGK **21**	11.42[c]	948.5	948.6
RRIYDLIEL **22**	45.33[b]	1190.4	1190.7

[a] MALDI or electrospray, m/z for $[M + H]^+$. [b] 70 : 30 Water–MeCN + 0.05% TFA. [c] 88 : 12 Water–MeCN + 0.05% TFA to 55 : 45 water–MeCN + 0.05% TFA over 30 min. [d] 99.95% Water + 0.05% TFA to 99.95% MeCN + 0.05% TFA over 60 minutes.



Scheme 7 Reagents and conditions: i. DCC, DMAP, DMSO, 2 days; ii. TFA, TES, phenol, H_2O.

a cell-surface class I MHC stabilisation assay; and a CD8 T-cell cytotoxicity assay. The first provides a measure of the peptide–MHC binding ability, while the second provides a measure of the ability of an epitope-specific T-cell clone to recognise the peptide–MHC complex displayed on the surface of a target cell.

Class I MHC stabilisation assays

The ability of the modified peptides and the native epitopes to bind class I MHC molecules was assessed by a class I MHC stabilisation assay,[26] as described in the Experimental section. Briefly, cells which are deficient in components of the peptide-loading machinery, T2-B*2705, mostly present empty HLA-B*2705 at the cell surface, which are unstable and are rapidly degraded. Upon binding of an appropriate peptide, the resultant complexes are stabilised and can be detected by a fluorescently labelled conformation-specific monoclonal antibody, W6/32. The fluorescence intensities of these labelled cells can then be determined by flow cytometric analysis, giving a measure of the ability of the peptide to stabilise the complex. This is expressed as MHC stabilisation efficiency (MSE), the percentage increase of the mean fluorescence above that of the negative control. All peptides that resulted in a fluorescence intensity greater than the mean + 3 standard errors of the mean (SEM) of the fluorescence intensity resulting from the T2-B*2705 cells in the absence of peptide at

26 °C (negative control) were considered to be positive binders. The results of one representative assay of three are presented in Chart 1. The peptides incorporating the isostere, **19** and **20**, stabilised free MHC molecules on the surface of T2-B*2705 cells (MSE 130.3 and 123.7 respectively). However, the modified peptides stabilised free MHC molecules less well than the native epitopes **21** and **22** (MSE 178.2 and 171.4 respectively), although both gave MSE values above the cut-off point for positive binders (DMSO + 3 SEM 109.8) and therefore do bind to HLA-B*2705.

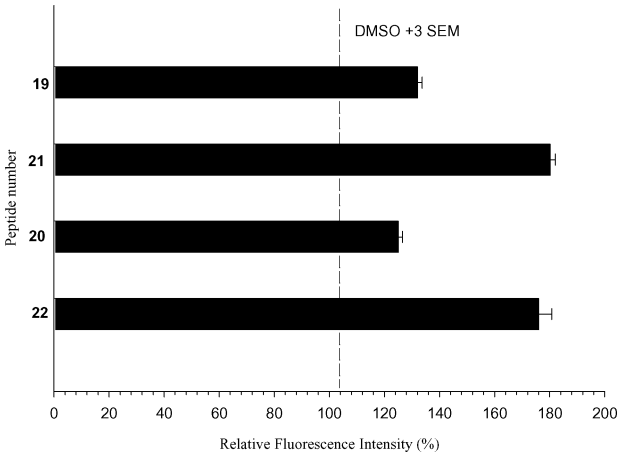


Chart 1 MHC stabilisation on T2-B*2705 cells using peptides **19–22**. The dotted line (mean + 3 SEM) indicates the background fluorescence intensity for T2-B*2705 cells incubated at 26 °C without peptide, which was the cut-off for a positive result.

^{51}Cr release assays

While cell-surface class I MHC stabilisation assays provide a measure of peptide–MHC binding ability, they do not give any information about the ability of the complex to be recognised by the immune response. Chromium release cytotoxicity assays can be used to determine the ability of peptide-loaded target cells to be recognised by $CD8^+$ T-cells.[27] The target cells are loaded with radioactive ^{51}Cr before being incubated with the peptide. On exposure to a T-cell clone specific for the particular peptide–MHC complex, the target cells are lysed, releasing ^{51}Cr which may be measured using a scintillation detector; the amount of ^{51}Cr released provides a measure of the response of the T-cell clone to the peptide–MHC complex. As we only had access to a T-cell clone specific for the RRIYDLIEL–HLA-B*2705 complex, our chromium release assays were limited to peptides **20** and **22**.

^{51}Chromium-loaded T2-B*2705 cells were used as target cells, and these were sensitised in the presence of peptides **20** and **22** by incubation for 1 hour at 37 °C in a 5% CO_2 humidified atmosphere, and then overnight at 26 °C. Following incubation, the peptide-sensitised cells were exposed to different concentrations of a T-cell clone specific for the native RRIYDLIEL peptide epitope and the resultant percentage specific cell lysis determined by measuring the released radioactivity.

Target cells sensitised with the native epitope **22** gave a high percentage (44%, 23% and 18%) specific cell lysis for the effector–target cell ratios 5 : 1, 2 : 1 and 1 : 1 respectively (Chart 2). Cells sensitised with the modified peptide epitope **20** gave 16% specific cell lysis at an effector–target cell ratio of 5 : 1, indicating that the complex formed between peptide **20** and HLA-B*2705 is recognised by the T-cell clone, but the modified peptide elicits only a weak response when compared to the native epitope.

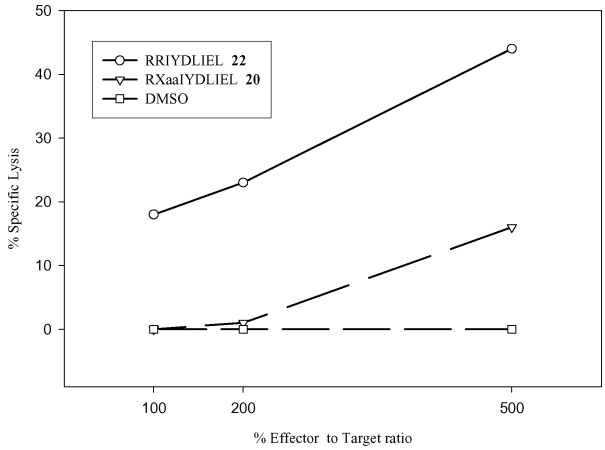


Chart 2 ^{51}Cr release assays results at three effector–target cell ratios, 1 : 1, 2 : 1 and 5 : 1, represented as percentages.

Conclusion

We have successfully synthesised an α-methylated β,γ-unsaturated arginine analogue and incorporated it at position 2 of two peptide epitopes presented by the class I MHC molecule HLA-B*2705. Class I MHC stabilisation assays indicated that the modified peptides have a lower affinity for HLA-B*2705 than the native epitopes, contrary to our expectations, and chromium release assays on one of the modified peptides confirm that it elicited a weaker cytotoxic response from cognate epitope-specific $CD8^+$ T-cells. It would appear that the incorporation of a double bond and an α-methyl group into the arginine analogue is deleterious to binding, although the presence of both features in the same analogue prevent us from determining the effect of each on peptide affinity for HLA-B*2705. It is possible that the effect of the α-methyl substituent is to distort the backbone conformation of the peptide, disrupting important binding interactions with the MHC molecule.[28] Further work will focus on peptidomimetics lacking this feature.

Experimental

General chemical procedures

THF and diethyl ether were distilled from sodium and benzophenone. Toluene, CH_2Cl_2, MeCN, triethylamine and diisopropylethylamine were distilled from calcium hydride.

^{1}H, ^{19}F, and ^{13}C NMR spectra (300, 282 and 75 MHz respectively) were recorded on a Bruker AC-300 spectrometer. HSQC, HMBC, COSY 90, ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX500 (500 MHz and 125 MHz for ^{1}H and ^{13}C) or a Bruker AMX400 spectrometer (400 MHz and 100 MHz for ^{1}H and ^{13}C). ^{19}F NMR spectra were referenced downfield from fluorotrichloromethane. ^{1}H and ^{13}C NMR spectra were recorded using deuterated solvent as the lock and were referenced downfield from tetramethylsilane. ^{13}C spectra NMR were recorded using the PENDANT pulse sequence. *J* values are reported in Hz. The

multiplicities of the spectroscopic signals are represented in the following manner; *s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *m* = multiplet, *br s* = broad singlet and *env* = overlapping signals.

Chemical ionisation (CI) and electron impact (EI) mass spectra were recorded on a VG Zabspec mass spectrometer or a VG Prospec mass spectrometer. Chemical ionisation (CI) methods used ammonia as the carrier gas. Liquid secondary ion mass spectrometry (LSIMS) was recorded using a VG Zabspec instrument. A Micromass LCT mass spectrometer was used for both low-resolution electrospray time of flight (ES-TOF) mass spectrometry (using a methanol mobile phase) and accurate mass measurement (using a lock mass incorporated into the mobile phase). Matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry was recorded using a Bruker Biflex IV instrument using either sinapinic acid or (α-cyano-4-hydroxy-*trans*-cinnamic acid–nitrocellulose [3 : 1]) as a thin layer matrix.

Thin layer chromatography was performed on precoated glass-backed silica gel plates supplied by ICN Ltd (Silica gel 60 F_{254}, thickness 0.25 mm). Column chromatography was performed on silica gel 40–63 μ 60A (Fluorochem Ltd).

All HPLC was performed using a Dionex Summit HPLC system with Chromeleon software. Analytical and semi-preparative HPLC were carried out using a Summit P580 quaternary low-pressure gradient pump with built-in vacuum degasser. A P580P high-pressure binary gradient pump with built-in vacuum degasser was employed for preparative HPLC. A UVD 170s UV–VIS multi channel detector was used to monitor all HPLC. Luna 10 μ columns supplied by Phenomenex containing C18 as the sorbent were used for all HPLC (250 × 4.6 mm, 250 × 10 mm and 250 × 21.2 mm columns were used for analytical, semi-preparative, and preparative HPLC respectively). Unless otherwise stated, all HPLC was performed using the following solvent mixtures: eluent A (water–TFA (99.95 : 0.05)); and eluent B (MeCN–TFA (99.95 : 0.05)).

Methyl (*S*)-2-(*tert*-butoxycarbonylamino)-3-hydroxy-2-methylpropanoate 3. (±)-α-Methylserine methyl ester hydrochloride[16] (21.2 g, 0.16 mol) and (+)-CSA (37.0 g, 0.16 mol) were heated at reflux in CH_2Cl_2 (200 mL) and acetone (23 mL) until a homogeneous solution formed. The solution was allowed to cool to room temperature and was left until crystallisation occurred. The crystals were collected by filtration and the fractional crystallisation procedure was repeated for a second time. The crystals (23.4 g, 64 mmol) were dissolved in dry CH_2Cl_2 (100 mL) and Et_3N (8.9 mL, 64 mmol). $(Boc)_2O$ (13.97 g, 64 mmol) was added, and the mixture was heated at reflux overnight. Following the addition of water (50 mL), the organic layer was separated, dried ($MgSO_4$) and the solvent removed *in vacuo* to yield the enantiomerically enriched alcohol **3** as a colourless oil (13.4 g, 68% overall yield, based on recovery of a single enantiomer). $[\alpha]_{589}^{25}$ +1.5 (*c* 1.0 in $CHCl_3$) (lit.[18] $[\alpha]_{589}^{18}$ +1.9 (*c* 0.54 in $CHCl_3$)), $[\alpha]_{578}^{25}$ +3.1 (*c* 1.0 in $CHCl_3$), $[\alpha]_{546}^{25}$ +14.1 (*c* 1.0 in $CHCl_3$), $[\alpha]_{463}^{25}$ +23.6 (*c* 1.0 in $CHCl_3$), $[\alpha]_{365}^{25}$ +25.5 (*c* 1.0 in $CHCl_3$); $\nu_{max}(CHCl_3)/cm^{-1}$ 3348, 2978, 1750, 1724, 1450, 1366, 1250, 1184, 1165, 1057; δ_H (300 MHz, $CDCl_3$) 1.37 (9H, *s*, $C(CH_3)_3$), 1.41 (3H, *s*, $NHCCH_3$), 3.60 (3H, *s*, OCH_3), 3.61 (1H, *d*, *J* 9.9, CH_2OH), 3.86 (1H, *d*, *J* 9.9, CH_2OH), 5.42 (1H, *s*, NH); δ_C (75 MHz, $CDCl_3$) 19.6 ($NHCCH_3$), 28.2 ($C(CH_3)_3$), 52.6 (OCH_3), 60.9 ($NHCCH_3$), 66.8 (CH_2OH), 80.2 ($C(CH_3)_3$), 155.4 ($NHCO_2{}^tBu$), 173.9 (CO_2Me); *m*/*z* (EI) 234 ($[M + H]^+$, 100%), 202 ($[M - CH_2OH]^+$, 40).

Methyl (*S*)-2-(*tert*-butoxycarbonylamino)-3-oxo-2-methylpropanoate 5. To a solution of pyridinium chlorochromate (2.77 g, 12.85 mmol) in CH_2Cl_2 (50 mL) was added celite to form a slurry, which was stirred vigorously for 5 minutes before being cooled to 0 °C. A solution of alcohol **3** (2.0 g, 8.6 mmol) in CH_2Cl_2 (20 mL) was added, and the mixture was stirred overnight at room temperature. Sodium metabisulfite (2 g) and Et_2O (50 mL) were added, and the mixture was stirred vigorously for 15 minutes. The mixture was then filtered through a short pad of silica, dried ($MgSO_4$) and concentrated *in vacuo* to yield a pale yellow oil. Purification by column chromatography (R_f = 0.42, petroleum ether–Et_2O 1 : 1) gave the aldehyde **5** as a colourless oil (1.07 g, 54%). $[\alpha]_D^{23}$ −11.4 (*c* 1.29 in $CHCl_3$); $\nu_{max}(CHCl_3)/cm^{-1}$ 3015, 2978, 1724, 1705, 1450, 1366, 1250, 1184, 1165, 1057; δ_H (300 MHz, $CDCl_3$) 1.39 (9H, *s*, $C(CH_3)_3$), 1.55 (3H, *s*, $NHCCH_3$), 3.74 (3H, *s*, CO_2CH_3), 5.62 (1H, *s*, $NHCCH_3$), 9.51 (1H, *s*, CHO); δ_C (75 MHz, $CDCl_3$) 19.2 ($NHCCH_3$), 28.1 ($C(CH_3)_3$), 52.3 (OCH_3), 66.6 ($NHCCH_3$), 80.8 ($C(CH_3)_3$), 154.6 ($NHCO_2{}^tBu$), 169.4 (CO_2Me), 194.0 (CHO); *m*/*z* (ES) 286 ($[M + Na + MeOH]^+$, 100%), 254 ($[M + Na]^+$, 20), 230 ($[M - H]^+$, 40).

Methyl (*E*,*S*)-2-(*tert*-butoxycarbonylamino)-5-oxo-2-methylpent-3-enoate 6. To a solution of the aldehyde **5** (1.8 g, 7.8 mmol) in benzene (50 mL) was added (triphenylphosphoranylidene)acetaldehyde (3.56 g, 11.7 mmol) and the mixture was stirred at room temperature for 4 days. Removal of the solvent *in vacuo* and purification of the residue by column chromatography (R_f = 0.35, petroleum ether–Et_2O 1 : 1) gave the unsaturated aldehyde **6** as a colourless oil (1.40 g, 70%). $[\alpha]_D^{23}$ −7.8 (*c* 0.36 in $CHCl_3$); $\nu_{max}(CHCl_3)/cm^{-1}$ 3018, 2974, 1713, 1706, 1518, 1425, 1216; δ_H (300 MHz, $CDCl_3$) 1.41 (9H, *s*, $C(CH_3)_3$), 1.60 (3H, *s*, $NHCCH_3$), 3.66 (3H, *s*, CO_2CH_3), 5.49 (1H, *s*, $NHCCH_3$), 6.19 (1H, *dd*, *J* 15.8 and 7.7, CH=CHCHO), 7.19 (1H, *d*, *J* 15.8, CH=CHCHO), 9.59 (1H, *d*, *J* 7.7, CHO); δ_C (75 MHz, $CDCl_3$) 24.6 ($NHCCH_3$), 28.4 ($C(CH_3)_3$), 53.4 (OCH_3), 60.2 ($NHCCH_3$), 80.9 ($C(CH_3)_3$), 131.1 (CH=CHCHO), 154.6 ($NHCO_2{}^tBu$), 156.3 (CH=CHCHO), 172.3 (CO_2Me), 193.6 (CHO); *m*/*z* (ES) 312 ($[M + Na + MeOH]^+$, 20%), 280 ($[M + Na]^+$, 100).

Methyl (*E*,*S*)-2-(*tert*-butoxycarbonylamino)-5-hydroxy-2-methylpent-3-enoate 7. To a solution of the unsaturated aldehyde **6** (3.19 g, 12.0 mmol) in MeOH (20 mL), $CeCl_3$ (2.98 g, 14.0 mmol) was added. The resultant mixture was stirred at room temperature for 15 minutes, then sodium borohydride (0.47 g, 12.4 mmol) was added and the reaction mixture was stirred overnight. Following evaporation of the solvent *in vacuo*, water (10 mL) was added, and the mixture was extracted with EtOAc (3 × 30 mL). The organic phase was washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to yield a yellow oil. Purification by column chromatography (R_f = 0.18, petroleum ether–Et_2O 1 : 2) gave the allylic alcohol **7** as a colourless oil (1.6 g, 50%). $[\alpha]_D^{23}$ −10.7 (*c* 0.46 in $CHCl_3$); $\nu_{max}(CHCl_3)/cm^{-1}$ 3364, 2978, 2950, 2869, 1712 (br), 1503, 1435, 1391, 1367, 1251, 1166, 1059; δ_H (300 MHz, $CDCl_3$) 1.41 (9H, *s*, $C(CH_3)_3$), 1.63 (3H, *s*, $NHCCH_3$), 3.71 (3H, *s*, CO_2CH_3), 4.17 (2H, *d*, *J* 4.6, CH_2OH), 5.29 (1H, *br s*, NH), 5.72 (1H, *dt*, *J* 15.8 and 4.6, CH=CHCH$_2$), 5.89 (1H, *d*, *J* 15.8, CH=CHCH$_2$); δ_C (75 MHz, $CDCl_3$) 23.2 ($NHCCH_3$), 28.3 ($C(CH_3)_3$), 52.8 (OCH_3), 59.7

(NHCCH$_3$), 62.8 (CH$_2$OH), 80.8 (C(CH$_3$)$_3$), 130.0 (CH=), 131.1 (CH=), 154.4 (CO$_2$^{t}Bu), 173.4 (CO$_2$Me); *m/z* (ES) 282 ([M + Na]$^+$, 100).

Methyl (*E*,*S*)-2-(*tert*-butoxycarbonylamino)-5-(9*H*-fluoren-9-ylmethoxycarbonylamino)-2-methylpent-3-enoate 8. To a solution of the allylic alcohol **7** (0.93 g, 3.59 mmol) in CH_2Cl_2 (20 mL), was added triethylamine (0.55 mL, 9.59 mmol) and freshly distilled methanesulfonyl chloride (305 μL, 3.95 mmol), and the mixture was stirred at room temperature overnight. Following the addition of water (10 mL), the mixture was extracted with CH_2Cl_2 (3 × 30 mL), washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to furnish the mesylate as a colourless oil that was used immediately.

To a solution of the crude mesylate (1.44 g) in CH_2Cl_2 (20 mL) was added ammonium hydroxide solution (28%, 10 mL), and the mixture was stirred at room temperature for 4 h before the solvent was removed *in vacuo.* The residue was dissolved in acetone–H_2O (10 : 1, 20 mL), $NaHCO_3$ solution (1 M, 3.48 mL) and *N*-(9*H*-fluoren-9-ylmethoxycarbonyloxy)succinimide (1.88 g, 5.6 mmol) were added, and the mixture was stirred at room temperature for 16 h. Following concentration *in vacuo*, the residue was partitioned between H_2O (50 mL) and CH_2Cl_2 (3 × 30 mL). The combined organic extracts were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo.* The residue was purified by column chromatography (R_f = 0.35, hexane–EtOAc 2 : 1) to yield an amorphous solid that was recrystallised from EtOAc–hexane 2 : 1 to give the orthogonally protected amino ester **8** as a white crystalline solid (0.67 g, 25% overall yield for 3 steps). Mp 110–112 °C; $[\alpha]_D^{23}$ −21.5 (*c* 0.02 in $CHCl_3$); ν_{max}($CHCl_3$)/cm^{-1} 3017, 2977, 2950, 1742, 1712, 1513, 1449, 1367, 1215, 1164; δ_H (300 MHz, $CDCl_3$) 1.42 (9H, *s*, C(CH$_3$)$_3$), 1.57 (3H, *s*, NHCCH$_3$), 3.73 (3H, *s*, CO$_2$CH$_3$), 3.80–3.88 (2H, *m*, CH$_2$NH), 4.17–4.25 (1H, *m*, CHCH$_2$O), 4.40 (2H, *d*, *J* 7.0, CHCH$_2$O), 4.73 (1H, *br s*, NH), 4.91 (1H, *br s*, NH), 5.68 (1H, *dt*, *J* 15.8 and 5.5, CH=CHCH$_2$), 5.87 (1H, *d*, *J* 15.8, CH=CHCH$_2$), 7.30 (2H, *t*, *J* 7.7, Ar–H), 7.39 (2H, *t*, *J* 7.7, Ar–H), 7.58 (2H, *d*, *J* 7.7, Ar–H), 7.75 (2H, *d*, *J* 7.7, Ar–H); δ_C (75 MHz, $CDCl_3$) 24.4 (NHCCH$_3$), 28.8 (C(CH$_3$)$_3$), 42.9 (CH$_2$NH), 47.7 (CHCH$_2$O), 52.9 (OCH$_3$), 58.0 (NHCCH$_3$), 67.3 (CHCH$_2$O), 80.2 (C(CH$_3$)$_3$), 120.5, 125.5, 127.6, 128.2 (overlapping 4 Ar–CH, CH=), 132.7 (CH=), 141.8 (Ar–C), 144.4 (Ar–C), 152.6 (C=O), 154.5 (C=O), 171.5 (CO$_2$Me); HRMS (ES) 503.2148 ($C_{27}H_{32}N_2O_6Na$ requires 503.2158); *m/z* (ES) 503 ([M + Na]$^+$, 100%), 447 ([M + Na–C_4H_8]$^+$, 80).

Methyl (*E*,*S*)-2-{[(*N*-*tert*-butoxycarbonyl)glycyl]amino}-5-(9*H*-fluoren-9-ylmethoxycarbonylamino)-2-methylpent-3-enoate 11. Amino ester **8** (0.35 g, 0.73 mmol) was deprotected by stirring in TFA–CH_2Cl_2 (1 : 2, 3 mL) overnight. Excess TFA was removed *in vacuo*, the residue dissolved in CH_2Cl_2 (10 mL), and Et_3N (0.21 mL, 1.48 mmol) was added. The reaction mixture was stirred for 10 minutes at room temperature before the addition of *N*-(*tert*-butoxycarbonyl)glycyl fluoride (0.14 g, 0.82 mmol) in CH_2Cl_2 (2 mL) over 60 seconds. The reaction was stirred at room temperature for 16 h, then water (10 mL) was added, the organic phase was separated and the aqueous phase extracted with CH_2Cl_2 (3 × 10 mL). The combined organic extracts were washed twice with 5% HCl, 10% $NaHCO_3$, water, dried ($MgSO_4$), and concentrated *in vacuo.* Purification by HPLC (eluent A–B 100 : 0 to 0 : 100 over 60 minutes) yielded the dipeptide **11** (t_r 49.37 minutes) as a white solid (0.26 g, 66%). $[\alpha]_D^{23}$ −25.9 (*c* 0.06 in CH_3CN); ν_{max}(film)/cm^{-1} 3066, 2980, 1700 (br), 1520, 1450, 1254, 1167; δ_H (300 MHz, CD_3CN) 1.40 (9H, *s*, C(CH$_3$)$_3$), 1.49 (3H, *s*, NHCCH$_3$), 3.60–3.62 (5H, *env*, CH$_2$NH, CO$_2$CH$_3$), 3.68–3.74 (2H, *m*, CH$_2$NH), 4.20–4.26 (1H, m, CHCH$_2$O), 4.33 (2H, *d*, *J* 7.0, CHCH$_2$O), 4.79 (1H, *br s*, NH), 5.55 (1H, *br s*, NH), 5.65 (1H, *dt*, *J* 15.4 and 4.8, CH=CHCH$_2$), 5.85 (1H, *d*, *J* 15.4, CH=CHCH$_2$), 6.95 (1H, *br s*, NH), 7.35 (2H, *dt*, *J* 8.1 and 1.0, Ar–H), 7.42 (2H, *dt*, *J* 8.1 and 1.0, A–Hr), 7.65 (2H, *d*, *J* 8.1, A–Hr), 7.83 (2H, *d*, *J* 8.1, A–Hr); δ_C (75 MHz, CD_3CN) 22.9 (NHCCH$_3$), 27.5 (C(CH$_3$)$_3$), 40.0 (CH$_2$), 41.7 (CH$_2$), 45.4 (CHCH$_2$O), 50.4 (OCH$_3$), 57.6 (NHCCH$_3$), 64.4 (CHCH$_2$O), (C(CH$_3$)$_3$) not detected, 119.9, 125.1, 127.1, 127.4, 127.7 (overlapping 4 Ar–CH, 2 CH=), 139.4 (Ar–C), 142.5 (Ar–C), 154.5 (C=O), 155.7 (C=O), 167.9 (C=O), 173.0 (CO$_2$Me); HRMS (ES) 560.2361 ($C_{29}H_{35}N_3O_7Na$ requires 560.2373); *m/z* (ES) 560 ([M + Na]$^+$, 100).

***N*,*N*′,*N*″-Tris(*tert*-butoxycarbonyl)arginine 13.** To solution of N^{α}-*tert*-butoxycarbonylornithine (50 mg, 0.2 mmol) and iPr_2EtN (37 μL, 0.2 mmol) in MeOH (2 mL) was added bis(*tert*-butoxycarbonyl)triflylguanidine (77 mg, 0.2 mmol) and the mixture was stirred for 3 days. The solvent was removed *in vacuo* and the residue purified by column chromatography (R_f = 0.42, chloroform–methanol 19 : 1) to afford the protected amino acid **13** as a colourless oil (81 mg, 76%). $[\alpha]_D^{23}$ −79.2 (*c* 0.014 in MeOH); ν_{max}($CHCl_3$)/cm^{-1} 3317, 2978, 1713, 1674, 1620, 1365, 1227, 1149; δ_H (300 MHz, CD_3OD) 1.44 (9H, *s*, C(CH$_3$)$_3$), 1.46 (9H, *s*, C(CH$_3$)$_3$), 1.52 (9H, *s*, C(CH$_3$)$_3$), 1.63–1.73 (3H, *env*) and 1.78–1.90 (1H, *m*) (CHCH$_2$CH$_2$), 3.37 (2H, *m*, CH$_2$NH), 4.09 (1H, *m*, COCH), 4.97 (3H, *br s*, 3 NH); δ_C (75 MHz, CD_3OD) 28.2 (CH$_2$), 29.7 (C(CH$_3$)$_3$), 30.1 (C(CH$_3$)$_3$), 30.2 (C(CH$_3$)$_3$), 31.5 (CH$_2$), 42.8 (CH$_2$), 56.2 (CH), 81.8 (C(CH$_3$)$_3$), 81.9 (C(CH$_3$)$_3$), 85.9 (C(CH$_3$)$_3$), 155.5 (C=O), 158.9 (C=O), 159.4 (C=O), 165.8 (C=N), 176.8 (CO$_2$H); *m/z* (ES) 497 ([M + Na]$^+$, 100), 475 ([M + H]$^+$, 5%).

Methyl (*E*,2*S*,2′*S*)-2-{[*N*,*N*′,*N*″-tris(*tert*-butoxycarbonyl)-argininyl]amino}-5-(9*H*-fluoren-9-ylmethoxycarbonylamino)-2-methylpent-3-enoate 14. Amino ester **8** (0.3 g, 0.63 mmol) was deprotected by stirring in TFA–CH_2Cl_2 (1 : 2, 3 mL) overnight. The solvent was removed *in vacuo*, iPr_2EtN (0.22 mL, 1.25 mmol) and THF (10 mL) were added, and the solution was stirred for 15 minutes. At the same time, the triprotected arginine **13** (61.39 mg, 0.39 mmol) was dissolved in THF (10 mL), and HATU (0.18 g, 0.69 mmol) and HOAt (94 mg, 0.69 mmol) were added. The solution was stirred for 15 minutes before being added dropwise to the solution of the deprotected amino ester, and the mixture was stirred at room temperature overnight. Following filtration and solvent removal *in vacuo*, the resulting residue was dissolved in EtOAc. The solution was washed with 1 M citric acid, water and brine, dried ($MgSO_4$) and concentrated *in vacuo.* Purification by HPLC (eluent A–B 100 : 0 to 0 : 100 over 60 minutes) yielded the dipeptide **14** (t_r 50.47 minutes) as a colourless oil (66 mg, 13%). $[\alpha]_D^{23}$ +26.7 (*c* 0.05 in CH_3CN); ν_{max}(neat)/cm^{-1} 3284, 3083, 2966, 1710, 1632, 1538, 1203, 1139; δ_H (300 MHz, CD_3CN) 1.39 (9H, *s*, C(CH$_3$)$_3$), 1.46–1.54 (21H, *env*, 2 C(CH$_3$)$_3$, CCH$_3$NH), 1.60–1.76 (4H, *env*, CHCH$_2$CH$_2$), 3.20–3.24 (1H, *m*, CHCH$_2$CH$_2$CHH), 3.37–3.45 (1H, *m*, CHCH$_2$CH$_2$CHH), 3.60 (3H, *s*, CO$_2$CH$_3$), 3.68–3.72 (2H, *m*, CH$_2$NH), 4.08–4.12 (1H, *m*, BocNHCH),

4.20–4.26 (1H, *m*, C*H*CH$_2$O), 4.31 (2H, *m*, CHC*H*$_2$O), 5.60–5.90 (4H, *env*, C*H*=CHCH$_2$, CH=C*H*CH$_2$ and 2 N*H*), 7.20–7.28 (1H, *m*, N*H*), 7.34 (2H, *t*, *J* 7.4, Ar–H), 7.42 (2H, *t*, *J* 7.4, Ar–H), 7.64 (2H, *d*, *J* 7.4, Ar–H), 7.83 (2H, *d*, *J* 7.4, Ar–H), 9.30–9.35 (1H, *m*, N*H*), 9.58–9.67 (1H, *m*, N*H*); δ_C (75 MHz, CD$_3$CN; some doubling of signals evident due to restricted rotation) 23.0, 24.3, 24.8, 25.2, 25.4, 25.9, 29.1, 29.6, 41.3, 42.1, 42.4, 47.6, 52.6, 54.0, 59.8, 66.7, 82.5, 85.1, 85.5, 118.4, 123.5, 125.2, 125.9, 126.1, 126.7, 139.6, 142.9, 151.9, 153.0, 153.1, 155.1, 159.7, 160.2, 171.9, 173.2; HRMS (ES) 859.4197 ($C_{43}H_{60}N_6O_{11}Na$ requires 859.4218); *m*/*z* (ES) 859 ([M + Na]$^+$, 50%), 557 (100).

(*E*,*S*)-5-Amino-2-{[(*N*-*tert*-butoxycarbonyl)glycyl]amino}-2-methylpent-3-enoic acid 15. The dipeptide **11** (115 mg, 0.71 mmol) was dissolved in MeCN (5 mL). LiOH (25.6 mg, 3.55 mmol) in H_2O (0.5 mL) was added, and the mixture was stirred at room temperature overnight. Removal of the solvent *in vacuo* and purification by HPLC (eluent A–B 100 : 0 to 0 : 100 over 60 minutes) yielded the dipeptide **15** (t_r 18.30 minutes) as a white solid (61 mg, 94%). $[\alpha]_D^{23}$ −54.8 (*c* 0.02 in D_2O); ν_{max}(KBr)/cm^{-1} 3281, 3088, 2968, 1675, 1633, 1536, 1457, 1203, 1139; δ_H (300 MHz, D_2O) 1.35 (9H, *s*, C(C*H*$_3$)$_3$), 1.48 (3H, *s*, NHCC*H*$_3$), 3.55 (2H, *d*, *J* 6.6, C*H*$_2$NH$_2$), 3.72 (2H, *s*, NHC*H*$_2$CO), 5.59–5.71 (1H, *m*, CH=C*H*CH$_2$), 6.23 (1H, *d*, *J* 15.8, C*H*=CHCH$_2$); δ_C (75 MHz, D_2O) 22.4, 26.5, 41.5, 58.3, 60.1, 85.3, 120.4, 134.7, 150.2, 165.4, 175.2; HRMS (ES) 324.1537 ($C_{13}H_{23}N_3O_5Na$ requires 324.1535); *m*/*z* (ES) 346 ([M − H + 2Na]$^+$, 40%), 324 ([M + Na]$^+$, 100), 302 ([M + H]$^+$, 20).

(*E*,2*S*,2′*S*)-5-Amino-2-{[(*N*$^{\alpha}$-*tert*-butoxycarbonyl)argininyl]-amino}-2-methylpent-3-enoic acid 17. The dipeptide **14** (66 mg, 0.08 mmol) was dissolved in MeCN (3 mL). LiOH (9.4 mg, 0.39 mmol) in water (1 mL) was added, and the mixture was stirred at room temperature overnight. Removal of the solvent *in vacuo* and purification by HPLC (eluent A–B 100 : 0 to 0 : 100 over 60 minutes) yielded the dipeptide **17** (t_r 26.24 minutes) as a white solid (30 mg, 94%). $[\alpha]_D^{23}$ −15.7 (*c* 0.02 in CH$_3$CN); ν_{max}(KBr)/cm^{-1} 3276, 3093, 2964, 1673, 1650, 1531, 1205, 1137; δ_H (300 MHz, CD$_3$OD) 1.45 (9H, *s*, C(C*H*$_3$)$_3$), 1.59 (3H, *s*, CC*H*$_3$NH), 1.60–1.74 (4H, *env*, CHC*H*$_2$C*H*$_2$), 3.17–3.21 (1H, *m*, CHCH$_2$CH$_2$C*H*H), 3.31–3.37 (1H, *m*, CHCH$_2$CH$_2$CH*H*), 3.55 (2H, *d*, *J* 6.3, C*H*$_2$NH$_2$), 4.03–4.10 (1H, *m*, BocNHC*H*), 5.65–5.80 (1H, *m*, CH=C*H*CH$_2$), 6.29 (1H, *d*, *J* 15.8, C*H*=CHCH$_2$); δ_C (100 MHz, CD$_3$OD; some doubling of signals evident due to restricted rotation) 23.5, 24.2, 25.6, 26.3, 28.1, 28.7, 29.8, 30.2, 41.8, 42.0, 42.2, 53.7, 55.7, 60.7, 81.0, 85.8, 122.5, 137.9, 153.6, 158.0, 161.7, 162.0, 174.1, 175.1; HRMS (ES) 401.2459 ($C_{17}H_{33}N_6O_5$ requires 401.2434); *m*/*z* (ES) 401 ([M + H]$^+$, 100%), 301 ([M + H–$C_5H_8O_2$]$^+$, 55).

(*E*,2*S*)-2{[(*N*-*tert*-Butoxycarbonyl)glycyl]amino}-5-{[(*tert*-butoxycarbonyl)amino-(*tert*-butoxycarbonylimino)methyl]amino}-2-methylpent-3-enoic acid 16. To a solution of the dipeptide **15** (40 mg, 0.13 mmol) and iPr$_2$EtN (23 μL, 0.13 mmol) in MeOH (2 mL) was added bis(*tert*-butoxycarbonyl)triflylguanidine (47 mg, 0.13 mmol), and the mixture was stirred at room temperature for 3 days. Removal of the solvent *in vacuo* and purification by HPLC (eluent A–B 100 : 0 to 0 : 100 over 60 minutes) yielded the dipeptide **16** (t_r 58.29 minutes) as a white solid (64 mg, 90%). $[\alpha]_D^{23}$ −14.8 (*c* 0.01 in MeOH); ν_{max}(KBr)/cm^{-1} 3434, 3057, 2924, 1722, 1601, 1451, 1273, 1117; δ_H (500 MHz, CD$_3$OD) 1.53 (27H, *br s*, 3 × C(C*H*$_3$)$_3$), 1.60 (3H, *s*, NHCC*H*$_3$), 3.92 (2H, *d*, *J* 5.9, CH=CHC*H*$_2$), 4.12 (2H, *s*, NHC*H*$_2$CO) 5.73 (1H, *dt*, *J* 15.8, 5.9 CCH=C*H*CH$_2$), 6.17 (1H, *d*, *J* 15.8, CC*H*=CHCH$_2$); δ_C (125 MHz, CD$_3$OD) 24.3 (NHCC*H*$_3$), 28.1 (3 C(*C*H$_3$)$_3$), 43.6 (*C*H$_2$), 44.7 (*C*H$_2$), 61.1 (NH*C*CH$_3$), 85.9 and 86.0 (3 *C*(CH$_3$)$_3$), 124.4 (*C*H=), 134.4 (*C*H=), 153.5, 155.5, 156.0, 162.1, 167.5 (4 *C*=O, *C*=N), 175.3 (*C*O$_2$H); *m*/*z* (ES) 566 ([M + Na]$^+$, 10%), 544 ([M + H]$^+$, 40), 444 ([M + H–$C_5H_8O_2$]$^+$, 100).

(*E*,2*S*,2′*S*)-2-{[(*N*$^{\alpha}$-*tert*-Butoxycarbonyl)argininyl]amino}-5-{[(*tert*-butoxycarbonyl)amino-(*tert*-butoxycarbonylimino)methyl]-amino}-2-methylpent-3-enoic acid 18. To a solution of the dipeptide **17** (49 mg, 0.12 mmol) and iPr$_2$EtN (21 μL, 0.12 mmol) in MeOH (2 mL), was added bis(*tert*-butoxycarbonyl)triflylguanidine (44 mg, 0.12 mmol), and the mixture was stirred at room temperature for 3 days. Evaporation of the solvent *in vacuo* and purification by HPLC (eluent A–B 100 : 0 to 0 : 100 over 60 minutes) yielded the dipeptide **18** (t_r 54.54 minutes) as a white solid (40 mg, 52%). $[\alpha]_D^{23}$ +33.9 (*c* 0.02 in CH$_3$OH); ν_{max}(KBr)/cm^{-1} 3273, 3082, 2964, 1670, 1628, 1531, 1203, 1137; δ_H (500 MHz, CD$_3$OD) 1.45 (9H, *s*, C(C*H*$_3$)$_3$), 1.53 (9H, *s*, C(C*H*$_3$)$_3$), 1.54 (9H, *s*, C(C*H*$_3$)$_3$), 1.59 (3H, *s*, CC*H*$_3$NH), 1.67–1.82 (4H, *env*, CHC*H*$_2$C*H*$_2$), 3.18–3.22 (1H, *m*, CHCH$_2$CH$_2$C*H*H), 3.35–3.39 (1H, *m*, CHCH$_2$CH$_2$CH*H*), 3.81–3.85 (1H, *m*, =CHC*H*H), 4.01–4.08 (2H, *env*, BocNHC*H*, =CHCH*H*), 5.65–5.73 (1H, *m*, CH=C*H*CH$_2$), 6.07–6.16 (1H, *m*, C*H*=CHCH$_2$); δ_C (125 MHz, CD$_3$OD) 24.2 (CC*H*$_3$NH), 24.8 (CHCH$_2$*C*H$_2$), 28.1 (C(*C*H$_3$)$_3$), 28.7 (C(*C*H$_3$)$_3$), 28.9 (C(*C*H$_3$)$_3$), 30.0 (CH*C*H$_2$), 42.2 (CHCH$_2$CH$_2$*C*H$_2$), 43.6 (=CH*C*H$_2$), 55.8 (BocNH*C*H), 60.8 (NH*C*CH$_3$), 81.0 (*C*(CH$_3$)$_3$), 85.8 (*C*(CH$_3$)$_3$), 85.9 (*C*(CH$_3$)$_3$), 124.1 (CH=*C*HCH$_2$), 134.7 (*C*H=CHCH$_2$), 153.5, 153.6, 155.5, 157.9, 162.9, 163.2 (4 *C*=O, 2 *C*=N), 174.2 (*C*O$_2$H); HRMS (ES) 643.3771 ($C_{28}H_{51}N_8O_9$ requires 643.3779); *m*/*z* (ES) 687 ([M − H + 2Na]$^+$, 60%), 665 ([M + Na]$^+$, 10), 643 ([M + H]$^+$, 100), 543 ([M + H–$C_5H_8O_2$]$^+$, 70).

Manual peptide coupling and cleavage procedure

To a solution of the dipeptide (30 μmol) in DMSO (150 μL) at room temperature were added DCC (7 mg, 33 μmol) and DMAP (0.4 mg, 0.3 μmol). The resultant solution was stirred for 15 minutes before being added to the resin-bound heptameric peptide (30 μmol; resin loadings were typically around 0.4 mmol g^{-1}). The reaction was heated at 60 °C for 2 days under an argon atmosphere. Upon completion of the reaction the resin was transferred to a cleavage vessel consisting of a quick fit glass tube with glass sinter and three-way tap.[17] The resin was washed with CH_2Cl_2 and then treated with reagent B (TFA–phenol–water–triisopropylsilane [88 : 5 : 5 : 2, 10 mL]) at room temperature for 2 hours. The cleavage cocktail was removed by filtration and the resin washed with TFA (2 × 5 mL). The combined filtrate was evaporated to dryness and the residue was triturated with Et_2O (5 mL). The resultant solid was dissolved in water (5 mL) and lyophilised to afford the fully deprotected crude peptide as a white solid.

Preparation of H–Gly–X$_{aa}$–Ala–Phe–Val–Thr–Ile–Gly–Lys–OH 19. Following manual coupling and cleavage, purification by HPLC (eluent A–B 100 : 0 to 0 : 100 over 60 minutes) gave peptide **19** (t_r 18.57 minutes) as a white solid (12 mg, 42%). *m*/*z*

(MALDI) 983.2 ([M + Na]$^+$, 100%), 961.2 ([M + H]$^+$, 40) (calc. 960.6 for [M + H]$^+$).

Preparation of H–Arg–X_{aa}–Ile–Tyr–Asp–Leu–Ile–Glu–Leu–OH 20. Following manual coupling and cleavage, purification by HPLC (eluent A–B 100 : 0 to 0 : 100 over 60 minutes) gave peptide **20** (t_r 31.87 minutes) as a white solid (17 mg, 47%). *m/z* (MALDI) 1202.6 ([M + H]$^+$, 100) (calc. 1202.7 for [M + H]$^+$).

Cell surface stabilisation assays

T2-B*2705 cells (2×10^5) were incubated in serum-free AIM-V medium in the presence of 100 μg mL^{-1} of the peptide for 14 to 16 h at 26 °C, after which the cells were incubated at 37 °C for 2 h prior to immunofluorescent staining. Cells were washed free of unbound peptide with growth medium prior to the addition of primary antibody. Anti-MHC allele-specific monoclonal antibody, W6/32, was added to the T2-B*2705 cells and incubated at 4 °C for 30 minutes. To detect binding of the W6/32 monoclonal antibody, these cells were washed and incubated with an anti-mouse fluorescein isothiocyanate labelled antibody at 4 °C for 30 minutes. Finally, cells were washed and resuspended in 500 μL of cold PBS supplemented with 1% FCS. A sample of T2-B*2705 cells was incubated with AIM-V medium alone (no peptide) at 26 °C for 14 to 16 h and served as a negative control. The second negative control comprised a sample of T2-B*2705 cells that had been cultured in growth medium without peptide at 37 °C. Fluorescence intensities of the T2-B*2705 cells were then measured using flow cytometry. The MHC stabilisation efficiency (MSE) for each peptide was calculated as the percentage increase of the mean fluorescence above that of the negative controls.

Cytotoxic assays (^{51}Cr)

Target cells were sensitised with the synthetic peptides **20** and **22** at a concentration of 5 μM during incubation with 0.7 mCi ^{51}Cr for 90 minutes at 37 °C. Following incubation, these cells were washed twice in growth medium and used as targets for cognate effector cells at three effector ratios 1 : 1, 2 : 1 and 5 : 1 in a standard 5 hour ^{51}Cr-release assay.[27]

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